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## RESEARCH ARTICLE

# Prostaglandin D<sub>2</sub> acts through the Dp2 receptor to influence male germ cell differentiation in the foetal mouse testis

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**ABSTRACT**

Through intercellular signalling, the somatic compartment of the foetal testis is able to program primordial germ cells to undergo spermatogenesis. Fibroblast growth factor 9 and several members of the transforming growth factor  $\beta$  superfamily are involved in this process in the foetal testis, counteracting the induction of meiosis by retinoic acid and activating germinal mitotic arrest. Here, using *in vitro* and *in vivo* approaches, we show that prostaglandin D<sub>2</sub> (PGD<sub>2</sub>), which is produced through both L-Pgds and H-Pgds enzymatic activities in the somatic and germ cell compartments of the foetal testis, plays a role in mitotic arrest in male germ cells by activating the expression and nuclear localization of the CDK inhibitor *p21<sup>Cip1</sup>* and by repressing pluripotency markers. We show that PGD<sub>2</sub> acts through its Dp2 receptor, at least in part through direct effects in germ cells, and contributes to the proper differentiation of male germ cells through the upregulation of the master gene *Nanos2*. Our data identify PGD<sub>2</sub> signalling as an early pathway that acts in both paracrine and autocrine manners, and contributes to the differentiation of germ cells in the foetal testis.

**KEY WORDS:** Prostaglandin D<sub>2</sub>, Germ cells, Mitotic arrest, Differentiation, Embryonic testis, Mouse

**INTRODUCTION**

In mammals, the formation of a functional testis involves two successive cellular determination processes that take place during embryonic and foetal life. The first of these occurs in somatic cells, and the second takes place in the germ cells; in both cases, the process involves a choice between male and female fates. In male mice, the somatic cell fate decision is effected by the *Sry* gene, which is expressed in the supporting cell lineage between embryonic stages E10.5 and E12.5. *Sry* gene expression leads to the upregulation of *Sox9* expression and the subsequent differentiation of these cells into Sertoli cells, which then influence the germ cell lineage (McClelland et al., 2012). In both sexes, primordial germ cells (PGCs) colonize the genital ridges at around E10.5, and continue proliferating until E13.5. The

sexual fate of the germ cells becomes apparent between E12.5 and E15.5. In the developing ovary, germ cells stop undergoing mitosis and enter the prophase of the first meiotic division at E13.5. In the testicular environment, the proliferation of germ cells gradually slows down and the cells ultimately reach quiescence, also called ‘mitotic arrest’, which corresponds to a block in the G<sub>0</sub>/G<sub>1</sub> phase. Male germ cells remain quiescent until shortly after birth, at which time they resume mitosis and then initiate meiosis at around 8 dpp (days post partum) (for a review, see Ewen and Koopman, 2010).

This male-specific quiescence is a crucial event in the establishment of the male germ cell fate and is tightly associated with the expression of G<sub>1</sub>/S phase checkpoint regulators such as the CDK inhibitors *p27<sup>Kip1</sup>* (*Cdkn1b* – Mouse Genome Informatics) and *p21<sup>Cip1</sup>* (*Cdkn1a* – Mouse Genome Informatics), cyclins E1, E2 and D3 (Spiller et al., 2009; Western et al., 2008), and the retinoblastoma 1 protein (Rb1) (Spiller et al., 2010). Concomitant with these events, male germ cell commitment is also associated with the repression of key regulators of pluripotency, including Oct4, Sox2 and Nanog; this repression is achieved by E15.5 (Western et al., 2010). Various factors are known to be involved in the regulation of these events in male germ cells. The transcription factor *Dmrt1* influences cell cycle arrest by directly regulating the expression of the CDK inhibitor *p19<sup>ink</sup>* (*Cdkn2a* – Mouse Genome Informatics) and the pluripotency marker *Sox2* (Krentz et al., 2009). In addition, the RNA-binding protein *Dnd1* (dead end homolog 1) permits *p21<sup>Cip1</sup>* expression by protecting its mRNA from degradation (Kedde et al., 2007); loss of *Dnd1* expression in male germ cells has multiple effects, including (1) the prevention of cells from entering mitotic arrest at G<sub>0</sub>, (2) the strong downregulation of the male germ cell fate factor *Nanos2*, (3) the ectopic upregulation of meiotic markers and (4) the maintenance of pluripotency genes (Cook et al., 2011). Multiple Tgf $\beta$  superfamily members have also been associated with male germ cell differentiation, including Tgf $\beta$ 2 (Miles et al., 2013; Moreno et al., 2010) and activin  $\beta_5$  (*Inhba* – Mouse Genome Informatics) (Mendis et al., 2011). These factors repress germ cell proliferation, and participate in the entry into quiescence (Moreno et al., 2010) and probably also in its maintenance (Mendis et al., 2011; Moreno et al., 2010). Notch pathway members also appear to be involved in male-specific differentiation, as their overexpression in foetal Sertoli cells can induce gonocytes to prematurely exit the quiescent state and enter meiosis (Garcia et al., 2013). However, the early-acting mechanisms that regulate and trigger these processes remain poorly understood (Western, 2009).

The decision between male and female germ cell fates in germ cells is known to depend on environmental signals (Adams and McLaren, 2002) that control the expression of two master genes: *Stra8* (stimulated by retinoic acid gene 8), which is required for

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the initiation of meiosis in females (Baltus et al., 2006), and *Nanos2*, which blocks *Stra8* expression in males and thereby prevents meiosis (Suzuki et al., 2010, 2012; Suzuki and Saga, 2008). Null mutations of *Nanos2* in males lead to germ cell death (Tsuda et al., 2003), to the transient upregulation of meiotic markers (Suzuki and Saga, 2008) and to defects in the upregulation of male-specific markers such as the DNA methylase *Dnmt3l* (Suzuki et al., 2012).

One important environmental factor known to play a role in female-specific development is retinoic acid (RA), which activates *Stra8* in female germ cells (Bowles et al., 2006; Koubova et al., 2006; Kumar et al., 2011). In the male, germ cells are protected from exposure to RA by *Cyp26b1*, an RA-metabolizing enzyme of the cytochrome P450 family that is produced by the Sertoli cells (Bowles et al., 2006; Koubova et al., 2006; MacLean et al., 2007) and the Leydig cells (Kashimada et al., 2011). This degradation of RA in males results in the suppression of meiosis after E13.5, thereby allowing mitotic arrest (Trautmann et al., 2008). However, despite the importance of RA inhibition in males, multiple lines of evidence indicate that additional secreted factors also play crucial roles (Best et al., 2008; Guerquin et al., 2010; Ohta et al., 2012). One candidate that has been proposed for such a secreted male-specific factor is *Fgf9*, as its secretion by differentiating Sertoli cells promotes the survival of germ cells after E12.5 (DiNapoli et al., 2006). In addition, *Fgf9* signalling maintains the expression of pluripotency-related genes, and actively suppresses entry into meiosis in male germ cells by activating *Nanos2* expression (Barrios et al., 2010; Bowles et al., 2010) via the transient activation of expression of the Cripto/Nodal pathway (Spiller et al., 2012). Indeed, this latter pathway displays an autocrine role in the inhibition of the meiotic entry in foetal XY germ cells (Souquet et al., 2012), a role that has also been observed with *Tgfb2* signalling (Miles et al., 2013). However, in double mutants for *Fgf9* and *Wnt4*, germ cells do not enter meiosis and the male marker *Dnmt3l* is still expressed (Jameson et al., 2012), suggesting that *Fgf9* is not the only signalling molecule involved in inducing these effects in male germ cells.

Considered together, these studies indicate that the crucial decision of the germ line to commit to either a male or a female fate involves a complex regulatory network, and that the previously identified factors and pathways are insufficient to explain fully this decision in males. Here, we highlight the role of an additional factor, prostaglandin D<sub>2</sub> (PGD<sub>2</sub>), in this process. PGD<sub>2</sub> has been known to act during Sertoli cell differentiation to induce the nuclear translocation of *Sox9* protein (Malki et al., 2005; Moniot et al., 2009, 2011), and to help maintain *Sox9* gene expression (Moniot et al., 2009; Wilhelm et al., 2005); its role in the male germ line, however, has not previously been established.

