Prostaglandin D2 (PGD2) signalling and male germ cell: differentiation in the mouse embryonic testis
Safdar Ali Ujjan

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TITRE DE LA THESE
Prostaglandin D2 (PGD2) Signalling and Male Germ Cell Differentiation in the Mouse Embryonic Testis

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ABSTRACT

The sex determination and subsequent germ cell differentiation is a highly ordered process that starts at embryonic stage and completes at adult life. In the embryonic gonads Sry expression followed by Sox9 expression initiates testis development while in the absence of Sry expression, genes associated to female fate initiate ovary development. The germ cells that migrated towards newly formed gonads continue extensive proliferation until they commit to the male or female pathway. The fate decision of germ cells as male or female does not depend only on germ cell chromosomal sex but also on gonadal micro-environment. If germ cells are in female gonad, they have to stop proliferation, pass through mitotic arrest and enter into meiosis to then arrest into prophase I. While if germ cells are in male gonad, they have to stop proliferation and enter into mitotic arrest.

Here we show that during embryonic sex determination, Prostaglandin D2 (PGD2) produced by each of the two enzymes: L-Pgds and H-Pgds in somatic cells and germ cells of testis participates in male germ cell differentiation program. PGD2 signaling supports mitotic arrest by activating the expression and nuclear localization of cell cycle inhibitor P21cip1 and by repressing pluripotency markers and PGD2 has negative effects on Stra8 expression. In addition PGD2 supports activation of male specific gene Nanos2. Hence these data suggest that PGD2 signaling through DP2 receptor is required for proper male germ cell differentiation.
ACKNOWLEDGEMENT

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I am thankful to professor Philippe Berta, Dr. Eric Pailhoux, Dr. Marie-Christine Chaboissier and Dr. Corinne Cotinot for accepting to be the members of my thesis jury. I thank you all for checking and critical reading of my thesis manuscript.

I also give thanks to all people of IGH, including animal care facility for providing L/H-Pgds double knockout mice for experiments, informatics department and team of Montpellier Rio Image facility for your valuable and generous help.

I also want to say thanks to all my friends and colleagues who shared their precious moments and made my life colourful. I am thankful to you Stephanie Martinetti for your help.

Finally, huge thanks for my whole family, my dear mother your prayers always guard me, my dear wife you always dreamed with me for my PhD from a highly reputed research institute, which came true and my son I also have many thanks for you because you were only of two months when I came to start my PhD and left you alone with your mom.
I dedicate this work to MY FAMILY.........
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Abbreviations

Sry/SRY  Sex-determining region Y
SF-1    Steroidogenic factor 1
NR5A1   Nuclear Receptor Subfamily5 Group A Member1
WT1     Wilms' tumour 1
Sox9/SOX9  Sry box-containing gene 9
AMH     Anti-Mullerian hormone
MIH     Mullerian-inhibiting hormone
Mis     Mullerian inhibiting substance
MIF     Mullerian inhibiting factor
PGD2    Prostaglandin D2
Nr0b1   Nuclear receptor subfamily 0, group B, member 1
Dax1    Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
DMRT1   Doublesex and mab-3-related transcription factor 1
FGF9    Fibroblast growth factor 9
FOG2    'Friend of Gata1' type 2
FOXl2   Forkhead box L2
GATA4   GATA-binding protein 4
RSPO1   R-spondin homologue 1
WNT4    Wingless-related MMTV integration site 4
NHR1    Nuclear hormone receptor
Dhh     Desert Hedgehog
Gadd45G Growth arrest and DNA damage-inducible gamma gene
Six1    SIX homeobox 1
Jmjd1a  Jumonji domain-containing protein 1A
Lhx9    Lim homeobox protein 9
KDM3A   lysine (K)-specific demethylase 3A
DSD     Disorders of sex development
SOX     SRY-related HMG box
HMG     High mobility group
<table>
<thead>
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<th>Description</th>
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<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>caM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>dpc</td>
<td>Days post coitum</td>
</tr>
<tr>
<td>SP1</td>
<td>Specificity protein1</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6J</td>
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<tr>
<td>CD</td>
<td>Campomelic dysplasia</td>
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<tr>
<td>Ods</td>
<td>Odd sex</td>
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<tr>
<td>TESCO</td>
<td>Testis specific enhancer of Sox9 core element</td>
</tr>
<tr>
<td>TES</td>
<td>Testis specific enhancer of Sox9</td>
</tr>
<tr>
<td>L-Pgds</td>
<td>Lipocalin-type prostaglandin D synthase</td>
</tr>
<tr>
<td>H-Pgds</td>
<td>hematopoietic-type prostaglandin D synthase</td>
</tr>
<tr>
<td>CHIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<td>LH</td>
<td>Luteinizing hormone</td>
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<td>Patch1</td>
<td>Patched homolog 1</td>
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<td>INLS3</td>
<td>Insulin-like growth factor 3</td>
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<td>DDS</td>
<td>Denys-Drash syndrome</td>
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<td>KTS</td>
<td>K-lysine, T-tyrosine, S-serine</td>
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<td>FTZ-F1</td>
<td>Fushi-tarazu factor 1</td>
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<td>PMDS</td>
<td>Persistent Mullerian Duct Syndrome</td>
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<td>TSP1</td>
<td>Thrombospondin type1 protein</td>
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<td>CTNNB</td>
<td>β-catenin</td>
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<tr>
<td>BPES</td>
<td>Blefarophimosis/ptosis/epicanthus inversus syndrome</td>
</tr>
<tr>
<td>Fst</td>
<td>Follistatin</td>
</tr>
<tr>
<td>DSS</td>
<td>Dosage sensitive sex reversal</td>
</tr>
<tr>
<td>PGCs</td>
<td>Primordial germ cells</td>
</tr>
<tr>
<td>Blimp1</td>
<td>B-lymphocyte induced maturation protein1</td>
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<tr>
<td>TGF-B</td>
<td>Transforming growth factor-β</td>
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<tr>
<td>BMPs</td>
<td>Bone morphogenetic proteins</td>
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<tr>
<td>SMAD</td>
<td>Drosophila Mothers against decapentaplegic</td>
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KL  Kit ligand
SCF  Stem cell factor
C-KIT  Cytokine Kit ligand, Proto-Oncogene Protein (Receptor for SCF)
MGF  Mast cell growth factor
PECAM-1  Platelet endothelial cell adhesion molecule-1
Tnap  Tissue non-specific alkaline phosphate
SSEA1  Stage Specific Embryonic Antigen-1
Gcna1  Germ cell nuclear antigen 1
Dazl  Deleted in azoospermia like
Gcl  Germ cell-less
ERR-B  Estrogen related receptor-β
Mvh  Mouse vasa homolog
Nanos2/3  Nanos homolog 2/3
RA  Retinoic acid
Aldh  Aldehyde dehydrogenase (enzymes family)
Stra8  Stimulated by Retinoic Acid gene 8
VAD  Vitamin A deficient
Syxcp3  synaptonemal complex protein 3
Dmc1  DNA meiotic recombinase 1
Oct4  Octamer-binding transcription factor 4
Pou5f1  POU class 5 homeobox 1
CYP26B1  Cytochrome P450, family 26, subfamily B, polypeptide 1
TUNEL  Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling
EG  Embryonic germ cells
ESCs  Embryonic stem cells
Dnmt  DNA methyltransferase
CDK  Cyclin-dependent kinase
RB  Retinoblastoma
Cdkn1b  Cyclin dependent kinase inhibitor 1B
DND1  Dead end homolog1
ICM  Inner cell mass
<table>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Dppa2</td>
<td>Developmental pluripotency-associated gene2</td>
</tr>
<tr>
<td>TGCTs</td>
<td>Testicular germ cell tumors</td>
</tr>
<tr>
<td>Bax</td>
<td>BCL2-associated X protein</td>
</tr>
<tr>
<td>Bcl-x</td>
<td>Bcl2-like 1</td>
</tr>
<tr>
<td>CIS</td>
<td>Carcinoma-in-situ</td>
</tr>
<tr>
<td>YST</td>
<td>Yolk sac tumours</td>
</tr>
<tr>
<td>Pten</td>
<td>Phosphatase and tensin homolog</td>
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<td>GDNF</td>
<td>Glial cell derived neurotrophic factor</td>
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<tr>
<td>MMAC1</td>
<td>Muted in multiple advanced cancers</td>
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<td>Teratocarcinoma derived growth factor 1</td>
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<td>PLD</td>
<td>Phospholipase D</td>
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<td>Non-steroidal anti-inflammatory drugs</td>
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<td>G Protein-coupled receptors</td>
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<td>D Prostanoid receptor 1</td>
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<td>Imp-B</td>
<td>Importin-β</td>
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<td>Ddx4</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 4</td>
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<td>EdU</td>
<td>5-ethynyl-2’-deoxyuridine</td>
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<td>phospho-Histone H3</td>
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<td>Mybl1</td>
<td>Myeloblastosis viral oncogene homolog-like 1</td>
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<tr>
<td>Erbb4</td>
<td>Erythroblast leukemia viral oncogene homolog 4</td>
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</table>
Trim71  Tripartite motif containing 71
Cpeb4   Cytoplasmic polyadenylation element binding protein 4
Rbm38   RNA binding motif protein 38
Peg3    Paternally expressed 3
Nr2c2   Nuclear receptor subfamily 2, group C, member 2
Lhx1    LIM homeobox 1
Sall1   Spalt-like transcription factor 1
Mtf2    Metal response element binding transcription factor 2
L1td1   LINE-1 type transposase domain containing 1
Tet1    Tet methylcytosine dioxygenase 1
Gtf3c3  General transcription factor IIC, polypeptide 3
INTRODUCTION
Chapter 1.

1.1. Introduction

The life of multicellular organism starts from a diploid cell, the fertilized egg called zygote. It is formed by the fusion of two haploid cells, the oocyte and spermatozoa, also called gametes. Zygote passes through a series of changes to develop into an adult. In bi-sexual organisms each individual organism is either male or female and upon maturity will produce gametes, spermatozoa and oocytes respectively. In mammals every individual organism inherits an X or a Y sex chromosome from father and an X sex chromosome from mother. In this way an embryo that inherits XY sex chromosomes will develop as a male whereas an embryo with XX sex chromosomes will develop as a female. Male and female gametes carry genetic information that is expressed during the life of an organism and is transmitted from generation to generation.

In adult animals, the female gametes are produced in ovary through oogenesis while male gametes are produced by spermatogenesis that takes place in testis and both the gametogenesis as well as gonadal differentiation starts from embryonic life. Fate of the developing embryo as male or female is particularly original because both of the gonads either a testis or an ovary develop from a same precursor tissue, the genital ridge. The sex determination process that takes place between 10.5dpc and 12.5dpc in mice and between 6-8 weeks in human will thus start by the differentiation of the somatic cells, in male, the Sertoli cells. On the other hand the fate of germ cells, as male or female does not depend on its own chromosomal sex but depends mainly on the signals they receive from the somatic supporting cells. Any disturbance or imbalance in molecular or cellular networks that regulate differentiation of embryonic gonads can cause disorders of sex development (DSD). Therefore sex differentiation is highly specialized and tightly regulated among species.

Here we will discuss history and research work about the gene networks that are involved in formation of testis and germ cell differentiation.
1.2. Somatic Sex Determination

1.2.1.1. SRY

Sry (sex determining region on Y chromosome) is the first member of the SOX (SRY-related HMG box) family, a family of transcription factors found throughout the animal kingdom. SOX family is characterized by the presence of a DNA-binding high mobility group (HMG) domain. These SOX proteins play a central role in many processes including embryogenesis and development of organs (Bowles et al., 2000). The HMG domain is highly conserved among species and is the site of almost all missense mutations. This domain also provides nuclear import and DNA bending (Harley et al., 2003). The HMG domain mediates DNA-binding of SRY and SOX proteins to regulate target genes (Bergstrom et al., 2000). The HMG domain is crucial for the function of SRY and SOX9, as in human XY females' mutations are found essentially in this domain (Harley and Goodfellow, 1994). SRY is a nuclear protein and two nuclear localization signal (NLS) motifs are located on its HMG domain that can independently direct the import of proteins into nucleus. These NLS motifs lie at each end of HMG domain, (N and C termini) (Poulat et al., 1995; Sudbeck and Scherer, 1997). The N-terminal, a bipartite NLS interacts with calmodulin (caM)(Hanover et al., 2009) while C-terminal NLS interacts with importin-β (nuclear import factor) (Forwood et al., 2001) and support in localization of SRY protein in the nucleus of Pre-Sertoli cells (Hanley et al., 2000). At the C-terminal end, mouse SRY protein has a glutamine rich region extended half of the protein. This glutamine rich domain has important role in mouse male sex determination, as XX embryo transgenic of Sry construct that encode proteins lacking the glutamine rich region were unable to effect male sex determination (Bowles et al., 1999).
Figure 1. Overview of sex determination in mice.

Chronological flow of early mouse sex differentiation; the grey area indicates the period of sex determination. During mouse embryogenesis, bi-potential gonads (yellow) arise from the genital ridges by 10.5 dpc. In somatic cells of XY genital ridges, Sry expression (shown in dark blue beneath the schematic) starts at 10.5 dpc, reaches a peak at 11.5 dpc and then wanes by 12.5 dpc. A few hours later, Sox9 expression (shown in light blue beneath the schematic) is upregulated to induce differentiation of Sertoli cells. Sox9 expression peaks at 11.5-12.5 dpc, continues to be expressed postnatally and is supported by several positive-feedback loops (including FGF9 and prostaglandin D2), and SOX9 subsequently activates many male-specific genes, including Amh. At 12.5 dpc, testis cords have formed, and morphological differences between testis (blue) and ovary (pink) are evident. In the absence of SRY, genes that are already expressed in undifferentiated gonads of both sexes such as Wnt4 and Rspos (Chassot et al., 2012) and Foxl2 are expressed in a female-specific manner and induce ovarian development, as characterized by the expression of follistatin and many other ovary-specific genes. **Abbreviations:** Amh, anti-Müllerian hormone; dpc, days post coitum; FGF9, fibroblast growth factor 9; FOXL2, forkhead box L2; PGD2, prostaglandin D2; Rspo1, R-spondin 1; SOX9, SRY box containing gene 9; SRY, sex-determining region on the chromosome Y; WNT4, wingless-type MMTV integration site family, member 4 (Kashimada and Koopman, 2010).
1.2.1.2 Sry Expression

Expression of Sry either from Y chromosome or from ectopic transgene directs testis formation from rudimentary gonad. Sry is considered as a genetic switch to determine the fate of the bipotential gonad: the introduction of Sry in XX mouse embryo is sufficient to induce testis differentiation (Koopman et al., 1991). In mice Sry is expressed in somatic cells of XY genital ridges (pre-Sertoli and Sertoli cells) for a short time window first detectable at 10.5dpc (days post coitum), reaches to peak at 11.5dpc and declines up to 12.5dpc (Hacker et al., 1995; Koopman et al., 1990). The expression of Sry initiates in the centre of mouse genital ridge then extends to both poles over several hours (Bullejos and Koopman, 2001). Duration of Sry expression varies between species, as in goats and humans SRY gene expression continues during gestational period and is even observed after birth (Hanley et al., 2000; Pannetier et al., 2006). The expression of Sry leads to the up-regulation of Sox9, a transcription factor that promotes testis differentiation in XY bipotential gonads (Sekido et al., 2004). The overview of Sry expression and sex determination in mice is shown in Fig. 1 (Kashimada and Koopman, 2010).

1.2.1.3 Sry Activation and Regulation

In the undifferentiated gonad, Sry gene is activated by steroidogenic factor 1 (SF-1), a component of the nuclear receptors family that binds specifically to the NHR1 (nuclear hormone receptor) site (Sekido and Lovell-Badge, 2008). Human SRY promoter has two SP1 (specificity protein1-transcription factor) binding sites that regulate SRY expression (Desclozeaux et al., 1998). Human SRY promoter responds to cAMP dependant PKA phosphorylation through SF-1 and Sp1 sites. Mutation of NHR1 site reduces promoter activity by 70% however additional mutation of Sp1 sites reduces promoter activity by nearly 90%, indicating that proximal NHR1 and Sp1 sites are essential for transcriptional activity of human SRY gene (de Santa Barbara et al., 2001). Sry gene is cell autonomously regulated by Wt1 (+KTS) (Wt1, Wilms tumor 1
homolog) as in \( Wt1 (+KTS) \)-null mouse XY gonads SRY protein level significantly
decreased per cell as well as number of SRY expressing cells reduced due to
decrease in proliferation of coelomic epithelial cells by 11.5dpc. In addition
Sertoli cell differentiation was blocked in XY \( Wt1 (+KTS) \)-null mouse gonads due
to loss of \( Sox9 \) and \( Fgf9 \) expression. However in \textit{ex vivo} gonad cultures addition
of recombinant \( Fgf9 \) rescued the mutant phenotype by enhancing Sertoli cell
differentiation (Bradford et al., 2009).
The polycomp group protein CBX2 (chromobox homolog 2; also known M33) is
involved in the regulation of \( Sry \) gene expression and testis differentiation. In
\( cbx2^{-/-} \) KO gonads the expression of \( Sry, Sox9 \) and other male fate genes was
affected. When \( cbx2 \) KO mice were crossed with transgenic mice displaying
forced expression of \( Sry \) or \( Sox9 \), which rescued male to female sex reversal but
testis remained hypoplastic, indicating that the size and the sex of the gonad are
determined by different sets of CBX2 target genes (Katoh-Fukui et al., 2012).

GATA4 and its co-factor FOG2 are required for regulation of \( Sry \) and mouse
gonadal differentiation in both sexes. Homozygous mutations in \( Gata4 \) or \( Fog2 \)
cause gonadal abnormalities in mouse embryo of both sexes (Tevosian et al.,
2002). The transcript level of \( Sry \) significantly reduced in XY \( Fog2 ^{-/-} \) gonads at
the time (11.5dpc) when \( Sry \) expression level reaches its peak (Manuyllov et al.,
2007; Tevosian et al., 2002). The genes required for normal Sertoli cell functions
such as \( Sox9 \), Mullerian inhibiting substance (\( Mis \)), Desert Hedgehog (\( Dhh \)) and
Leydig cell biosynthetic enzymes were not expressed in XY \( Fog2 ^{-/-} \) gonads
whereas ovary development gene \( Wnt4 \) was expressed ectopically (Tevosian et
al., 2002).
The transcription factors \( Six1 \) and \( Six4 \) participate in male gonadal
differentiation by regulating \( Sry \) expression and by inducing gonadal precursor
cell formation. Loss of both \( Six1 \) and \( Six4 \) (not only one) resulted in a male to
female sex reversal with failure in \( Sry \) activation. Decrease in gonadal precursor
cell formation with reduced gonadal size was observed in mutant embryos of
both sexes and forced \( Sry \) transgene expression in XY \( Six1 \) and \( Six4 \) mutant
gonads rescued testicular development. \( Six1 \) and \( Six4 \) act through two
downstream targets \( Fog2 \) and \( Sf-1 \) in gonadal development (Fujimoto et al.,
2013).
Mitogen-activated protein kinase (MAPK) signaling is required for male sex determination in mouse, as homozygous mutation in \textit{Map3k4}^{-/-} caused XY gonadal sex reversal in C57BL/6J (B6) genetic background, due to failure in mesonephric cell migration and reduction in \textit{Sry} expression. Normally Map3k4 activates P38 in the coelomic region of the XY gonads at 11.5dpc to promote gonadal somatic cell proliferation and regulation of \textit{Sry} expression (Bogani et al., 2009). \textit{Gadd45G} (growth arrest and DNA damage) gene is expressed in somatic cells of developing gonads of both sexes at 10.5dpc but maintained in XY gonads from 10.5-12.5dpc (Warr et al., 2012). Gadd45G is essential for primary sex determination, male fertility and testis development (Johnen et al., 2013). Gadd45G positively regulates Map3k4 mediated P38 MAPK signaling in XY embryonic gonadal somatic cells as requirement for testis determination. Mice lacking \textit{Gadd45G} also exhibit XY gonadal sex reversal caused by disruption of \textit{Sry} expression as in \textit{Map3k4}^{-/-} gonads. Gadd45G and Map3k4 genetically interact during sex determination, and transgenic overexpression of \textit{Map3k4} rescues gonadal defects in \textit{Gadd45G}^{-/-} deficient embryos (Warr et al., 2012). Histone demethylation has important roles in mammalian sex determination and gene expression, as \textit{Jmjd1a/Kdm3a} gene directly and positively controls \textit{Sry} expression. In XY mice homozygous mutant of H3K9 demethylase Jmjd1a, male to female sex reversal is observed (Kuroki et al., 2013).

1.2.1.4 \textit{Sry} Function

The process of Sex determination in mammals starts during embryonic life. The choice of male or female sex determination fate depends upon the presence of the gene \textit{Sry}, located on the Y chromosome (Koopman et al., 1991). In mice \textit{Sry} gene is expressed at a stage of male gonadal development, which is deleted in XY female mice mutant for \textit{Tdy}, indicating its role in testis determination (Gubbay et al., 1990). The \textit{Y} specific new identified gene is conserved among a wide range of mammals including humans and encodes a protein with conserved DNA-binding motif present in the nuclear HMG1 and HMG2 (Sinclair et al., 1990). \textit{SRY}
identification as a gene responsible for initiating male sex determination was reported in patients where mutations in SRY resulted in 46 XY female sex reversals (Berta et al., 1990). The loss of function of SRY in humans is associated with sex reversal (Domenice et al., 2004). Sry knock out mice had female external and internal genitalia, female level of blood testosterone, showed estrous cycle and performed copulatory behavior as females but infertile or reduced fertility (Kato et al., 2013). The transgenic Sry expression in chromosomally XX female resulted in development of male mouse (Koopman et al., 1991). After expression of Sry, the earliest identified change observed in XY genital ridges, is the male specific proliferation of coelomic epithelial cells, which is not detected, in XX genital ridges (Schmahl et al., 2000). At the molecular level, the primary function of Sry is to initiate Sox9 gene expression to promote Sertoli cell fate. Any delayed or reduced Sry expression can cause XY sex reversal by failure to boost Sox9 expression (Albrecht and Eicher, 2001).

1.2.2. Sox9

SOX9 (Sry box containing gene 9) is a gene located on the long arm (q) of chromosome 17 (17q24.3) in humans that plays important roles in the normal skeletal development and male sex determination. SOX9 heterozygous mutations develop into skeletal malformation syndrome called campomelic dysplasia (CD)(Bi et al., 2001) and 75% XY patients exhibit male to female sex reversal in humans (Mansour et al., 1995; Wagner et al., 1994). The null mutation of mouse Sox9 causes male to female sex reversal (Barrionuevo et al., 2006; Chaboissier et al., 2004) however the mouse heterozygous Sox9 mutation causes skeletal abnormalities but do not develop into XY sex reversal. The skeletal abnormalities observed in Sox9+/− mice resemble to that of CD patients (Campomelic Dysplasia) and are caused by delayed or defective pre cartilaginous condensation. Furthermore premature mineralization observed in many bones is consequence of allele insufficiency of Sox9 expression (Bi et al., 2001). Sox9 gain of function experiments in mice, have reported sex reversal from female to male in XX gonads (Vidal et al., 2001). Not only the presence but also sufficient Sox9 expression is
required for normal development and male sex differentiation. XX embryos with Sox9 upstream insertion mutation (XX ods/+ ) develop as sterile XX males without presence of Sry. Sox9 expression was upregulated and maintained in these XX ods/+ sterile males (Bishop et al., 2000). Qin and Bishops showed that in Y chromosomal Sry mutated (XY^a, ~9kb Sry mutation on Y chromosome) embryos, when Sox9 was mutationally and heterozygously activated (Sox9 XY^a ods/+ ) (ods= odd sex), it produced fertile males but with testis smaller in size and irregular testis specific vascularization. These males were fertile in the beginning but fertility was lost up to six months, suggesting insufficient Sox9 expression. When Sox9 expression was increased with homozygous activation condition (XY^a ods/ods), spermatogonial defects were rescued with gain of fertility and normal testis vasculature (Qin and Bishop, 2005).

