

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; Chaboissier 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

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(Kim et al., 2007; Schmahl et al., 2004). Conditional deletion of *Fgfr2* in mouse embryonic gonads confirmed the distinct roles of FGF9 in Sertoli cell proliferation and differentiation (Bagheri-Fam et al., 2008; Kim et al., 2007).

Adams and McLaren have previously shown that the gene encoding the enzyme lipocalin-type prostaglandin D2 synthase (L-PGDS, PTGDS - Mouse Genome Informatics), which converts prostaglandin H2 into prostaglandin D2 (PGD2), is male-specifically expressed at early stages of mouse gonadogenesis (Adams and McLaren, 2002). Moreover, PGD2 has a masculinizing effect on cultivated XX gonadal explants (ectopic testicular cord formation and expression of AMH) (Adams and McLaren, 2002). This effect of PGD2 is mediated by stimulating the transcription of the *Sox9* gene (Wilhelm et al., 2005). PGD2 also has an effect at the posttranslational level on nuclear translocation of the SOX9 protein (Malki et al., 2005b): SOX9 is excluded from the nucleus in the genital ridge of both sexes prior to sex determination (de Santa Barbara et al., 2000; Morais da Silva et al., 1996) via a nuclear export signal (NES) located in its HMG domain (Gasca et al., 2002), and is retained in the cytoplasm possibly via its interaction with microtubules (Malki et al., 2005a). Upon sex determination, SOX9 nuclear translocation is strongly stimulated by PGD2 signaling, transduced by the cAMP/PKA pathway (Malki et al., 2005b).

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 prospermatogonia 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/Sox9^{fllox/fllox}* and *Amh-Cre/Sox9^{fllox/fllox}* 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-acting *L-Pgds/Sox9* and *Fgf9/Sox9* regulatory loops, contributing to the final differentiation of Sertoli cells.

MATERIALS AND METHODS

Mouse 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; Sox9^{fllox/fllox}*, *Amh-Cre; Sox9^{fllox/fllox}* and *Fgf9^{-/-}* mutants has been previously described (Barrionuevo 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 μ m 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-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 blotting (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 mesonephroi 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). Reverse transcriptions were carried out with 500 ng of total RNA previously treated with DNase I (Promega), 250 ng of random primers (Invitrogen) and SuperScript III (Invitrogen), following manufacturers' recommendations.

Resulting cDNAs were then subjected to quantitative real-time PCR in a LightCycler 480 (Roche) apparatus using the QuantiTect SYBR Green PCR Kit (Qiagen). Gene expression levels of *L-Pgds*, *Sox9*, *Amh*, *Fgf9* and *Gapdh* were investigated using the following pairs of primers (from 5' to 3'): *L-Pgds* Forward, GGCTCCTGGACACTACACCT; *L-Pgds* Reverse, ATAGTTGGCCCTCCACCACTG; *Sox9* Forward, TCGGACACG-GAGAACC; *Sox9* Reverse, GCACACGGGAACCTTATCTT; *Amh* Forward, GGGGAGACTGGAGAACAGC; *Amh* Reverse, AGAGCTCG-GGCTCCCAT; *Fgf9* Forward, TGCAGGACTGGATTTCATTTAG; *Fgf9* Reverse, CCAGGCCACTGCTATACTG; *Gapdh* Forward, TGGCAA-AGTGGAGATTGTTGCC; *Gapdh* Reverse, AAGATGGTGATGGGCTCCCG.

Each sample was measured in triplicate in three separate experiments. Quantification was performed using a second derivative calculation method with LC480 software version 1.5 (Roche) using *Gapdh* as the reference gene. 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 prospermatogonia 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* (*Sox9^{fllox/fllox}*) 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*; *Sox9^{fllox/fllox}* mice) (Barrionuevo et al., 2006). Real-time RT-PCR