PGD<sub>2</sub> is produced in the developing mouse testes by two enzymes: lipocalin-type prostaglandin D<sub>2</sub> synthase (*L-Pgds* or *Ptgds*), an enzyme that is expressed specifically in males at E12.5 by Sertoli cells and by differentiating germ cells (Adams and McLaren, 2002); and hematopoietic *Pgds* (*H-Pgds* or *Ptgds2*), which is expressed in both sexes (Moniot et al., 2011). In this study, using multiple approaches [*in vivo* analysis of double-knockout *L/H-Pgds* (*L/H-Pgds*<sup>-/-</sup>, i.e. depleted for all PGD<sub>2</sub>) and *Dp2*<sup>-/-</sup> gonads; *ex vivo* gain-of-function studies on isolated germ cells, mixed somatic and germ cell cultures; and transcriptome analysis of E13.5 wild type and *L/H-Pgds*<sup>-/-</sup> testes] we show that both somatic- and germ cell-produced PGD<sub>2</sub>, acting in both a paracrine and an autocrine manner, play a role in the regulation of male foetal germ cell differentiation.

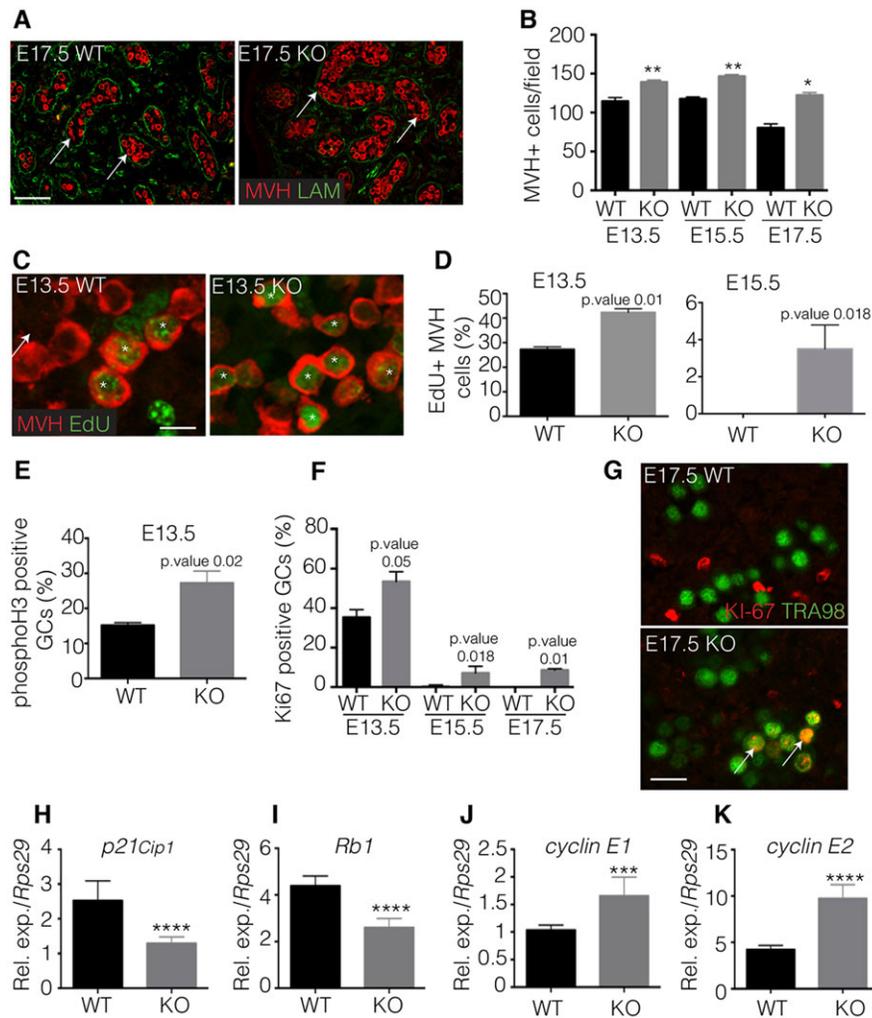
## RESULTS

### Germ cells in PGD<sub>2</sub>-depleted foetal testes proliferate abnormally

As both of the prostaglandin D synthases are expressed in both the somatic and the germ cell lineages (Adams and McLaren, 2002; Moniot et al., 2009, 2011), we analysed gonads from double *L*- and *H-Pgds* (*L/H-Pgds*<sup>-/-</sup>) mutant embryos (Qu et al., 2006). At the somatic level, their phenotype was similar to that previously reported for *L-Pgds*<sup>-/-</sup> testes (Moniot et al., 2009). Both *Sox9* action and testis cord organization were delayed in mutant gonads up to E13.5, but both were achieved by late E17.5 (supplementary material Fig. S1A). In addition, the level of *Sox9* and *Amh* transcripts were significantly lower in the mutant E13.5 gonads than in wild type, with *Sox9* expression remaining affected up to E17.5. By contrast, the expression of *Fgf9* and *Dmrt1* was not modified in the mutant gonads, although the expression of another somatic factor, *Notch1*, was significantly reduced in E13.5 mutant gonads (supplementary material Fig. S1B).

In the mutant testes, we also observed that the number of cells positive for *Mvh* (mouse vasa homolog, a germ cell marker; Ddx4 – Mouse Genome Informatics) was significantly higher than it is in the wild-type gonads from stages E13.5 to E17.5 (Fig. 1A,B). This result was specific to the *L/H-Pgds*<sup>-/-</sup> germline, as the phenotype of the single mutant *L-Pgds*<sup>-/-</sup> or *H-Pgds*<sup>-/-</sup> gonads was similar to that of the wild type (supplementary material Fig. S2A,B). We thus hypothesized that the proliferation rate of germ cells might be modified in the double mutant gonads. Indeed, co-staining for *Mvh* together with EdU detection (S-phase) or phospho-Histone H3 (M-phase) (pH3) in wild-type and *L/H-Pgds*<sup>-/-</sup> gonads showed that, at E13.5, the percentage of S-phase positive germ cells in the PGD<sub>2</sub>-depleted mutant gonads was increased to 42% compared with 27% in wild type (1.5 fold) (Fig. 1C,D); however, only a small number of S-phase-positive germ cells was detected in the mutant gonads at E15.5 (Fig. 1D). Immunofluorescence staining for pH3 also showed a twofold higher percentage of mutant germ cells in M-phase at E13.5 than in wild type (Fig. 1E), whereas no pH3 staining was detected in mutant E15.5 germ cells (not shown). In addition, single *L-Pgds*<sup>-/-</sup> or *H-Pgds*<sup>-/-</sup> mutant gonads had the same pH3 expression pattern as wild-type gonads (supplementary material Fig. S2C). This increased proliferation appeared to be limited to the germ cells, and the proliferation of the Sertoli cells at E13.5 was not modified in the *L/H-Pgds*<sup>-/-</sup> gonads (supplementary material Fig. S3A). Finally, an immunofluorescence experiment against the proliferation marker Ki-67, which is expressed in all phases of the cell cycle except for G<sub>0</sub>, detected 38% Ki-67-positive germ cells in wild-type gonads and 58% positive cells in the mutant E13.5 gonads (Fig. 1F). At E15.5 and even E17.5, 8–10% of the mutant germ cells were still Ki-67 positive (Fig. 1F,G), showing that a significant proportion of the mutant germ cells were not mitotically arrested and were still engaged in the cell cycle.

We thus surmised that PGD<sub>2</sub> signalling might control the expression of cell cycle genes as several key regulators of the G<sub>1</sub>/S phase checkpoint are known to be transcriptionally regulated in the male germ line during mitotic arrest (Spiller et al., 2010; Western et al., 2008). Indeed, we observed a halving in the mRNA level of both *p21<sup>Cip1</sup>* and *Rb1* in *L/H-Pgds*<sup>-/-</sup> testes (KO) at E13.5 compared with wild type (Fig. 1H,I), as well as a significant increase in the expression of cyclin E1 (*Ccne1*) and cyclin E2 (*Ccne2*) at the same stage (Fig. 1J,K). These findings were consistent with the enhanced proliferation observed in the mutant germ cells at E13.5. Finally, we did not observe any differences in the number of apoptotic cells between wild-type and mutant gonads, as measured by TUNEL at



**Fig. 1. PGD<sub>2</sub> signalling controls the germline proliferation in the embryonic testis.**

(A) Co-immunofluorescence of Mvh (red) and laminin (green) on E17.5 KO and wild-type testes. Arrows indicate Mvh-positive germ cells. Scale bar: 200  $\mu$ m. (B) Quantification of germ cells within the testis cords of KO and wild-type testes at stages E13.5, E15.5 and E17.5 is represented as numbers of germ cells per field from independent gonads ( $n=10$ ). \* $P<0.05$ ; \*\* $P<0.01$ . (C) Germ cell proliferation in E13.5 KO and wild-type testes was evaluated by co-immunofluorescence using Mvh (red) and EdU (green). Asterisks indicate proliferative germ cells. Scale bars: 50  $\mu$ m. (D-F) Quantification of Mvh germ cells that are positive for EdU in E13.5 and E15.5 (D) and for phospho-histone H3 in E13.5 (E) KO and wild-type gonads; quantification of Tra98<sup>+</sup> KI-67<sup>+</sup> cells on E13.5, E15.5 and E17.5 KO and wild-type gonads (F). Data are represented as the percentage of proliferating (EdU, pH3 or KI-67 positive) germ cells among Mvh- (D,E) or Tra98- (F) positive germ cells. (G) Co-immunofluorescence on E17.5 testis sections with KI-67 and Tra98. Arrows indicate proliferating germ cells. Scale bars: 100  $\mu$ m. (H-K) Expression of cell cycle genes *p21<sup>Cip1</sup>* (H), *Rb1* (I), cyclin E1 (J) and cyclin E2 (K) is studied by RT-qPCR in E13.5 KO and wild-type gonads, and normalized to *Rps29*. Error bars indicate s.d. of assays carried out in triplicate ( $n=4$ ). \*\*\* $P<0.001$ ; \*\*\*\* $P<0.0001$ .

the E13.5 stage (data not shown). Taken together, our results suggested that PGD<sub>2</sub>, produced by germ cells and/or surrounding cells, is involved in the control of cell cycle genes in the foetal testis, acting to slow down germ cell proliferation within this tissue.

