

# Sexually dimorphic development of mouse primordial germ cells: switching from oogenesis to spermatogenesis

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## SUMMARY

During embryogenesis, primordial germ cells (PGCs) have the potential to enter either spermatogenesis or oogenesis. In a female genital ridge, or in a non-gonadal environment, PGCs develop as meiotic oocytes. However, male gonadal somatic cells inhibit PGCs from entering meiosis and direct them to a spermatogenic fate. We have examined the ability of PGCs from male and female embryos to respond to the masculinising environment of the male genital ridge, defining a temporal window during which PGCs retain a bipotential fate. To help understand how PGCs respond to the male gonadal environment, we have identified molecular differences between male PGCs that are committed to spermatogenesis and bipotential female PGCs. Our results suggest that one way in which PGCs

respond to this masculinising environment is to synthesise prostaglandin D<sub>2</sub>. We show that this signalling molecule can partially masculinise female embryonic gonads in culture, probably by inducing female supporting cells to differentiate into Sertoli cells. In the developing testis, prostaglandin D<sub>2</sub> may act as a paracrine factor to induce Sertoli cell differentiation. Thus part of the response of PGCs to the male gonadal environment is to generate a masculinising feedback loop to ensure male differentiation of the surrounding gonadal somatic cells.

Key words: Primordial germ cells, Testis development, Sertoli cells, Prostaglandin D synthase, Prostaglandin D<sub>2</sub>, Sex reversal, Mouse

## INTRODUCTION

In the mouse embryo, primordial germ cells (PGCs) have the potential to develop either as meiotic oocytes, progressing through the first meiotic prophase and arresting in dictyate just after birth, or as prospermatogonia, mitotically arrested in G1/G0 until a few days after birth, when they resume proliferation (Hilscher et al., 1974; McLaren, 1984). This developmental switch, which has occurred by 13.5 days post coitum (dpc) is dependent on the sex of the gonadal somatic cells, rather than the chromosomal sex of the PGCs: XY PGCs can develop as oocytes in female embryos and XX PGCs can develop as prospermatogonia in male embryos (Ford et al., 1975; Palmer and Burgoyne, 1991). Somatic sex in most mammals is determined by the presence or absence of *Sry*, a dominant male-determining gene located on the Y chromosome (Lovell-Badge and Robertson, 1990; Koopman et al., 1991). In mice, *Sry* is expressed between 10.5 dpc and 12.0 dpc (Koopman et al., 1990; Hacker et al., 1995) in the supporting cell lineage of the indifferent gonad (Palmer and Burgoyne, 1991), before any overt sexual differentiation of the gonad has occurred.

Somatic cell types in the indifferent gonad also have bipotential, sexually dimorphic fates, and in response to appropriate *Sry* expression, these cell types follow a male rather than a female developmental pathway. The supporting cell lineage differentiates into Sertoli rather than follicle cells,

and the steroidogenic precursor cells differentiate into Leydig rather than theca cells (reviewed by McLaren, 1991; Capel, 2000). Testis development, which is driven by the Sertoli cells, entails increased somatic cell proliferation, cell migration from the mesonephros into the *Sry*-expressing genital ridge, the development of testis-specific vasculature and the formation of testis cords. There appears to be no ovarian cell type that is analogous to testicular peritubular myoid cells, which are derived from the immigrant mesonephric cells, and there is no major cell migration from the mesonephros into the female genital ridge (Martineau et al., 1997). In a normal mouse embryonic testis, the prospermatogonia are clustered together in the centre of the testis cords, surrounded by a layer of Sertoli cells, with peritubular myoid cells enclosing the entire cord. The testosterone-producing Leydig cells are located in the interstitial tissue between the cords (reviewed by McLaren, 1998; Capel, 2000).

Germ cells seem to play a more active role in ovary development than they do in testis development. In the absence of PGCs, supporting cells in the ovary differentiate into pre-follicle cells that aggregate into mesenchymal condensations, but these eventually degenerate leaving only stromal tissue. However, in the presence of meiotic oocytes, the pre-follicle cells can differentiate into follicle cells surrounding the oocyte. If oocytes are subsequently lost from these follicles, the supporting cells transdifferentiate into Sertoli cells and form structures resembling testis cords. The

involvement of germ cells in the differentiation and maintenance of the male-supporting cell lineage is less obvious, as germ cells are required neither for the differentiation of Sertoli cells nor for the assembly of testis cords (reviewed by McLaren, 1991).

PGCs in a female genital ridge do not seem to require specific signals from the surrounding somatic cells to enter oogenesis: they will develop as oocytes, entering meiosis with apparently normal timing, outside the genital ridge but within the embryo (Zamboni and Upadhyay, 1983), or outside the embryo in a cultured lung aggregate (McLaren and Southee, 1997), or outside the embryo in a simple cell culture system (Chuma and Nakatsuji, 2001). However, it has been proposed that an as yet uncharacterised signal produced by the somatic cells in a male genital ridge inhibits PGCs from entering meiosis, arresting them in G1/G0, and directing them towards spermatogenesis (Dolci and De Felici, 1990; McLaren and Southee, 1997). PGCs in male genital ridges can be rescued from this signal if they are removed from the genital ridges at 11.5 dpc, and will develop as oocytes in lung aggregates. However, by 12.5 dpc, PGCs isolated from male genital ridges are committed to spermatogenesis (McLaren and Southee, 1997).

We have characterised the ability of PGCs from female embryos to respond to the masculinising signal that emanates from the somatic cells in a male genital ridge. Furthermore, we have identified molecular differences between PGCs in male and female embryos that reveal how PGCs may not only respond to the masculinising environment of the developing testis, but may also contribute to it.

## MATERIALS AND METHODS

### Mice

All mice were from naturally mated MF1×MF1 strains, unless otherwise stated, with noon on the day the vaginal plug was found being termed 0.5 dpc. MF1×129/Sv-TgR(ROSA26)26Sor natural matings (MF1×ROSA) were used to obtain cells carrying a ubiquitously expressed *lacZ* transgene (Friedrich and Soriano, 1991). *W<sup>e</sup>/W<sup>e</sup>* homozygotes (14.5 dpc) were identified by their anaemic appearance and gonadal morphology in matings between *W<sup>e</sup>/+* heterozygotes (Lyon et al., 1996). Pregnant mice were killed by cervical dislocation and the embryos removed into PB1 for dissection (Whittingham, 1971).

