Sox10 gain-of-function causes XX sex reversal in mice: implications for human 22q-linked disorders of sex development

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INTRODUCTION

Disorders of sex development (DSD) encompass a wide spectrum of congenital conditions in which the chromosomal, gonadal or anatomic sex is atypical (1). Many DSDs result from either gain or loss-of-function of genes that regulate sex determination, the suite of molecular and cellular events that lock the embryonic bipotential gonad into a testicular or an ovarian fate (2,3). In most mammals, including humans, the Y-chromosomal master gene that controls testis fate is Sry. The ability of Sry to trigger testis formation was demonstrated by the addition of Sry function to an XX transgenic mouse, which developed testes as a result (4). Mutations in human SRY result in 46,XY DSD (XY femaleness) (5), although translacation of SRY onto an X-chromosome or autosome is the most common cause of 46,XX DSD (XX maleness) (6,7).

The action of SRY protein results in the up-regulation of Sox9 (Sry-related HMG-box gene 9) (3,8) which, like Sry, is a member of the Sox gene family (9). Previous studies have established a pivotal role for Sox9 in male sexual development. Ablation of Sox9 in mice causes male-to-female phenotypic sex reversal (10,11); in humans, SOX9 mutations cause campomelic dysplasia (OMIM® 114290), a skeletal disorder associated with 46,XY DSD in ~75% of XY patients (12–15). Conversely, SOX9 gain-of-function, such as duplication of the SOX9 locus in humans and over-expression in transgenic mice, causes testis formation in XX individuals (16,17), demonstrating that Sox9 is not only necessary but also sufficient to drive testis formation.

Aside from translocation of SRY to an X-chromosome, few causes of 46,XX DSD have been identified in humans. In theory, these can be caused by loss-of-function of female sex-determining genes, as is the case with mutations in R-spondin1 (RSPO1) (18,19), or gain-of-function of male sex-determining genes, as has been described for a patient mosaic for a duplication of SOX9 (17).

Masculinized or incompletely feminized XX patients have been described in a number of cases with full or partial duplication of chromosome 22. Nicholl et al. (20) described a masculinized XX trisomy 22 patient with male external genitalia including micropenis, but internally presenting a small...
vagina and uterus. Both gonads were located in the abdominal cavity and contained testicular tissue. Triploidy for 22q12–22qter has been described in a girl with ovarian dysgenesis (21). Another patient with an inverted duplication of 22q13.1–22qter presented externally with ambiguous genitalia, and internally with rudimentary vagina and uterus, accompanied with gonadal asymmetry, one gonad containing ovarian tissue and the other testis-like structures (22). Finally, duplication of 22q11.2–22q13 was reported in a patient with almost complete XX sex reversal, showing only mild hypospadias (23). The genetic basis of the DSD phenotype has not been established in any of these cases.

SOX10, a gene closely related to SOX9 and SOX8 (9), maps to chromosome 22q13.1 in humans and has been suggested as a candidate for 46,XX DSD (23). SOX10 is known to be involved in neural crest and glial development (24), and is mutated in Waardenburg–Shah syndrome (OMIM® 277580; Ref. 25), Yemenite deaf-blind hypopigmentation syndrome (OMIM® 601706; Ref. 26) and Waardenburg syndrome type IIE (OMIM® 611584; Ref. 27). However, a functional role for Sox10 in gonadal development has not been established, presumably because loss-of-function during gonadal development is likely to be masked by the action of Sox9 and/or Sox8. Here, we demonstrate, using a transgenic mouse approach, that Sox10 over-expression causes XX sex reversal, suggesting that human SOX10 is involved in 22q13-linked 46,XX DSD.

RESULTS

Sox10 expression in developing gonads supports a role in testis differentiation

We first investigated the expression of endogenous Sox10 during gonad development in wild-type mice. Whole mount in situ hybridization (WISH) and quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR) analyses showed that, similar to Sox9 (28) and Sox8 (29), Sox10 is expressed at low levels in both XX and XY mouse gonads at 10.5 days post coitum (dpc), prior to sex differentiation (Fig. 1A). Sox10 expression became XY-specific in gonads from 11.5 dpc, and was associated with testis cords once these had differentiated at 12.5 and 13.5 dpc (Fig. 1A and B). Sox10 protein was detected in nuclei of Sertoli cells (Fig. 1C), WISH analysis of testes from W/W° mice (Fig. 1D and E), which lack germ cells (30), further indicated that Sox10 is expressed by Sertoli cells in the testis cords. However, expression of Sox10 mRNA was weaker than that of Sox9 or Sox8, judged by quantitative real-time RT–PCR using primers of similar efficiency for all three genes (Fig. 1F). The Sertoli cell-specific expression of Sox10 after 11.5 dpc suggests a role in testis differentiation, although the expression in both sexes at earlier stages is consistent with the hypothesis that duplication of SOX10 causes expression of this gene in XX genital ridges to exceed a threshold required for testis development.