#### LH-Pgds depletion leads to an altered transcriptional profile of germ cell-specific and cell cycle genes

To identify genes regulated by PGD<sub>2</sub>, we performed an RNA sequencing analysis from wild-type and *L/H-Pgds*<sup>-/-</sup> E13.5 testes. We identified 2829 genes that were differentially expressed between the two types ( $P<0.01$ ), including 1484 genes upregulated and 1345 genes downregulated in mutant compared with wild-type gonads (supplementary material Table S1). We identified genes involved in the cell cycle and cell proliferation regulation (Table 1), as well as genes involved in the regulation of germ cell differentiation and pluripotent marker expression (Table 2). We validated the expression of 29 differentially expressed genes by real time RT-PCR, obtaining results similar to those observed with the RNA-seq experiments (supplementary material Fig. S4). In mutant gonads, we observed a significant decrease in the expression of the cell cycle inhibitors *p21<sup>Cip1</sup>* and *p57<sup>Kip1</sup>*, and an increase in cell cycle activators such as the retinoblastoma-like gene *p130 (Rb1)* and in oncogenes such as *Kit*, *Myb11* and *ErbB4*. Furthermore, several regulators of *p21<sup>Cip1</sup>* expression were either upregulated [*Elavl2* (Wisniak et al., 2011; Yoon et al., 2012), *Trim71* (Chang et al., 2012), *Pten* (Luo et al.,

2013) and *Cpeb4* (Novoa et al., 2010)] or downregulated [*Rbm38* (Feldstein et al., 2012) and *Dnd1* (Zhu et al., 2011) (supplementary material Fig. S4B)]. Significantly, the crucial male germ cell gene *Nanos2* (Suzuki and Saga, 2008) was downregulated in the absence of PGD<sub>2</sub>. Moreover, numerous regulators of pluripotency, such as *Sox2* (Takahashi and Yamanaka, 2006), *Peg3* (Jiang et al., 2007), *Nr2c2* (Wagner and Cooney, 2013), *Trim71* (Chang et al., 2012), *Lhx1* (Birk et al., 2000), *Sall1* (Karantzali et al., 2011), *Mtf2* (Zhang et al., 2011), *L1td1* (Narva et al., 2012), *Tet1* (Vincent et al., 2013) and *Gtf3c3* (Luzzani et al., 2011), were upregulated in the absence of PGD<sub>2</sub> (Table 2; supplementary material Fig. S4A). These data have been deposited in the Gene Expression Omnibus database (Edgar et al., 2002) and are accessible through GEO Series accession number GSE55744 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55744>). In view of these observations, we next investigated germline differentiation in *L/H-Pgds*<sup>-/-</sup> foetal testes in more detail.

#### PGD<sub>2</sub> regulates the cell cycle inhibitor *p21<sup>Cip1</sup>* expression

As the CDK inhibitor *p21<sup>Cip1</sup>*, a potential regulator of the mitotic arrest process (Western et al., 2008), was found to be downregulated in mutant E13.5 *L/H-Pgds*<sup>-/-</sup> gonads, we next evaluated the direct action of PGD<sub>2</sub> on *p21<sup>Cip1</sup>* expression in the male germ line. Germ cells from E12.5 and E13.5 testes cultured in the presence of exogenous PGD<sub>2</sub> revealed that the levels of *p21<sup>Cip1</sup>* mRNA increased 3- and 3.5-fold, respectively, at E12.5 and E13.5

**Table 1. Cell cycle and cell proliferation genes up- and downregulated in E13.5L/H-Pgds mutants relative to wild-type testes**

Gene symbol	Fold change mutant/wild type	Description
<i>Cpeb4</i>	12.29	Cytoplasmic polyadenylation element-binding protein 4
<i>Mybl1</i>	11.12	Myeloblastosis oncogene-like 1
<i>Rcor1</i>	10.80	REST co-repressor 1
<i>Pde3a</i>	9.18	Phosphodiesterase 3A, cGMP inhibited
<i>ErbB4</i>	8.30	v-erb-a erythroblastic leukaemia viral oncogene homolog 4
<i>Crebbp</i>	6.14	CREB-binding protein
<i>ErbB3</i>	5.72	v-erb-b2 erythroblastic leukaemia viral oncogene homolog 3
<i>Atm</i>	2.14	Ataxia telangiectasia mutated homolog
<i>Arid4a</i>	2.08	AT rich interactive domain 4A (Rbp1 like)
<i>Cdk17</i>	2.02	Cyclin-dependent kinase 17
<i>Cdkn2aip</i>	1.94	CDKN2A interacting protein
<i>Pak3</i>	1.92	p21 protein (Cdc42/Rac)-activated kinase 3
<i>Rbl1</i>	1.88	Retinoblastoma-like 1 (p107)
<i>Rbl2</i>	1.78	Retinoblastoma-like 2 (p130)
<i>Cdk12</i>	1.78	Cyclin-dependent kinase 12
<i>Pkd2</i>	1.78	Polycystic kidney disease 2
<i>Ccnl1</i>	1.74	Cyclin L1
<i>Pkd1</i>	1.69	Polycystic kidney disease 1 like 3
<i>Cdk13</i>	1.67	Cyclin-dependent kinase 13
<i>Ccnd2</i>	1.67	Cyclin D2
<i>Ccng2</i>	1.64	Cyclin G2
<i>Cdk8</i>	1.55	Cyclin-dependent kinase 8
<i>Cdk4</i>	0.65	Cyclin-dependent kinase 4
<i>Cdk5</i>	0.62	Cyclin-dependent kinase 5, regulatory subunit 2 (p39)
<i>Cdk2ap2</i>	0.58	CDK2-associated protein 2
<i>Camk1</i>	0.56	Calcium/calmodulin-dependent protein kinase
<i>Cdc37</i>	0.54	Cell division cycle 37 homolog (-like 1)
<i>Cdkn1c</i>	0.48	Cyclin-dependent kinase inhibitor 1C (P57)
<i>Cdkn1a</i>	0.47	Cyclin-dependent kinase inhibitor 1A (P21)

(Fig. 2A). In addition, PGD<sub>2</sub> significantly increased the level of p21<sup>Cip1</sup> protein when applied to cultured E13.5 germ cells (Fig. 2B,C). The effect of PGD<sub>2</sub> on p21<sup>Cip1</sup> protein expression was confirmed in E13.5 whole gonads as p21<sup>Cip1</sup> protein expression was significantly decreased in mutants compared with wild-type gonads (Fig. 2B,C). Also, we found that PGD<sub>2</sub> was able to directly regulate the expression of other cell cycle genes in cultured germ cells, such as *p57<sup>Kip1</sup>* and *Ccne1* and *Ccne2* (Fig. 2D).

Depending on its subcellular localization, p21<sup>Cip1</sup> displays diverse activities with respect to proliferation (Romanov et al., 2012). Using co-immunofluorescence with the germ cell marker Tra98, we found that p21<sup>Cip1</sup> was present in both the cytoplasmic and nuclear compartments with a cytoplasm:nuclei ratio of close to 1:1 (Fig. 2F) in the wild-type E13.5 germ cells, with nuclear localization occurring only in some germ cells (Fig. 2E). By contrast, p21<sup>Cip1</sup> was mainly found in the cytoplasm, with a cytoplasm:nuclei ratio of 2:1 (Fig. 2F) in *L/H-Pgds*<sup>-/-</sup> germ cells (Fig. 2E); this result indicated that, in the absence of PGD<sub>2</sub>, foetal XY germ cells display a proliferative pattern of p21<sup>Cip1</sup> subcellular localization. Furthermore, after culturing purified E12.5 germ cells in the presence of PGD<sub>2</sub>, we found that their proliferation rate was significantly lower than that of the culture without PGD<sub>2</sub>, confirming the direct effect of PGD<sub>2</sub> on the proliferation rate of the male germline (Fig. 2G).

