Functional analysis of *Sox8* and *Sox9* during sex determination in the mouse

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Summary

Sex determination in mammals directs an initially bipotential gonad to differentiate into either a testis or an ovary. This decision is triggered by the expression of the sex-determining gene *Sry*, which leads to the activation of male-specific genes including the HMG-box containing gene *Sox9*. From transgenic studies in mice it is clear that *Sox9* is sufficient to induce testis formation. However, there is no direct confirmation for an essential role for *Sox9* in testis determination. The studies presented here are the first experimental proof for an essential role for *Sox9* in mediating a switch from the ovarian pathway to the testicular pathway. Using conditional gene targeting, we show that homozygous deletion of *Sox9* in XY gonads interferes with sex cord development and the activation of the male-specific markers *Mis* and *P450scc*, and leads to the expression of the female-specific markers *Bmp2* and follistatin. Moreover, using a tissue specific knock-out approach, we show that *Sox9* is involved in Sertoli cell differentiation, the activation of *Mis* and *Sox8*, and the inactivation of *Sry*. Finally, double knock-out analyses suggest that *Sox8* reinforces *Sox9* function in testis differentiation of mice.

Key words: Sex determination, Gonads, Conditional gene targeting, Mouse

Introduction

SOX genes are developmental regulators characterized by the presence of an HMG (high mobility group) DNA-binding domain with >50% homology to the sex-determining gene *SRY*. The SOX gene family can be further subdivided into twelve subgroups defined by additional homologies outside of the DNA-binding domain (Bowles et al., 2000). The E subgroup of SOX genes consists of three members: *SOX8*, *SOX9* and *SOX10*. The gene studied in most detail in this subgroup is *SOX9*, heterozygous mutations of which cause campomelic dysplasia (CD) in humans, a syndrome characterized by abnormal bone development, perinatal lethality and other abnormalities (Houston et al., 1983). Seventy-five percent of XY *SOX9* heterozygous individuals with campomelic dysplasia also show defects in testicular differentiation or complete sex reversal (i.e. ovaries instead of testes). In addition, a chromosomal duplication encompassing the *SOX9* locus has been found in one patient with XX sex reversal further supporting a role for *SOX9* during the sex determination process in man (Huang et al., 1999).

Campomelic dysplasia is caused by heterozygous loss-of-function mutations typically affecting the DNA-binding or transcriptional-activation domain (HMG box) of *SOX9* (Foster et al., 1994; Wagner et al., 1994). In addition, translocations or inversions with breakpoints several hundred kilobases (kb) upstream of *SOX9* have been identified (Wagner et al., 1994; Wirth et al., 1996), and transgenic analysis suggested that upstream sequences contain important regulatory elements for the *SOX9* gene (Bishop et al., 2000; Wunderle et al., 1998). Studies in vitro and in vivo have demonstrated that *SOX9* can bind and bend DNA, and can act as a transcriptional activator on target promoters (Bell et al., 1997; Lefebvre et al., 1997). Consistent with these findings, mutations in the transactivation domain at the C terminus have been found in individuals with campomelic dysplasia (Sudbeck et al., 1996). Moreover, recent in vitro data suggest that this gene may have an additional function during pre-mRNA splicing (Ohe et al., 2002).

Sex determination in the mouse occurs at E11.5, when an initially bipotential gonad becomes determined to develop either along the male or female axis depending on the presence or absence of the Y chromosome-encoded gene *Sry*. The expression of *Sry* is highly dynamic: very low levels of *Sry* transcripts can be detected as early as E10.5, which then sharply increase to a peak at E11.5, only to become repressed again by E12 (Hacker et al., 1995; Jeske et al., 1995). The regulation of *Sry* is largely unknown, but it has been shown that the Wilms' tumour suppressor *Wt1* is required for the presence of high levels of *Sry* RNA (Hammes et al., 2001;

Hossain and Saunders, 2001). Moreover, steroidogenic factor 1 (Sf1; Nr5a1 – Mouse Genome Informatics) seems to be able to activate the *Sry* promoter at least in pig (Pilon et al., 2003).

One of the first genes activated in male gonads after the expression of *Sry* is *Sox9*. Although small amounts of cytoplasmic Sox9 can be detected in both male and female gonads at E10.5, the production of high levels of nuclear Sox9 protein is triggered through the expression of Sry in XY gonads (de Santa Barbara et al., 2000; Morais da Silva et al., 1996). Based on this early appearance of *Sox9* after the onset of *Sry*, its association with sex reversal in man, and as it can functionally substitute for *Sry* in transgenic mice (Vidal et al., 2001), it has been proposed that Sry directly regulates *Sox9* (Canning and Lovell-Badge, 2002).

Sox9 can function as a transcriptional activator and there is strong evidence that it regulates the Mullerian-inhibiting substance *Mis* (*Amh* – Mouse Genome Informatics). First, co-transfection experiments show that Sox9 can bind and transactivate the *Mis* promoter, at least in vitro (De Santa Barbara et al., 1998). Second, mutations in the potential Sox9-binding site upstream of the *Mis* coding sequence result in a lack of *Mis* expression in vivo (Arango et al., 1999). Interestingly, a recent in vitro study by Schepers et al. indicated that Sox8, a close homologue of Sox9, can also activate the *Mis* promoter, albeit to a somewhat lower extent (Schepers et al., 2003). *Sox8* also shows a Sertoli cell-specific expression pattern in the developing testis, with an onset of expression at E12, just after the activation of *Sox9*. However, homozygous *Sox8* knock-out mice did not show a gonadal phenotype, suggesting either functional redundancy with another gene or that Sox8 does not play a role in the development of this organ (Sock et al., 2001).

Heterozygous mutations in the human *SOX9* gene are sufficient to induce sex reversal. By contrast, sex determination in heterozygous *Sox9* knock-out mice occurs normally, suggesting that this pathway is less sensitive to gene dosage in mice than in humans (Bi et al., 2001). We were interested in investigating the role of *Sox8* and *Sox9* during sex determination and testis differentiation in more detail. The perinatal lethal phenotype of heterozygous *Sox9* knock-out mice precludes the generation of homozygous null animals using standard genetic approaches. To overcome this problem we have generated a conditional knock-out allele of *Sox9* (*Sox9^{flox}*) (Akiyama et al., 2002). Using transgenic lines expressing the Cre recombinase either in the germline or within the developing gonad, we show that *Sox9* is essential for Sertoli cell differentiation and seminiferous tubule formation. The Sertoli cell-specific markers *Mis* and *Sox8* directly depend on *Sox9* levels, whereas high *Sry* expression persists in *Sox9* knock-out mice, indicating that *Sox9* activation leads to the downregulation of this gene. Finally, experiments with *Sox8/Sox9* double knock-out mice suggest that *Sox8* reinforces *Sox9* function in testis formation.

Materials and methods

Construction of the *Sf1-Cre* plasmid

A 674 bp promoter fragment of the *Sf1* gene (Wilhelm and Englert, 2002) was amplified by PCR using the primers 5′-TTATCA-TGCCATGGTCACACCCTTAGCCCAGCAGTC-3′ and 5′-TTATC-ATGCCATGGTCCCAGGCCTCAGGTAGGGCA-3′, and cloned in front of the gene encoding the Cre-recombinase from the plasmid pPGKcrebpA (Gu et al., 1993). Microinjections in the pronucleus of one-cell embryos heterozygous for the *Sox9^{flox}* allele (Akiyama et al., 2002) were carried out as described (Hogan et al., 1994).

Mouse strains and genetic background

Sox8 and *Sox9* knock-out mice were kept on a mixed 129/C57Bl6 background. The *Sf1:Cre* transgene was injected into fertilized oocytes derived from C57Bl6/CBA females mated with C57Bl6/CBA males, or with males homozygous for the *Sox9^{flox}* allele (mixed 129/C57Bl6 background). Prm1:Cre and Zp3:Cre mice were maintained on a 129/C57Bl6 mixed background and C57Bl6 inbred background, respectively. Mice homozygous for the *Sox8* mutation and heterozygous for the *Sox9* deletion (Fig. 9) were generated using either the Prm1:Cre or the deleter:Cre strain (Schwenk et al., 1995). Both crosses gave similar results.

