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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

(Agilent Technologies, Santa Clara, CA 95051, USA). All RNAs were found to have A^{260}/A^{280} ratios>1.97, A^{260}/A^{230} ratios>1.19, and RIN scores>8.9 (Schroeder et al., 2006). cDNA was generated using 300 ng of total RNA and the GeneChip WT cDNA Synthesis and Amplification Kit (Affymetrix, Santa Clara, CA 95051, USA). cDNA was fragmented and end-labelled using the GeneChip WT Terminal Labelling Kit (Affymetrix) and hybridized to the Affymetrix Mouse Gene 1.0 ST Array at 45 °C for 17 h. Hybridized arrays were washed and stained in a GeneChip Fluidics Station 450 and scanned with a GeneChip Scanner 3000 7G (Affymetrix) using default settings.

Expression data analysis

Data analysis was performed using the AMEN (Annotation, Mapping, Expression and Network analysis) suite of tools (Chalmel and Primig, 2008). Data from triplicate XY Sox9 mutant, XY wild-type and XX wild-type samples and 11 control tissues (brain, embryo, heart, kidney, liver, lung, ovary, skeletal muscle, spleen, testicle and thymus) provided by Affymetrix were normalized using the Robust Multi-Array Average (RMA) method (Irizarry et al., 2003). Statistical filtration and classification was performed for the three following comparisons: XY_WT versus XY KO, XX WT versus XY KO, and XX WT versus XY WT (Supplemental Fig. 2a). Probe sets yielding signals ≥6.935 (median of the normalized dataset) and a fold change ≥2.0 were selected in each of the three comparisons. A limma statistical test (F-value adjusted with False Discovery Rate ≤0.05) was used to identify significantly differentially expressed probe sets which were subsequently classified into two groups using the k-means algorithm (k=2).

MIAME compliance

Raw data CEL files are available at the NCBI's GEO certified public repository via the accession number GSE21650.

Gene ontology enrichments

The gene ontology (GO) over-representations were estimated with the Fisher exact probability, using the Gaussian Hypergeometric Test. A GO term was considered to be significantly enriched in a given group of genes when the *p*-value corrected with the FDR method was ≤0.01 and ≥3 genes within the group were associated with the annotation term.

Q-PCR analysis

Ovaries (n=6) were collected from XX *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{flox/flox}$ or XX *Sox9*$^{flox/flox}$ females at the age of four weeks, fat and oviducts were removed and samples were snap frozen in dry ice and stored at −80 °C. RNA was extracted using the RNeasy kit mini kit according to the manufacturer's instructions (Qiagen, Germany). cDNA synthesis was performed as previously described (Chassot et al., 2008). Primers and probes were designed by the Roche Assay design center (https://www.rocheappliedscience.com/sis/rtpcr/upl/adc.jsp): *Sox9* (NM_011448.4) 5′-CAGCAAGACTCTGGGCAAG-3′ and 5′-TCCACGAAGGGTCTCTTCTC-3′ (probe 66). *Hprt1* (NM_013556.2) 5′-TCCTCCTCAGACCGCTTTT-3′ and 5′-CCTGGTTCATCATCGCTAATC-3′ primers and probe set (probe 95) were used to normalize the Q-PCR assays for *Sox9*. Statistical analysis was performed using the Prism Graphpad software.

Superovulation and oocyte retrieval

Superovulation was induced in XY *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{flox/flox}$ females by intra peritoneal injection of 5 IU of eCG (Intervet UK Ltd., Cambridge, UK) followed by injection of 5 IU of hCG (Intervet) 44–48 later. Females were mated with B6DBA males yielding a C57BL/6JxDBA F1 background. One-cell stage embryos were recovered from oviducts at E1 21–23 h after hCG injection. Two-cell stage embryos were isolated by flushing oviducts 32–34 h after hCG injection. Morulas were obtained by flushing both oviducts and uterus at E3 following hCG injection. M2 (Sigma) and KSOM medium (Specialty Media) were used in our studies. Culture dishes with embryo-containing microdrops were incubated under oil at 37 °C in an incubator equilibrated with 5% CO_2 atmosphere.

Immunostaining of oocytes and embryos

Oocytes and embryos were fixed in 4% paraformaldehyde in PBS containing 1% BSA for 30 min and washed twice in fresh PBS. Fixed embryos were blocked and permeabilized in PBS with 0.25% Tween-20 and 0.1% BSA for 15 min and incubated at room temperature with a monoclonal mouse anti-β-Tubulin antibody (Sigma) at 1/100 followed by incubation with Alexa Fluor rabbit anti-mouse antibody (Invitrogen) at 1/500. Between each step the embryos were washed 3 times with PBS. Samples were mounted on glass slides with Vectashield mounting medium containing DAPI and examined on an AxioImager Z.I fluorescence microscope (Zeiss).

Results

Conditional inactivation of Sox9 in XY gonads

Sox9 is required for various processes of organogenesis and previous mouse models lacking *Sox9* displayed an embryonic lethal phenotype due to expression of the CRE recombinase in tissues other than gonads (Barrionuevo et al., 2006; Chaboissier et al., 2004). This problem is avoided by expressing CRE recombinase using the *Sf1* promoter with is active in the pituitary, spleen, adrenal and gonad (Bingham et al., 2006). To evaluate whether the *Sf1:Cre* transgene mediated *Sox9* loss-of-function in the XY gonads, we generated XY *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{flox/flox}$ embryos (referred to as XY mutants throughout the text) and analysed their phenotypes at E11.5 during the onset of *Sox9* expression and at E13.5 when *Sox9* expression is strongly induced. The number of embryos recovered (n=8) was normal in comparison to XX control females implying that ablation of *Sox9* in the cells expressing *Sf1:Cre* has no deleterious effect on the development of *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{flox/flox}$ embryos. At E11.5, whole mount *in situ* hybridizations with a *Sox9* probe showed its expression in the urogenital ridges of XY control embryos while XX gonads yielded no signal (Fig. 1). At this stage, *Sox9* expression was weak or absent in XY mutant urogenital ridges indicating that *Sox9* was deleted in the transgenic gonads. Next, immunostaining of E11.5 gonadal sections with SOX9 antibody clearly detected SOX9 in XY gonads, but not in XX gonads and in only a few gonadic cells in XY mutant embryos. In contrast, SPRRD2 was expressed at higher levels in XX or XY mutant urogenital ridges than in XY gonads (Fig. 1). By E13.5, neither Sox9 nor AMH were detected in XY mutant or XX gonads while it was strongly expressed in XY gonads. We conclude that *Sox9* was successfully deleted in XY mutant gonads at this stage (Fig. 1). We then examined the expression of FOXL2, a marker of pre-follicular cells. The protein was detected in XX and XY mutant but not XY wild-type samples (Fig. 1). Taken together, these results are consistent with the idea that *Sox9* loss-of-function in XY gonads promotes ovarian development.