SOX10 protein can regulate transcriptional targets of SOX9 in vitro

Given the similarity between SOX9 and SOX10 proteins, and the expression of these proteins in the same cell type in the developing testis, we next investigated whether SOX10 is able to regulate targets of SOX9 in vitro. Amh, encoding anti-Müllerian hormone, is important for male development, and its transcription is directly regulated by SOX9 and steroidogenic factor-1 (SF1) in vitro (31) and in vivo (32). Using transient transfection assays in HEK-293 cells, we found that Sox10, in synergy with SF1, strongly trans-activated the Amh promoter (Fig. 2A). In addition, recent evidence suggests that SRY activates Sox9 transcription by means of a testis-specific enhancer (TES) upstream of Sox9, and that TES is bound also by Sox9, which cooperates with SF1 to maintain Sox9 expression in a positive feedback loop (8). We found that the core element of TES (TESCO) was activated by Sox10 in vitro (Fig. 2B), less robustly than the activation by Sox9, but more so than SRY. Together, these results demonstrate that Sox10 is able to induce the expression of genes important for male development.

Sox10 overexpression causes XX sex reversal in transgenic adult mice

Having shown that Sox10 has similar expression and biochemical characteristics to Sox9, we next sought to determine whether over-expression of Sox10 might be sufficient to induce testis differentiation on an XX background. To this end, we over-expressed Sox10 under the control of the regulatory region of the Wilms’ tumor suppressor gene Wt1 using bacterial artificial chromosome (BAC) recombineering (33) and mouse transgenesis (Supplementary Material, Fig. S1). We chose the regulatory region of Wt1 because this gene is expressed in XX and XY genital ridges from an early stage (34), and because these sequences have been used to demonstrate XX sex reversal resulting from Sox9 over-expression (16). In this way, we generated two transgenic mouse lines (tg° and tg°°) that stably transmitted a Wt1:Sox10-ires-eGFP transgene. Both lines expressed the transgene in developing gonads from 10.5 dpc (Supplementary Material, Fig. S2A), and co-expressed the eGFP and SOX10 proteins (Supplementary Material, Figs S2 and S3).

Complete female-to-male sex reversal was observed in adult XX Wt1:Sox10 transgenic mice (n = 8/25 for tg°, n = 12/12 for tg°°). The complete sex reversal in both tg° and tg°° lines was characterized by male external genitalia (penis, scrotum, anogenital distance) that were indistinguishable from those of wild-type XY mice (Fig. 3A and B), and the presence of testes and male reproductive tracts (epididymes, vasa deferentia, seminal vesicles; Fig. 3E and F). Testes had well-defined cords (Fig. 31 and J) and interstitial Leydig cells expressing the marker p450Scc (Scc; Fig. 3M and N), but lacked germ cells, a phenotype also observed in sex-reversed XX mice that either express Sry (4) or Sox9 (16) as a transgene.