The mice conditional Sertoli mutant of Sox9 by 14.0dpc that have normal embryonic testis development and are initially fertile, display dysfunctional spermatogenesis and become sterile after 5 months. When this Sox9 conditional mutation was generated on Sox8^−/− mutant background, the differentiation of testis cords into seminiferous tubules ceased after P6 day (p= postnatal) while on Sox8^−/− mutant background the testis cord differentiation ceased after P0 and produced complete primary infertility, indicating that Sox8 compensates for the absence of Sox9 during embryonic testis differentiation. In addition Sox8, Sox9 double mutant testes show upregulation of early ovary-specific markers and downregulation of Sertoli intercellular junctions by 15.5dpc but still very low levels of Amh cause complete regression of the Mullerian duct with reduced penetrance. Therefore testis cord differentiation by 14.0dpc is independent of Sox9 and that Sox9 in concert with Sox8 is essential for the maintenance of testicular function (Barrionuevo et al., 2009).

Before the expression of Sry/SRY, Sox9/SOX9 is expressed at low level in both XX and XY genital ridges in mice and human embryos respectively (Morais da Silva et al., 1996a). Upon expression of Sry, Sox9 expression is increased in pre-Sertoli and Sertoli cells of XY gonads (Sekido et al., 2004). In mice, the ability of Sry to induce testis development is limited to approximately 11.0dpc to 11.25dpc, a time window of only 6 hours after the onset of Sry expression in normal XY gonads. Delayed Sry activation (after 11.3dpc) does not initiate Sox9 expression resulting
in ovarian development (Hiramatsu et al., 2009). In mice, Sry cooperate with steroidogenic factor 1 (SF-1) to initiate the expression of Sox9 through their binding to the gonad specific enhancer of the Sox9 gene (TESCO-testis-specific enhancer of Sox9 core element) (Sekido and Lovell-Badge, 2008). Chromatin immunoprecipitation (CHIP) assays showed that Sry and Sf-1 directly bind several sites within TES (testis specific enhancer of Sox9) and mutation in these sites decreased the enhancer activity suggesting that Sry and Sf-1 synergistically upregulate the enhancer of Sox9 (Sekido and Lovell-Badge, 2009). Sox9 expression after the downregulation of Sry suggests that Sox9 is able to sustain its own expression once initiated (Kashimada and Koopman, 2010). Indeed, Sox9 creates a feed forward pathway, interacting with Sf-1 and binding to TESCO in order to maintain its own expression (Sekido and Lovell-Badge, 2008).

Other factors are also suggested for Sox9 activation as Ectopic activation of Sox9 in XX Wt1: Sox9<sub>Tba</sub> transgenic mice induces female-to-male sex reversal in adult stage. Initially these transgenic XX Wt1: Sox9<sub>Tba</sub> mice display ovotestis differentiation with testicular central region and one or both ovarian poles, suggesting that Wt1: Sox9 is not as efficient as Sry to induce male development. The cells of ovarian region of ovotestis undergo apoptosis during late embryogenesis leading to complete female-to-male sex reversal of the transgenic mice at birth (Gregoire et al., 2011).

Sox9 also makes positive feedback loop to Fgf9 (fibroblast growth factor 9) to maintain its expression (Kim et al., 2006b). The assessment of Fgf9<sup>−/−</sup> mutant gonads indicates that Sox9 initial expression and upregulation was normal between 11.5dpc-12.0dpc but by 12.5dpc Sertoli precursor cells did not organize into testis cord structures, indicating that Fgf9 is required for maintenance of Sox9 expression in Sertoli precursor cells (Kim et al., 2006b). Fgf9 expression like Sry and Sox9, initiates in a wave pattern in center zone of gonad and expands to poles of gonad. If this center zone of XY gonad is removed before expansion of Fgf9 signals, the pole regions of gonads will be unable to form testis cords (Hiramatsu et al., 2010).

SOX9 protein is a member of the SOX family of transcription factors. SOX9 protein is involved in the activation of several direct target genes that are known to participate in testicular development such as Amh (De Santa Barbara et al., 1998),
Dhh (Clark et al., 2000) and L-Pgds (Wilhelm et al., 2007). SOX9 harbour a sex-specific subcellular translocation from the cytoplasm to the nucleus in male genital ridge cells at the time of testis differentiation to induce AMH expression (De Santa Barbara et al., 1998; de Santa Barbara et al., 2000; Gasca et al., 2002). The importance of SOX9 shuttling for its function can be seen from the experiment, when SOX9 nuclear export was inhibited by leptomycin B (nuclear export inhibitor) in the mouse cultured XX gonads, it induced a sex reversal phenotype characterized by nuclear SOX9 and anti-Mullerian hormone expression (Gasca et al., 2002).

1.2.3.1 Bipotential primordial gonad and Testis Formation

The gonad formation depends on finely controlled interactions between the various types of somatic cells and the germ cells. Gonadal primordia, called the genital ridges, arise at 10.0dpc. At this time, genital ridges of male and female embryos have no structural or functional difference and the somatic precursor cells can differentiate to either Sertoli cells (testis formation) or Granulosa cells (ovary formation).

Insulin receptor (INSR) and the IGF type I receptor (IGF1R) signaling is essential for adrenogenital development and primary sex determination of both gonads. Constitutive ablation of insr; igf1r signaling resulted in reduced proliferation rate of somatic progenitor cells in gonads of both sexes prior to sex determination. Consequently embryos lacking functional insulin/IGF signaling exhibit complete agenesis of the adrenal cortex, embryonic XY gonadal sex reversal due to delayed Sry upregulation and delay in ovarian differentiation. The insr; igf1r mutant gonads irrespective of genetic sex remained in an extended undifferentiated state finally ovarian differentiation program initiated at around 16.5dpc (Pitetti et al., 2013).

In bipotential gonadal primordia, lineage specific genes are sex specifically depleted and enriched. Sexually undifferent cell and supporting cell progenitors show lineage priming, where germ cell progenitors are primed with a bias towards the male fate, in contrast supporting cells are primed with a female bias, indicating that robust repression program is involved in the
commitment to XY supporting cell fate (Jameson et al., 2012b). So the decision to adopt the male fate does not depend only on expression of male fate genes such as Sox9 but also on active repression of female fate genes such as Wnt4 (Jameson et al., 2012a). For male fate development program Sry expression in the somatic cells of XY genital ridges is required for the testis formation.

1.2.3.2 Sertoli cells and Testis cords Compartments

Sertoli cells are called supporting cells because they nourish and support the developing germ cells and all other testicular cell types differentiate as a result of signaling from Sertoli cells. These cells were discovered by eponym Enrico Sertoli an Italian physiologist while studying medicine in 1865. Sertoli cells arise from coelomic epithelial cells. The earliest Sry product induced male specific cell proliferation that proceeds in two steps. First, before 11.5dpc, proliferation takes place in SF-1 positive cells that will give raise to Sertoli cells whereas the second wave of proliferation takes place in SF-1 negative cell population that will give raise to interstitial cells (Schmahl et al., 2000). Both Wnt4 and Rspo1, which control WNT/CTNNB1 signaling for female fate of embryo, are also important for earlier coelomic cell proliferation required for male gonadal development. As ablation of Wnt4 and Rspo1 in XY embryos reduced number of Sertoli cells and the formation of a hypoplastic testis with few seminiferous tubules (Chassot et al., 2012).

This proliferation of coelomic epithelial cells stops at 12.5dpc (Karl and Capel, 1998). The second Sry associated earliest event observed in gonadogenesis, is the migration of cells from mesonephros to form testis cords (Capel et al., 1999). Endothelial cell migration is required for testis cord formation because inhibition of endothelial cell migration prevents this process (Combes et al., 2009). In male bipotential gonads, supporting cells differentiate into Sertoli cells that will organize the embryonic testis into two compartments, the first compartment consists of germ cells surrounded by Sertoli cells. Peritubular myoid cells enclose and guard both cell types and together with Sertoli cells secrete components of the basal membrane to form testis cords. The second compartment is testis interstitium, which has steroidogenic Leydig cells and
testis vasculature (McClelland et al., 2012). Live imaging showed that XY gonads
developed dynamically in a 24h time-lapse system and all testicular
morphological structures were clearly identifiable, including testis cords and the
coelomic vessel from 11.5 to 12.5dpc (Coveney et al., 2008).
In addition to support male germ cell differentiation, Sertoli cells also stimulate
formation of androgen producing Leydig cells, testis vasculature and other
interstitial cell types (Kashimada and Koopman, 2010).

1.2.3.3 Testis Blood Vasculature
Another important change observed in XY gonads at 12.5dpc, is the presence of a
male specific blood vasculature under the coelomic epithelium. This vessel is
identical in both sexes until 11.5dpc but soon after Sry expression, a large
number of endothelial cells are recruited from the mesonephros to build male
specific vasculature, which is totally absent in XX gonads. The specification of
this blood vessel in XY gonads is required for developmental patterns and testis
cord formation (Brennan et al., 2002). Platelet derived growth factors (PDGFs)
and their receptors (Pdgfrs) play important roles in testis organogenesis. Pdgfra
knockout mouse embryo displayed disruption in the organization of the
vasculature and in the partitioning of interstitial and testis cord compartments
(Brennan et al., 2003).

1.2.3.4 Leydig Cells
The specification of testicular somatic cells, such as Leydig cells is crucial for the
production of androgen hormones. Leydig cells are also called interstitial Leydig
cells because they are present in interstitial spaces of testis, between testis
cords. They are named after German zoologist and anatomist Franz Leydig who
first identified them in 1850. The fetal Leydig cell population arises, around
12.5dpc in mice and around 6 weeks of gestation in humans (O’Shaughnessy et
al., 2006). The primary function of these cells is to produce androgens that
induce male sexual differentiation in the embryo, development of secondary sex
characters at puberty and maintenance of spermatogenesis in the adult (Wu et al., 2007). Unlike the adult population of Leydig cells, the fetal Leydig cells in the mouse do not require luteinizing hormone (LH) to stimulate androgen production. Desert hedgehog (Dhh) gene expression initiates in pre-Sertoli cells shortly after activation of Sry and persists in adult testis. The receptor of Dhh, Patched homolog 1 (Patch1) is expressed on the Leydig cells and peritubular cells (Bitgood et al., 1996; Clark et al., 2000). The DHH/PATCH1 signaling positively regulates the differentiation of Leydig cells in fetal testis (Bitgood et al., 1996). The DHH/PATCH1 signaling triggers Leydig cell differentiation by upregulating Steroidogenic Factor 1 and P450 side chain cleavage enzyme expression in Patch1-expressing precursor cells (Yao et al., 2002). In Dhh-/ XY mice, abnormal peritubular tissues and severely impaired spermatogenesis are observed. The ectopic activation of the Hedgehog pathway in the Steroidogenic factor 1 (SF-1) positive somatic ovarian cells, transformed these ovarian cells into functional fetal Leydig cells. These ectopic fetal Leydig cells produced androgens and insulin-like growth factor 3 (INLS3) that caused pseudo-hermaphroditic condition (Barsoum et al., 2009). The Notch signaling pathway has also an importance in the regulation of fetal Leydig cells during testis development. The blockage of the Notch signaling resulted in the increase of the number of Leydig cells in the testis. On the other hand, active Notch signaling in gonadal somatic progenitor cells causes loss of Leydig cells with an increase in undifferentiated mesenchymal cells (Tang et al., 2008). Hence Leydig cell differentiation and proper functioning is essential for male sex differentiation program.
1.3. Genes Involved In Male Somatic cell Fate Program

1.3.1 Wilms tumor suppressor gene 1 (Wt1)

In human, \textit{WT1} gene is present on chromosome 11 short arm (p) and encodes a zinc finger containing DNA-binding protein. Similarly in mice \textit{Wt1} (Wilms tumor 1 homolog) gene encodes a zinc finger transcriptional activator (Shimamura et al., 1997). WT1 has essential roles in development of urogenital system and specific mutation in this gene causes two urogenital abnormalities called Denys-Drash syndrome (DDS) and Frasier syndrome (Barbaux et al., 1997a; Pelletier et al., 1991). Symptoms of these syndromes are male to female sex reversal, male pseudo-hermaphroditism and cryptorchidism. WT1 regulates \textit{SRY} expression in the initial sex determination in human gonads and activates a cascade of genes that lead to the complete organogenesis of the testis (Hossain and Saunders, 2001). Wilms tumor 1 (WT1) and NR5A1 (commonly known steroidogenic factor1, SF-1) are known to regulate \textit{SRY} expression through SP1 promoter binding sites. Transfected cell studies show that upon de-phosphorylation WT1 and SF-1 can bind and activate human \textit{SRY} promoter (de Santa Barbara et al., 2001; Hossain and Saunders, 2001; Shimamura et al., 1997). In the gonad, alternative splicing of exon 9 of Wt1 results in the formation of two isoforms with insertion or deletion of three amino acids lysine (K), tyrosine (T) and serine (S), KTS between two zinc finger motifs. These Wt1 variants are important regulators for Sry in the sex determination pathway (Hammes et al., 2001). \textit{In vitro} studies show that WT1 isoforms are able to transactivate human \textit{SRY} promoter (Hossain and Saunders, 2001). WT1 haploinsufficiency results in XY sex reversal in human patients due to reduced levels of WT1+KTS (Barbaux et al., 1997b). Similarly in mice, Wt1-KTS isoform shows a complete sex reversal (Hammes et al., 2001). During testis development, Wt1 inhibits B-catenin signaling in Sertoli cells that promotes ovary development and Wt1 deletion results in up-regulation of B-catenin in Sertoli cells (Chang et al., 2008).
1.3.2. Steroidogenic Factor 1 (SF-1)/Nuclear Receptor Subfamily 5 Group A Member 1 (NR5A1)

The mouse *Sf-1* gene, the homolog of the Drosophila Fushi-tarazu factor 1 (FTZ-F1) is located to the proximal quarter of chromosome 2 and in human its homolog *SF-1* gene is located to chromosome 9q33 with seven exons (Taketo et al., 1995). Human SF-1 protein contains at least two *in vitro* cAMP-dependent protein kinase A (PKA) phosphorylation sites; phosphorylation modified its DNA-binding activity and interaction with general transcriptional factors (de Santa Barbara et al., 2001). SF-1 protein is expressed in male gonadal somatic cells before Sry expression and sex cord formation (de Santa Barbara et al., 2000). SF-1 is a component of the sex determination cascade that binds specifically to the Nuclear Hormone Receptor 1 (NHR1) site on the SRY promoter to activate SRY expression (de Santa Barbara et al., 2001). The expression of *Sf-1* in the embryonic gonads is sexually dimorphic, initially expressed in sexually indifferent genital ridges but later on *Sf-1* transcripts disappear from the ovary with reappearance at late gestation (Ikeda et al., 1994). Numerous SF-1 stained cells are observed in the coelomic epithelium and in the somatic component of the gonadal primordia (de Santa Barbara et al., 2000). SF-1 regulates the transcription of many genes by binding to specific response elements on their promoters (Achermann et al., 2001). In embryonic Sertoli cells, SF-1 regulates the expression of Mullerian inhibiting substance (MIS) that regresses Mullerian ducts in male embryo (Shen et al., 1994). In addition Steroidogenic factor SF-1 regulates transcription of genes involved in reproduction, steriodogenesis (such as biosynthesis of steroid hormones) and male sex differentiation. SF-1 is essential for survival of primary steroidogenic organs and in male development; SF-1 interacts with SRY to initiate the onset of the Sox9 gene expression in the embryonic testis (Sekido and Lovell-Badge, 2008). Male to female sex reversal is observed in human with a mutation in the *SF-1* gene. In phenotypically female patients (XY karyotype) adrenal glands failed in the first two weeks of life. Streak like gonads contained poorly differentiated tubules with removal of connective tissues. This mutation is in a 2 base pair (GGC→GAA) of exon3 that encodes part of DNA binding domain of SF-1 protein (Achermann et al., 1999). The
heterozygous point mutations in human SF-1 gene are implicated with reproductive disorders also known as disorders of sex development (Lin et al., 2007). These missense mutations cause partial loss of function in SF-1 and are associated with bilateral anorchia (testicular regression syndrome) and micropenis in humans (Philibert et al., 2007). SF-1 knockout mice also show improper development of adrenal glands and gonads, with male-to-female sex reversal of internal and external genitalia (Parker and Schimmer, 1997).

1.3.3 Anti-Müllerian Hormone (AMH)

Anti-Müllerian hormone (AMH) also known as Mullerian inhibiting factor (MIF), Mullerian-inhibiting hormone (MIH) and Mullerian-inhibiting substance (MIS), is a protein that is encoded by the AMH gene in humans (Cate et al., 1986). AMH gene is located on the tip of the short arm of chromosome 19 (Cohen-Haguenauer et al., 1987). AMH gene is the first Sox9 target gene, as within the human AMH proximal promoter the canonical SOX-binding site allows the binding of SOX9 to activate AMH expression, where as SOX9 can cooperate with SF-1 in this activation process (De Santa Barbara et al., 1998). The cytosolic expression of AMH protein is only observed in the Sertoli cells in human embryo after the expression of SOX9 protein in the nucleus (de Santa Barbara et al., 2000). AMH expression is observed in embryonic Sertoli cells at around 6.5 weeks in humans (de Santa Barbara et al., 2000) and at 12.5 dpc in the mouse and plays an important role in male sex differentiation by inhibiting the development of the Mullerian ducts (paramesonephric ducts) (Behringer, 1994). Mullerian ducts in females differentiate into fallopian tubes, the uterus and the upper part of vagina. Mice homozygous mutant of the SOX-binding site on Amh promoter, where Mis transcription did not initiate, developed into pseudohermaphrodites with the presence of Mullerian ducts derived organs (Arango et al., 1999). In case of mutation in AMH or AMH receptor 2 (AMHR2) gene, the failure of Mullerian duct regression results in persistence of the uterus and Fallopian tubes in males, resulting in the Persistent Mullerian Duct Syndrome (PMDS). Patients with PMDS are associated with unilateral
Fig. 2 Human R-Spondin proteins

Coding exon organization and domain structure of human R-spondins. The four human R-Spondin protein genes share a common organization, each consisting of five coding exons corresponding to predicted structural domains. Predicted domains include a leading signal peptide, furin-type type Cys-rich domains, a thrombospondin-type domain and a C-terminal basic region that scores as a nuclear localization signal. Black and blue vertical lines indicate conserved cysteine residues and exon boundaries respectively (Kim et al., 2006a).
cryptorchidism (failure of descend of one testis in scrotum) (Josso et al., 2005). The AMH precursor protein, a homodimer linked by disulphide bonds, is proteolytically cleaved to yield a 110kDa inactive N-terminal fragment and a biologically active 25kDa C-terminal fragment (Josso et al., 2005).

1.4 Genes Involved In Female Somatic Cell Fate Program

1.4.1 RSP01

*R-spondin 1 (Rspo1)* gene is expressed in the undifferentiated gonads of both sexes, suggesting a role in the early gonadal development. However, by 12.5dpc this expression increased in XX gonads and by 14.5dpc, its expression was five fold higher in XX gonads in comparison to XY gonads. The sex specific expression in 12.5dpc gonads was only observed in somatic cells and no staining was found in mesonephros or in Mullerian ducts (Parma et al., 2006).

RSP01, a secreted protein is the member of mammalian RSPO protein family that includes four independent gene products (Fig. 2) (Kim et al., 2006a). Each RSPO protein contains a N-terminal signal peptide, two adjacent cysteine rich furin-like domains, one thrombospondin type1 protein (TSP1) domain and C-terminal with nuclear localization signal domain (Kazanskaya et al., 2004).

In mice, Rspo1 expression induces activation of B-catenin in XX gonads (Chassot et al., 2008) and subsequent transcriptional activation of target genes. All four human Rsps family members are also capable of inducing activation of B-catenin and proliferative effects on gastrointestinal tract of mouse (Kim et al., 2006a) and play important roles in embryogenesis. Mice lacking R-spondin1 or Wnt4 display XX sex reversed phenotype. R-spondin 2 is exclusively expressed in the oocytes of ovarian follicles and directs primary follicle development to the second stage in a paracrine pattern. R-spondin 3 plays a dominant role during development of the placenta. In R-spondin 3 knockout animals embryos die at around 10.0dpc because of insufficient penetration of fetal blood vessels and improper alignment with maternal blood sinuses. R-spondin 4-gene mutation in

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human results in congenital anonychia, the lack of nails on fingers and toes (details review in (de Lau et al., 2014)).

Human *R-spondin 1 (RSP01)* is the gene disrupted in a recessive syndrome characterized by XX sex reversal, palmoplantar hyperkeratosis and predisposition to squamous cell carcinoma of the skin (Parma et al., 2006).

*Rspo1*−/− mutant mice show masculinized gonads with male like vascularization and steroidogenesis. Germ cells in *Rspo1*−/− knockout embryos display changes in cellular adhesion and fail to enter female specific meiosis (Chassot et al., 2008). *Rspo1* is required not only for ovarian differentiation but also for the testis differentiation. The *XY Wnt4*−/− *Rspo1*−/− double mutant embryos develop hypoplastic testis due to decreased number of seminiferous tubules. On the other hand, the coelomic epithelial cell proliferation decreased severely in early *Rspo1*−/− gonads while germ cells also have proliferation defects, indicating that *Rspo1* and *Wnt4* are two regulators of cell proliferation in the early gonad regardless of its sex (Chassot et al., 2012; Chassot et al., 2011).

### 1.4.2 Wnt4

*Wnt4* (*wingless-type MMTV integration site family, member4*) gene is expressed in mesenchyme along the mesonephros and coelomic epithelium but not in mesonephric tubules. *Wnt4* expression is visible by 11.0dpc in indifferent gonads of both sexes and mesonephros but absent in mesonephric tubules and Wolffian duct. Initial *Wnt4* expression is observed in both sexes but later on *Wnt4* expression becomes sex specific in gonads: it is maintained in the female gonads but downregulated in the male gonads (Vainio et al., 1999). *Wnt4* represses the testis formation and acts by antagonism with *Fgf9* (Kim et al., 2006b). *Wnt4* signaling is crucial for female sex development, XX embryos heterozygous mutant of *Wnt4*+/− are similar to their wild type siblings but homozygous mutant of *Wnt4*−/− are masculinized with the absence of Mullerian ducts and development of Wolffian ducts. *Wnt4* in the developing ovary appears to suppress the development of Leydig cells as *Wnt4* mutant females ectopically activated testosterone biosynthesis. *Wnt4* may also be required for maintenance of the
female germ line (Vainio et al., 1999). Wnt4 represses mesonephric endothelial and steroidogenic cells migration in the XX gonads to prevent the male-specific coelomic blood vessel formation and the production of steroids (Jeays-Ward et al., 2003). In XX gonads, the expression of Wnt4 and Foxl2 (forkhead box L2) at 11.5-12.5dpc up-regulates expression of female program specific genes (such as follistatin) which lead to differentiation of Granulosa and theca cells and formation of ovarian follicles (Kashimada and Koopman, 2010).

