Transient development of ovotestes in XX *Sox9* transgenic mice

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ABSTRACT

The sex of an individual results from the paternal transmission of the *SRY* gene located on the Y chromosome. In turn, SRY initiates *Sox9* expression, a transcription factor required for testicular differentiation. Ectopic activation of SOX9 in XX *Wt1:Sox9* transgenic mice induces female-to-male sex reversal in adult mice. Here we show that complete sex reversal is preceded by a transient phase of ovotestis differentiation with XX *Wt1: Sox9* transgenic gonads containing a testicular central region and one or both ovarian poles indicating that *Wt1:Sox9* is not as efficient as *Sry* to induce male development. In XX *Wt1:Sox9^{Tg/+}* gonads, transgenic *Sox9* is expressed earlier than *Sox9* in XY gonads and is able to induce the expression of EGFP, knocked into the 3′ UTR of *Sox9* indicating that SOX9 is involved in the initiation and maintenance of its own expression. However, the delayed onset of expression of endogenous Sox9–EGFP suggests that this activation requires other factors, whose expression depends on SOX9. In the testicular regions of the XX *Wt1:Sox9* ovotestes, proliferation of the XX fetal germ cells is hampered and they differentiate as pro-spermatogonia. This indicates that XX germ cells are not competent to respond to proliferative signals released from a testicular environment. In the ovarian regions, despite the continuous mRNA expression of the *WT1:Sox9* transgene, the SOX9 protein does not accumulate suggesting that regulation of this gene in ovarian cells involves post-transcriptional mechanisms. Finally, ovarian cells of the XX *Wt1:Sox9* ovotestis undergo apoptosis during late embryogenesis leading to complete female-to-male sex reversal of the transgenic mice at birth.

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Introduction

In human and mice, paternal transmission of the Y chromosome initiates sex determination. This is due to the expression of the *SRY/Sry* gene located on this chromosome and whose expression promotes testis differentiation of the bipotential gonad (Gubbay et al., 1990; Sinclair et al., 1990). In the absence of *SRY*, the XX bipotential gonad develops as an ovary. At a molecular level, this requires the activation of the Wnt/β-catenin signaling pathway by the secreted protein RSPO1 (Chassot et al., 2008; Parma et al., 2006). These first steps trigger complex molecular pathways leading to sexual differentiation (for review, see Sekido and Lovell-Badge, 2009).

In mouse gonads, SRY specifies the fate of the gonad in synergy with SF1. Whereas *Sf1* is expressed in the XX and XY bipotential gonad, the expression of *Sry* is initiated in the precursor cells of the supporting cell lineage in the center of the XY gonad (Bullejos and

Koopman, 2001; Sekido et al., 2004; Wilhelm et al., 2005). Within the next few hours, *Sry* expression extends to the anterior and then the posterior poles. Although its expression commences at E10.5, SRY becomes only functional between E11.0–11.25 and expression ceases at E12.5 (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001; Hiramatsu et al., 2009). During this short period, SF1/SRY upregulates the *Sox9* gene by binding to TESCO (testis specific enhancer core) upstream of the *Sox9* promoter (Sekido and Lovell-Badge, 2008).

In human, *SOX9* mutations are responsible for the congenital disorder campomelic dysplasia, a syndrome characterized by skeletal malformations, absence of olfactory bulbs and tracts, heart and kidney disorders and mental retardation. Up to two-thirds of XY patients exhibit a range of genital defects and often complete sex reversal (Foster et al., 1994). Moreover, female-to-male sex reversal has been found in an XX patient with a duplication of *SOX9* (Huang et al., 1999). Mouse models have shown that *Sox9* is required and sufficient to induce testis development (Barrionuevo et al., 2006; Chaboissier et al., 2004; Vidal et al., 2001). SOX9 induces Sertoli cell differentiation, the supporting cell lineage of spermatogenesis. At a molecular level, expression of *Sox9* triggers upregulation of a variety of genes involved in male sexual development, including the direct targets *Amh* (Arango et al., 1999;

De Santa Barbara et al., 1998) and *Pgds* (Wilhelm et al., 2007) as well as genes that are known to be critical for male development such as *Fgf9* (Kim et al., 2006).

From E12.5, the testicular environment influences germ cells to become gonocytes (Best et al., 2008; McLaren and Southee, 1997). In XY gonads, proliferation of primordial germ cells is followed by their blockage in G0/G1 phase at E14.5 (Western et al., 2008) and these gonocytes will remain quiescent until after birth when spermatogenesis commences. In XX gonads, the germ cells enter meiosis from E13.5 and become blocked in prophase I around birth. Follicular cells then organize around the oocyte, a process driven by the expression of the transcription factor *Foxl2* (Schmidt et al., 2004; Uda et al., 2004).

Thus, sex determination results from the initial switch of either the testis differentiating pathway or the ovarian determining molecular cascade in an exclusive manner (Kim et al., 2006). However, both pathways can be activated in the same gonad resulting in the development of ovotestes. The vast majority of these organs exhibit a central testicular region flanked by one or two polar ovarian tissues (Eicher and Washburn, 2001). Ovotestis differentiation results from defects in XY gonad differentiation. This can be caused by a low or delayed expression of the *Sry* gene in XY gonads (Buaas et al., 2009; Bullejos and Koopman, 2005). XY ovotestes have been also described in conditional *Fgfr2* mutants (Bagheri-Fam et al., 2008; Kim et al., 2007). In this case, it has been suggested that the poles of the XY gonads might be more sensitive to loss of a paracrine signal. FGFR2 is a receptor of FGF9 and recent data show the requirement of FGF9 to mediate the formation of sex cords from the center to the poles of the XY gonads (Hiramatsu et al., 2010).

We have previously generated the transgenic mouse line *Wt1: Sox9*$^{Tg/+}$, in which ectopic expression of *Sox9* results in the development of XX *Wt1:Sox9*$^{Tg/+}$ sterile adult males (Vidal et al., 2001). In this study, we have performed a detailed phenotypic and molecular analysis of this transgenic line. We show that in XX gonads, transgenic *Sox9* is expressed earlier than endogenous *Sox9* in XY wild-type testis and transgenic *Sox9* can activate endogenous *Sox9*. XX *Wt1: Sox9*$^{Tg/+}$ gonads differentiate as ovotestis with a central testicular region. Strikingly, XX germ cells in the testis parts do not proliferate but differentiate like XY gonocytes. This indicates that proliferation and sexual differentiation of XX fetal germ cells are independent, with the proliferation depending on their sex chromosome constitution and differentiation being controlled by the somatic environment. During late embryogenesis, the ovarian regions of XX *Wt1:Sox9*$^{Tg/+}$ gonads undergo apoptosis thus leading to a complete sex reversal in transgenic adults.

Materials and methods

Genotyping of embryos and mice

The experiments herein 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 number of tail somite (Ts) was determined by counting them from the end of the tail until the middle of hind limb. This corresponds to 2 Ts more than when counting them until the posterior to the hind limb. The presence of the Y chromosome was determined using primers Zfy5′ (5′-GACTAGACATGTCTTAA-CATCTGTCC-3′) and Zfy3′ (5′-CCTATTGCATGGACTGCAGCTTATG-3′). *Wt1:Sox9* transgene, *Sox9–EGFP* knock in and *Foxl2*^{lacZ} were identified as published previously (Vidal et al., 2001; Nel-Themaat et al., 2009; and Schmidt et al., 2004, respectively). *Axin2*$^{+/LacZ}$ transgenic mice were generated by Lustig et al. (2002) and genotyped using forward *Axin2* primer 5′-AAGCTGCGTCGGATACTTGAGA-3′, reverse *Axin2* primer 5′-AGTCCATCTTCATTCCGCCTAGC-3′ and reverse *LacZ* primer 5′-TGGTAATGCTGCAGTGGCTTG-3′ (sequences kindly provided by Michel V. Hadjihannas).

Histological and immunological analyses

Embryonic samples from timed matings (day of vaginal plug E0.5) were collected, fixed in 4% paraformaldehyde overnight at 4°C, followed by paraffin embedding. Microtome sections of 5-μm thickness were stained with HE according to standard procedures. Immunofluorescence analysis was performed after antigene retrieval in 10 mM Na citrate (pH 6) for 2 min in a pressure cooker. Sections were incubated for 60 min in blocking solution (3% BSA, 10% donkey serum, 0.1% Triton) at room temperature. Blocking solution was replaced by a solution of primary antibodies prepared in 3% BSA, 3% donkey serum, 0.1% Triton at the following concentrations: SOX9 (1:1000) (Stolt et al., 2003, 2005), AMH (C-20: sc-6886, Santa-Cruz, 1:100), DDX4/MVH (ab13840, Abcam, 1:200), FGF9 (PA0152, Cell Sciences, 1:400), FOXL2 (ab5096, Abcam, 1:100). Relevant Cy3- or FITC-conjugated anti-rabbit or anti-goat antibodies (1:150, Jackson Laboratories) were used for detection of primary antibodies. Slides were mounted using vectashield with a Leica microscope DMLB, and pictures were taken with a spot RT-slider camera (Diagnostic instruments) and processed with Adobe Photoshop.

RT-PCR using the LightCycler™ system and TaqMan probe system

Embryonic gonads were collected at E12.5, immediately snap frozen in dry ice and stored at −80°C. RNA was extracted using the RNeasy mini kit (cat. No. 7404, QIAGEN GmbH, D-40725 Hilden, Germany) according to the manufacturer's instructions. cDNA synthesis was performed as previously described (Chassot et al., 2008). Primers and probes were designed by the Roche Assay design center (https://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp): *Sox9* (NM_011448.4) 5′-cagcaagactctgggcaag-3′ and 5′-tccacgaagggtctcttctc-3′ (probe 66), *Fgf9* (NM_013518.3) 5′- tgcaggactggatttcatttag-3′ and 5′-ccaggcc-cactgctatactg-3′ (probe 60), *Pdgs* (NM_008963.1) 5′-ggctcctggacacta-caccta-3′ and 5′-atagttggcctccaccactg-3′ (probe 89). Real-time PCR assays were performed on three embryos ($n=6$ gonads) of each genotype and compared to standards. *Hprt1* (NM_013556.2) 5′-tcctcctcagaccgcttttt-3′ and 5′-cctggttcatcatcgctaatc-3′ (probe 95) primer and probe set were used to normalize the quantitative real-time PCR assays for *Sox9*, *Fgf9* and *Pdgs*. Statistical analysis was performed using the Prism Graphpad software.