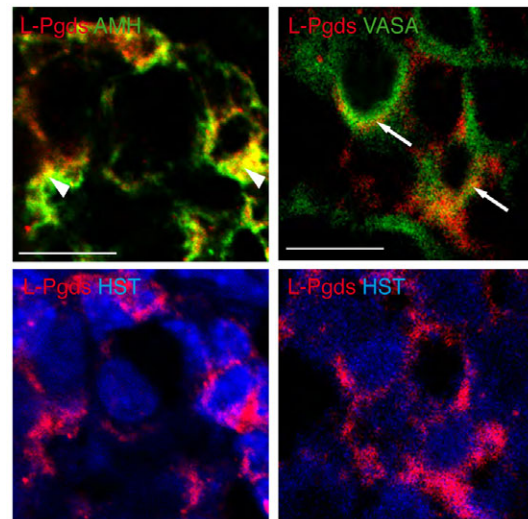


Fig. 1. Confocal analysis of co-immunofluorescence experiments on XY 12.5 dpc gonads. Top: frozen sections were stained for L-PGDS (red), together with AMH (left) or VASA (right, green), and analyzed by laser confocal microscopy. Bottom: Hoechst staining (HST, blue) labeled nuclei. Sections (250-500 nm) were routinely visualized and panels that are shown represent a unique z-section. Arrowheads highlight L-PGDS and AMH overlapping staining and arrows indicate L-PGDS within the cytoplasm of germ cells. Scale bars: 10 μ m.

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*; *Sox9^{fllox/fllox}* mutant embryos. In these mutants, *Sox9* is completely deleted in 14.5 dpc Sertoli cells (Barrionuevo 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 ($XY^{+/+}$ or $XX^{+/+}$) 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*

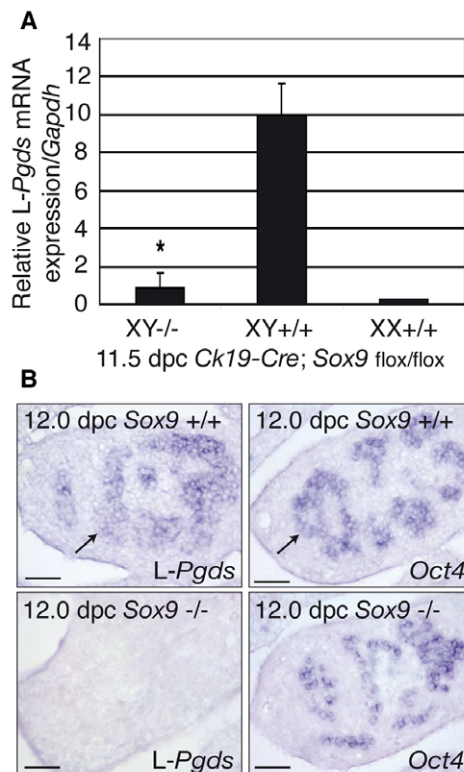


Fig. 2. Initiation of *L-Pgds* expression is dependent on SOX9. (A) Real-time RT-PCR analysis of *L-Pgds* expression in 11.5 dpc wild-type male (XY^{+/+}), female (XX^{+/+}) and *Ck19-Cre*; *Sox9*^{flox/flox} mutant male (XY^{-/-}) gonads. Datasets ($n=5$) were normalized to *Gapdh* expression and averaged. 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 μ m.

gene expression during testis cord differentiation. Together with published results showing that SOX9 binds to the 5' 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 coupled to immunofluorescence staining for SOX9; at this stage, SOX9 is transiently expressed in pre-Sertoli cells (Morais da Silva et al., 1996) under the control of SRY. *L-Pgds* expression was similar in both wild-type and *Fgf9*^{-/-} mutant gonads (Fig. 4A, left panels) and SOX9-expressing Sertoli cells also expressed *L-Pgds* (Fig. 4A, right