Genotyping of embryos

The presence of the Y chromosome was determined using primers for the *Zfy* and *Sry* genes (Hogan et al., 1994). Wild-type and *Sox9^{flox}* alleles were identified using the primers 5′-GGGGCTTGTCTC-CTTCAGAG-3′, or 5′-ACACAGCATAGGCTACCTG-3′ and 5′-TG-GTAATGAGTCATACACAGTAC-3′, respectively. The *Sox9* knock-out allele was identified using the primers: 5′-GTCAAGCGACCC-ATG-3′ and 5′-TGGTAATGAGTCATACACAGTAC-3′. Genotyping for the *Sox8* knock-out allele was performed as described (Sock et al., 2001). The presence of the *Sf1-Cre* transgene was determined using primers specific for the *Cre* gene, 5′-CAATTTACTGACCGTACAC-3′ and 5′-AGCTGGCCCAAATGTTGCTG-3′.

Histological analysis

Visceral organs were dissected free of the urogenital system that remained associated with the carcass, fixed overnight at 4°C in Bouin's solution and embedded in paraffin wax. Sections were cut at 5-6 μm thickness and stained with haematoxylin and eosin.

Whole mount in situ hybridization

Embryos were fixed with 4% paraformaldehyde (PFA) in 1×PBS at 4°C overnight. Further processing of embryos and in situ hybridization were carried out essentially as described (Wilkinson, 1992). *Sox9* riboprobes were synthesized according to Morais da Silva et al. (Morais da Silva et al., 1996).

Quantitative RT-PCR analysis

Individual urogenital ridges (mesonephros+gonad) were dissected in PBS from E13.5 embryos and immediately frozen at –70°C. RNA was prepared using the Absolutely RNA RT-PCR kit (Stratagene). Each sample was divided into two aliquots, one of which was reverse transcribed using the MMLV (Gibco), following the manufacturers instructions. The second aliquot was used as a control (RT-PCR without reverse transcription) to identify samples with DNA contamination that could not be included in the analysis of the expression from the intron-less *Sry* gene. Primers and probes for all other genes were designed to cover introns. All real time PCR assays were carried out using the LC-Faststart DNA Master kit (Roche). A standard curve for each gene was generated using serial dilutions of cDNA. Relative expression levels of each sample were determined in the same run and normalized by measuring the amount of *Gapdh* (*Gapd* – Mouse Genome Informatics) cDNA.

Primers for real-time PCR analysis were:

Gapdh cDNA, 5′-ATTCAACGGCACAGTCAAGG-3′ and 5′-TG-GATGCAGGGATGATGTTC-3′, hybridization probes 5′-CCAGAA-GACTGTGGATGGCCCCT-X and LC-Red640-TGGAAAGCTGT-GGCGTGATGGC-p;

Sox9, 5′-GACAAGCGGAGGCCGAA-3′ and 5′-CCAGCTTGCA-CGTCGGTT-3′, hybridization probes 5′-CTTGCAGCGCCTTGAA-

GATAGCATTAG-X and LC-Red640-GAGATGTGAGTCTGTTCC-GTGGCCTC-p; and

Mis, 5′-GTCCTACATCTGGCTGAAGTGAT-3′ and 5′-CCGAG-TAGGGCAGAGGTTCT-3′ (overlapped the junction between exon 1 and 2, and exon 2 and 3, respectively), hybridization probes 5′-GGC-CCTGTTAGTGCTATACTCTGGACC-X and LC-Red640-GCCCC-CAGGTCACAGTCACAGG-p.

We also used the following primers:

Sf1, 5′-TTCTGAGAGCCCGCTAGCC-3′ and 5′-CCTCGTCGTA-CGAGTAGTCCATG-3′, hybridization probes 5′-CCTGGTGTCC-AGTGTCCACCCT-X and LC-Red640-TCCGGCTGAGAATTCTC-CTTCCG-p;

Sox8, 5′-CAGAGCTCAGCAAGACCCTA-3′ and 5′-GGGTGGT-GGCCCAGTT-3′, hybridization probes 5′-TTACAAATACCAGC-CAAGGCGAAG-X and LC-Red640-AAGAGTGTGAAGACTGGC-CGGAGC-p; and

Sry, 5′-AGCCTATGTGTAGTTCCTTGGTC-3′ and 5′-TGCATA-AGGAGTCACATTTTGCT-3′, hybridization probes 5′-CAATCTG-GCAGTTGAGTTAATGTGCAGAT-X and LC-Red640-CCATTCAT-TCATCCCACATATACTTGCCC-p.

Detection of the linear *Sry* transcript was performed as described (Toyooka et al., 1998). Each sample of RNA was checked for DNA contamination using the same amount of starting material without reverse transcription.

Immunohistochemistry

Tissues were isolated at E15.5, fixed for 2 hours in 4% PFA, washed twice for 5 minutes in 1×PBS and, after equilibration in 30% sucrose, frozen in OCT on dry ice. Sectioning and staining were carried out as described by Hammes et al. (Hammes et al., 2001). The following dilutions of primary antibodies were used: Sox9 (provided by Michael Wegner), 1:500; Mis (C-20, cat# sc-6886, Santa Cruz Biotechnology), 1:100; Wt1 (C-19, cat# sc-192 Santa Cruz Biotechnology), 1:100; and laminin (L-9393 Sigma), 1:50.

For laminin staining of cultured gonads, tissues were fixed with 4% PFA overnight and paraffin embedded. Sections were generated at 7 μm. After re-hydration, slides were placed in boiling 10 mM sodium citrate (pH 6.0) in a microwave oven for 20 minutes for antigen retrieval, and soaked in 3% hydrogen peroxide in methanol for 10 minutes at room temperature to block endogenous peroxidases. Immunodetection was performed by Vectastain Elite ABC Kit (PK-6100, Vector Laboratories) and substrate development by AEC Substrate System (K3464, DakoCytomation). Laminin α-1 antibody (M-20, cat# sc-6017 Santa Cruz Biotechnology) was used as the primary antibody at a 1:350 dilution.

In vitro organ cultures

The culture medium was Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, 0.1 mM 2-mercaptoethanol, 2 mM glutamine, 0.5 mM pyruvate, 100 units/ml penicillin and 0.05 mg/ml streptomycin (McLaren and Southee, 1997). The urogenital organs from $Sox9^{flox/\Delta}$ and $Sox9^{\Delta/\Delta}$ embryos were dissected in the culture media containing 20 mM HEPES. The isolated organs were cultured on polycarbonate membranes (Transwell #3403, Coster, NY) coated with 1% agarose in phosphate-buffered saline (PBS), with 500 μl of culture media per well at 37°C with 5% CO_2 in air for 2 or 3 days. After culture the organs were fixed in 4% PFA at 4°C overnight. For histological analysis, the fixed tissues were paraffin wax-embedded and sectioned at 7 μm.

Results

Heterozygous *Sox9* mutations in mice cause perinatal lethality due to severe bone malformations and respiratory deficiency. To analyze *Sox9* function during gonad formation we made use of our recently generated conditional $Sox9^{flox}$ allele (Akiyama et al., 2002). Two complementing strategies were employed. First, we produced *Sf1:Cre* transgenic lines, which allowed the tissue-specific analysis of *Sox9* in the developing gonad. Second, we generated homozygous knock-out animals by employing transgenic lines expressing the CRE recombinase within the male/female germ cell lineage, thus avoiding the lethality associated with heterozygous mutations in *Sox9*.