1.4.3 Wnt4, Rspos1 and Wnt/β-Catenin Signaling

Rspo1 and Wnt4 promote ovarian differentiation by activating WNT/CTNNB1 signaling (Chassot et al., 2012). For detailed role of R-spondin 1, Wnt4 and the β-catenin signaling on ovarian differeniation review in (Chassot et al., 2014). β-catenin signaling is required for female sex differentiation and antagonizes the establishment of the testis pathway. The ectopic stabilization of β-catenin in XY gonads is sufficient to disrupt the male pathway and promote ovarian development resulting in a male to female sex reversal (Maatouk et al., 2008). In culture experiments, the activation of the Wnt/β-catenin pathway inhibits the expression of Sox9 and Amh without altering mRNA and protein levels of SRY and steroidogenic factor (SF-1). However ectopic activation of Wnt/β-catenin signaling in male gonads led to a loss of SF-1 binding to the Tesco enhancer resulting in no expression of Sox9 (Bernard et al., 2012). Moreover ectopic Wnt/β-catenin signaling induced the expression of the female somatic cells markers, Bmp2 and Rspo1 as a consequence of the loss of Sox9 (Bernard et al., 2012) and male to female sex reversal (Maatouk et al., 2008). Formation of testis specific coelomic vessel, appearance of androgen-producing adrenal like cells and loss of female germ cells are observed in ovaries that lack β-catenin signaling (Liu et al., 2009).
1.4.4 Foxl2

Foxl2 (forkhead box l2) is a gene that is essential for granulosa cell differentiation and ovary maintenance (Schmidt et al., 2004). Sequence comparison of Foxl2 has shown that this gene is highly conserved among several vertebrate species (Uhlenhaut and Treier, 2006). The distinction between Foxl2 and other early ovary genes like Wnt4 and Rspo1 is that the other ovary genes are mainly expressed in the somatic cells of the bipotential gonads of both sexes; but Foxl2 is only expressed in differentiated female somatic cells (Garcia-Ortiz et al., 2009). Foxl2 is required for proper reproductive functions in females and represses the male pathway while its lack activates the genetic program for somatic testis differentiation (Ottolenghi et al., 2005). The two genes Wnt4 and Foxl2 are required to initiate or maintain all major aspects of female sex determination in mammals. When two genes were inactivated, produced testis differentiation in XX mice, resulting in the formation of testis tubules and spermatogonia. However forced expression of Foxl2 impairs testis tubules differentiation in XY transgenic mice (Ottolenghi et al., 2007).

In goat Foxl2 views to be an anti-testis gene rather than as a female promoting gene because when Foxl2 was absent in XX goat gonads 163 genes were deregulated, among them two-third were corresponding to testicular genes that were upregulated and only 19 female associated genes were downregulated. In addition Dmrt1 was upregulated ahead of Sox9 suggesting that Sox9 primary upregulation may require Dmrt1 in goat (Elzaiat et al., 2014).

In humans, the mutation in FOXL2-/- causes an autosomal dominant disorder, known as Blepharophimosis-ptosis/epicanthus inversus syndrome (BPES), associated with premature ovarian failure (De Baere et al., 2001). In mice, Foxl2-/- mutation displays craniofacial malformation and female infertility showing the involvement of Foxl2 gene in ovary development especially in granulosa cell function. The granulosa cells are ovarian somatic cells that surround and nourish the oocyte and are essential in follicle formation and activation (Uhlenhaut and Treier, 2006). In Foxl2-/- homozygous mutant mouse ovaries, granulosa cells do not complete the transition from squamous to cuboidal secondary follicles causing oocyte atresia. These results indicate that granulosa cell function is crucial for oocyte growth (Schmidt et al., 2004).
The combined mutation of both genes Foxl2\(^{-/-}\) and Rspo1\(^{-/-}\) promotes sex reversal, detectable at earlier stages than in XX Rspo1\(^{-/-}\) embryos. Moreover ectopic development of the steroidogenic lineage is more noticeable in XX Foxl2\(^{-/-}\) Rspo1\(^{-/-}\) double mutant embryos than in XX Rspo1\(^{-/-}\) embryos, indicating that Foxl2 is involved in preventing ectopic steroidogenesis in fetal ovaries (Auguste et al., 2011).

In goat Foxl2 loss of function (Foxl2\(^{-/-}\) mutation, also known as polled intersex syndrome mutation) is sufficient to cause an XX female-to-male sex reversal in goat (Boulanger et al., 2014). This gonadal sex reversal was initially detectable between 36-40dpc and mainly characterized by the reduction of the ovarian cortex and organization of seminiferous cord due to upregulation of Sox9 and Amh. Additionally 50-75% germ cells disappeared from testicular cords (PIS\(^{-/-}\) gonad) around 70dpc (Pailhoux et al., 2002).

Foxl2 is also important for adult ovary maintenance, as ovarian phenotype is an active process throughout life. Foxl2 prevents trans-differentiation of an adult ovary to a testis. Inducible deletion of Foxl2 in adult ovarian follicles results in immediate upregulation of testis-specific genes including Sox9 and reprogramming of granulosa and theca cell lineages into Sertoli like and Leydig like cell lineages with testosterone levels comparable to those of normal XY male littermates (Uhlenhaut et al., 2009).
**Fig. 03. Follicular development in mammalian ovary**

Primordial follicle having oocyte is covered by one or two layers of granulosa cells arrange into primary follicle. The secondary follicle has multiple layers of granulosa cells and theca cells around the basement membrane of follicle. In the third stage follicles develop a fluid filled antrum, now follicle is also called Graafian follicle and can ovulate the oocyte (Hirshfield, 1991).
1.4.5 Development of Mammalian Ovary and Source of Granulosa Cells

Genital ridges are the gonadal primordia that arise at 10.0dpc in the mouse embryo of both sexes. Initially genital ridges of both sexes are bipotential and their somatic precursor cells can differentiate into Sertoli cells or granulosa cells. In absence of Sry female fate genes drive the fate of bipotential genital ridge towards the ovary formation. In the mouse embryo ovarian differentiation appears at 13.5dpc when oogonia enter into meiosis and arrest into prophase I that continues until 17.0dpc. The meiosis process completes on maturation of oocyte that takes place at the time of fertilization (McLaren and Southey, 1997; Speed, 1982). The oocytes that are arrested in diplotene of prophase I are surrounded by pregranulosa cells to form primordial follicles (Merchant-Larios and Chimal-Monroy, 1989). Germ cells are not required for Sertoli cell differentiation however their presence is necessary for follicle formation as absence of germ cells in XX gonads results in streak like gonads (McLaren, 1991). The supporting cells of ovary are called Granulosa cells, which play important role in the growth and development of oocytes in fetal and adult ovarian follicles. The follicle is the basic unit of ovary, which consists of germ cells surrounded by granulosa and theca cells. Follicle passes through three maturation phases before oocyte can be able to fertilize. In the initial primordial follicle, granulosa cells arrange into one or two layers to become primary follicle. The primary follicle turns into secondary stage, characterized by multiple layers of granulosa cells, having theca cells around the basement membrane of follicle. In the third stage follicles develop a fluid filled antrum and can ovulate the oocyte, where as oocytes are mature, can be fertilized and resume meiosis (Hirshfield, 1991). Follicle development in mammalian ovary is shown in Fig. 03.

Ultrastructural studies of diverse mammalian ovary models suggest the origin of granulosa cells from three sources: already present supporting cell precursor of bipotential gonad, ovarian surface epithelial cells and the rete ovarii (review in (Auersperg et al., 2001; Liu et al., 2010)). The granulosa cells that arise from bipotential supporting precursor cells do not develop into adult granulosa cells but instead contribute to the subset of follicles in the medullary region of the
ovary, activated immediately after birth and reach to antral stage before puberty hence majority of these follicles are promoted to be lost by atresia (degeneration) or converted into the interstitial tissues. The granulosa cells that populate the primordial follicles of cortical region of ovary arise from surface epithelium at around birth and enter into mitotic arrest at that stage and are activated in adult life. By postnatal day 7 (P7), when granulosa cells individually encapsulated most of surviving oocytes, the surface epithelial cells dropped to undetectable level (Mork et al., 2012).
Fig. 4. **The mouse testis- and ovary-determining pathways and their mutual antagonism.**

Genes required for normal *Sry* expression are shown in the top left, including a GADD45γ/MAP3K4/ p38/GATA4 pathway (grey arrow). Within the large blue arrow are shown the core testis-determining genes *Sry, Sox9, Fgf9* and *Fgfr2* and their positive (arrow) or negative (hammered line) interactions. These negative (inhibitory) interactions are almost exclusively with elements of the ovary-determining pathway of *Rspo1, Wnt4* /β-catenin and *Foxl2* (large pink arrow), which themselves inhibit the testis-determining genes, primarily *Sox9*. The existence of possible additional targets, or uncertainty concerning an interaction, is shown by a question mark next to an arrow or hammered line. Arrows and hammered lines do not necessarily indicate a direct interaction between the named genes/proteins (Carre and Greenfield, 2014).


1.5 Gene antagonism or Mis-Expression and Sex Reversal

Several cascades of genes that are regulated in an antagonistic fashion during sex determination control the fate of embryo between male and female. The genetic antagonism between male and female pathways is illustrated in Fig. 4 (Carre and Greenfield, 2014).

In the mid of 20th century, ovarian development was considered as a default pathway in mammalian sex development but now it is reconciled with occurrence of complete or partial XX male sex reversal due to loss of function mutations in genes such as Wnt4, Rspo1 and Foxl2 (Sekido and Lovell-Badge, 2009). In the mouse gonads of both sexes, Fgf9 is expressed prior to sex determination (Kim et al., 2006b) and loss of Fgf9 leads to XY sex reversal (Colvin et al., 2001). The loss and gain-of-function experiments show that Fgf9 and Wnt4 act as opposing signals to regulate sex determination. Loss of Fgf9 leads to XY sex reversal, whereas loss of Wnt4 results in partial testis development in XX gonads. In the XY mouse gonads, Sry induces Sox9 and Fgf9 feed forward loop up regulating the expression of Sox9 and suppressing Wnt4. Surprisingly, loss of Wnt4 in XX gonads is sufficient to upregulate Sox9 and Fgf9 without expression of Sry (Jameson et al., 2012a; Kim et al., 2006b; Vainio et al., 1999).

Dmrt (double sex and mab3 related transcription factor) a family of genes that are highly expressed in vertebrates, are involved in mammalian sexual development and function in both testis and ovaries. In addition to Dmrt1, other members of DM domain genes are expressed in embryonic gonads, as Dmrt4 is expressed at similar levels in gonads of both sexes, Dmrt3 is highly expressed in males and Dmrt7 is more highly expressed in females (Kim et al., 2003). DMRT1 is specifically expressed in genital ridges of human males during embryonic sex determination (Moniot et al., 2000). Dose-sensitive Dmrt1 expression has also been shown to be required for mouse fetal male germ cell differentiation and mitotic arrest (Krentz et al., 2009). Dmrt1 is also required at postnatal level in male gonads for survival and differentiation of both somatic and germ-line cells. In Dmrt1-/- mutant postnatal testis Sertoli cells failed to differentiate and germ
cells reduced greatly by P10, a time when meiosis begins normally and by P14 germ cells were absent (Raymond et al., 2000).

Dmrt1 and Foxl2 also act antagonistically to control and maintain gonadal sex in adult gonads. Loss of transcription factor Dmrt1 in mouse adult Sertoli cells activates Foxl2 leading to the reprogramming of Sertoli cells into Granulosa cells (Matson et al., 2011). On the other hand in Foxl2\(^{-/-}\) mutant XX gonads, the adult ovary transdifferentiated into testis with upregulation of Sox9 and Dmrt1. The ovarian Granulosa cells reprogrammed into Sertoli like cells and theca cells into Leydig like cells (Uhlenhaut et al., 2009).

On the other hand, Dax-1 also called nuclear receptor subfamily 0 group B member 1 (Nr0B1), a X-linked gene, was considered as an anti-testis gene because its duplication or over expression in XY human individuals causes male to female sex reversal, known as dosage sensitive sex reversal (DSS) (Bardoni et al., 1994; Sukumaran et al., 2013). This Dax1 over expressed XY female sex reversal can be found in mice when Sry expression is delayed transgenetically (Ludbrook and Harley, 2004). However, Dax1 was also found to be required for testis differentiation (Meeks et al., 2003b). Dax1 is expressed in both Leydig cells and Sertoli cells and targeted mutation of Dax1 in both Sertoli cells and Leydig cells of mice causes primary gonadal defect resulting in germ cell death and infertility (Meeks et al., 2003a).
Fig. 5. Mouse PGC ontogeny.
Developmental timing of PGC specification, migration, proliferation, apoptosis, colonization of the genital ridges, genetic reprogramming, X-chromosome activity and sexual differentiation (Ewen and Koopman, 2010).
1.6 Germ Cell Sex Differentiation

1.6.1 From PGC Specification to Colonization

Germ cells are unique from other cell lines at least in two respects; they are the source of transmission of genetic information from a generation to the next generation and have ability of pluripotency even they give rise to only one-cell line gonocytes or oocytes. From specification to final fate they pass through a series of genetic, epigenetic and morphogenetic changes (Fig. 5) either imposed by cellular or gonadal environment or autonomously by germ cells themselves (Ewen and Koopman, 2010).

Germ cells arise from epiblast derived extra embryonic mesoderm as primordial germ cells (PGCs) and develop into either spermatozoa in males or oocytes in females (Ginsburg et al., 1990; Lawson and Hage, 1994). By arising from epiblast, cells are not pre-programmed for PGC fate because they can develop into somatic or germline cells. By 6.25dpc the expression of B-lymphocyte induced maturation protein1 Blimp1/Prdm1 specifies PGCs from somatic differentiation program to germline program (Saitou et al., 2005). In Blimp1−/− mutant embryos, PGC like cells clustered, did not display PGC specific migration and proliferation and did not repress somatic cell lineage genes (Saitou et al., 2005). Some signaling molecules like transforming growth factor β (TGF-B) super family members, Bone morphogenetic protein 2, 4, 8b (BMPs) and their signaling transducers, Drosophila Mothers against decapentaplegic (SMADs)-1 and -5, are produced around 6.0dpc to induce PGC lineage in mouse (Arnold et al., 2006; Lawson et al., 1999; Tremblay et al., 2001; Ying et al., 2000; Ying and Zhao, 2001). These molecules are required for proliferation of PGCs, while BMP4 provides localization and survival to primordial germ cells (Fujiwara et al., 2001).

During the entire migration up to genital ridges, PGCs receive somatic signals that are thought to be necessary for directional migration (De Felici, 2000). In vitro evidences suggest that genital ridges of 10.5dpc mouse embryo secrete TGF-B1 like diffusable protein to attract PGCs (Godin et al., 1991). The Cytokine kit ligand (KL, also known as stem cell factor “SCF” or mast cell growth factor “MGF”), a somatic factor that binds to tyrosine kinase receptor (encoded by c-kit)
on germ cells, is required for normal proliferation and migration of germ cells (Runyan et al., 2006). BMP signals are crucial not only for PGC specification but also for directional migration into genital ridges because both migratory PGCs and somatic cells of genital ridges respond to endogenous BMP by elevated phosphorylation of Smad 1/5/8 (the intracellular transducers of BMP signals). In vitro treatment with BMP antagonist Noggin resulted in failure of PGCs to colonize the genital ridges (Dudley et al., 2007). Cell to cell adhesion plays important role in PGC migration especially from hindgut to genital ridges (Gomperts et al., 1994); in this regard, E-cadherin is crucial as it is expressed by PGCs when they leave the hindgut (Bendel-Stenzel et al., 2000). Migratory PGC plasma membrane at 9.0dpc expresses immunoglobulin super family member, Platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31). Suggested role of this molecule is to mediate PGC cell-cell adhesion for migration and colonization in genital ridges (Wakayama et al., 2003). Thus, once specified, primordial germ cells proliferate and at 10.5dpc when they enter into genital ridges for colonization, their number reaches nearly 1000 PGCs. After colonization, germ cells proliferate for two to three days and at the time of their entry into mitotic arrest in male or meiosis in female, they reach approximately 25000 germ cells (Tam and Snow, 1981).

### 1.6.2 Change in Genetic Machinery on arrival into Genital Ridges

After their colonization of genital ridges, Primordial Germ Cells are called Germ cells or gonocytes. At this time, germ cells are no more able to move because their motile machinery is inactivated and they make tight clusters (Gomperts et al., 1994). Germ cell colonization into new environment, the newly formed gonads, brings changes in the expression of genes. Previously expressed markers are down regulated including tissue non-specific alkaline phosphate (Tnap) (Chiquoine, 1954), SSEA-1 (Kanai et al., 1992) and Nanos3 (Tsuda et al., 2003) with upregulation of new sets of gene markers. The new set of gene markers include germ cell nuclear antigen 1 (Gcna1) (Enders and May, 1994), deleted in azoospermia like (Dazl) (Saunders et al., 2003), germ cell-less (Gcl)(Kimura et al.,
1999) and orphan nuclear receptor, estrogen related receptor-β (ERR-B) that is required for GC proliferation (Mitsunaga et al., 2004). Mouse vasa homolog (Mvh) expression is required in both male and female germ cells and its deletion (Mvh−/−) causes reproductive defects (Tanaka et al., 2000b). In XY gonads, Mvh seems to predominantly involve in germ cell proliferation and differentiation however in XX gonads, Rspo1 is required to promote proliferation of these cells (Chassot et al., 2011). Nanos2 and Nanos3 genes are differentially expressed in germ cells. Nanos3 is predominantly expressed in migratory PGCs of both sexes before colonization, elimination of Nanos3 results in complete loss of germ cells in both sexes. However Nanos2 is expressed after colonization, only in differentiated male GC germ cells and elimination of Nanos2 results in loss of spermatogonia (Tsuda et al., 2003). Wilm’s tumor suppressor gene 1 (Wt1) upregulation is suggested important for male germ cell survival and differentiation after colonization (Natoli et al., 2004).

1.6.3 Germ Cell Male Fate

Many early events of germ cell development are known but we still lack complete knowledge about gene regulatory networks and signaling controls that participate in male/female germ cell development (Ewen and Koopman, 2010). In the mouse embryo, the germ cell progenitors are primed with a bias towards the male fate (Jameson et al., 2012b) but instead the fate of germ cells as male or female does not depend only on germ cell chromosomal sex but also on gonadal environment (Ewen and Koopman, 2010; McLaren, 1995). During embryogenesis, if germ cells enter into ovary they must have to enter into meiosis for correct oogenesis while in male embryonic gonads germ cells do not enter in meiosis and arrest into mitosis for normal male differentiation program (Bowles and Koopman, 2007). Germ cell commitment to spermatogenesis starts from 12.5dpc. At this stage, Sertoli cells are differentiated and testis cords are formed. If germ cells are removed before this time and mixed with either 12.5dpc female genital ridge cells or fetal lung cells they will enter into meiosis.
Retinoic acid (RA) an active derivative of vitamin A is known to induce germ cell entry into meiosis in the developing ovary. RA is produced by the aldehyde dehydrogenase enzymes family (Aldh) in mesonephri of both sexes and then transferred into gonads (Bowles et al., 2006). RA signaling is required to induce the expression of Stra8 (Stimulated by Retinoic Acid gene 8), which is required for meiotic initiation in germ cells of both sexes. In embryonic ovary due to RA signaling, Stra8 is expressed just before meiotic initiation while in testis first detected after birth (Koubova et al., 2006). Male and female Stra8+/− heterozygous mutants were fertile but in homozygous Stra8−/− mutants, both male and female were infertile. In adult homozygous Stra8−/− mutants, gonads were observed with marked reduction in size while at the 8th week, ovaries contained no oocytes or follicles and testicular germ cells severely reduced in number (Baltus et al., 2006). Exogenous RA signaling is sufficient to induce Stra8 expression in embryonic testis and in vitamin A deficient (VAD) adult testis in vivo (Koubova et al., 2006). In culture experiments of urogenital ridges, retinoic acid induced expression of premeiotic marker Stra8, meiotic markers Symp3 and Dmc1 with suppression of pluripotency marker Oct4 (Pou5f1). This indicates that exogenous RA stimulates the entry of germ cells into meiosis (Bowles et al., 2006). In embryonic testis, P450 enzyme CYP26b1 (cytochrome P450, family 26 subfamily B, polypeptide 1) is produced and is responsive for the degradation of RA (Bowles et al., 2006). Cyp26b1 is initially expressed in gonads of both sexes but at 12.5dpc it becomes male specific with a peak of expression at 13.5dpc in Sertoli cells (Bowles et al., 2006). The role of Cyp26b1 in the inhibition of meiosis is evident from Cyp26b1−/− mutant mice, where the expression of Stra8 was upregulated and germ cells entered into meiosis. In addition to the inhibition of meiosis, Cyp26b1 also provides germ cell survival by preventing them from cell apoptosis (MacLean et al., 2007). The role of retinoic acid is also questioned; germ cells in the intervening mesonephric area tend to enter mitotic arrest despite their exposure to RA (McLaren, 1984). The data published by Bowles et al (2006) (Bowles et al., 2006) about cultured gonads does not support these data because female gonads cultured with retinoic acid receptor antagonist do not appear to contain spermatogonia; however, male gonads cultured with Cyp26b1 antagonist do
not appear to contain meiotic oocytes and germ cells display characteristics of
dying cells (Best et al., 2008). When male embryonic gonads were cultured with
exogenous RA, they did not initiate meiosis but induced cell death with reduction
in Sertoli cell and germ cell number (Li and Kim, 2004). Similarly, Ketokonazole
inhibition of Cyp26b1 in Raldh2−/− testis (where RA is not produced) allows RA-
independent induction of Stra8 but only when mesonephros remained attached
to gonads, indicating that Stra8 expression and subsequent onset of meiosis
requires a substrate from mesonephros that should be other than RA (Kumar et
al., 2011).

The molecular events and factors that participate in male germ cell fate program
and their contribution are given below.

1.6.3.1 Dazl

Dazl (deleted in azoospermia like) is a homologue of the human Y-linked DAZ
gene, an autosomal gene, located on chromosome 17 of the mouse (Cooke et al.,
1996). Dazl is first expressed at 11.5dpc in post-migratory germ cells of male and
female gonads (Seligman and Page, 1998). In Dazl deficient C57BL/6 (B6)
embryos, XX and XY PGCs migrate normally to the gonads but post migratory
germ cells remain in a sexually undifferentiated state because they do not
respond to sexual cues from ovary or testis. Thus Dazl is giving license to germ
cells to pass through an active developmental transition that enables them to
respond to feminizing and masculinizing cues produced by the fetal ovary or
testis, to adopt oogenesis or spermatogenesis (Gill et al., 2011).
Dazl is required for embryonic development and survival of XY germ cells. In
homozygous Dazl−/− mutant male gonads in B6 mice, the expression of germ cell
markers reduced by 14.5dpc, however by 15.5dpc most of germ cells exhibited
apoptotic morphology and gonads contained increased number of TUNEL-
positive cells. The germ cells persisted until birth, resembled to 13.5dpc wild
type germ cells. Dazl expression is also required in adult male mice because
disruption in Dazl gene expression leads to loss of spermatogonia and complete
absence of gamete production (Ruggiu et al., 1997). Dazl is also required for female germ cells because Dazl-/- mutant ovaries at 15.5dpc displayed abnormal chromosomal condensation and reduction in Stra8 expression (Lin et al., 2008).

1.6.3.2 Nanos3

Nanos3 (Nanos homolog 3) is found in migrating PGCs of both sexes and its expression is detected in PGCs from 9.5dpc and later stages (Tsuda et al., 2003). However Yamaji et al showed that Nanos3 is expressed in PGCs as early as 7.25dpc and this expression continued in female germ cells until around 14.5dpc while in males declined after 16.5dpc (Yamaji et al., 2010). Nanos3 expression is involved in the maintenance of PGCs by supporting their proliferation or by suppression of cell death and its elimination results in the complete loss of germ cell in both sexes at 15.5dpc (Tsuda et al., 2003).