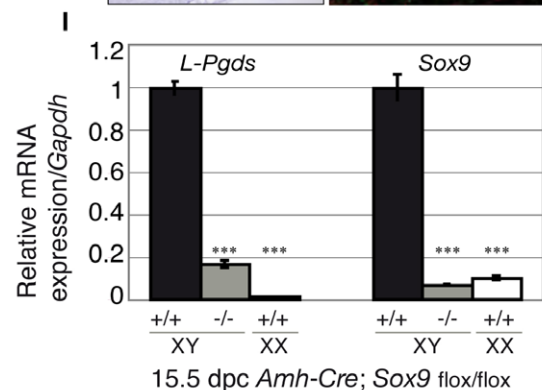
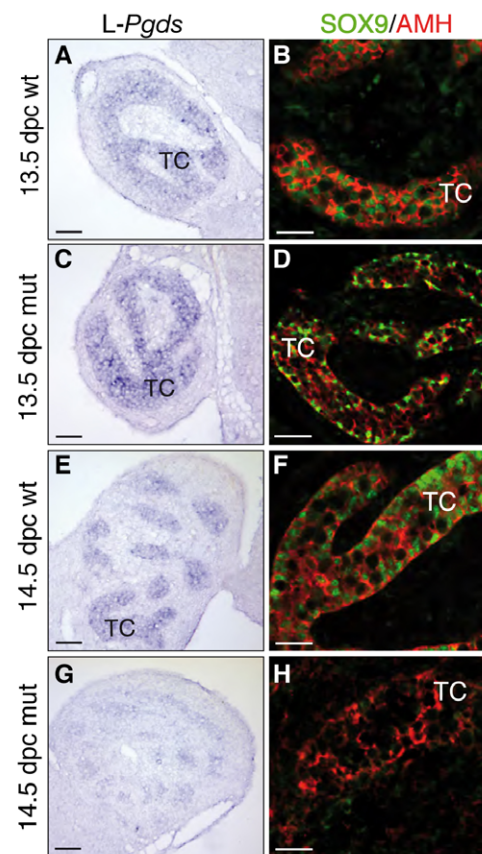


Fig. 3. Maintenance of *L-Pgds* expression is dependent on SOX9. (A-H) mRNA in situ hybridization for *L-Pgds* was performed on 13.5 dpc (A, C) and 14.5 dpc (E, G) gonads from wild-type (A, E) and *Amh-Cre*; *Sox9*^{flox/flox} mutant (C, G) XY embryos. In parallel to *L-Pgds* in situ hybridization, co-immunofluorescence 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 μ m in A, C, E, G; 50 μ m 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*; *Sox9*^{flox/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.

panels, arrows). We subsequently also wanted to examine the reciprocal dependence, namely whether *Fgf9* expression depends on *L-Pgds* status. Real-time RT-PCR analysis of RNAs extracted from 12.5 dpc *L-Pgds* mutant (XY^{-/-}) and heterozygous (XY^{+/-}) gonads