Not all XX tg° mice were completely masculinized. These mice (n = 17/25) exhibited a number of sex differentiation anomalies involving external and internal genitalia, including a closed vagina and distended uterine horns (Fig. 3C and D). Gonadal development was asymmetrical in these animals: their left gonads were either atrophic or partially masculinized, although their right gonads were ovary-like but containing many conspicuous hemorrhagic follicles (Fig. 3G and H), a reduced number of follicles and oocytes (Fig. 3K and L),
Figure 1. Expression of Sox10 in developing mouse gonads. (A) qRT–PCR analysis of Sox10 expression in wild-type XX (pink bars) and XY (blue bars) gonads at 10.5, 11.5 and 12.5 dpc. Data are from three independent samples each assayed in triplicate. Expression levels were measured relative to 18S, and are shown with levels of Sox10 expression in 12.5 dpc testes set to 100%. Error bars represent one standard deviation. (B) Whole-mount in situ hybridization (WISH) for Sox10 from 11.5 to 13.5 dpc (g, gonad; m, mesonephros). (C) Immunohistochemistry for SOX10 protein at 13.5 dpc on testis sections from wild-type XY mice. Arrowheads indicate SOX10 in nuclei of Sertoli cells (Scale bar, 100 μm). (D) WISH analysis comparing Sox10 expression in 13.5 dpc testes from wild-type (Wt) and W/W XY testes lacking germ cells. (E) qRT–PCR analysis of Sox10 (grey bars) and Oct4 (germ cell marker, red bars) expression in 13.5 dpc wild-type (Wt) and W/W XY gonads by qRT–PCR. Levels of Sox10 and Oct4 expression in 13.5 dpc wild-type testes set to 100%. (F) Sox9 is expressed at relatively higher levels than Sox8 and Sox10 in testes from wild-type mice. Levels of Sox9 in 13.5 dpc testes set to 100%. qRT–PCR data were obtained from three independent samples, each assayed in triplicate. Expression levels were normalized using 18S. Error bars represent one standard deviation.

Figure 2. SOX10 regulates transcriptional targets of SOX9 in vitro. (A) Co-transfections of mouse SOX9, SOX8, SOX10, SRY and SF1 expression constructs with an Amh luciferase-reporter construct into HEK-293 cells. Expression constructs transfected are indicated at left. SOX10, -9 and -8 synergized with SF1 to trans-activate the Amh promoter with similar efficiency. Fold activation was calculated relative to the luciferase activity obtained from transfection with pGL3-Amh-LUC alone. (B) SOX10 synergizes with SF1 to trans-activate the testis-specific enhancer core (TESCO) of Sox9. SOX9 trans-activated more efficiently than SRY, SOX8 or SOX10. Fold activation was relative to transfection with pTESCO-51-LucII only. All data were compiled from three independent experiments, each performed in triplicate. Error bars show one standard deviation.
and absence of corpora lutea (Fig. 3O and P). In the absence of complete XX sex reversal, ectopic expression of Sox10 had evidently interfered with the development and hormonal cycle of the ovaries, thereby preventing normal female differentiation.

