The PGD2 pathway, independently of FGF9, amplifies SOX9 activity in Sertoli cells during male sexual differentiation

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Activation by the Y-encoded testis determining factor SRY and maintenance of expression of the Sox9 gene encoding the central transcription factor of Sertoli cell differentiation are key events in the mammalian sexual differentiation program. In the mouse XY gonad, Sox9 upregulates Fgf9, which initiates a Sox9/Fgf9 feedforward loop, and Sox9 expression is stimulated by the prostaglandin D2 (PGD2) producing lipocalin prostaglandin D synthase (L-PGDS, or PTDGS) enzyme, which accelerates commitment to the male pathway. In an attempt to decipher the genetic relationships between Sox9 and the L-Pgds/PGD2 pathway during mouse testicular organogenesis, we found that ablation of Sox9 at the onset or during the time window of expression in embryonic Sertoli cells abolished L-Pgds transcription. By contrast, L-Pgds−/− XY embryonic gonads displayed a reduced level of Sox9 transcript and aberrant Sox9 protein subcellular localization. In this study, we demonstrated genetically that the L-Pgds/PGD2 pathway acts as a second amplification loop of Sox9 expression. Moreover, examination of Fgf9−/− and L-Pgds−/− XY embryonic gonads demonstrated that the two Sox9 gene activity amplifying pathways work independently. These data suggest that, once activated and maintained by SOX9, production of testicular L-PGDS leads to the accumulation of PGD2, which in turn activates Sox9 transcription and nuclear translocation of SOX9. This mechanism participates together with FGF9 as an amplification system of Sox9 gene expression and activity during mammalian testicular organogenesis.

KEY WORDS: SOX9, PGD2, FGF9, Sertoli cell, Testis organogenesis, Mouse

INTRODUCTION

In mammals, Sertoli cell differentiation and embryonic testicular organogenesis are controlled by the master effector gene Sox9, which encodes a transcription factor that belongs to the HMG superfamily (Foster et al., 1994; Wagner et al., 1994). Its embryonic male-specific gonadal activation depends directly on another Sox-related gene, the Y chromosome-encoded testis determining factor SRY. In mice, the Sry gene is expressed for a short period (10.5-12.5 dpc) (Sinclair et al., 1990), suggesting that SRY acts within a brief window of time to induce the initial transcriptional pulse of the Sox9 gene via a direct interaction with an enhancer sequence 14 kb upstream of Sox9 (Sekido and Lovell-Badge, 2008). Lineage tracing of Sry-expressing cells demonstrated that once testis differentiation is established, Sertoli cells that express Sox9 are descendants of cells that have expressed Sry (Sekido et al., 2004), suggesting that activation of Sox9 is a cell-autonomous effect of SRY. In the mouse testis, this activation of Sox9 has at least two functions: (1) the initiation of its own transcription via a feedback loop and (2) the activation of a network of genes driving Sertoli cell differentiation.

Testicular Sox9 regulation is crucial for the reproductive capacity of the species. Indeed, loss of function mutation (Wagner et al., 1994) or deletion (Barrionuevo et al., 2006; Chaboisser et al., 2004) of Sox9 induces male-to-female sex reversal (XY female), whereas duplication (Huang et al., 1999) or ectopic expression of Sox9 in females (Bishop et al., 2000; Vidal et al., 2001), induces female-to-male sex reversal (XX male). In all cases, individuals are sterile because of the discrepancy between the germline chromosomal content and the gonadal environment. Despite its pivotal role in male sex differentiation, the regulation of Sox9 expression within the developing testis remains poorly understood. In mouse, the expression of Sry in the male genital ridge occurs only in a very narrow time window at ~11.5 dpc (Hacker et al., 1995), and a central question still remains: besides a putative autoregulatory loop in which Sox9 activates itself (Sekido and Lovell-Badge, 2008), how is the expression of Sox9 maintained after Sry has been turned off?