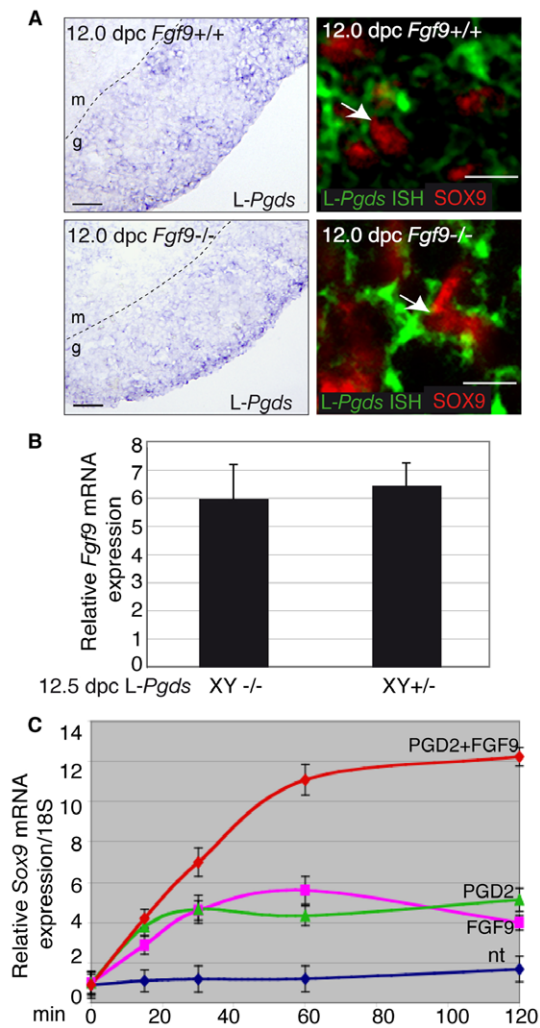


Fig. 4. *L-Pgds* expression is independent of FGF9 signaling. (A) mRNA in situ hybridization for *L-Pgds* (left) was performed on frozen sections from wild-type (*Fgf9*^{+/+}, top) and mutant (*Fgf9*^{-/-}, bottom) 12 dpc male gonads. Following *L-Pgds* in situ hybridization, the sections were submitted to immunofluorescence for SOX9 (red) and *L-Pgds* in situ hybridization is represented in green (right). Arrows highlight SOX9 and *L-Pgds* expressing cells. g, gonad; m, mesonephros. Scale bars: 50 μ m in left, 10 μ m in right. (B) Real-time RT-PCR analysis of *Fgf9* expression was performed on 12.5 dpc male heterozygous (XY^{+/-}) and homozygous (XY^{-/-}) *L-Pgds* mutant gonads. For each point, datasets ($n=5$) were normalized to *Gapdh* expression and averaged. Normalized relative expression shows that *Fgf9* expression is similar in both genotypes. Error bars indicate s.d. of triplicate experiments. (C) Real-time RT-PCR analysis of *Sox9* expression was performed on NT2/D1 cells treated with PGD2, FGF9, PGD2+FGF9 or with vehicle (ethanol, nt) for 20-120 minutes. Datasets ($n=5$) were normalized to *18S* expression and averaged. Normalized *Sox9* relative expression revealed that FGF9 and PGD2 signaling molecules cooperate to additively upregulate expression of *Sox9*.