### Aggregate cultures

Urogenital ridges were isolated from embryos as described (McLaren and Southee, 1997). Embryos (11.5 dpc) were sexed by PCR genotyping of *UbeX1* (Chuma and Nakatsuji, 2001), urogenital ridges from 12.5 dpc and older embryos were sexed by their appearance. Aggregate cultures were performed essentially as described (McLaren and Southee, 1997). Briefly, PGCs were isolated from urogenital ridges (MF1×ROSA) by pricking with a hypodermic needle after incubation in EDTA (De Felici and McLaren, 1982). This technique generally results in PGC suspensions that have less than 20% somatic cell contamination (mostly erythrocytes). Four recipient urogenital ridges (MF1×MF1) were disaggregated to a single cell suspension with trypsin, mixed with the PGC suspension, then pelleted in a microfuge tube. A typical suspension contained a few thousand donor cells, and a few hundred thousand recipient cells. The aggregated tissue was dislodged from the bottom of the tube, transferred onto 2% agar blocks, and cultured as described (McLaren and Southee, 1997). Aggregates containing 11.5 dpc donor PGCs were cultured for 4 days,

those containing 12.5 dpc or 13.5 dpc donor PGCs were cultured for 3 days.

Intact urogenital ridges were similarly cultured on agar blocks using the same culture conditions. Prostaglandin D<sub>2</sub> (Sigma) was added to the culture medium at a concentration of 100–500 ng/ml. For routine histology, tissue was fixed with Bouin's solution and embedded in wax. Sections were cut at 5 µm, stained with Ehrlich's Haematoxylin and Eosin, and mounted under a glass coverslip with DPX (Merck).

Immunohistochemistry for anti-Müllerian hormone was performed essentially as described (Rey et al., 1996), except that peroxidase-coupled anti-rabbit IgG (Jackson Immunoresearch) was used as a secondary antibody, and tyramide signal amplification was performed according to manufacturer's instructions (NEN Life Science). 3,3'-diaminobenzidine was used to visualise the signal (FAST DAB tablets, Sigma), and the sections counterstained with Haematoxylin and mounted with DPX.

### Combined β-galactosidase histochemistry and germ cell histology

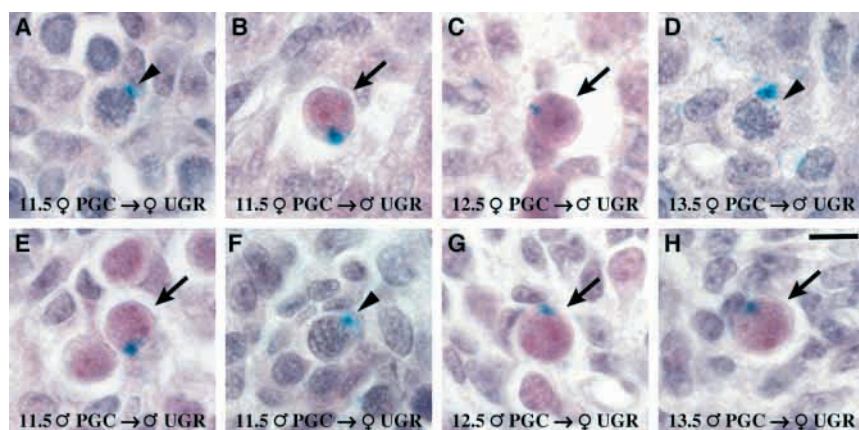
At the end of the culture period, aggregates were washed twice with PBS containing 2 mM MgCl<sub>2</sub>, then fixed for 45 minutes on ice with pre-chilled fixative (100 mM acetate buffer, 2 mM MgCl<sub>2</sub>, 5 mM EGTA, 0.5% glutaraldehyde, pH 5.5). The tissue was then rinsed three times with rinse buffer [phosphate-buffered saline (PBS) containing 2 mM MgCl<sub>2</sub>, 5 mM EGTA, 0.01% sodium deoxycholate and 0.02% Nonidet P-40], and stained overnight at 37°C in staining solution (rinse buffer with 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide and 1 mg/ml X-gal). The tissue was washed extensively with rinse buffer, dehydrated in ethanol, cleared in xylene and embedded in Paraplast plus wax (Sigma). Sections were cut at 5 µm, stained with Ehrlich's Haematoxylin and Eosin, and mounted under a glass coverslip with Canada balsam (Sigma).

### cDNA synthesis and representational difference analysis

PGCs were isolated from around 20 sexed 12.5 dpc urogenital ridges by incubating them in EDTA, then pricking them with a hypodermic needle (De Felici and McLaren, 1982). PGCs were further enriched by magnetic immunoaffinity isolation using antibodies against stage-specific embryonic antigen 1 as described (Pesce and De Felici, 1995). The resulting populations of around 10,000 PGCs were around 95% pure, as judged by alkaline phosphatase staining (data not shown). Poly A<sup>+</sup> RNA was isolated from the PGCs using oligo dT-coupled magnetic beads (Dynal), and the eluted RNA used for reverse transcription and SMART cDNA synthesis according to manufacturers instructions (Clontech). PCR amplification of the SMART cDNA was performed as described by the manufacturer (Clontech), with care being taken to ensure that the PCR did not reach the plateau phase. Representational difference analysis (RDA) was used to identify differences between the two cDNA populations (Lisitsyn et al., 1993; Hubank and Schatz, 1994). *DpnII*-digested PGC cDNA (0.2 µg) was used for RDA essentially as described by O'Neill and Sinclair (O'Neill and Sinclair, 1997) except that oligonucleotides were gel-purified and Qiaquick columns (Qiagen) were used to purify cDNA. After three rounds of RDA, five discrete bands could be seen by gel electrophoresis. These bands were excised, cloned and sequenced.

### In situ hybridisation

Nucleotides 1–637 of the *Ptgds* cDNA (Genbank Accession Number, AB006361) were amplified by PCR from PGC cDNA and blunt-end cloned into pBluescript II SK+ (Stratagene). The resulting plasmids were sequenced, then linearised and used to generate digoxigenin-labelled antisense riboprobes using T3 RNA polymerase according to suppliers instructions (Roche). Whole-mount in situ hybridisation on isolated urogenital ridges was performed as described (Henrique et al., 1995). BM Purple (Roche) was used to visualise the signal.



**Fig. 1.** Development of PGCs in urogenital ridge aggregates. Donor PGCs carrying a *lacZ* transgene were isolated from 11.5 dpc (A,B), 12.5 dpc (C) or 13.5 dpc (D) female genital ridges, and from 11.5 dpc (E,F), 12.5 dpc (G) or 13.5 dpc (H) male genital ridges, as indicated. The donor PGCs were aggregated with male (B-E) or female (A,F,G,H) 12.5 dpc recipient urogenital ridges (UGR) and cultured for 3–4 days. Donor PGCs are identified by a cyan perinuclear dot. Donor PGCs developing as oocytes (arrowheads) have condensed chromatin staining. Donor PGCs developing as prospermatogonia (arrows) have diffuse chromatin staining and prominent nucleoli. Scale bar: 10  $\mu$ m.