**Sox10 causes complete or partial primary sex reversal in fetal XX gonads**

To investigate the etiology of Sox10-induced sex reversal and address the mechanism of SOX10 action during sex development, we analyzed the effect of the Wt1:Sox10 transgene on primary differentiation of the fetal gonads (Fig. 4). At 13.5 dpc, 100% of XX tg<sup>W</sup> mice showed complete sex reversal (Fig. 4B) and wild-type XX mice at 6 weeks of age. External genitalia were identical between XY (A) and some XX transgenic mice (XX tg<sup>W</sup> complete) (B). Other XX tg<sup>W</sup> mice showed partial sex reversal (C), resembling wild-type XX mice (D) in urogenital distance but exhibiting an imperforate vagina. (E, F) Reproductive tracts dissected from wild-type and Wt1:Sox10 transgenic mice. XX tg<sup>W</sup> mice with complete sex reversal (F) showed the entire characteristics of the male phenotype, comprising a fully differentiated testis, epididymides, vasa deferentia and seminal vesicles, as found in XY males (E). XX tg<sup>W</sup> partially sex-reversed mice exhibited abnormal distended uterine horns along with several hemorrhagic follicles exclusively in the right gonad (G and inset). The left gonad was small compared with wild-type ovaries (H), resembled an ovotestis and lacked an oviduct (G, inset). (I–L) Hematoxylin and eosin histological analysis of gonadal sections. Low magnification image at left (scale bar, 200 μm), higher magnification at right (scale bar, 100 μm). XY testis (I) shows seminiferous tubules with presence of several stages of male gametogenesis, contrasting with XX tg<sup>W</sup> complete sex reversal testes lacking germ cells (J). XX ovaries (L) exhibit several stages of follicular genesis along with a number of oocytes. XX tg<sup>W</sup> partial sex reversal gonads showed a reduced number of oocytes, and abundant hemorrhagic follicles in the right gonad (K). Occasionally some oocytes were still present within these damaged follicles. (M–P) Section in situ hybridization for detection of Scc mRNA, a marker of Leydig cells (63). XY testis (M) and XX tg with complete sex reversal (N) expressed Scc abundantly in Leydig cells of the interstitium, adjacent to seminiferous tubules (scale bar, 100 μm). In XX adult ovary (P), theca and granulosa cells normally express Scc (64). During ovarian follicle maturation the granulosa cells differentiate into highly steroidogenic luteal cells (scale bar, 200 μm). XX transgenic gonads with partial sex reversal (O), did not show expression of Scc in granulosa cells nor development of corpora lutea. This phenotype was consistent in both the hemorrhagic right ovary (not shown) and in the atrophic left gonad shown (O; scale bar, 200 μm) (B, bladder; G, gonad; Gr, granulosa cells; Hf, hemorrhagic follicle; L, left; Le, Leydig cells; Lu, corpus luteum; O, ovary; Oc, oocyte; R, right; St, seminiferous tubules; Sv, seminal vesicle; T, testis; Th, theca cells; U, uterus).
Figure 4. Markers of sexual development in fetal gonads of Wt1:Sox10 transgenic mice. (A–D) Double immunofluorescence for SOX9 (green; Sertoli cells) and E-cadherin (E-CAD; red; germ cells, mesonephric tubules) on sagittal sections of gonads from wild-type fetuses (XY, XX), and XX transgenic fetuses from the W and D lines (XX tg^W, XX tg^D) at 13.5 dpc. Wild-type XY and XX tg^D testes showed conspicuous testis cords (arrows) with strong expression of SOX9 protein, a marker of Sertoli cell development. Some SOX9 expression was observed in XX tg^W gonads. (E–H) Immunofluorescence analysis of FOXL2 (green), a marker of the ovarian pathway. FOXL2 was not detected in XX tg^D testes, but was expressed in XX tg^W intersex gonads. (I–L) Detection of Scc transcripts, a marker of fetal Leydig cell differentiation, by SISH. Scc transcripts were observed XX tg^D testes, but not XX tg^W gonads. (M–P) Double immunofluorescence for SCP3 (green; meiotic germ cells) and OCT4 (red; germ cells) showed that some germ cells had started to enter meiosis in wild-type XX ovaries (P). XX tg^W gonads showed reduced expression of SCP3 (O) and no SCP3 expression is detected in XX tg^D testes (N). Scale bar, 100 μm.
Fig. S2E), did not form well-defined testis cords (Fig. 4C), and contained a large number of FOXL2-expressing cells similar to those seen in wild-type XX ovaries (Fig. 4G). However, these gonads also showed clustered distribution of germ cells surrounded by SOX9-positive cells (Fig. 4C), indicative of Sertoli cell differentiation and therefore partial primary sex reversal. However, Leydig cells expressing Sox9 were absent at this stage (Fig. 4K). Fewer germ cells expressing SCP3 than in wild-type XX gonads were also observed (Fig. 4O and P). These observations suggest that a lower number of SOX9-expressing cells during fetal stages of XX tg_w mice compared with XX tg_D mice may explain the differences in phenotypes observed postnatally.

Quantitation of male and female markers by qRT–PCR at 13.5 dpc confirmed that XX tg_D testes (complete sex reversal) expressed Sox9 at similar levels to those seen in wild-type XY testes, whereas XX tg_w gonads (partial sex reversal) expressed Sox9 at a much lower level (Fig. 5A). In agreement with our immunofluorescence analyses, Foxl2 expression levels in XX tg_D gonads were negligible, comparable with levels seen in wild-type XY gonads, but were only partially reduced in XX tg_w gonads compared with wild-type XX gonads (Fig. 5B). Sox9 target genes such as Amh (31,32) and Ptgds (37) were expressed in XX tg_D but not in XX tg_w gonads, suggesting that SOX9 expression did not reach the threshold level necessary for the activation of these markers (Fig. 5C and D). Interestingly, expression levels of Amh and Ptgds were elevated in XX tg_D and XY tg_D gonads beyond the levels seen in wild-type XY gonads (Fig. 5C and D), presumably as a result of the additional Sox10 transgene expression, implying that SOX10 protein can directly up-regulate these transcriptional targets of SOX9 in vivo. Similarly, SOX10 also substituted for SRY in triggering the up-regulation of Sox9.