In situ hybridization

In situ hybridizations on paraformaldehyde-fixed/paraffin-embedded sections or on whole-mount embryos were carried out essentially as described in Chassot et al. (2008) and Parma et al. (2006). *Sox9* riboprobes were synthesized according to Morais da Silva et al. (1996). The plasmids used to synthesize *Rspo1*, *Nanos2*, *Cyp26b1* and *Stra8* digoxigenin riboprobes were provided by Parma et al. (2006); Chassot et al. (unpublished data), and Peter Koopman and David Page, respectively.

Whole-mount X-Gal staining and fluorescent imaging

X-Gal staining was performed according to Moore et al. (1998). SOX9–GFP gonads were dissected and fluorescent signal were analyzed directly with a Leica microscope MZFLIII.

Counting of germ cells and proliferation

Urogenital organs were dissected, fixed in 4% paraformaldehyde overnight and processed to obtain 5-mm paraffin sections as described above. For each genotype, 4 sections of at least 3 embryos (6 gonads per genotype) were processed for immunohistological experiments. DDX4/MVH antibody was used to identify germ cells and AMH antibody to determine testis versus ovarian parts. Total germ cells proliferating (both BrdU- and MVH-positive cells) were quantified within a predefined

square area using AxioVision digital image processing software. This square area was the same to count germ cells either in ovarian and testicular regions of the XX transgenic ovotestes and also in XY or XX wild-type gonads. Then for each genotype, the mean and standard deviation of these percentages were calculated and reported on a graph after statistical analysis. The results were analyzed using Sigma plot and Graphpad for statistical relevance.

Germ cell proliferation analysis was performed on the same embryos using the 5-bromo-2′-deoxy-uridine (BrdU) labeling and detection kit (Roche). This entailed injecting BrdU into pregnant females 4 h before collecting the fetal gonads. Total germ cells and proliferating germ cells (both BrdU- and MVH-positive cells) per predefined square area were counted on testis, ovarian parts, and controls using a Zeiss microscope Imager.Z1. For all gonads, the percentage of BrdU-positive germ cells versus total germ cells was determined. The results were analyzed using Sigma plot and Graphpad for statistical relevance.

Apoptosis analysis was performed with the *In Situ* Cell Death Detection kit, TMR red (cat. 11 684 795 910, Roche). Apoptotic (TUNEL-positive cells) were quantified on the entire section (4 sections of 6 gonads per genotype). Statistical analyses were performed as described above.

Results

Ectopic SOX9 in Wt1:Sox9^{Tg/+} XX gonads is functional at early stages of gonadal development

Wt1 is required for gonad formation (Kreidberg et al., 1993) and is expressed in all somatic cells of the bipotential gonad from the beginning of its formation at E9.5 (Pelletier et al., 1991). The *Wt1:Sox9* transgene places *Sox9* expression under the control of *Wt1* regulatory regions suggesting that *Sox9* expression might start earlier in *XX Wt1: Sox9*^{Tg/+} gonads than in XY wild-type gonads. At E11 (ts14–16), low levels of *Sox9* expression were detected in wild-type XX and XY gonads (Fig. 1Aa, b). In contrast, a robust staining was present in the XX *Wt1:Sox9*^{Tg/+} gonads indicating premature upregulation of *Sox9* (Fig. 1Ac). At E11.5, whereas *Sox9* mRNA was mostly present in the central region of XY gonads, transgenic *Sox9* expression could be detected throughout the XX *Wt1:Sox9*^{Tg/+} gonads (Fig. 1Ad–f). This indicates that the central to poles gradient of *Sox9* expression in the XY gonad does not occur in XX transgenic gonads. This is likely caused by the different regulatory elements controlling *Sox9* expression in both cases, i.e., *Sox9* elements in XY gonads and *Wt1* elements in XX *Wt1:Sox9*^{Tg/+} gonads. At E11.5, immunostaining showed SOX9
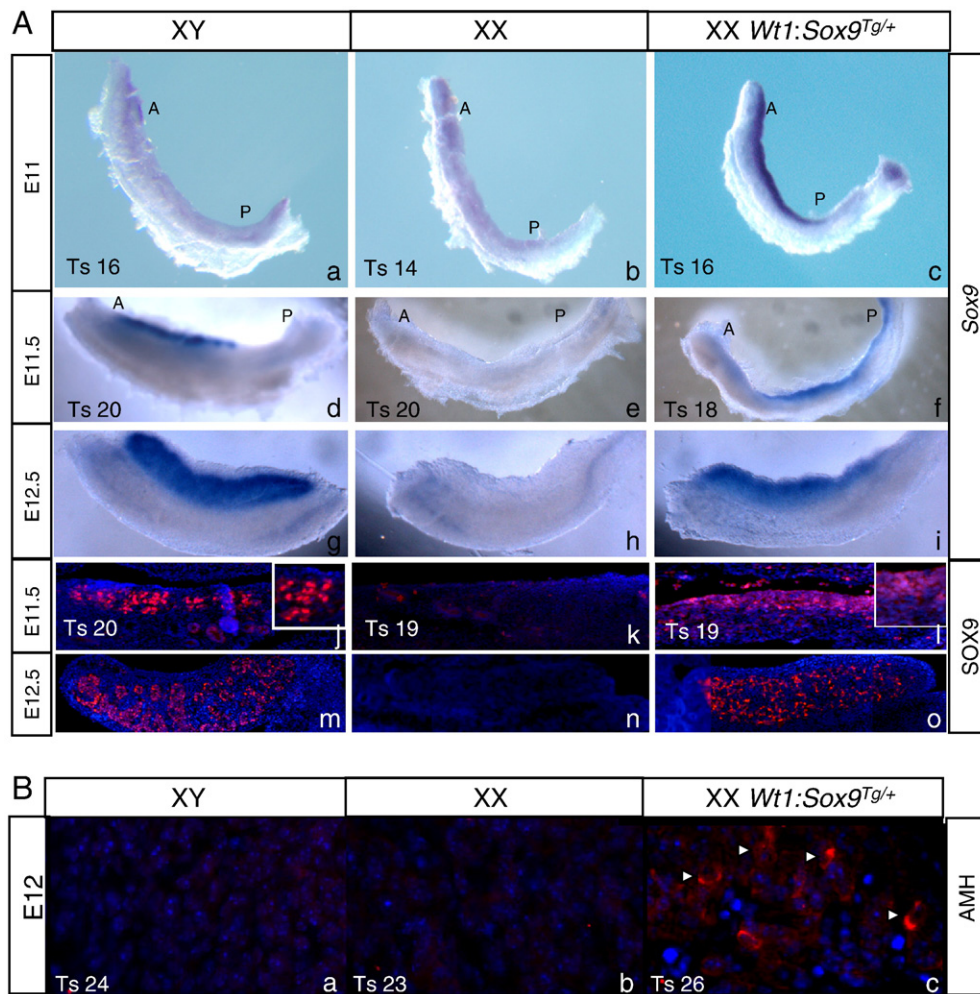