1.6.3.3 Nanos2

Another member of Nanos family, Nanos2 is predominantly expressed in post migratory male germ cells, plays critical role in the differentiation of male germ cells (Tsuda et al., 2003). In the absence of Nanos2 male germ cells undergo apoptosis from 15.5dpc and most are subsequently lost by birth. Nanos2 blocks the male gonocytes from entry into meiosis as in Nanos2 null male embryos, Stra8 expression upregulated in gonocytes at 15.5dpc. In this way Nanos2 maintains the suppression of meiosis by preventing Stra8 expression and promotes male specific genetic program (Suzuki and Saga, 2008; Tsuda et al., 2003).

1.6.3.4 Vasa/Mvh

Mouse vasa homolog (Mvh) gene, a homolog to drosophila Vasa gene, encodes a DEAD-box family protein in mice. MVH protein is expressed in germ cells of both sexes that have recently colonized embryonic gonads. Mvh expression is not detected in pluripotent cell lines of embryonic germ cells (EG), embryonic stem
cells (ES) and migrating PGCs (Fujiwara et al., 1994). Mvh is involved in the regulation of male germ cell proliferation and differentiation as homozygous Mvh+/− mutant male mice display reproductive deficiency (infertility). No sperm production in the testis as premeiotic germ cells cease differentiation by the zygotene stage and undergo apoptosis, additionally proliferative activity of PGCs remarkably decreased (Tanaka et al., 2000b). Mvh is believed to act as a positive translational regulator of various factors involved in the determination and maintenance of germline cells. It is an essential factor in the piRNA-processing pathway in germ cells of male mice and plays a role in ping-pong amplification cycle as two PIWI family members MILI and MIWI2 bound to Mvh (Kuramochi-Miyagawa et al., 2010).

1.6.3.5 Basonuclin 2 (BNC2)
The zinc-finger protein BNC2 is found in males but not in females and is thought to bind to DNA. BNC2 is required for proper mitotic arrest, prevention of premature meiotic initiation and meiotic progression in mouse male germ cells. In Bnc2+/− mutant gonads germ cells multiply exclusively by 14.5dpc and re-enter cell cycle prematurely while also engage in abnormal meiosis at 17.5dpc by expressing synaptonemal complex protein (SYCP3). Moreover at the time of birth, Bnc2−/− prospermatogonia accumulate large amount of non-filamentous SYCP3 appearing to be blocked in leptonema. They do not undergo proper male differentiation due to lack of Dnmt3l mRNA and increased level of Srta8 mRNA. Bnc2−/− embryos produce prospermatogonia but due to premature meiotic entry they undergo strong apoptotic death during meiotic prophase (Vanhoutteghem et al., 2014).

1.6.3.6 Epigenetic Regulation
Germ cell genome demethylation not only helps to erase imprints during genetic reprogramming but also plays an important role in regulation of differentiation in proliferating germ cells. DNA methylation has essential role in mammalian
development and DNA methyltransferase enzymes (Dnmts) contribute to the establishment and maintenance of methylation patterns in male germ cells and their expression is tightly regulated during spermatogenesis. In mouse embryo, homozygous mutation of DNA methyltransferase genes caused abnormal development and embryonic lethality (Li et al., 1992).

Dnmt1, Dnmt3a, Dnmt3b and Dnmt3l are expressed in male and female germ cells. Both Dnmt3a and Dnmt3l enzymatic activities interact during prenatal germ cell development and act for the de novo DNA methylation in males while Dnmt3b and Dnmt3l are also both expressed in males shortly after birth and maintain DNA methylation. In mice, the erasure of DNA methylation patterns occurs in male and female germ cells between 10.5dpc- 12.5dpc as PGCs enter the gonads (Trasler, 2006).

During DNA remethylation, Dnmt1 that maintains DNA methylation in male germline, is downregulated between 14.5dpc - 18.5dpc and is absent at the time of initiation of DNA methylation (La Salle et al., 2004). Then DNA methylation patterns are reestablished, beginning at around 15.5dpc and complete after birth (La Salle et al., 2007; Reik et al., 2001).

1.7 Important Steps During Male Germ Cell Fate Program

1.7.1 Mitotic Arrest

The cell reproduction or cell cycle includes DNA replication, nuclear division and partition of cytoplasm to produce two daughter cells. The process of mitosis is divided into G1, S, G2 and M phases and two restriction points G1-S and G2-M, which are also called checkpoints. These restriction points allow the repair of DNA damage and replication errors. At each restriction point, cell cycle is strictly regulated by cyclin/cyclin-dependent kinase (CDK) complexes. Among these
Fig. 6. Different phases of cell cycle and corresponding Cyclins, CDKs and their inhibitor proteins.

Cyclin and CDK complexes promote cell cycle while checkpoint regulator protein RB at its hypophosphorylated form, suppression of cyclin E1, E2 and D3 and activation of P15, P16, P18, P19, P27, P21 and P57 inhibit cell cycle by blocking cyclin-CDK complex formation (Spiller et al., 2009b; Western et al., 2008).
complexes, Cyclin-D-Cdk4/6 and CyclinE-Cdk2 are required to continue cell cycle. The phases of cell cycle, cyclin/CDK complexes and their inhibitors are illustrated in Fig. 6 (Spiller et al., 2009b; Western et al., 2008). These complexes inactivate the checkpoint regulator protein retinoblastoma (RB) by its hyperphosphorylation. In response to anti-proliferation signals, the inhibitor proteins blocks cyclin-CDK complex and hypophosphorylate retinoblastoma to activate G1-S phase checkpoint in order to block cell cycle in mitotic arrest (G1-G0 arrest)(Massague, 2004; Western et al., 2008). The fate of germ cell as male starting from 12.5dpc is characterized by entry of germ cells into mitotic arrest, which continues throughout gestation period (Western et al., 2011). Entry of male germ cells into mitotic arrest is key point of male germ cell fate program that requires expression of cell cycle inhibitor proteins, P27Kip1 (Cdkn1b, cyclin dependent kinase inhibitor 1B) P15INK4B (Cdkn2b, cyclin dependent kinase inhibitor 2B), P16INK4A (Cdkn2A, cyclin dependent kinase inhibitor 2A) and activation of G1-S phase checkpoint protein RB (Retinoblastoma). In addition regulated expression of the cell cycle inhibitors p21Cip1, p27Kip1, and p57Kip2 is also essential in the control of G1/G0 arrest (Western et al., 2008).

The TGF-B-Activin signaling through their receptors ALK4/5/7 are required for the regulation of testis cord formation and male germ cell development (Miles et al., 2013). TGF-B signaling controls numerous cellular process including cell proliferation, differentiation and apoptosis during embryogenesis and adulthood. In male germ cells TGF-B signaling is involved in the regulation of male gametogenesis as a direct anti-proliferative and anti-apoptotic factor. In knockout condition, animals die during fetal life however surviving adults show reduced pool of spermatogonial stem/progenitor cells and become sterile with time (Moreno et al., 2010). When XY gonads were cultured with inhibitors of TGF-B-Activin signaling, the testis cord formation was prevented and germ cells at 11.5dpc entered into meiosis suggesting that this signaling is required for male germ cell mitotic arrest (Miles et al., 2013). DND1 (dead end homolog1) is known to regulate mitotic arrest in male germ cells through transcriptional regulation of cell cycle genes. DND1 is a RNA binding protein that directly binds to transcripts of negative regulators of cell cycle including P27Kip1 and P21cip1.
(both are cell cycle inhibitors). In case of $Dnd^{1ter/ter}$ mutation, both proteins are significantly decreased in germ cells of all strain backgrounds, suggesting an essential role in mitotic arrest (Cook et al., 2011). Dmrt1 (doublesex and mab-3 related transcription factor 1) is not only expressed in Sertoli cells, but also in germline and $Dmrt1$ mutant 129Sv mouse embryos, male germ cells failed to arrest in mitosis and highly expressed Ki67 (proliferation marker) at 16.5 dpc. $Ki67$ expression is normally down regulated at 13.5 dpc, in mitotically arrested male germ cells. On the other hand, mRNA expression of $P18^{INK4c}$ (Cdkn2c) and $P19^{INK4d}$ (Cdkn2d) decreased at 15.5 dpc in $Dmrt1$ mutant testis, suggesting that Dmrt1 directly affects mitotic proliferation (Krentz et al., 2009).

1.7.2 Meiosis Inhibition

The process of meiosis is highly specialized among mammals, which enables diploid germ cells to generate haploid gametes in order to maintain constant chromosome number. The process of meiosis is divided into two divisions meiosis I division and meiosis II division. Each meiotic division consists of four phases, prophase, metaphase, anaphase and telophase. The prophase of meiotic I cell division is the longest phase that accounts 90% of meiosis during which DNA replication takes place and DNA material is reduced from diploid to haploid hence this division is also called reduction division. It is further divided into five, leptotene, zygotene, pachytene, diplotene and diakinesis, which are defined by recombination events (developmental biology 6th edition, page 533, meiosis). Germ cells in fetal ovary enter meiotic prophase I by 13.5 dpc that continues until 17.0 dpc while meiosis process completes on maturation of oocyte at the time of fertilization (McLaren, 1995; Speed, 1982). During prophase I, female germ cells pass through leptotene, zygotene and pachytene stages before arresting in diplotene at about birth (McLaren, 2003). However in male genital ridges germ cells do not precede into meiosis, Stra8 is not up-regulated like in XX gonads and germ cells enter into mitotic arrest as prospermatogonia (McLaren, 1984). The production of retinoic acid (RA) in association with Dazl (deleted in azoospermia) is required to activate $Stra8$ expression to promote entry of female
germ cells into meiosis (Lin et al., 2008). Before sex differentiation, *Cyp26b1* is expressed in mouse gonads of both sexes, but during gonadal differentiation *Cyp26b1* expression becomes only expressed in male gonads. In testis, *Cyp26b1* inhibits male germ cells from entering into meiosis by degrading RA (Bowles et al., 2006). The role of *Cyp26b1* in the inhibition of meiosis is confirmed from *Cyp26b1*<sup>−/−</sup> mutant mice, where germ cells expressed *Stra8* and entered into meiosis (MacLean et al., 2007).

Similarly Fgf9 (fibroblast growth factor9) expression is also required for male germ cell survival and the balance between expression of RA and Fgf9 influences female/male germ cell fate, where Fgf9 acts as a meiosis inhibiting substance and makes germ cells less responsive for RA (Bowles et al., 2010; Dinapoli et al., 2006). In both male and female primordial germ cells and premeiotic spermatogonia, Fgf9 acts as an inhibitor of meiosis through the upregulation of Nanos2 (Barrios et al., 2010). It is also shown that Fgf signaling is required to repress female genes during testis development but it is not required to maintain Sox9 or not to regulate male germ cell development or activate male vascular development and steroidogenesis. Because in Fgf9 or Fgfr2 mutant XX gonads additional deletion of Wnt4 rescued the male-to-female sex reversal phenotypes in somatic and germ cells while in XX Wnt4 mutant gonads, deletion of Fgf9 could not rescue the sex reversal phenotype, indicating that the partial male sex reversal in XX gonads does not depend on the up-regulation of Fgf9 and if Fgf9 has any role on germ cells it should be indirect (Jameson et al., 2012a).

The Nodal pathway and its co-receptor Cripto are expressed in male but not in female germ cells during mouse gonadal development and Nodal signaling is triggered when somatic signals, including Fgf9, induce testicular germ cells to upregulate Cripto. Mice mutant of Nodal-Cripto signaling showed premature differentiation with depressed pluripotency marker expression and reduced ability to form embryonic germ cell colonies in vitro (Spiller et al., 2012). Nodal signaling prevents meiosis and reinforces male fate in fetal male germ cells and plays potential role as a gatekeeper of male differentiation. When Tgf-B/Nodal/Activin signaling was blocked in 11.5dpc testis in organotypic culture experiment, it disrupted normal male germ cell development and induced *Stra8* expression without making any change in the expression of *Cyp26b1* or Fgf9 and
induced germ cell entry into meiosis. On the other hand, when 11.5dpc ovaries were cultured with Nodal, it prevented Stra8 expression and germ cell entry into meiosis, indicating that Nodal signaling has autocrine role during the development of germ cells and that members of Tgf-b family may reinforce the male fate and prevent meiosis in embryonic germ cells (Souquet et al., 2012).
Fig. 7. Temporal expression of pluripotency core genes during mouse embryonic development (Avilion et al., 2003; Western et al., 2010; Yamaguchi et al., 2005).
1.7.3 Regulation of Pluripotency During Embryogenesis

Mammalian germ cells are derived from the pluripotent epiblast and share features with pluripotent stem cells, including the expression of key genes that regulate developmental potency (Tesar et al., 2007). Inner cell mass (ICM) derived embryonic stem cells (ESCs) are pluripotent as they are able to differentiate into all derivates of the three primary germ layers (ectoderm, endoderm and mesoderm)(McElroy and Reijo Pera, 2008). Apart from signaling molecules, some transcription factors maintain or protect the pluripotent state of the ICM (Avilion et al., 2003). The transcription factors Oct-4, Sox2 and Nanog are well known as they transcriptionally control genes to regulate pluripotency in embryonic stem cells and germ cells and their persistence in differentiated germ cells has potential in occurrence of germline tumors in humans (Boyer et al., 2005; Rajpert-De Meyts et al., 2003). The temporal expression of pluripotency core genes is shown in Fig. 7 (Avilion et al., 2003; Western et al., 2010; Yamaguchi et al., 2005) and their further role is described in the following text.

1.7.3.1 OCT-3/4

OCT-4 (octamer-binding transcription factor 4) also known as POU5F1 (POU domain, class 5, transcription factor 1) is expressed in the ICM, epiblast and later in primordial germ cells. Oct-4 maintains potency of stem cells and germline cells by preventing all other differentiation pathways (Pesce et al., 1998a). Oct-4 expression is observed in epiblast of developing embryo, and is later on repressed in the epiblast to become confined to the newly established primordial germ cells (Scholer et al., 1990; Yeom et al., 1996). Oct3/4 acts as a master regulator of pluripotency and its repression induces loss of pluripotency (Niwa et al., 2000). In vivo ablation of Oct-4 causes early embryonic lethality, as embryonic cells differentiated into trophectoderm, which normally would give rise to pluripotent inner cell mass of the blastocyst (Pesce and Scholer, 2000). Oct-4 is essential for the identity of the pluripotent founder cell population in the mammalian embryo (Nichols et al., 1998). Loss of Oct4 function leads to
apoptosis of PGCs, suggesting function of Oct4 in maintaining viability of mammalian germline (Kehler et al., 2004). Oct-4 expression is observed in PGCs as they proliferate and migrate into genital ridges. In female PGCs, Oct-4 is repressed by the onset of meiotic prophase-I and re-expressed after birth; however in male germ cells Oct-4 expression continues throughout fetal development. After birth it is maintained in proliferating gonocytes, spermatogonia and later in undifferentiated spermatogonia (Pesce et al., 1998b; Tadokoro et al., 2002).

1.7.3.2 Sox2

The transcription factor Sox2 (SRY-box2) belongs to Sox family, a family of transcription factors with HMG DNA-binding domain (Wegner, 1999). Sox2 like Oct-4 is expressed in the ICM, epiblast and germ cells however it is also expressed in extra embryonic ectoderm. Sox2 marks the pluripotent lineage of the early mouse embryo being essential for maintaining pluripotency of undifferentiated embryonic stem cells (Avilion et al., 2003). The interaction between Sox2 and Oct-3/4 has been linked closely to the transcription of the Fgf4 gene. Sox2 and Oct-3/4 bind adjacent sites within FGF4 enhancer to form a protein-DNA complex for its activation (Yuan et al., 1995). An essential enhancer, located in the untranslated region of the third exon, 3kb downstream of the transcription start site, controls expression of the FGF4 gene, (Curatola and Basilico, 1990). FGF4 gene expression in embryonic carcinoma cells requires a synergistic interaction between Oct-3/4 and Sox2 on the FGF4 enhancer. The zygotic Sox2 expression is also required for the ICM/epiblast because in the absence of zygotic Sox2, there is no equivalent rapidly proliferating cell population, the only surviving cells being trophoblast giant cells and extra embryonic endoderm (Avilion et al., 2003). Sox2 homozygous null embryos failed to survive and died just after implantation, reflecting the unique expression of Sox2 in the inner cell mass and epiblast (Avilion et al., 2003; Pevny et al., 1998).
1.7.3.3 Nanog

Nanog (Nanog homolog) is a homeodomain-bearing transcription factor, expressed in pluripotent cells in mouse pre-implantation embryo, embryonic stem cells (ES), embryonic germ cells (EG) and embryonic carcinoma cells (Chambers et al., 2003; Mitsui et al., 2003). Nanog is required for the maintenance of pluripotency as Nanog deficient embryonic stem cells lost pluripotency and differentiated into extra-embryonic endoderm lineage. Homozygous Nanog−/− mutation caused embryonic lethality due to lack of epiblast (Mitsui et al., 2003). The zygotic Nanog was initially detected at the morula stage, the inner cell mass of blastocyst and epiblast of 6.5dpc and 7.5dpc embryo but not detected in primordial germ cells of the early post-implantation embryo (Hatano et al., 2005). It is suggested that Nanog transcription is controlled by the synergistic action of Oct-4 and Sox2. Double staining with antibodies to Nanog and Oct-4 revealed that the two signals were overlapping in the majority of colonies (Hatano et al., 2005). Nanog promoter assays show that the Octamer and Sox elements located at 5′ of the transcription start site regulate pluripotent cell-specific expression of Nanog. This promoter is highly conserved among the mouse, monkey and humans (Kuroda et al., 2005). Nanog expression is detected in migrating and gonadal PGCs from 7.75dpc-12.5dpc embryos and then was downregulated in 13.5dpc-14.5dpc female germ cells during meiosis entry and in 14.5dpc-16.5dpc male germ cells at the stage of mitotic arrest. Nanog expression was not detectable in the adult testicular or ovarian germ cells (Yamaguchi et al., 2005).

1.7.3.4 Combined action of OCT-4, SOX2 and NANOG and their role in differentiating germ cells

OCT4, SOX2 and NANOG collaborate to form regulatory circuitry (regulatory pathways) that includes autoregulatory and feedforward loops. OCT4, SOX2 and NANOG co-occupy the promotors of 353 genes in human embryonic stem cells. Surprisingly more than 90% of promoter regions bound by both Oct4 and Sox2
were also occupied by Nanog and their binding sites occurred in close proximity in nearly all occupied genes. In this way, the core elements Oct4, Sox2 and Nanog contribute to pluripotency and self-renewal by activating their own genes and gene encoding components and by repressing genes of developmental pathways (Boyer et al., 2005). The repression of this core machinery that regulates pluripotency is required for fetal germ cell differentiation of both sexes. In mouse female gonads, at the time of germ cell entry into meiosis, the transcription of Sox2, Oct4 and Nanog are strongly repressed between 12.5dpc and 15.5dpc. During mitotic arrest the regulators of pluripotency Oct4, Sox2 and Nanog are suppressed and male fate genes Nanos2 (Nanos homolog 2) and Dnmt3l (DNA cytosine-5-methyltransferase 3-like) are activated (Sakai et al., 2004; Suzuki and Saga, 2008; Western et al., 2010). Due to male specific methylation of Nanog and Sox2 promoters, these genes are suppressed during early male germ cell differentiation however Oct4 translation is suppressed post-transcriptionally as germ cells enter mitotic arrest. The suppression of pluripotency regulating machinery indicates that this event is robustly involved in male germ cell differentiation program and failure in this system increases germ cell susceptibility to tumor formation (Western et al., 2010). The reprogramming of pluripotency in germ cells and prevention of tumor formation requires cell cycle arrest. Dnd1 regulates cell cycle arrest in male germ cells through translational regulation of cell cycle genes. Dnd1Ter/Ter mutation on 129/Svj genetic background showed strong down regulation of male differentiation genes; germ cells failed to enter into mitosis and did not down regulate pluripotency regulators Nanog, Sox2 and Oct4 (Cook et al., 2011). Dmrt1 controls expression of pluripotency regulators in the embryonic testis by transcriptional suppression. In Dmrt1−/− mutant mouse embryos on 129Svj genetic background, germ cells showed defect in restriction of pluripotency, and testis developed embryonic carcinoma cell clusters with increased expression of Nanog, Sox2 and Oct4 at birth (Krentz et al., 2009).
Except these core regulators, other genes are also involved in the regulation of pluripotency, like \textit{Dppa2} (developmental pluripotency-associated gene 2) and \textit{Dppa4}. \textit{Dppa2} and \textit{Dppa4} are expressed in inner cell mass of blastocyst and then downregulated, which after gastrulation are re-expressed only in the developing germline. \textit{Dppa4} expression was detected by 10.5dpc while \textit{Dppa2} expression was detected by 11.5dpc in germ cells. In differentiating germ cells, these pluripotency markers are downregulated as female germ cells enter into meiosis between 12.5dpc-13.5dpc and in male germ cells between 12.5dpc-15.5dpc soon after commitment to male germ cell differentiation pathway (Maldonado-Saldivia et al., 2007). The signaling through Nodal (member of TGFβ morphogen family) and its co-receptor Cripto, is shown active during male germ cell development. Male germ cells of Nodal-Cripto signaling mutant mice display premature differentiation with reduced expression of pluripotency markers and reduced ability to form embryonic germ cell colonies in vitro. NODAL-CRIPTO signaling was upregulated in human testicular tumor cells and CIS cells; normally both are not expressed in human adult testes (Spiller et al., 2012).

\subsection*{1.7.4 Germ Cell Apoptosis (Programmed Cell Death)}

Programmed cell death or apoptosis is important to prevent tumor development caused by aberrantly controlled germ cells (Runyan et al., 2008) and to ensure high genomic integrity of surviving gonadal germ cells (Bristol-Gould et al., 2006). Germ cells that are left in ectopic locations (hindgut, midline and surrounding organs) during and after germ cell migration are eliminated around 10.5dpc (Runyan et al., 2006; Stallock et al., 2003). The apoptosis of pronephros occurs at 12dpc however the apoptosis of the distal mesonephros occurs primarily at 13dpc and completes at 14dpc (Pole et al., 2002). Regardless of their genetic sex, non-gonadal germ cells enter into meiosis and finally go through apoptosis (McLaren, 1983; Molyneaux et al., 2001). In failure to apoptosis, gonadal and non-gonadal germ cells give rise to tumors (Schneider et al., 2001). This failure to apoptosis of germ cells particularly in testis results in CIS (carcinoma in situ) and TGCTs (testicular germ cell tumors) formation (Bartkova
et al., 2003; Spiller et al., 2009a) indicating that apoptosis is an effective mechanism for maintaining cellular integrity (Matsui, 1998). Calcium-signaling pathway has been identified in both XX and XY germ cells, which extensively regulates cell survival within XY germ cell populations (Spiller et al., 2009a). Gonadal germ cell survival and death is controlled by expression of two members of Bcl-2 family, Bcl-2-like 1 (Bcl-x) anti-apoptotic (cell survival factor) and Bcl-2-associated X (Bax) pro-apoptotic (cell death factor) (Ratts et al., 1995; Rucker et al., 2000). The pro-apoptotic gene Bax is required for germ cell death during later stages of their development in gonads. Bax is also expressed during germ cell migration and induces apoptosis of germ cells that are left in ectopic locations (extra-gonadal) during and after germ cell migration. Bax inactivation protects germ cells against rapid cell death in culture experiments (Stallock et al., 2003). Homozygous Bax−/− mutant young female mice possess three fold more primordial germ cells in their ovarian reserve than their Bax+/+ wild-type sisters (Perez et al., 1999). According to (Rucker et al., 2000) male null mutant mice of Bcl-x (Bcl-x−/−) lack spermatogonia and are sterile whereas females with reduced oocytes and greatly impaired fertility. However deleting both copies of Bax gene (Bax−/−) resulted in increased number of follicles even more than control and could restore germ cell survival (Rucker et al., 2000). Bax works under the cell survival signaling interaction mediated by steel factor/receptor interaction (Stallock et al., 2003). Steel factor (also known as cytokine kit ligand-KL-survival factor) is required for normal proliferation and migration of germ cells and its changing expression is required for normal midline germ cell death (Runyan et al., 2006). Decreased expression of KL increases apoptosis of ectopic Primordial Germ Cells and Germ Cells, through Bax pathway. As in case of mutation of Kl (steel−/−) and its PGC receptor c-kit (c-kit−/−), very few germ cells arrive to the genital ridges in comparision to Wild type germ cells (Buehr et al., 1993; Mintz and Russell, 1957).
1.8 Formation of Germ Cell Tumors

Proper regulation of cell cycle arrest and reprogramming of pluripotency in germ cells are essential steps in prevention of tumor formation (Cook et al., 2011). In mouse, male germ cell tumor formation is strain-specific, with the inbred strain 129Sv/J being the most susceptible (Cook et al., 2011; Krentz et al., 2009). Male germ cells in B6, CD1 (tumor non-susceptible strains) and 129T2/SvJ (tumor susceptible strain) genetic backgrounds entered into mitotic arrest at same developmental time but there were significant differences in the cross sectional size and germ cell content of testis cords; including differences in total cord number, average cord area, germ cells per cord and germ cells per section. These differences may affect the susceptibility of germ cell tumor formation in these strains (Western et al., 2011). In human, like gonadal germ cell tumors, non-gonadal germ cell tumors are derived from PGCs, as ectopic germ cells that fail to die by apoptosis will develop as tumors (Schneider et al., 2001). Germ cell tumors are divided into different subtypes; Type I germ cell tumors (infantile germ cell tumors) arise from early PGCs and develop as teratoma and yolk sac tumors in neonates and children and often arise at extragonadal locations from migrating PGCs (van de Geijn et al., 2009); Type II TGCTs arise from fetal gonadal germ cells and develop as Carcinoma-in-situ (CIS) (Skakkebaek, 1972) and Type III germ cell tumors arise from differentiated spermatogonia/spermatocytes and develop as spermatocytic seminomas in adult men (Visfeldt et al., 1994).

