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SOX4 regulates gonad morphogenesis and promotes male germ cell differentiation in mice

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Abstract

The group C SOX transcription factors SOX4, -11 and -12 play important and mutually overlapping roles in development of a number of organs. Here, we examined the role of *SoxC* genes during gonadal development in mice. All three genes were expressed in developing gonads of both sexes, predominantly in somatic cells, with *Sox4* being most strongly expressed. *Sox4* deficiency resulted in elongation of both ovaries and testes, and an increased number of testis cords. While female germ cells entered meiosis normally, male germ cells showed reduced levels of differentiation markers *Nanos2* and *Dnmt3l* and increased levels of pluripotency genes *Cripto* and *Nanog*, suggesting that SOX4 may normally act to restrict the pluripotency period of male germ cells and ensure their proper differentiation. Finally, our data reveal that SOX4 (and, to a lesser extent, SOX11 and -12) repressed transcription of the sex-determining gene *Sox9 via* an upstream testis-specific enhancer core (TESCO) element in fetal gonads, raising the possibility that SOXC proteins may function as transcriptional repressors in a context-dependent manner.

Introduction

In mice, gonadal development starts around 10.5 days *post coitum* (dpc) when the coelomic epithelium overlying the ventromedial surface of the mesonephros thickens to form a pair of sexually bipotent genital ridges. In XY genital ridges, the subsequent expression of the Y-linked male sex-determining gene *Sry* (Gubbay et al., 1990; Koopman et al., 1991; Sinclair et al., 1990) in somatic precursor cells marks the onset of testis differentiation. SRY activates its key target *Sox9* by binding to an upstream testis-specific enhancer core (TESCO) element (Sekido and Lovell-Badge, 2008). SOX9 directs somatic precursor cells to differentiate into

Sertoli cells, which in turn orchestrate the development of other testicular cell lineages, including fetal Leydig cells (Svingen and Koopman, 2013). In the absence of *Sry*, XX somatic precursor cells differentiate towards granulosa cells under the influence of unopposed RSPO1/WNT4/ß-Catenin and FOXL2 signalling (Nicol and Yao, 2014). As a result, XX genital ridges form ovaries.

Accompanying the differentiation of testicular cell lineages, mouse fetal testes undergo morphological changes from 11.5 dpc, including rapid growth, testis cord formation and vascularisation (Svingen and Koopman, 2013; Ungewitter and Yao, 2013). Along with the rapid growth, the shape of fetal testes changes significantly. Width of the testes increases ~4-fold (Nel-Themaat et al., 2009) by 13.5 dpc due to a combination of somatic cell proliferation (Schmahl et al., 2000) and cell migration from the mesonephroi and coelomic epithelium (Karl and Capel, 1998; Martineau et al., 1997). At the same time, fetal testes become shorter (Nel-Themaat et al., 2009), presumably due to cells migrating more towards the centre of the gonad (Nel-Themaat et al., 2010). In contrast, fetal ovaries show no dramatic morphological changes within this time window apart from a slight increase in width and a reduction in length.

Depending on the sexual fate choice of gonadal somatic cells, fetal germ cells adopt male or female fate. In fetal ovaries, germ cells enter meiosis in response to retinoic acid, and express meiotic markers including *Stra8* and *Sycp3* (Bowles et al., 2006; Koubova et al., 2006). In contrast, germ cells in fetal testes avoid entering meiosis and are instead mitotically arrested (McLaren, 1984). They transiently activate the *Nodal*/*Cripto* signalling pathway (Spiller et al., 2012), down-regulate pluripotency genes such as *Nanog*, *Sox2* and *Oct3/4* (*Pou5f1*) (Western et al., 2010), and begin to express genes marking the commitment to

spermatogenesis, including *Nanos2* (Suzuki and Saga, 2008) and *Dnmt3l* (Bourc'his and Bestor, 2004).

SRY and SOX9 belong to the multi-member SOX family of transcription factors defined by the presence of an SRY-type HMG (high-mobility group) box (SOX) DNA-binding domain. Based on the sequence homology of the HMG box, the 20 mammalian SOX proteins can be classified into groups A-H (Bowles et al., 2000). The SOXC group comprises three members, SOX4, SOX11 and SOX12. All possess a transactivation domain at the carboxyl-terminus, with SOX11 being the strongest transactivator (Dy et al., 2008). *SoxC* genes are expressed widely in the early mouse embryo and have been shown to redundantly function in a dose-dependent manner in the development of multiple organs (Bhattaram et al., 2010). *Sox4* $^{-/-}$ mice die at 14.5 dpc due to a heart outflow tract malformation (Schilham et al., 1996). *Sox11* $^{-/-}$ mice develop multiple organ defects and die at birth with similar heart malformations but of less severity compared with *Sox4* $^{-/-}$ mice (Sock et al., 2004). *Sox12* $^{-/-}$ mice are grossly normal (Hoser et al., 2008).

Northern blotting has previously revealed strong *Sox4* expression in adult mouse thymus, ovaries and testes (van de Wetering et al., 1993), prompting us to investigate whether this gene might also play a role in fetal gonad development. In the present study, we show that *Sox4* is highly expressed in both sexes in gonadal supporting cells, the organising centre of gonad organogenesis. By analysing *Sox4* $^{-/-}$ embryos, we found that *Sox4* loss of function resulted in a dysregulation of the organ shape of both ovaries and testes. Although the specification of gonadal somatic cell lineages and female germ cells appeared unperturbed in *Sox4* $^{-/-}$ fetal gonads, the spermatogenic differentiation of male germ cells was significantly compromised. In addition, our results suggest that SOX4 (and possibly SOX11/12) may

function as a transcriptional repressor in fetal testes, contributing to the precise regulation of *Sry* and *Sox9*. Taken together, our data indicate that SOX4 plays important roles in mouse gonad development by modulating gonad morphogenesis and promoting male germ cell differentiation *in vivo*.

Materials and methods

Mice

All animal procedures were approved by the Animal Care Committee at the Atlantic Veterinary College, University of Prince Edward Island or the University of Queensland Animal Ethics Committee. *Sox4* $^{+/-}$ strain (Schilham et al., 1996), Wt1-RG red-green reporter strain (Zhao et al., 2014b), and W^e strain (Buehr et al., 1993) have been described previously. Embryos were collected from timed matings of various strains, with noon of the day on which the copulatory plug was observed designated 0.5 dpc.

In situ *hybridisation*

Section *in situ* hybridisation was performed following a previously described protocol (Wilhelm et al., 2007) with modifications: 7-μm paraformaldehyde-fixed paraffin sections were dewaxed, rehydrated, proteinase K-treated, refixed, acetylated and pre-hybridised as described (Wilhelm et al., 2007). Digoxigenin (DIG)-labelled riboprobes were heat denatured at 85 °C for 5 min and cooled on ice before adding to the hybridisation buffer to a final concentration of 1 μg/ml. Hybridisation was carried out at 65 °C overnight. Slides were subsequently washed in 5× SSC for 25 min at 65 °C and NTE buffer (10 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 1 mM EDTA, pH 8) for 15 min at room temperature before RNase A (5 μg/ml) treatment in NTE buffer at 37 °C for 2× 15 min. Slides were washed sequentially in 2× SSC at 65 °C for 10 min, 0.5× SSC at 65 °C for 30 min and room temperature for 15 min, MAB buffer (0.1 M Maleic acid, 0.15 M NaCl, pH 7.5) for 10 min, and MABT buffer (MAB buffer containing 0.1% Tween-20) for 10 min, before incubating for 45 min in MABT buffer containing 2% Blocking reagent (Roche) and 5% heat-inactivated horse serum. Anti-DIG-AP antibody (Roche) at 1:1000 dilution in MABT buffer was added to the slides and incubated at room temperature for 1 h. Slides were subsequently washed two times in MABT buffer and

three times in NTT buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, and 0.1% Tween-20) before incubating with BM purple solution (Roche) until optimal staining was achieved. The probe sequences of *Sox4*, *Sox11*, and *Sox12* have been described previously (Huang et al., 2013).