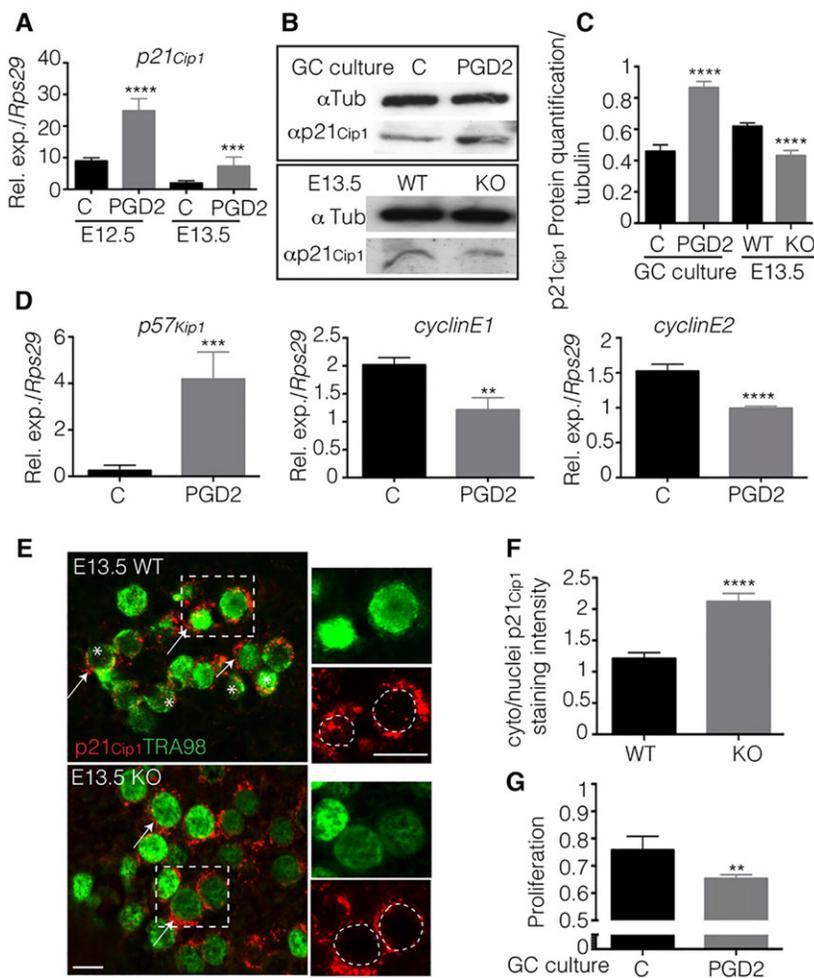
**Table 2. Germ cells and pluripotency genes up- and downregulated in E13.5L/H-Pgds mutants relative to wild-type testes**

Gene symbol	Fold change Mutant/wild type	Description
<i>Peg3</i>	11.99	Paternally expressed 3
<i>Rcor1</i>	10.80	REST co-repressor 1
<i>Trim71</i>	8.98	Tripartite motif-containing 71
<i>Nr2c2</i>	8.80	Nuclear receptor subfamily 2, group C, member 2
<i>Ror1</i>	8.29	Receptor tyrosine kinase-like orphan receptor 1
<i>Lhx1</i>	4.06	LIM homeobox protein 1
<i>Rif1</i>	3.35	Rap1 interacting factor 1 homolog (yeast)
<i>Sall1</i>	3.42	Sal-like 1 ( <i>Drosophila</i> )
<i>Tet1</i>	2.76	Tet methylcytosine dioxygenase1
<i>Elavl2</i>	2.31	ELAV (embryonic lethal, abnormal vision (Hu antigenB))
<i>Mtf2</i>	2.19	Metal response element binding transcription factor 2
<i>Dazl</i>	2.11	Deleted in azoospermia-like
<i>L1td1</i>	2.10	LINE-1 type transposase domain containing 1
<i>Kit</i>	1.99	Kit oncogene
<i>Pten</i>	1.96	Phosphatase and tensin homolog
<i>Sox2</i>	1.90	SRY-box containing gene 2
<i>Gtf3c3</i>	1.86	General transcription factor IIIC, polypeptide 3
<i>Ddx4</i>	1.84	DEAD (Asp-Glu-Ala-Asp) box polypeptide 42
<i>Rbm38</i>	0.65	RNA-binding motif protein 38
<i>Dnd1</i>	0.52	Dead end homolog 1 (zebrafish)
<i>Nanos2</i>	0.13	Nanos homolog 2 ( <i>Drosophila</i> )

### PGD<sub>2</sub> signalling regulates the expression of pluripotency factors

As the level of *Sox2* mRNA was increased in mutants (Table 2), we next compared the level of pluripotent gene expression in *L/H-Pgds*<sup>-/-</sup> and wild-type testes at E17.5, a stage when these markers are normally fully repressed. First, we found that the level of *Sox2* mRNA was increased by 2.5-fold in the mutant testes when compared with wild-type testes, whereas the levels of *Nanog* and *Oct4* mRNAs remained unchanged (Fig. 3A). By culturing male germ cells at the E12.5 and E13.5 stages in the presence of PGD<sub>2</sub>, we observed a direct repressive effect of PGD<sub>2</sub> on *Sox2* mRNA level in germ cells at both stages (Fig. 3B). Furthermore, using mixed cultures of somatic and germ cells from wild-type and mutant E13.5 gonads, we determined that PGD<sub>2</sub> produced by both the somatic and germ cell compartments contributes to the full repression of *Sox2* expression (Fig. 3C). We also confirmed that PGD<sub>2</sub> is produced by both the somatic and germ cell lineages, following chemical fixation of PGD<sub>2</sub> on its production site and immunofluorescence experiments (Fig. 3D).

Using immunofluorescence we then observed the expression of the *Sox2*, *Oct4* and *Nanog* proteins in mutant testes at E17.5 (Fig. 3E). Notably, for all three of the factors, their subcellular localization was mainly restricted to the cytoplasmic compartment at this stage, whereas under identical conditions the same three proteins were fully nuclear in wild-type and mutant E13.5 gonads (supplementary material Fig. S5). Furthermore, the L-Pgds and H-Pgds enzymatic activities were complementary for this phenotype as in either single mutant L- or H-Pgds gonads, *Nanog* (supplementary material Fig. S2D), *Oct4* and *Sox2* (data not shown) expression was normally downregulated. Our data therefore show that PGD<sub>2</sub> is involved in the downregulation of the



**Fig. 2. PGD<sub>2</sub> regulates the cell cycle p21<sup>Cip1</sup> inhibitor towards the mitotic arrest.** (A) Purified XY E12.5 or E13.5 germ cells were cultured in the presence of PGD<sub>2</sub> (5 ng/ml) or not (C=control). RT-qPCR was used to evaluate the expression of p21<sup>Cip1</sup> normalized to *Rps29* expression. Error bars represent s.d. of assays carried out in triplicate ( $n=3$ ). (B,C) Protein levels of p21<sup>Cip1</sup> ( $\alpha$ -p21<sup>Cip1</sup>) were analysed by western blot and compared with the ubiquitous tubulin ( $\alpha$ -Tub) protein in E13.5 male germ cells cultured with PGD<sub>2</sub> or not (C=control), and in E13.5 KO and WT gonads. (C) p21<sup>Cip1</sup> protein expression levels were quantified ( $n=3$ ) and are represented on the corresponding graph. (D) Purified XY E12.5 germ cells were cultured for 18 h in the presence of PGD<sub>2</sub> (5 ng/ml) or not (C=control). RT-qPCR evaluated the expression of *p57<sup>Kip1</sup>*, *Ccne1* and *Ccne2* normalized to *Rps29* expression. Error bars represent s.d. of assays carried out in triplicate ( $n=3$ ). (E) Immunofluorescence analysis of p21<sup>Cip1</sup> and Tra98 proteins in E13.5 wild-type and *L/H-Pgds*<sup>-/-</sup> gonads (KO). Asterisks highlight nuclear p21<sup>Cip1</sup> and arrows indicate cytoplasmic p21<sup>Cip1</sup>. Outlined areas are enlarged in the right-hand panels, in which dotted circles delineate nuclei. Scale bars: 25  $\mu$ m. (F) Cytoplasmic and nuclear signals were quantified on five different gonads (70 and 90 germ cells) for each genotype using the ImageJ software and were represented as the ratio of cytoplasmic/nuclei staining. (G) Proliferation of E12.5 germ cells cultured in the presence of PGD<sub>2</sub> (PGD<sub>2</sub>) or not (C) for 24 h was measured using the cell proliferation assay. \*\* $P<0.01$ ; \*\*\* $P<0.001$ ; \*\*\*\* $P<0.0001$ .

pluripotency factors Sox2, Oct4 and Nanog, and in the subcellular distribution of these proteins during male germ-line differentiation, acting through different transcriptional (*Sox2*) or translational (Nanog and Oct4) and putative post-translational mechanisms.