Several extracellular signaling pathways, including the fibroblast growth factor 9 (FGF9) pathway (for a review, see Ross and Capel, 2005) downstream of SRY, have been shown to play a role during the early stages of sexual development and have been implicated in the control of cell migration, proliferation and differentiation underlying mammalian gonadal development. Mutations in Fgf9 result in XY partial (Colvin et al., 2001) or complete (Schmahl et al., 2004) sex reversal in mixed (129;C57BL/6) or uniform (C57BL/6) genetic backgrounds, respectively. Loss of FGF9 in XY gonads does not affect the expression of Sry or the initial upregulation of Sox9 (Kim et al., 2006); however, after 12.5 dpc, testis differentiation is aborted owing to the lack of the maintenance of Sox9 expression. Nevertheless, Sox9 mutant gonads do not express Fgf9 at 11.5 dpc, demonstrating a positive feedforward loop between FGF9 and SOX9 in XY gonads (Kim et al., 2006). Interestingly, FGF9 might act through its putative receptor FGFR2 in Sertoli cell precursors.
L-Pgds expression was described as a dynamic wave-like expression pattern closely resembling those of Sry and Sox9 in the embryonic testis (Wilhelm et al., 2007). L-Pgds transcripts were detected in the center of the testis at 17 tail somite stage (ts), shortly after the onset of Sox9 expression at 15 ts. L-Pgds was found to be expressed only in the Sertoli cells between stages 11.5 and 14.5 dpc (Wilhelm et al., 2007) but in both Sertoli cells and spermatagonia by 13.5 dpc (Adams and McLaren, 2002). Expression of L-Pgds in both germ cells and Sertoli cells has also been reported in the adult rat testis (Samy et al., 2000). In the adult mouse testis, positive immunostaining for L-PGDS was also detected within the interstitial tissue, including the Leydig cells (Gerena et al., 2000), suggesting that this protein plays an integral role in both adult testicular development and function.

In this study, after establishing the precise expression pattern of L-Pgds from embryonic to adult stages, we investigated the in vivo regulation of this gene. By analyzing Ck19-Cre/Sox9lox/lox and Amh-Cre/Sox9lox/lox mutant XY gonads, we show that the onset and maintenance of L-Pgds expression is completely dependent on Sox9, thus definitively refuting the hypothesis that L-Pgds is an SRY target gene. Moreover, the onset of L-Pgds expression was not affected in Fgf9+/- mutant XY gonads, showing that FGF9 is not required for L-Pgds gene activation. L-Pgds+/- mutant XY gonads exhibited a delay in Sertoli cell differentiation up to 13.5 dpc. This was associated with a delay in the nuclear translocation of SOX9 and a reduced level of Sox9 gene expression and subsequent Amh gene expression, thereby confirming in vivo the existence of an amplification loop between the Sox9 and L-Pgds genes. Thus, following its nuclear translocation, SOX9 is implicated in parallel-acquiring L-Pgds/Sox9 and Fgf9/Sox9 regulatory loops, contributing to the final differentiation of Sertoli cells.

MATERIALS AND METHODS

Mice and cell lines

L-Pgds KO mice generated at Osaka Bioscience Institute (Osaka, Japan) (Eguchi et al., 1999), were back-crossed to the C57BL/6 strain for 20 generations; they were kept at the IGH animal care facility. The generation of Ck19-Cre;Sox9lox/lox, Amh-Cre;Sox9lox/lox and Fgf9+/- mutants has been previously described (Barriónuevo et al., 2006; Barrionuevo et al., 2008; Schmahl et al., 2004). NT2/D1 cells were grown as previously described (Malki et al., 2005b); for PGD2 (500 ng/ml) and FGF9 (25 ng/ml) stimulation, cells were starved in serum free medium without Phenol red for 18 hours (with 0.01% BSA and non essential amino acids) and treated for 20-120 minutes, before RNA extraction.

In situ hybridization

The antisense L-Pgds RNA probe described previously (Adams and McLaren, 2002) was PCR-amplified from embryonic mouse cDNAs, cloned in a pCRII-TOPO vector (Invitrogen) and sequenced using an ABI automatic sequencer. Digoxigenin-labeled riboprobes were synthesized using a digoxigenin RNA labeling kit, following the manufacturer’s instructions (Roche Diagnostics). In situ hybridization was carried out essentially as previously described (Moniot et al., 2008).