Fig. 1. (A) Early expression of *Sox9*/SOX9 in XX *Wt1:Sox9*^{Tg/+} gonads. Whole-mount *in situ* hybridizations using a *Sox9* antisense probe at E11 (a–c)–E11.5 (d–f) and E12.5 (g–i). At E11, *Sox9* is expressed at a low level in XY (a) and XX (b) control gonads and mesonephros and is upregulated in XX *Wt1:Sox9*^{Tg/+} gonads and mesonephros (c). At E11.5, *Sox9* expression is detected in the central and anterior regions of XY gonads (d) and is expressed in the entire XX *Wt1:Sox9*^{Tg/+} gonads (f). At E12.5, *Sox9* expression is similar in XY (g) and XX *Wt1:Sox9*^{Tg/+} gonads (i). No expression is observed in XX gonads (e, h). Ts: tail somites. A: anterior pole, P: posterior pole. Immunodetection of SOX9 in XY, XX and XX *Wt1:Sox9*^{Tg/+} gonads at E11.5 (j–l) and E12.5 (m–o). At E11.5, some cells show nuclear SOX9 in XY gonads (j and inset), whereas the signal seems predominantly cytoplasmic in XX *Wt1:Sox9*^{Tg/+} gonads (l and inset). At E12.5, SOX9 is expressed in XY (m) and XX *Wt1:Sox9*^{Tg/+} gonads (o). SOX9 is not synthetized in XX gonads (k, n). DAPI (blue) was used to detect nuclei. Posterior poles are on the right side. (B) Early function of *Wt1:Sox9* transgene. Immunostainings against AMH, a direct target of SOX9. At E12, XY (a) and XX (b) controls are negative for AMH (red), while XX *Wt1:Sox9*^{Tg/+} gonads show AMH-positive cells (c and white arrowheads) (magnification ×40). DAPI (blue) was used to detect nuclei.
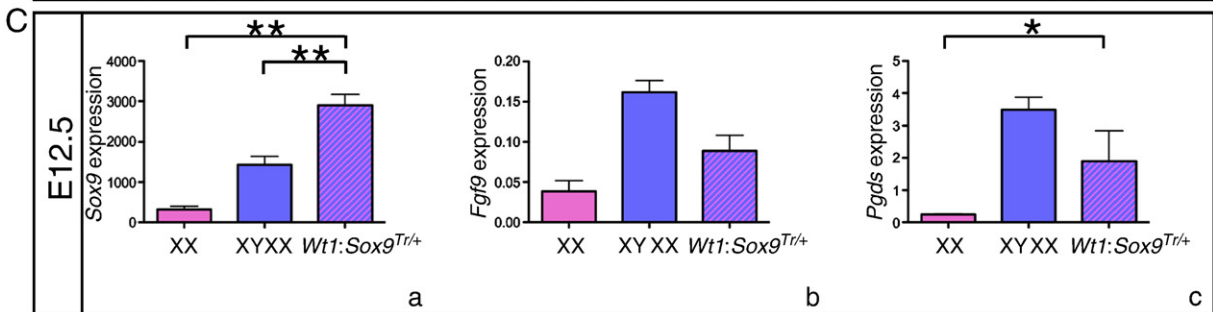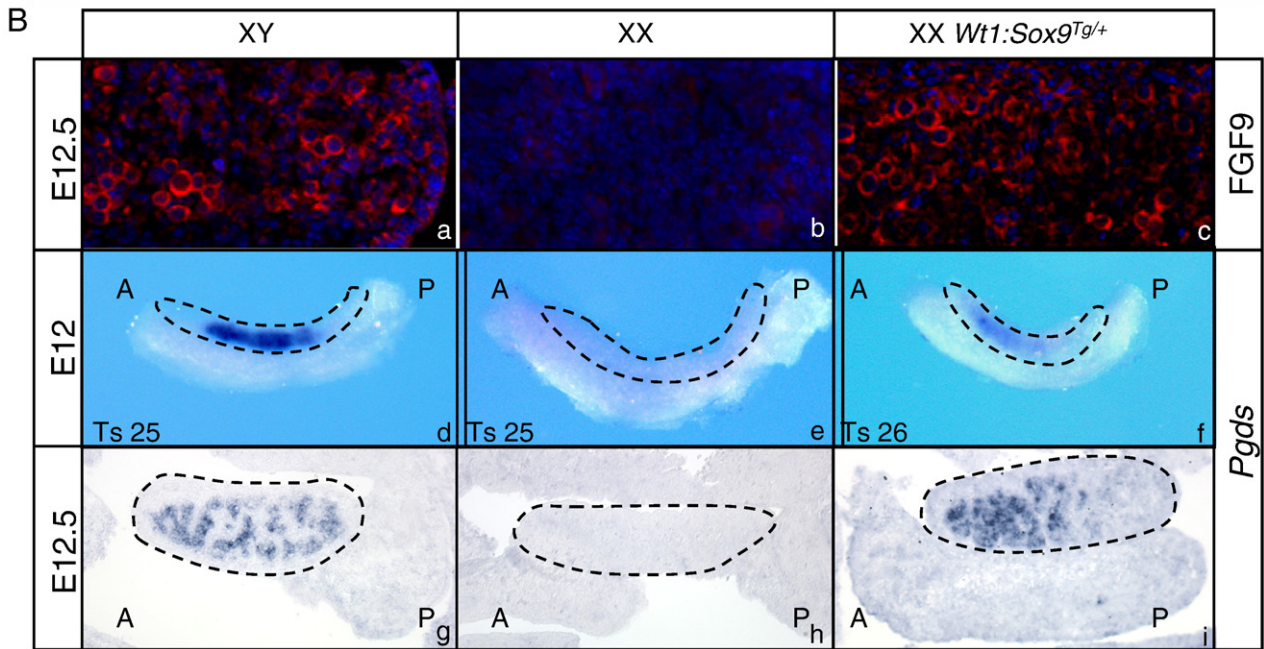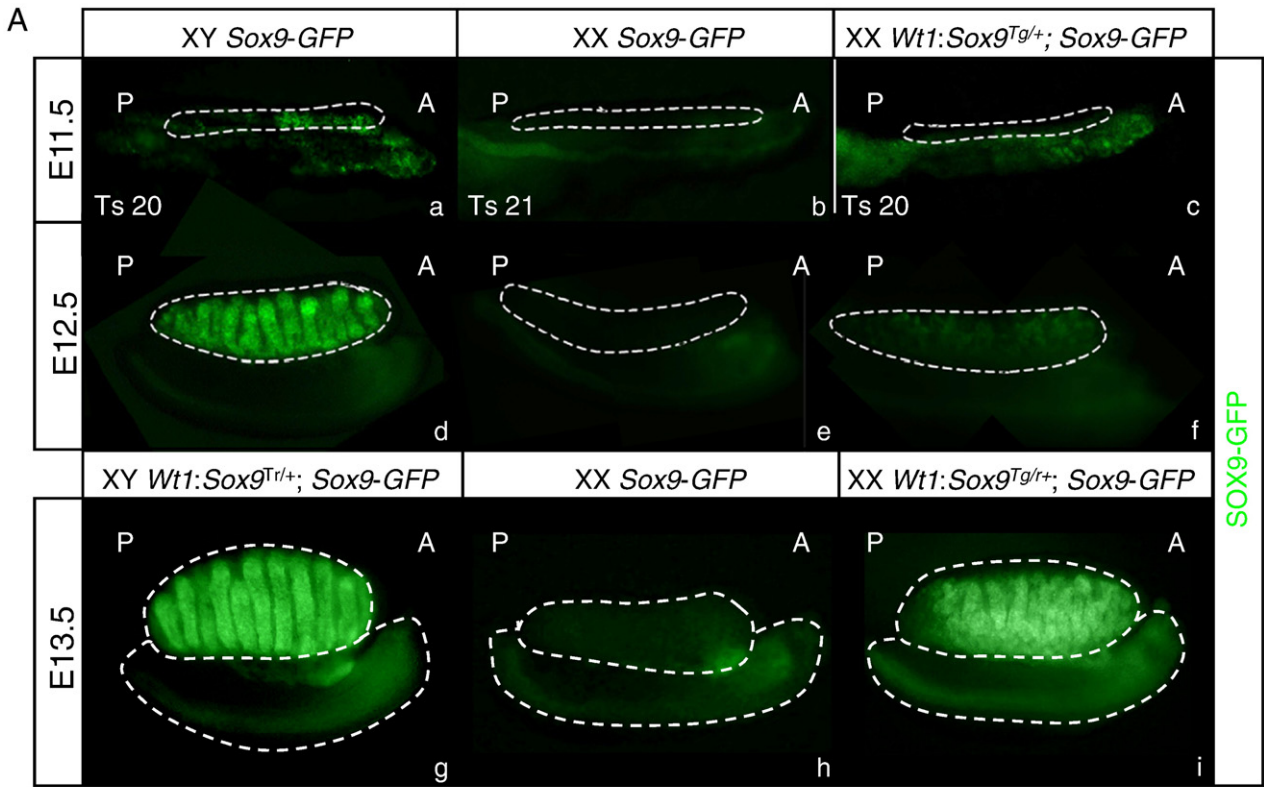
localization in the nucleus of cells of the XY gonads (Fig. 1Aj–l). In XX $Wt1:Sox9^{Tg/+}$ gonads, SOX9 protein was mostly detected in the cytoplasm. By E12.5, the pattern of $Sox9$ expression was similar in XX $Wt1:Sox9^{Tg/+}$ gonads and XY controls (Fig. 1Ag–i). QPCR experiments indicated that the level of $Sox9$ expression was higher in XX transgenic gonads in comparison to XY controls (Fig. 2Ca). At this stage, nuclear SOX9 was robustly detected in both XX $Wt1:Sox9^{Tg/+}$ gonads and XY gonads (Fig. 1Am–o).

Since we detected strong $Sox9$ expression in XX $Wt1:Sox9^{Tg/+}$ gonads at an earlier stage than in XY gonads, we next asked whether transgenic $Sox9$ in XX gonads is functional at early stages of gonadal development. To test this, we studied the expression of AMH, a direct target of SOX9 (De Santa Barbara et al., 1998). Immunostaining experiments showed that AMH was synthesized in XX $Wt1:Sox9^{Tg/+}$ gonads at E12 (ts23–26) (Fig. 1B). At ts22–24, AMH was not detected in XY littermate gonads. These data show that transgenic SOX9 is able to activate AMH in XX gonads from E12.

$Wt1:Sox9^{Tg/+}$ activates endogenous $Sox9$ in XX gonads

SF1 and SRY synergistic binding to TESCO is sufficient for the gonadal expression of SOX9 (Sekido and Lovell-Badge, 2008). Once SOX9 is activated, maintenance of its expression is critical to promote testis differentiation (Kim et al., 2006). However, at E12.5, SRY expression ceases and in vitro experiments suggest that SOX9 could maintain its own expression in vivo (Sekido and Lovell-Badge, 2008). This can be tested in $Wt1:Sox9^{Tg/+}$ animals by studying whether transgenic SOX9 can activate the endogenous $Sox9$ gene. For this, we employed the $Sox9$–$EGFP$ knock-in mouse line (Nel-Themaat et al., 2009). Previous experiments showed that SOX9 and EGFP co-localize indicating that EGFP follows the expression of SOX9 (Nel-Themaat et al., 2009).

At E11.5, XY $Sox9$–$EGFP$ testis exhibited green fluorescence and no EGFP signal was visualized in XX $Wt1:Sox9^{Tg/+}$; $Sox9$–$EGFP$ and XX $Sox9$–$EGFP$ gonads (Fig. 2Aa–c). At E12.5, green fluorescence was observed in XX $Wt1:Sox9^{Tg/+}$; $Sox9$–$EGFP$ gonads, but the intensity of the fluorescence was lower than in XY $Sox9$–$EGFP$ testis (Fig. 2Ad–f). By E13.5, green fluorescing sex cords were observed in both XY $Wt1:Sox9^{Tg/+}$; $Sox9$–$EGFP$ and XX $Wt1:Sox9^{Tg/+}$; $Sox9$–$EGFP$, but not in XX $Sox9$–$EGFP$ gonads (Fig. 2Ag–i). Taken together, these results show that SOX9 can initiate and maintain its own expression, but activation of $Sox9$–$EGFP$ is less efficient and delayed when compared to XY control gonads. At E12.5, this delay of endogenous $Sox9$ activation is not due to a lack of SOX9 expression, since overall expression of SOX9 (transgenic and endogenous) was comparable in XX $Wt1:Sox9^{Tg/+}$ and XY wild-type gonads at E12.5 (Fig. 1Am–o).

Whereas initially $Sox9$ expression requires SRY for its activation in XY gonads, the two independent downstream signaling pathways FGF9/FGFR2 and PGD2/PGDS are involved in its maintenance (Kim et al., 2006; Moniot et al., 2009). To test whether these pathways were upregulated in XX $Wt1:Sox9^{Tg/+}$ gonads, we performed QPCR and in situ hybridization experiments with a probe for $Pgds$. At E12, $Pgds$ was expressed in XX $Wt1:Sox9^{Tg/+}$ gonads. However, the signal appeared stronger in XY controls at this stage (Fig. 2Bd–f). By E12.5, QPCR analysis showed no significant difference in $Pdgs$ expression between XY gonads and XX $Wt1:Sox9^{Tg/+}$ gonads (Fig. 2Cc). In addition, the level of $Pgds$ hybridization signal was shown to be similar on XY and

XX $Wt1:Sox9^{Tg/+}$ gonad sections (Fig. 2Bg–i); however, this signal was restricted to the central region of the XX $Wt1:Sox9^{Tg/+}$ gonads. At E12.5, FGF9 was also expressed at an equivalent level in XY and XX $Wt1:Sox9^{Tg/+}$ sex cords (Fig. 2Ba–c and Cb). These results show that transgenic SOX9 is able to induce upregulation of $Pgds$ and FGF9. Thus, these two pathways could be involved in the establishment and maintenance of robust expression of SOX9–EGFP.