The degree of sex reversal correlates with Sox10 expression levels

To determine the molecular basis of the differences in penetrance and expressivity between the two transgenic lines, we examined expression levels of the transgene in each line. Fluorescence resulting from expression of eGFP (included in the transgenic constructs as IRES-eGFP, Supplementary Material, Fig. S1) was more intense in XX tg_D gonads compared with XX tg_w (Supplementary Material, Fig. S2C and D). Further, qRT–PCR confirmed that total (transgenic + endogenous) Sox10 expression in XX tg_D gonads was approximately 5-fold higher than in XX tg_w gonads (Fig. 5E), demonstrating that the system is sensitive to dosage and that higher levels of Sox10 cause more complete sex reversal.
DISCUSSION

In the present study, we demonstrate that Sox10 over-expression causes complete or partial XX sex reversal in transgenic mice, assessed at anatomical, histological and molecular levels. The degree of sex reversal correlated with expression levels of the Sox10 transgene, and SOX10 was shown to activate directly components of the male sex-determining pathway. These findings have several implications for our understanding of normal sex development and of the potential causes of human DSD.

Previous analysis of gene expression in gonads of Sox9 conditional knockout mice has revealed SOX9-dependent expression of Sox8 (11). In the peripheral nervous system, conserved enhancers of Sox10 can be bound directly by SOX9 protein (38), which raises the possibility that Sox10 may be transcriptionally regulated by SOX9 in developing testes. Here, we showed that the reverse is also true: SOX10 and SOX8 proteins individually can synergize with SFI1 to trans-activate a testis-specific enhancer of Sox9. Furthermore, our XX Sox10-overexpressing mice showed expression of endogenous Sox9, indicating that SOX10 protein activated Sox9 transcription in vivo. Our data, combined with published evidence, suggest that a complex positive feedback network involving Sox8, -9 and -10 is active during testis determination, perhaps providing a reinforcement or canalization mechanism for testis development.

Our data extend a previous report of Sox10 expression in the developing mouse testis (39), and indicate that Sox10 is expressed with a similar temporal profile and cell type-specificity to Sox9 (28) and Sox8 (29) during testis development. The overlapping male-specific expression patterns of Sox9, -8 and -10 in mice, along with their close phylogenetic relationship, raise the possibility that a common mechanism may be involved in the coordinated up-regulation of these three genes in the developing gonads, perhaps initiated by SRY and then maintained by a positive feedback between Sox8, -9 and -10 once SRY expression has ceased.

Our data also suggest that these three genes may act redundantly in testis development, activating a similar suite of target genes. However, Sox9 loss-of-function has a severe testis phenotype in mice (10), whereas Sox8 (40) and Sox10 (41) loss-of-function does not. We found that Sox9 is expressed at higher levels than Sox8 and Sox10, which may explain the relative importance of Sox9 in testis determination. It will be of interest to determine whether the relative expression levels of the three genes are similar in developing human testes, given that 50% reduction of SOX9 levels causes XY sex reversal in humans (13,42), whereas more than 70% reduction in Sox9 levels is necessary to generate XY sex reversal in mice (10,43). It also remains to be tested whether the differences in Sox8, -9 and -10 mRNA levels are translated into differences in their respective protein levels. Alternatively, the apparently greater importance of Sox9 may indicate that it has one or more key targets that are not shared by Sox8 or -10, or is more efficient than either Sox8 or -10 in activating common targets.

Chaboissier et al. (11) showed that incomplete deletion of Sox9 in mice resulted in only partial XY femalelessness, but when the same mutation was combined with a Sox8-null background, complete sex reversal was observed, confirming genetic cooperation between Sox9 and Sox8. Our observations raise the possibility that Sox10 may have a similar ancillary or backup role to that proposed for Sox8 (44). Double knockout mice lacking the function of both Sox9 and Sox8 in the gonads retain some expression of the male-specific SOX9/SOX8 target gene Amh (45), and our present data suggest that this residual Amh expression is likely caused by Sox10 in these gonads. In a similar manner, gene redundancy involving SOX8 and/or SOX10 may contribute to the incomplete penetrance of XY femaleness caused by mutations in SOX9 in campomelic dysplasia patients (12,13,15). The present study provides an example of how association between a given gene and human disease might be established using over-expression analysis, in a situation where loss-of-function analysis in mouse models is unlikely to be informative.