1.8.1 CIS Formation

An hypothesis is that carcinoma-in-situ (CIS) germ cells are malignant gonocytes, which arise from fetal germ cells that fail to differentiate correctly and that CIS is the precursor cell of type II TGCTs (Skakkebaek et al., 1987). TGCTs account to more than 90% of all testicular tumors and are the most common cancer in men between the ages of 20 and 39 (Richardson et al., 2008). Carcinoma-in-situ (CIS) the precursor of many tumors originates from fetal gonocytes and will give rise
Fig. 8. Developmental origin of human type II TGCT.

During normal germ cell development pluripotency genes are turned off as fetal germ cells differentiate into spermatogonia. After puberty spermatogenesis begins and sperm is produced. Under pathological conditions, germ cells that fail to undergo correct spermatogenic differentiation and instead retain expression of pluripotency markers, develop into the precursor lesion carcinoma in situ (CIS). CIS cells remain dormant until puberty when they begin transformation into either seminoma (SE) or non-seminomas (NS), or both. Seminomas display fetal germ cell characteristics. NS include the most common subtype, embryonal carcinoma (EC), as well as yolk sac tumours (YST), teratomas (TE), Choriocarcinomas (CH) or a mixture of these subtypes (mixed non-seminomas; mNS) (Spiller et al., 2013).
to testicular germ cell tumors (TGCTs) (Ulbright et al., 2000). Testicular germ cell transformation into CIS is illustrated in Fig. 8 (Spiller et al., 2013). CIS germ cells are malignant gonocytes with the capacity to regress into totipotent embryonic cells that can give rise to all types of non-seminomatous germ cell tumors (Skakkebaek et al., 1987). The testicular teratomas that develop in 129sv mouse strain have power of progressive growth and are composed of variety of embryonic and adult tissues, which are not found in a normal testis (Stevens and Little, 1954). Many risk factors are identified for testicular germ cell tumors such as prenatal conditions, which are related to environmental influences or lifestyle at the time of early development. The prenatal conditions associated with testicular germ cell tumors are undescended testis (cryptorchidism), familial testis cancer and gonadal dysgenesis (Dieckmann and Pichlmeier, 2004).

High incidences of teratomas in 129Sv/j mouse strain have already been reported. Mutation in some genes increases tumor susceptibility, like Dnd1 (Dead end 1) (Cook et al., 2011; Youngren et al., 2005), Dmrt1 (Double sex and mab-3 related transcription factor) (Krentz et al., 2009) and Pten (Phosphatase and tensin homolog) (Kimura et al., 2003) but less is known about genetic and environmental conditions of germ cells in teratoma susceptible 129Sv/j strain and teratoma non susceptible mouse strains, they will be discussed below.

Mouse strain 129Sv/J shows germline abnormality and develops spontaneous teratomas (Stevens, 1984). The introduction of several mutant genes in the 129Sv/j genetic background can induce incidence of tumors (Stevens, 1981). A single autosomal recessive mutation Ter (teratoma) causes germ cell deficiency in both sexes and an extremely high incidence of congenital testicular teratoma in 129/Sv-Ter/Ter males. All 129/Sv-Ter/Ter males were completely sterile, whether or not their testes were destroyed by teratomas (Noguchi and Noguchi, 1985). Ter was mapped to mouse chromosome 18 and positional cloning revealed that ter is a nonsense point mutation in the third exon of the dead end homolog 1 (Dnd1Ter) that causes significant loss of protein by inducing a premature stop codon. Dnd1 is expressed in the fetal gonads during the critical period when TGCTs originate (Youngren et al., 2005).
The loss of *Dnd1*ter/ter in germ cells of 129Sv/J mouse strain, results in a strong
downregulation of male differentiation genes, including Nanos2, ectopic
upregulation of meiotic markers, maintenance of pluripotency genes and failure
to enter in G0 mitotic arrest. Mouse Dnd1 binds to transcripts of cell cycle genes,
including p27Kip1 and p21Cip1, which significantly decreased in *Dnd1*ter/ter mutant
of all strain backgrounds, suggesting that Dnd1 regulates mitotic arrest in male
germ cells through translational regulation of cell cycle genes. While C57Bl/6j
(B6) mice do not form tumors as the pluripotency markers NANOG and SOX2
were successfully downregulated and negative regulators of cell cycle over
expressed at 14.5dpc stage (Cook et al., 2011).
The expression of DMRT1 in mice is limited to gonads and essential for postnatal
differentiation of germ cells and Sertoli cells. Loss of DMRT1 results high
incidence of teratoma in 129Svj mice and no teratoma but testicular dysgenesis
in C57BL/6j (B6) postnatal testis. As a tumor suppressor, DMRT1 suppresses the
expression of pluripotency regulators specifically Sox2 through activating GDNF
co-receptor Ret (Krentz et al., 2009).
*PTEN* (phosphatase and tensin homolog deleted on chromosome ten) also
known as *MMAC1* (muted in multiple advanced cancers) is a tumor suppressor
gene located on human chromosome 10q23 (Li et al., 1997; Steck et al., 1997). In
addition to suppression of cancers, *Pten* gene regulates cell signaling, growth,
migration and apoptosis (Yamada and Araki, 2001). *Pten* is also involved in the
embryonic development as homozygous Pten−/− mutant mouse embryos die by
9.5dpc due to defect in chorio-allantoic development and defective placentation
(Suzuki et al., 1998). Pten is essential for the correct differentiation of PGCs to
mature germ cells. In case of PGC specific Pten−/− mutation in mice, the percentage
of apoptotic cells highly increased in male gonads after 14.5dpc, male mice
exhibited bilateral testicular teratoma due to impaired mitotic arrest and out
growth of cells with immature characters. In culture experiments, Pten-null PGCs
displayed greater proliferative capacity and enhanced pluripotent embryonic
germ (EG) cell colony formation (Kimura et al., 2003).
Nodal-cRIPTO signaling is essential to control the regulation of pluripotency and
male germ cell differentiation, as this signaling is active in XY germ cells during
the period of sexual fate determination in mouse embryo (Spiller et al., 2012).
Nodal, a member of the TGF-B family binds to Activin receptors (Alk4/7, ActR2A/B) in the presence of the obligate receptor, Cripto (also known as teratocarcinoma derived growth factor 1-TDGF-1) to induce the phosphorylation (activation) of Smad2 and Smad4 transcription factors towards the transcriptional regulation of target genes (Schier and Shen, 2000). In male mice, germ cells mutant of Nodal signaling showed premature differentiation, reduced expression of pluripotency markers and a reduced ability to form embryonic germ cell (EG) colonies in vitro. On the other hand NODAL-CRIPTO signaling was upregulated in human testicular tumors, which normally is not expressed in adult human testes (Spiller et al., 2012).

### 1.9 PGD2 Pathway

#### 1.9.1 Fatty Acids and Phospholipase Enzymes

Free polyunsaturated fatty acids are required for the formation of eicosanoids, which are provided by the action of phospholipases (PL) especially PLA2 and others like Phospholipase D (PLD) and diacylglycerol (DAG) (Massey and Nicolaou, 2011). Polyunsaturated fatty acids are released by cell membrane, which serves as pool for lipids. The derivatives of three fatty acids with 20-carbon acyl chains, arachidonic acid (AA, C20: 4n-3), eicosapentaenoic acid (EPA, C20: 5n-3) and dihomo-gamma linolenic acid (DGLA, C20: 3n-6) are called eicosanoids (Greek word eikosi, means twenty for the 20 carbon atoms in fatty acid). Among them arachidonic acid is the best-known and studied lipid.
1.9.2 COX Enzymes and Arachidonic Acid

The enzyme COX (cyclooxygenase) has two isoforms COX1 and COX2 that convert arachidonic acid to prostaglandins precursors (Smith, 2008). COX enzymes share the same functional domains and are located in the lumen of the nuclear envelope and endoplasmic reticulum (Simmons et al., 2004). The Cox-derived mediators belong to the family of eicosanoids and are collectively known as prostanoids (Massey and Nicolaou, 2011). Each COX is involved in distinct physiological processes. Cox1 is a housekeeping gene and its expression is constitutive while Cox2 gene expression is inducible. The two COXs might have evolved partly to permit prostaglandin formation at different tissue sites. The constitutively expressed COX1 is involved in maintaining physiological homeostatic functions, while the inducible Cox2 is believed to be involved primarily in the inflammatory response (Smith, 2008). Cox1 enzyme is constitutively expressed in many tissues including: kidney, lungs, stomach and whole intestine of the rat, dog, Rhesus monkey and human (Kargman et al., 1996). Various intracellular and extracellular stimuli including cytokines, mitogens, growth factors and tumor promoters induce rapidly Cox2 expression in different cell lines (Cha et al., 2006). Cox2 pathway is an important source of prostanoid formation in inflamed tissues and in cancer (Hida et al., 1998). Arachidonic acid under the action of Cox1 and Cox2 enzymes is converted into unstable cyclic endoperoxides PGG2 and PGH2 (Harizi et al., 2001). This conversion is done through two successive reactions: in the first bis-oxygenation and cyclization of arachidonic acid results to the formation of PGG2 and then peroxidation of PGG2 forms PGH2 (Smith, 2008; Smith et al., 2000).
Figure 9: The prostanoid biosynthesis pathway (modified from Nicolaou et al., 2014). COX, cyclooxygenase; PGES, prostaglandin E synthase; PGDS, prostaglandin D synthase; PGFS, prostaglandin F synthase; PGIS, prostacyclin synthase; TXS, thromboxane synthase.
1.9.3 Prostaglandin Formation

PGH2 is then converted to structurally related prostanoids, prostaglandins including PGE2, PGD2, PGI2, PGF2α and a TxA2 (Thromboxane A2) by the action of tissue specific prostaglandin (PG) synthases (Funk, 2001). The prostanoid biosynthesis pathway is illustrated in Figure 9 (modified from Nicolaou et al., 2014)).

Prostaglandins play important roles in various physiological processes in invertebrates and vertebrates including mammals, indicating that these bioactive lipid molecules are evolutionary conserved (Cha et al., 2006). Aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) were shown to inhibit the activity of COX enzymes and to block formation of prostaglandins, which normally cause inflammation, swelling, pain and fever.
Figure 10. Prostanoids and their receptors and tissues or cellular processes in which they are involved. From (Cha et al., 2006).
1.9.4 Prostanoid Receptors

The bioactive prostanoids effects are mediated through G protein-coupled receptors (GPCRs). In mammals, prostanoid receptors are classified into eight groups (EP1, EP2, EP3, EP4, DP1, FP, IP and TP) and more recently another receptor (DP2) was identified (Hirai et al., 2001; Narumiya, 2007). PGE2 binds to four receptors EP1-EP4, PGD2 binds to two receptors DP1 and DP2 and PGF2α binds to FP, PGI2 to IP, TxA2 to TP (Breyer et al., 2001; Woodward et al., 2011). The prostanoid receptors and their involvement in various tissues or cellular processes are illustrated in Figure 10 (Cha et al., 2006). All these receptors are G protein-coupled rhodopsin-type receptors, with seven trans-membrane domains and a separate gene encodes each receptor (Narumiya, 2007).

The PGD2 actions are through two receptors, the first one is the classical receptor called D prostanoid receptor (DP1) also called PTGDR (prostaglandin D2 receptor) (Boie et al., 1995), the second one is the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2), a surface marker of human Th2 cells, also called DP2 receptor (Hirai et al., 2001). Dp1 is coupled with a Gsα protein that increases intracellular cAMP concentration (Boie et al., 1995), however DP2 is coupled with Giα protein, which increases calcium and decreases cAMP concentration (Hirai et al., 2001). DP2 receptor is widely expressed; its high expression is observed in human stomach, small intestine, heart and thymus; intermediate expression in the colon, spinal cord and blood; and low expression in the brain, skeletal muscles and spleen (Sawyer et al., 2002).

1.9.5 L-PGDS and H-PGDS, Two enzymes involved in PGD2 Formation

PGD2 synthases (PGDS) catalyse the isomerization of PGH2, a common precursor of various prostanoids, to produce PGD2 in the presence of sulphydryl compounds. Two types of PGDS are involved in this process, the lipocalin-type PGDS (L-PGDS) and the hematopoietic-type PGDS (H-PGDS) (Urade and Hayaishi,
2000b). Experiments on gene knockout mice and enzyme-overexpressing transgenic mice, indicate that the two enzymes are quite different from each other, in terms of their amino acid sequence, tertiary structure, evolitional origin, chromosomal and cellular localization, tissue distribution and also in functional relevance (Urade and Eguchi, 2002).

1.9.5.1 L-PGDS

The \textit{L-PGDS} gene (also known \textit{PTGDS}) was cloned from rat, human and mouse and extended on about 3kb with seven exons and six introns. In mouse, the gene is mapped to chromosome 2B-C1 and in human it is mapped to chromosome 9q34.2-34.3. \textit{L-Pgds} knock out mice grow normally but show several functional abnormalities in nociception (feeling pain) and sleep (Yoshihiro Urade, 2000). L-PGDS is a N-glycosylated protein with a molecular weight (M\textsubscript{r}) of 26000 (26KDa), which is similar to that of B-TRACE, a major protein of human cerebrospinal fluid (Urade and Hayaishi, 2000a). L-PGDS belongs to the lipocalin a family of secretory proteins that are involved in the binding and transport of small hydrophobic molecules. L-PGDS is the first member of its family to be recognized as an enzyme and as a highly glycosylated protein (Urade and Eguchi, 2002). L-Pgd is localized in the central nervous system and male genital organs of various mammals. It is expressed in meningeal cells, epithelial cells of the choroid plexus and oligodendrocytes in the brain and epithelial cells of the epididymis and Leydig cells in the testis and is secreted into the cerebrospinal and seminal plasma (Beuckmann et al., 2000; Gerena et al., 2000; Kanaoka and Urade, 2003). The L-Pgd concentrations in these body fluids are useful for the diagnosis of several neurological disorders, dysfunction of sperm formation and cardiovascular and renal diseases (Urade and Hayaishi, 2000a). L-Pgds is glutathione (GSH) independent Pgd (Urade and Hayaishi, 2000a). Due to functional coupling between L-Pgds and cyclooxygenases, L-Pgds is a dual functional protein, acting as a PGD2-producing enzyme within cells and functioning as a lipophilic ligand-binding protein after secretion into the extracellular space into various body fluids (Urade and Eguchi, 2002).
1.9.5.2 H-PGDS

The human and mouse H-PGDS genes (official name PTGDS2) were cloned and their chromosomal localization were mapped. The Human H-PGDS is a 41kb gene located on chromosome 4q21-22 and mouse H-Pgd is a 28kb gene located on chromosome 3D-E, both consisting of six exons and five introns. H-Pgd is a member of the glutathione S-transferase (GST) family (the family consists of Sigma class GSTs found in invertebrates, such as: squid, nematodes (C. elegans) and flies). H-PGDS genes are expressed in a highly species-specific manner; in human, H-PGDS gene is widely distributed while in mouse, the gene is detected in oviduct and skin (Kanaoka et al., 2000) in somatic and germ cells of embryonic gonads of both sexes (Moniot et al., 2011). In adult mouse ovary, H-Pgd gene is detected in granulosa cell of the growing follicle from primary to the pre-ovulatory stage and in the corpus luteum (Farhat et al 2011).

H-PGDS is a 26KDa cytosolic protein, producing PGD2 in immune and inflammatory cells. It is expressed in various adult rat tissues including thymus, stomach, skin and small intestine (Ujihara et al., 1988; Urade et al., 1990). It is also expressed in rat histiocytes and dendritic cells of the spleen, thymus, skin, in Peyer’s patch of intestine and Kupffer cells in the liver (Urade et al., 1989) and human Th2 cells (Tanaka et al., 2000a). H-Pgd is an enzyme that is strongly homologous to the several members of the GST family and there is no homology between L-Pgd and H-Pgd (Kanaoka et al., 2000). The enzyme is the first member of the GST family to be produced in vertebrates and possesses cleft as an active site, not found in other family members (Kanaoka et al., 1997). The human and the mouse open reading frame encode 199 amino acid proteins that share 78.5% identity. However the amino acid sequences of human and mouse H-PGDS were 81% and 93.5%, respectively identical to sequences of rat H-PGDS (Kanaoka et al., 1997).

1.10 Prostaglandin D2 (PGD2) and its Different Roles

Prostaglandin D2 (PGD2) is actively produced in a variety of tissues as a major prostanoid and is involved in numerous physiological and pathological functions
(Urade and Hayaishi, 2000a). The presence of PGD2 was identified in most of the adult rat tissues, such as the spleen, intestine, bone marrow, and lung, less extended in the epididymis, skin, thymus and brain (Ujihara et al., 1988).

PGD2 is involved in regulation of sleep-awake, inhibition of platelet aggregation, relaxation of smooth muscles, vasodilation and attraction of inflammatory cells, such as Th2 cells, eosinophil’s and basophils (Hirai et al., 2001), release of hormones (Tasaka et al., 1983), regulates body temperature by decreasing it (Ueno et al., 1982) and also released in asthma allergies (Matsuoka et al., 2000). PGD2 is further dehydrated (shown in Figure 8) to produce the J series of prostaglandins, such as PGJ2, Δ12PGJ2 and 15-deoxy- Δ12, 14 PGJ2 (Urade and Eguchi, 2002). This hydrolysis is a non-enzymatic reaction and gives rise to anti-inflammatory products (Surh et al., 2011). PGD2 also seems to be involved in implantation of human embryo, through CRTH2 (DP2), significantly accumulated at the materno-fetal interface (implantation site) in decidua. Trophoblasts, uterine epithelium, and endometrial glands all express H-Pgds (Michimata et al., 2002). As PGD2 is involved in increase of blood flow, inhibition of platelet aggregation and smooth muscle contraction and relaxation, PGD2 may also controls menstrual blood loss (Saito et al., 2002). The L-PGDS protein in bovine seminal plasma is associated with fertility (Gerena et al., 1998).

L-PGDS mRNA expression is male specifically detected in human and mice embryonic and adult gonads (Adams and McLaren, 2002; Tokugawa et al., 1998). L-PGDS mRNA expression has been detected in human male genital organs including testis, prostate, epididymis and Leydig cells (Tokugawa et al., 1998), however H-Pgds mRNA expression is detected in adult female mouse granulosa cells, where PGD2 produced by H-Pgds increased FshR and LhR receptor expression, suggesting important role of PGD2 in FSH signaling. PGD2 produced by H-Pgds is also involved in the regulation of follicular growth by inhibiting granulosa cell proliferation (Farhat et al., 2011). Similarly, H-Pgds mRNA is highly expressed in rat female reproductive system (oviduct) (Kanaoka et al., 1997), indicating that PGD2 may have important roles in oocyte migration and capture, sperm migration and maturation, fertilization and early embryonic development but direct evidences are not yet obtained (Gerena et al., 1998).
Fig. 11. Maintenance of Sox9 expression by Positive feedback loops

After cease of Sry expression, Sox9 makes positive feedback loops to maintain its expression. Except maintaining its own expression, Sox9 also makes positive feedback loops with Fgf9 and PGD2 (Kim et al., 2006b; Moniot et al., 2009; Sekido and Lovell-Badge, 2009; Wilhelm et al., 2007).
1.11 PGD2 and Embryonic Development, including Contribution of my Group

1.11.1 PGD2 signaling and maintenance of the Sox9 expression after down regulation of Sry Expression

In the undifferentiated bipotential gonad, the appropriate Sry expression determines the fate of supporting cells as Sertoli cells, by inducing the expression of the sex determining effector, Sox9 (Sekido 2008). In mice after the downregulation of Sry expression, the maintenance of a sufficient Sox9 expression is crucial for normal development and Sertoli cell differentiation. Since Sry and Sox9 share the same DNA-binding motif, Sox9 was shown to maintain its own regulation (Sekido and Lovell-Badge, 2009). Besides this autoregulatory loop, Fgf9/Fgfr2 an independent signaling pathway maintains the Sox9 expression to promote testicular development (Kim et al., 2006b).

PGD2/L-Pgds is another independent signaling pathway that maintains the Sox9 expression to promote testicular development. Sox9 maintenance after downregulation of Sry expression is shown in Fig 11. In XY gonads, PGD2 independently of Fgf9 stimulates the expression of Sox9 to promote Sertoli cell differentiation (Wilhelm et al., 2007; Wilhelm et al., 2005). Cell mixing culture experiments indicate that PGD2 a paracrine signaling molecule is able to induce Sox9 expression in XX gonadal cells. Similarly in XX cultured gonads PGD2 upregulated Sox9 and Amh mRNA relative level even higher than normally seen in XY gonads at the same developmental stage (Wilhelm et al., 2005). The prostaglandin D synthase (L-Pgds) is produced in Sertoli cells shortly after the onset of Sox9 expression and its expression is in a similar dynamic wave pattern like expression of Sry and Sox9 (Wilhelm et al., 2007). L-Pgds male specific weak expression is detected from late 11.5dpc while strong expression is first detected in 12.5dpc and 13.5dpc testis (Adams and McLaren, 2002). Promoter analysis identified a paired SOX/SRY binding site on Pgds promoter, where only Sox9 was able to bind as a dimer to trans-activate Pgds. The ablation of Sox9 expression in embryonic Sertoli cells abolished L-Pgds transcription (Moniot et al., 2009). However Pgds expression is not affected by ectopic Sry expression
(Wilhelm et al., 2007). In this way L-Pgds/PGD2 is second amplification loop of Sox9 expression, where Sox9 initially activates L-Pgds to produce PGD2, then PGD2 activates Sox9 transcription and its nuclear translocation (Malki et al., 2005b) (Moniot et al., 2009).