Histochemical and immunofluorescence staining and antibodies

Gonads were dissected from staged embryos, fixed in 4% paraformaldehyde, then processed and cut into 12 µm thick cryosections, as previously described (Gasca et al., 2002; Malki et al., 2005b). For immunolabeling, slides were dried, rehydrated, rinsed in PBS and incubated with different antibodies, as previously described (Gasca et al., 2002). SOX9 was detected using a 1:200 dilution of a rabbit polyclonal antibody raised against the N-terminal part of the human protein (Notarnicola et al., 2006). The basal lamina surrounding testis cords was detected using a 1:400 dilution of a mouse monoclonal anti-laminin antibody (Sigma-Aldrich, clone Lam-89). Cell types in the gonad were detected using dilutions of goat anti-AMH (Santa Cruz Laboratories, C20; 1:100), mouse anti-OCT4 (Santa Cruz Laboratories C10; 1:50), rat anti-PECAM (BD Pharmingen, MEC 13.3; 1:100), rabbit anti-VASA (Abcam, ab13840; 1:300) and mouse anti-α-tubulin (Sigma-Aldrich, 1:500) antibodies. The appropriate secondary antibodies (Alexa-Ig, Molecular Probe) were used (dilution 1:800). The L-PGDS antibody was raised in rabbit, against the entire mouse L-PGDS protein coupled to GST protein. The rabbit serum was purified first through a GST column to remove anti-GST antibodies and was recovered through a L-PGDS protein-coupled column. The purified antibody was characterized by western blotted (dilution 1:500) on protein extracts of HeLa cells transfected with empty pcDNA vector (EV) or L-Pgds cDNA-pcDNA vector and extracts from 13.5 dpc male and female gonads (without mesonephros). The antibody was also characterized by immunofluorescence (dilution 1:500) on cryosections from 13.5 dpc male and female gonads. Immunostaining experiments were analyzed by fluorescent microscopy (Leica DMRA2) or by laser confocal microscopy (Leica SP2).

Reverse transcription and real-time PCR

During dissection, embryonic gonads were separated from mesonephros and conserved in RNAlater Solution (Qiagen) until genotyping for sex. Gonads were then pooled (10 pairs of gonads per experiment) and RNA extraction was performed using the RNeasy Mini Kit (Qiagen). Gene expression levels of L-Pgds was considered statistically significant at a P<0.05. Statistical analysis was performed using Student’s t test, and results were considered statistically significant at a P<0.05.
RESULTS

Cell type-specific expression of L-Pgds

The L-Pgds gene was originally identified as a gene differentially expressed in Sertoli cells and germ cells during sex determination in embryonic mouse gonads (Adams and McLaren, 2002) but later studies have reported that L-Pgds is expressed only in differentiating Sertoli cells starting at stage 11.5 dpc (17 ts stage) shortly after the onset of Sox9 expression (Wilhelm et al., 2007). Moreover, the postnatal rat testis was shown to express L-Pgds in both germ cells and Sertoli cells (Samy et al., 2000). To resolve these discrepancies, we developed an antibody raised against mouse L-PGDS protein and analyzed its expression in developing gonads, together with a Sertoli cell marker (AMH) and a germ cell marker (VASA), using confocal microscopy. The characterization of this antibody is presented in Fig. S1 in the supplementary material. L-PGDS is indeed expressed in differentiating 12.5 dpc Sertoli cells together with AMH (Fig. 1, top left panel, arrowheads), and is also in the cytoplasm of some VASA-positive cells identified as germ cells (Fig. 1, top right panel, white arrows), although some germ cells are negative for L-PGDS. By contrast, L-PGDS protein could not be detected at any stages in female gonads (data not shown).

Using in situ hybridization to detect L-Pgds transcript coupled to immunofluorescence to detect SOX9 protein, we also showed that in the newborn testis, L-Pgds expression was limited to the germ cell lineage (see Fig. S2 in the supplementary material, top panels) whereas in the adult testis, L-Pgds was found in the interstitial compartment containing Leydig cells, in the basal germ cell layer containing spermatagonia and in the Sox9-expressing Sertoli cells (see Fig. S2 in the supplementary material, bottom panels).

To summarize, L-Pgds transcripts are expressed from embryonic stages until birth in both Sertoli and germ cells whereas in newborns, the expression is turned off in Sertoli cells and stays on in germ cells. In the fertile adult, L-Pgds expression is present in SOX9-expressing Sertoli cells and in cells in the early stages of spermatogenesis (spermatogonia) but has also shifted to interstitial Leydig cells.