Whole-mount *in situ* hybridisation with a DIG-labelled riboprobe for the *myoD* gene (Ashe et al., 2012) was performed essentially as described (Hargrave et al., 2006).

Cell sorting

Fetal testes and ovaries (with mesonephros tissue removed) were dissected from Wt1-RG embryos at 13.5 dpc and pooled ($n = 4$). Gonad tissue was incubated with 0.05% trypsin for 5 min at 37 °C followed by mechanical dissociation with 18G and 23G needles sequentially. Cells were sorted for mCherry and EGFP fluorescence on an Influx Flow Sorter (BD Biosciences). Two cell populations were isolated from ovarian tissue, namely RG^{+} ($mCherry^{+}EGFP^{+}$) somatic cells and $mCherry^{-}EGFP^{-}$ (RG^{-}) germ cells (Supplementary Fig. S1). Three distinct populations were isolated from fetal testes: RG^{Hi} Sertoli cells, RG^{Lo} interstitial cells (including fetal Leydig cells), and RG^{-} germ cells (Supplementary Fig. S1).

Histology

Embryos were fixed overnight in 4% paraformaldehyde and embedded in paraffin. Briefly, 7-μm paraffin sections were de-waxed in xylene, rehydrated through an ethanol series, then stained sequentially in haematoxylin and eosin solution. Images were captured on a BX-51 microscope (Olympus).

Immunofluorescence

Immunofluorescence staining was performed on 7-μm paraformaldehyde-fixed paraffin sections as described (Zhao et al., 2015). Images were captured on a LSM710 confocal microscope (Zeiss). Primary antibodies were used at the following dilutions: goat-anti-AMH (Santa Cruz C-20), 1:500; rabbit anti-HSD3β (Transgenic Inc. KAL-KO607), 1:500; rabbit anti-FOXL2 (Polanco et al., 2010), 1:500; rabbit anti-Laminin (Sigma L9393), 1:300; rabbit anti-DDX4 (Abcam ab13840), 1:800; mouse anti-SCP3 (Abcam ab97672), 1:200; mouse anti-DDX4 (Abcam ab27591), 1:500; rabbit anti-ARL13B (Proteintech 17711-1-AP), 1:300; rabbit anti-SRY (Wilhelm et al., 2005), 1:100; mouse anti-SOX9 (Abnova H00006662-M01), 1:200; rabbit anti-cleaved Caspase-3 (Cell Signaling #9664), 1:600; rabbit anti-Ki67 (Abcam ab15580), 1:100.

Quantitative RT-PCR (qRT-PCR)

mRNA expression analysis was performed as described (Bagheri-Fam et al., 2015). Embryos at 13.5 dpc were collected and stored in RNAlater solution (Qiagen). Fetal gonads were then dissected in RNAlater solution with mesonephros removed. Total RNA was extracted using RNeasy Micro kit (Qiagen) and cDNA synthesized using a high-capacity cDNA kit (Life Tech). Quantitative PCR was conducted with SYBR Green mix (Life Tech) on a ViiA7 machine (Life Tech). Primer sequences have been described previously (Huang et al., 2013; Zhao et al., 2015). Relative expression was calculated using ΔCt method with *Tbp* as the normalizing control (Svingen et al., 2009). Multiplicity-adjusted *P* values were calculated using GraphPad Prism 7.

Luciferase reporter assays

Luciferase reporter assays were conducted as described (Zhao et al., 2014a). Briefly, HEK293 cells were co-transfected with the TESCO-Luc construct (Sekido and Lovell-Badge, 2008), an empty pcDNA3 or a pcDNA3-Sf1 construct, an empty vector or an expression construct containing *Sry* or *Sox9* coding sequence, in combination with an expression construct containing coding sequence of *Sox4*, *11* or *12* (Huang et al., 2013). A CMV-renilla luciferase plasmid was included as a control for transfection efficiency. Cell lysates were harvested 48 h post-transfection and luciferase activities measured using a Dual Luciferase kit (Promega) on a POLARstar Omega luminometer (BMG Labtech). Statistical significance was determined with one-way ANOVA with Tukey's range test for multiple comparisons performed using GraphPad Prism 7.

Image quantification

For morphometric analysis of fetal urogenital organs, fetal gonads and kidneys were dissected from 13.5 dpc embryos and imaged with a Leica EC3 digital camera attached to a Leica MZ7.5 stereomicroscope. Length and width of each organ were measured using ImageJ software. Embryo trunk length was measured using ImageJ on embryos previously stained by whole-mount *in situ* hybridisation with a *myoD* riboprobe as described (Wainwright et al., 2014). Number of external testis cords (those running immediately beneath the coelomic epithelium) were counted on the central sagittal section of the fetal testis (the largest plane). Multiplicity-adjusted *P* values were calculated using GraphPad Prism 7.

For quantifying apoptosis, the number of $CC3^+$ cells in a whole gonadal section from each of several embryos of each genotype was manually counted and the area of each section measured with ImageJ. For quantifying cell proliferation and SRY-expressing cells, the

number of $Ki67^+$ or SRY^+ cells in three randomly chosen areas of arbitrary and identical size was manually counted in a whole gonadal section from each of several embryos of each genotype.

Results

SoxC *genes are predominantly expressed in the fetal gonadal somatic cell lineages*

Sox4 has previously been shown to be strongly expressed in adult mouse ovaries and testes (van de Wetering et al., 1993). However, detailed information about its expression pattern during mouse gonadogenesis is lacking. We therefore analysed the expression of *Sox4*, *Sox11* and *Sox12* in the developing fetal gonads from 10.5 to 14.5 dpc. qRT-PCR analysis (Fig. 1A) showed that all three genes were expressed in CD1 mouse fetal gonads, with similar levels in XX and XY gonads during this period. Expression of all three genes gradually decreased during the time course, with *Sox4* expression appearing to plateau after 12.5 dpc.

We further analysed the spatial expression pattern of *SoxC* genes using *in situ* hybridisation on sagittal sections of 13.5 dpc CD1 mouse embryos (Fig. 1B). In the fetal ovary, *Sox4* was strongly expressed in the domain close to mesonephros (future ovarian medulla), while *Sox11* and *Sox12* were expressed throughout the tissue. In the fetal testis, intensive staining of *Sox4* mRNA was observed within testis cords comprising cluster of germ cells and surrounding Sertoli cells. *Sox12* expression was detected both within testis cords and in the interstitium. In contrast, staining of *Sox11* transcripts appeared to be restricted to the interstitium. In accordance with these observations, elevated levels of *SoxC* genes were detected by qRT-PCR in W^{e} fetal gonads which lack germ cells, possessing an increased proportion of somatic cells (Buehr et al., 1993), compared with wild type gonads (Fig. 1C). These results indicate that *SoxC* genes are predominantly expressed in the somatic compartment of fetal gonads.

To further delineate the cell lineages expressing *SoxC* genes, we analysed their expression in 13.5 dpc fetal gonads from the Wt1-RG (red-green) mouse strain carrying an mCherry-EGFP transgene under the control of *Wt1* regulatory sequence (Zhao et al., 2014b). Gonadal cells from Wt1-RG fetal ovaries were sorted into RG^{+} ($mCherry^{+}$ $EGFP^{+}$) and RG^{-} ($mCherry^{-}$ $EGFP^{-}$) cell populations, whereas cells from Wt1-RG testes were sorted into three distinct populations based on their fluorescence intensities: RG^{Hi}, RG^{Lo} and RG^{-} (Supplemental Fig. S1). qRT-PCR analyses of lineage marker genes indicate that XX RG^{+} and RG^{-} cell populations were enriched for granulosa and germ cells respectively, whereas XY RG^{Hi}, RG^{Lo}, and RG^{-} populations were enriched for Sertoli, interstitial (including fetal Leydig cells) and germ cells respectively (Supplemental Fig. S1).