1.11.2 PGD2 signaling and subcellular localization of Sox9 expression

Like SRY, each SOX protein including SOX9 protein has two nuclear localization signal (NLS) motifs, located on its HMG domain. These NLS motifs lie at each end of HMG domain, (N terminal and C terminal) and support in DNA binding and protein transport in nucleus (Malki et al., 2010; Poulat et al., 1995; Sudbeck and Scherer, 1997). The N-terminal, a bipartite NLS interacts with calmodulin (Hanover et al., 2009) while C-terminal NLS interacts with importin-β (nuclear import factor) (Forwood et al., 2001).

Similarly, Sox9 transcriptional activity depends on SOX9 cytoplasmic-nuclear transport and fine balance between import and export determines the level of transcriptionally active Sox9 in the nucleus. Mutations in both N and C-terminal NLSs of SOX9 protein that blocked its nuclear localization, resulted in sex reversal, indicate the importance of SOX9 nuclear localization in male sex differentiation (Sim et al., 2008).

Before sexual differentiation, SOX9 protein is initially found in the cytoplasm of gonads of both sexes. But at the time of testis differentiation, it becomes localized to the nuclear compartment in males whereas it is down regulated in females. Cytoplasmic SOX9 protein is translocated to the nucleus where it activated target genes to induce programmed cell differentiation in humans (de Santa Barbara et al., 2000) and in mice (Morais da Silva et al., 1996b). The male-specific reorganization of the microtubules in Sertoli cells of embryonic gonads at the time of sexual differentiation and testis cord formation facilitates SOX9 cytoplasmic retention (Malki et al., 2005a).
Figure 12. Model for the regulation of SOX9 cytoplasmic retention and nuclear localization through activation of PGD2/PKA pathway. (Malki et al., 2005b).
The role played by PGD2 in the nuclear localization of SOX9 protein to promote male germ cell differentiation program is discussed below.

PGD2 via its receptor DP1, mediates SOX9 nuclear translocation, where PGD2 through cAMP-dependent protein kinase A (PKA) induces phosphorylation of Sox9 on its two PKA sites and promotes nuclear localization by its binding to the nucleocytoplasmic protein importin-β (Malki et al., 2005b) (Figure 12).

Mouse embryonic gonads null for \textit{L-Pgds}\textsuperscript{-/-} displayed reduced level of \textit{Sox9} transcripts and aberrant SOX9 protein subcellular localization up to 13.5dpc (Moniot et al., 2009).

As \textit{L-Pgds} expression is downstream of Sox9, so PGD2 produced by L-Pgds cannot induce SOX9 protein initial subcellular localization. Hence for the initial subcellular localization of SOX9 protein, another enzyme hematopoietic Pgds (H-Pgds) produces PGD2. \textit{H-Pgds} is expressed in somatic and germ cells of the embryonic gonads of both sexes at 10.5dpc before the onset of L-Pgds. When H-Pgds activity was inhibited by HQL-79 inhibitor (specific inhibitor of H-Pgds activity), it caused impaired nuclear translocation of SOX9 protein in 11.5dpc Sertoli cells.

These results are confirmed in \textit{H-Pgds}\textsuperscript{-/-} mutant male embryonic gonads at 11.5dpc, suggesting a role for H-Pgds-produced PGD2, in the initial nuclear translocation of SOX9 and subsequent mouse testicular differentiation (Moniot et al., 2011).

In this way, \textit{Sox9} transcriptional regulation is controlled in three phases (i) \textit{Sry} dependent initiation (ii) \textit{Sry} independent upregulation (iii) maintenance after the downregulation of \textit{Sry} expression (Sekido and Lovell-Badge, 2009).

These data indicate that PGD2 signaling is necessary for SOX9 nuclear translocation (Moniot et al., 2009; Moniot et al., 2011) and is sufficient in \textit{Sry} negative cells to upregulate Sox9 expression for differentiation into Sertoli cells (Wilhelm et al., 2005).
OBJECTIVE OF
THEESIS
OBJECTIVE OF THIS WORK

In the life of multicellular organisms, sexual reproduction is a vital process, which helps to propagate the generation of an individual for the continuity of life. In multicellular vertebrates, including mammals, the development of male or female sex, takes place during embryonic time of life, but “how an individual’s sex is determined” is a question, scientists are engaged to answer. In mammals the sexual development of embryo is achieved by the differentiation of gonad and subsequent germ cell differentiation. During embryonic stage gonadal primordium is a common tissue, which can develop into either a testis or an ovary (Brennan and Capel, 2004). The Y chromosome located gene Sry directs the development of gonad to differentiate into either a testis or an ovary, which will then direct sexual development of the rest of the embryo (Koopman et al., 1991). Sry activates the expression of Sox9 in somatic cells of XY embryo and then Sox9 induces the differentiation of gonadal somatic cells into Sertoli cells (Sekido et al., 2004) and secretion of hormones that favor testis cord formation and development of sex accessory organs (Behringer, 1994). In mice, the gene Sry is expressed for very short time from 10.5dpc to 12.5dpc (Kashimada and Koopman, 2010) and Sox9 maintained expression is required for normal gonadal development; hence Sox9 takes help from other signaling molecules to maintain its expression. Among these molecules, Fgf9 (fibroblast growth factor 9) is one of them that makes a positive feedback loop with Sox9 to maintain its expression; (Kim et al., 2006b).

Since many years my group is engaged in discovering roles of Sox9 in sex development. They found that PGD2 produced in embryonic gonads by L-Pgds and H-Pgds, is a signaling molecule that makes a positive feedback loop with Sox9, acting in parallel to the Fgf9/Sox9 loop and maintains the Sox9 expression throughout the embryonic stages. The shuttling between cytoplasm and nucleus is also important for Sox9 functions. This nuclear localization of SOX9 protein is induced with the help of
two nuclear localization signals located on HMG domain N-terminal NLS and C-terminal NLS.

My group has discovered that PGD2 provides nuclear localization to Sox9, where PGD2 is first produced by H-Pgds and then by L-Pgds. The production of PGD2 by prospermatogonia during sex differentiation has already been shown by Adams and McLaren in 2002, Moniot et al 2009 and Moniot et al 2011 but its function in germ cell differentiation is not yet known.

My project was to investigate the role of PGD2 produced by both H-Pgds and L-Pgds in the Sertoli cells and germ cells of XY embryonic gonads, for the differentiation of germ cells.

In this project we have used mice knockout for both L-Pgds and H-Pgds genes and mice knockout for PGD2 receptor (DP2). We also have used chemicals to block PGD2 receptors to find out the role of PGD2 in germ cell differentiation.

We used mouse model for this work because it has many advantages, such as enough data is available for mouse model concerning to this work, large number of verified antibodies available in market, Short gestation period, enough number of babies per litter, easy to handle and not expensive. We used fetal stages as germ cell differentiation process starts from embryonic life.
RESULTS
CHAPTER 2. RESULTS

Introduction

Delayed Sry expression or insufficient Sox9 expression does not guarantee for testis formation and male sex development and will allow somatic cells of primordial gonad to differentiate into Granulosa cells and promote female sex development (Albrecht and Eicher, 2001; Qin and Bishop, 2005). Once female fate is decided, germ cells in the embryonic ovary stop proliferation and enter into meiosis by 13.5dpc (Bowles and Koopman, 2007). In the developing ovary the presence of RA induces the expression of Stra8 gene, which is known as gatekeeper for entry of germ cells in meiosis (Bowles et al., 2006; Koubova et al., 2006). On the other hand if male fate is decided, germ cells in the developing testis stop proliferation and enter into quiescence by 12.5dpc, which is also called mitotic arrest (Hilscher et al., 1974). In the developing testis, germ cells cannot enter into meiosis because Sertoli cells produce Cyp26b1 enzyme that degrades RA and does not allow the induction of Stra8 expression (MacLean et al., 2007). Mitotic arrest is an important event of male germ cell differentiation program, during which various G1/S phase checkpoint regulators are shown active. In mice during male germ cell mitotic arrest, Retinoblastoma 1 (RB1), a potent cell cycle regulator is required for correct germ cell entry into G1/G0 arrest (Spiller et al., 2010). Germ cell mitotic arrest also requires the upregulation of various cell cycle inhibitors P21cip1 (Spiller et al., 2009a), P27kip1, P15INK4b and P16INK4a and suppression of cell cycle activators CyclinE1, CyclinE2 and CyclinD3 (Western et al., 2008). Male germ cell differentiation is supported by the activation of genes, where Nanos2 plays critical role in the maintenance of male germ cells (Suzuki and Saga, 2008) and its loss results in complete loss of germ cell due to apoptosis (Tsuda et al., 2003). Loss of Dnd1 gene results in severe germ cell deficiency (Youngren et al., 2005). The up-regulation of the de novo DNA methyltransferase genes is required for male germ cell development, where Dnmt3a and Dnmt3b levels increased by 10-fold between 13.5dpc to 15.5dpc while Dnmt3l transcription increased more than 100 fold only in male germ cells (Western et al., 2010). The male germ cell differentiation requires the
suppression of pluripotency. During male germ cell differentiation, the pluripotency regulators Sox2 and Nanog are transcriptionally suppressed while Oct4 is post transcriptionally suppressed (Western et al., 2010). Today we know gene networks that regulate cell cycle and participate in germ cell differentiation but still we lack complete knowledge about the inducer(s) of these processes. We do not completely know about mis-regulations that cause abnormal cell cycle and development of testicular tumors. Many genes are shown to regulate cell cycle and loss of their function is involved in testicular germ cell tumor development.

PGD2 is already shown to be expressed in Sertoli cells and germ cell at the time of somatic sex determination and germ cell differentiation. PGD2 is shown to be involved in male somatic sex determination but we are interested to know the role of PGD2 played in male germ cell differentiation.

Previously my group has shown that Prostaglandin D2 (PGD2), produced in mouse embryonic testis during Sertoli cell differentiation, participates in male sex development by inducing nuclear translocation of SOX9 protein and by maintaining Sox9 gene expression. Using L-Pgds-/- or H-Pgds-/- mouse models, they showed that Sox9 initially activates L-Pgds to produce PGD2, and then PGD2 activates Sox9 transcription and its nuclear translocation (Malki et al., 2005b; Moniot et al., 2009; Moniot et al., 2011).

Here in this study we have investigated the role of PGD2 in male germ cell differentiation. In the developing mouse testis PGD2 is produced by the two enzymes, Lipocalin-type prostaglandin D2 synthase (L-Pgds) and Hematopoietic-type prostaglandin D2 synthase (H-Pgds) in Sertoli cells and differentiating male germ cells (Adams and McLaren, 2002; Moniot et al., 2009). By using L/H-Pgds double knockout, PGD2 receptor DP2 knockout mice and by ex vivo culture experiments on isolated germ cells and somatic cells, we show that PGD2 signaling produced by L-Pgds and H-Pgds in Sertoli cells and germ cell is required for proper male fetal germ cell differentiation.
MANUSCRIPT
Prostaglandin D$_2$ 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 β 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$_2$ (PGD$_2$), 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$^{Cip1}$ and by repressing pluripotency markers. We show that PGD$_2$ 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$_2$ 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$_2$, 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$_0$/G$_1$ 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$_2$–S phase checkpoint regulators such as the CDK inhibitors p27$^{Kip1}$ (Cdkn1b – Mouse Genome Informatics) and p21$^{Cip1}$ (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 Dmr1 influences cell cycle arrest by directly regulating the expression of the CDK inhibitor p19$^{ink}$ (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$^{Cip1}$ 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$_0$, (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 Tgfβ superfamily members have also been associated with male germ cell differentiation, including Tgfβ2 (Miles et al., 2013; Moreno et al., 2010) and activin βs (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 stage 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
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; Gnerquin 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 regulation of male foetal germ cells and 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). 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 Tgfβ2 signalling (Miles et al., 2013). However, in double mutants for *Fgf9* and *Wnt4*, germ cells do not enter meiosis and the male marker Dnm3l 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 D2 (PGD2), in this process. PGD2 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.

PGD2 is produced in the developing mouse testes by two enzymes: lipocalin-type prostaglandin D2 synthase (L-Pgds or Pgds), 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 Pgds2), 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−/−, i.e. depleted for all PGD2*) and *Dp2−/−* 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−/−* tests) we show that both somatic- and germ cell-produced PGD2, 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 PGD2-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−/−) mutant embryos (Qu et al., 2006). At the somatic level, their phenotype was similar to that previously reported for L-Pgds−/− tests (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 Amb 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 *Dnr1* 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−−/− germine, as the phenotype of the single mutant L-Pgds−−/− or H-Pgds−−/− 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−−/− gonads showed that, at E13.5, the percentage of S-phase positive germ cells in the PGD2-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−−/− or H-Pgds−−/− 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−−/− 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 G0, 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 PGD2 signalling might control the expression of cell cycle genes as several key regulators of the G1/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*−/− and *Rb* in L/H-Pgds−−/− tests (KO) at E13.5 compared with wild type (Fig. 1H,I), as well as a significant increase in the expression of cyclin E1 (*Ccn1*) and cyclin E2 (*Ccn2*) 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...
the E13.5 stage (data not shown). Taken together, our results suggested that PGD2, 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.

**L/H-Pgds depletion leads to an altered transcriptional profile of germ cell-specific and cell cycle genes**

To identify genes regulated by PGD2, we performed an RNA sequencing analysis from wild-type and L/H-Pgds−/− 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-qPCR, 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\(^{Cip1}\) and p57\(^{Kip2}\), and an increase in cell cycle activators such as the retinoblastoma-like gene p130\(^{(Rb)}\) and in oncogenes such as Kit, Mybl1 and Erbb3. Furthermore, several regulators of p21\(^{Cip1}\) expression were either upregulated \( (Elavl2 \text{ (Wisniak et al., 2011; Yoon et al., 2012)}, \text{Trim71} \text{ (Chang et al., 2012)}, \text{Pten} \text{ (Luo et al., 2013)} \text{ and } \text{Cpeb4} \text{ (Novoa et al., 2010)) or downregulated } [\text{Rbm38 (Feldstein et al., 2012)} \text{ and } \text{Dmd1} \text{ (Zhu et al., 2011)}] \text{ (supplementary material Fig. S4B)}. Significantly, the crucial male germ cell gene Nanos2 (Suzuki and Saga, 2008) was downregulated in the absence of PGD2. Moreover, numerous regulators of pluripotency, such as Sox2 (Takahashi and Yamanaka, 2006), Pog2 (Jiang et al., 2007), Nr2c2 (Wagner and Cooney, 2013), 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 PGD2 (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−/− foetal testes in more detail.

**PGD2 regulates the cell cycle inhibitor p21\(^{Cip1}\) expression**

As the CDK inhibitor p21\(^{Cip1}\), a potential regulator of the mitotic arrest process (Western et al., 2008), was found to be downregulated in mutant E13.5 L/H-Pgds−/− gonads, we next evaluated the direct action of PGD2, on p21\(^{Cip1}\) expression in the male germ line. Germ cells from E12.5 and E13.5 testes in more detail.

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**Fig. 1. PGD2 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 μm. (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<0.001. (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 μm. (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\(^{Kl-67}\) 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 μm. (H-K) Expression of cell cycle genes p21\(^{Cip1}\), 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.
Table 1. Cell cycle and cell proliferation genes up- and downregulated in E13.5L/H-Pgds mutants relative to wild-type testes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change mutant/wild type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cpeb4</td>
<td>12.29</td>
<td>Cytoplasmic polyadenylation element-binding protein 4</td>
</tr>
<tr>
<td>Myb1</td>
<td>11.12</td>
<td>Myeloblastosis oncogene-like 1</td>
</tr>
<tr>
<td>Rcor1</td>
<td>10.80</td>
<td>REST co-repressor 1</td>
</tr>
<tr>
<td>Pd3a</td>
<td>9.18</td>
<td>Phosphodiesterase 3A, cGMP inhibited</td>
</tr>
<tr>
<td>Erbb4</td>
<td>8.30</td>
<td>v-erb-b2 erythroblastic leukemia viral oncogene homolog 4</td>
</tr>
<tr>
<td>Crebbp</td>
<td>6.14</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>Erbb3</td>
<td>5.72</td>
<td>v-erb-b2 erythroblastic leukemia viral oncogene homolog 3</td>
</tr>
<tr>
<td>Atm</td>
<td>2.14</td>
<td>Ataxia telangectasias mutated homolog</td>
</tr>
<tr>
<td>Arid4a</td>
<td>2.08</td>
<td>AT rich interactive domain 4A (Rbp1 like)</td>
</tr>
<tr>
<td>Cdk17</td>
<td>2.02</td>
<td>Cyclin-dependent kinase 17</td>
</tr>
<tr>
<td>Cdkn2aip</td>
<td>1.94</td>
<td>Cdns2A interacting protein</td>
</tr>
<tr>
<td>Pak3</td>
<td>1.92</td>
<td>p21 protein (Cdc42/Rac)-activated kinase 3</td>
</tr>
<tr>
<td>Rb1t</td>
<td>1.88</td>
<td>Retinoblastoma-like 1 (p107)</td>
</tr>
<tr>
<td>Rb2t</td>
<td>1.78</td>
<td>Retinoblastoma-like 2 (p130)</td>
</tr>
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</tr>
<tr>
<td>Cdk5</td>
<td>0.65</td>
<td>Cyclin-dependent kinase 4</td>
</tr>
<tr>
<td>Cdk6</td>
<td>0.52</td>
<td>Cyclin-dependent kinase 5, regulatory subunit 2 (p39)</td>
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<td>Cdk2ap2</td>
<td>0.58</td>
<td>Cdns2-associated protein 2</td>
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<td>Camk1</td>
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<td>Calcium/calmodulin-dependent protein kinase</td>
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<tr>
<td>Cdc37</td>
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<td>Cell division cycle 37 homolog (-like 1)</td>
</tr>
<tr>
<td>Cdkn1c</td>
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<td>Cyclin-dependent kinase inhibitor 1C (P57)</td>
</tr>
<tr>
<td>Cdkn1a</td>
<td>0.47</td>
<td>Cyclin-dependent kinase inhibitor 1A (P21)</td>
</tr>
</tbody>
</table>

PGD₂ 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 E12.5 and E13.5 stages in the presence of PGD₂, we observed a direct repressive effect of PGD₂ 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 PGD₂ produced by both the somatic and germ cell compartments contributes to the full repression of Sox2 expression (Fig. 3C). We also confirmed that PGD₂ is produced by both the somatic and germ cell lineages, following chemical fixation of PGD₂ 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 PGD₂ is involved in the downregulation of the
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.

**PGD2 signalling contributes to the establishment of the male cell differentiation**

We next asked whether PGD2 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 to E17.5 was lower in mutant than in wild-type gonads (Fig. 4A).

Despite this low level of the *Nanos2* transcript level at E13.5, E15.5 and E17.5, we had observed changes in the expression of genes known to be important for male germ-cell development, such as *Nanos2* and *Cyp26b1*, another male-specific gene. As PGD2 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 PGD2 produced by both the somatic and the germ cell compartments was necessary for the full activation of the *Nanos2* expression (Fig. 4C). *Dmnt3l*, 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 PGD2 treatment directly repressed the expression of the *Stra8* transcript (Fig. 4I). Furthermore, PGD2 was able to diminish the activation of *Stra8* by RA by 50%, confirming the negative effect of PGD2 on *Stra8* expression (Fig. 4I). However, we cannot exclude the possibility that PGD2 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 PGD2 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*−/− XY gonads from E13.5 to E17.5 (Fig. 4K).

**PGD2 signals through its Dp2/Crth2 receptor to control the male germ line differentiation**

As PGD2 can act on both the Dp1 and Dp2/Crth2 G-protein-associated receptors (Matsuoka et al., 2000; Nagata and Hirai, 2007; et al., 2007; Suzuki and Saga, 2008), we next tested whether their increased 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 PGD2 treatment directly repressed the expression of the *Stra8* transcript (Fig. 4I). Furthermore, PGD2 was able to diminish the activation of *Stra8* by RA by 50%, confirming the negative effect of PGD2 on *Stra8* expression (Fig. 4I). However, we cannot exclude the possibility that PGD2 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 PGD2 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*−/− XY gonads from E13.5 to E17.5 (Fig. 4K).
2003), we first determined the localization of the Dp1 (Pgdr) and Dp2 (Pgdrr2) 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, whereas Dp1 was only expressed in 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, the different percentages of EdU-positive germ cells observed in wild-type testes (Moniot et al., 2009, 2011; Wilhelm et al., 2007). Our analysis of gonads from double L- and H-Pgds (L/H-Pgds−/−) and Dp2−/− mutant embryos has shown that PGD2, acting through its receptor CAY10471, significantly extends 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).
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$_2$ is indeed synthesized by both the somatic and germ cell populations and provide evidence that both sources of PGD$_2$ 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$^{cip1}$ 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 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$_2$.

In the absence of PGD$_2$, 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$_2$ signalling pathway contributes to mitotic arrest in male foetal germ cells. This impaired mitotic arrest in the L/H-Pgds$^{-/-}$ testes was similar to that observed in mutant gonads for Dmnt1 (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$^{cip1}$, a key regulator of the G1-S phase checkpoint (Fotedar 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$_2$. Furthermore, in L/H-Pgds$^{-/-}$ germ cells, we detected the p21$^{cip1}$ protein mainly within the cytoplasm, suggesting a role for PGD$_2$ in the post-transcriptional regulation of p21$^{cip1}$ and potentially explaining the reduced cell-cycle inhibitory activity of p21$^{cip1}$ observed in the absence of PGD$_2$ (Starosta et al., 2010; Wu et al., 2011). As Dnd1 expression is downregulated in L/H-Pgds$^{-/-}$
gonads, PGD2 signalling might affect p21Cip^1 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 regulation (Jung et al., 2010), both at the transcriptional (Jung et al., 2010), by activating Pde3 and Pten, and post-transcriptional levels (Elavl2, Dnd1, Rbm38, Dds4 and Trim71). Thus, these data show that PGD2 might be a pathway acting early at multiple levels of p21^Cip^1 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 PGD2 appears to be independent of Dmrt1 (Krentz et al., 2009) as Dmrt1 expression was not modified by PGD2. 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 PGD2 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 PGD2 and might participate in this regulation. In the absence of PGD2, Nanog, Oct4 and Sox2 are ectopically expressed in mutant foetal testes and display cytoplasmic localisation, suggesting that PGD2 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 PGD2 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-Chr10^MOLF/Fe (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 PGD2 target gene Lifid1 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 PGD2 pathway as one of the earliest signalling pathway involved in the male germ cell
determination, showing that PGD₂ is a male fate-promoting factor. As PGD₂ 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_Crhi² 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-ethyl-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)-PGD₂, 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 (Alcya-Ig, Molecular Probe) were used. Histology images were captured with a Leica DM6000 fluorescent microscope or with a Leica SP6-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 (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 SP6-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 S2, as previously described (Woodward et al., 2011). See methods in the supplementary material for further details.

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 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.

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 benzonase (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 electrophoblated 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.

Acknowledgements

We thank the staff of the IGH animal care facility, particularly Elodie Gavios, Frédéric Gallardo and Florence Arnal. We thank Dr Julien Cau and Amélie Sarrazin from the Imagery platform of IGH (MRI Montpellier) for their help in imagery and quantification analysis with Imagej. We are grateful to Prof. Gabriel Liva, Dr Peter Follette and Dr Rosemary Kieran for critical reading of the manuscript.

Competing interests

The authors declare no competing financial interests.

Author contributions


References


Supplementary Material

Supplementary Methods

In vivo EdU incorporation and CAY10471 treatments

EdU (5-ethynyl-2′-deoxyuridine) solution (10mM) (Sigma Aldrich) was made in DMSO and aliquots were kept at -20°. 350µl of a 3-fold dilution in phosphate-buffered saline (PBS) was intraperitoneally injected into the pregnant female, 2h before dissecting the embryos. Detection of the EdU positive cells on testis sections was performed using Alexa Fluor Azide 488 with the Click-iT® EdU Assay accordingly to the supplier’s instructions (Invitrogen).