XY Sf1:Cre$^{Tg/+}$; *Sox9*$^{flox/flox}$ *mutant gonads display an intermediate expression signature as compared to XY Sox9*$^{flox/flox}$ *and XX Sox9*$^{flox/flox}$ *wild-type controls*

To investigate the genome-wide transcriptional signature in XY mutant mice versus XX and XY wild-type controls we performed an expression profiling analysis of E13.5 gonads using mouse GeneChips (Materials and Methods). The mRNA profiles from triplicate XY *Sf1:*

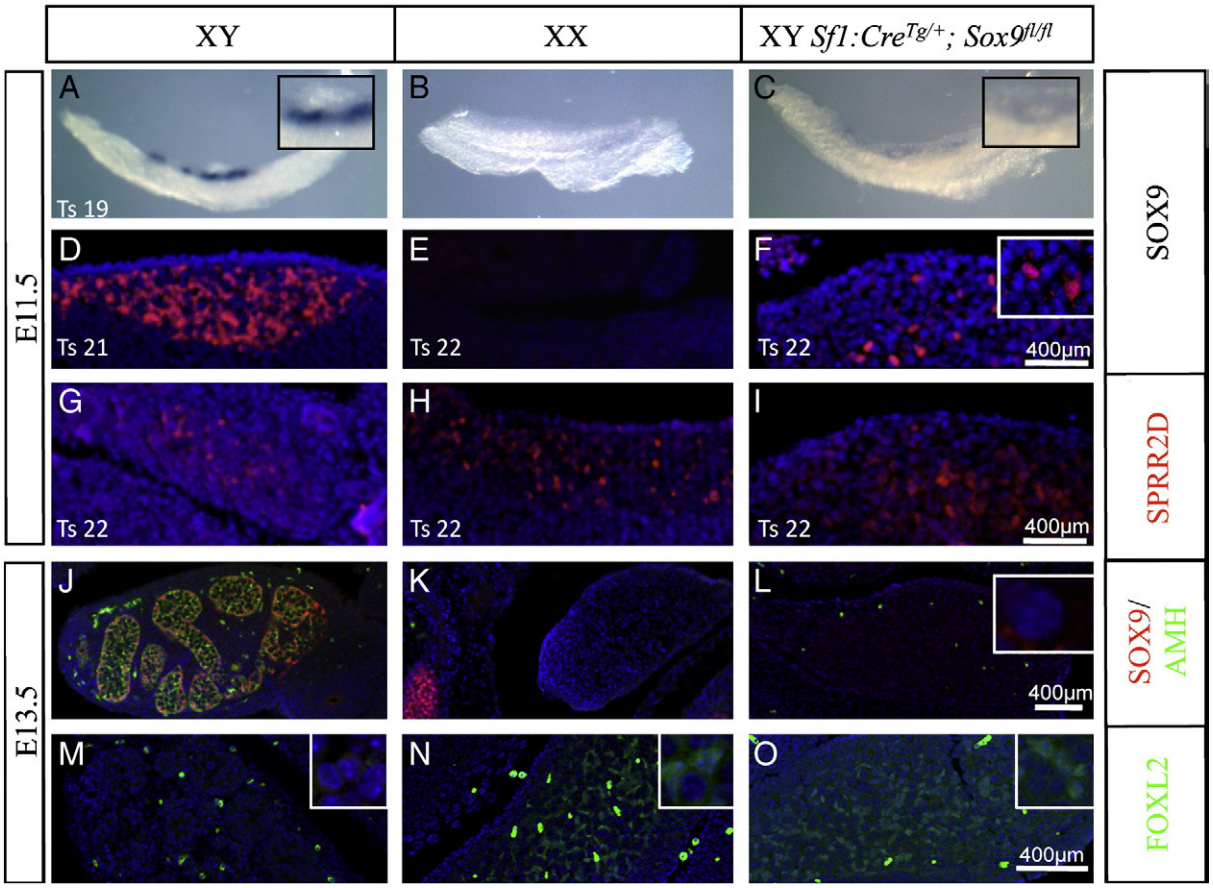


Fig. 1. *Sox9* expression analysis in XY *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{flox/flox}$ embryonic gonads. (A–C) Whole mount *in situ* hybridization analysis using a *Sox9* antisense probe at E11.5 in (A) XY control urogenital ridge, (B) an XX control and (C) an XY mutant. Immunofluorescence detection of SOX9 protein in E11.5 (D) XY control urogenital ridges, XX controls (E) and (F) XY mutant urogenital ridges. SPRRD2, a gonad specific marker shows expression in the contiguous section to those shown in D,E,F. At E11.5 XY gonads (G) show polar expression whereas SPRRD2 pattern of expression is more even in XX wild-type (H) and XY mutant (I). (J-L) SOX9 and its direct target AMH are expressed in XY wild-type E13.5 gonads (J) but absent in XX (K) and XY mutant gonads (L). The ovarian FOXL2 protein was detected in E13.5 XX (N) and XY mutant gonads (O) but absent in the XY wild-type gonad (M) indicating that complete sex reversal has occurred.

Cre$^{Tg/+}$; *Sox9*$^{flox/flox}$ (XY_KO), XY *Sox9*$^{flox/flox}$ (XY_WT) and XX *Sox9*$^{flox/flox}$ (XX_WT) gonads were determined and compared to each other. Standard data quality control procedures revealed homogeneous hybridization patterns (Supplemental Fig. 1A) and the expected range of signal distributions across the sample set (Supplemental Fig. 1B). We analysed global expression signals using a distance matrix and a dendrogram and found the sample triplicates to be grouped together; overall, XY mutant and XY wild-type transcriptomes were clustered together and XX wild-type was separated (Supplemental Fig. 1C).

To carry out three two-way comparisons (XY_KO versus XX_WT; XY_KO versus XY_WT; XY_WT versus XX_WT) we first identified for each sample pair the probe sets for which signals above the cut-off were observed (6.935 log2 units; Supplemental Fig. 2A). We next selected genes significantly differentially expressed ($\geq$2-fold change; Limma test, FDR adjusted p-value$\leq$0.05) and split them into two groups showing increasing or decreasing signals (wild-type versus mutant) and increasing signals in XX or XY samples using the k-means clustering algorithm (n = 2). We found 379 genes significantly differentially expressed between XY wild-type and mutant gonads. Among them 151 genes showed increased signals and 228 genes showed decreased signals, respectively, in the XY mutant as compared to the XY control (Supplemental Fig. 2A, middle column). XY mutant versus XX wild-type identified 270 genes significantly differentially expressed including 108 showing stronger and 160 showing weaker signals in the XY mutant (Supplemental Fig. 2A, left). Finally, we identified 1047 genes significantly differentially expressed between XX and XY wild-type samples including 428 which display stronger signals in males and 619 genes which display stronger signals in females (Supplemental Fig. 2A, right).

Selected genes required for ovarian differentiation like *Foxl2*, *Wnt4*, *Rspo1*, *Bmp2*, and *Fst* showed stronger signals in XY *Sf1: Cre*$^{Tg/+}$; *Sox9*$^{flox/flox}$ gonads in comparison to XY gonads indicate ongoing female differentiation; they are shown in Table 1A. To validate the expression profiling analysis we examined the transcription of selected genes by whole mount *in situ* hybridization. Four genes expressed in Sertoli cells (*Sox8*, *Sox10*, *Pgds*, and *Fgf9*), and one each expressed in Leydig cells (*P450Scc/Cyp11a1*) and pro-spermatogonia (*ErbB3*) yielded positive signals in XY gonads but not in XX wild-type and show an intermediate level of expression in XY mutant samples (Table 1D). Consistently, mRNA from loci known to be expressed in females (*Rspo1*, *Bmp2*, *Emx2*, *Phf19* and *Stra8*) were detected in XX wild-type and in XY mutant gonads (Supplemental Fig. 3).