Male-specific onset of L-Pgds expression is controlled by SOX9

Recent studies showed that SOX9 and SRY are able to bind to a conserved paired Sox binding site at ~400 bp downstream of the L-Pgds promoter. The binding of SOX9 but not of SRY was sufficient for transactivation of the L-Pgds promoter following transfection of cultured cells (Wilhelm et al., 2007). Furthermore, ectopic expression of Sry in a Hsp70.3 promoter/Sry transgenic mouse line did not modify the expression pattern of L-Pgds in XX transgenic gonads compared with that of wild-type XY gonads, suggesting that L-Pgds is not directly regulated by SRY (Wilhelm et al., 2007). However, even though ectopic Sry expression precedes Sox9 expression by at least 8 hours in this transgenic mouse line, the absence of a modified expression pattern of L-Pgds might be due to the absence of a specific SRY protein partner at this stage in XX gonads, rather than the absence of a functional interaction between SRY and the L-Pgds promoter.

To define the in vivo genetic relationships between Sry, Sox9 and L-Pgds, we used real-time RT-PCR and in situ hybridization to analyze the expression of L-Pgds in Sox9 homozygous mutant XY gonads, generated by crossing mice homozygous for a conditional null allele of Sox9 (Sox9flo/fl) with mice carrying CRE recombinase under the control of the cytokeratin K19 (Ck19, or Krt19 - Mouse Genome Informatics) gene that is expressed throughout the postimplantation mouse embryo (Ck19-Cre; Sox9flo/fl mice) (Barriónuevo et al., 2006). Real-time RT-PCR analysis on RNA extracted from 11.5 dpc XY mutant (XY−−) and wild-type (XY++) gonads indicated that L-Pgds expression was almost completely abolished in mutant gonads, with L-Pgds transcript levels comparable to those in XX gonads (Fig. 2A). In situ hybridization on gonad sections confirmed that mutant XY gonads essentially lack L-Pgds expression, whereas the germ cell marker OCT4 was equally expressed in mutant and wild-type gonads (Fig. 2B), confirming that the initiation of L-Pgds expression is under the exclusive control of SOX9 and not of SRY.

Maintenance of L-Pgds expression is dependent on SOX9

In order to evaluate the possible role of SOX9 in the maintenance of L-Pgds expression after the onset of testis differentiation, L-Pgds expression was monitored in XY gonads from Amh-Cre; Sox9flo/fl mutant embryos. In these mutants, Sox9 is completely deleted in 14.5 dpc Sertoli cells (Barriónuevo et al., 2008). By using in situ hybridization, we detected L-Pgds expression in 13.5 dpc mutant gonads; at this time point, SOX9 is still expressed together with AMH at a level similar to that observed in wild-type gonads (Fig. 3A-D). This is consistent with the fact that expression of the human AMH-driven transgene occurs one or two days later than the endogenous mouse Amh gene (Lecureuil et al., 2002). By contrast, SOX9 had almost completely disappeared in 14.5 dpc mutant gonads, which show reduced levels of AMH and dramatically downregulated L-Pgds expression (Fig. 3E-H). Clearly in these experiments the level of L-Pgds transcript correlates directly with that of nuclear SOX9 protein. Moreover, the fact that L-Pgds is detected in the 13.5 dpc mutant gonad shows that normal activation occurs at an early stage. Real-time RT-PCR analysis on RNAs extracted from 15.5 dpc XY mutant (XY−−) and wild-type (XY++) gonads indicated that L-Pgds and Sox9 expression were almost completely abolished in mutant XY gonads (Fig. 3I). Thus, we conclude that SOX9 is required for the maintenance of L-Pgds
gene expression during testis cord differentiation. Together with published results showing that SOX9 binds to the 5′/H11032 region of the L-Pgds gene in vivo and activates its transcription in vitro (Wilhelm et al., 2007), our results provide genetic evidence for a pathway ordered from Sry → Sox9 → L-Pgds, rather than from Sry → L-Pgds → Sox9.