Sox9 ablation in developing gonads interferes with Sertoli cell differentiation

To address *Sox9* function in gonad development in vivo we deleted the $Sox9^{flox}$ allele in a tissue specific manner. To direct Cre expression to the developing gonad, we chose regulatory regions of the *Sf1* gene, which in vivo is expressed in the gonadal primordium from E9.5 onwards. Using a 674 bp promoter fragment that has previously been shown to direct gonad-specific expression in transgenic mice (Wilhelm and Englert, 2002), 17 *Sf1:Cre* transgenic animals were generated. Expression in a number of these transgenic lines was early/ectopic, which resulted in complete deletion of the *loxP*-flanked *Sox9* gene and concomitant perinatal lethality in the heterozygous state (*Sf1:Cre;* $Sox9^{flox}$/+). However, bigenic mice from several lines survived and line *Sf1:Cre (5)* was selected for all following analysis based on its efficient ablation of *Sox9* expression (Fig. 1).

Sf1:Cre (5) transgenic animals were bred with $Sox9^{flox}$/+ animals. A small proportion of double heterozygous mice (*Sf1:Cre;* $Sox9^{flox}$/+) died soon after birth with a phenotype similar to campomelic dysplasia, suggesting early excision of the floxed *Sox9* allele in these animals. Surviving *Sf1:Cre;* $Sox9^{flox}$/+ animals were further bred with homozygous $Sox9^{flox}/Sox9^{flox}$ mice, and embryos analyzed for deletion of the floxed allele on the genomic level (Fig. 1A,B) and for *Sox9* expression by in situ hybridization (Fig. 1C-E). Detection of *Sox9* mRNA in *Sf1:Cre;* $Sox9^{flox}/Sox9^{flox}$ embryos was variable ranging from almost normal expression to a complete absence of signals at E13.5, reflecting the efficiency of Cre-mediated $Sox9^{flox}$ excision in individual gonads (Fig. 1). Similarly, real-time PCR analysis performed on individual urogenital ridges at E13.5 (left or right) showed variable *Sox9* expression ranging from 50% of wild-type male expression to amounts comparable to that found in female mice (see later).

At E13.5, male testis development can be easily recognized by the formation of sex cords (Fig. 2B). In XY *Sf1:Cre;* $Sox9^{flox}/Sox9^{flox}$ gonads we always detected some sex cord formation, although they appeared highly abnormal in a proportion of animals (Fig. 2C). Older embryos (E18.5) showed testicular descent, indicating the production of sufficient male hormones in these mice (data not shown). To analyze the effect of *Sox9* ablation on testicular differentiation we performed histological analysis at day E13.5, E15.5 and E18.5. All XY gonads analyzed showed the development of male sex cords, and the general organization of germ cells within the seminiferous tubules seemed to be normal. However, gonads appeared to have a reduced number of seminiferous cords and the outline of these cords was irregular (Fig. 3G,H).

Meiosis of germ cells occurs at different time points during male and female gonad development. In wild-type female mice, first meiotic figures can be detected from E15 onwards, whereas meiosis in males occurs from 8 days after birth. Surprisingly, when we analyzed gonads of XY *Sf1:Cre;*

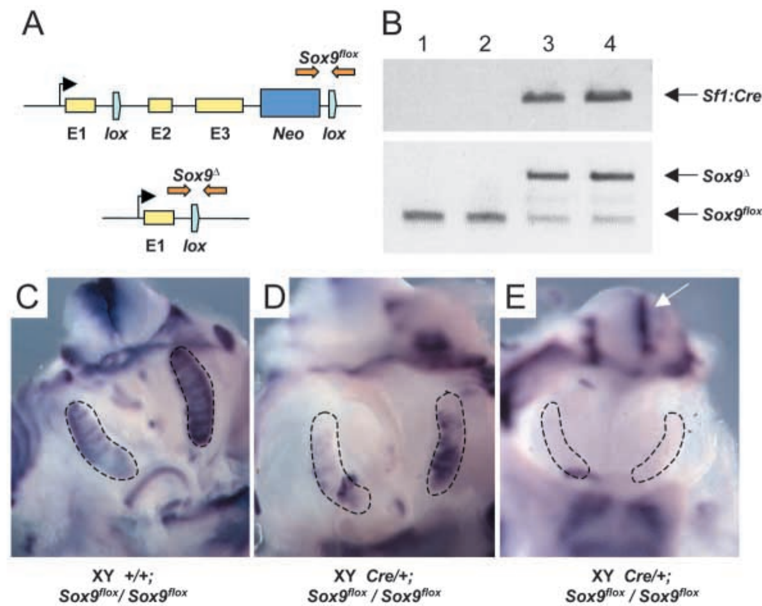

Fig. 1. Analysis of tissue specific deletion of *Sox9* in *Sf1:Cre; Sox9*^{flox}*/Sox9*^{flox} animals. Transgenic lines carrying the Cre recombinase under a 674 bp *Sf1* promoter fragment were crossed onto the homozygous *Sox9*^{flox}*/Sox9*^{flox} background. (A) The floxed and deleted *Sox9* locus. Orange arrows indicate the position of primers used for the detection of the *Sox9*^{flox} and the *Sox9*$^{\Delta}$ allele. (B) Detection of *Sox9* deletion in urogenital ridges (E13.5) on the genomic level. Only mice carrying the *Sf1:Cre* transgene (lanes 3 and 4) show the deleted band *Sox9*$^{\Delta}$. Incomplete deletion of the floxed allele may be due to inefficient expression of *Sf1:Cre* or may reflect the contamination of cells from the mesonephros. (C-E) In situ hybridization analysis for *Sox9* at E13.5. Dotted lines indicate the outline of the developing gonads. Embryos homozygous for the *Sox9*^{flox} allele and carrying the *Sf1:Cre* transgene (Cre) show reduced (D) or absent (E) expression of *Sox9* in the developing gonad. Note the persistent signal of *Sox9* in other tissues (e.g. arrow in panel E indicates expression in the neural tube).

Sox9^{flox}*/Sox9*^{flox} at E18.5 a significant number of meiotic gonocytes (prophase) were detectable (Fig. 3I). Interestingly, these cells seemed to be located within seminiferous tubules next to quiescent gonocytes. Primordial germ cells (PGCs) develop according to their somatic cell environment and it has been proposed that an as yet unidentified signal produced by the Sertoli cells inhibits PGCs from entering meiosis (Adams and McLaren, 2002; Dolci and De Felici, 1990; McLaren and Southee, 1997). The presence of meiotic cells in XY testes of the tissue-specific knock-out may suggest that *Sox9* is also required for proper functioning of Sertoli cells, including for the suppression of meiosis in male gonocytes.

Molecular analysis of *Sox9* knock-out gonads

To investigate the effect of *Sox9* ablation on the expression of genes involved in the sex determination process, we performed real-time PCR analyses with RNA isolated from individual E13.5 urogenital ridges. First we analyzed the expression of the Mullerian-inhibiting substance (*Mis*/*Amh*), which has been suggested to be a direct target of *Sox9* (Arango et al., 1999; De Santa Barbara et al., 1998). Indeed, *Mis* expression in our conditional knock-out mice showed a linear relationship with *Sox9* expression (Fig. 4A; r=0.9905; P<0.0001), supporting the theory that *Mis* represents a direct target of this transcription factor. Similarly, *Sox8*, which belongs to the same subgroup of Sox genes as *Sox9*, and which also shows Sertoli cell-specific expression from E12.5 days onwards (Schepers et al., 2003), was directly dependent on the level of *Sox9* (Fig. 4B; r=0.8444; P<0.0001). Remarkably, even in cases where *Sox9* expression was as low as that of wild-type female gonads, both the expression of *Mis* and *Sox8* in XY knock-out gonads was still higher than in XX tissues. By contrast, expression of *Sf1*, which in vitro can be activated by the Sox9 protein (Shen and Ingraham, 2002), did not show a clear dependence on *Sox9* expression levels (Fig. 4C; P=0.27), which may partly be due to the expression of *Sf1* in other cell types, including progenitor cells and Leydig cells.