We subsequently correlated expression signatures with male, female and XY mutant phenotypes by organising these genes into 11 classes using Venn diagrams (Supplemental Fig. 2B). The first four classes comprise genes that show peak signals in XX wild-type, intermediate signals in the XY mutants and the weakest signals in XY wild-type samples (Fig. 2A). Consistently, Class 1 (124 genes) is enriched for genes bearing the Gene Ontology annotation terms such as *Chromosome organization involved in meiosis* (GO:0070192, 4 observed/0 expected by chance, p-value 2.75×10^{-5}), and *Meiosis*

Table 1
Comparative expression analysis of genes involved in ovarian and testicular development.

Gene name	Entrez gene ID	XY_KO vs XY_WT	XY_KO vs XX_WT	XY_WT vs XX_WT
(A) Expression of genes involved in ovarian development at E13.5				
Foxl2	26927	1.21	−0.08	1.29
Wnt4	22417	1.13	0.28	0.85
Rspo1	192199	1.38	−0.12	1.50
Bmp2	12156	2.14	−0.53	2.67
Fst	14313	3.61	−0.28	3.89
(B) Expression of genes involved in germ cell development at E13.5				
Rec8	56739	2.57	−0.43	3.00
Stra8	20899	3.32	−0.45	3.77
Sycp3	20962	2.33	−1.78	4.11
Dmc1	13404	2.01	−1.40	3.41
Dazl	13164	0.28	−0.11	0.39
Nanos3	244551	−0.52	0.27	−0.79
Pou5f1/Oct4	18999	−0.58	0.71	−1.29
Nanos2	378430	−1.20	0.62	−1.82
Tdrd1	83561	−0.23	−0.08	−0.15
Dnmt3l	54427	−0.21	0.28	−0.49
(C) Expression of Y chromosome genes at E13.5				
Ube1y1	22202	0.41	5.40	−4.99
		0.17	4.37	−4.20
		0.03	2.49	−2.46
Rbmy1a1	19657	−0.04	2.41	−2.45
		−0.10	1.60	−1.70
Sry	21674	0.70	0.51	0.19
Zfy1	22767	0.63	1.68	−1.05
Zfy2	22768	0.22	0.37	−0.14
(D) Expression of genes involved in testis differentiation				
Sox8	20681	−1.03	0.3	-1.33
Sox10	20665	−0.47	0.11	−0.58
Ptgds/Pgds	19215	−1.98	0.2	−2.18
Fgf9	14180	−0.57	0.23	−0.80
Cyp11a1/P450Scc	13070	−4.65	1.7	−6.35
ErbB3	13867	−1.35	0.37	−1.72
Dhh	13363	−2.21	0.21	−2.42
Dmrt1	50796	−0.30	0.30	−0.6
Sry	21674	0.70	0.51	0.19
Amh	11705	−4.34	1.26	−5.60
Tmem184a/Sdmg1	231832	−2.40	2.67	−2.67
Ptch1	19206	−1.57	−0.04	−1.53
Hsd17b1	15485	−1.04	0.76	−1.80
Hsd3b1	15492	−2.40	0.22	−2.62

The given values are the log2 of the indicated fold change.

(0007126, 5/1, 1.79×10^{-3}). Class 2 (362 genes) is also enriched for *Chromosome organization involved in meiosis* (0070192, 3/0, 5.6×10^{-3}) as well as *Reproduction* (0000003, 25/9, 5.31×10^{-5}), *Female gamete generation* (0007292, 5/1, 4.85×10^{-3}), *Regulation of signal transduction* (0009966, 23/9, 1.15×10^{-3}) and *Negative regulation of apoptosis* (0043066, 11/3, 5.5×10^{-3}). Class 3 contains only 18 genes but is still enriched for *Chromosome organization involved in meiosis* (0070192, 3/0, 2.8×10^{-7}), *Meiosis* (0007126, 4/0, 6.7×10^{-7}), and *Gamete generation* (0007276, 5/0, 6.27×10^{-6}) while Class 4 (125 genes) is enriched only for *Meiosis* (0007126, 7/0, 3.7×10^{-6}).

The next three classes shown in Fig. 2B identify genes for which we find the strongest signals in XY wild-type and the weakest in XX wild-type samples while XY mutants show intermediate signals (C5 and C6) or equivalent signals as the XY wild-type (C7). Class 5 (212 genes) is enriched, among others, for *Sex differentiation* (0007548, 8/1, 10.0×10^{-4}), *Spermatogenesis* (0007283, 8/3, 6.8×10^{-3}), *Steroid metabolic process* (0008202, 10/2, 1.7×10^{-5}), and *Regulation of apoptosis* (0042981, 13/5, 2.8×10^{-3}) and Class 6 (155 genes) was found to be enriched for *Embryonic morphogenesis* (0048598, 10/3, 1.3×10^{-3}), *Cell differentiation* (0030154, 25/10, 2.5×10^{-4}) and also *Steroid metabolic process* (0008202, 6/1, 1.8×10^{-3}). Class 7 contained 64 genes but no GO term was found to be enriched.

Finally, we determined three classes shown in Fig. 2C that display insignificant signal differences between male and female gonads (which distinguishes this group from the others shown in Figs. 2A and B) and for which RNA concentrations in the XY mutant were either found to be closer to female (C8 and C10) or male (C9 and C11) gonads. Class 8 (9 genes) and Class 11 (46 genes) were not enriched for any GO terms. Class 9 (17 genes) and Class 10 (17 genes) were found to enriched for genes associated with processes such as *Cellular macromolecule metabolic process* (0044260, 7/2, 9.9×10^{-3}), *Cell proliferation* (0008283, 3/0, 5.15×10^{-3}) and *Anatomical structure morphogenesis* (0009653, 4/1, 9.52×10^{-3}).

Sex reversal of newborn and adult XY Sf1:Cre$^{Tg/+}$; Sox9$^{flox/flox}$ mice

Previous analyses using XY *Sox9* mutants could not be performed after E15.5 because of the early lethality of the *Sox9* mutant alleles used in these studies. In our model, however, we were able to investigate how XY mutant gonads develop at P0. Histological analysis of XY mutant gonads did not show sex cord formation as compared to the XY wild-type control (Fig. 3A). In addition, Sertoli cells were detected in the testicular tubules of XY control gonads but no signal was apparent in XX or XY mutant gonads using SOX9 or SDMG1 which are nuclear and cytoplasmic markers of Sertoli cells, respectively (Fig. 3A). In contrast, FOXL2 immunostaining showed follicular cell differentiation in XX ovaries and in XY mutant gonads indicating that they develop an ovarian structure at P0 (Fig. 3A).

At E13.5, genes involved in the onset of meiosis such as *Rec8* and *Stra8* were up-regulated in XY mutant gonads in comparison to XY controls (Table 1). At P0, histological analysis revealed the presence of meiotic germ cells in XX wild-type and XY mutant gonads whereas XY quiescent gonocytes were found within the testicular cords in XY gonads. This is consistent with the outcome of our expression profiling analysis which indicated that meiosis was initiated in XX wild-type and XY mutant germ cells (Fig. 3A).

XY mutant mice exhibited female external genitalia once they reached adulthood but were otherwise healthy (Fig. 4). Macroscopic analysis of the urogenital system of seven-week-old mice revealed vaginas, uteri, oviducts and ovaries like XX females and no seminal vesicles, prostates, vas deferens, epididymes or testes as expected in males. Moreover, histological analysis showed that XY mutant gonads did not develop seminiferous tubules as expected in testis but rather ovarian follicles at different stages of maturation (Fig. 4). Oocytes were observed in some of the pre-ovulatory follicles. Furthermore some corpus luteum was present in XY mutant mice suggesting that ovulation might have occurred. Taken together, these data indicate sex reversal of XY mutant mice.