**L-Pgds expression is independent of FGF9**

One recent study showed that SOX9 is essential for Fgf9 male-specific upregulation, and that FGF9 in turn maintains expression of Sox9, generating a positive feedforward loop (Kim et al., 2006). We wondered whether the FGF9 pathway could be required together with SOX9 for initial activation of the L-Pgds gene. To test this hypothesis, we analyzed L-Pgds expression in 12.0 dpc XY wild-type or Fgf9−/− mutant gonads by in situ hybridization. L-Pgds antisense riboprobe revealed that L-Pgds expression within the testis cords (arrows) of wild-type (Sox9+/+) gonads is abolished in mutant (Sox9−/−) gonads, whereas Oct4 expression is similar in both Sox9 genotypes. Scale bars: 50 μm.

**Fig. 2. Initiation of L-Pgds expression is dependent on SOX9.**

(A) Real-time RT-PCR analysis of L-Pgds expression in 11.5 dpc wild-type male (XY+/+), female (XX+/+) and Ck19-Cre; Sox9flox/flox mutant male (XY−/−) gonads. Datasets (n=5) were normalized to Gapdh expression and averaged. Normalized relative expression revealed that L-Pgds expression is completely abolished in mutant (XY−/−) compared with wild-type (XY+/+) gonads (*, P value <0.05). Error bars indicate s.d. of triplicate experiments. (B) mRNA in situ hybridization for L-Pgds (left) and germ cell marker Oct4 (right) on frozen sections from wild-type (Sox9+/+, top) and mutant (Sox9−/−, bottom) 12.0 dpc male gonads. L-Pgds antisense riboprobe revealed that L-Pgds expression within the testis cords (arrows) of wild-type (Sox9+/+) gonads is abolished in mutant (Sox9−/−) gonads, whereas Oct4 expression is similar in both Sox9 genotypes. Scale bars: 50 μm.

**Fig. 3. Maintenance of L-Pgds expression is dependent on SOX9.**

(A-H) mRNA in situ hybridization for L-Pgds was performed on 13.5 dpc (A,C) and 14.5 dpc (E,G) gonads from wild-type (A,E) and Amh-Cre; Sox9flox/flox mutant (C,G) XY embryos. In parallel to L-Pgds in situ hybridization, co-immunofluorescence staining for SOX9 (green) and AMH (red) was performed on wild-type (B) and mutant (D) 13.5 dpc XY gonads, and wild-type (F) and mutant (H) 14.5 dpc XY gonads. TC, testis cords. Scale bars: 75 μm in A,C,E,G; 50 μm in B,D,F,H. (I) Real-time RT-PCR analysis of L-Pgds and Sox9 expression in 15.5 dpc wild-type male (XY+/+), female (XX+/+) and Amh-Cre; Sox9flox/flox mutant male (XY−/−) gonads. Normalized relative expression revealed that L-Pgds and Sox9 expression is significantly reduced in mutant (XY−/−) compared with wild-type (XY+/+) gonads (***, P<0.01). Error bars indicate s.d. of triplicate experiments.
(Fig. 4B) showed similar levels of Fgf9 expression, confirming that the pathways do not interact genetically. If the PGD2 pathway is not required for Fgf9 expression and vice versa, we wanted to investigate whether both pathways could have an additive effect on Sox9 gene expression. For that purpose, we performed an in vitro assay using the human NT2/D1 cell line, which displays cellular characteristics of embryonic Sertoli cells (Knower et al., 2007). A time course of Sox9 transcript expression upon stimulation by FGF9 and/or PGD2 showed that both signaling molecules cooperate to additively upregulate Sox9 expression (Fig. 4C).