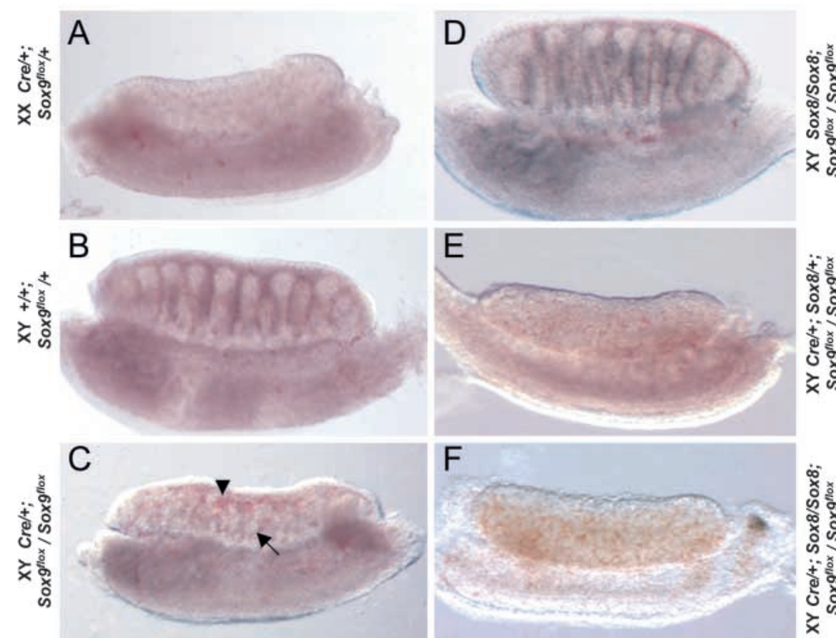

Fig. 2. *Sox9* or *Sox8/Sox9* knock-out mice show defects in sex cord formation. E13.5 gonads from *Sox9* tissue-specific knock-out animals show a variable degree of sex cord formation ranging from normal (not shown) to severely abnormal (arrow in C). Note the presence of abnormal vascularization (arrowhead in C). *Sox8/Sox8* knock-out gonads develop normal testis cords (D). By contrast, Sox9 tissue-specific knock-out animals (*Cre/+ Sox9*^{flox}*/Sox9*^{flox}) either heterozygous (E) or homozygous (F) for the *Sox8* knock-out allele often show complete absence of sex cord formation. (For frequencies of abnormal gonadal development see Table 1.)

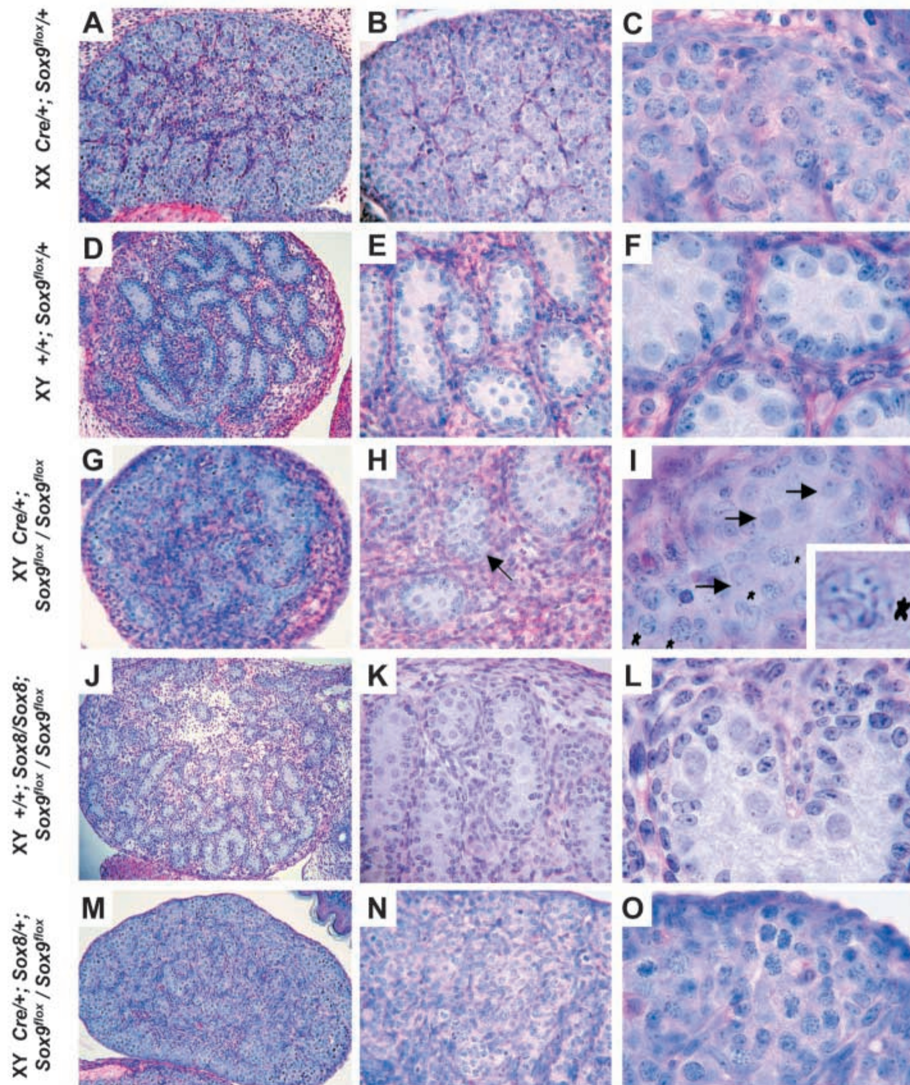

Fig. 3. Histological analysis of tissue-specific knock-out gonads at E18.5. (A-C) Control females. In contrast to control males (D-F), *Sox9* knock-out animals show somewhat irregular and poorly differentiated sex cords (G, and arrow in H). Next to quiescent gonocytes (arrows in I), several gonocytes in meiotic prophase could be detected (asterisks), suggesting differentiation of these primordial germ cells along the female pathway (compare with C). (J-L) Mice homozygous for the *Sox8/Sox8* knock-out allele, but without the *Sox9* tissue-specific knock-out showed normal sex cord formation and no sign of meiosis. By contrast, embryos heterozygous for the *Sox8* knock-out and homozygous for the *Sox9* knock-out (M-O) often showed complete sex reversal (compare with female development in A-C). See Table 1 for the frequencies of the sex reversal phenotype.

In wild-type mice, expression of *Sry* shows a very dynamic pattern with upregulation at E10.5, a peak at E11.5 and almost complete repression at E12.5 (Hacker et al., 1995). Hence, the rapid decrease of *Sry* expression occurs just after *Sox9* activation and it has been suggested that *Sry* repression is mediated by expression of this gene. Indeed, *Sry* expression in our knock-out mice was significantly increased over wild-type levels. It should be noted that *Sry* repression was not linearly dependent on *Sox9* expression, but occurred only in mice were *Sox9* transcripts were significantly reduced (Fig. 4D).