Sox9 is dispensable for ovarian function

Sox9 expression has been detected in the inner layer of the theca cells suggesting a potential function for SOX9 in the ovary (Notarnicola et al., 2006). Moreover the *Sf1:Cre* transgene is expressed in the theca cells of the ovaries (Bingham et al., 2006) suggesting that *Sox9* could be depleted in ovarian cells. As a consequence we examined the level of *Sox9* expression in XX *Sox9$^{flox/flox}$* and XX mutant ovaries of four-week-old females using Q-PCR. We found that *Sox9* expression was significantly reduced in XX mutant ovaries as compared to XX *Sox9$^{flox/flox}$* controls (Fig. 3B).

We sought to estimate the fertility of the XX mutants in comparison to XX *Sox9$^{flox/flox}$* control females. To this end, XX mutant and XX *Sox9$^{flox/flox}$* females were split into two groups bases on their age, i.e. 1.5 to 3 months and 3 to 6 months (Fig. 3C). The average number of offspring obtained from XX mutant females was similar to XX controls with an average of 8 pups per litter in the four groups of females

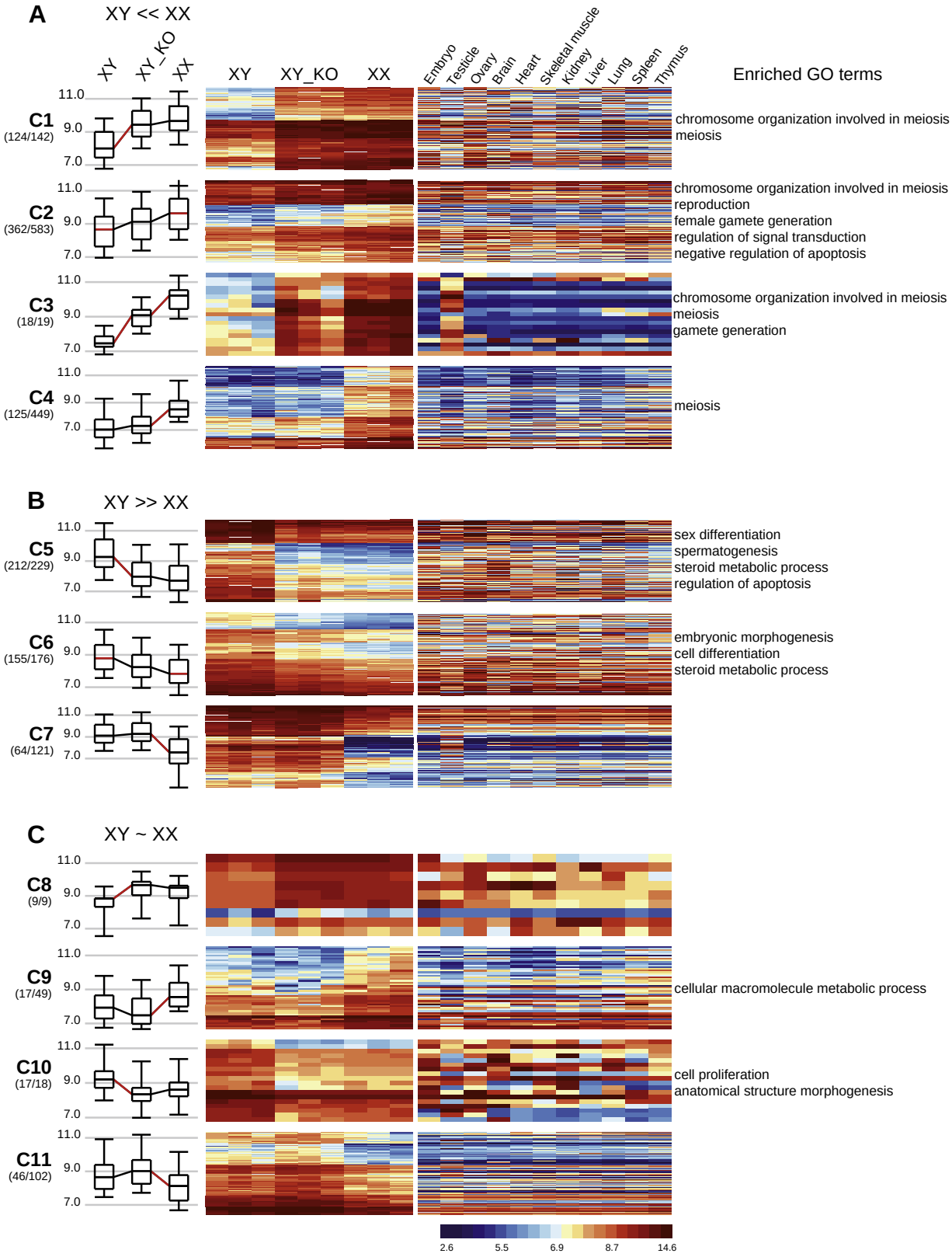


Fig. 2. Expression profiling of XX and XY wild-type versus XY mutant gonads. (A) A heatmap is shown for triplicate samples from XY wild-type (XY), XY mutant (XY_KO) and XX wild-type (XX) samples. Four classes of genes are shown for which signals are weaker in XY wild-type than in XX wild-type (XY<XX). (B) Three classes are given for which signals are stronger in XY wild-type than in XX wild-type (XY>XX). (C) Four classes are shown for which no significant difference was observed between XY and XX wild-type samples (XY~XX). Expression classes are denominated as C1 to C11. The number of genes versus corresponding probe sets displayed in each class is given in parenthesis. Box plots display the log2 signal distributions within three samples as indicated at the top. A black line in the box represents the median. Red lines indicate significant differential expression. Data from external somatic and adult testis samples (left panel) are indicated at the top. Examples of enriched GO terms are shown. A log2 scale is given.