Stained tissue was sectioned as required after embedding in paraffin wax. Sections were counterstained with Neutral Red to visualise the nuclei.

## RESULTS

### PGCs from female genital ridges can develop as prospermatogonia

The first series of experiments was designed to assay the ability of 11.5 dpc female PGCs to respond to the masculinising environment of a 12.5 dpc male genital ridge. In these experiments, donor PGCs are aggregated with recipient urogenital ridge tissue, then cultured to allow the PGCs to differentiate into either mitotically arrested prospermatogonia or meiotic oocytes. The donor germ cells are distinguished from the germ cells in the recipient urogenital ridge tissue by the presence of a *lacZ* transgene. Previous experiments had shown that the distinctive histological nuclear morphology of meiotic oocytes and resting prospermatogonia was not preserved after standard  $\beta$ -galactosidase histochemistry (McLaren and Southee, 1997). Fluorescent labelling of donor germ cells had similarly proven to be unsuitable for this purpose (Dolci and De Felici, 1990). We therefore developed a method that allowed  $\beta$ -galactosidase histochemistry to be combined with germ cell histology. Under these conditions,  $\beta$ -galactosidase activity in germ cells could be visualised as a cyan perinuclear dot of X-gal precipitate, and the developmental status of the germ cells was apparent from their nuclear morphology: as meiosis progresses into zygotene and

pachytene, the chromatin staining becomes increasingly condensed (Fig. 1A,D,F), whereas prospermatogonia have dispersed interphase chromatin and prominent nucleoli (Fig. 1B,C,E,G,H).

11.5 dpc PGCs carrying a *lacZ* transgene were isolated from female MF1 $\times$ ROSA embryos and mixed with disaggregated 12.5 dpc urogenital ridges from male MF1 $\times$ MF1 embryos. The recipient MF1 $\times$ MF1 urogenital ridge tissue does not carry a *lacZ* transgene. This cell suspension was pelleted, and the resulting aggregate cultured for 4 days. In these aggregates, most of the *lacZ*-bearing germ cells were prospermatogonia (Table 1, Fig. 1B). In control experiments, most female 11.5 dpc *lacZ*-bearing PGCs developed as oocytes when cultured in female 12.5 dpc urogenital ridge aggregates (Table 1, Fig. 1A). This experiment shows that 11.5 dpc female PGCs have not yet become committed to oogenesis and are able to respond to masculinising signals from a male urogenital ridge and develop as prospermatogonia.

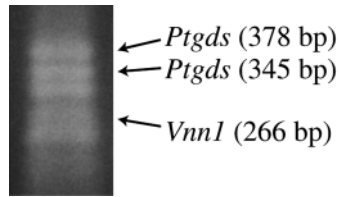
We similarly confirmed that 11.5 dpc male *lacZ*-bearing PGCs would develop as oocytes when aggregated with 12.5 dpc female urogenital ridges, and as prospermatogonia when aggregated with 12.5 dpc male urogenital ridges (Table 1, Fig. 1E,F). The differentiation of 11.5 dpc male PGCs into oocytes when aggregated with female urogenital ridges is consistent with previous observations that 11.5 dpc male PGCs develop as oocytes in lung aggregates (McLaren and Southee, 1997). Thus, both male and female PGCs are sexually dimorphic with respect to their developmental fate at 11.5 dpc.

Previous experiments have shown that by 12.5 dpc, PGCs in a male genital ridge become committed to develop as

**Table 1.** The effect of the somatic environment on PGC development

Donor PGCs	Gender of recipient of 12.5 dpc urogenital ridges	Donor PGCs (number of aggregates)	Percentage of donor PGCs developing as prospermatogonia	Percentage of donor PGCs developing as oocytes
11.5 dpc female	Female	260 (2)	7%	93%
11.5 dpc female	Male	172 (4)	85%	15%
12.5 dpc female	Male	1063 (2)	78%	22%
13.5 dpc female	Male	796 (2)	18%	82%
11.5 dpc male	Male	148 (2)	89%	11%
11.5 dpc male	Female	271 (4)	16%	84%
12.5 dpc male	Female	177 (2)	79%	21%
13.5 dpc male	Female	2076 (2)	90%	10%

Donor PGCs were aggregated with recipient urogenital ridges as described in the text. The total number of donor PGCs that were clearly developing as either prospermatogonia or oocytes in each aggregate was counted.



**Fig. 2.** Representational difference analysis of 12.5 dpc PGC cDNA. RDA was used to identify cDNAs that were differentially expressed in male and female PGCs at 12.5 dpc. Five male-enriched bands were present on a gel

after three rounds of RDA. The identity and size of three of these bands are shown.

prospERMATOGONIA (McLaren and Southee, 1997). However, it is not clear if PGCs in female genital ridges similarly become committed to oogenesis by 12.5 dpc, or if they still retain the ability to differentiate into prospermatogonia. Therefore, we performed a further series of experiments using *lacZ*-bearing 12.5 dpc donor PGCs aggregated with 12.5 dpc recipient urogenital ridges. PGCs from 12.5 dpc female embryos were still able to respond to a male somatic environment and developed as prospermatogonia when aggregated with 12.5 dpc male urogenital ridges (Table 1; Fig. 1C). However, 12.5 dpc PGCs from male embryos developed as prospermatogonia when aggregated with female 12.5 dpc urogenital ridge tissue (Table 1; Fig. 1G). Thus, PGCs in male genital ridges become committed to spermatogenesis between 11.5 dpc and 12.5 dpc. By contrast, PGCs in female genital ridges still retain bipotential sexually dimorphic fates at 12.5 dpc and can develop as either oocytes or prospermatogonia, depending on their surrounding environment.

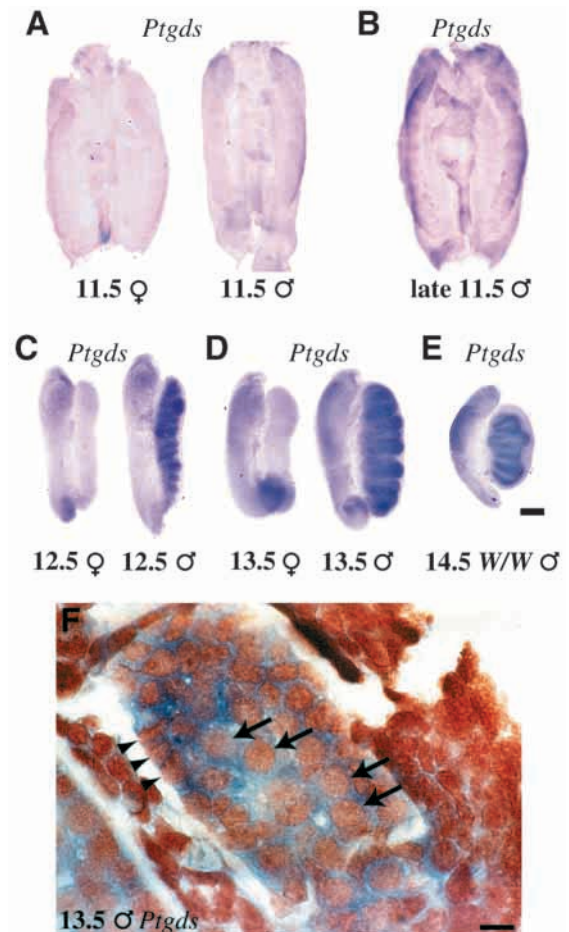
We next tested if PGCs from 13.5 dpc female genital ridges would be able to respond to the male gonadal environment and enter spermatogenesis. Female PGCs (13.5 dpc) developed as oocytes when aggregated with male 12.5 dpc urogenital ridges (Table 1; Fig. 1D). Thus, between 12.5 dpc and 13.5 dpc, PGCs in female urogenital ridges lose their ability to respond to masculinising signals from male urogenital ridges and become committed to oogenesis.