(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

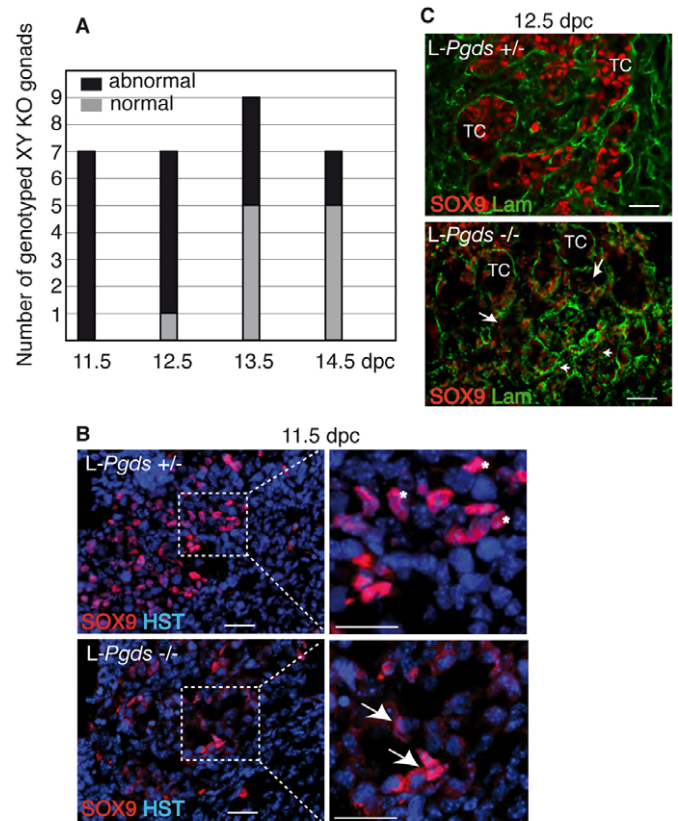


Fig. 5. Sertoli cell differentiation and testis cord formation is delayed in the absence of *L-Pgds*. (A) Seven to nine *L-Pgds*^{-/-} male gonads were analyzed at developmental stages 11.5 dpc, 12.5 dpc, 13.5 dpc and 14.5 dpc in terms of their SOX9 phenotype (SOX9 subcellular localization and number of SOX9-expressing cells) by immunofluorescence. 'Normal' (gray) corresponds to a phenotype with exclusively nuclear SOX9 and a number of SOX9-expressing cells identical to that observed in *L-Pgds*^{+/+} gonads. 'Abnormal' (black) corresponds to the presence of cytoplasmic and nuclear SOX9 and/or a number of SOX9-expressing cells decreased by 25% compared with that observed in *L-Pgds*^{+/+} gonads. (B,C) Immunofluorescence of frozen sections from 11.5 dpc and 12.5 dpc *L-Pgds*^{+/+} (top) and *L-Pgds*^{-/-} (bottom) male gonads, stained for SOX9 (red; B,C) and laminin (green, C), and with Hoechst dye (HST, blue; B) to label nuclei. In B, enlarged panels (right) highlight cytoplasmic SOX9 (arrows) in *L-Pgds*^{-/-} XY gonads compared with exclusive nuclear SOX9 (asterisks) in *L-Pgds*^{+/+} gonads. In C, SOX9 and laminin staining revealed a low level of SOX9 expression and impaired testis cord formation (long arrows) in *L-Pgds*^{-/-} gonads, compared with *L-Pgds*^{+/+} gonads. Short arrows indicate SOX9-positive cells lying outside testis cords and TC indicates completely formed testis cords. Scale bars: 50 μ m in B (left), 20 μ m in B (right), 50 μ m in C.

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. 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 PGD2-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-Pgds* promoter (Wilhelm et al., 2007). In this study, we confirmed genetically that the *L-Pgds*/PGD2 pathway forms an amplification loop with the *Sox9* gene, whereby SOX9 activates and maintains *L-Pgds* gene transcription and PGD2 participates in the maintenance of *Sox9* expression during Sertoli cell differentiation.