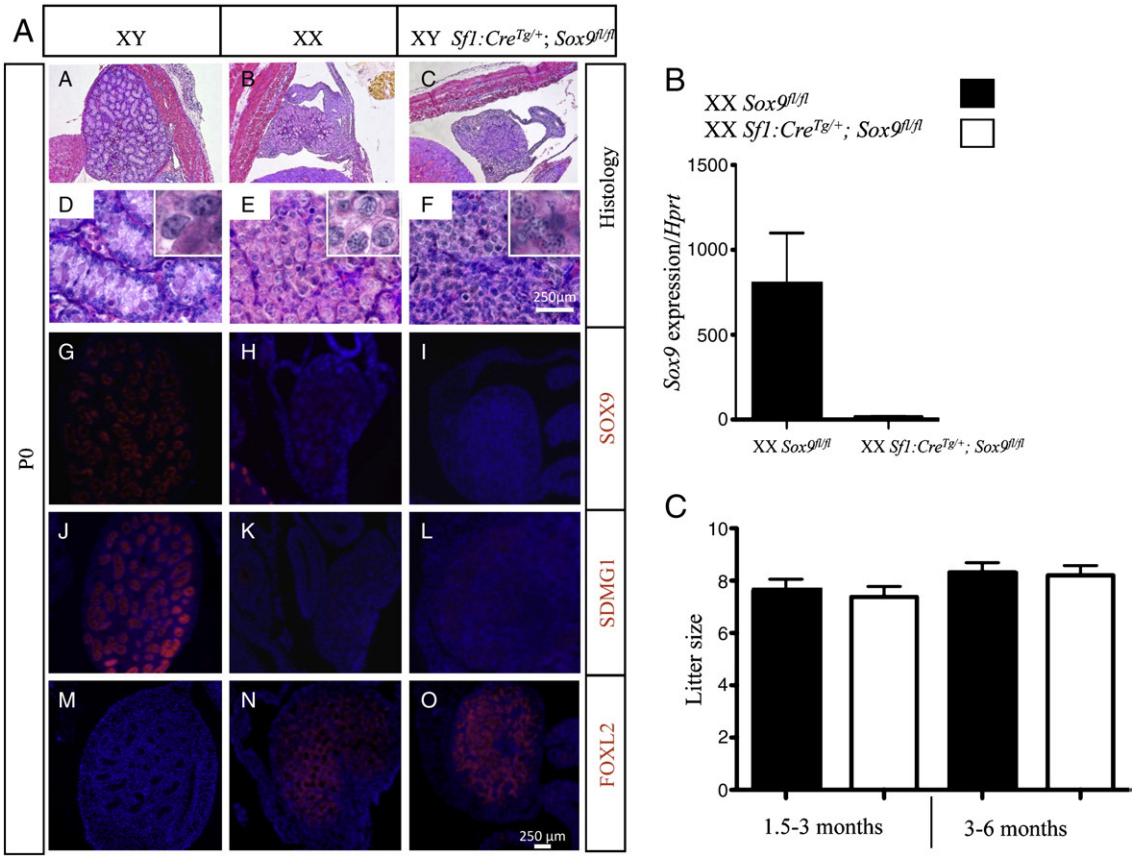


Fig. 3. Histological and immunofluorescence analysis on sagital sections of gonads from wild-type XY, XX and XY *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{flox/flox}$ mice. (A)–(A–F) Haematoxylin and eosin histological analysis of gonadal sections. Low magnification ×10 (A–C). High magnification ×100 (D–F). XY testes (A) shows the presence of seminiferous tubules with sex cords containing quiescent germ cells as shown in inset. In contrast, XY mutant gonads (C) resemble ovaries (B) both consisting of meiotic germ cells and highlighted in insets (E, F). (G–R) Immunofluorescence analysis using male and female specific markers. (G–I) Immunofluorescence for SOX9 (red) a marker of Sertoli cell nuclei was detected in the XY controls (G) but not in XX (H) or transgenic gonads (I). (J–L) Detection of SDMG1 (red), a marker of Sertoli cell cytoplasm was evident in the XY controls (J) but not in XX (K) or transgenic gonads (L). (M-O) FOXL2 (red) a marker, of ovarian somatic cells was detected in XX wild-type gonads (N) and not in XY control gonads (M). In gonads of XY mutant newborns (O) FOXL2 was detected, showing a less organized pattern of expression compared to XX control gonads. *Sox9* is dispensable for ovarian function(B) Q-PCR of *Sox9* expression in XX mutant ovaries (n = 6) as compared to XX *Sox9*$^{flox/flox}$ controls (n = 6) ($P = 0.0339$). (C) The litter size obtained from 1.5 to 3 month or 3–6 months old XX *Sox9*$^{flox/flox}$ female (n = 31 and 25, respectively) versus XX mutant females (n = 32 and 34 respectively) (P = 0.691) and (P = 0.831).

(Fig. 3C). This suggests that *Sox9* expression in ovaries and more generally in *Sf1:Cre* expressing cells is not required for fertility.

XY Sf1:Cre$^{Tg/+}$; *Sox9*$^{flox/flox}$ *females are subfertile*

We next investigated whether the XY mutant females are fertile. Ten XY mutant animals were crossed with a XY *Sox9*$^{flox/flox}$ wild-type male and three females produced one pup, two pups and two litters of four and two pups each. The genotype of the surviving mice was XX *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{flox/flox}$ (2), XX *Sox9*$^{flox/flox}$ (6) and XY *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{flox/flox}$ (2). One of the XX mutant mice was used for subsequent mating experiments and was found to produce two viable litters. This shows that XY mutant females are fertile, albeit at a reduced level.

XY Sf1:Cre$^{Tg/+}$*Sox9*$^{flox/flox}$ *females produce a reduced number of oocytes*

We subsequently investigated the causes for the poor fertility observed in XY mutant females. MVH (Mouse Vasa Homologous), a marker of XX and XY germ cells (Tanaka et al., 2000), confirmed the presence of germ cells in XY mutant gonads that were mainly localized around the cortical region of the gonads in comparison to XX controls at P0 and P7 but not at E17.5 (Fig. 5A). In addition, quantification of germ cells showed fewer of them in XY mutant gonads than in XX controls at P0 and P7 (Fig. 5B). In summary, although XY mutant gonads develop into ovaries, they, however, contain a lower number of germ cells than normal control ovaries.

To determine if XY mutant females are able to produce fertilized oocytes, ovarian stimulation was performed using three groups consisting of five XX *Sox9*$^{flox/flox}$ females, six XX mutant females and six XY mutant females each (Fig. 5D); all animals were seven weeks old. Although we found variations in ovarian size between different XY mutant females, the ovaries of these females were smaller than XX mutant ovaries (Fig. 5C). This is likely due to the reduced number of oocytes present in them (Fig. 5A). A total of 126 (from five XX *Sox9*$^{flox/flox}$ females), 160 (six XX mutant females) and 56 (six XY mutant females) oocytes or embryos were collected. On average nine oocytes or fertilized embryos were recovered per XY mutant female two days after the super-ovulation (Fig. 5D). In comparison, we recovered an average of 25 and 26 oocytes or embryos from XX *Sox9*$^{flox/flox}$ and XX mutant females respectively. We conclude that XY mutant females produce substantially fewer oocytes or embryos after super-ovulation than XX controls.

The development of embryos from XY Sf1:Cre$^{Tg/+}$; *Sox9*$^{flox/flox}$ *females is partially impaired*

Using B6.Y^{TIR} male-to-female sex reversal mice it was shown that XY oocytes do not complete Meiosis II resulting in infertility of these XY females (Obata et al., 2008). Consistently, 95% of *in vitro* cultured XY