#### PGD<sub>2</sub> signalling contributes to the establishment of the male cell differentiation

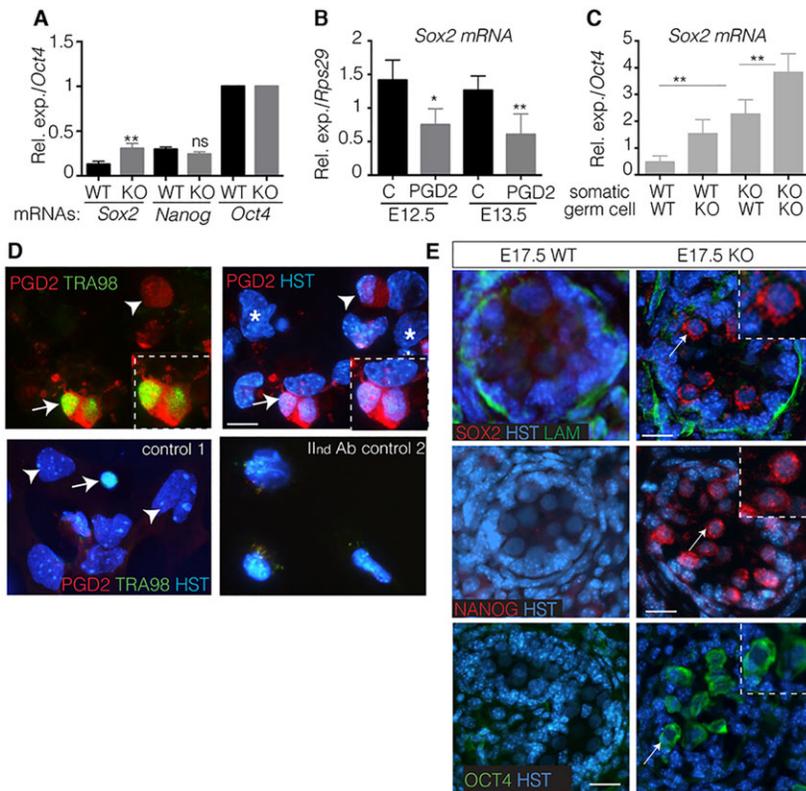
We next asked whether PGD<sub>2</sub> signalling could have a role in the regulation of male germ cell differentiation. In our RNA-seq screen, we had observed changes in the expression of genes known to be important for male germ-cell development, such as *Nanos2*. First, we confirmed that the *Nanos2* transcript level at E13.5, E15.5 and E17.5 was lower in mutant than in wild-type gonads (Fig. 4A). Using cultures of isolated male germ cells obtained from E13.5 gonads, we showed that this effect of PGD<sub>2</sub> on *Nanos2* was direct, because upon PGD<sub>2</sub> treatment *Nanos2* levels were 2.5-fold higher in treated than in non-treated cells (Fig. 4B). Then, using mixed cultures of somatic and germ cells from WT and mutant E13.5 gonads, we showed that PGD<sub>2</sub> produced by both the somatic and the germ cell compartments was necessary for the full activation of *Nanos2* expression (Fig. 4C). *Dnmt3l*, another male-specific gene, was also expressed at lower levels in mutant gonads, but only at the E17.5 stage (Fig. 4D); *Cyp26b1* was downregulated in mutant male gonads at E13.5 and E15.5 (Fig. 4E). However, *Nodal* expression level was similar in mutant and wild-type gonads (Fig. 4F).

As the expression of *Nanos2* and *Cyp26b1* is directly or indirectly linked to the repression of *Stra8* in male foetal germ cells (MacLean

et al., 2007; Suzuki and Saga, 2008), we next tested whether their decreased expression in mutant male gonads led to the activation of meiotic markers. In E13.5, E15.5 and E17.5 testes, *Stra8* mRNA levels were significantly higher in mutant gonads than in wild-type gonads (Fig. 4G), although their levels in the male mutant gonads were still 40-fold lower than in female gonads at E13.5 (Fig. 4G). Despite this low level of the *Stra8* transcript, clear expression of the *Stra8* protein was detected in mutant male gonads at E17.5 (Fig. 4H). Using cultures of germ cells from E12.5 XY gonads, we also observed that PGD<sub>2</sub> treatment directly repressed the expression of the *Stra8* transcript (Fig. 4I). Furthermore, PGD<sub>2</sub> was able to diminish the activation of *Stra8* by RA by 50%, confirming the negative effect of PGD<sub>2</sub> on *Stra8* expression (Fig. 4I). However, we cannot exclude the possibility that PGD<sub>2</sub> acts by directly upregulating *Nanos2*. Finally, using mixed cultures of somatic and germ cells from wild-type and mutant E13.5 gonads, we showed that PGD<sub>2</sub> produced by either the somatic or the germ cell compartments was sufficient to repress *Stra8* expression (Fig. 4J). However, the mutant germ cells did not show a reversed sexual fate; they did not overcome the block to meiosis entry as the recombination marker *Dmc1* was absent in *L/H-Pgds*<sup>-/-</sup> XY gonads from E13.5 to E17.5 (Fig. 4K).

#### PGD<sub>2</sub> signals through its Dp2/Crth2 receptor to control the male germline differentiation

As PGD<sub>2</sub> can act on both the Dp1 and Dp2/Crth2 G-protein-associated receptors (Matsuoka et al., 2000; Nagata and Hirai,



**Fig. 3. PGD<sub>2</sub> regulates the expression of pluripotency genes.**

(A,B) RT-qPCR analysis of the expression of the pluripotent genes *Sox2* (A,B), *Nanog* and *Oct4* (A) in KO and wild-type gonads at E17.5 (A) or in E13.5 germ cells cultured with PGD<sub>2</sub> (5 ng/ml) or not (C=control) (B) using *Oct4* (A) or *Rps29* (B) as the normalization control gene. Error bars indicate s.d. of assays carried out in triplicate ( $n=5$ ).

(C) Somatic and germ cells from wild-type and mutant E13.5 gonads were mixed and cultured for 24 h; *Sox2* expression levels were quantified by RT-qPCR relative to *Oct4* expression. Error bars indicate s.d. of assays carried out in triplicate ( $n=2$ ). \* $P<0.05$ ; \*\* $P<0.01$ .

(D) Dissociated somatic/germ cells from E13.5 wild-type testes were cultured for 24 h; PGD<sub>2</sub> was fixed using the EicosaCell protocol (upper panels and control 2) or cells were fixed in 4% PFA (control 1) and co-immunofluorescence with anti-PGD<sub>2</sub> (red) and anti-Tra98 (green) antibodies was performed (upper panels and control 1); control 2 was only incubated with secondary antibodies. HST, Hoechst dye. Arrows and arrowheads show germ cells and somatic cells expressing PGD<sub>2</sub>, respectively. Asterisks show somatic cells that weakly produce PGD<sub>2</sub>. Germ cells producing PGD<sub>2</sub> are outlined. Scale bar: 25  $\mu$ m. (E) Immunofluorescence with anti-*Sox2* (red), anti-*Nanog* (red) and anti-*Oct4* (green) antibodies, and Hoechst dye (HST, blue) on sections of E17.5 KO and wild-type testes. Arrows indicate *Sox2*, *Nanog* and *Oct4* staining (enlarged in the insets). Scale bars: 50  $\mu$ m.

2003), we first determined the localization of the Dp1 (Ptgdr) and Dp2 (Ptgdr2) receptors in E13.5 male gonad. Using co-immunofluorescence experiments with Tra98 staining, we found that Dp2 was expressed in both germ cells and somatic compartments, whereas Dp1 was only expressed in somatic cells (Fig. 5A). These Dp1 and Dp2 patterns were confirmed by purifying somatic and germ cell fractions from male E13.5 gonads: the germ cell fraction only expressed *Dp2* mRNA (Fig. 5B), whereas the somatic cell fraction expressed both *Dp2* and *Dp1* mRNAs (Fig. 5B). In the same experiments, we confirmed that both *L-Pgds* and *H-Pgds* mRNAs were expressed in both somatic and germ cell compartments, although the somatic expression was higher than that in the germ cells (Fig. 5B). Furthermore, using the PGD<sub>2</sub>-biotin tool followed by immunofluorescence, we were able to visualize the binding of PGD<sub>2</sub> to Tra98-expressing germ cells (Fig. 5C). These data confirmed that germ cells expressing only Dp2 are effectively able to bind PGD<sub>2</sub> produced by both germ cells and somatic cells, as shown in Fig. 3D.