Ovotestes formation in XX $Wt1:Sox9^{Tg/+}$ embryos

While performing the above experiments, it was observed that the XX $Wt1:Sox9^{Tg/+}$ gonads exhibit a polar region negative for $Pgds$ (Fig. 2Bi) or for GFP in XX $Wt1:Sox9^{Tg/+}$; $Sox9$–$EGFP$ (Fig. 2Ai). To better visualize the cellular organization of the XX $Wt1:Sox9^{Tg/+}$ gonads, we performed histological analyses at E14.5 and E15.5 (data not shown and Fig. 3A). Similar to XY wild-type gonads, XX $Wt1:Sox9^{Tg/+}$ gonads contained testicular cords and gonocytes arrested in G0/G1 located within these cords (Fig. 3Aa, d and c, f). However, a region located at the poles of transgenic gonads did not contain testicular cords, but instead meiotic germ cells reminiscent of XX control ovaries (Fig. 3Ab, e and c, g). In most of the histological sections, some condensed nuclei reminiscent of apoptotic figures were also present in ovarian regions of the XX transgenic gonads (Fig. 3Ag) (see further).

To determine the identity of this polar region, we studied the expression of two genes specifically upregulated in early ovarian differentiation, $Foxl2$ and $Rspo1$ (Parma et al., 2006; Schmidt et al., 2004). To detect $Foxl2$ expression, we made use of a previously generated $Foxl2:LacZ$ knock-in allele and control ovaries showed strong staining throughout the gonad. β-Galactosidase staining in XX $Wt1:Sox9^{Tg/+}$; $Foxl2^{+/LacZ}$ embryos at E13.5 and E15.5 showed that while the central region was devoid of staining, one or both poles of the gonads expressed $Foxl2^{+/LacZ}$ (Fig. 3Ba–f). Similarly, $Rspo1$ in situ hybridizations on E13.5 sections of XY, XX and XX $Wt1:Sox9^{Tg/+}$ gonads showed that $Rspo1$ was expressed not only in XX controls but also at the poles of the XX $Wt1:Sox9^{Tg/+}$ gonads (Fig. 3Bg–i). Taken together these results suggest that ectopic expression of $Sox9$ can lead to the formation of an ovotestis in XX gonads during the fetal period.

Post-transcriptional regulation of $Sox9$ in ovarian cells of the ovotestis

To study whether FOXL2 and SOX9 are expressed in a mutually exclusive manner, immunostainings were performed using XY, XX controls and XX $Wt1:Sox9^{Tg/+}$ ovotestes. At E14.5 FOXL2 was expressed in the ovarian regions of the XX $Wt1:Sox9^{Tg/+}$ gonads and in the XX controls, whereas SOX9 was detected in the testicular regions of the XY and $Wt1:Sox9^{Tg/+}$ gonads (Fig. 4Aa–c). However, closer examination and longer immunostaining showed faint staining of nuclear SOX9 also in ovarian regions of XX $Wt1:Sox9^{Tg/+}$ gonads (Fig. 4Ba–d).

A potential reduction of $Sox9$ expression in the ovarian regions seems surprising given that the $Wt1$ regulatory region is expected to drive expression in both XY and XX gonads. We therefore studied whether $Sox9$ is transcribed in the whole gonads of XX $Wt1:Sox9^{Tg/+}$ ovotestes at E13.5. Whole-mount in situ hybridizations using a $Sox9$ probe confirmed that $Sox9$ is expressed along the entire length of the gonad in XX $Wt1:Sox9^{Tg/+}$ fetuses, as previously shown (Fig. 4Be–g–1A).

Fig. 2. (A) Regulation of SOX9–EGFP by $Wt1:Sox9$ transgene. The green fluorescence of Sertoli cells is synthetized from SOX9–EGFP (a to i). At E11.5, green fluorescent cells are not detected in XX $Wt1:Sox9^{Tg/+}$ gonads (c) compared to XY gonads (a) (magnification ×5). At E12.5, SOX9–EGFP is weakly expressed in XX $Wt1:Sox9^{Tg/+}$ (f) in comparison to XY $Sox9$–$EGFP$ gonads (f) (magnification ×5) and is expressed similarly in XY $Wt1:Sox9^{Tg/+}$; $Sox9$–$EGFP$ (g) and XX $Wt1:Sox9^{Tg/+}$; $Sox9$–$EGFP$ (i) gonads at E13.5, indicating that the expression of SOX9–EGFP is delayed in XX $Wt1:Sox9^{Tg/+}$; $Sox9$–$EGFP$ gonads compared to XY $Sox9$–$EGFP$ gonads at E12.5. SOX9–GFP is not detected in XX $Sox9$–$EGFP$ (b, e, h) gonads. P: posterior pole, A: anterior pole. (B) FGF9 and $Pgds$ are upregulated in XX $Wt1:Sox9^{Tg/+}$ gonads. Upper panel: immunodetection of FGF9 in gonads at E12.5. FGF9 is robustly expressed in XY (a) and XX $Wt1:Sox9^{Tg/+}$ (c) gonads (magnification ×40) but no in XX gonads (b). DAPI (blue) was used to detect nuclei. Middle and lower panels: whole-mount and in situ hybridization on sections using a $Pgds$ antisense probe. At E12 $Pgds$ is expressed at a low level in XX $Wt1:Sox9^{Tg/+}$ gonads (f) in comparison to XY gonads (d). At E12.5, $Pgds$ is expressed similarly in XY (g) and XX $Wt1:Sox9^{Tg/+}$ gonads but $Pgds$ expression is restricted to the anterior and central parts of the $Wt1:Sox9^{Tg/+}$ gonad. No expression was detected in XX gonads (e, h). P: posterior pole, A: anterior pole. Ts: tail somites. (C) Quantitative RT-PCR analysis of $Sox9$, $Fgf9$ and $Pgds$. For each genotype, $n = 6$ gonads were used.
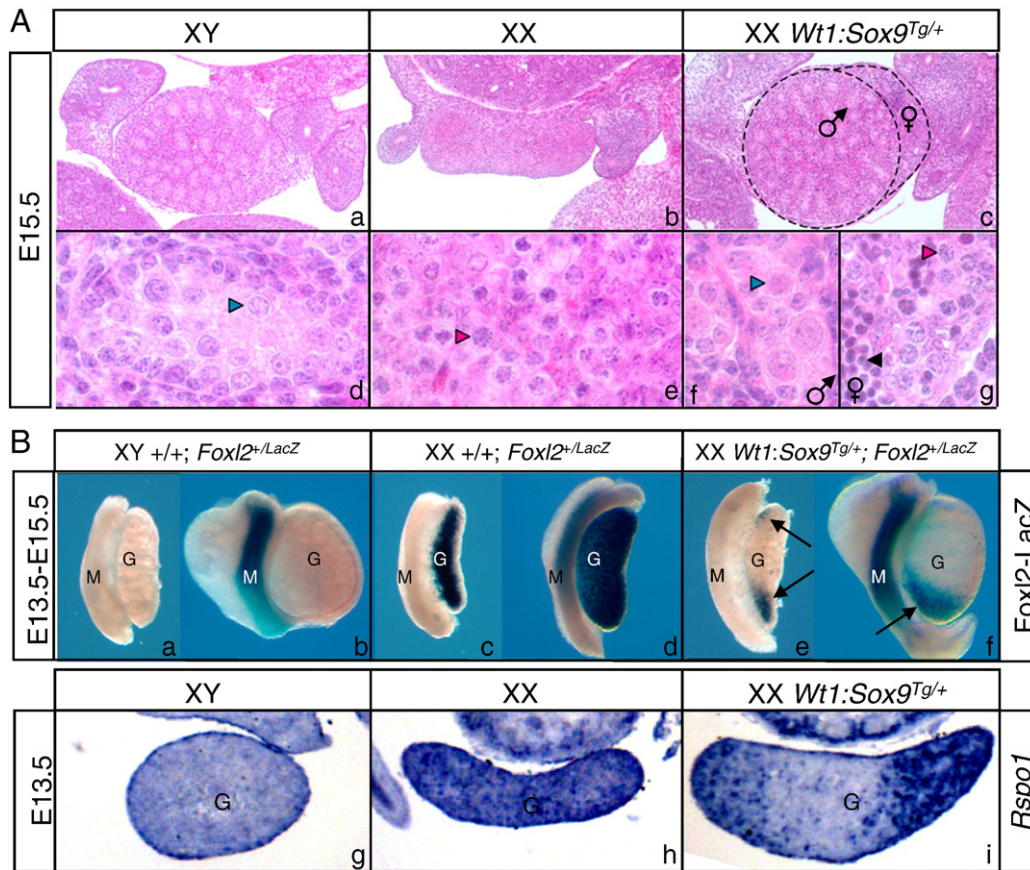
Fig. 3. Ovotestis development in XX *Wt1:Sox9*^{Tg/+} gonads. (A) Hematoxylin and eosin stainings of E15.5 embryos gonadal sections (magnification ×10 and ×100). In XX *Wt1:Sox9*^{Tg/+} gonads (c, f, g), the anterior region contains testis cords with G0/G1 quiescent gonocytes (c, f) (blue arrowheads) like in XY gonads (a, d). The posterior region of XX *Wt1:Sox9*^{Tg/+} gonads contains meiotic germ cells (c, g) like XX gonads (b, e) (pink arrowheads) and apoptotic cells (g) (black arrowhead). Male and female symbols in f and g indicate the testicular or ovarian tissues of the transgenic gonads. (B) Poles of XX *Wt1:Sox9*^{Tg/+} gonads develop as ovarian tissue. Upper panel: X-Gal whole-mount staining at E13.5 (a, c, e) and E15.5 (b, d, f). Strong blue staining is representative of *Foxl2–LacZ* expression. Blue staining is detected in XX *Foxl2*^{+/LacZ} gonads (c, d) indicating ovarian differentiation in the anterior and posterior regions (e) and in the posterior region (f) of XX *Wt1:Sox9*^{Tg/+}; *Foxl2*^{+/LacZ} gonads (Arrows). This shows that ovarian poles are present in XX transgenic gonads. XY gonads are devoid of staining (a, b). The posterior poles are the lower poles. Lower panel: *in situ* hybridizations on sections with *Rspo1* probe (g, h, i) confirm that XX *Wt1:Sox9*^{Tg/+} gonads contain ovarian tissues.