Homozygous deletion of *Sox9* interferes with sex cord formation and the expression of male specific markers

Although our real-time PCR analysis at E13.5 suggested that, at least in some gonads, *Sox9* expression was very low and comparable to the level of expression in female gonads, we could not exclude the possibility that the lack of sex reversal in our tissue-specific knock-out animals was due to incomplete deletion of *Sox9* at the time of sex determination (E11.5). To address this possibility we generated homozygous knock-out animals, making use of germ-line specific Cre transgenic mouse lines. Males carrying the spermatogenesis-specific Prm1:Cre transgene (O'Gorman et al., 1997) on a floxed *Sox9* background (Prm1:Cre; $Sox9^{flox}/Sox9^{flox}$) were crossed with female $Sox9^{flox}/Sox9^{flox}$ mice transgenic for the oocyte-specific ZP3:Cre transgene (de Vries et al., 2000) (Fig. 5). Unfortunately, homozygous *Sox9* knock-out animals die at E11.5 as a result of cardiac failure (Fig. 6A-D) (H. Akiyama et al., unpublished), which prohibited the in vivo analysis of the fate of XY gonads in these embryos at later stages. Hence, to investigate the function of *Sox9* during sex determination, gonads were isolated at E11.5, placed into organ culture and analyzed after two to three days in culture using in situ hybridization or immunohistochemistry, respectively.

As expected, cultured XY gonads heterozygous for the *Sox9* deletion ($Sox9^{flox/\Delta}$) formed sex cords, which could be visualized using laminin antibodies (Fig. 6E,I), and expressed the Sertoli cell-specific marker *Mis* and the Leydig cell-specific marker *P450scc* (also known as *Cyp11a1*) (Fig. 7A,E). XX gonads expressed the female-specific marker *Bmp2* and follistatin (*Fst*) (Fig. 7K,O). By contrast, XY gonads carrying a homozygous deletion of *Sox9* showed no signs of sex cord formation (Fig. 6F,J), and did not express any of the male-

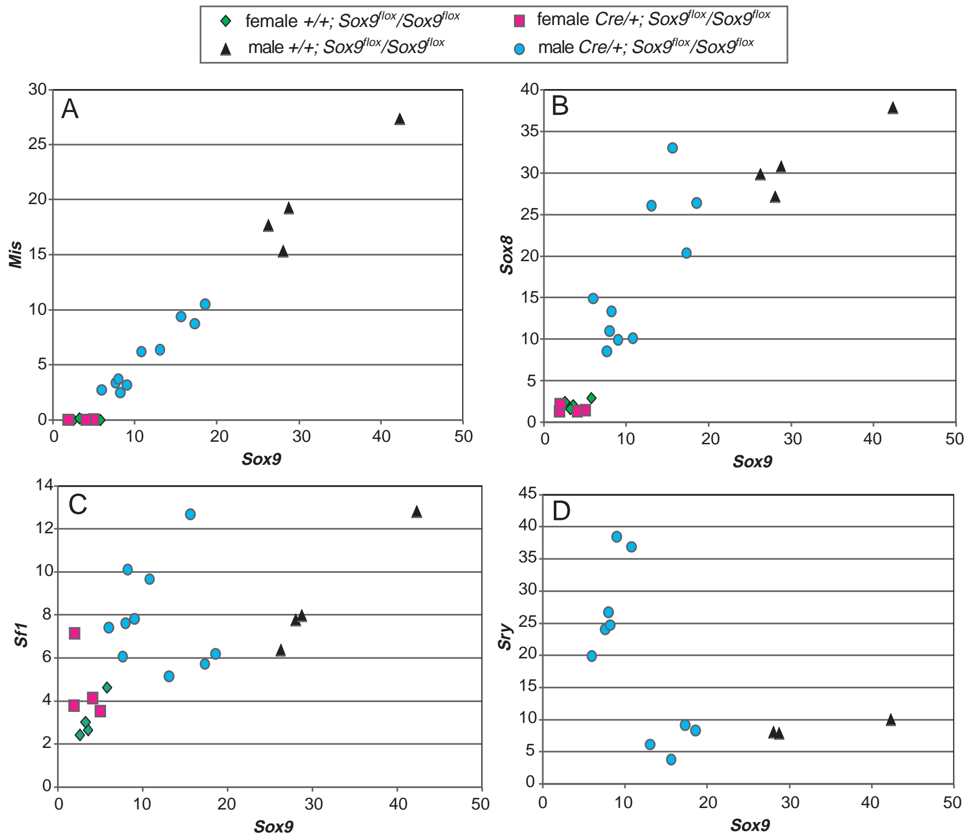

Fig. 4. Real-time RT-PCR analysis of markers involved in the sex determination process. *Sox9* expression (shown on the X axis) in individual E13.5 knock-out gonads was variable with levels in some gonads being comparable to those in female mice. (A) *Mis* expression was directly dependent on *Sox9* levels indicating direct activation of its promoter (correlation coefficient r=0.9905; P<0.0001). Note that *Mis* expression in mice with low levels of *Sox9* was still higher than that in control females. (B) Expression of *Sox8* also showed a clear dependence on *Sox9* levels (r=0.8444; P<0.0001). (C) No such direct relationship for the steroidogenic factor Sf1 was observed (r=0.3173; P=0.2689). (D) Gonads with very low levels of *Sox9* showed persistent expression of the *Sry* gene.

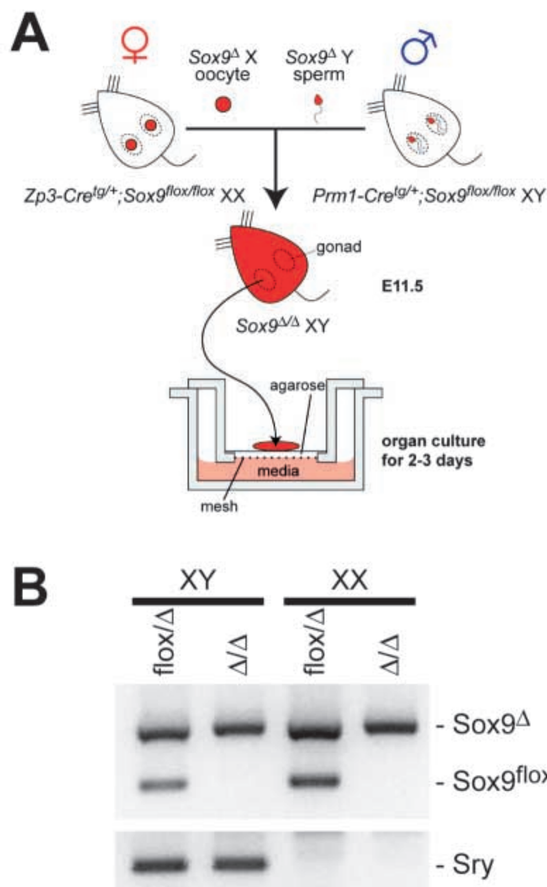

Fig. 5. Conditional knock-out of *Sox9* in male and female germ cells. (A) Schematic diagram of breeding strategy. Although *Sox9* was deleted in almost all oocytes of $Sox9^{flox/flox}$; *Zp3:Cre* females, *Sox9* was deleted in only about half of the sperm of in $Sox9^{flox/flox}$; *Prm:Cre* males. Therefore, when *Prm:Cre* males and *Zp3:Cre* females were mated, $Sox9^{\Delta/\Delta}$ and $Sox9^{flox/\Delta}$ embryos were obtained at a 1:1 ratio (n=251). Only one $Sox9^{flox/flox}$ embryo was obtained (0.4%). (B) PCR genotyping of *Sox9* mutants obtained from the germ cell knock-out. No mosaicism was observed in homozygous *Sox9* mutants. $Sox9^{flox}$, conditional (active) *Sox9* allele; $Sox9^{\Delta}$, recombined (inactive) *Sox9* allele.

specific marker analyzed (Fig. 7B,F). Instead, expression of *Bmp2* and follistatin was clearly activated in these gonads (Fig. 7J,N), indicating differentiation along the female pathway. Taken together, these data demonstrate that phenotypically, as well as on the molecular level, expression of *Sox9* is required for male sex determination and testis differentiation.