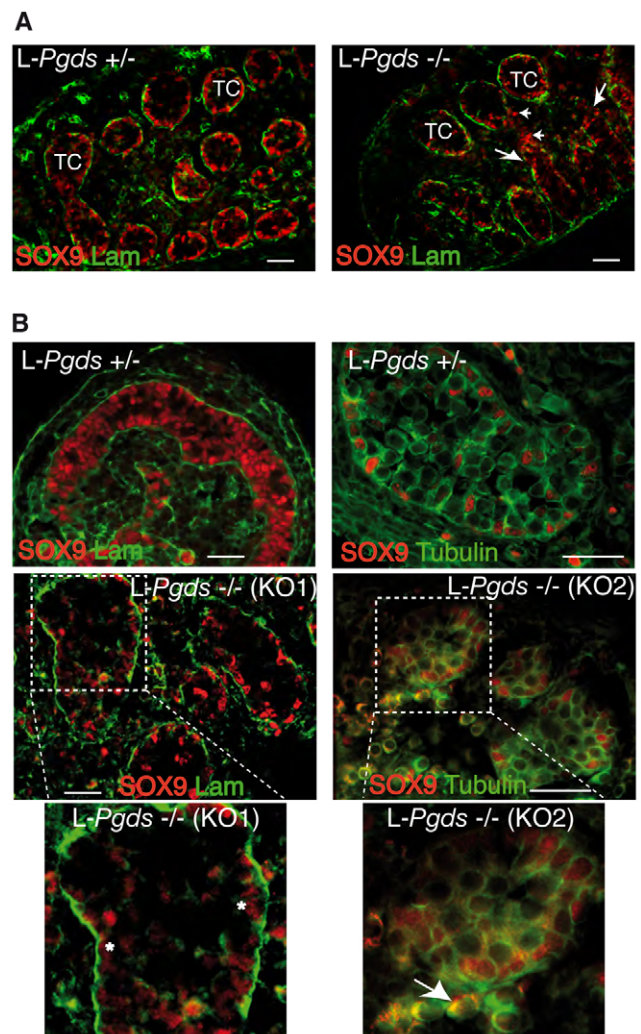


Fig. 6. Phenotype of 13.5 dpc XY *L-Pgds*^{-/-} gonads. (A) Sagittal cryosections of 13.5 dpc XY *L-Pgds*^{+/-} (left) and *L-Pgds*^{-/-} (right) gonads were submitted for SOX9 (red) and laminin (green) co-immunofluorescence. Long arrows show impaired testis cord organization, short arrows indicate SOX9-positive cells lying outside testis cords in *L-Pgds*^{+/-} gonads, normal testis cords are highlighted by TC. (B) Two 13.5 dpc XY *L-Pgds*^{-/-} gonads (KO1, left and KO2, right) with different abnormal phenotypes are shown. Frozen sections were stained for SOX9 (red) and laminin (left) or tubulin (right, green). Top: XY *L-Pgds*^{+/-} gonads. Asterisks and arrows indicate nuclear and cytoplasmic SOX9, respectively. Bottom panels are enlarged from middle panels. Scale bars: 100 μm in A, 50 μm in B (KO1), 30 μm in B (KO2).

In the original article describing *L-Pgds* expression during testicular differentiation, expression was found in both somatic (presumably pre-Sertoli) cells and in germ cells (Adams and McLaren, 2002). By contrast, recent data described *L-Pgds* expression uniquely in the somatic lineage of 14.5 dpc male embryonic gonads (Wilhelm et al., 2007). Using immunofluorescence/in situ hybridization and co-immunofluorescence coupled with confocal analysis, we found that *L-Pgds* was expressed in Sertoli cells (together with AMH) and also in germ cells. In this cell type, we found that *L-Pgds* was weakly expressed compared with Sertoli cells and that not all germ cells are positive for *L-Pgds*. *L-Pgds* expression status in germ cells might depend on their proliferation/differentiating state or might depend on

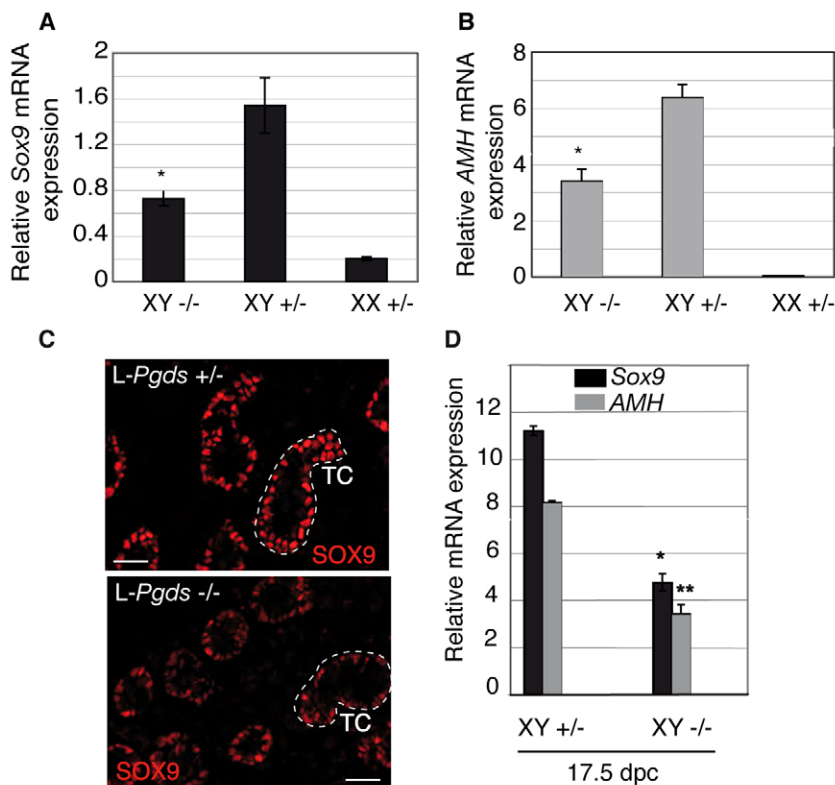


Fig. 7. Quantification of *Sox9* and *Amh* transcript levels in *L-Pgds*^{-/-} gonads and phenotype of 17.5 dpc *L-Pgds*^{-/-} gonads.