SoxC genes were expressed at high levels in the somatic cell compartment (RG^{+} population in fetal ovaries; and RG^{Hi} and RG^{Lo} populations in fetal testes) with much lower levels in RG^{-} germ cells (Fig. 1D), in agreement with our *in situ* hybridisation and W^{e} qRT-PCR results. In 13.5 dpc fetal testes where Sertoli and fetal Leydig cells have developed by this stage, *SoxC* genes showed clearly diverged expression patterns (Fig. 1D): *Sox4* was expressed at high levels in RG^{Hi} Sertoli cells and slightly lower levels in RG^{Lo} interstitial cells; *Sox12* was expressed at roughly equal levels in RG^{Hi} Sertoli cells and RG^{Lo} interstitial cells. In contrast, *Sox11* was predominantly expressed in RG^{Lo} interstitial cells with substantially lower levels in RG^{Hi} Sertoli cells.

Common somatic progenitor cells are believed to give rise to sex-specific somatic cell lineages (granulosa or Sertoli; stromal or interstitial) in the fetal testis and ovary (Albrecht and Eicher, 2001; Burgoyne et al., 1988; Palmer and Burgoyne, 1991a, b). It is thus highly likely that the expression of *SoxC* genes in ovarian somatic cell lineages (granulosa and

stromal) follows the same patterns in their corresponding testicular lineages. This extrapolation is supported by a previously published mouse fetal gonadal expression dataset conducted on sorted cell lineages (Supplemental Fig. S2; Jameson et al., 2012).

SOX4 ***influences the organ shape of fetal gonads in mice***

Based on the identified expression patterns of *SoxC* genes in mouse fetal gonads, we next focused our study on *Sox4*, as it is highly expressed in the supporting cell lineage that orchestrates fetal gonad development. While *Sox12* is also expressed in the supporting cells, it is the weakest transactivator amongst the SOXC factors (Dy et al., 2008), and has been shown previously to be dispensable for mouse embryonic development and fertility in adult mice (Hoser et al., 2008).

The gross morphology of both XX and XY *Sox4*$^{-/-}$ fetal gonads at 13.5 dpc largely resembled that of wild type (Fig. 2A): testis cords formed in *Sox4*$^{-/-}$ testes, while no overt morphological changes were noticed in *Sox4*$^{-/-}$ ovaries. Mutant embryos were indistinguishable from their littermates macroscopically (Supplemental Fig. S3A). However, *Sox4*$^{-/-}$ fetal gonads were significantly longer and thinner than wild type in both sexes (Fig. 2A,C,D): on average, *Sox4*$^{-/-}$ ovaries were ~27% longer and ~20% thinner than wild type, and *Sox4*$^{-/-}$ testes ~24% longer and ~9% thinner. The effect of SOX4 appears to be specific to fetal gonads, as neither the length of embryo trunk axis (Supplemental Fig. S3B) nor the shape of the fetal kidney, another organ originating from the urogenital ridge (Fig. 2B,E,F), were altered in *Sox4*$^{-/-}$ embryos.

Anti-laminin immunofluorescence revealed significantly more external testis cords (those running immediately beneath the coelomic epithelium; Combes et al., 2009) in *Sox4*$^{-/-}$ testis than wild type at 14.5 dpc (Fig. 3), consistent with a previously reported correlation between the gonad length and the number of testis cords (Nel-Themaat et al., 2009; Wainwright et al., 2014). Since the number of external testis cords decreases from 12.5 to 14.5 dpc in wild type testes due to extensive cord remodelling (Combes et al., 2009; Nel-Themaat et al., 2009), the observed increase in testis cord number in *Sox4*$^{-/-}$ testis could be caused by either excess formation of new cords or stabilisation of existing ones. Future experiments examining the dynamics of cord formation and remodelling in *Sox4*$^{-/-}$ fetal testes across 12.5 to 14.5 dpc should help to clarify this issue.

SOX4 is likely to modulate gonad shape through mechanisms common to both testes and ovaries, as *Sox4*$^{-/-}$ testes and ovaries underwent similar shape changes. We therefore reason that the increase in cord number was unlikely to drive the elongation of the gonad domain in *Sox4*$^{-/-}$ fetal testes, since cord-like structures were absent in *Sox4*$^{-/-}$ fetal ovaries. Instead, we suggest that the increased number of testis cords in *Sox4*$^{-/-}$ fetal testis may be a result of the extended gonad domain available for cord formation, in agreement with a previously proposed model that the number of testis cords may be determined by the space available in the gonadal field (Wainwright et al., 2014).

We have recently reported that one cause of increased gonad length is a defect in *Ift144*, a gene encoding a component of the primary cilium (Wainwright et al., 2014). We therefore analysed the presence of primary cilia in *Sox4*$^{-/-}$ fetal gonads using immunofluorescence. No apparent difference in primary cilia distribution between *Sox4*$^{-/-}$ and wild type fetal gonads was observed (Supplemental Fig. S4). Although we cannot rule out that *Sox4* regulates

primary cilium-mediated signalling to affect gonad length without affecting the formation and distribution of primary cilia, our results do not support an active involvement of primary cilia signalling in this phenotype.

We next analysed cell apoptosis and proliferation, since *SoxC* genes have been shown to be essential for the survival of mesenchymal and neural progenitor cells (Bhattaram et al., 2010). Immunostaining for the apoptosis marker cleaved Caspase-3 and the mitosis marker Ki-67 showed no significant changes in cell survival or proliferation in *Sox4* $^{-/-}$ gonads compared with wild type (Supplemental Figs. S5-S6), suggesting that SOX4 influences the shape of fetal gonads *via* other cellular and molecular mechanisms.

SOXC factors have been shown to directly regulate *Tead2* (Bhattaram et al., 2010; Poncy et al., 2015), a co-activator of the Hippo pathway, which plays a critical role in controlling organ shape and size in development and cancer (Zhao et al., 2011). We found that *Tead2* was significantly downregulated in XY but not XX mutant gonads at 13.5 dpc (Fig. 2G). Similar to previous studies (Bhattaram et al., 2010; Poncy et al., 2015), no significant changes in expression of other Hippo pathway genes, including *Amotl2*, *Ctgf* and *Cyr61*, were observed in *Sox4* $^{-/-}$ fetal gonads (Fig. 2H-J).

Sox4 loss-of-function does not perturb the specification of somatic cell lineages in mouse fetal gonads

Sox4 has been shown to control cell fate determination in neural progenitor cells (Bergsland et al., 2006) and skeletogenesis (Bhattaram et al., 2014). We therefore sought to determine whether specification of major somatic cell lineages was affected in the developing *Sox4* $^{-/-}$

fetal gonads. Histological examination of gonad sections at 14.5 dpc showed no apparent difference between the mutant and wild type gonads (Fig. 4A). Immunofluorescence analysis of several markers of the major gonadal cell lineages, including AMH (a marker of Sertoli cells), HSD3β (a steroidogenic enzyme expressed by fetal Leydig cells), and FOXL2 (a marker of granulosa cells) showed overall similar expression patterns in *Sox4*$^{-/-}$ and wild type gonads (Fig. 4B): AMH-expressing Sertoli cells and HSD3β-expressing fetal Leydig cells were present in *Sox4*$^{-/-}$ fetal testes and were absent in *Sox4*$^{-/-}$ ovaries. Conversely, FOXL2-positive granulosa cells were only detected in *Sox4*$^{-/-}$ ovaries but not in *Sox4*$^{-/-}$ testes. Quantitative analysis of these marker genes at 13.5 dpc using qRT-PCR confirmed similar expression levels of these marker genes in *Sox4*$^{-/-}$ and wild type gonads (Supplemental Fig. S7A–C).