Signals in testicular cords were darker likely due to an additive expression of the transgenic and the endogenous *Sox9* gene. Indeed, in situ hybridizations on XX *Wt1:Sox9*^{Tg/+}; *Sox9–EGFP* sections with a *Gfp* probe reveal a strong staining of *Gfp* in testicular cords likely mimicking endogenous *Sox9* expression (Fig. 4Bh–j). These results suggest that *Sox9* transcription in ovarian cells is not sufficient to induce/maintain sex reversal after E13.5.

In XY gonads, ectopic activation of the Wnt/β-catenin pathway results in male-to-female sex reversal (Maatouk et al., 2008) by disrupting the maintenance of the testis-determining pathway (Chang et al., 2008). Interestingly, *in vitro* data show that β-catenin can interact with SOX9 to induce its degradation (Akiyama et al., 2004). To test whether β-catenin stabilization may be involved in feminization of the poles in the *Wt1:Sox9*^{Tg/+} gonad, we made use of the *Axin2:LacZ* strain, a universal reporter of β-catenin stabilization (Lustig et al., 2002). Indeed, β-catenin was stabilized in the ovarian regions of XX *Wt1:Sox9*^{Tg/+} ovotestes (Fig. 4C). This suggests that failure of SOX9 accumulation in ovarian areas of the transgenic gonads may be caused by activation of β-catenin, which in turn may inhibit SOX9 by a post-transcriptional mechanism.

Germ cell proliferation is controlled by their sex chromosome constitution in XX Wt1:Sox9^{Tg/+} *gonads*

Germ cells proliferate until E12.5 and E14.5 in XX and XY gonads, respectively (Western et al., 2008). In XXY testes, germ cells undergo degeneration when spermatogenesis initiates after birth (Hunt et al., 1998). However, double immunostainings for AMH and MVH (germ cell marker) showed that less germ cells were present in the testicular region of the transgenic ovotestes as early as E14.5 (Fig. 5A). To determine whether germ cell numbers may be affected in our transgenic model, we counted germ cells at E12.5, E13.5 and E14.5 in XY and XX *Wt1:Sox9*^{Tg/+} gonads. At E12.5, there was no significant difference between the number of germ cells in both genotypes (Fig. 5Ba, blue histograms), but at E13.5 and E14.5, we found a dramatic reduction in the testicular region of XX transgenic compared to XY wild-type gonads (Fig. 5Bb, c, blue histograms). At E14.5, the numbers of germ cells between control ovaries and the ovarian region of XX *Wt1:Sox9*^{Tg/+} ovotestes were similar (Fig. 5Bd, pink histograms), but less germ cells were observed in the testicular compared to the ovarian regions in the transgenic gonads (Fig. 5Be, pink histograms).

Low numbers of germ cells in testicular regions of XX transgenic embryos were not linked to germ cell apoptosis at E12.5 (data not shown) or E14.5 (Fig. 7B). Thus, we speculated that germ cell proliferation might be impaired in testicular regions of XX *Wt1:Sox9*^{Tg/+} gonads. To check this hypothesis, we performed BrdU incorporation experiments at E12.5 and E13.5. At E12.5, the numbers of BrdU-positive germ cells were equivalent in XX transgenic gonads and XY controls (Fig. 5Ca, b, e), whereas the number of BrdU-positive germ cells was reduced in XX *Wt1:Sox9*^{Tg/+} gonads compared to XY gonads (Fig. 5Cc, d, f). Taken together, these results show that XX germ cell proliferation is impaired in testicular regions of the transgenic gonads.
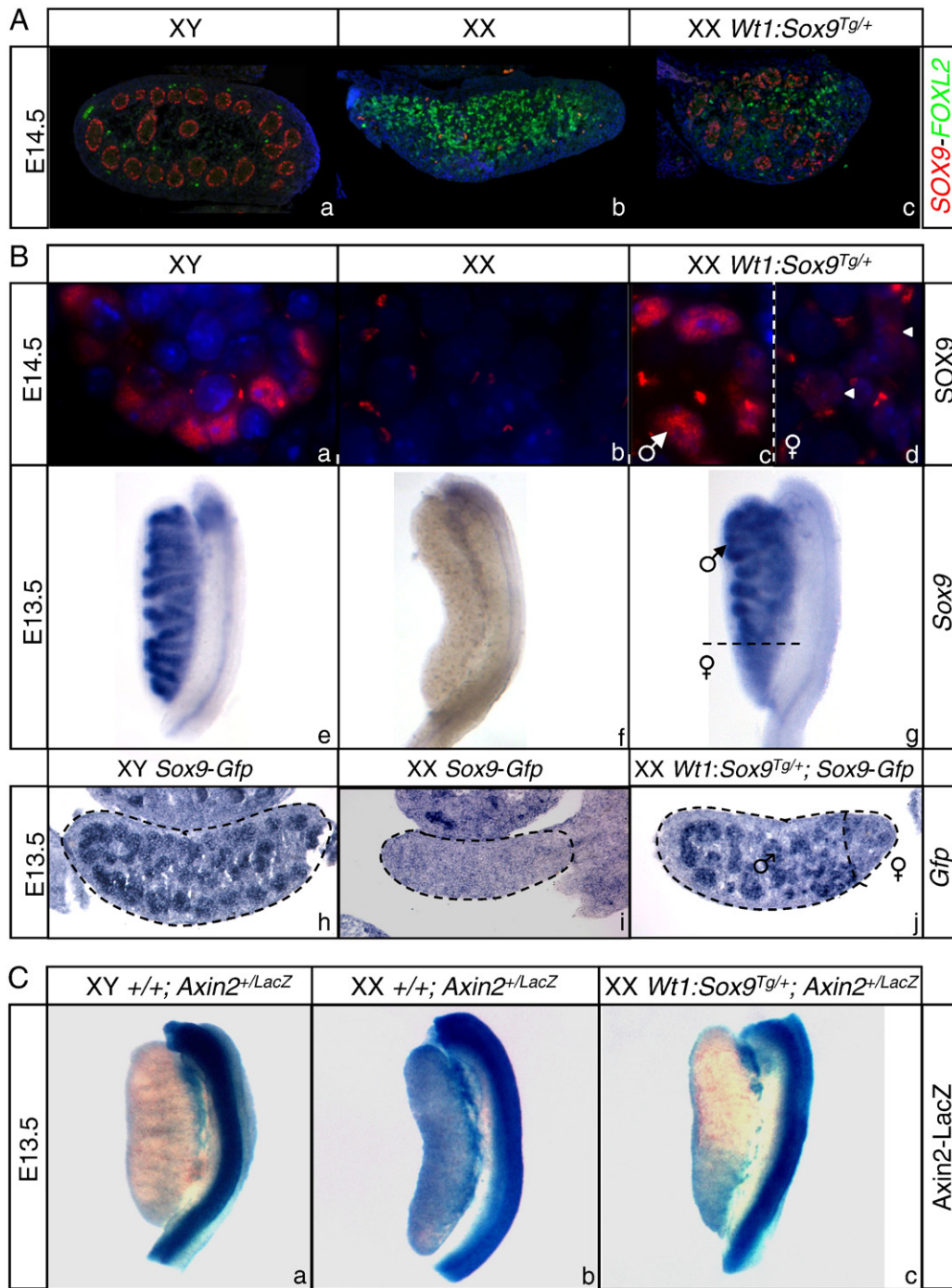
Fig. 4. *Sox9* post-transcriptional regulation in XX *Wt1:Sox9^{Tg/+}* gonads. (A) Immunodetection of SOX9 (red) and FOXL2 (green) in E14.5 gonads (magnification ×20). Whereas SOX9 is strongly expressed in XY gonads (a) and FOXL2 in XX gonads (b), both proteins are detected in XX *Wt1:Sox9^{Tg/+}* gonads (c) but seem not to be expressed in the same cells. Posterior poles are on the right side. DAPI (blue) was used to detect nuclei. (B) Upper panel: high magnification (×100) of XX *Wt1:Sox9^{Tg/+}* gonads shows low level of nuclear SOX9 in ovarian parts (d) compared to high level of SOX9 expression in testicular regions (c) or in XY gonads (a). Middle panel: whole-mount *in situ* hybridizations of *Sox9* at E13.5 in XY (e), XX (f) and XX transgenic gonads (g). The blue staining corresponds to whole *Sox9* expression (transgene plus gene) and shows *Sox9* transcription in testicular and ovarian parts of XX *Wt1:Sox9^{Tg/+}* gonads (g) and in XY gonads (e). Male and female symbols in g indicate the testicular or ovarian tissues of the transgenic gonads. Lower panel: *in situ* hybridizations on sections of XY (h) and XX SOX9–EGFP (i) and XX *Wt1:Sox9^{Tg/+}*, SOX9–EGFP gonads (j), using a *Gfp* probe, indicate that *Sox9–Gfp* endogenous expression is restricted to sex cords (h, j). Male and female symbols in j indicate the testicular or ovarian tissues of the transgenic gonads. (C) β-Catenin activity in the XX *Wt1:Sox9^{Tg/+}* ovarian poles. X-Gal (*Axin2–LacZ*) whole-mount staining of gonads at E13.5 (a–c). Blue staining is representative of *Axin2* expression, a gene upregulated after β-catenin activation. The staining is located in the ovarian region of XX *Wt1:Sox9^{Tg/+}*; *Axin2^{+/LacZ}* and in XX *Axin2^{+/LacZ}* gonads. The posterior poles are the lower ones.

Although *Wt1* has been shown to be expressed in germ cells (Natoli et al., 2004), we did not detect ectopic expression of SOX9 in germ cells (data not shown), excluding a direct effect of the transgene in germ cells. These data rather imply that the somatic environment of XX germ cells does not regulate their proliferation and this is controlled by their sex chromosome constitution.