### Molecular differences between male and female PGCs at 12.5 dpc

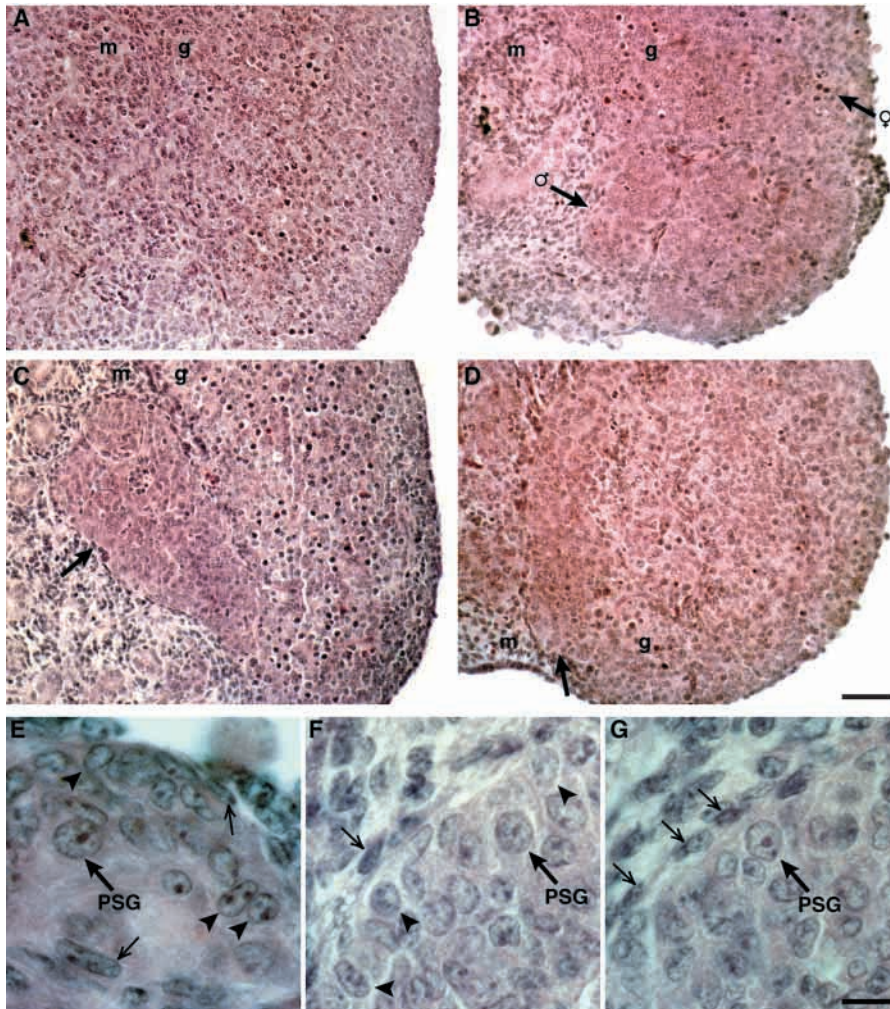
The experiments described in the previous section show that at 12.5 dpc, PGCs in male gonads have responded to their somatic environment and are committed to spermatogenesis, whereas PGCs in female gonads have the ability to respond to the masculinising signal, but have not been exposed to it. Although PGCs in 12.5 dpc male and female genital ridges differ with respect to their developmental potential, they are both in a transient postmitotic state and are histologically indistinguishable from each other. We therefore sought to identify molecular differences between male and female PGCs at 12.5 dpc to help to understand how the PGCs respond to the male somatic gonadal environment and become committed to spermatogenesis. PGCs were isolated from either male or female 12.5 dpc urogenital ridges and used to synthesise cDNA. The PGC cDNAs were then subjected to representational difference analysis (RDA) to identify differences between the male and female populations (see Materials and Methods).

RDA resulted in five discrete male-enriched bands on a gel (Fig. 2). This paper is concerned with the characterisation of one of the genes isolated in this screen; the identity of the other

genes will be reported elsewhere (I. R. A. and A. M., unpublished). One of these bands contained sequences identical to nucleotides 321 to 586 of *Vnn1* cDNA (Accession Number, NM\_011704). *Vnn1* encodes a glycoposphoinositol-anchored glycoprotein, vanin 1, which is involved in thymocyte migration (Aurrand-Lions et al., 1996). *Vnn1* has previously been isolated in two differential screens to identify genes involved in sex determination and testis development, and is highly expressed in the Sertoli cells in male genital ridges from 12.5 dpc onwards (Bowles et al., 2000; Grimmond et al., 2000). As the sexually dimorphic expression of *Vnn1* has already been established, this gene was not studied further. The isolation of



**Fig. 3.** Expression of *Ptgds* in embryonic gonads. Whole-mount in situ hybridisation for *Ptgds* was performed on isolated 11.5 dpc (A,B), 12.5 dpc (C) or 13.5 dpc (D) male and female urogenital ridges, as indicated. *W<sup>e</sup>/W<sup>e</sup>* testes (14.5 dpc) were used in (E). At 11.5 dpc, paired urogenital ridges with intervening mesenchyme are shown with the genital ridges lying on top of the mesonephroi along the left and right edges of the tissue. At 12.5-14.5 dpc, single urogenital ridges are shown, orientated with the mesonephros on the left and the genital ridge on the right. Sections of 13.5 dpc male urogenital ridges after whole-mount in situ hybridisation for *Ptgds* are shown in (F). Sertoli cells are marked with arrowheads, prospermatogonia with arrows. Sections are stained with neutral red to show the nuclei. Transcript expression is visualised by a dark blue/purple precipitate. Scale bars: in E, 200  $\mu$ m in A-E; in F, 10  $\mu$ m.