To evaluate the function of Dp2 within the gonad, we orally administered the specific Dp2 antagonist CAY10471 (Royer et al., 2007) to pregnant females and analysed the proliferation rate of the developing germ cells. At E13.5, 10% of the germ cells were positive for EdU in the control gonads, compared with 28% in the CAY10471-treated gonads (supplementary material Fig. S6A,B); at the same time, no modified proliferation of the Sox9-expressing Sertoli cells was detected upon CAY10471 treatment (supplementary material Fig. S6C,D). The different percentages of EdU-positive germ cells compared with those obtained with E13.5 *L/H-Pgds*<sup>-/-</sup> testes (Fig. 1D) were probably due to the different genetic backgrounds, namely CD1 (CAY experiments) and C57BL/6 (*L/H-Pgds*<sup>-/-</sup>) (Western et al., 2011). This effect on proliferation was specific to the Dp2 receptor, as oral administration of the Dp1 antagonist BWA868C did not significantly modify the number of proliferating germ cells,

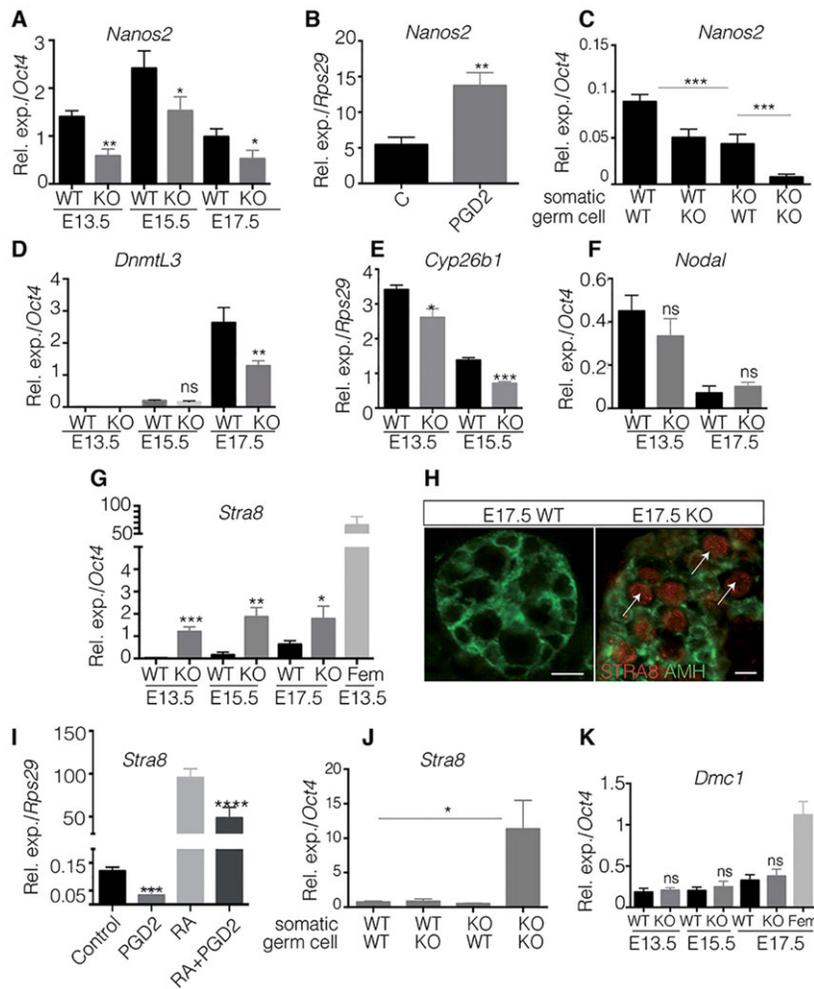
whereas the Dp2 agonist 15R-PGD<sub>2</sub> potentiated the effect of endogenous PGD<sub>2</sub> (supplementary material Fig. S6B). Furthermore, upon CAY10471 treatment, a decreased level of expression of *p21<sup>Cip1</sup>* and *Nanos2* was found (supplementary material Fig. S6E). Together, these data showed that the administration of the Dp2 antagonist mimicked the loss of PGD<sub>2</sub> signalling at E13.5.

To confirm these data, we next analysed E13.5 *Dp2*<sup>-/-</sup> testes (Satoh et al., 2006). We observed that the numbers of Ki-67 proliferating (Fig. 5D,E) and pH3 mitotic (Fig. 5F) germ cells were significantly higher in *Dp2* mutant gonads than in wild type (45%/28% and 24%/17%, respectively). These differences were in the same range as those described above for the *L/H-Pgds*<sup>-/-</sup> testes. Furthermore, E13.5 *Dp2*<sup>-/-</sup> testes expressed the p21<sup>Cip1</sup> protein within the cytoplasm of germ cells (Fig. 5G), and at E17.5 the germ cells ectopically expressed the pluripotent marker *Nanog* (Fig. 5H) and the premeiotic marker *Stra8* (Fig. 5I), similarly to what was observed in *L/H-Pgds*<sup>-/-</sup> mutant germ cells.

The function of Dp2 was confirmed by culturing purified E12.5 germ cells in the presence of PGD<sub>2</sub> with either the Dp1 antagonist BWA868C or the Dp2 antagonist BAY-U3450: the repression of *Stra8* and *Sox2* expression and the activation of *Nanos2* and *p21<sup>Cip1</sup>* expression observed in the presence of PGD<sub>2</sub> was partially reversed only in the presence of BAY-U3450 (supplementary material Fig. S6F-I). These data confirmed that PGD<sub>2</sub> signalling indeed acts on male foetal germ cells through the Dp2 receptor.

## DISCUSSION

In this study, we have investigated the role of PGD<sub>2</sub> in the differentiation of male foetal germ cells, significantly extending earlier findings on the role of this signalling molecule in the biology of the foetal testis (Moniot et al., 2009, 2011; Wilhelm et al., 2007). Our analysis of gonads from double L- and H-*Pgds* (*L/H-Pgds*<sup>-/-</sup>) and *Dp2*<sup>-/-</sup> mutant embryos has shown that PGD<sub>2</sub> acting through its