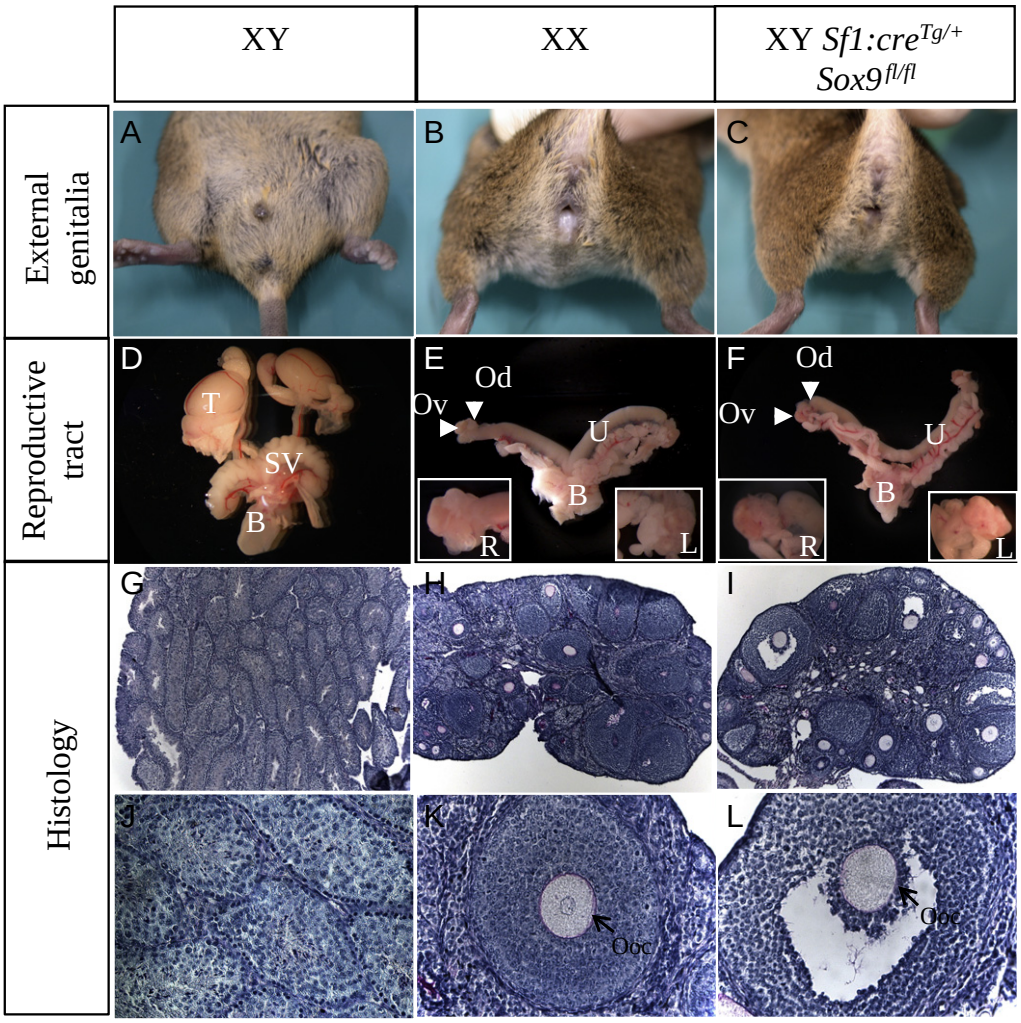


Fig. 4. Sex reversal in adult XY *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{flox/flox}$ transgenic mice.(A, B, C) External genitalia of 7-week-old control XY, XX mice and XY mutant transgenic mice respectively. (D, E, F) Reproductive tracts dissected from control XY, XX mice and XY mutant transgenic mice respectively. (F) showed the entire characteristics of XX females (E); vagina, uterus, oviducts and ovaries but no seminal vesicles, prostate, vas deferens, epididymis or testis as expected in male (D). T: testis, SV: seminal vesicle, B: bladder, Ov: ovary, Od: oviduct, U: uterus, R: right ovary, L: left ovary. (G–L) Haematoxylin and eosin histological analysis of gonadal sections. Low magnification X10 (G–I). High magnification ×100 (J–L). (G) shows seminiferous tubules of the XY testes and (J) the presence of spermatogenic cells. XX gonads (H) show all stages of folliculogenesis including oocytes (K) as observed in the XY mutant transgenic mice (I, M).

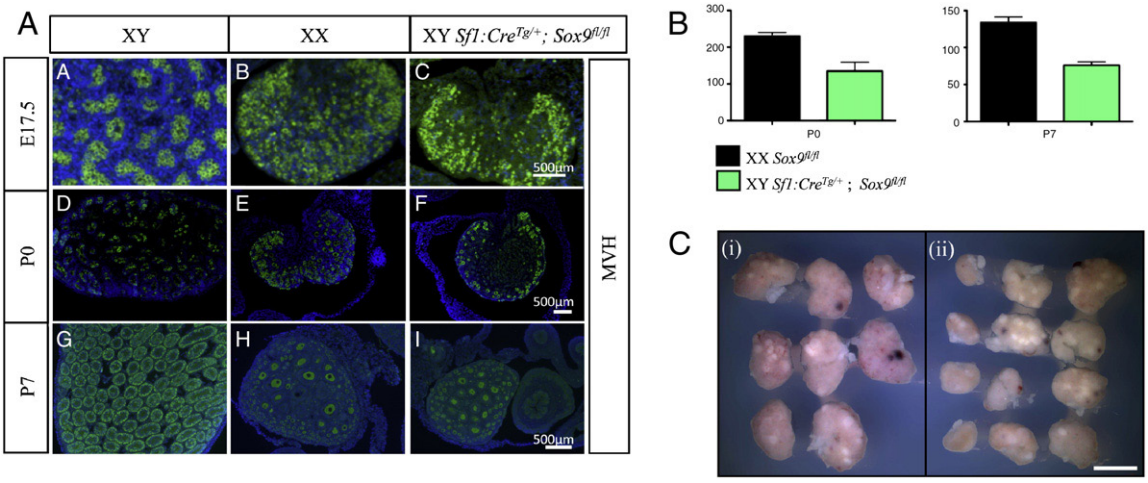
oocytes from this line did not reach the two-cell stage embryo (Villemure et al., 2007). Therefore we investigated oocyte development in XY mutant females. Oocytes/embryos from super-ovulated XX *Sox9*$^{flox/flox}$ and XY *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{flox/flox}$ mutant mice were collected at 1.5 day post-hCG injection (dpi). XY mutant oocytes showed more morphological abnormalities than XX *Sox9*$^{flox/flox}$ oocytes; we observed a high degree of vacuoles, granulation, fragmentation and abnormal division (Fig. 6D) as compared to XX *Sox9*$^{flox/flox}$ oocytes (Fig. 6A). To visualise microtubules and chromosomes oocytes were stained with α-Tubulin and DAPI (Figs. 6B and E). At Meiosis II normal barrel-shaped spindle formation around the second metaphase chromosomes was observed in XX *Sox9*$^{flox/flox}$ (Fig. 6B) and XY mutant oocytes as previously described in the B6.Y^{TIR} background (Villemure et al., 2007).

We subsequently investigated whether oocytes from XY mutant females were able to develop into two-cell stage embryos after fertilization. Out of 126 XX *Sox9*$^{flox/flox}$ and 160 XX mutant oocytes/embryos we analysed, 102 (81%) and 137 (86%) were scored as being at two-, to eight-cell stages, respectively (Fig. 5D). In contrast, 41 (73%) embryos from XY mutant had reached at least the two-cell stage. The embryos were cultured for another 24 h and out of 102 XX *Sox9*$^{flox/flox}$, 137 XX mutant and 41 XY mutant embryos recovered, 57 (56%), 73 (53%) and two (5%) reached the morula-, or blastocyst stage *in vitro*, respectively (Fig. 5D).

Finally, two-cell stage embryos from super-ovulated XY mutant females and XX *Sox9*$^{flox/flox}$ controls were collected at 1.5 dpi and cultured over night prior to immunostaining. While most XY mutant oocytes progressed through Meiosis II, abnormal two-cell stage division defects were prevalent. We also observed large nuclei, blastomeres with multiple nuclei, cleavage abnormalities and nuclear material which appeared to be unable to condense (Figs. 6F–I) as compared to XX *Sox9*$^{flox/flox}$ (Fig. 6C). Taken together, these results show that XY mutant ovaries contain fewer ovulated oocytes in comparison to XX controls and that a population of embryos from XY females cannot develop beyond the two-cell stage.