Germ cells differentiate according to their somatic environment in XX *Wt1:Sox9^{Tg/+}* gonads

It has been shown previously that XX germ cells differentiate predominantly as gonocytes in XX/XY chimeric testes (Palmer and Burgoyne, 1991) suggesting that XX germ cells differentiate according

to their somatic environment. To determine the fate of germ cells in XX $Wt1:Sox9^{Tg/+}$ gonads, we performed *in situ* hybridization experiments using *Stra8*, a marker of meiotic commitment (Baltus et al.,

2006). Wild-type ovaries and ovarian regions of XX $Wt1:Sox9^{Tg/+}$ ovotestes exhibited staining indicating the presence of meiotic oocytes (Fig. 6a–c). In contrast, control testes and the testicular
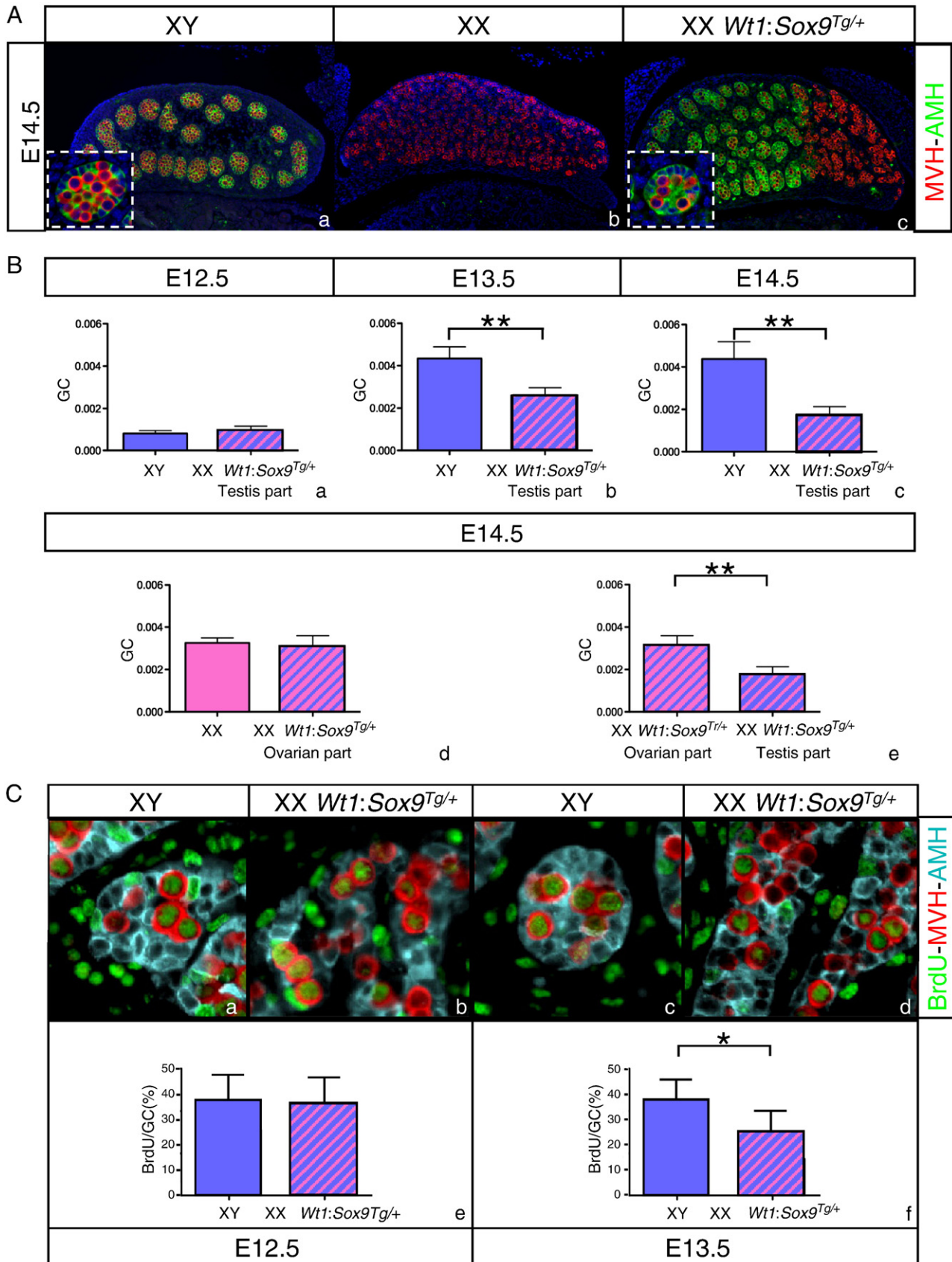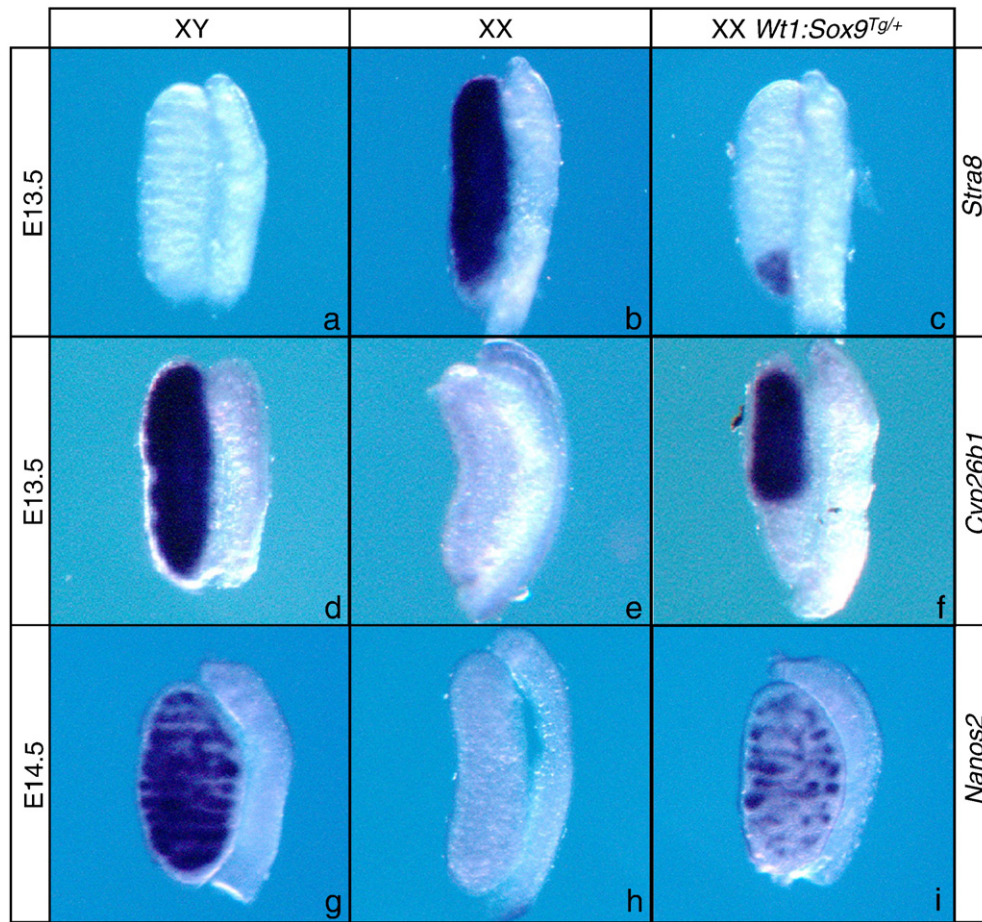
Fig. 6. Germ cells differentiation in XX *Wt1:Sox9*$^{Tg/+}$ ovotestis depends on their somatic environment. Upper panel: whole-mount *in situ* hybridizations using *Stra8* as a probe at E13.5 (a, b, c) show a strong expression in XX gonads and XX *Wt1:Sox9*$^{Tg/+}$ ovarian parts indicating meiosis commitment of germ cells. Middle panel and lower panel: whole-mount *in situ* hybridizations using *Cyp26b1* (d, e, f) and *Nanos2* (g, h, i) as probes at, respectively, E13.5 and E14.5 show XX gonocytes differentiation in both XX *Wt1:Sox9*$^{Tg/+}$ testicular regions and in XY gonads. Note the weak level of *Nanos2* expression likely due to low number of germ cells.

regions of XX *Wt1:Sox9*$^{Tg/+}$ ovotestes lacked staining indicating that meiotic germ cells were mostly present in the ovarian regions of the transgenic gonads. Meiotic commitment is controlled by various stimuli including the retinoic acid (RA) pathway (Bowles et al., 2006; Koubova et al., 2006). In embryonic testes, low levels of RA are required for gonocytes to become quiescent (Trautmann et al., 2008). RA levels are controlled by the activity of the testis-specific RA degrading enzyme, CYP26B1. When using *Cyp26b1* as a probe, we found strong expression in the testicular regions of XX *Wt1:Sox9*$^{Tg/+}$ ovotestes at levels comparable to those of wild-type testes (Fig. 6d–f). Thus, the quiescence of XX gonocytes in XX *Wt1:Sox9*$^{Tg/+}$ gonads is likely to be associated with the activity of CYP26B1.

Nanos2 is also a key regulator of XY gonocyte differentiation (Suzuki and Saga, 2008), and as such, it is specifically expressed in XY gonocytes. To determine whether *Nanos2* is expressed in the XX gonocytes located in the sex cords of the XX *Wt1:Sox9*$^{Tg/+}$ embryos, we carried out *in situ* hybridizations. A robust staining was visualized

in XY wild-type gonads and a weaker signal, likely to be due to a low number of germ cells, was detected in XX *Wt1:Sox9*$^{Tg/+}$ testicular regions (Fig. 6g–i).

Taken together, our results show that whereas XX germ cell proliferation depends on their genotype, germ cells differentiate according to their somatic environment in XX *Wt1:Sox9*$^{Tg/+}$ fetus. This indicates that both mechanisms are independent.

Regression of the ovarian regions of XX Wt1:Sox9$^{Tg/+}$ *gonads*

Although we have shown that ovarian poles differentiate in XX *Wt1:Sox9*$^{Tg/+}$ fetal gonads, we have not observed ovarian tissues in adult mice and transgenic gonads were azoospermic testis (Vidal et al., 2001). At birth, histological analyses confirmed the absence of forming follicles or meiotic germ cells indicating that XX *Wt1:Sox9*$^{Tg/+}$ gonads were testes (Fig. 7A).