Over-expression of Sry (4), Sox9 (16) or Sox10 (this study) is able to elicit XX sex reversal in mice, and is likely that other SOX factors could trigger testis differentiation, provided that they are expressed at sufficient levels within a given window in the genital ridge (3,46). In support of this possibility, over-expression of a transgene carrying a replacement of the Sry HMG box with that of Sox3 also results in female-to-male sex reversal (47). Together, these studies suggest that genetic lesions resulting in over-expression of other SOX genes during gonadal development have the potential to cause human 46,XX DSD, and may contribute to the 20% of cases of human SRY-negative XX maleness (7).

The data reported here indicate that levels of expression are important in determining the gonadal phenotype in situations involving SOX10 gain-of-function: the mouse line exhibiting higher level of Sox10 expression (tg10) was 100% efficient in triggering complete testis differentiation, whereas the tgW line, expressing lower levels of the transgene, showed complete sex reversal in only 30% of mice postnatally. However, all tgW fetuses were able to initiate Sertoli cell differentiation to some extent, as judged by the presence of SOX9-expressing cells in XX transgenic gonads at 13.5 dpc. In these gonads, cells committed to the female pathway, identified by expression of FOXL2, were found to be intermingled with SOX9-positive cells as was recently described in human ovotestes (48), and in a mouse model of ovotestis development (49). The final sex of the gonad is likely to depend on the balance of cells committed to the male and female pathways (50), with intermediate and atrophic phenotypes possible where neither pathway dominates. In that light, expression levels of the Sox10 transgene in tgD mice were evidently high enough to act dominantly on promoting testis differentiation, whereas in tgW fetuses, Sox10 expression levels appeared to be within the range of competition between the male and female pathways, a competition that was not resolved at 13.5 dpc but later resulted in a range of phenotypes observed postnatally.

A number of studies have described increased copy number involving chromosome 22q in human XX DSD (20–23). In particular, the region of overlap between two patients duplicated for 22q13.1–22qter (22) and 22q11.2–22q13 (23), respectively, implicates a gene or genes in 22q13. The study by Seeherunvong et al. (23) showed that a 22q13 marker (STS WI-941 or D22S688), adjacent to the telomere and
distal to **SOX10**, and a 22q13.1 marker (D22S691), proximal to **SOX10**, were duplicated in this patient, whereas a 22q11.2 marker (D22S75), was not duplicated.

Barring the possibility of an interstitial deletion of **SOX10** in this patient in addition to the large duplication, these mapping data indicate that **SOX10** lies within the duplicated region in this patient.

Although it is difficult to positively identify any one gene as being responsible for an XX DSD phenotype associated with increased copy number of the gene-rich chromosomal region 22q13, several predictions can be made that must apply to any candidate. First, the gene would be expected to be expressed at low levels during female gonadal development and at higher levels during male gonadal development, so that increased copy number in females causes expression to exceed a threshold required to induce testis development. Secondly, the gene must act in a dosage-sensitive manner. Thirdly, the product of the gene must be demonstrably capable of activating components of the male sex-determining pathway. Finally, gain-of-function of that gene alone would be predicted properties of a gene for which gain-of-function would likely cause XX DSD.

Importantly, over-expression of **SOX10** alone in mice was able to mimic the XX DSD phenotypes associated with duplication of human chromosome 22 (20–23). The spectrum of pathways. Finally, gain-of-function of that gene alone would be expected to result in a phenotype resembling increased copy number of the chromosomal region in which the gene resides. Our present results establish that **SOX10** has all the predicted properties of a gene for which gain-of-function would likely cause XX DSD.

**Materials and Methods**

**Mouse strains and husbandry**

Protocols and use of animals in this project were undertaken with approval by the Animal Welfare Unit of the University of Queensland, registered as an institution that uses animals for scientific purposes under the Queensland Animal Care and Protection Act (2001). Timed matings were carried out with 0.5 dpc designated as noon on the day of detection of the copulatory plug. For expression studies, Swiss Quackenbush outbred females were mated with males expressing an X-linked eGFP transgene (53) for sexing embryos at 11.5 dpc. Other developmental stages were obtained from Swiss CD1 outbred mice. *We/We* (30) were maintained as an inbred colony.