The specific DP₂ antagonist CAY10471, ((+)-3-[(4-fluorophenyl-sulfonyl] methyl amino]-1,2,3,4-tetrahydro-9H-carbazole-9-acetic acid, Cayman Chemical) (Ulven et al., 2006; Woodward et al., 2011) was made up to a stock solution of 5mg/ml in ethanol and was stored at -20°C. Before use, CAY10471 was diluted four fold in PBS. Mice were treated with oral doses of 5 mg/kg/day CAY10471 (150 µg per 30g mouse) from the E10.5 embryonic stage to E12.5 and E13.5 as indicated in the text. Mice were also treated from E10.5 to E13.5 with oral doses of the DP₂ agonist, 9α, 15R-dihydroxy- 11- oxo- prosta- 5Z, 13E- dien- 1- oic acid (15(R)-PGD₂) or DP₁ antagonist, 3- [(2-cyclohexyl- 2- hydroxyethyl)amino]- 2, 5- dioxo- 1- (phenylmethyl)- 4- imidazolidineheptanoic acid (BW A868C), (Cayman Chemical); 15(R)-PGD₂ and BW A868C were administrated at 5 mg/kg/day.

Control mice were treated with an equivalent volume of 20% ethanol in PBS.

With the CAY10471 antagonist, the following were analyzed: at E12.5, two control mice (14 male embryos) and two CAY-treated mice (13 male embryos); at E13.5, five control mice (30 male embryos) and 8 CAY-treated mice (44 male embryos). For the 15(R)-PGD₂ and BW A868C experiments, 3 treated mice (E13.5) were analyzed for each treatment (23 and 15 male embryos, respectively). Mesogonads or mesonephros-free gonads were pooled within each litter and processed for immunofluorescence or RNA work, respectively.
**Immunomagnetic germ cell and somatic cell isolation, culture, expression and proliferation studies**

For each experiment, 40 to 90 male E12.5 or E13.5 gonads (without mesonephros) from 5 to 12 littermates were pooled and were dissociated according to Guerquin et al. (2010). Germ cell isolation using the Ssea-1 antigen was performed as previously described (Moniot et al., 2011), using magnetic sorting (column MS MACS, Miltenyi Biotech, Germany). The SSEA1-negative cell fraction containing the somatic cells was also recovered.

To analyze *Dp1*, *Dp2*, *L-Pgds* and *H-Pgds* expression, purified E13.5 germ cell and somatic fractions were prepared through three independent experiments from dissected male gonads (from 46 to 54 gonads). Total RNA was prepared by the TRIZOL technique and was submitted to RT-qPCR as described previously.

For *in vitro* cultures in the presence or absence of PGD2, 10⁴ male germ cells purified from E12.5 or E13.5 gonads were plated in a 48-well plates using a previously described technique (Bowles et al., 2010). After 18h of culture, cells were lysed in 20 µl and processed towards RNA and cDNA using the RealTime Ready Cell Lysis kit (Roche). Proliferation of cultured germ cells in the described conditions was measured in triplicates after 24h, using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega).

For culture of mixed purified cells, E12.5 *L/H-Pgds*⁻/⁻ and WT testes were dissected and dissociated according to Munger et al. (Munger et al., 2013); somatic and germ cells were purified using magnetic sorting for each genotype and co-cultures of 100000 somatic (SOM) cells (WT or mutant) and 30000 germ (GC) cells (WT or mutant) were performed for 48h. Four kinds of co-cultures were performed: SOM WT+ GC WT: the normal situation; SOM KO+ GC WT: situation in which PGD₂ is only produced by germ cells; SOM WT+ GC KO: situation in which PGD₂ is only produced by somatic cells; SOM KO+ GC KO: the *L/H-Pgds* KO situation. Total RNA was prepared by the TRIZOL technique and was submitted to RT-qPCR as described previously.
Detection of produced PGD$_2$ using the EicosaCell technique

For intracellular detection of prostanoid-synthesizing compartments of the cells, the EicosaCell protocol was performed on dissociated E12.5 wild type XY embryonic cells. 250000 cells were cultured on glass coverslip in a 24-wells plate for 24h (Munger et al., 2013). The covalent cross-link of the lipid mediators at their sites of synthesis by the EDAC product (ethyl-3-(3-dimethylaminopropyl) carbodiimide) was performed after treatment of the cells by 0.2% EDAC in Hanks-buffered salt solution for 30min at 37°C (Bandeira-Melo et al., 2011). EDAC immobilizes newly synthesized prostanoids by cross-linking carboxyl groups to the amine of adjacent proteins localized at eicosanoid-synthesizing compartments. Cross-linked PGD$_2$ was identified using immunofluorescence experiments with a specific anti-PGD$_2$ antibody with germ cell marker TRA98.

mRNA expression profiling and analysis

Male gonads from six WT and six mutant littermates were collected and were pooled by two litters each (between 10 to 18 male gonads per pool). Three biologically separated pools from WT and three from mutant litters were made, total RNA was extracted using the TRIZOL reagent. Libraries were generated using the « Truseq RNA sample prep kit V2 » from Illumina (RS-122-2001). Briefly, the polyadenylated RNA were isolated from 600ng of total RNA using oligo(dT) magnetic beads. Isolated mRNAs were fragmented and primed using random hexamer before reverse transcription. A second strand synthesis was performed. Double stranded cDNA was repaired resulting in blunt ends double stranded cDNA. Both 3' ends were adenylated and Illumina's indexed adapters were ligated to the cDNA. A 15 cycles PCR was performed for each library using Illumina's PCR primers. Libraries were validated using a DNA1000 chip on a Bioanalyzer (Agilent) and quantified by qPCR using the « Library Quantification Kit - Illumina/Universal » from KAPA (KK4824). Prior to clustering, 2 sets of 3 libraries were equimolarly pooled. Each pool was denatured using NaOH and diluted to 8 pM. Clustering and 50nt single read sequencing were performed on a single lane per pool following the manufacturer's instructions.

Image analyses and basecalling were performed using the HiSeq Control Software (HCS 1.5.15) and Real-Time Analysis component (RTA 1.13.48). The quality of the data was assessed using fastqc from Development | Supplementary Material
the Babraham Institute (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and the Illumina software SAV (Sequence Analysis Viewer). Demultiplexing, alignment and RNA counting was performed using CASAVA 1.8.2 (Illumina). Alignment was made with eland_rna on the mm9 version of mouse genome and on several contaminants (the ribosomal RNA sequences 28S, 18s and 5s, the mitochondrial chromosome, the PhiX genome and the Illumina adaptors). Differentially expressed genes were identified using the Bioconductor (Gentleman et al., 2004) package edgeR(2-2) (v2.6.2) (Anders and Huber, 2010). Genes with less than 15 reads (cumulating the two analysed samples) were filtered and thus removed from the analysis. Data were normalized using Upper Quartile normalization factors (Bullard et al., 2010). Genes with adjusted p-value less than 1% (according to the FDR method from Benjamini-Hochberg) were declared differentially expressed. To perform the functional analysis with the Gene Ontology (GO) annotations, Bioconductor packages were used. The analysis of Functional Profiles for the 3 ontologies was performed with the goProfiles package. To identify the over-represented GO terms, a Fisher's exact test was used with the weight method implemented in the topGO (Alexa et al., 2006) package. As confidence threshold we used a p-value of 1%. The differentially expressed genes were compared with all genes on the genome. The GO categories were found in the Org.Mm.db.eg package based on the gene reporter EntrezGeneID.

Statistical analysis

For quantitative analysis, each sample was measured in triplicate in three separate RT reactions performed from at least 3 different gonad pools and quantification was performed using a second derivative calculation method with LC480 software version 1.5 (Roche). Statistical analysis with PRISM 6 software (GraphPad Software) was performed using the Student’s t-test (comparison of two individual data) or an ANOVA test with the Geisser-Greenhouse correction (when comparing more that two data) (Figures 3C, 4J, 4K) for the qPCR experiments, and using the Fisher’s exact test for the cell counting experiments, and the results were considered statistically significant at a P<0.05. For whole gonad tissue (E13.5, E15.5 and E17.5), statistical significance was assessed between the double KO mutant and the wild type samples. For the germ cell culture studies, statistical significance was assessed between the non-treated and (PGD2, RA, BW A868C, BAY-U3405) treated cultures. For
the germ cell proliferation studies using immunofluorescence experiments on whole gonads, 110 to 1200 MVH- or TRA98-positive germ cells were counted on different fields of 4 to 10 different gonads and their proliferate states were reported on a graph, representing the percentages of proliferating cells. Figure 1D: E13.5 KO n=350, WT n=400 and E15.5 KO n=450, WT n=400; Figure 1E: E13.5 KO n=810, WT n=750; Figure 1F: E13.5 KO n= 950, WT n=1200, E15.5 KO n=1200, WT n=850 and E17.5 KO n=730, WT n=540; Figure 5E: KO n= 460, WT n=770; Figure 5F: KO n=540, WT n= 310.

Supplementary References


Supplementary Figures

Fig. S1. Somatic cells differentiate normally in male L/H-Pgds\textsuperscript{−/−} gonads. (A) Co-immunofluorescence of SOX9 (red) and AMH or TRA98 (green) on E13.5 and E17.5 KO and WT testes. Scale bars represent 100 \( \mu \)m. (B) RT-qPCR analysis of sex differentiating Sox9, Amh, Dmrt1, Fgf9 and Notch1 gene expression in E13.5 and E15.5 or E17.5 KO and WT gonads. Rps29 RNA was used as the normalization control. Data are represented as mean±/− SEM, n=3.

Fig. S2. Somatic and germ cells phenotype is normal in L-Pgds\textsuperscript{−/−} and H-Pgds\textsuperscript{−/−} gonads. (A, D, E) Co-immunofluorescence of SOX9 (red), TRA98 (green) and Laminin (blue) (A), NANOG (red), TRA98 (green) and Laminin (blue) (D) on E17.5 wild type (WT), L-Pgds\textsuperscript{−/−}, H-Pgds\textsuperscript{−/−} and L/H-Pgds\textsuperscript{−/−} testes, and STRA8 (red), TRA98 (green) and Hoescht dye (HST, blue) (E) on E17.5 L-Pgds\textsuperscript{−/−} and L/H-Pgds\textsuperscript{−/−} testes. Bars=100\( \mu \)m. Arrows indicate cytoplasmic NANOG staining (D) or nuclear STRA8 staining (E) on enlarged panels. (B) Quantification of germ cells within the testis cords of E17.5 WT, L-Pgds\textsuperscript{−/−}, H-Pgds\textsuperscript{−/−} and L/H-Pgds\textsuperscript{−/−} testes, is represented as numbers of TRA98 positive germ cells per field from different independent gonads (n=10). (C) Quantification of TRA98 germ cells that are positive for mitotic marker phosphoHistoneH3 in E13.5 WT, L-Pgds\textsuperscript{−/−}, H-Pgds\textsuperscript{−/−} and L/H-Pgds\textsuperscript{−/−} testes, is represented in percentage of pH3 positive cells amongst TRA8 germ cells (n= 110 to 150).

Fig. S3. Proliferation of somatic cells is not affected in L/H-Pgds\textsuperscript{−/−} and Dp2\textsuperscript{−/−} gonads. Germ cell proliferation in E13.5 L/H-Pgds\textsuperscript{−/−} (KO) (A) or Dp2\textsuperscript{−/−} (B) and wild-type (WT) testes is evaluated by co-immunofluorescence using Sertoli marker SOX9 (red) and EdU (green) (A) or GATA4 (green) and phosphoH3 (red) (B). Arrows indicate proliferative Sertoli cells (A-B). Bars=75\( \mu \)m. The corresponding graphs on the right, indicate quantification of SOX9 positive cells (KO, n= 250; WT, n=300) that are positive for EdU in E13.5 L/H-Pgds\textsuperscript{−/−} mutant and wild type gonads, represented as
percentages of EdU positive cells (A) and GATA4 positive cells (Dp2−/−, n=150; WT, n=125) that are positive for pH3 in E13.5 Dp2−/− and wild type, represented as percentages of pH3 positive cells (B).

**Fig. S4. PGD₂ signaling regulates germ cell-specific and cell cycle genes.** (A-B) RT-qPCR analysis of stem cell (A) and cell cycle (B) gene expression levels in E13.5 WT and L/H-Pgds mutant testes. Oct4 RNA was used as the normalization control. Data are represented as means of fold activation of L/H-Pgds mutant expression versus wild type gonads (expression in WT was set at 1) and SD (error bars) from three independent experiments. Statistical significance was calculated on raw expression values from each experiment: d: p < 0.05; c: p < 0.01; b: p < 0.001; a: p < 0.0001; ns = not significant. (Elval2): Embryonic lethal abnormal vision Hu antigen, (Trim71): Tripartite motif-containing 71, (Pten): Phosphatase and tensin homolog, (Rbm38): RNA binding motif protein 38, (Dnd1): dead end homolog 1, (Mybl1): myeloblastosis oncogene-like1, (Erbb4): erythroblastemic leukemia viral oncogene, (Peg3): paternally expressed-3, (Nr2c2): nuclear receptor subfamily2, (Lhx1): LIM homeobox protein1, (Sall1): Sal-like 1, (Mtf2): Metal response element binding transcription factor 2, (L1td1): LINE-1 type transposase domain containing 1, (Tet1): methylcytosine dioxygenase, (Gtf3c3): general transcription factor IIIc, (Hdac4): Histone deacetylase 4, (Pde3): phosphodiesterase3, (Arid4a or RBBP1): Retinoblastoma-binding protein 1.

**Fig. S5. Pluripotent markers are expressed in the nucleus of the germline in L/H-Pgds−/− as in WT gonads.** Co-immunofluorescence with anti-NANOG or anti-SOX2 (red), TRA98 (green) and laminin (blue), or anti-OCT4 (green) and SOX9 (red) antibodies on sections of E13.5 L/H-Pgds−/− and WT testes. Enlarged panels are shown on the NANOG panels. Arrows indicated SOX2, NANOG and OCT4 nuclear staining. Bars=100 µm for NANOG panels and =25µm for SOX2 and OCT4 panels.

**Fig. S6. Administration of the DP₂ antagonist CAY10471 mimicked the loss of PGD₂ signalling at E13.5.** Co-immunofluorescence of MVH (A) or SOX9 (C) (red) with EdU (green) on CAY10471 treated or non-treated (control) E13.5 male gonads. * indicate proliferating MVH germ cells (A) and
arrows indicate proliferating SOX9 Sertoli cells (C). Bars=50 μm (A) and =75μm (C). (B, D) Quantification of germ cells (C, n=1200; CAY, n=1100; 15R-PGD2, n=1100; BWA868C, n= 950) (B) and Sertoli cells (C, n= 550; CAY, n= 700) (D) that were positive for EdU in E13.5 testes that had been treated with CAY10471 (B, D), the DP$_2$ agonist 15(R)-PGD$_2$ or the DP$_1$ antagonist BWA868C (B). Data are represented as percentage of proliferating cells among the MVH- or SOX9-positive cells. (E) RT-qPCR analysis of Nanos2 and p21Cip1 expression levels in CAY10471 treated (CAY) or not treated (C=Control) E13.5 gonads. Data are represented using Rps29 as the normalization gene; error bars represent S. D. of assays done in triplicate, n= 3 independent experiments. (F-I) Isolated germ cells from E12.5 testes were cultured without (Control) or with PGD$_2$ (5ng/ml) and/or the DP$_1$ antagonist BWA868C (200nM) or DP$_2$ antagonist BAY-U3450 (100nM). RT-qPCR analysis of Stra8 (F), Nanos2 (G), p21Cip1 (H) and Sox2 (I) expression is represented using Rps29 as the normalization gene. Error bars indicate S. D. of assays done in triplicate on two independent samples.

Table S1. Genes differentially expressed in mutant compared to wild type E13.5 testes.

Table S2. Antibodies used in immunofluorescence and Western blotting.

Table S3. Oligonucleotides used for qPCR.
Figure S1
Figure S2
Figure S3
Figure S4

Levels of expression (log2) on KO in rel. to WT (WT= 1)

A

B

Development | Supplementary Material
Figure S5
Figure S6
Table S1. Genes differentially expressed in mutant compared to wild type E13.5 testes.

Download Table S1

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Table S2: Antibodies used in Immunofluorescence and Western blotting
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Table S3. Oligonucleotides used for qPCR. For each gene, the upper oligonucleotide corresponds to the forward oligonucleotide and the lower to the reverse oligonucleotide.
GENERAL DISCUSSION AND PERSPECTIVES
GENERAL DISCUSSION

Here we present the role of PGD2 signaling in male germ cell differentiation in fetal testis. Previously we showed that PGD2 a signaling molecule is produced during sex determination in fetal gonads to maintain Sox9 expression after the cease of Sry expression and to provide nuclear localization of Sox9 protein in order to promote differentiation of gonadal somatic cells into Sertoli cells (Malki et al., 2005b; Moniot et al., 2009; Moniot et al., 2011). We show that PGD2 signaling produced by L-Pgds and H-Pgds in Sertoli cells and germ cells is required for proper male germ cell differentiation in fetal testis. The analysis of mutant testis showed that PGD2 signaling controls proliferation of male germ cells in fetal testis for timely happening mitotic arrest.

The germ cells that enter into gonads at 10.5dpc extensively proliferate for two to three days to increase their number before differentiation (Tam and Snow, 1981). In fetal testis germ cells must have to stop proliferation and enter into quiescence stage, which is also called mitotic arrest or G0/G1 arrest (McLaren, 1984). The male germ cell mitotic arrest in fetal testis starts from 12.5dpc and all germ cells are mitotically arrest by 14.5dpc (Western et al., 2008). The analysis of L/H-Pgds double mutant and DP2 mutant testis showed prolonged proliferation of germ cells from 13.5dpc to 17.5dpc. This prolonged germ cell proliferation might be due to decreased expression of cell cycle inhibitor P21cip1, observed in PGD2 mutant gonads at 13.5dpc. In differentiating testis P21cip1 expression is required for mitotic arrest of male germ cells, which is detected from 12.5dpc and 13.5dpc (Cook et al., 2011; Western et al., 2008). PGD2 direct actions on germ cells to express P21 are observed in isolated male germ cells when cultured in the presence of PGD2, where P21 mRNA level increased. The data suggests that PGD2 directly activates P21 expression to inhibit cell cycle in male fetal germ cells. There is one more possibility that PGD2 might indirectly regulates P21 expression by activating DND1 expression as we observed DND1 decreased expression in PGD2 mutant male germ cells. However P21 decreased expression is already shown in DND1−/− mutant germ cells (Cook et al., 2011). Except inducing cell cycle arrest, P21cip1 expression is also shown involved in either tumourigenesis or in tumor suppression. Mice knockout of P21−/−
displayed spontaneous tumor growth (Martin-Caballero et al., 2001). P21 protein can be within nucleus and cytoplasm and its subcellular localization is shown to influence its function and is associated with cancer development. P21 localization can be influenced by post-translational modification (Piccolo, 2012). PGD2 is also involved in post-transcriptional regulation of P21 protein as P21 protein is detected within the cytoplasm of L/H-Pgds mutant germ cells. P21 protein when present in the nucleus is associated to the cell cycle arrest and in the cytoplasm it blocks apoptosis (Piccolo, 2012).

Cyclins E1, E2 and D3 the proteins that make complex with CDK2 and CDK4/6 respectively, to control the progression of cell cycle and promote transition from G1 to S phase, are expressed by 12.5dpc in male germ cells at the time of mitotic arrest (Spiller et al., 2009a; Western et al., 2008). The increased expression of cyclin E1 and E2 in L/H-Pgds mutant germ cells by 13.5dpc in comparison to WT germ cells indicates abnormal cell cycle regulation in absence of PGD2.

At the time of male germ cell differentiation, Nanos2 and Dnmt3l are upregulated to support male germ cell differentiation program (Western et al., 2011). However in PGD2 depleted gonads Nanos2 mRNA level decreased by each stage 13.5dpc, 15.5dpc and 17.5dpc. Normally Nanos2 is highly expressed in male germ cells by 14.5dpc and 15.5dpc (Western et al., 2011). As Nanos2 suppresses Stra8 expression and prevents male germ cell entry into meiosis (Suzuki and Saga, 2008), Possibly in L/H-Pgds mutant gonads Stra8 expression should increase due to decreased expression of Nanos2. Similarly in L/H-Pgds mutant male gonads Stra8 expression significantly increases by 13.5dpc, 15.5dpc and 17.5dpc in mutant male germ cells but still this Stra8 expression was 40 folds lower than in normal female gonads and was not sufficient to induce meiosis. Nanos2 \(-/-\) mutant XY germ cells die by apoptosis (Tsuda et al., 2003) but we did not find any apoptotic difference in WT and L/H-Pgds mutant gonads. On the other hand PGD2 negative effects on Stra8 expression are detected when germ cells were cultured in the presence of RA alone and PGD2+RA, where PGD2 50% decreased Stra8 expression induced by RA.

Another male germ cell fate marker Dnmt3l, which is responsible for the global DNA methylation in mouse male germ cells, is expressed between 14dpc and 18dpc (Sakai et al., 2004). However loss of Dnmt3l causes loss of spermatogonia
and DNA methylation defects but at postnatal level (La Salle et al., 2007). In *L/H-Pgds* mutant male germ cells *Dnmt3l* expression was downregulated but only after 17.5dpc.

The core regulators of pluripotency Sox2, Oct4 and Nanog are essential for maintaining pluripotency, self-renewal and early embryonic development (Avilion et al., 2003; Mitsui et al., 2003; Nichols et al., 1998), which are also expressed by germ cells; are male specifically methylated and suppressed by 15.5dpc in differentiating male germ cells (Western et al., 2010). In *L/H-Pgds* double mutant gonads we observed increased mRNA level of Sox2. The Sox2 expression is shown directly repressed by *Dmrt1* (Krentz et al., 2009) but it seems that PGD2 effects on Sox2 expression are independently of Dmrt1 because in *PGD2* mutant gonads *Dmrt1* expression was not affected.

This indicates that Prostaglandin D2 produced through both L-Pgds and H-Pgds in the somatic cells and germ cells of the fetal testis, is required for proper male germ cell differentiation and PGD2 actions like: activation of P21^cip1^ and suppression of pluripotency markers for mitotic arrest of germ cells, are through DP2 receptor.
**Role of PGD2 in control of male germ cell proliferation and differentiation in embryonic mouse testis**

In mouse embryonic testis PGD2 produced by H-Pgd and L-Pgd in Sertoli cell and germ cells is required for control of germ cell proliferation and proper differentiation. PGD2 activates P21^{Cip1} directly or by DND1 or by both ways to control germ cell proliferation and cell cycle arrest. PGD2 activates Cyp26b1 enzyme to degrade RA and activates male fate gene Nanos2 to suppress Stra8 expression in order to induce meiotic inhibition. PGD2 also suppresses regulators of pluripotency Oct4, Sox2 and Nanog, as their silence is required for differentiation of germ cell.
Perspectives

1. To analyze old L/H-Pgds KO animals around 1 year, to look for tumours in the testes; preliminary analysis by a pathologist revealed a hyperplasia of the germ line with increased number of total germ cells in the testicular tubules with an increase of the diameter of these tubules.

2. As the number of germ cells increased in embryonic testicular tubules, it is required to investigate the role of PGD2 in spermatogenesis in mice.