Sox8 reinforces *Sox9* action in testis formation

As described above, *Sox9* is essential for male differentiation of XY gonads. Given the dosage sensitivity of male sex determination for *SOX9* in humans with campomelic dysplasia, we were surprised that despite the very low levels of *Sox9* expression in some of our tissue-specific knock-out animals (see Figs 1, 4), all of them developed sex cords at least to some extent (Fig. 2C; Fig. 3H). We therefore speculated that another member of the Sox gene family might take over some of the function of *Sox9* in our tissue-specific knock-out animals. *Sox8* shows a high degree of homology to *Sox9* (53% amino-acid identity; 67% homology) (Schepers et

al., 2000) and is switched on in the male gonad soon after the onset of *Sox9* expression (Schepers et al., 2003). Moreover, *Sox8* knock-out mice do not show gonadal defects, which may also suggest functional redundancy with other genes (Sock et al., 2001). Our real-time PCR analysis indicated that, although *Sox8* levels depend on the expression of *Sox9*, tissue-specific knock-out mice still express significantly more *Sox8* mRNA than XX littermates (Fig. 4B). To determine whether this *Sox8* expression may have compensated for *Sox9* function, we crossed the *Sox8* knock-out allele onto the $Sox9^{flox}$ background. As expected, *Sox8* knock-out mice homozygous for the conditional $Sox9^{flox}$ allele but lacking Cre recombinase developed normal testes (Fig. 2D; Fig. 3J,K). By contrast, animals carrying the *Sox8* knock-out allele in addition to the tissue-specific *Sox9* knock-out (*Sf1:Cre; Sox8/+;* $Sox9^{flox}/Sox9^{flox}$ or *Sf1:Cre; Sox8/Sox8;* $Sox9^{flox}/Sox9^{flox}$) showed a much more dramatic phenotype, and several gonads developed very few or no sex cords (Fig. 2E,F; Table 1). Immunohistochemical analysis at E15.5 confirmed this observation, and demonstrated a complete absence of *Sox9* and *Mis* expression in some of these gonads (Fig. 8H). Macroscopic analysis at E18.5 showed that these knock-out gonads did not descend caudally, suggesting reduction or a complete lack of male hormone production. Finally, histological analysis showed the absence of Sertoli and Leydig cells, and the presence of ovarian differentiation (Fig. 3M-O). Thus, on all accounts these gonads appeared to be completely sex reversed. Not surprisingly, the phenotype was not completely penetrant (~40% sex reversal; Table 1), as *Sox9* expression in some gonads remained high because of inefficient Cre-mediated deletion of the $Sox9^{flox}$ allele. Importantly, sex reversal was only detected in mice

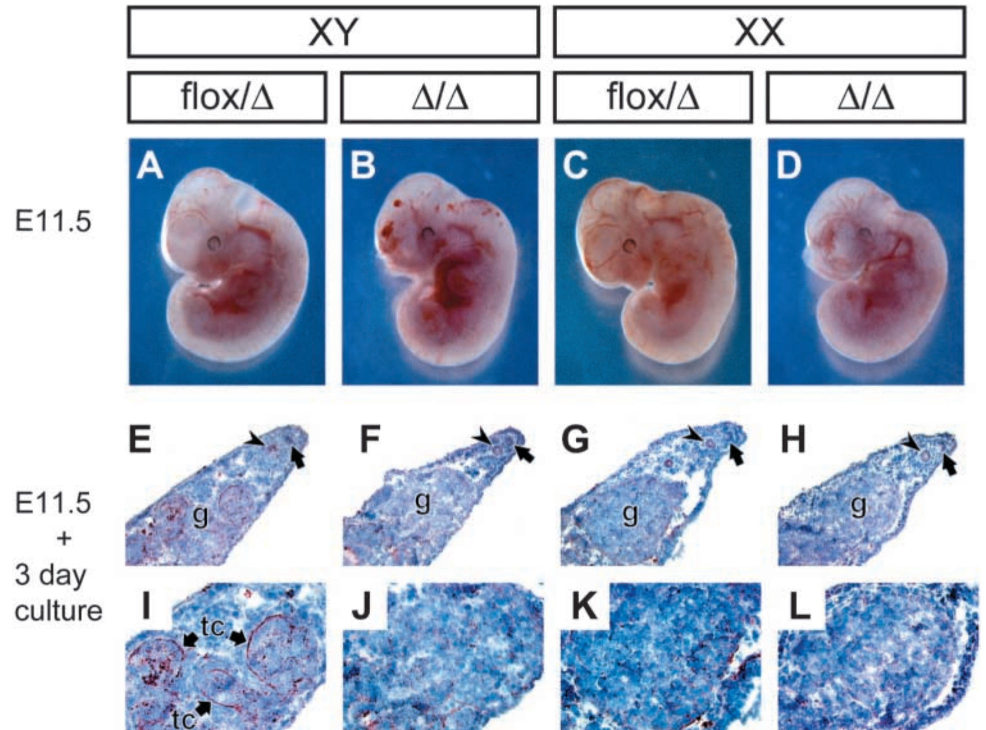

Fig. 6. Organ culture of urogenital tissues from *Sox9* mutants. (A-D) Lateral view of embryos at E11.5. (E-H) Laminin immunostaining of sectioned cultured urogenital systems. (I-L) High magnification of the gonad of cultured urogenital systems. No cord formation was observed in gonads from XY $Sox9^{\Delta/\Delta}$ embryos. g, gonad; tc, testis cords; arrowhead, Wolffian duct; arrow, Müllerian duct.

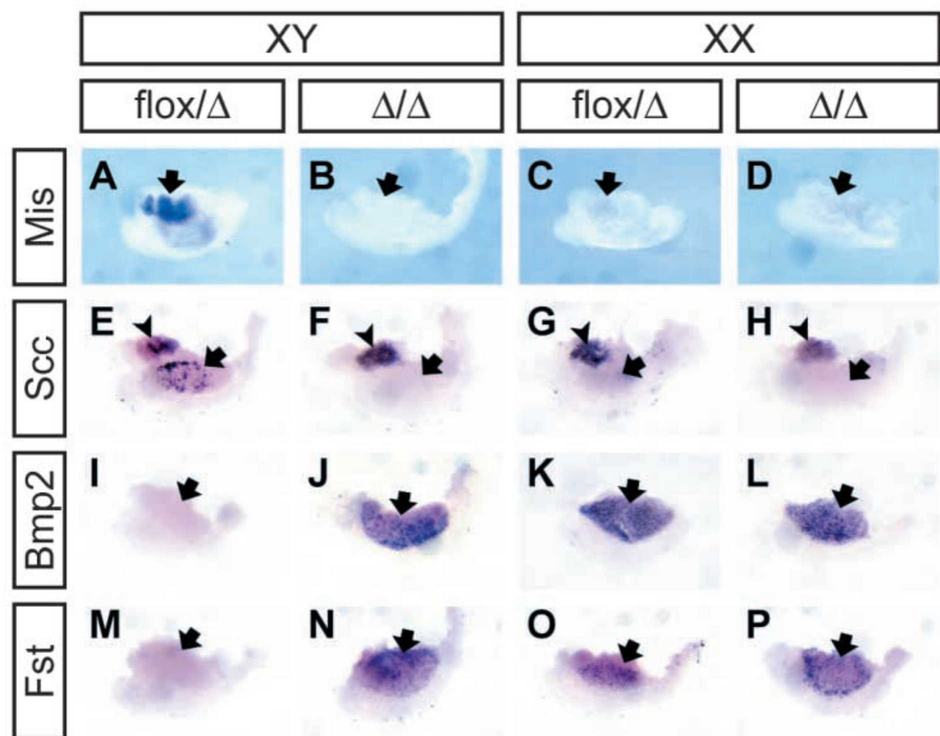

Fig. 7. Whole-mount in situ hybridization of cultured urogenital systems. *Mis* (A-D), *P450-Scc* (E-H), *Bmp2* (I-L) and follistatin (M-P). Arrows indicate the gonad. (E-H) Arrowheads indicate *Scc* expression in the adrenal gland. The cultured XY $Sox9^{\Delta/\Delta}$ gonad is sex reversed to female.