Discussion

The present study used the Cre/*loxP* recombination system to delete *Sox9* from embryonic XY gonads and we present several pieces of evidence at both the RNA and protein level at different developmental stages that the *Sox9* locus was indeed efficiently and specifically removed from the target tissue (see Fig. 1). In contrast to



D

XX *Sox9*$^{fl/fl}$				XX *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{fl/fl}$				XY *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{fl/fl}$			
Hours after oviduct flushing (Embryo stage)	24h (2.5dpc)	48h (3.5dpc)	72h (4.5dpc)	Hours after oviduct flushing (Embryo stage)	24h (2.5dpc)	48h (3.5dpc)	72h (4.5dpc)	Hours after oviduct flushing (Embryo stage)	24h (2.5dpc)	48h (3.5dpc)	72h (4.5dpc)
2-cell	24	8	2	2-cell	32	8	8	2-cell	32	22	9
4/8- cell	78	11	10	4/8- cell	105	25	2	4/8- cell	9	19	8
Morula	0	57	14	Morula	0	65	4	Morula	0	1	0
blastocyst	0	0	9	blastocyst	0	8	23	blastocyst	0	1	1
undivided	4	3	1	undivided	0	0	0	undivided	6	3	0
Abnormaly divided	16	32	47	Abnormaly divided	17	31	55	Abnormaly divided	6	5	9
lysed	4	15	43	lysed	6	23	68	lysed	3	5	30

Fig. 5. XY *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{flox/flox}$ *females are subfertile.*(A) Reduction and sequestering of XY mutant ovarian germ cell to the cortical region of the XY ovary. Immunofluorescence staining showed a reduction of MVH positive cells in the central region of XY mutant ovary compared to XX wild-type ovary at E17.5, P0 and P7 (A–I). (C) Reduction of the number of germ cells in the whole ovary of XY mutant (P0 and P7; n = 3) when compared to XX controls.(C) Age matched ovaries from XX mutant females and XY mutant females were examined after dissection. (D) Oocytes from age matched XX *Sox9*$^{flox/flox}$, XX mutant and XY mutant females were collected 2.5dpi and cultured *in vitro* for up to 72 h. A total of 126, 160 and 56 oocytes/embryos were collected from XX *Sox9*$^{flox/flox}$, XX mutant and *XY* mutant females, respectively. An average of 9, 25 and 26 oocytes was collected from XY mutant, XX *Sox9*$^{flox/flox}$ and XX mutant females.

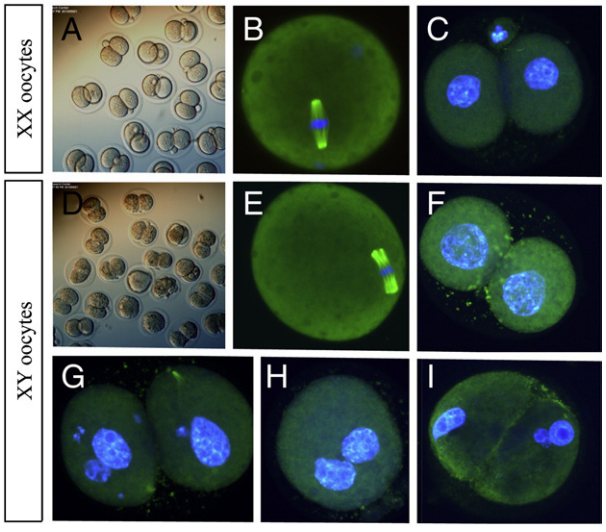


Fig. 6. *In vitro* monitoring of *XX Sox9*$^{flox/flox}$ and XY *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{flox/flox}$ oocytes by immunocytochemical staining. The morphology of fertilized XY mutant oocytes at 2.5 dpi showed the presence of a high level of vacuoles, granulation, fragmentation and abnormal division (D) compared to XX *Sox9*$^{flox/flox}$ oocytes (A). Oocytes prior to the two-cell stage development were isolated from XX controls and XY mutant females followed by immunocytochemical staining with tubulin and DAPI (B and E). At Meiosis II normal barrel-shaped spindle formation around the second metaphase chromosomes were observed in XX *Sox9*$^{flox/flox}$ (B) and XY mutant oocytes (E). While most XY mutant oocytes progressed through Meiosis II, abnormal two-cell stage division defects were more prevalent, large nuclei/inability of nuclear material to condense (F), multinuclear blastomeres (G), cleavage abnormalities (H and I).

previous studies (Barrionuevo et al., 2006; Chaboissier et al., 2004), the combination of the *Sf1:Cre* transgene (Bingham et al., 2006) with the conditional knockout allele of *Sox9* did not result in a lethal phenotype. In addition to the gonad, the *Sf1:Cre* transgene is also active in the anterior pituitary, spleen, and the adrenal gland (Bingham et al., 2006). *Sox9* expression has not been described in spleen and the adrenal gland (Wright et al., 1995) implying that it plays no role in these organs. Although *Sox9* is expressed in the pituitary (Fauquier et al., 2008), ablation of *Sox9* using the *Sf1:Cre* does not seem to affect this tissue in *XY* mutant mice. This enabled us to study the fate of the XY bipotential gonad in the absence of *Sox9* during adult development.

Early ablation of *Sox9* in XY gonads prevents testis cord formation and XY mutant gonads express genes that are up-regulated in XX wild-type gonads like *Wnt4* or *Fst*. It has been shown genetically that *Wnt4* is an antagonist of *Fgf9*, a gene involved in ovarian and testicular differentiation (Kim et al., 2006). *Wnt4* is up-regulated by the Rspo1/β-catenin signalling pathway in ovaries (Chassot et al., 2008) while *Fgf9* up-regulation requires *Sox9* expression in the XY gonads (Kim et al., 2006). In addition β-catenin and SRY/SOX9 are antagonists during sex determination (Chassot et al., 2008; Maatouk et al., 2008; Bernard et al., 2008) indicating that ablation of *Sox9* allows WNT/β-catenin signalling to promote ovarian formation. This is accompanied by a down-regulation of genes required for testis development.

In XY *Sox9* loss-of-function mutant gonads, Sertoli cell differentiation defects were revealed by reduced expression of Sertoli cell markers. Although *Sox9* expression is restricted to the Sertoli cell lineage, male steroidogenic cell markers were not expressed which

implies that Leydig cells fail to differentiate in XY mutant gonads. The DHH/PTCH1 signaling pathway was shown to be important for fetal Leydig cell development (Yao et al., 2002). Thus, lack of *Dhh* transcripts in XY mutant gonads is likely responsible for impaired Leydig cell differentiation. As a consequence, testicular hormones which are secreted by Leydig cells are absent and male reproductive organ development is not stimulated. Instead, the Mullerian ducts develop into female genitalia. This is promoted by the absence of the anti mullerian hormone normally expressed in Sertoli cells (Arango et al., 1999) in a manner dependent upon SOX9 (De Santa Barbara et al., 1998).