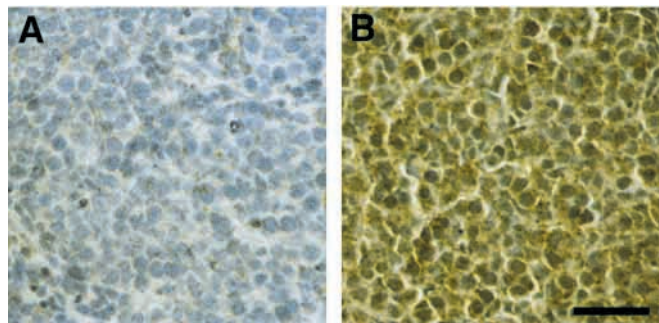
**Fig. 4.** Exogenous prostaglandin D<sub>2</sub> masculinises female urogenital ridges in culture. Female urogenital ridges were cultured with or without exogenous prostaglandin D<sub>2</sub> in the culture medium, fixed in Bouin's solution and sections stained with Haematoxylin and Eosin. (A-D) Sections are oriented with the mesonephros (m) on the left and the genital ridge (g) on the right. (A) Control female urogenital ridge cultured without prostaglandin D<sub>2</sub>. (B-D) Female urogenital ridges cultured with prostaglandin D<sub>2</sub>. (B) Disorganised cord-like structures in the masculinised region of the genital ridge (♂), and the ovarian region (♀) of the genital ridge. (C) Eosinophilic cells bulging from the genital ridge into the mesonephros (arrow). (D) The mass of gonadal eosinophilic cells is located along the boundary with the mesonephros (arrow). (E-G) Higher magnification of female urogenital ridges cultured with prostaglandin D<sub>2</sub>. Thick arrows labelled PSG indicate prospermatogonia, peritubular myoid-like cells are indicated with small arrows and Sertoli-like cells are indicated with arrowheads. (E) A disorganised cord in the masculinised region of the urogenital ridge shown in B. (F,G) Sections located at the edge of the bulge of eosinophilic cells spilling into the mesonephros, from the urogenital ridge shown in C. Scale bars: 50 µm in A-D; 10 µm in E-G.

*Vnn1*, which is reported to be expressed in Sertoli cells, suggests that although we attempted to bias the screen in favour of genes expressed by the PGCs, sex-specific differences in gene expression between the contaminating somatic cell populations might also be identified.

Two further male-enriched bands were identified as prostaglandin D synthase (*Ptgds*, also known as  $\beta$ -trace). These two RDA sequences were different sizes, but correspond to the same region of the prostaglandin D synthase cDNA (nucleotides 336-713 of *Ptgds* cDNA, Accession Number, AB006361). The smaller of the two RDA sequences lacks nucleotides 641-673 in the 3' untranslated region of the *Ptgds* cDNA, presumably as a result of alternative splicing. Prostaglandin D synthase has been suggested to have dual functions: it catalyses the final step in prostaglandin D<sub>2</sub> synthesis from a common prostaglandin precursor; and may be involved in binding and transporting small hydrophobic ligands into cells (reviewed by Urade and Hayaishi, 2000). *Ptgds* expression has been demonstrated in the testis cords and in the developing leptomeninges of the brain and spinal cord in 14.5 dpc mouse embryo sections (Hoffmann et al., 1996). *Ptgds* has been reported not to be expressed in ovaries at this stage. We examined the expression of *Ptgds* in developing male and female urogenital ridges from 11.5 to 13.5 dpc by whole-mount

in situ hybridisation. Expression of *Ptgds* was not detected in male or female 11.5 dpc genital ridges (Fig. 3A). However late 11.5 dpc male urogenital ridges showed some expression of prostaglandin D synthase in the forming testis cords (Fig. 3B). Strong *Ptgds* expression was detected in 12.5 dpc and 13.5 dpc testes but not ovaries (Fig. 3C,D). Prostaglandin D synthase expression was restricted to the testis cords, suggesting Sertoli cell and/or germ cell expression. Sections of the 13.5 dpc testis after whole-mount in situ hybridisation revealed that prostaglandin D synthase was expressed throughout the testis cords, by both Sertoli cells and prospermatogonia (Fig. 3F). Expression of prostaglandin D synthase in both germ cells and Sertoli cells has also been demonstrated in postnatal rat testis (Samy et al., 2000).

We also examined whether germ cells might induce expression of prostaglandin D synthase in the Sertoli cells. Testis cords in male *W/W* embryos have a relatively normal morphology, possessing peritubular myoid cells and Sertoli cells, but they lack prospermatogonia (reviewed by McLaren, 1991). In situ hybridisation of 14.5 dpc *W<sup>e</sup>/W<sup>e</sup>* testis, has shown that prostaglandin D synthase is expressed in the testis cords in the absence of germ cells (Fig. 3E). Thus, Sertoli cells do not require germ cells to initiate prostaglandin D synthase expression.



**Fig. 5.** Exogenous prostaglandin D<sub>2</sub> induces expression of AMH in cultured female urogenital ridges. Contralateral urogenital ridges from a female 11.5 dpc embryo were cultured without prostaglandin D<sub>2</sub> (A), or with prostaglandin D<sub>2</sub> (B). AMH expression was then examined by immunohistochemistry. Positive signal is shown by the brown precipitate, nuclei were counterstained blue with Haematoxylin. Images are from the genital ridge regions. Scale bar: 50  $\mu$ m.

### Prostaglandin D<sub>2</sub> induces masculinisation of female urogenital ridges in culture

To investigate the function of prostaglandin D synthase in testis development, we examined the effect of adding exogenous prostaglandin D<sub>2</sub> to female urogenital ridges in culture. Female urogenital ridges (11.5 dpc) were cultured for 4 days in the presence or absence of prostaglandin D<sub>2</sub>. In four separate experiments, a total of eight out of ten female urogenital ridges cultured with prostaglandin D<sub>2</sub> exhibited varying degrees of partial masculinisation (Fig. 4). We have never seen masculinisation in large numbers of female urogenital ridges cultured in the absence of prostaglandin D<sub>2</sub>. The most extensive masculinisation of the prostaglandin D<sub>2</sub>-treated urogenital ridges resulted in one female urogenital ridge developing an ovotestis-like appearance (Fig. 4B). Most of the germ cells in this urogenital ridge were in the ovarian region, developing as meiotic oocytes. The testicular region contained eosinophilic cells and disorganised cord-like structures (Fig. 4B). The few germ cells that were found in the testicular region were usually prospermatogonia (Fig. 4E). Peritubular myoid-like and Sertoli-like cells were also present in these disorganised cords (Fig. 4E). Urogenital ridges from control female littermates cultured without prostaglandin D<sub>2</sub> developed as apparently normal ovaries (Fig. 4A).