We further analysed additional marker genes of the gonadal somatic cell lineages at 13.5 dpc using qRT-PCR. In line with our immunofluorescence analysis showing the presence of the major somatic cell lineages in *Sox4*$^{-/-}$ gonads, *Sf1* (also known as *Nr5a1*), a gene required for the differentiation of somatic cell lineage in both sexes (Luo et al., 1994), showed no significant expression changes between *Sox4*$^{-/-}$ and wild type gonads (Fig. 4C). Testicular cell lineage markers, including *Dhh* (Sertoli), *Cyp11a1* and *Star* (fetal Leydig), showed similar expression levels in *Sox4*$^{-/-}$ and wild type testes (Fig. 4D–F). Similarly, expression of *Fst*, a marker of granulosa cells and a downstream target of FOXL2 (Blount et al., 2009; Kashimada et al., 2011), remained unaltered in *Sox4*$^{-/-}$ ovaries compared with wild type (Fig. 4H). Interestingly, we found that *Wnt4*, a key driver of fetal ovarian development (Vainio et al., 1999), was slightly up-regulated in *Sox4*$^{-/-}$ fetal ovaries compared with wild type ovaries (Fig. 4G), suggesting that SOX4 may directly or indirectly repress *Wnt4* transcription in fetal ovaries.

Together, these data do not support an active role of *Sox4* in cell fate determination of fetal gonadal somatic cell lineages, in accordance with previous findings that various marker genes of embryo patterning and cell lineage specification are expressed normally in *SoxC* mutant embryos (Bhattaram et al., 2010).

SOX4 negatively regulates Sry and Sox9 in mouse fetal testes

Complex interplay among different SOX proteins has been well documented (Kamachi and Kondoh, 2013; Wegner, 2010). For instance, SOX4 and SOX9 cooperate to control bile duct development (Poncy et al., 2015), whereas SOXC proteins antagonise SOX9 activity in skeletogenesis (Bhattaram et al., 2014). Therefore, to identify potential regulatory interactions of SOX proteins, we analysed *Sox4*$^{-/-}$ fetal gonads for the expression of multiple *Sox* genes, including *SoxC* (*Sox4/11/12*), *Sry* and *SoxE* (*Sox8/9/10*) genes.

Levels of *Sox4* transcripts were substantially decreased but detectable using qRT-PCR at 13.5 dpc in *Sox4*$^{-/-}$ fetal gonads (~35% of that in wild type; Supplemental Fig. S7E), suggesting that the transcription termination conferred by the single polyadenylation signal inserted upstream of the *Sox4* coding region in this allele (Schilham et al., 1996) may not be complete. Expression of *Sox11* and *Sox12* remained largely unchanged in *Sox4*$^{-/-}$ fetal gonads compared with wild type, with the exception that *Sox11* was slightly up-regulated in *Sox4*$^{-/-}$ ovaries (Supplemental Fig. S7F,G), suggesting *Sox4* may cross regulate *Sox11* transcription in fetal ovaries.

In mice, *Sry* is expressed in XY genital ridges within a very short time window from 10.5 to 12.5 dpc, with its expression almost completely extinguished by 13.5 dpc (Bullejos and Koopman, 2001; Hacker et al., 1995; Jeske et al., 1995; Koopman et al., 1990; Wilhelm et al., 2005). We observed no obvious changes in SRY protein expression or the number of SRY-expressing cells in XY *Sox4*$^{-/-}$ genital ridges at 11.5 dpc compared with wild type (Supplemental Fig. S8), indicating that SOX4 may not contribute to the induction of *Sry* expression in fetal testes. Interestingly however, we found that *Sry* transcripts were clearly detectable in *Sox4*$^{-/-}$ fetal testes at 13.5 dpc (~6 fold higher than the background level in wild type testes; Fig. 5A), albeit at much lower levels than its peak expression at 11.5 dpc (~8% of peak levels, data not shown). This result raises the possibility that *Sox4* may contribute to down-regulating *Sry* in the mouse fetal testis.

Of the three SOXE group members, SOX9 and SOX8, cooperate to maintain the male sexual fate at both the fetal (Barrionuevo et al., 2009; Georg et al., 2012) and adult stages (Barrionuevo et al., 2016). The other *SoxE* gene, *Sox10*, is also expressed in Sertoli cells and has been shown to be able to sex reverse XX mice when overexpressed (Polanco et al., 2010). We found that the expression of *Sox8* and *Sox10* remained unchanged in *Sox4*$^{-/-}$ fetal testes at 13.5 dpc (Supplemental Fig. S7H,I). In contrast, *Sox9* mRNA levels were increased by ~50% in the mutant testes compared with wild type (Fig. 5B), indicating that SOX4 may selectively repress the expression of *Sox9* but not *Sox8 or Sox10*.

Given the critical involvement of the *Sox9*-TESCO enhancer in the initiation and maintenance of *Sox9* expression in the fetal testis (Sekido and Lovell-Badge, 2008), we next examined whether SOXC factors regulate TESCO activity, using a well-established *in vitro* reporter assay system (Polanco et al., 2010; Sekido and Lovell-Badge, 2008; Uhlenhaut et al.,

2009; Zhao et al., 2014a). We found that SOX4 potently repressed the activation of TESCO by SF1, SRY + SF1, or SOX9 + SF1 (~90% repression in each situation; Fig. 5C), despite a ~3-fold activation of TESCO on its own (Supplemental Fig. S9). SOX11 and SOX12 manifested weaker repressor activities compared with SOX4 (~70% or ~50% repression respectively; Fig. 5C) in the presence of SF1 and/or SRY/SOX9 (Fig. 5C). However, SOX11 or -12 alone activated TESCO more strongly than SOX4 (Supplemental Fig. S9). Together, these results reveal a context-dependent role for SOXC proteins in negatively regulating *Sox9* expression *via* repressing the TESCO enhancer in mouse fetal testes.

SOX4 promotes spermatogenic differentiation of male fetal germ cells

Fetal germ cells adopt either male or female sexual fate depending on the molecular cues provided by their surrounding somatic cells (Bowles and Koopman, 2013; Spiller and Bowles, 2015). In the fetal ovary they switch on *Stra8* and enter meiosis in response to retinoic acid, but in contrast, in the fetal testis germ cells are mitotically arrested and up-regulate male fate markers including *Nanos2*, *Dnmt3l* and $p15^{INK4b}$ (*Cdkn2b*).

We sought to determine whether the disruption of *Sox4* might affect fetal germ cell development. qRT-PCR analysis showed that upregulation of *Stra8* at 13.5 dpc was similar in $Sox4^{-/-}$ and wild type fetal ovaries (Fig. 6F), indicating that the initiation of meiosis of female germ cells had proceeded normally in $Sox4^{-/-}$ ovaries. Immunofluorescence for SYCP3, a marker of meiosis, revealed comparable expression patterns at 14.5 dpc in wild type and $Sox4^{-/-}$ ovaries (Fig. 6G), confirming the entry of meiosis of fetal germ cells was unperturbed in the mutant ovaries.

Consistent with the unperturbed differentiation of Sertoli and fetal Leydig cells in *Sox4*$^{-/-}$ fetal testes (Fig. 4B), germ cells in these mutant testes adopted male fate, and did not up-regulate meiosis markers *Stra8* and SYCP3 (Fig. 6F and data not shown). Instead, expression of *Nanos2* and *Dnmt3l*, markers of spermatogenic differentiation, were detected in the mutant testes at 13.5 dpc (Fig. 6A,B), confirming that germ cells in *Sox4*$^{-/-}$ fetal testes had embarked on spermatogenesis. However, both *Nanos2* and *Dnmt3l* were expressed at levels substantially lower than those in wild type testes (Fig. 6A,B), indicating that the differentiation of male germ cells was severely impaired in *Sox4*$^{-/-}$ testes.

As male germ cell fate commitment hinges on a fine balance between maintaining pluripotency and initiating spermatogenic differentiation, we analysed the expression of several genes that play key roles in maintaining the pluripotency of male germ cells, including *Nodal*, *Cripto*, *Nanog*, *Sox2* and *Oct3/4* (Spiller et al., 2012; Western et al., 2010). Our data revealed that the levels of *Cripto*, and its downstream target *Nanog* (Miles et al., 2013) were doubled at 13.5 dpc in *Sox4*$^{-/-}$ testes compared with wild type (Fig. 6D,E). Expression levels of *Nodal*, *Sox2*, and *Oct3/4* remained unchanged (Supplemental Fig. S7J-L). No significant change in *Fgf9*, encoding a Sertoli cell-secreted factor pivotal in promoting male germ cell fate (Barrios et al., 2010; Bowles et al., 2010), was found (Supplemental Fig. S7D).