**Plasmid and BAC sources**

Plasmids pcDNA.HA-**SOx10**, pcDNA.HA-**Sox9** (54), pcDNA.HA-**Sox8** (29), pcDNA.HA-**Sry** (54) and pcDNA.**Sf1** (29) were obtained by direct cloning of their respective cDNAs in the expression vector pIRES2-EGFP (Clontech), reporter vector pGL3-basic (Promega) and pTK-beta-galactosidase control vector. Plasmid pTESCO-51-LucII was kindly provided by Robin Lovell-Badge and Ryohei Sekido (MRC National Institute for Medical Research, London, UK). *Wt1* BAC, RP24-67H19, was obtained from the Children’s Hospital Oakland Research Institute (CHORI, USA). This BAC-*Wt1* contains the 160 223 bp *Wt1* gene locus (nucleotide position 104 821 343 bp to 104 981 566 bp, on mouse chromosome 2), encompassing the 5’-regulatory region, coding sequence and 3’-UTR. Plasmid pGalK was obtained from the National Cancer Institute, at Frederick USA, as part of a kit designed for recombineering (33).

**Construct BAC-**Wt1p-HASox10-IRES-EGFP**

BAC-*Wt1* was modified by recombineering techniques that use the **galK** gene as reported by Warming et al. (33), to generate an HA-tagged version of **Sox10** followed by an IRES-eGFP under the control of the *Wt1* regulatory region (Supplementary Material, Fig. S1). Protocols 1 and 3 were followed as instructed on the recombineering website of National Cancer Institute, at Frederick USA (http://recombineering.ncifcrf.gov/Protocol.asp). As a first step, BAC-*Wt1*-GalK was generated by insertion of **galK** gene using 50 bp homology arms (Primer pair A, Supplementary Material, Table S1). **galK** gene insertion was accompanied by a deletion of 453 bp, from the start codon of **Wt1** up to 60 nt inside of intron 1. A shuttle vector (pW1), derived from pIRES2-EGFP, was designed containing about 500 bp homology arms aligning to **Wt1** on the same DNA sequences used to insert **galK** gene (Supplementary Material, Fig. S1B). **Sox10** cDNA was cloned into pWT1 (Supplementary Material, Fig. S1C), to generate the counter-selection cassette for a second round of recombineering to replace the **galK** gene (33), generating BAC-*Wt1p-HASox10-IRES-EGFP*.

**Generation of BAC transgenic lines**

BAC-*Wt1p-HASox10-IRES-EGFP* was extracted and purified with NucleoBond Xtra Maxi columns (Macherey-Nagel). Standard pronuclear microinjections into zygotes of CBA × C57BL/6 crosses were used to generate transgenic founders. Microinjection buffer composition was 10 mM Tris–HCl, pH7.5, 0.1 mM EDTA, 30 μM spermine, 70 μM spermidine, 100 mM NaCl. This polyamines-containing buffer is more likely to produce transgenic mice with intact, unfragmented large DNA molecules (55).
Genotyping

In order to identify mice transgenic for BAC-Wt1p-HA-Sox10-1RES-EGFP, mice were genotyped for the presence of eGFP gene (Primer pair B, Supplementary Material, Table S1), and for the presence of WT1-Sox10 (Primer pair C, Supplementary Material, Table S1) at 58°C annealing, in presence of 5% (v/v) DMSO for the WT1-Sox10 PCR. In addition, genetic sexing of embryos was performed by amplification of the Zfy gene (56) and of Ube-X gene (57).

Immunofluorescence staining of embryo sections

Embryos were dissected at 13.5 dpc and fixed in 4% (w/v) paraformaldehyde (PFA) at 4°C overnight, and then washed with 1× phosphate buffered saline (PBS). Embryos were embedded in either paraffin or OCT (Tissue Tek) as previously described (58). Paraffin sections (7 μm) or cryo-sections (10 μm) were mounted on Superfrost Plus slides (Menzel-Glaser), and processed for immunofluorescence as previously described (58). When immunohistochemistry was carried out with sections, detection was performed with VECTASTAIN Elite ABC Kit (PK-6100, Vector) and DAB substrate kit (SK-4100, Vector) according to manufacturer’s guidelines. Primary antibodies against endogenous mouse antigens used for this study were: mouse anti-OCT4 (SC-5279, Santa Cruz), mouse anti-E-Cadherin (Becton Dickinson), rabbit anti-SCP3 (ab15092, ABCAM), rabbit anti-FoxL2 generated as described (58). Paraffin sections (7 μm) or cryo-sections (10 μm) were mounted on Superfrost Plus slides (Menzel-Glaser), and processed for immunofluorescence as previously described (58). When immunohistochemistry was carried out with sections, detection was performed with VECTASTAIN Elite ABC Kit (PK-6100, Vector) and DAB substrate kit (SK-4100, Vector) according to manufacturer’s guidelines. Primary antibodies against endogenous mouse antigens used for this study were: mouse anti-OCT4 (SC-5279, Santa Cruz), mouse anti-E-Cadherin (Becton Dickinson), rabbit anti-SCP3 (ab15092, ABCAM), rabbit anti-FoxL2 generated as described in (59), rabbit anti-GAP (A-6455, Invitrogen), rat anti-HA (11867423001, Roche), rabbit anti-SOX9 (58) and guinea pig anti-SOX10 (a kind gift of Michael Wegner). Secondary antibodies were anti-mouse Alexa 594 (A-11005, Invitrogen), anti-rabbit Alexa 488 (A-11034, Invitrogen), goat anti-guinea pig Alexa 594 (A-11076, Invitrogen), biotinylated anti-goat (705-065-003, Jackson Laboratories) and biotinylated anti-rabbit (RPN480, Amersham). 4′,6-Diamidino-2-phenylindole (DAPI, from Molecular Probes, 2 ng/μl in PBS), was used to visualize nuclear DNA in immunofluorescence using a Zeiss LSM 510 Meta confocal microscope.