In three other female urogenital ridges cultured with prostaglandin D<sub>2</sub>, the gonad appeared to have a relatively normal ovarian appearance, with germ cells developing as meiotic oocytes (Fig. 4C). However, a mass of eosinophilic cells, originating from the gonad, could be seen to have breached the boundary between the gonad and the mesonephros, bulging into the mesonephric tissue (Fig. 4C). Peritubular myoid-like cells could be seen around the edges of this bulge, often adjacent to polarised Sertoli-like cells (Fig. 4F,G). Few germ cells were observed in this bulge region, but occasionally prospermatogonia could be seen in contact with the Sertoli-like cells towards the edges of these structures (Fig. 4F,G). Thus segments at the edge of the bulge of eosinophilic cells invading the mesonephros possessed cells that histologically resembled the three cell types in an embryonic testis cord.

The least extensively masculinised urogenital ridges contained a mass of eosinophilic cells within an otherwise apparently normal ovary (Fig. 4D). These eosinophilic cells were typically located close to the edge of the gonad at the boundary with the mesonephros. The eosinophilic cells did not have any distinctive histological features. Few germ cells were found within the mass of eosinophilic cells, most of the germ cells were located in the rest of the gonad and were developing as meiotic oocytes.

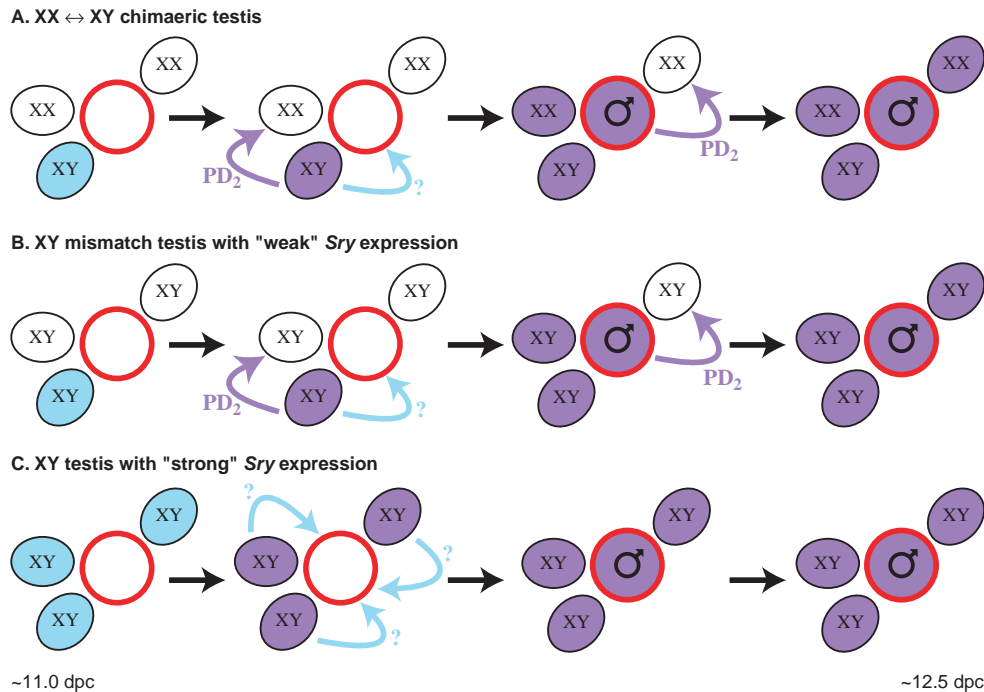
We confirmed the apparent masculinising influence of prostaglandin D<sub>2</sub> on female urogenital ridges by examining the expression of anti-Müllerian hormone (AMH), a Sertoli cell marker. During embryonic development, AMH is expressed in Sertoli cells from 11.5 dpc onwards, but is not expressed in female embryonic gonads (Münsterberg and Lovell-Badge, 1991; Hacker et al., 1995). Immunohistochemistry of cultured female urogenital ridges showed that AMH expression is induced in female urogenital ridges in response to prostaglandin D<sub>2</sub> (Fig. 5A,B). AMH expression was strongest along the region of the gonad closest to the mesonephros. Thus, prostaglandin D<sub>2</sub> can induce gonadal somatic cells that lack *Sry* to differentiate into Sertoli-like cells in this culture system.

## DISCUSSION

### PGCs and the masculinising environment of the male genital ridge

In this study, we have examined the ability of PGCs to respond to the masculinising environment of the developing testis. Previous work has shown that the sex chromosome constitution of the PGCs does not influence their decision to enter spermatogenesis or oogenesis, rather the PGCs are directed along one or other of these developmental pathways by their surrounding somatic environment (McLaren, 1995). We have shown that the somatic environment up to 11.5 dpc does not restrict the ability of the PGCs to develop as either oocytes or prospermatogonia. Between 11.5 dpc and 12.5 dpc, PGCs in a male genital ridge respond to the masculinising somatic environment and become committed to spermatogenesis, although female PGCs retain their developmental bipotency at this stage. By 13.5 dpc, however, PGCs in female genital ridges have become committed to oogenesis and do not develop as prospermatogonia if cultured in male urogenital ridge tissue. These experiments define a temporal window during which PGCs must be exposed to the male gonadal environment if they are to develop as prospermatogonia. It may be significant that the temporal window during which PGCs are competent to respond to the male gonadal environment correlates with the period when PGCs stop dividing mitotically and enter a transient postmitotic state.

A small proportion (7%) of the 11.5 dpc female donor PGCs developed as prospermatogonia when aggregated with 12.5 dpc female urogenital ridges (Table 1). A similar proportion of recipient PGCs also developed as prospermatogonia in these aggregates (I. R. A. and A. M., unpublished). This result was somewhat unexpected, given the lack of male cells in the experiment. Intact female urogenital ridges cultured, fixed and processed in the same way as the aggregates do not contain prospermatogonia (I. R. A. and A. M., unpublished). Perhaps the small proportion of prospermatogonia in the female