Table 1. Overview of gonads analyzed

	Sox9^{flox}/+	*Sox9*^{flox}/ *Sox9*^{flox}	*Sox8*/+; *Sox9*^{flox}/+	*Sox8*/+; *Sox9*^{flox}/*Sox9*^{flox}	*Sox8/Sox8*; *Sox9*^{flox}/+	*Sox8/Sox8*; *Sox9*^{flox}/*Sox9*^{flox}
XX	40	28	9	1	5	18
XY	33	31	13	7	8	6
XX (*Sf1:Cre*)	37	27	6	4	7	9
XY (*Sf1:Cre*)	30	31	11	9 (4SR)	5	3 (1SR)

Gonads were analyzed at various stages of development and judged by the presence of sex cords (E13.5), or their external appearance and descent status (E15.5 and E18.5). Sex-reversed gonads or those with abnormal appearance were sectioned (cryostat or paraffin-wax embedded) to confirm the finding histologically. Complete sex reversal (SR) was only detected in mice homozygous for the *Sox9* knock-out and heterozygous/homozygous for the *Sox8* mutation. Note, table only includes gonads with complete sex reversal (no sex cords); gonads with present but abnormal sex cords were scored as testes.

homozygous for the *Sox9* mutation and at least heterozygous for the *Sox8* knock-out.

To further support the hypothesis that functional complementation rather than genetic background caused the sex reversal in our *Sox8/Sox9* double knock-out model, we investigated whether the Sox8 mutation would provoke a gonadal phenotype in a *Sox9* heterozygous knock-out background. *Sox9* heterozygous-mutant mice wild-type for *Sox8* did not show a gonadal phenotype (Bi et al., 2001). Similarly, *Sox8* knock-out mice wild-type for *Sox9* showed normal sex cord development and a clearly outlined coelomic vessel (Fig. 9B). By contrast, three out of three animals (6 gonads) heterozygous for the *Sox9* deleted allele, and homozygous for the *Sox8* mutation (*Sox8/Sox8; Sox9*$^{\Delta}$/+), showed abnormal sex cord formation and defects in coelomic vessel formation (Fig. 9C). Moreover, *Sox8/Sox8; Sox9*$^{\Delta}$/+ testes at E15.5 showed a reduced number of seminiferous tubules and contained areas that resembled an ovary lacking Mis expression (Fig. 9F).

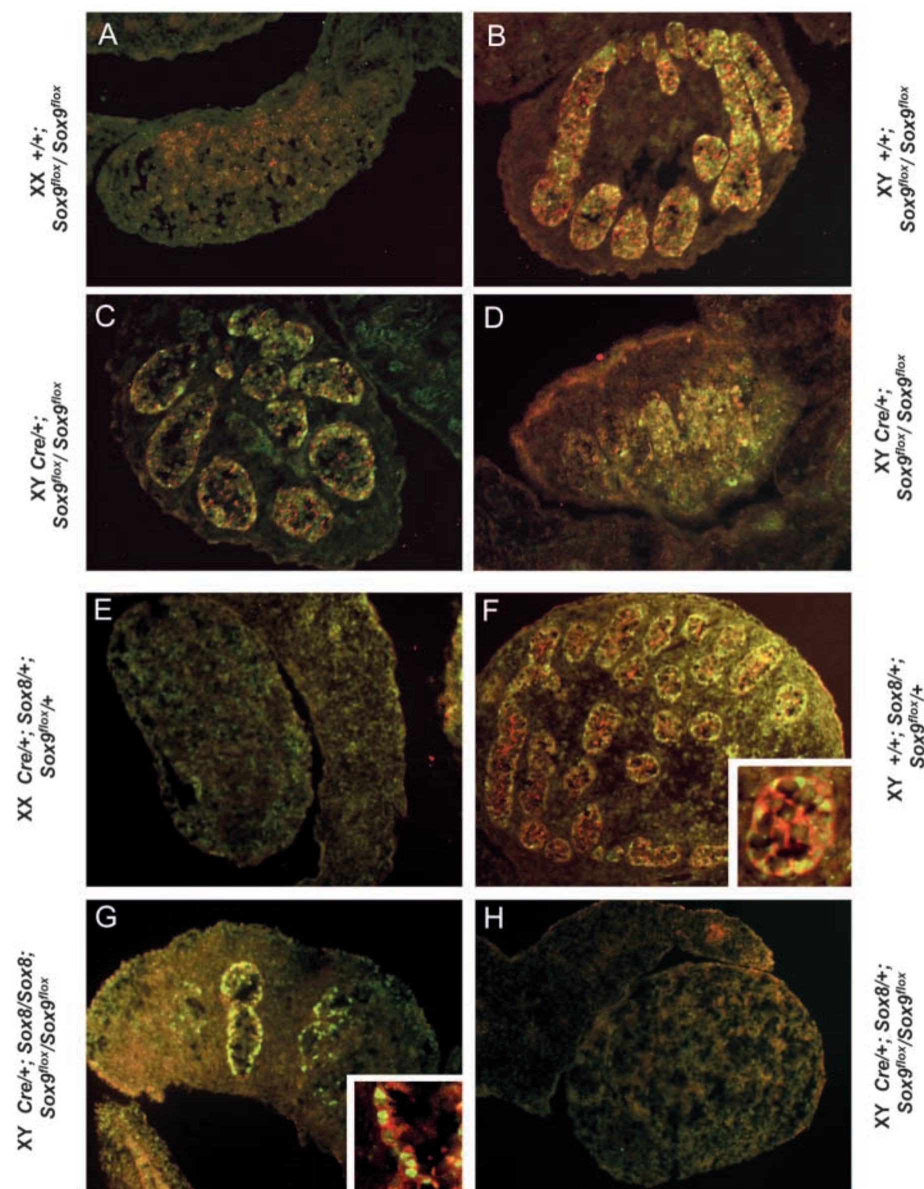

Fig. 8. Immunofluorescent analysis of *Sox8*, *Sox9* double knock-out mice. Double staining of E15.5 gonads for Mis (red) and Sox9 (green). (A,B) Control ovary and testis. *Sf1:Cre; Sox9/Sox9* knock-out mice (C,D) showed a varying degree of sex cord formation (D shows the most severe phenotype observed during this analysis). (E,F) Control ovary and testis. By contrast, *Sox8/Sox9* double knock-out mice showed a much more severe phenotype, often resulting in complete sex reversal (G,H). The formation of sex cords and the expression of Mis was restricted to cells expressing *Sox9*. Note, embryos shown in A-D were derived from a different litter to those shown in E-H.

Discussion

Since its cloning *Sox9* has attracted considerable interest because of its association with campomelic syndrome in man (Foster et al., 1994; Wagner et al., 1994). Although there has been a substantial amount of evidence supporting an important role for *Sox9* in the sex determination process (Kent et al., 1996; Morais da Silva et al., 1996; de Santa Barbara et al., 2000; Hanley et al., 2000; Huang et al., 1999; Bishop et al., 2000; Vidal et al., 2001), direct proof of its essential function during sexual differentiation has been missing.