**L-Pgds ablation perturbs Sox9 transcript expression and SOX9 protein subcellular localization**

We previously reported the role of PGD2 in the nuclear translocation of SOX9 (Malki et al., 2005b), and an activating effect of PGD2 on Sox9 transcription has also been reported (Wilhelm et al., 2007). To go further, we asked whether L-Pgds mutant XY gonads show defects in Sox9 expression, SOX9 subcellular localization and testis cord formation.
formation. The global morphology of the mutant gonads of all stages analyzed was similar to that of wild-type gonads, with levels of Sox9 expression and subcellular localization of SOX9 comparable between L-Pgds+/− XY and wild-type gonads (data not shown). We performed analysis of L-Pgds+/− and L-Pgds−/− XY gonads from 11.5 dpc up to 14.5 dpc, a time when testis cords are fully formed in the wild-type testis, and at the 17.5 dpc late embryonic stage. At 11.5 dpc (18-20 ts stage), all L-Pgds−/− XY gonads (n=7) showed an abnormal SOX9 cellular localization pattern (Fig. 5A). Cytoplasmic SOX9 remained in all samples even though some cells also expressed SOX9 in their nuclear compartment (Fig. 5B). By contrast, in heterozygous XY gonads, all cells expressed SOX9 exclusively in the nucleus (Fig. 5B). Gonads from 12.5 dpc, 13.5 dpc and 14.5 dpc L-Pgds−/− embryos show a variable SOX9 expression pattern and sex cord formation phenotype ranging from normal to severely abnormal. Moreover, a temporal recovery process occurred: 14% (1/7) of the mutant gonads revealed a normal phenotype similar to that of wild-type gonads at 12.5 dpc, 55% (5/9) at 13.5 dpc and 72% (5/7) at 14.5 dpc, suggesting a delay in testicular organogenesis (Fig. 5A).

The abnormal global phenotype of 13.5 dpc XY L-Pgds−/− gonads was characterized by co-immunofluorescence with SOX9 and laminin to delineate testis cords. In L-Pgds−/− gonads testis cords are completely formed, whereas in mutant gonads formation of some testis cords was impaired (Fig. 6A, long arrows) with several SOX9-positive cells lying outside of the cords (Fig. 6A, short arrows). The same phenotype was seen in 12.5 dpc L-Pgds−/− XY gonads (Fig. 5C, long and short arrows). SOX9 activation was itself delayed, with the overall number of SOX9-expressing cells and SOX9 protein levels in these gonads reduced at 12.5 dpc (Fig. 5C) and at 13.5 dpc (Fig. 6B, left panels, asterisks), and some gonads still showing cytoplasmic SOX9 (Fig. 6B, right panels, arrow). Laminin or tubulin staining confirmed incomplete testis cord formation in some mutant gonads (Fig. 6B). Sox9 and Amh mRNA levels were quantified by real-time RT-PCR from 10 L-Pgds−/− and 10 L-Pgds+/− XY pairs of gonads at the 12.5 dpc stage. Sox9 and Amh (a direct target gene of SOX9) expression levels were significantly reduced by 50% in 12.5 dpc L-Pgds−/− compared with those in 12.5 dpc L-Pgds+/− and wild-type (data not shown) XY gonads (Fig. 7A,B).

The process of testis cord organization was delayed up to 14.5 dpc but was universally complete by the late embryonic stages (17.5 dpc). At this later stage, SOX9 was always localized in the nucleus, even though up to 17.5 dpc the level of Sox9 expression remains lower in L-Pgds−/− than in L-Pgds+/− XY gonads (Fig. 7C). Real-time RT-PCR confirmed the significant decreased Sox9 and Amh expression levels in 17.5 dpc L-Pgds−/− compared with L-Pgds+/− and wild-type (data not shown) XY gonads (Fig. 7D).

**DISCUSSION**

Prostaglandin D synthase displays a male-specific expression pattern during mammalian testicular differentiation (Adams and McLaren, 2002). By investigating the function of the PGD2 signaling pathway in mammalian sexual differentiation, we have previously shown that SOX9 nuclear localization is regulated via its L-Pgd-initiated PKA phosphorylation (Malki et al., 2005b). Moreover, in vitro luciferase assays and chromatin immunoprecipitation experiments performed on embryonic gonads have shown that SOX9 can bind and activate the L-Pgs promoter (Wilhelm et al., 2007). In this study, we confirmed genetically that the L-Pgs/PGD2 pathway forms an amplification loop with the Sox9 gene, whereby SOX9 activates and maintains L-Pgs gene transcription and PGD2 participates in the maintenance of Sox9 expression during Sertoli cell differentiation.
Here, we observed that L-Pgds male gene expression, which could later be maintained by SOX9 established. One possibility is that SRY protein initially activates agreement with the fact that SOX9 protein binds to the cells. Examination of XX gonads from double knockout embryos the somatic environment suggesting that, via PGD2, the germinal PGD2-producing cells could participate in the masculinization of Rspo1 knockout mice, XX–/– mutants develop In rspondin1 (Adams and McLaren, 2002). have shown that PGD2 production by PGCs is a response to a environment during testicular differentiation? Adams and McLaren regulatory controls.