The most important outcome of the mRNA profiling experiment was the finding that XY mutant gonads at E13.5 show an intermediate transcript signature resembling both XY wild-type and XX wild-type gonads. Our data indicate that *Sox9* removal was likely complete in the tissues investigated; as a consequence this pattern possibly indicates that the male developmental pathway is not completely shut down at this stage (E13.5) while the female one is not completely active in the XY mutant. It is a non-trivial task to interpret the output of a GeneChip experiment which analyses complex tissue samples including distinct cell types since mRNA concentrations depend both on transcriptional events and fluctuating cell populations. It is, however, possible to discern overall patterns as we have recently demonstrated in a study of late onset infertility caused by Sertoli cell specific deletion of *Sox9* (Lardenois et al., 2010).

It is noteworthy that some transcripts are present in the XY mutant sample at concentrations very close to what is observed in XX wild-type (C1 and C3), while others are more similar to XY wild-type samples (C6 and C7). Specifically, XY mutant gonads show significantly weaker mRNA concentrations of Sertoli cell marker genes such as *Sox9* (Kent et al., 1996; Morais da Silva et al., 1996), *Dhh* (Bitgood et al., 1996), *Tmem184a*/*Sdmg1* (Best et al., 2008), *Amh* (Behringer et al., 1990), and *Sox8* (Sock et al., 2001). Moreover, weaker signals were found for genes involved in Leydig cell differentiation like *Ptch1* (Bitgood et al., 1996) or in Leydig cell steroidogenesis like *Cyp17a1*, Cyp11a1, *Hsd17b,* Hsd17b3 and *Hsd3b1* (Heikkila et al., 2005). In contrast, genes expressed in somatic ovarian cells showed stronger signals in XY mutant gonads and in the XX controls compared to XY controls like *Rspo1* (Parma et al., 2006), *Wnt4* (Vainio et al., 1999), *Fst* (Yao et al., 2004) and *Foxl2* (Pailhoux et al., 2002) (Table 1). This was also the case of genes involved in meiosis such as *Rec8, Spo11, Tex11, Msh4, Msh5* and *Stra8* (Baltus et al., 2006). Note that *Cyp26b1*, which has been implicated in the direct and indirect control of meiosis at different developmental stages displays a significantly weaker signal in the XY mutant sample as compared to the XY wild-type (Class 5) (Bowles et al., 2006; Kumar et al., 2011; MacLean et al., 2007). This is consistent with its role in maintaining male germ cells in an undifferentiated state (Li et al., 2009). Taken together, these data are consistent with the notion that germ cells in XY mutant gonads have initiated (the female form of) meiosis as part of their sex reversal phenotype.

Previous work showed that *Sox9* expression occurs transiently in steroidogenic cells of the ovaries (Notarnicola et al., 2006). However, depletion of *Sox9* in XX mutant ovaries has no dramatic effect on the fertility. While *Sox9* is essential for murine testis differentiation, we show here that it is dispensable for functional ovarian development. At E13.5, germ cells have entered meiosis in ovaries in contrast to prospermatogonia in foetal testis which are quiescent from E14.5 onwards (Western et al., 2008). Indeed meiotic mRNAs were found at higher concentrations in XY mutant gonads in comparison to XY $Sox9^{flox/flox}$ gonads indicating that XY germ cells had initiated meiosis. It is conceivable that the intermediate expression pattern observed for XY mutants is a reflection of inefficient female germ cell development. Indeed, at birth, the number of oocytes is lower in XY mutant ovaries in comparison to XX controls. This reduction in the number of oocytes has been described in ovaries in B6.Y^{TIR} sex-reversed mice (Alton et al., 2008) and in XO females (Burgoyne and Baker, 1981; Burgoyne and Baker, 1985). This occurs around birth when oocytes are at the pachytene stage. Both X chromosomes are active during prophase and this is maintained through processes involving homologous synapsis (Burgoyne et al., 2009). Lack of homologous synapsis due to a single X chromosome in XO or XY oocytes might promote silencing of that single X chromosome which might affect oocyte survival. It is conceivable that selfsynapsed X chromosomes escape silencing thereby enabling a sub-population of XO and XY oocytes to persist.

In females, oocytes promote follicle formation (Guigon and Magre, 2006). Oocytes can form independently of their sex chromosome constitution. Indeed, the Y chromosome has been visualized in oocytes of XXY females (Palmer and Burgoyne, 1991). When the capacity of XY oocytes to be fertilized was analysed using B6.Y^{TIR} mice it was found that XY oocytes were incompatible with normal fertilization and development: the oocytes eventually enter interphase but rarely reach the two-cell stage due to deficient Meiosis II spindle assembly (Obata et al., 2008; Villemure et al., 2007). This defect was overcome by transfer of the nucleus of XY oocytes into wild-type XX ooplasm suggesting that the XY ooplasm is incompetent for fertilization in B6.Y^{TIR} mice. The B6.Y^{DOM} sex-reversed mice (from the cities of Poschavinus, B6.Y^{POS} or Tirano B6.Y^{TIR}) were generated by introducing different variants of the Y chromosome of *Mus domesticus* into a *M. musculus* C57BL/6J background (Eicher and Washburn, 2001). The Y chromosome in our XY mutant females (C57BL/6J; 129SV mixed background) does not seem to have a similar effect on XY ooplasm because the dramatic loss of embryos in these females occurs at later stage than in B6.Y^{TIR} which might be a consequence of the different strain backgrounds used. Indeed 73% fertilized oocytes from XY mutant females reached at least two-cell stage, in comparison to 81% or 86% of the embryos coming from XX $Sox9^{flox/flox}$ and XX mutant females respectively. Thus most of the XY oocytes from XY mutant females are able to pass meiosis but fail to develop further than the first embryonic divisions.

Although defects in sex chromosomal segregation result in lethal aneuploidy in XY oocytes or XO oocytes, additional defects have been found. XO mice can be also poorly fertile because the two-cell stage embryos from XO oocytes cannot pass further divisions and become abnormal (Burgoyne and Biggers, 1976). This suggests that both X chromosomes are required for the first stages of embryonic development. Indeed, XY mutant females exhibit also a poor oocyte quality which precludes the first mitotic divisions. When comparing XXY and XYY females (Mahadevaiah et al., 1993) found that XXY females were more fertile than XYY suggesting that the Y chromosome has a negative effect on the capacity of XY oocytes to undergo embryonic development. Altogether this suggests that XY mutant female fertility is hampered because of X dosage deficiency and potentially by the presence of the Y chromosome. Interestingly, a recent study has shown that the X chromosome appeared to interfere with genome-wide expression by regulating hundreds of autosomal genes through epigenetic processes (Wijchers et al., 2010). Expression of maternal factors is required for early embryonic development but whether the expression of these genes is influenced by the sex chromosome remains to be elucidated (Zheng et al., 2010).

In spite of their infertility phenotype some XY mutant females are able produce offspring: two out of 10 females gave birth to a single pup and one female had two litters of four and two pups each. This is comparable to XY females since three out of twelve females produced at least one pup (Mahadevaiah et al., 1993). Some XY fertile females have also been found in mare (Kent et al., 1988) and while this phenomenon is rarely found in humans one case was recently reported: retarded sexual development of her daughter motivated a patient to undergo a genetic analysis which showed that the karyotype of the daughter was 46,XY (100% of the blood cells tested) and 46,XY (99.25%), 45,X (0.75%) in the gonads. The mother's karyotype was 46,XY (100% of the blood cells) and 46,XY (92.9%), 45,X (5.9%), 46,XX (0.6%), 47,XXY (0.6%) in gonadal cells suggesting

that this XY female represents an example for an individual who was able to procreate (Dumic et al., 2008).

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