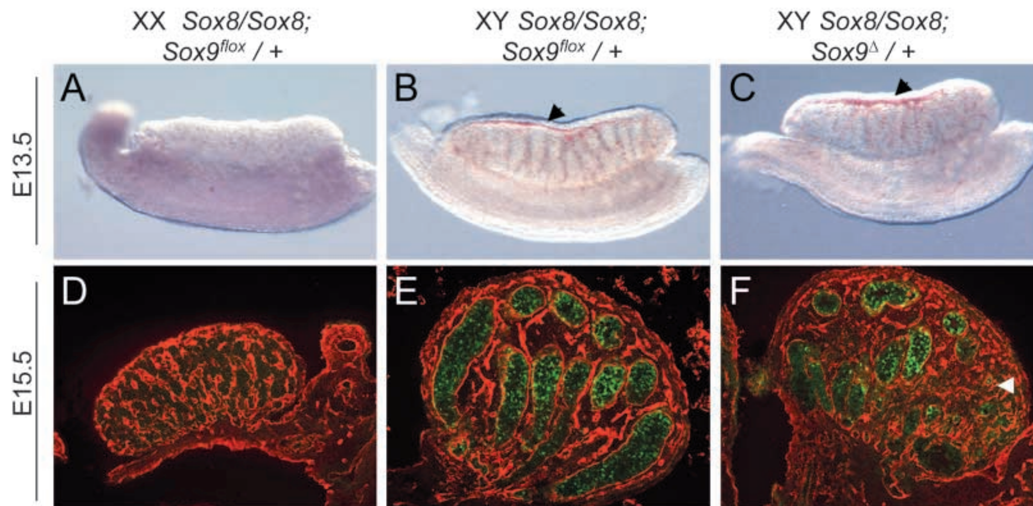

Fig. 9. Defects in testis formation in *Sox9* heterozygous knock-out mice on a *Sox8/Sox8* knock-out background. (A-C) Urogenital ridges isolated at E13.5 were analyzed for the presence of sex cords. Cords in *Sox8/Sox8; Sox9*$^{\Delta}$/+ gonads were poorly outlined and the formation of the coelomic vessel was disturbed. (D-F) Immunofluorescent analysis of gonads at E15.5 (laminin, red; Mis, green). *Sox8/Sox8; Sox9*$^{\Delta}$/+ gonads show a reduced number of sex cords, reduced expression of Mis and areas with poor differentiation (white arrowhead).

Here, we have used conditional gene targeting to analyze the developmental function of *Sox9* during mouse testis formation. Gonads completely lacking *Sox9* expression do not show any signs of male specific differentiation, lack the expression of testis-specific genes, such as *Mis* and *Scc*, and instead express the female-specific markers *Bmp2* and follistatin (Fig. 7). These data, together with our earlier studies showing that expression of *Sox9* in XX gonads can substitute for *Sry* function in male sex determination, demonstrate that *Sox9* is both required and sufficient for male testis differentiation in mice. These findings are also in agreement with a model in which Sry serves simply as a molecular switch to activate the *Sox9* gene, which in turn directs gonadal cells to differentiate into Sertoli cells leading to the formation of a testis. However, the regulation of *Sox9* is likely to be more complex than a simple activation through Sry, as mice lacking the orphan nuclear receptor gene *Dax1* (*Nr0b1* – Mouse Genome Informatics) on a *Mus poschiavinus* background do not express *Sox9*, despite apparently normal levels of *Sry* (Meeks et al., 2003). A detailed analysis will require the identification of gonad-specific regulatory elements at the *Sox9* locus.

The fact that we did not observe a complete sex reversal in our tissue-specific knock-out mice using the *Sf1:Cre* transgene was probably due to incomplete inactivation of *Sox9* at the time of sex determination, suggesting that the remaining small amounts of this protein may have been sufficient to activate male-specific genes. However, these variations in *Sox9* levels allowed us to draw a number of important conclusions. First, using real-time PCR analysis, we could relate the expression of varying amounts of *Sox9* with the expression of several genes known to be involved in the process of sexual differentiation. This analysis demonstrated a clear dependence of *Mis* expression on *Sox9* levels, thus supporting in vivo the findings of earlier studies that described *Mis* as a direct transcriptional target of Sox9 (Arango et al., 1999; De Santa Barbara et al., 1998). Similarly, the transcription factor *Sox8* directly depends on expression levels of *Sox9*. At present we cannot clearly distinguish whether this dependence is due to a direct regulation of *Sox8* by Sox9, or whether it simply reflects a reduction of the number of Sertoli cells in knock-out animals.

In contrast to the direct dependence of *Mis* and *Sox8* on *Sox9* expression levels, *Sry* expression was higher in gonads with low levels of *Sox9*. At present there is no evidence for a repressive function of the Sox9 protein, and the downregulation of *Sry* may occur through the interaction of Sox9 with other factors, or through the activation of a transcriptional repressor. The fact that *Sry* levels were only increased in cases where *Sox9* expression dropped below a certain threshold may suggest that persistent expression is an indirect event, possibly because of a continuous presence of *Sry*-positive Sertoli precursor cells originating from the coelomic epithelium (Karl and Capel, 1998; Schmahl et al., 2000) that fail to differentiate.

The incomplete inactivation of *Sox9* in our tissue-specific analysis also allowed us to look at the differentiation of testes with reduced expression levels of *Sox9* at later stages of development. In our histological analysis of E18.5 XY gonads, we observed meiotic gonocytes, a hallmark of female differentiation. This may suggest that *Sox9* is important to maintain a signal that suppresses meiosis in XY gonocytes. Further analysis may allow us to identify this signal.

Maybe the most surprising finding in our studies was the absence of sex reversal in tissue-specific knock-out animals (*Sf1:Cre; Sox9*^{flox}/*Sox9*^{flox}), despite the significant reduction of *Sox9* expression levels. This suggests that *Sox9* expression levels are much less critical in mice than in humans. By contrast, the additional deletion of at least one knock-out allele *Sox8* can lead to XY male to female sex reversal, suggesting that important functions of *Sox9* can be taken over by its close homologue *Sox8*. While this study was in progress, a paper by Schepers et al. was published describing Sox8 as a potential activator of the *Mis* promoter (Schepers et al., 2003). The authors demonstrated that although Sox8 can activate the *Mis* promoter region, this activation was much weaker than that

seen following *Sox9* induction. Based on their studies, Schepers et al. (Schepers et al., 2003) speculated that *Sox8* expression in the gonad might only represent an evolutionary remnant of a duplicated gene, which is in the process of adopting new functions. However, another possible explanation would be that upregulation of *Sox8* by Sox9 (directly or indirectly) soon after the commencement of sex determination is used to reinforce and imprint male sex determination and testis differentiation on the developing gonad. This hypothesis is further strengthened by the fact that *Sox8* expression seems to depend on Sox9 levels rather than the expression of Sry, which may suggest that the male-specific regulatory elements of *Sox8* were acquired independently of that of *Sox9*.

When interpreting results in a tissue-specific knock-out it is important to consider the inactivation of Sox9 on a cellular level. In any given cell, Sox9 is either wild-type (100%), heterozygous (50%) or homozygous (0%) for the mutation. Cells that are heterozygous for the *Sox9* knock-out allele seem to differentiate normally into Sertoli cells (at least in mice), as heterozygous mutants do not show a gonadal phenotype (Bi et al., 2001). However, in the case of an additional heterozygous or homozygous deletion of *Sox8*, the overall Sox8/Sox9 gene dosage in a given cell would be further reduced, which may interfere with the activation of their transcriptional targets and, as a consequence, block the differentiation of this cell into a Sertoli cell.

Taken together, our data suggest the following model. *Sox9* is activated through the expression of Sry, and is both essential and sufficient to induce testis formation. Sox9, either directly or indirectly, represses expression of *Sry* and activates *Sox8*. A certain threshold of Sox8 and Sox9, or Sox9 on its own, is then required for gonadal precursor cells to differentiate into Sertoli cells.

Our finding that *Sox8* may reinforce *Sox9* function during gonad formation may also have an impact on human genetics. Only 75% of XY individuals with campomelic dysplasia show sex reversal. The reason for this variable penetrance of the phenotype is presently unknown. Our data suggest that the gonadal phenotype in individuals with campomelic dysplasia may be influenced by the expression of *SOX8*, which could be variable because of either mutations at the *SOX8* locus or polymorphism of *SOX8*, which influence its expression level or function. Moreover, given its ability to at least partially take over SOX9 function, it is conceivable that ectopic activation of *SOX8* in XX gonads may cause sex reversal in human patients similar to findings for the *SOX9* gene.

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