What is the impact of germ cell PGD2 production on the somatic environment during testicular differentiation? Adams and McLaren (2002). In rspoind1 (Rspo1) knockout mice, XX+− mutants develop ovotestes with masculinized XX germ cells expressing high levels of L-Pgds at later stages (18.5 dpc) (Chassot et al., 2008). These PGD2-producing cells could participate in the masculinization of the somatic environment suggesting that, via PGD2, the germinal lineage may have an important influence on surrounding somatic cells. Examination of XX gonads from double knockout embryos for L-Pgds and Rspo1 should test this hypothesis.

These expression studies showing that L-Pgds transcript is always co-expressed in differentiating Sertoli cells with SOX9 are in good agreement with the fact that SOX9 protein binds to the L-Pgds promoter region (Wilhelm et al., 2007). However, the position of the L-Pgds gene in the sex-determining cascade was not clearly established. One possibility is that SRY protein initially activates L-Pgds gene expression, which could later be maintained by SOX9. Here, we observed that L-Pgds expression is abolished in 11.5-12.0 dpc male Sox9+− gonads (Ck19-Cre; Sox9floxflox mice), at stages in which the SRY protein is fully expressed, showing that SOX9 is required for the initiation of L-Pgds gene expression and genetically confirming that L-Pgds is not a target for the SRY protein. Moreover, ablation of Sox9 after the onset of L-Pgds expression (~13.5-14.5 dpc, Amh-Cre; Sox9floxflox) also induces a strong downregulation of L-Pgds transcript expression, demonstrating the requirement for SOX9 protein in the maintenance of L-Pgds gene expression in embryonic Sertoli cells. A recent study, analyzing the effects of Dax1 (Nr6b1 - Mouse Genome Informatics) knockout on testicular differentiation in various genetic backgrounds, reported that the expression of Sox9 transcript was not altered in a Dax1−/−129 background displaying testicular abnormality but no sex reversal (Park et al., 2008). In this model, L-Pgds expression was significantly decreased, strongly suggesting that DAX1 protein could participate in the regulation of the L-Pgds gene. Further experiments such as chromatin immunoprecipitation for DAX1 could investigate binding to the L-Pgds promoter to address this hypothesis.

We and others have shown that signaling by PGD2 increases the transcription of the Sox9 gene in mouse (Wilhelm et al., 2005) and chicken (Moniot et al., 2008) cultivated explant gonads, and induces nuclear translocation of the SOX9 protein (Malki et al., 2005b) leading to the model: PGD2→SOX9. To genetically confirm this hypothesis, we examined L-Pgds+− XY gonads and observed that testicular organogenesis was affected in the early developmental stages, with visible defects in Sertoli cell differentiation. Moreover, expression of the Sox9 transcript was diminished by nearly 50%, showing that the PGD2 pathway participates in maintaining Sox9 expression in the differentiating testis. Amh was also expressed at lower levels in L-Pgds+− XY compared with wild-type gonads, indicating that direct target genes are also affected by a reduction in SOX9 protein levels. SOX9 subcellular localization was disturbed, confirming the role of PGD2 in its nuclear translocation. However, no sex reversal was observed in the L-Pgds+− mutant mice, and

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)
Sex-specific regulation of gonad organogenesis is the result of the cell-autonomous response of some cells to the sex-determining switch SRY directly activating Sox9 expression (Sekido and Lovell-Badge, 2008). Besides this, feedforward pathways of Sox9 gene expression with the classic FGF9 signaling pathway (Kim et al., 2006) and, more recently, with a high-glucose metabolic state through an extracellular matrix (ECM) (Matoba et al., 2008), were shown to be necessary for the maintenance of Sox9 transcription and subsequently for Sertoli cell differentiation and testis cord formation. In this study, we have revealed that the PGD2 signaling pathway is likely to act independently of FGF9, thus implicating two independent feedforward loops between Sox9/Fgf9 (Kim et al., 2006) and Sox9/L-Pgds in the coordination of growth, cell differentiation and morphogenesis of the gonad. Why such a complex regulation? The control of Sox9 gene expression in Sertoli cells is crucial for the mammalian species in which cases of misregulation lead to a sex reversal and sterility. These three additive pathways are probably the best way of ensuring that the Sox9 gene is transcribed efficiently.

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Supplemental material
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