**Fig. 6.** The role of prostaglandin D<sub>2</sub> signalling in testis development. (A) In a XX ↔ XY chimaeric testis, a mixture of XX and XY supporting cells (ovoid cells) can be in contact with a bipotential primordial germ cell (outlined in red). XY-supporting cells express *Sry* (blue), and differentiate into pre-Sertoli cells. The pre-Sertoli cell produces the uncharacterised masculinising signal (blue arrow, ?) that acts on PGCs directing them to develop as prospermatogonia. The pre-Sertoli cell also expresses prostaglandin D synthase (purple). Prostaglandin D<sub>2</sub> acts as a local paracrine factor to induce nearby XX supporting cells to differentiate into pre-Sertoli cells (purple arrow, PD<sub>2</sub>). Commitment of PGCs to prospermatogonial development results in the prospermatogonia initiating expression of prostaglandin D synthase (purple). Prostaglandin D<sub>2</sub> from the prospermatogonia induces nearby XX supporting cells to differentiate into pre-Sertoli cells (purple arrow). (B) In XY mismatch testis with 'weak' *Sry* expression and an early acting programme for ovarian differentiation, some XY supporting cells express *Sry* effectively (blue), while others do not (white). Testis development proceeds similarly to the chimaeric testis, with prostaglandin D<sub>2</sub> inducing undifferentiated supporting cells to differentiate into pre-Sertoli cells. (C) In XY testis with 'strong' *Sry* expression, *Sry* is expressed effectively in all the supporting cells (blue), and the paracrine prostaglandin D<sub>2</sub> signal for inducing pre-Sertoli cell differentiation is somewhat redundant.

aggregates are induced by the disaggregation and reaggregation of the urogenital ridge tissue, which destroys the normal partitioning of mesonephric and gonadal cells. In the aggregates, peritubular myoid cell precursors, which presumably exist in the female mesonephros, could come into contact with undifferentiated supporting cells from the female genital ridge. The interaction between these cell types, which would normally only happen in a male genital ridge owing to *Sry*-dependent mesonephric cell migration, may be enough to induce *Sox9* expression in the supporting cells (Tilman and Capel, 1999). Expression of *Sox9* is sufficient to induce Sertoli cell differentiation and testis development in the absence of *Sry* (Vidal et al., 2001).

#### PGCs respond to the male genital ridge by expressing prostaglandin D synthase

We attempted to identify how PGCs respond to the male gonadal environment by characterising differences in gene expression between male and female 12.5 dpc PGCs. One of the genes that we identified was prostaglandin D synthase, an enzyme that catalyses the final step in the synthesis of prostaglandin D<sub>2</sub>. Prostaglandin D synthase is expressed in both Sertoli cells and prospermatogonia, and its expression appears to be initiated between 11.5 dpc and 12.5 dpc, around

the time of onset of cord formation. The onset of *Ptgds* expression in the male genital ridge is approximately coincident with the onset of *Amh* expression in Sertoli cells (Münsterberg and Lovell-Badge, 1991; Hacker et al., 1995). The promoter region of *Amh* contains binding sites for transcriptional regulators involved in sex determination (Shen et al., 1994; De Santa Barbara et al., 1998). Sequence comparison of the *Amh* promoter and that of prostaglandin D synthase revealed a partial match to the *Sox9*-binding site (CTTTGTG in *Ptgds*, CTTTGAG in mouse and bovine *Amh*), and a match to the steroidogenic factor 1 binding site (CAAGGTC in both *Ptgds* and *Amh*). These sites have both been shown to be required for normal expression of AMH in Sertoli cells in vivo (Arango et al., 1999). However, the promoter elements responsible for driving *Ptgds* expression in prospermatogonia are presently unknown.

#### Prostaglandin D<sub>2</sub> acts as a paracrine factor for pre-Sertoli cell differentiation

The addition of exogenous prostaglandin D<sub>2</sub> to cultures of 11.5 dpc female urogenital ridges in culture resulted in partial masculinisation of the female gonad. Prostaglandin D<sub>2</sub> did not appear to have a direct effect on PGC development. Even in the most masculinised urogenital ridges most of the PGCs

developed as oocytes, although these were mainly located in the ovarian regions. Very few germ cells were found in the masculinised regions of the urogenital ridges, and these were usually prospermatogonia. Meiotic oocytes can be found within testis cords in *XXSxr* mice (McLaren, 1981), suggesting that meiotic oocytes can survive and develop normally in a male environment *in vivo*. Therefore it is not clear at present why there is a paucity of germ cells in the masculinised regions of prostaglandin D<sub>2</sub>-treated urogenital ridges, and if this is caused by localised germ cell death, segregation of male and female cells within the gonad, preferential masculinisation in regions of the gonad with few germ cells or some other mechanism.

The primary effect of prostaglandin D<sub>2</sub> on the female genital ridge seemed to be the induction of a large number of eosinophilic cells in the gonad. Different urogenital ridges responded to these eosinophilic cells in slightly different ways, perhaps depending on the precise developmental status of the urogenital ridges. When these eosinophilic cells were able to make contact with mesonephric cells, either through inducing mesonephric cell migration into the gonad (Fig. 4B) or by bulging into the mesonephros (Fig. 4C), cells resembling peritubular myoid cells and morphologically normal Sertoli cells could be seen. Furthermore, the presence of prospermatogonia in these regions is indicative of the presence of functional Sertoli cells. Thus, the prostaglandin D<sub>2</sub>-induced eosinophilic cells are presumably pre-Sertoli cells that require interaction with peritubular myoid cells to become polarised and exhibit a distinctive Sertoli cell morphology. Similar AMH-expressing morphologically indistinct pre-Sertoli cells have previously been reported in cultures of male 11.5 dpc genital ridges dissected away from their mesonephros (Buehr et al., 1993a; Merchant-Larios et al., 1993). Thus, exogenous prostaglandin D<sub>2</sub> can induce pre-Sertoli cell differentiation in female urogenital ridges. We propose that the role of prostaglandin D<sub>2</sub> in male gonadal development is to induce pre-Sertoli cell differentiation in a paracrine manner.

Palmer and Burgoyne (Palmer and Burgoyne, 1991) examined the proportion of XX and XY cells in different cell types in male XX ↔ XY chimeras. Sertoli cells were the only cell type to show a strong bias towards being XY, suggesting that *Sry* acts in a cell-autonomous manner in the supporting cell lineage to induce Sertoli cell differentiation. However, a small number of XX Sertoli cells were also seen, showing that a paracrine factor that can induce Sertoli cell differentiation must exist in the developing testis. Prostaglandin D<sub>2</sub> is a good candidate to be that paracrine factor (Fig. 6A). Normal non-chimaeric XY male foetuses will of course contain no XX cells, but *Sry* may not be expressed effectively in all the cells of the supporting cell lineage. This is particularly so when 'weak' *Sry* alleles, which are expressed at a slightly later time or at a lower level than normal, are mismatched with an early acting programme for ovarian determination (Eicher and Washburn, 1986). In such mismatched XY embryos, some of the supporting cells differentiate into Sertoli cells, while others follow the female developmental pathway, causing the gonads to develop as ovotestes before birth. Most of these ovotestes will be resolved as either ovaries or testes after birth, depending on the relative amounts of each tissue. The presence of a masculinising paracrine factor in the developing testis could help to protect XY embryos from mismatch situations in a

genetically heterogeneous wild population (Fig. 6B). The relative importance of the prostaglandin D<sub>2</sub> paracrine signal for Sertoli cell differentiation for testis development may depend on how effectively *Sry* is expressed in the supporting cells (Fig. 6B,C).