Fig. 5. Defects of XX germ cell proliferation in XX *Wt1:Sox9*$^{Tg/+}$ testicular parts. (A) Immunodetection of AMH (green) and MVH (red) in E14.5 gonads (magnification ×20). DAPI (blue) was used to detect nuclei. Germ cells seem to be less abundant in testicular region of XX transgenic gonads (c) compared to XY gonads (a). The posterior poles are on the right side. (B) Numbers of germ cells in XX, XY controls (pink and blue histograms, respectively) and in ovarian and testicular regions of XX *Wt1:Sox9*$^{Tg/+}$ gonads (shaded pink and shaded blue histograms, respectively). The values are number of germ cells per square area. At E12.5, no significant differences are observed between XX *Wt1:Sox9*$^{Tg/+}$ ovarian tissues ($n=4$ gonads) and XY gonads ($n=6$) (a) whereas at E13.5 and E14.5, germ cells are highly significantly less abundant in XX *Wt1:Sox9*$^{Tg/+}$ testicular tissues ($n=10$ and 6, respectively) compared to XY controls ($n=7$ and 4, respectively) (b, c). At E14.5, whereas no significant differences are observed between germ cell numbers in XX ($n=3$) and ovarian regions of XX transgenic gonads ($n=4$), germ cells are less abundant in testicular parts compared to ovarian parts of XX transgenic gonads (e). (C) Percentages of proliferating germ cells in XY gonads and testicular parts of XX *Wt1:Sox9*$^{Tg/+}$ gonads (blue and striped blue pink histograms, respectively). Immunostainings (a, b, c, d) using anti-BrdU (green), MVH (red), AMH (blue) allowed to identify the sex cords of the testicular regions of XX transgenic gonads (magnification ×40). At E12.5 (e), no significant difference was observed, whereas at E13.5, there were significantly less BrdU-positive germ cells in XX *Wt1:Sox9*$^{Tg/+}$ gonads compared to XY controls (f). At 12.5 XY gonads ($n=4$), XX *Wt1:Sox9*$^{Tg/+}$ ovotestis ($n=2$). At E13.5, XY gonads ($n=5$) and XX *Wt1:Sox9*$^{Tg/+}$ ovotestis ($n=6$).
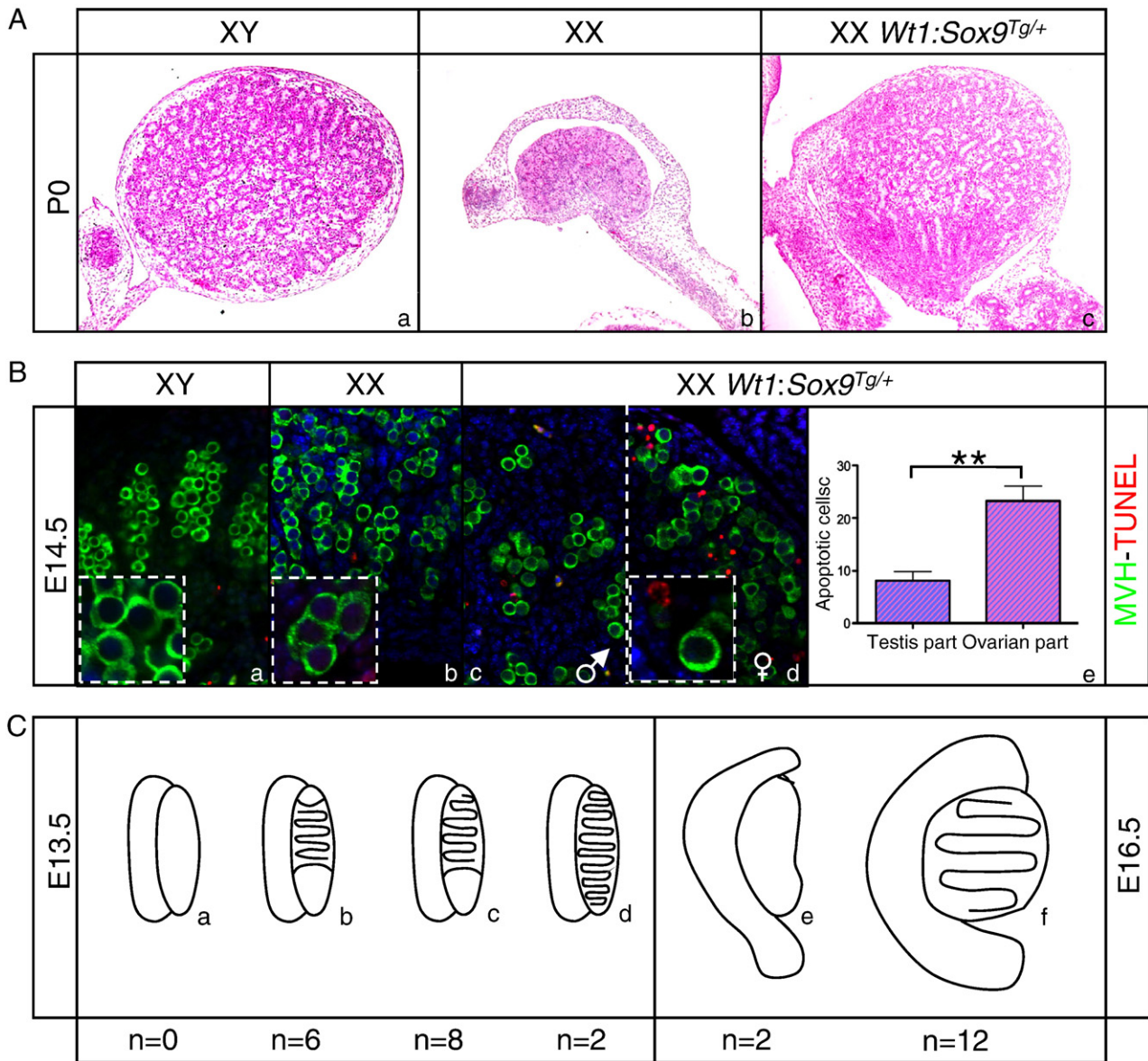
Fig. 7. Regression of ovarian regions in XX *Wt1:Sox9*$^{Tg/+}$ ovotestis. (A) Hematoxylin and eosin staining of P0 gonadal sections. XX *Wt1:Sox9*$^{Tg/+}$ gonads show no obvious ovarian parts. (B) TUNEL experiments show higher number of apoptotic cells in the ovarian parts (d) compared to the testicular parts of the XX *Wt1:Sox9*$^{Tg/+}$ gonads (c) or to XY (a) or XX (b) control gonads (magnification ×40). Male and female symbols in c and d indicate the testicular or ovarian tissues of the transgenic gonads. MVH (green). TUNEL (red). DAPI (blue) was used to detect nuclei. Quantification of the number of apoptotic cells in XX *Wt1:Sox9*$^{Tg/+}$ ovotestis (n = 6) (e) shows that high levels of apoptotic cells are detected in ovarian parts of the XX transgenic gonads in comparison to the testicular parts. (C) Regression of the ovarian tissues of XX *Wt1:Sox9*$^{Tg/+}$ gonads between E13.5 and E16.5. XY *Wt1:Sox9*$^{Tg/+}$; *Axin2*$^{+/LacZ}$ or XY *Wt1:Sox9*$^{Tg/+}$; *Foxl2*$^{+/LacZ}$ males (mixed background) were mated to C57BL/6 females. X-Gal staining allowed to visualize the ovarian regions in F1 fetal gonads at E13.5 and E16.5. Tail biopsies were used for genotyping. The gonads were scored according to their morphology (n = number of ovaries, ovotestes with one or two ovarian poles and testes). The drawing shows the testicular regions containing sex cords. Although each transgenic gonad was scored, the structural pattern was always similar between both gonads of the embryo, suggesting no left–right asymmetric effects. At E16.5, no ovotestes were observed in XY *Wt1:Sox9*$^{Tg/+}$; *Axin2*$^{+/LacZ}$ fetus.

Histological analysis of XX transgenic gonads at E15.5 showed apoptotic cells in the ovarian regions of the ovotestes (Fig. 3Ag). Moreover, at this stage, the ovarian regions were very small or absent in most of the transgenic gonads (data not shown). Indeed, TUNEL experiments confirmed the presence of apoptotic nuclei in the ovarian regions at E14.5 (Fig. 7Ba–d). Quantification of the number of apoptotic cells in ovarian and testicular regions of the XX *Wt1:Sox9*$^{Tg/+}$ ovotestis showed that there was a significant increase of apoptotic cells in the ovarian regions of the ovotestes (Fig. 7Be). This suggests that the ovarian regions that initially form in XX *Wt1:Sox9*$^{Tg/+}$ gonads subsequently regress by apoptosis.

We next analyzed whether XX *Wt1:Sox9*$^{Tg/+}$ gonads are initially developing as ovotestes and whether specific regression of the ovarian regions may be responsible for testis development. For this,

we analyzed the morphology of 16 and 14 XX *Wt1:Sox9*$^{Tg/+}$ gonads at E13.5 and E16.5, respectively. The ovarian regions were identified by X-Gal staining using XX *Wt1:Sox9*$^{Tg/+}$; *Foxl2:LacZ* or XX *Wt1:Sox9*$^{Tg/+}$; *Axin2:LacZ* embryos and gonads were scored according to their structure, i.e., ovary, both ovarian poles, one ovarian pole, no ovarian tissue (Fig. 7C). At E13.5, 87.5% (14/16) of the gonads showed one or two ovarian poles and 12.5% (2/16) showed no X-Gal staining implying that they were fully differentiated as testis. This variability of the gonadal phenotypes of the XX *Wt1:Sox9* transgenic fetus may be due to the mixed genetic background of this line.

At E16.5, 14.3% (2/14) showed X-Gal staining along the entire gonads indicating that they were ovaries. Both gonads were from the same embryo and might be due to an epigenetic silencing of the transgene. At this stage, 85.7% (12/14) XX transgenic gonads were

devoid of X-Gal staining, indicating that by this time, all ovarian tissue had become eliminated. Taken together, these data suggest that while XX *Wt1:Sox9*$^{Tg/+}$ gonads initially differentiate as ovotestes, specific apoptosis within the ovarian portion of the gonad leads to testis development by E16.5.