Another male germ cell fate marker, *p15*^{INK4b}, is up-regulated in male germ cells at 14.5 dpc and considered to contribute to the mitotic arrest of male germ cells (Spiller et al., 2012; Western et al., 2008). Interestingly, *p15*^{INK4b} was upregulated precociously in *Sox4*$^{-/-}$ testes at 13.5 dpc (Fig. 6C), suggesting that male differentiation and mitotic arrest of germ cells may be uncoupled.

In summary, these data indicate that although SOX4 does not seem to affect the cell fate commitment of fetal germ cells, it plays an important role in promoting the spermatogenic differentiation of male germ cells.

Discussion

SOX transcription factors play crucial roles in a wide variety of developmental contexts (Kamachi and Kondoh, 2013). In the developing mouse gonads, two SOX transcription factors, SRY and SOX9, play instructive roles in male sex determination and fetal testis development (Koopman et al., 1991; Vidal et al., 2001). We show in the current study that another SOX protein, SOX4, also plays important roles in mouse fetal gonad development by modulating morphogenesis of the developing gonads and promoting male germ cell differentiation.

SOX4 modulates morphogenesis of both fetal testes and ovaries

Organ shape and size are determined through tightly regulated and highly stereotypic processes. While some progress has been made in unravelling developmental pathways regulating organ and embryo size, little is understood regarding how organ shape is regulated. Our morphometric analysis of fetal gonads reveals that both *Sox4* $^{-/-}$ testes and ovaries were longer and thinner compared with wild type at 13.5 dpc. Since mouse fetal gonads become plumper and shorter from 11.5 to 13.5 dpc (Nel-Themaat et al., 2009), one explanation is that *Sox4* inactivation may have simply caused a delay in gonad development, either cell-autonomously or secondarily to developmental defects in other organs, such as the heart. However, a number of somatic marker genes, including *Star*, *Cyp11a1*, and *Hsd3b*, that are dynamically expressed during this period (Büdefeld et al., 2009; Yao et al., 2002), showed similar expression levels in *Sox4* $^{-/-}$ and wild type gonads at 13.5 dpc, arguing against a developmental delay.

We suggest that SOX4 is part of the mechanism by the shape of fetal gonads is determined. Supporting this hypothesis, *SoxC* genes have been shown to be essential for organogenesis in mouse embryos by ensuring the survival of neural and mesenchymal progenitor cells (Bhattaram et al., 2010). *Tead2*, a direct target of SOXC proteins and a Hippo/YAP pathway component, has been suggested to mediate the pro-survival activity of SOXC proteins (Bhattaram et al., 2010). We found that *Tead2* was down-regulated only in *Sox4*$^{-/-}$ fetal testes but not ovaries, suggesting that *Tead2* may not be the main target required for mediating SOX4's activity. Alternatively, SOX4 may regulate different targets in testes (namely *Tead2*) and ovaries (other unidentified targets) to achieve similar outcomes.

Consistent with our observation that neither cell survival nor proliferation was significantly altered in *Sox4*$^{-/-}$ fetal gonads, estimates based on gonad length and width suggested that the gonad volume was not altered substantially in *Sox4*$^{-/-}$ embryos, despite significant changes observed in external profile. We therefore speculate that SOX4 modulates the gonad shape by regulating genes involved in extracellular matrix remodelling, cell-cell interaction and cell migration. Supporting this concept, genes encoding cell adhesion molecule E-Cadherin and metalloprotease ADAM19 have been identified as SOXC direct targets in heart outflow tract remodelling (Paul et al., 2014).

Mouse ovaries and testes undergo gradual changes in shape during fetal development and become oval-shaped before they descend to their final location in later embryonic life, namely the lower abdomen for ovaries and the scrotum for testes. It is conceivable that the changes in shape of gonads may facilitate their translocation across the abdominal cavity (and the abdominal wall in the case of testis descent). However, the embryonic lethality of *Sox4*$^{-/-}$ embryos at 14.5 dpc precludes us from testing this hypothesis. Conditional *Sox4* knockout

mouse models should help to evaluate this hypothesis by allowing the examination of the shape and position of gonads at later developmental stages.

Sox4 regulates male fetal germ cell development

Our data indicate that *Sox4* is required for proper progression of male germ cell differentiation but appears dispensable for female germ cell development. We found that disruption of *Sox4* in fetal testes leads to up-regulation of genes involved in maintaining pluripotency, including *Cripto* and its downstream target *Nanog*, and down-regulation of spermatogenic differentiation markers *Nanos2* and *Dnmt3l*. These results suggest SOX4 plays an important role in restricting the period of pluripotency in male germ cells by repressing the *Cripto*/*Nodal* pathway. Reduced activities of *Cripto* and its target *Nanog* should allow male germ cells to proceed with spermatogenic differentiation (Spiller et al., 2012).

How does SOX4 regulate male germ cell development? We found that *Sox4* is expressed highly in Sertoli and interstitial cells, and at a much lower level in germ cells. It is possible that SOX4 in Sertoli/interstitial cells regulates the secretion of certain signalling molecules to regulate *Cripto* expression in germ cells. Such an example has recently been described. *Emx2*, a gene expressed in Sertoli cells, regulates *Fgf9*, a Sertoli cell-secreted molecule crucial in inducing both the pluripotency genes and male differentiation markers (Barrios et al., 2010; Bowles et al., 2010; Spiller et al., 2012), to modulate male germ cell development (Tian-Zhong et al., 2016). However, *Fgf9* does not seem to be a SOX4 target, as its expression appeared unchanged in *Sox4*$^{-/-}$ fetal testes. It is possible that SOX4 may regulate unidentified signalling molecules in Sertoli/interstitial cells to fulfil this role. Alternatively,

SOX4 in germ cells, albeit at very low levels, may cell-autonomously restrict *Cripto* expression. Further studies involving Sertoli and germ cell-specific conditional *Sox4* knockout models are required to elucidate the molecular mechanisms underlying its ability to regulate *Cripto* and male germ cell differentiation.

SOXC proteins as context-dependent transcriptional repressors

Our results uncover a hitherto unappreciated role of SOXC proteins in transcriptional repression of *Sox9*, adding an extra level of control of *Sox9* expression in fetal gonad development. The negative regulation of *Sox9* by SOX4, and possibly SOX11 and -12, in fetal Sertoli cells may contribute to the homeostasis of *Sox9*. Since *SoxC* genes are highly expressed in female somatic cells, SOXC proteins may also contribute to repression of *Sox9* in fetal ovaries. However, no apparent up-regulation of *Sox9* was observed in *Sox4*$^{-/-}$ fetal ovaries. Two possibilities may account for this. First, other mechanisms to repress *Sox9* expression involving the RSPO1/WNT4/ß-Catenin or FOXL2 are in place in the fetal ovary (Greenfield, 2015). Second, SOX11/12 may function redundantly in this context to repress *Sox9*, as has been described in other developing tissues (Bhattaram et al., 2010).

SOXC proteins possess a single transactivation domain at the carboxyl-terminus and predominantly function as transcriptional activators (Wegner, 2010). Our *in vitro* reporter assays show that SOXC proteins induce TESCO activity on their own but potently repress the activation of TESCO-mediated transcription in the presence of SF1 (either SF1 alone or combined SF1 and SRY/SOX9), indicating SOXC proteins may function as transcriptional repressors in a context-dependent manner. It is unclear how SOXC proteins exert repressor activity in the absence of a transcriptional repressor domain. SOXC factors may directly

interact with SF1 and repress its ability to activate TESCO-mediated transcription. It is also possible that the formation and stabilisation of a repressor complex containing SOXC proteins and partner co-repressors (Koumangoye et al., 2015) may be facilitated by SF1. Alternatively, the binding of SOXC proteins to SOX binding motifs within TESCO may sterically hinder the binding of SF1 to its nearby binding sites. In the presence of SRY or SOX9, SOXC proteins may also compete with SRY/SOX9 for DNA binding.