Luciferase reporter assays

A 360 bp Amh promoter cloned into pGL3-basic (29), and the TES core enhancer (TESCO) cloned in p-κB1-LuciII (8), were used in co-transfection assays to test luciferase reporter activity mediated by transcription factors. Transfections in HEK-293-T cells (human embryonic kidney cells) for the assay with Amh promoter and COS-7 (African green monkey kidney cells) for the assay with TESCO enhancer, were performed in triplicate in 12-well plates, using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. About 0.4 μg of reporter plasmid (pGL3-Amh-LUC or pTESCO-κB1-LuciII respectively) was co-transfected with equal amounts (0.1 μg for Amh promoter and 0.2 μg for TESCO) of full-length expression constructs (pcDNA.HA-Sox9, pcDNA.HA-Sox8, pcDNA.HA-Sox10, pcDNA.HA-Sry and pcDNA-Sf1), along with 0.8 μg pTK-beta-galactosidase per well. The final amount of plasmid DNA was adjusted to 1.6 μg per well, using empty pcDNA.HA vector. Cells were harvested and assayed after 48 h using the Luciferase Reporter Gene Assay High Sensitivity (Roche). Luciferase reporter activity was normalized using a beta-galactosidase assay with o-nitrophenyl-β-D-galactoside (ONPG) (60). Data compiled from three independent experiments performed in triplicates are reported.

Quantitative real-time RT–PCR and data analysis

Total RNA from gonad pairs was extracted and DNase-treated with a Qiagen RNeasy Micro kit according to the manufacturer’s instructions. Typically 0.4–0.7 μg total RNA was reverse transcribed using random hexamers (Promega) and Superscript III reverse transcriptase (Invitrogen). Target cDNA levels were analyzed by the comparative cycle time (Ct) method of real time RT–PCR with reactions including 2X SYBR Green PCR Master Mix (ABI) and 150 nM each of forward and reverse primers. Triplicate assays were carried out on an ABI Prism 7000 Sequence Detector System and the mean relative level of expression and associated standard deviations were calculated. 18S rRNA was used to normalize gene expression (Primer pair D, Supplementary Material, Table S1). Gene expression levels of Sox8, Sox9, Sox10, Amh, Ptgsd and Foxl2 were determined (Primer pairs E, F, G, H, I, respectively, Supplementary Material, Table S1). Gene expression (Primer pair D, Supplementary Material, Table S1). Gene expression levels of Sox8, Sox9, Sox10, Amh, Ptgsd and Foxl2 were determined (Primer pairs E, F, G, H, I, respectively, Supplementary Material, Table S1).

In situ hybridization

Probes used were Sox10 (U66141, nt 1029–1789) and Scc (NM_019779, nt 25–824). For WISH, dissected gonads/mesonephroi were fixed in 4% PFA in PBTX (PBS containing 0.1% Triton X-100) for several hours at 4°C. WISH with DIG-labeled RNA probes were carried out as described elsewhere (61). RNA probe was detected by incubation with BM Purple, AP Substrate (Roche). For sections in situ hybridization (ISH), whole embryos were processed and mounted in paraffin. SISH was performed on 7 μm sagittal sections as described previously (62).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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