Discussion

Genes involved in sexual differentiation are expressed at a low level in the bipotential gonad (Kim et al., 2006). Activation of either the testis-determining pathway, with SRY being the main switch in the XY gonad, or the ovarian differentiating pathway, with *Rspo1* activating the Wnt/β-catenin signaling pathway, induces sex determination.

SOX9 regulation in XX transgenic gonads

In mice, proper testis differentiation is temporally tightly controlled. *Sry* function is effective between E11.00 and 11.25 with expression being initiated in the central region and reaching the poles of the gonads at E11.25 (Hiramatsu et al., 2009). SRY directly activates *Sox9* expression (Sekido and Lovell-Badge, 2008), and FGF9 signals are required to maintain *Sox9* expression and thus allow progression of sex cord formation from the center to the poles (Hiramatsu et al., 2010). Our analysis suggests that the overall level of *Sox9* transcript is similar or even higher in XX *Wt1:Sox9*$^{Tg/+}$ gonads than in XY gonads. However, since *Sox9* expression is controlled by the *Wt1* regulatory region in XX *Wt1:Sox9*$^{Tg/+}$ gonads, its expression is not restricted to the Sertoli cell lineage. Moreover, at E11.5, cells with nuclear SOX9 are more abundant in XY gonads than in XX transgenic gonads. Thus it is likely that in the cells that will eventually differentiate as Sertoli cells in XX *Wt1:Sox9*$^{Tg/+}$ gonads, the level of *Sox9* expression per cell is lower compared to those in Sertoli cells in the XY gonads. We conclude that the *Wt1:Sox9* transgene is not as efficient as *Sry* to induce testis development and thus results in ovotestis formation. XX *Wt1:Sox9*$^{Tg/+}$ ovotestes showed a similar organization than XY ovotestes with testicular cords in the central region and ovarian tissues at the poles of the gonads (Eicher and Washburn, 2001). In XY gonads, SRY induces a central to pole wave of Sertoli cell differentiation. Our results show that during sex reversal of XX gonads such a wave of differentiation also exists despite the fact that the *Wt1:Sox9*$^{Tg/+}$ transgene is evenly expressed throughout the entire XX gonad. Since the transgene is unable to promote sex cord formation at the poles, this promotes an opposite wavelike ovarian differentiation from the poles to the center. Thus, the overall lower cellular expression of *Sox9* from the transgene appears to be insufficient to prevent ovarian differentiation.

Whereas early expression of *Sox9* is induced by SRY in XY gonads, *Sox9* remains expressed at E12.5 when SRY expression has ceased. SRY and SOX9 belong to the same family of genes, the SOX genes (Wegner, 1999), and has been suggested that they may serve similar functions during testis formation. For instance, transgenic SRY, SOX9 and SOX10 are able to induce female-to-male sex reversal (Koopman et al., 1991; Polanco et al., 2010; Vidal et al., 2001; and the present data). Thus SOX9 could take over SRY function and promote its own expression at E12.5. We have shown here that in XX *Wt1:Sox9*$^{Tg/+}$; *Sox9–EGFP* gonads, the *Sox9* transgene can induce the expression of EGFP knocked into the *Sox9* locus implying that SOX9 can activate its own expression. Earlier ChIP experiments (Sekido and Lovell-Badge, 2008) showed that SRY immuno-precipitated fragments in TESCO were different from that precipitated by SOX9 antibodies, a sequence also pulled down with SF1. This suggests that *Sox9* regulation by SRY is mechanistically different from a potential autoregulation of SOX9. Whereas *Wt1:Sox9* is robustly expressed (at the level of transcript and protein) at E12.5, it fails to upregulate efficiently *Sox9–EGFP* at this

stage. Taken together these data suggest that SOX9 might not directly regulate its own expression in XX *Wt1:Sox9*$^{Tg/+}$ gonads.

In mice, mutations in *Rspo1* or *Wnt4*, two genes of the ovarian differentiation pathway, trigger sex reversal of XX embryonic gonads and *Sox9* becomes upregulated in these tissues (Chassot et al., 2008; Vainio et al., 1999). *Sox9* upregulation can also be achieved by grafting embryonic ovaries under the kidney capsule. In this case, a reporter of TESCO expression (TESCO-CFP) becomes weakly expressed 10 days after the grafts, and high levels of CFP are detected 30 days post-grafts (Sekido and Lovell-Badge, 2008). How SOX9 becomes activated without the presence of SRY is presently unknown. A potential mechanism could involve the FGF9/FGFR2 and PDG2/PGDS signaling pathways, both of which are required for SOX9 maintenance (Kim et al., 2006; Moniot et al., 2009). The delayed onset of endogenous *Sox9* expression in XX *Wt1:Sox9*$^{Tg/+}$ gonads suggests that SOX9 by itself is insufficient to induce its own expression. By contrast, forced expression of SOX9 induces robust upregulation of *Pgds* and FGF9 at E12.5, which is likely to contribute to the induction of *Sox9–EGFP* expression. Since FGFR2 has been detected in the nucleus of Sertoli cells (Schmahl et al., 2004), it is conceivable that nuclear FGFR2 may directly contribute to *Sox9* activation.

Regression of ovarian regions leads to full testis development in XX Wt1: Sox9$^{Tg/+}$ mice

Ectopic expression of SOX9 in XX gonads triggers full sex reversal of adult mice. This can be achieved either by transgenesis (Vidal et al., 2001) or caused by mutations in the upstream region that lead to activation of the endogenous *Sox9* gene (Bishop et al., 2000). Thus, it is surprising that before full sex reversal becomes apparent, there is an embryonic period (E13.5–E16.5) when XX *Wt1:Sox9*$^{Tg/+}$ gonads develop as ovotestes. At E13.5, the ovarian regions are variable, i.e., they are present either at one or both poles of the XX transgenic gonads or are absent. This indicates that the *Wt1:Sox9* transgene efficiency is variable and this is likely associated to variability of the XX background (Munger et al., 2009) resulting in incomplete penetrance of sex reversal. *Sox9* expression from the transgene occurs at E11 and persists in the entire gonad at E11.5–12.5 and E13.5 (Vidal et al., 2001, and present results). Although, *Sox9* transcripts are evenly expressed in the transgenic gonads, the protein does not accumulate in the ovarian poles of the transgenic ovotestis and thus leads to ovarian differentiation. In wild-type testis *Fgf9* signaling plays an important role in the maintenance of *Sox9* expression, which involves the control at the level of transcription (Kim et al., 2006). In *Wt1: Sox9* mice, the *Sox9* transgene is not regulated by its own promoter and thus is maintained throughout the gonad. The fact that we could not detect SOX9 protein at the poles despite the persistence of RNA expression may suggest that post-transcriptional regulation is also involved in preventing SOX9 accumulation. Stabilization of β-catenin is required for ovarian differentiation (Chassot et al., 2008). Ectopic β-catenin activation in XY gonads prevents testicular development (Maatouk et al., 2008) and suppression of β-catenin signaling is essential for proper testis formation (Chang et al., 2008). *In vivo*, SOX9 prevents β-catenin accumulation during cartilage differentiation, as well as within the intestinal epithelium (Akiyama et al., 2004; Bastide et al., 2007). It is possible that a similar mechanism of SOX9 degradation promotes ovarian cell maintenance during normal differentiation and that initial stabilization of β-catenin leads to SOX9 protein degradation at the poles of XX *Wt1:Sox9*$^{Tg/+}$ ovotestes. However, the ovarian poles in XX *Wt1:Sox9*$^{Tg/+}$ gonads seem to be transiently present. At E13.5, 87.5% of XX *Wt1:Sox9*$^{Tg/+}$ gonads are ovotestes and 85.7% are testes at E16.5. Apoptosis of the ovarian regions occurs at E14.5, indicating ovarian pole regression in XX embryonic *Wt1: Sox9*$^{Tg/+}$ ovotestes. Interestingly, the apoptotic cells detected in this analysis were somatic cells. This has not be described in XY ovotestes (Eicher and Washburn, 2001), suggesting that the antagonism between β-catenin and continual expression of *Sox9* cause somatic cells to undergo apoptosis. Although we did not detect germ cell apoptosis at E14.5 in

transgenic ovotestes, it has been shown that germ cell undergo degeneration at birth in XXY testis (Hunt et al., 1998). This suggests that degeneration could also occur in XX *Wt1:Sox9*$^{Tg/+}$ testes. Indeed, we did not observe germ cells in adult transgenic animals (Vidal et al., 2001).

XX germ cell proliferation is uncoupled from their differentiation in XX Wt1:Sox9$^{Tg/+}$ *ovotestes*

Previous data showed that the RNA helicase MVH is expressed in XX and XY germ cells but is only required for germ cell proliferation in embryonic testis (Tanaka et al., 2000) indicating that germ cell proliferation depends on different signals in XX and XY gonads. In testicular regions of the XX *Wt1:Sox9*$^{Tg/+}$ gonads, germ cell proliferation is lower than in XY controls. Moreover, the number of germ cells in testicular parts of XX transgenic ovotestis is lower than in the ovarian parts, suggesting that XX germ cells in testicular regions fail to proliferate as efficiently as in ovarian tissues. Thus XX germ cells are not fully competent to respond to proliferative stimulating signals from the testicular somatic environment suggesting that the proliferation of germ cells in fetal testis depends at least in parts on their genotype.

Transgenic experiments have shown that *Eif2s3y* transgenes rescue the spermatogonial proliferation failure of mutants bearing a chromosomal deletion of the Y chromosome. Thus genes on the Y chromosome are essential for spermatogenesis (Mazeyrat et al., 2001). Our results suggest that some Y factors may also be required for pro-spermatogonia proliferation. Whether these genes are the same as those involved for proliferation at beginning of spermatogenesis remain to be elucidated.

In XX/XY chimeric gonads or XX *Wt1:Sox9*$^{Tg/+}$ gonads, XX germ cells adopt the fate of their environment, i.e., become oogonia in ovarian regions and pro-spermatogonia in testicular regions of the ovotestes (Palmer and Burgoyne, 1991, and present results). Whereas FGF9 is required for germ cell survival and differentiation in XY gonads, it is not involved in their proliferation (Barrios et al., 2010) (DiNapoli et al., 2006). Thus these data suggest that germ cell proliferation and differentiation are two independent mechanisms.

Acknowledgments

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