### Germ cells can influence pre-Sertoli cell differentiation

Does the paracrine signal for Sertoli cell differentiation emanate from the Sertoli cells, the prospermatogonia, or both? Prostaglandin D synthase is expressed in Sertoli cells and germ cells, suggesting that both cell types could produce the paracrine signal. Genetic evidence that germ cells may help to masculinise the developing testis comes from the previously mentioned mismatch situations with 'weak' *Sry* alleles: homozygosity or heterozygosity for semidominant alleles of either *Sl* or *W* have been shown to greatly enhance feminisation of such mismatched mice (Cattanach et al., 1988; Burgoyne and Palmer, 1991; Nagamine and Carlisle, 1996). Similarly, *W* mutations can feminise *XXSxr* embryos, which also develop ovotestes before birth (Nagamine et al., 1998). The *W* and *Sl* loci encode a cell surface receptor and its ligand involved in the proliferation and survival of PGCs. *Sl*/+ and *W*/+ heterozygotes have small testes, with reduced numbers of germ cells, and homozygosity for *Sl* or *W* prevents almost all PGCs from reaching the genital ridge (Buehr et al., 1993b). If the prospermatogonia in the testicular region of a developing ovotestis produce prostaglandin D<sub>2</sub> as a masculinising paracrine signal, a reduction in their number might well tip the balance towards ovarian development.

*W/W* or *Sl/Sl* XY embryos with no mismatch develop normal testes, with Sertoli cells and testis cords, even in the absence of germ cells. This apparently normal development of germ cell-free testes has often been cited as evidence that male germ cells play little part in the differentiation of the testis (McLaren, 2000). If, however, the germ cells respond to the *Sry*-derived masculinising environment of the male genital ridge by producing a factor that will induce Sertoli cell differentiation, this will ensure that if the germ cells develop as male, the surrounding somatic cells will also develop as male. This type of feedback loop would also help to consolidate gonadal sex determination, particularly in situations where *Sry* is not expressed effectively in all the supporting cells.

### The role of prostaglandin D<sub>2</sub> in testis development

If prostaglandin D<sub>2</sub> acts as a paracrine signal for Sertoli cell differentiation, should testis development be disrupted in the absence of this signal? The genes encoding prostaglandin D synthase (*Ptgds*), one of the prostaglandin D<sub>2</sub> receptors (*Ptgdrr*), and two intracellular receptors for naturally occurring metabolites of prostaglandin D<sub>2</sub> (PPAR $\gamma$  and NF- $\kappa$ B) have been knocked-out in mice, with no reported effects on testis development (Sha et al., 1995; Barak et al., 1999; Eguchi et al., 1999; Matsuoka et al., 2000). However the presence of multiple prostaglandin D synthase enzymes, multiple receptors and possible redundancy with other prostaglandins makes it difficult to know if this putative paracrine signal for Sertoli cell differentiation has been completely removed. Indeed, other prostaglandin synthases also appear to exhibit sexually dimorphic expression in developing embryonic gonads (I. R. A. and A. M., unpublished). Mice carrying mutations in genes



required for prostaglandin biosynthesis (*Cox1<sup>-/-</sup> Cox2<sup>-/-</sup>*) have also been generated which would presumably lack all prostaglandins. These mice die perinatally from failure to close the ductus arteriosus, but there is no reported defect in testis development (Loftin et al., 2001). However, if Sertoli cell differentiation is induced primarily by cell autonomous expression of *Sry* in the supporting cells (Palmer and Burgoyne, 1991), paracrine induction of Sertoli cell differentiation would probably play only a minor role in testis development (Fig. 6C). Therefore removing this paracrine signal by genetic manipulation may have little phenotypic consequence to the developing gonad in a genetic background where *Sry* is expressed effectively in all the supporting cells.

Genetic analysis of a putative prostaglandin signal for Sertoli cell differentiation is further complicated by the possibility of redundancy with other paracrine factors for Sertoli cell differentiation. Fibroblast growth factor 9 (Fgf9), which appears to act downstream of *Sry* in testis development (Colvin et al., 2001), is a good candidate to act as such a factor. Fgf9 is a signalling molecule that can induce mesonephric cell migration when added exogenously to XX urogenital ridges in culture, and *Fgf9<sup>-/-</sup>* mice show XY sex reversal, but it is not yet clear if Fgf9 can induce Sertoli cell differentiation (Colvin et al., 2001). Another potential source of a paracrine signal for Sertoli cell differentiation are peritubular myoid cells. If, as discussed earlier, immigrant peritubular myoid cells in a genital ridge can induce *Sox9* expression and Sertoli cell differentiation in undifferentiated supporting cells, this potential feedback loop could also act redundantly with the germ cell-derived prostaglandin D<sub>2</sub> signal to help consolidate sex determination in mismatch situations.

Prostaglandins generally signal through binding to a seven-pass transmembrane receptor on the cell surface (reviewed by Narumiya et al., 1999). These receptors can activate heterotrimeric G proteins that can signal through a variety of secondary messengers. Different prostaglandins signalling through different heterotrimeric G proteins coupled to different receptors can often induce antagonistic effects on the secondary messengers. Two molecules are reported to act as cell surface receptors for prostaglandin D<sub>2</sub>, *Ptgdr* and *Crth2* (*Grp44* – Mouse Genome Informatics) and these are coupled to different G-proteins (Hirata et al., 1994; Hirai et al., 2001). Furthermore, naturally occurring metabolites of prostaglandin D<sub>2</sub> can enter cells and either signal through the nuclear receptor PPAR $\gamma$ , or influence the transcriptional activator NF- $\kappa$ B (Forman et al., 1995; Kliewer et al., 1995; Rossi et al., 2000; Straus et al., 2000). It is not clear at present how the supporting cells respond to the prostaglandin D<sub>2</sub> signal in order to become pre-Sertoli cells. In this respect, it may be mechanistically relevant that prostaglandin F<sub>2 $\alpha$</sub> , which acts antagonistically to prostaglandin D<sub>2</sub> during adipogenesis (Reginato et al., 1998), has been shown to increase expression of *Dax1* (*Nr0b1* – Mouse Genome Informatics), an anti-testis gene that is thought to repress Sertoli cell gene expression (Swain et al., 1998), in adult rat ovaries (Sandhoff and McLean, 1999). Perhaps prostaglandin D<sub>2</sub> in the developing testis analogously decreases expression of *Dax1* to help promote pre-Sertoli cell differentiation. Further investigation into the role of prostaglandins in gonad development may allow greater understanding of the mechanism of Sertoli cell differentiation, and provide further insight into how bipotential cell types in a

bipotential organ interact with each other to ensure a single developmental fate.

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