We found that the down-regulation of *Sry* was delayed in *Sox4* $^{-/-}$ fetal testes. In mice, *Sry* expression is switched off only 2 days after reaching its peak (Kashimada and Koopman, 2010). The molecular mechanisms underlying the down-regulation of *Sry* are not fully understood (Larney et al., 2014). SOX9 appears to be involved, as prolonged *Sry* expression has been observed in conditional *Sox9* knockout gonads (Barrionuevo et al., 2006; Chaboissier et al., 2004). However, down-regulation of *Sry* also occurs in the ovarian portion of ovotestes where *Sox9* is not expressed, suggesting that additional unidentified factors may also contribute to down-regulating *Sry* (Wilhelm et al., 2009). We suggest that SOX4 may be a contributing factor, possibly *via* the repressor function revealed in this study.

Among SOXC proteins, SOX11 is the strongest transactivator with its transactivating capacity several times more potent than that of SOX4 (Dy et al., 2008and the present study). However, the transactivating capacity of SOXC proteins does not necessarily correlate with the severity of certain organ defects in mice deficient for *SoxC* genes. For example, the defects in heart outflow tract are more severe in *Sox4* $^{-/-}$ mice than those in *Sox11* $^{-/-}$ or *Sox4* $^{+/-}$ *Sox11* $^{+/-}$ mice (Bhattaram et al., 2010; Schilham et al., 1996; Sock et al., 2004). It is thus puzzling why *Sox4* deficiency causes the most severe phenotype if transactivation is the main function of SOXC proteins *in vivo*. It has been postulated that SOX4 may be present and

function in non-cardiac neural crest, non-mesodermal cells (that do not express *Sox11*), e.g. pharyngeal endoderm cells, during outflow tract development but direct evidence is lacking (Paul et al., 2014).

Our results from TESCO-luciferase reporter assays show that SOX4 is the most potent transcriptional repressor among SOXC factors, followed by SOX11 and SOX12. Interestingly, this order mimics the order of phenotypic severity of heart outflow tract malformation in *SoxC* knockout mouse models, raising the possibility that SOXC proteins may function as transcriptional repressors in other developmental contexts *in vivo*, and their repressor activities may contribute to the different phenotypic outcomes in knockout mouse models. Supporting this hypothesis, knockout of *Sox4* in primary mouse limb bud cells resulted in upregulation of a number of genes (Bhattaram et al., 2010); similarly, overexpression or knockdown of *Sox4* in various cancer cell lines caused down- or up-regulation of many downstream genes respectively (Vervoort et al., 2013). We suggest that mouse models carrying mutated *SoxC* alleles with the native transactivation domain replaced by an ectopic transactivation or repressor domain such as the VP16 or engrailed repressor domain (Bergsland et al., 2006; Zhao et al., 2014a), should help to solve this puzzle.

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Supporting information

Supplementary data associated with this article are available online.

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Figure legends

Fig. 1. *Sox4, Sox11 and Sox12* are expressed in the developing mouse fetal gonads. (A) qRT-PCR analyses of *SoxC* genes in CD1 mouse fetal gonads from 10.5 to 14.5 dpc. Mean ± s.e.m; $n = 3$. (B) *In situ* hybridisation of sagittal sections of XX or XY CD1 embryos at 13.5 dpc. Dashed lines delineate testis cords. Scale bar, 100 μm. (C) qRT-PCR analyses of *SoxC* genes on wild type (WT) or W^e fetal gonads at 14.5 dpc. Mean ± s.e.m; $n = 4$. (C) qRT-PCR analyses of *SoxC* genes on sorted somatic and germ cell populations from Wt1-RG fetal gonads. Cells from multiple pairs of fetal gonads were pooled and sorted for mCherry and EGFP (see also Supplemental Fig. S1). Mean ± s.e.m of triplicate qPCR reactions.

Fig. 2. *Sox4* affects the morphology of mouse fetal gonads. (A,B) Bright-field images of 13.5 dpc gonads (A) or kidneys (B) from wild type (WT) or *Sox4*$^{-/-}$ embryos. Representative images from littermate WT and *Sox4*$^{-/-}$ embryos were shown. Scale bars, 0.5 mm. (C-F) Quantitation of the length or width of fetal gonads (C,D) or kidneys (E,F). Mean ± s.d.; $n = 9$, 11, 6 or 7 (XY WT, XX WT, XY *Sox4*$^{-/-}$ or XX *Sox4*$^{-/-}$). *$P<0.05$, **$P<0.01$, multiple *t* tests. (G-J) qRT-PCR analyses of *Hippo* pathway genes on 13.5 dpc fetal gonads. *Tead2*, a known *Sox4* target gene, was significantly down-regulated in *Sox4*$^{-/-}$ testes compared with wild type (WT) controls. Mean ± s.e.m; $n = 5$. *$P<0.05$ (multiple *t* tests); ns, not significant.

Fig. 3. *Sox4*$^{-/-}$ fetal testes develop significantly more testis cords. The largest sagittal sections of 14.5 dpc wild type (WT) or *Sox4*$^{-/-}$ testes were stained with an anti-laminin antibody to reveal testis cords. (A) Representative images from littermate wild type (WT) and *Sox4*$^{-/-}$ embryos were shown. Scale bar, 100 μm. (B) Quantitation of testis cords. Mean ± s.d.; $n = 6$ (WT) or 5 (*Sox4*$^{-/-}$). **$P<0.01$, Mann-Whitney test.

Fig. 4. The differentiation of gonadal somatic cell lineages appeared unperturbed in *Sox4*$^{-/-}$ fetal gonads. (A) Histological analysis of gonadal sections at 14.5 dpc using Hematoxylin and Eosin staining. (B) Immunofluorescence analyses of 14.5 dpc gonadal sections. Sertoli cells (marked by AMH, green) and fetal Leydig cells (marked by HSD3β, magenta) were present in XY *Sox4*$^{-/-}$ testes, while granulosa cells (marked by FOXL2, magenta, bottom panel) were present in XX *Sox4*$^{-/-}$ ovaries. Nuclei were counterstained with DAPI (blue). Dashed lines delineate gonad perimeters. Scale bars (A,B), 50 μm. (C-H) qRT-PCR analyses of additional somatic cell lineage markers at 13.5 dpc. Expression of testicular somatic cell markers (C-F) and an ovarian marker *Fst* (H) remained unchanged in *Sox4*$^{-/-}$ gonads compared with wild type (WT). Another ovarian marker *Wnt4* (G) was slightly up-regulated in *Sox4*$^{-/-}$ ovaries. Mean ± s.e.m; $n = 5$. **P<0.05* (multiple *t* tests); ns, not significant.

Fig. 5. SOX4 negatively regulates *Sry* and *Sox9* in mouse fetal gonads. (A,B) qRT-PCR analyses on 13.5 dpc gonads revealed significantly higher levels of *Sry* (A) and *Sox9* (B) in *Sox4*$^{-/-}$ testes compared to wild type. Mean ± s.e.m; $n = 5$. **$P<0.01$, ***$P<0.001$, multiple *t* tests. (C) SOXC proteins strongly repressed the activation of TESCO by SF1, SRY and SOX9. Reporter assays were performed in HEK293 cells. The activity of TESCO-Luc co-transfected with the empty vector in the absence of SF1 was set to 1. For simplicity, only the +SF1 data are presented here, as mean ± s.e.m ($n = 3$). **$P<0.01$, one-way repeated measures ANOVA with Holm-Sidak multiple comparisons test.

Fig. 6. *Sox4* regulates differentiation of male fetal germ cells. (A-F) qRT-PCR analyses of markers of germ cell development on 13.5 dpc gonads. Mean ± s.e.m; $n = 5$. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (one-way ANOVA with Sidak multiple comparisons test); ns, not significant. (G) Immunofluorescence showing germ cells (marked by DDX4, magenta) in

both wild type and *Sox4* $^{-/-}$ ovaries expressed SYCP3 (green), a meiotic marker. Nuclei were counterstained with DAPI (blue). Scale bar, 50 μm.