(A,B,D) Relative expression of *Sox9* (black bars; A,D) and *Amh* (gray bars; B,D) mRNA in male (XY^{+/+}) and female (XX^{+/+}) heterozygous and in male homozygous (XY^{-/-}) mutant gonads collected from 12.5 dpc and 17.5 dpc embryos, normalized to *Gapdh* mRNA levels. Error bars indicate s.d. of triplicate experiments performed on 10 individual gonads. (*, $P < 0.05$ in A,B and ***, $P < 0.025$ in D). (C) SOX9 immunostaining (red) was performed on frozen sections from 17.5 dpc *L-Pgds*^{+/+} (top) and *L-Pgds*^{-/-} (bottom) male gonads. Testis cords (TC) are highlighted by dashed lines. Scale bars: 50 μ m.

paracrine signals from the neighboring somatic cells. Moreover, our results also demonstrate that germ cell expression of *L-Pgds* is not dependent on the presence of Y chromosome genes that could be expressed in primordial germ cells (PGCs). Interestingly, whereas *Sox9* and *L-Pgds* are always co-expressed during fetal life in the differentiating Sertoli cells, at the newborn stage only *Sox9* expression remains in the Sertoli cells and *L-Pgds* transcript is found in the germ cell lineage, meaning that at these stages regulation of this transcript by SOX9 can occur in a paracrine manner or must be under different regulatory controls.

What is the impact of germ cell PGD2 production on the somatic environment during testicular differentiation? Adams and McLaren have shown that PGD2 production by PGCs is a response to a somatic masculinizing environment (Adams and McLaren, 2002). In *rspondin1* (*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; Sox9*^{fllox/fllox} 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; Sox9*^{fllox/fllox}) 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* (*Nr0b1* - Mouse Genome Informatics) knockout on testicular differentiation in various genetic backgrounds, reported that the expression of *Sox9* transcript was not altered in a *Dax1*^{-Y}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

examination of 17.5 dpc XY gonads revealed normal testicular cords even though the level of the *Sox9* transcript remained lower than in wild-type gonads.

These observations suggest that the expression level of the *Sox9* transcript is not solely maintained by the *L-Pgds*/PGD2 pathway, and that other signaling pathways compensated for impaired early Sertoli cell differentiation in the *L-Pgds* mutants. FGF9 represents a good candidate for fulfilling this role, as the FGF9 pathway was shown to maintain *Sox9* expression in Sertoli precursor cells that, in turn, maintained a high level of *Fgf9* expression (Kim et al., 2006). However, in vivo, in the absence of FGF9, the PGD2 pathway has only a marginal effect on testicular differentiation: *Fgf9*^{-/-} male gonads display variable phenotypes, from disorganized testis on mixed genetic background (129; C57/BL6) (Colvin et al., 2001), to complete sex reversal on a pure background (C57/BL6) (Schmahl et al., 2004). Moreover, we show in the present study that despite the initiation of *L-Pgds* expression by SOX9 in *Fgf9*^{-/-} gonads at 12.0 dpc, *Sox9* expression was not maintained. By contrast, FGF9 may compensate for the lack of PGD2 in *L-Pgds* mutant gonads to maintain a minimal level of SOX9 protein able to induce testicular differentiation. Interestingly, decreased levels of *Sox9* transcript in 13.5 dpc *Dax1* knockout XY embryonic gonads is associated with sex reversal in a mixed genetic background (129; C57/BL6) (Park et al., 2008). In the present study however, the reduction in *Sox9* transcript levels in 13.5 dpc *L-Pgds*^{-/-} XY gonads on a pure background (C57/BL6) was not associated with sex reversal. This discrepancy could be explained by the fact that DAX1 protein is critical at multiple steps of the sex-determining cascade, leading to additional defects in the pathway.

In developing male gonads, conditional inactivation of the FGF9 receptor *Fgfr2* induces partial sex reversal (Bagheri-Fam et al., 2008). A normal *L-Pgds* transcript level was detected in the testicular region of the ovotestis, suggesting that *L-Pgds* expression could be independent of the FGF9 pathway. In the present study, we show that *L-Pgds* expression is indeed induced in *Fgf9*^{-/-} gonads downstream of the short burst of SOX9 protein at 12.0 dpc, confirming that activation of *L-Pgds* is independent of the FGF9 pathway. Moreover, the level of *Fgf9* transcript was not altered in *L-Pgds*^{-/-} XY gonads. Thus, we conclude that the FGF9 pathway has no interconnection with the PGD2 pathway at the transcriptional level. However, PGD2 and FGF9 signaling molecules might act together to maintain *Sox9* gene expression as suggested in vitro using NT2/D1 cells. Nevertheless, we cannot exclude that PGD2 and FGF9 signaling could act at different levels of *Sox9* transcript homeostasis such as transcription and/or mRNA stability.