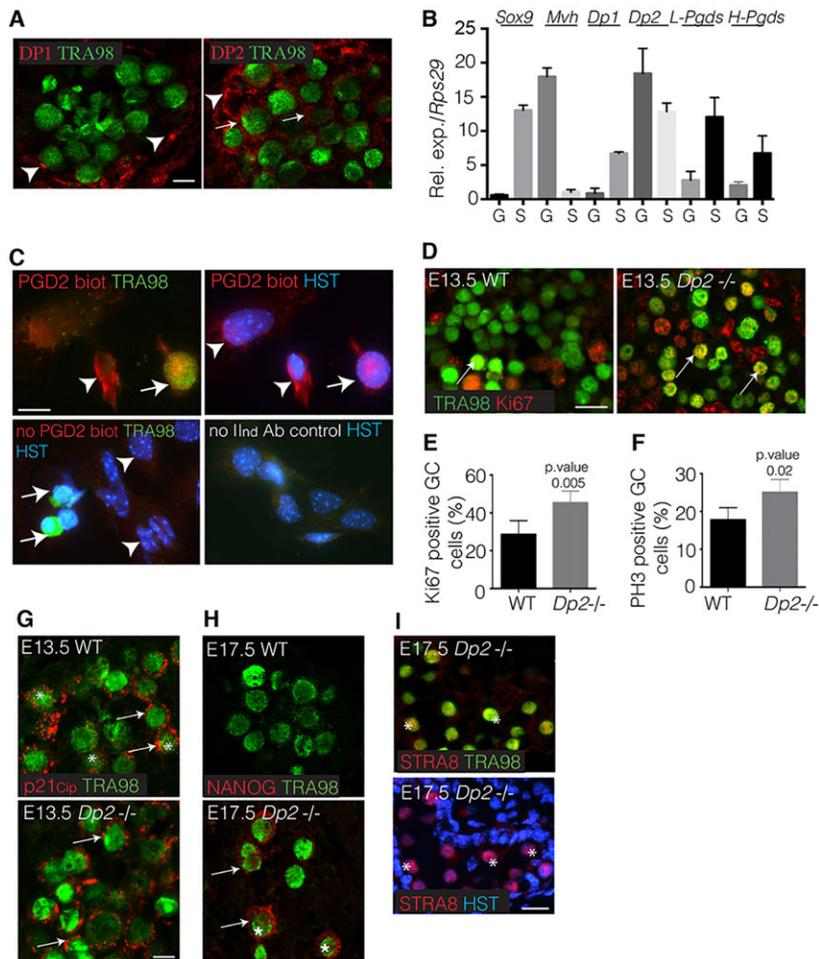
**Fig. 4. PGD<sub>2</sub> contributes to the male germ cell differentiation.** (A,D-G,K) RT-qPCR analysis of *Nanos2* (A), *DnmtL3* (D), *Cyp26b1* (E), *Nodal* (F), *Stra8* (G) and *Dmc1* (K) expression levels in E13.5, E15.5 (A,D-G,K) and E17.5 (A,D,F,G,K) KO and wild-type male gonads and in E13.5 wild-type ovaries (*Stra8*, G; *Dmc1*, K). Error bars indicate s.d. of assays carried out in triplicate ( $n=4$ ). (B) Germ cells were isolated from E12.5 testes and cultured with PGD<sub>2</sub> (5 ng/ml) or not (C=control). RT-qPCR analysis of *Nanos2* expression is represented using *Rps29* as the normalization gene; error bars represent s.d. of assays carried out in triplicate ( $n=4$ ). (C) Somatic and germ cells from wild-type and mutant E13.5 gonads were mixed and cultured for 24 h; *Nanos2* expression levels were quantified by RT-qPCR relative to *Oct4* expression. Error bars indicate s.d. of assays carried out in triplicate ( $n=2$ ). (H) Co-immunofluorescence of pre-meiotic marker *Stra8* (red) with *Amh* (green) on E17.5 KO and wild-type testes. Arrows highlight the expression of *Stra8*. Scale bars: 50  $\mu$ m (wild type) and 25  $\mu$ m (KO). (I) Isolated germ cells from E12.5 testes were cultured with PGD<sub>2</sub> (5 ng/ml) and/or retinoic acid (RA, 10 nM) (C=control). RT-qPCR analysis of *Stra8* expression is represented using *Rps29* as the normalization gene. Error bars indicate s.d. of assays carried out in triplicate on three independent samples. (J) Somatic and germ cells from wild-type and mutant E13.5 gonads were mixed and cultured for 24 h; *Stra8* expression levels were quantified by RT-qPCR relative to *Oct4* expression. Error bars indicate s.d. of assays carried out in triplicate ( $n=2$ ). \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$ ; \*\*\*\* $P<0.0001$ .

Dp2 receptor, is involved in processes required for proper male germ cell differentiation: slowing down proliferation to reach the mitotic arrest, inhibiting the expression of pluripotency master genes and upregulating male germ cell genes.

Consistent with previous studies (Adams and McLaren, 2002; Moniot et al., 2009, 2011), we have confirmed that *L-Pgds* and *H-Pgds* are both expressed at early stages in the male germline and have found that both enzymatic activities act in a complementary manner with respect to germ cell differentiation, as *L-Pgds* and *H-Pgds* single mutants have a normal phenotype. Furthermore, we have demonstrated that PGD<sub>2</sub> is indeed synthesized by both the somatic and germ cell populations and provide evidence that both sources of PGD<sub>2</sub> work in concert to effect germ cell differentiation. This effect might be the result of a direct action on germ cells to activate expression of the male germ cell marker *Nanos2* and of *p21<sup>Cip1</sup>* and to downregulate pluripotency markers; this might also result from indirect effects by reinforcing the male phenotype in somatic cells through the activation of Notch signalling and *Cyp26b1* expression. Further work will decipher the respective contribution of both cell types in the PGD<sub>2</sub>-mediated germ cell differentiation using germ cell- and Sertoli cell- specific *Dp2* receptor knockout strains. Adding to our knowledge of Fgf9 (Bowles et al., 2010; Spiller et al., 2012), Notch (Garcia et al., 2013) and Tgfb $\beta$  signalling (Miles et al., 2013) in Sertoli cells and of *Dmrt1* (Krentz et al., 2009) and *Dnd1* (Cook et al., 2011) in germ cells, we have thus identified the PGD<sub>2</sub> signalling pathway, expressed in both

compartments and active in the germline in both a paracrine and an autocrine manner, as a new factor contributing to the male germ cell differentiation process. In contrast to Fgf9 (Bowles et al., 2010) and Nodal signalling (Souquet et al., 2012; Spiller et al., 2012), however, suppressing PGD<sub>2</sub> signalling alone is not sufficient to allow progression into meiosis; this is presumably because these two other pathways, and also *Cyp26b1* and *Nanos2*, continue to exert their strong repressive influences even in the absence of PGD<sub>2</sub>.

In the absence of PGD<sub>2</sub>, we observed an increase in the proliferation rate at E13.5; even at E17.5, 10% of the mutant gonocytes remained in the cell cycle, strongly suggesting that the PGD<sub>2</sub> signalling pathway contributes to mitotic arrest in male foetal germ cells. This impaired mitotic arrest in the *L/H-Pgds*<sup>-/-</sup> testes was similar to that observed in mutant gonads for *Dmrt1* (Krentz et al., 2009) and for *Dnd1* (Cook et al., 2011), where persistent Ki-67-positive and pH3-negative cells remained at E17.5. Our experiments showed that the gene coding for *p21<sup>Cip1</sup>*, a key regulator of the G1-S phase checkpoint (Fotadar et al., 2004) that is specifically expressed in the male germ line at the time of mitotic arrest (Western et al., 2008), is directly upregulated by PGD<sub>2</sub>. Furthermore, in *L/H-Pgds*<sup>-/-</sup> germ cells, we detected the *p21<sup>Cip1</sup>* protein mainly within the cytoplasm, suggesting a role for PGD<sub>2</sub> in the post-transcriptional regulation of *p21<sup>Cip1</sup>* and potentially explaining the reduced cell-cycle inhibitory activity of *p21<sup>Cip1</sup>* observed in the absence of PGD<sub>2</sub> (Starostina et al., 2010; Wu et al., 2011). As *Dnd1* expression is downregulated in *L/H-Pgds*<sup>-/-</sup>



**Fig. 5. The Dp2 receptor is expressed in the male germ line and functions to control proliferation and differentiation of the germ line.**

(A) Co-immunofluorescence of Dp1 or Dp2 (red) and germ cells stained with Tra98 (green). Arrows and arrowheads indicate germ and somatic cells, respectively. Scale bar: 25  $\mu$ m. (B) RT-qPCR analysis of *Dp1*, *Dp2*, *L-Pgds* and *H-Pgds* expression in E13.5 germ cells (G) and somatic (S) cell fractions. *Mvh* and *Sox9* expression is shown to assess the purity of both somatic and germ cell fractions. (C) Dissociated somatic/germ cells from E13.5 wild-type testes were cultured for 24 h and were treated by PGD<sub>2</sub>-biotin (5 ng/ml) for 10 min. Germ cells able to bind PGD<sub>2</sub>-biotin were visualised by immunofluorescence (IF) using streptavidin-Texas Red antibody (red) together with anti-Tra98 (green) (upper panels). Two controls (lower panels) were performed: one was not treated with PGD<sub>2</sub>-biotin but was submitted to immunofluorescence and one was treated by PGD<sub>2</sub>-biotin but submitted to immunofluorescence without secondary antibodies. HST, Hoechst dye. Scale bar: 50  $\mu$ m. Arrow and arrowheads indicate germ cell and somatic cells, respectively. (D) Co-immunofluorescence with Ki-67 (red) and Tra98 (green) on E13.5 wild-type and *Dp2* mutant (*Dp2*<sup>-/-</sup>) testes. Arrows indicate proliferating germ cells. Scale bar: 50  $\mu$ m. (E,F) Quantifications of Ki-67 proliferating germ cells (E) and p3H mitotic germ cells (F) are represented as percentages of total Tra98+ germ cells in the E13.5 *Dp2*<sup>-/-</sup> and wild-type testes. (G-I) Co-immunofluorescence of p21<sup>Cip1</sup> (G), Nanog (H), Stra8 (I) (red) and Tra98 (green) on E13.5 (G) or E17.5 (H-I) wild-type (WT) and *Dp2*<sup>-/-</sup> testes. Asterisks indicate nuclear staining of p21<sup>Cip1</sup> (G), Nanog (H) or Stra8 (I); arrows indicate cytoplasmic localisation of p21<sup>Cip1</sup> (G) and Nanog (H). Scale bars: 25  $\mu$ m in G,H; 50  $\mu$ m in I.

gonads, PGD<sub>2</sub> signalling might affect *p21*<sup>Cip1</sup> expression, indirectly by activating *Dnd1* expression (Cook et al., 2011; Kedde et al., 2007). Furthermore, the RNA-seq analysis highlighted differences in a number of genes known to be involved in the complex p21<sup>Cip1</sup> regulation (Jung et al., 2010), both at the transcriptional (*Hdac4*, *Pde3* and *Pten*) and the post-transcriptional levels (*Elavl2*, *Dnd1*, *Rbm38*, *Ddx4* and *Trim71*). Thus, these data show that PGD<sub>2</sub> might be a pathway acting early at multiple levels of *p21*<sup>Cip1</sup> regulation during the mitotic arrest within male germ cells, through still unknown mechanisms.