Highlights

- *SoxC* genes are expressed in mouse fetal gonads, predominantly in somatic cells.
- SOX4 activity influences organ shape in developing ovaries and testes.
- SOX4 activity also promotes the differentiation of male germ cells.
- SOX4 both activates and represses transcription depending on context.

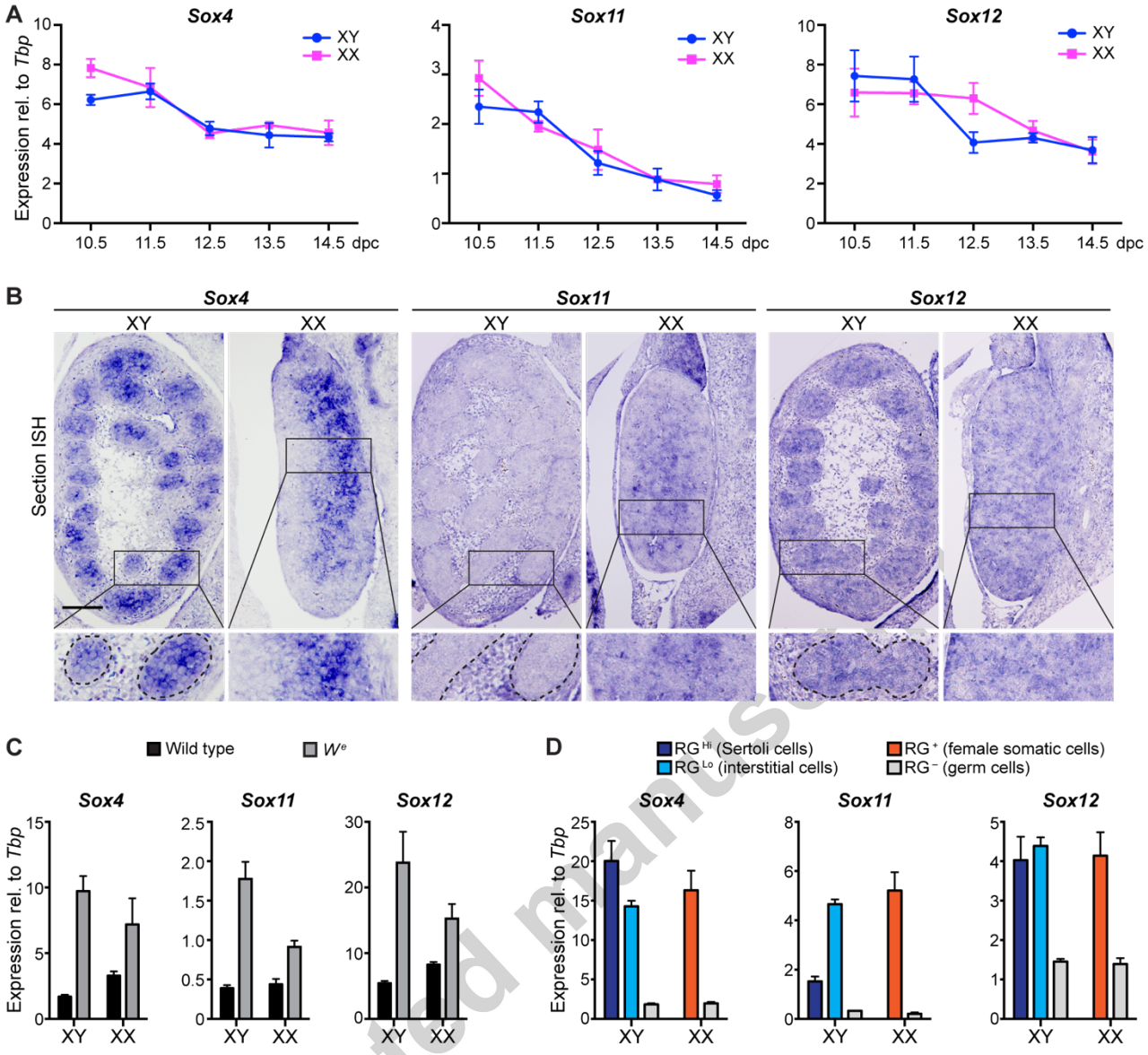

Figure 1

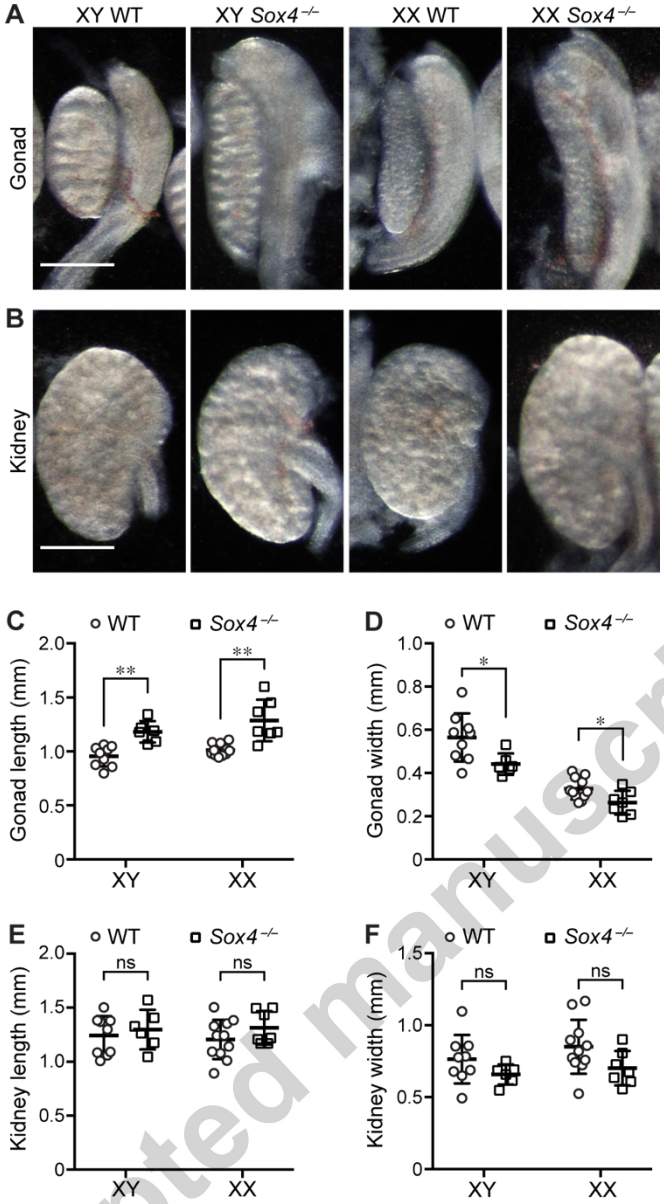

Figure 2

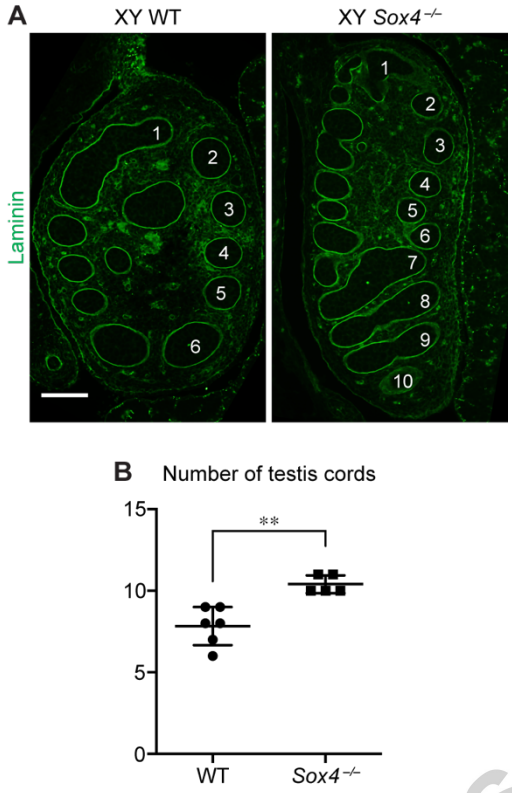

Figure 3

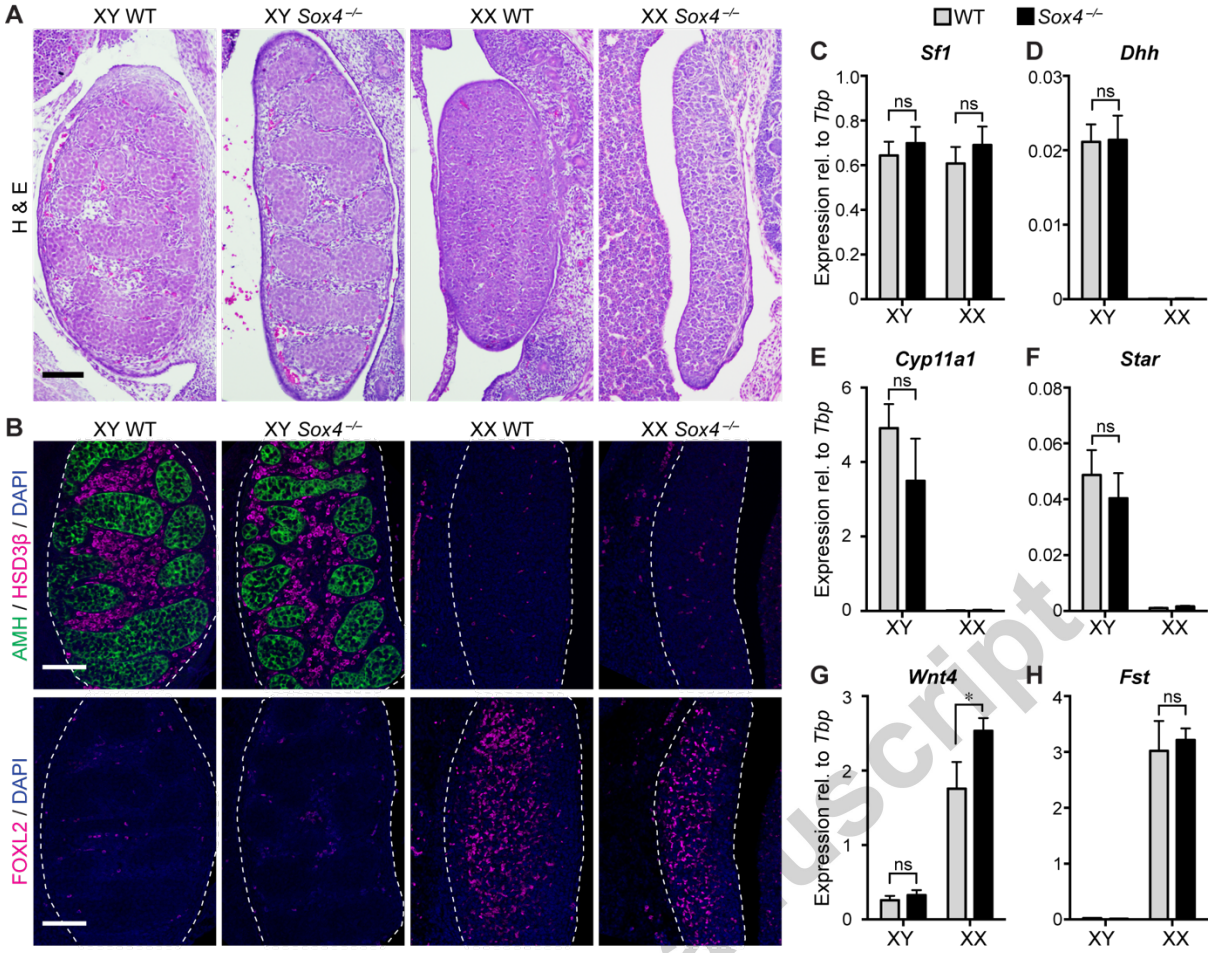

Figure 4

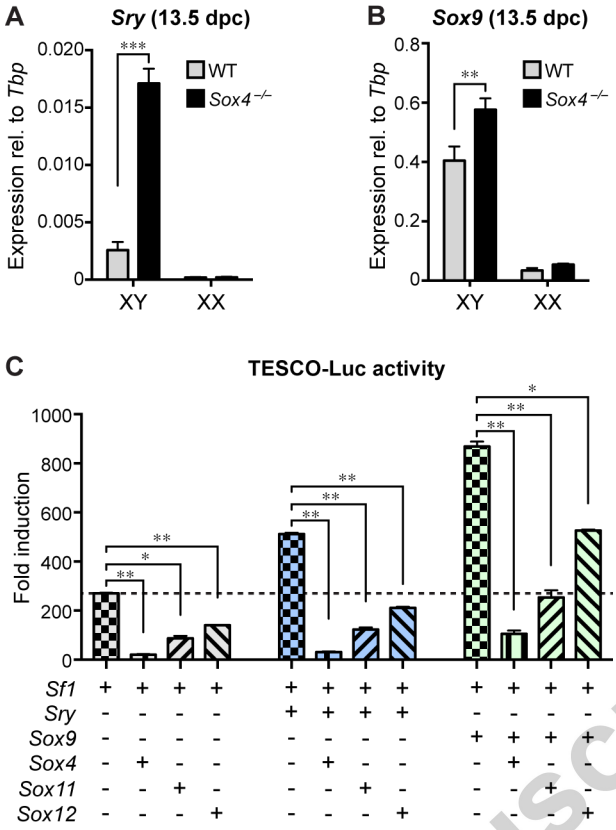

Figure 5

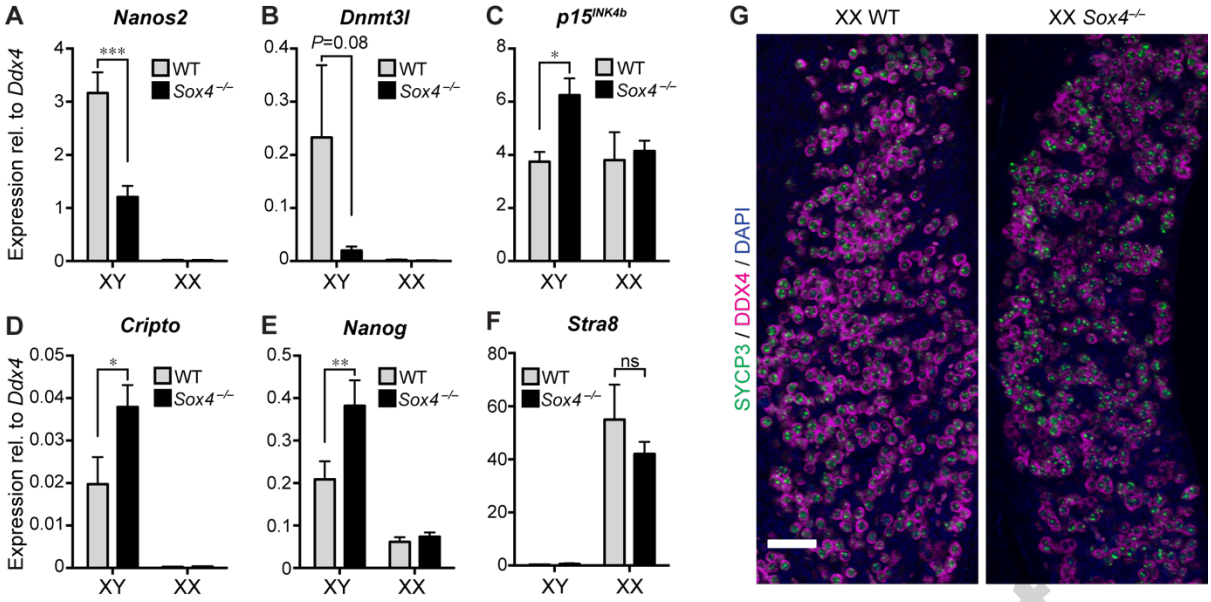

Figure 6