This study raises several questions concerning the role of the PGD2 pathway in mammalian testicular organogenesis, such as how is *Sox9* transcription upregulated in response to PGD2 and which transcription factors relay the PGD2 signal? The SOX9 protein is likely to be involved in this mechanism through its phosphorylation by the cAMP pathway (Malki et al., 2005b) and its capacity to activate the testicular enhancer sequence (TES) of its own gene (Sekido and Lovell-Badge, 2008). However, PGD2 activation of *Sox9* transcription in 11.5 dpc XX gonadal explants (Wilhelm et al., 2005) suggests that other pathways, such as CREB, could be involved. In support of this, a cAMP response sequence has been described in the 5' proximal region of the *Sox9* gene (Kanai and Koopman, 1999). However, the mechanism involved in FGF9 induction of *Sox9* expression is still unclear. Male-specific nuclear translocation of the IIIc variant of FGFR2 receptor (Kim et al., 2007; Schmahl et al., 2004) may have a key role, although this mechanism remains to be elucidated.

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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Supplementary material

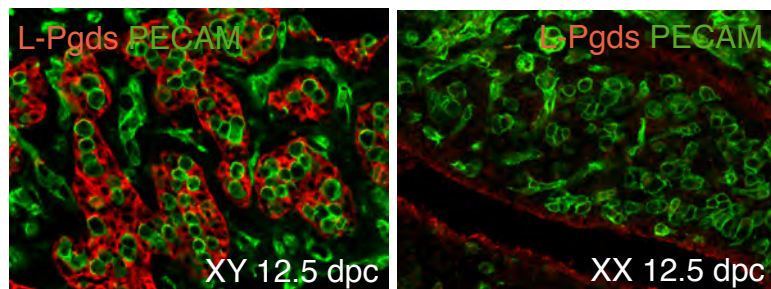
Supplementary material available online at
<http://dev.biologists.org/cgi/content/full/136/11/1813/DC1>

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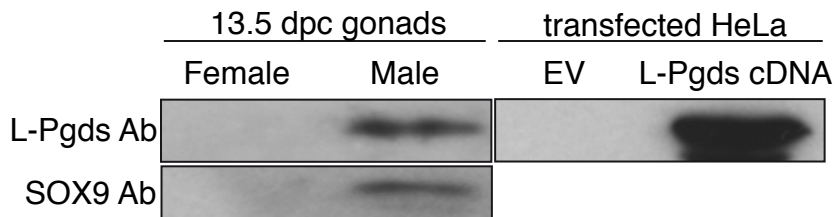
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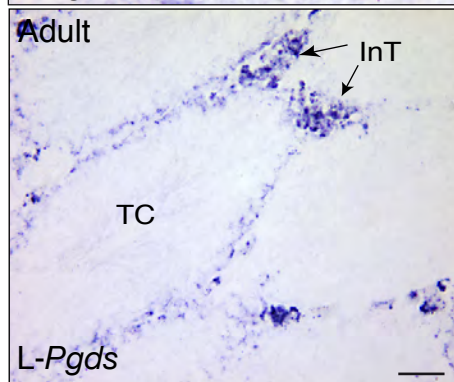
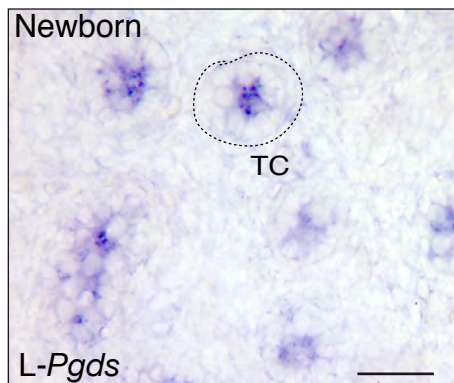
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