Concomitant with the impaired mitotic arrest observed in mutant germ cells at E17.5, the pluripotent proteins Oct4, Sox2 and Nanog were still present, even though only *Sox2* was upregulated at the mRNA level. The regulation of *Sox2* by PGD<sub>2</sub> appears to be independent of *Dmrt1* (Krentz et al., 2009) as *Dmrt1* expression was not modified by PGD<sub>2</sub>. After E12.5, the downregulation of the Fgf9 (Bowles et al., 2010) and Cripto/Nodal (Spiller et al., 2012) pathways, together with the upregulation of *L-Pgds* (Moniot et al., 2009), can explain the slow decrease in *Sox2* mRNA that is observed in the male germ line (Western et al., 2010). Here, we also observed that PGD<sub>2</sub> could post-transcriptionally regulate the expression of Oct4 and Nanog at the level of mRNA translation or protein stability (or both). Interestingly, numerous RNA-binding proteins such as *L1td1* (Iwabuchi et al., 2011) are upregulated in the absence of PGD<sub>2</sub> and might participate in this regulation. In the absence of PGD<sub>2</sub>, Nanog, Oct4 and Sox2 are ectopically expressed in mutant foetal testes and display cytoplasmic localisation,

suggesting that PGD<sub>2</sub> might also participate in the nuclear translocation of these transcription factors as has been previously observed for *Sox9* (Malki et al., 2005). This phenotype might reflect a transient and incomplete differentiation of the germline, as described in embryonic stem cells (da Cunha et al., 2013; Elatmani et al., 2011) and in a variety of cancer cells (Gu et al., 2012; Guo et al., 2011).

Our ablation of the PGD<sub>2</sub> pathway in the teratoma-resistant C57Bl/6 (B6) background induced phenotypes that were similar to those that have been observed in the teratoma-susceptible strains 129/SvJ or 129-Chr19<sup>MOLF/Ei</sup> (Heaney et al., 2012), likely related to the impaired mitotic arrest. Along these lines, questions related to germ cell differentiation and mitotic arrest can have clear implications for human health, as germ cells that are not controlled appropriately during foetal life can later transform into carcinoma *in situ* (CIS), the precursor for testicular germ cell tumours (Kristensen et al., 2008). Furthermore, testicular cancers commonly include molecular abnormalities such as mutations in cell cycle regulators (Bartkova et al., 2000), and the PGD<sub>2</sub> target gene *L1td1* is highly expressed in seminomas and testicular germ cell tumours (Narva et al., 2012). Further work will determine whether the double *L/H-Pgds* mutation can lead to a high incidence of germ-line tumours in the 129sv background, as has been described for *Dmrt1* (Krentz et al., 2009, 2013), *Pten* (Kimura et al., 2003) and *Dnd1* (Cook et al., 2011) mutants.

In summary, the present study identifies the PGD<sub>2</sub> pathway as one of the earliest signalling pathways involved in the male germ cell

differentiation, showing that PGD<sub>2</sub> is a male fate-promoting factor. As PGD<sub>2</sub> is a potential target for endocrine disruptors (ED) (Kristensen et al., 2011), our findings thus open new perspectives for future investigations into how germ cell development can be perturbed by the external environment.

## MATERIALS AND METHODS

### Mice

*L-Pgds* KO (Eguchi et al., 1999) and *H-Pgds* KO (Trivedi et al., 2006) mice were generated at Osaka Bioscience Institute (Osaka, Japan) using the C57BL/6 strain. They were cross-bred to generate the *L/H-Pgds* double KO mice that were used in this work. *L/H-Pgds* double KO animals were kept and bred at the IGH animal care facility under controlled environmental conditions. *Dp2/Crth2* mice were generated at BioMedical Laboratories (Saitama, Japan) (Satoh et al., 2006) and were transferred into the C57BL/6 genetic background. For the pharmaceutical experiments, wild-type CD1 E10.5 females were purchased from Charles River Laboratories. All animal uses were conducted according to procedures approved by the Réseau des Animaleries de Montpellier (RAM) (agreement number 34-366 for B.B.-B.) and by the Regional Ethics committee.

### In vivo EdU incorporation and treatments

EdU (5-ethynyl-2'-deoxyuridine) was intraperitoneally injected into pregnant females, 2 h before dissecting the embryos. Detection of the EdU-positive cells on testis sections was performed using the Click-iT EdU Assay, according to the supplier's instructions (Invitrogen). The Dp2 antagonists CAY10471 and BAY-U3450, the Dp2 agonist 15(R)-PGD<sub>2</sub>, and the Dp1 antagonist BW A868C were administered as previously described (Woodward et al., 2011). See methods in the supplementary material for further details.

### Immunofluorescence

Dissected gonads from staged embryos were processed into cryosections and immunofluorescence was performed using the primary antibodies that are listed in supplementary material Table S2, as previously described (Malki et al., 2005; Moniot et al., 2009). The appropriate secondary antibodies (Alexa-Ig, Molecular Probe) were used. Histology images were captured with a Leica DM6000 fluorescent microscope or with a Leica SP8-UV Confocal microscope.

### RNA isolation and real-time PCR analysis

Embryonic gonads were dissected and separated from mesonephros and were then pooled by sex within each litter (between four and seven pairs of gonads). RNA extraction was performed using the TRIZOL technique (Invitrogen). Real-time RT-PCR was performed as previously described (Moniot et al., 2009, 2011) using primers listed in supplementary material Table S3. *Oct4* or *Rps29* were used as the normalization gene in experiments with whole gonads, and *Rps29* was used with isolated germ cells.

### Immunomagnetic germ cell and somatic cell isolation and culture

Germ cell isolation using the Ssea-1 antigen was performed as previously described (Moniot et al., 2011), using magnetic sorting. *In vitro* cultures of germ cells and mixed somatic and germ cells were performed using previously described techniques (Bowles et al., 2010; Munger et al., 2013). Dissociated gonadal cells (250,000 cells) were also cultured on glass coverslips in 24-well plates for 24 h (Munger et al., 2013) to detect intracellular production of PGD<sub>2</sub> using the Eicosacell technique (Bandeira-Melo et al., 2011) and to analyse the PGD<sub>2</sub> binding on embryonic cells using the PGD<sub>2</sub>-biotin tool. For details, see methods in the supplementary material.

### Protein extracts and western blots

Protein extracts from E13.5 testes or from cultured germ cells were prepared in Tris buffer (pH 8) with 25 U benzamide (Sigma-Aldrich) and protein contents were quantified using the micro BCA protein assay kit (Thermo Scientific). Proteins (20 µg) were electrophoresed in SDS/PAGE gels and

then electroblotted onto nitrocellulose membranes. Membranes were incubated with primary antibodies (see supplementary material Table S1 for concentration), followed by HRP-conjugated secondary antibodies. Signal was detected using the Chemiluminescent Substrate detection kit (Thermo Scientific).

### Statistical analysis

Statistical analysis with PRISM 6 software (GraphPad Software) was performed using the Student's *t*-test to compare two groups in a independent experiments or the ANOVA test with the Geisser-Greenhouse correction for multiple comparisons (qPCR experiments) and using the Fisher's exact test (cell counting experiments), and the results were considered statistically significant at  $P < 0.05$ . Asterisks indicate the level of statistical significance: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$  (Student's or ANOVA tests) or *P*-values are indicated on each graph (Fisher's exact test); ns indicates not significant. For details of the analysis, see methods in the supplementary material.

### mRNA expression profiling and analysis

Whole-transcriptome analysis of E13.5 wild-type and mutant male gonads was performed using RNA-seq experiments, as described in detail in the methods in the supplementary material.

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### Competing interests

The authors declare no competing financial interests.

### Author contributions

B.M., S.U., J.C., F.P. and B.B.-B. performed experiments. E.D. and S.N. performed RNA-seq experiments and analysis. K.A. and Y.U. provided *L/H-Pgds* KO mice. H.H., K.N. and M.N. provided *Dp2* knockout mice. F.P. and B.B.-B. designed the experiments and wrote the paper.

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### Supplementary material

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