3. On C57Bl/6 (B6) genetic background, PGD2 actions on regulation of pluripotency and mitotic arrest are same like DND1 and Dmrt1 actions on 129/SvJ genetic background. In $DND1^{-/-}$ and $Dmrt1^{-/-}$ mutant gonads, high incidence of teratoma development is found on 129/SvJ background, where germ cells failed to down-regulate Sox2 expression and did not enter into mitotic arrest due to significant decrease in P21 protein. These tumors do not develop on tumor non-susceptible C57Bl/6 (B6) genetic background (Cook et al., 2011; Krentz et al., 2009). Therefore it is required to further investigate the role of PGD2 signaling on 129/SvJ genetic background that can be helpful in further understanding mitotic arrest required for male germ cell differentiation and tumor suppression.
PUBLICATION 2
Publication 2

The multiples roles of the Prostaglandin D2 signaling pathway in reproduction

The signaling molecule PGD2 and its different roles in various body systems, are already discussed in chapter one introduction. Here we review multiple roles of PGD2 in reproduction with respect to both embryonic and adult testicular development. By discussing our previous and current work, we review the involvement of two enzymes L-Pgds and H-Pgds and the PGD2 in different aspects of reproduction, including testicular formation, function, Sertoli cell and germ cell differentiation and fertility. Hopefully this review paper will increases and updates our knowledge about multiple roles of PGD2 signaling in reproduction and will help in planning future experiments for those who are working on infertility and testicular cancers.
Multiple roles of the prostaglandin D₂ signaling pathway in reproduction

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Abstract

Prostaglandins signaling molecules are involved in numerous physiological processes. They are produced by several enzyme-limited reactions upon fatty acids, which are catalyzed by two cyclooxygenases and prostaglandin synthases. In particular, the prostaglandins E₂ (PGE₂), D₂ (PGD₂), and F₂ (PGF₂a) have been shown to be involved in female reproductive mechanisms. Furthermore, widespread expression of lipocalin- and hematopoietic-PGD synthases in the male reproductive tract supports the purported roles of PGD₂ in the development of both embryonic and adult testes, sperm maturation, and spermatogenesis. In this review, we summarize the putative roles of PGD₂ signaling and the roles of both PGD₂ synthases in testicular formation and function. We review the data reporting the involvement of PGD₂ signaling in the differentiation of Sertoli and germ cells of the embryonic testis. Furthermore, we discuss the roles of lipocalin-PGD synthase in steroidogenesis and spermatogenesis, in terms of lipid molecule transport and PGD₂ production. Finally, we discuss the hypothesis that PGD₂ signaling may be affected in certain reproductive diseases, such as infertility, cryptorchidism, and testicular cancer.

Introduction

Prostaglandins (PGs) derived from polyunsaturated fatty acids belong to the superfamily of eicosanoids. The eicosanoid cascade starts with the activation of phospholipases A2 and C that release arachidonic acid from the cellular membrane. Arachidonic acid is oxidized and then reduced by the enzymes cyclooxygenases 1 and 2 (COX1 and COX2, also referred to as prostaglandin endoperoxidase H synthase 1 and 2 (PTGS1 and PTGS2)), to be converted into PGG₂ and PGH₂. The COXs are key enzymes in PG biosynthesis and differ in their expression levels and tissue distribution; COX1 is constitutively expressed, whereas expression of COX2 is induced (Simmons et al. 2004). PGH₂, the unstable reaction intermediate, is then converted into either PGD₂, PGE₂, PGF₂a or prostacyclin (PGI₂) and thromboxane A₂ (TxA₂), by the action of specific terminal PG synthases: prostaglandin D synthase (PGDS), prostaglandin E synthase (PGES), prostaglandin F synthase (PGFS) or prostacyclin synthase (PGIS), or thromboxane synthase (TXS) respectively (Fig. 1 and Table 1) (Cha et al. 2006). PGs are rapidly inactivated by oxidation by the NAD+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) (Fincham & Camp 1983). PGs are involved in the cardiovascular, gastrointestinal, genitourinary, endocrine, respiratory, immune, and nervous systems (Hata & Breyer 2004). These molecules act locally in an autocrine and/or paracrine manner and their actions are complex, not least because, given the structural similarity of these molecules and their receptors, PGs may have synergistic or antagonistic effects upon the same physiological processes (Woodward et al. 2011, Tootle 2013).

PGD₂ is actively produced in many organs, and is the most abundant prostanoid in the CNS (Urade & Hayaishi 2000a) and in the respiratory tract and airways of asthmatic patients (Oguma et al. 2008). PGD₂ has essential roles in various physiological processes (Matsuoka et al. 2000, Kobayashi & Narumiya 2002, Qu et al. 2006, Huang et al. 2007, Taniguchi et al. 2007, Oguma et al. 2008, Gao et al. 2009, Nieves & Garza 2014), and particularly in several steps of the reproductive function (this function will be discussed further in the following paragraphs). Also, PGD₂ together with the prostaglandins PGE₂ and PGI₂, in conjunction with other mediators such as histamine, are involved in the inflammation process (Hata & Breyer 2004, Herlong & Scott 2006). Hematopoietic PGDS (H-PGDS) is the key enzyme in the synthesis of PGD₂ in the immune system and mast cells (Urade & Hayaishi 2000a, Kanaoka & Urade 2003). Furthermore, the resolution of inflammation is accompanied by a shift from the biosynthesis...
The synthesis of PGD₂ is under the specific control of two PGDS, the L-PGDS (or PTGDS), and the H-PGDS (or PTGDS2) (Urade & Eguchi 2002). Originally identified in the rat brain, L-PGDS, whose function is independent of the tripeptide glutathione, is part of the lipocalin protein superfamily, the members of which are secreted into the extracellular space (Urade et al. 1985, Urade & Hayaishi 2000a). This enzyme is produced in the CNS (brain, spinal cord, dorsal root ganglia), in the male genitalia (testes, epididymides, prostate) (Fouchecourt et al. 2002), and in the heart (Uguchi et al. 1997). It has been suggested that L-PGDS has dual functions. Associated with the endoplasmic reticulum and the outer nuclear membrane, it catalyzes the final step in PGD₂ synthesis from a common PG precursor. Secondly, as L-PGDS is secreted in many fluids (cerebrospinal fluid, seminal plasma, ascites, serum, urine, and amniotic fluid), it has been proposed to have a role in binding and transporting small hydrophobic ligands such as retinol, β-lactoglobulin, bile pigments, and thyroid hormones (Urade & Hayaishi 2000b, Fouchecourt et al. 2002).

The expression of L-Pgds is also under the control of many regulatory factors, protein kinase C (PKC) (Fujimori et al. 2005), estrogens (Mong et al. 2003), IL1β, RasGRP4...
(Li et al. 2003), each being highly cell-type specific. PGD₂ itself induces L-Pgds expression through binding of the Nrfl2 factor on the L-Pgds promoter region in macrophages (Kim et al. 2013). *In vitro* primary cultures of rat Sertoli cells also show the activation of L-PGDS protein expression after treatment with progesterone or retinoic acid (RA) (Samy et al. 2000). RA strongly induces the accumulation of L-PGDS mRNA in human 3AO ovarian cancer cells, leading to the inhibition of their proliferation (Su et al. 2003). Furthermore, in the embryonic male gonad, L-PGDS expression is initiated and maintained by the testis differentiating factor SOX9 (Moniot et al. 2009) (see below).

Originally identified in the rat spleen, H-PGDS is a member of the class of glutathione-S-transferase enzymes, which are cytosolic and play a role in detoxification. Bivalent Ca²⁺ and Mg²⁺ ions increase the activity of H-PGDS; however, only Mg²⁺ increases its affinity for glutathione (Inoue et al. 2003). Despite the high homology of the primary sequence in different species, the tissue expression profile is highly variable. Expression is high in the peripheral tissue, spleen, thymus, bone marrow, gastrointestinal tract, and oviduct of rats (Kanaoka & Urade 2003). In the mouse, expression is predominant in the skin, oviduct (Kanaoka et al. 2000), and granulosa cells of the postnatal and adult ovary (Farhat et al. 2011). However, in humans, expression is found in the placenta, lung, fetal liver, heart, brain, mastocytes, lymphocytes, th2 cells, and antigen-presenting cells (Kanaoka et al. 2000, Tanaka et al. 2000).

PGD₂ is dehydrated in *vitro* and in *vivo* by a nonenzymatic process to produce PGs of the J series, PGJ₂, and 15-deoxy-12,14-PGJ₂ (15-d PGJ₂) (Shibata et al. 2002). These PGJ₂ metabolites can also influence diverse cellular functions. In particular, H-PGDS was shown to control the onset and resolution of acute inflammation through PGD₂ and 15-d PGJ₂ (Rajakariar et al. 2007).

### PGD₂ signal transduction

PGs are secreted and activate nine different receptors (*Fig. 1*): DP₁ and DP₂ or chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH₂) for PGD₂, EP₁, EP₂, EP₃, FP for PGF₂α, IP for PGI₂, and TP for TXA₂ (Breyer et al. 2001). PG receptors are categorized as three clusters of a distinct subfamily of the G protein-coupled receptor (GPCR) superfamily of seven transmembrane-spanning proteins (Coleman et al. 1994). The only exception is DP₂, a member of the chemoattractant receptor subgroup. These receptors transduce different signals via the production of second messenger cAMP or IP₃/diacylglycerol/Ca²⁺⁺ (Woodward et al. 2011) (*Fig. 1*).

Thus, PGD₂ may bind to two receptors, the DP₁ receptor (Boie et al. 1995) and/or the DP₂ receptor CRTH₂ (Hirai et al. 2001). Activation of the DP₁ receptor, coupled to a Gαs protein, induces the production of the second messenger cAMP, which stimulates protein kinase A (PKA) and also induces an influx of Ca²⁺⁺ (Boie et al. 1995). The activation of CRTH₂ or DP₂ receptors coupled to a Gαi protein inhibits cAMP production (Hirai et al. 2001) and induces intracellular Ca²⁺⁺ mobilization caused by the production of inositol triphosphate (Woodward et al. 2011). On the other hand, the PGD₂ metabolite 15d-PGJ₂, was identified as a ligand for the peroxisome proliferator-activated receptor gamma (PPARγ), a member of the nuclear receptor family (Forman et al. 1995) and for DP₂.

### PGD₂ and reproduction in adult gonads

#### Female reproduction

Few studies have evaluated the involvement of PGD₂ in female reproduction. H-PGDS and both DP₁ and CRTH₂ receptors are expressed in the placenta and L-PGDS is present in amniotic fluid, indicating a role in the regulation of placental communication (Lumsden et al. 1986, Saito et al. 2002). H-Pgds mRNA was localized in the granulosa cells from primary to pre-ovulatory follicles of the mouse adult ovary (Farhat et al. 2011). In this tissue, H-PGDS-induced PGD₂ interferes with FSH signaling through increased Fshr and Lhgr (LhR) receptor expression, leading to the activation of steroidogenic Cyp11a1 and Star gene expression, and subsequently to progesterone secretion. Furthermore, H-PGDS-induced PGD₂ is involved in the regulation of follicular growth through inhibition of granulosa cell proliferation in growing follicles (Farhat et al. 2011).

However, numerous roles of other PGs, PGE₂, PGF₂α, and PGI₂ have been highlighted in different stages of blastocyst implantation: vascular permeabilization, stromal decidualization, blastocyst growth and development, leukocyte recruitment, embryo transport, trophoblast invasion, and extracellular matrix remodeling (Salleh 2014). The respective contribution of these PGs in female reproduction was highlighted through the analysis of the Cox₁ and/or Cox₂-knockout mice (Morita 2002). In particular, *Cox₂* gene-deficient mice have defective ovulation, fertilization, and implantation mechanisms (Loftin et al. 2001). This phenotype was mimicked in *Ep₂*⁻/⁻ receptor mice, demonstrating the involvement of PGE₂ in these processes. COX₂, PGE₂, and EP₂, synthesized in follicular cumulus cells in response to gonadotropins, induce the follicle and oocyte maturation necessary for fertilization and ovulation (Kobayashi & Narumiya 2002). Furthermore, mice with targeted disruption of the *Cox₁* gene have delayed parturition resulting in neonatal death, demonstrating the role of *Cox₁* for the initiation of labor (Gross et al. 1998). PGF₂α, which is highly expressed in the uterus, is involved in this process via the FP receptor. Moreover, PGF₂α expression in the corpus luteum of the ovary is also
involved in the apoptosis of these cells in the absence of gestation (Hasumoto et al. 1997).

Male reproduction
In the male, COX1−/− and COX2−/− mouse models do not exhibit perturbed reproductive processes. However, L-PGDS is widely expressed in the testis and caput epididymis of bull and mouse models (Gerena et al. 2000a, b). L-PGDS is detected in bovine and human seminal plasma (Gerena et al. 1998, Tokugawa et al. 1998) and its concentration is lower in oligozoospermic than in normozoospermic men (Tokugawa et al. 1998), suggesting that this protein plays a role in both the development and maturation of sperm and emphasizes the role of L-PGDS in spermatogenesis. L-Pgds mRNA expression is found mainly in Leydig cells (Baker & O’Shaughnessy 2001), prospermagonia, and SOX9-expressing Sertoli cells (Moniot et al. 2009) of the adult mouse testis. In rat, L-PGDS was detected in the Sertoli and germ cells of the adult testis (Samy et al. 2000). In humans, L-PGDS, H-PGDS, and DP1 receptor are also expressed in the interstitial compartments of testes with normal and impaired spermatogenesis (Schell et al. 2007). L-PGDS and H-PGDS are expressed in Leydig cells and mast cells, respectively, along with COX2, in testes with impaired spermatogenesis. COX1 and COX2 are shown to be absent in normal human testes, whereas they are highly expressed in testicular cancer, and act to induce the growth of testicular cancer cells (Hase et al. 2003). The expression of COX2 in testicular biopsies from patients with mixed atrophy is correlated with H-PGDS expression in the mast cells of these testes (Welter et al. 2011). The major function of L-PGDS in spermatogenesis may be related with its role in the supply of retinoids, thyroid hormones, and essential fatty acids for the development of germ cells in the seminiferous tubules and maturing spermatozoa in the epididymides (Urade & Hayashi 2000a, b). However, the role of L-PGDS in male reproduction remains unclear (Leone et al. 2002). H-PGDS expression in the male gonad is not well documented. The expression was detected in the Leydig cells and mast cells of the testes of human patients with impaired spermatogenesis (Schell et al. 2007); however, in the germ cells of murine testes (personal data not shown), its role in reproduction is unknown.

15-Deoxy PGJ2, a metabolite of PGD2 influences the expression of differentiation markers (SMC, smooth muscle actin) and the contractility of the human peritubular cells of the testes (Schell et al. 2010) and thus, may be involved in infertility (Welter et al. 2013). COX2 mRNA expression was greatly increased in experimental cryptorchid testes, when compared with contralateral testes. Furthermore, in the spermatocytes of the cryptorchid testes the COX2 protein was specifically upregulated, thus protecting germ cells against apoptosis and disturbance of spermatogenesis (Kubota et al. 2011).

Furthermore, PGD2 induced testosterone production in Leydig cells isolated from hamster testes (Schell et al. 2007). On the other hand, COX2 activity was shown to reduce steroidogenesis by decreasing Star gene expression in MA-10 mouse Leydig cells (Wang et al. 2003). However, the role of PGD2 in the steroidogenesis process is still unclear because other reports using organotypic cultures of adult human gonads did not find a link between the effect of analgesics on PG synthesis and inhibition of testosterone production (Albert et al. 2013).

PGD2 and the formation of embryonic male gonads
PGD2 signaling components are expressed in embryonic testes
Amongst the male-enriched bands, identified by representational difference analysis (RDA) at embryonic stage E12.5, the gene encoding for L-PGDS was identified (Adams & McLaren 2002). The expression of L-Pgds mRNA in developing urogenital ridges was first detected in the Sertoli cells and prospermagonia of late E11.5 male genital ridges (Adams & McLaren 2002). The expression of L-Pgds mRNAs in both somatic and germ cell compartments was confirmed at E13.5, although somatic expression was higher than that of germ cells (Moniot et al. 2014). L-Pgds showed similar expression profiles to Sox9 and Fgf9, with expression starting at mid-late E11.5 and progressing to a plateau at E12.5 (Wilhelm et al. 2005). L-Pgds expression was described as a dynamic wave-like expression pattern, closely resembling that of Sry and Sox9 in the embryonic testis. L-Pgds transcripts were detected in the center of the testis at the 17 tail somites (Ts) stage, shortly after the onset of Sox9 expression at 15 Ts, and were shown to be upregulated at 21 Ts (Wilhelm et al. 2007). L-PGDS protein expression was evident in the E12.5 male gonads, in both the Sertoli and germ cells (Moniot et al. 2009). L-Pgds mRNA expression shifts from the seminiferous tubules in the embryonic gonad and neonatal testis to the interstitial compartment, particularly the Leydig cells in the adult testis (Baker & O’Shaughnessy 2001, Moniot et al. 2009).

Concerning the second PGD2-inducing H-Pgds enzyme, H-Pgds mRNA expression in both germ and somatic cells was found in E11.5–E17.5 gonads. In addition, the H-PGDS protein is also expressed in both cell types (Moniot et al. 2011, 2014). Production of PGD2 by both the somatic and germ cell lineages was confirmed, using chemical fixation of PGD2 on its production site (Bandeira-Melo et al. 2011) followed by immunofluorescence analysis, suggesting that both L-PGDS and H-PGDS enzyme capabilities are active within the embryonic gonad (Moniot et al. 2014). On the other hand, in the E13.5 male gonad, the DP1 receptor is only expressed in somatic cells whereas the
DP$_2$ is expressed in both germ cells and somatic compartments, at the mRNA and protein levels (Moniot et al. 2014).

**PGD$_2$ signaling is involved in somatic differentiation**

In most mammals, somatic sex determination in males is initiated in undifferentiated embryonic gonads by the expression of the Sry gene, which occurs at stages E10.5–E12.5 in mice, initiating testis differentiating Sox9 gene expression (Sekido & Lovell-Badge 2008). The master effector gene Sox9 encodes a transcription factor that belongs to the HMG superfamily (Wagner et al. 1994). Before sex determination and before the peak of Sry expression at E11.5, SOX9 is excluded from the nucleus in the genital ridge of both sexes (Morais da Silva et al. 1996, de Santa Barbara et al. 2000), via a nuclear export signal (NES), located in its HMG domain (Gasca et al. 2002), and is retained in the cytoplasm, possibly via its interaction with microtubules (Malki et al. 2005a). Upon sex determination, the SOX9 protein is transported into the nucleus in the male gonad. PGD$_2$ signaling via its DP$_1$ receptor and stimulation of the cAMP pathway induce SOX9 nuclear translocation via PKA phosphorylation in NT2/D1 cells (Malki et al. 2005b) (Fig. 2). Indeed, in L-Pgds$^{-/-}$ gonads, SOX9 subcellular localization and testis cord formation were impaired up to E13.5, even though a variable SOX9 expression pattern and sex cord formation phenotype, ranging from normal to severely abnormal, were found (Moniot et al. 2009). Furthermore, the PGD$_2$-producing H-PGDS enzyme is expressed in the embryonic gonad at mid E11.5 (16–17 Ts), despite L-PGDS not being expressed. Inhibition of H-Pgds enzymatic activity by the specific HQL-79 inhibitor impairs nuclear translocation of the SOX9 protein in E11.5 pre-Sertoli cells, a phenotype that was also found in H-Pgds$^{-/-}$ XY gonads (Moniot et al. 2011), suggesting that an initial H-PGDS-mediated PGD$_2$ signal could participate in the SOX9 nuclear translocation necessary for the process of Sertoli cell differentiation (Fig. 2).
Moreover, PGD2 has a masculinizing effect on cultivated XX gonadal explants (ectopic testicular cord formation and expression of AMH) (Adams & McLaren 2002) through the stimulation of Sox9 gene expression (Wilhelm et al. 2005), as Sox9 can directly bind to and activate the L-Pgds promoter (Wilhelm et al. 2007). L-Pgds expression was indeed abolished in E12.0 male Sox9 knockout gonads (Ck19-Cre; Sox9floxed/floxed) mice, confirming that Sox9 is required for the initiation of L-Pgds gene expression, as L-Pgds is a direct target gene for SOX9. Moreover, ablation of Sox9 after the onset of L-Pgds expression (E13.5–E14.5 Amh-Cre; Sox9floxed/floxed) also induces a strong downregulation of L-Pgds expression, demonstrating the requirement for the SOX9 protein in the maintenance of L-Pgds gene expression in embryonic Sertoli cells (Moniot et al. 2009). Altogether, these data show that L-Pgds and Sox9 genes are part of a regulatory loop, initiating and maintaining L-Pgds expression and upregulating Sox9. This regulatory loop is independent on the fibroblast growth factor 9 (Fgf9)/Sox9 regulatory loop previously identified (Kim et al. 2006). Indeed, the onset of L-Pgds expression was not affected in Fgf9−/− (Moniot et al. 2009) or Fgf9 receptor R2 (FgfR2−/−) (Kim et al. 2007) mutant XY gonads and Fgf9 mRNA expression was not modified in E12.5 L-Pgds−/− gonads confirming that both pathways do not interact genetically. However, both FGF9 and PGD2 signaling molecules cooperate to additively upregulate Sox9 expression in the Sertoli-like NT2D1 cell line (Moniot et al. 2009; Fig. 2). Many endocrine disruptors (phtalates, bisphenol) and several NSAIDS that inhibit COXs activities reduce PGD2 production in the SC5 mouse Sertoli cell line and in cultured rat fetal testes (Kristensen et al. 2011a,b, 2012), leading to reduced testosterone production. However, the role of PGD2 in the onset of the steroidogenesis process remains unclear. Unlike adult human or rat testes, ex vivo exposure of embryonic human testis to paracatamol, aspirin, and indomethacin has no effect either on the production of PGD2 or on the concentration of testosterone (Mazaud-Guittot et al. 2013).

PGD2 is also involved in the process of testicular descent in mice, because adult L-Pgds−/− mice present unilateral cryptoorchidism without impaired androgen signaling, but rather a decrease in the INSL3 receptor Rxipt2 mRNA expression in the gubernaculum (Philibert et al. 2013). The use of NSAIDS, which inhibit COXs enzymes, during the second trimester of pregnancy is associated with an increased risk of cryptorchidism in humans (Jensen et al. 2010, Kristensen et al. 2011a); however, the nature of the PGs involved in this phenotype is unknown.

PGD2 signaling is involved in germ line differentiation

The differentiated Sertoli cells will then influence the germ cell lineage to differentiate (Svingen & Koopman 2013). In mice, primordial germ cells (PGCs) colonize the genital ridge at around E10.5 and continue proliferating until E13.5 (Mclaren 2000). At this time, in the developing ovary, germ cells enter prophase of the first meiotic division after the upregulation of the premeiotic gene Stra8 (Ewen & Koopman 2010). In contrast, in the testis, germ cells stop proliferating and fully enter the G0/G1 phase of the cell cycle by E15.5 (Western et al. 2008); meanwhile, pluripotient marker expression is repressed (Western et al. 2010) and male germ cell markers such as Nanos2 are upregulated, which actively inhibits meiosis entry and thus contributes to the differentiation of the germline (Suzuki & Saga 2008). Male germ cells remain quiescent until shortly after birth, at which point they resume mitosis and then initiate meiosis around 8 days post partum (dpp) (Ewen & Koopman 2010).

In vivo analysis of double-knockout L/H-Pgds (L/H-Pgds−/−) mice demonstrated that the proliferation rate of E13.5 mutant germ cells was increased by 1.5-fold compared with WT germ cells. At E15.5 and even E17.5, nearly 10% of the mutant germ cells were still Ki-67 positive, suggesting that a significant proportion of the mutant germ cells were not mitotically arrested and were still engaged in the cell cycle at a time which should be quiescent (Moniot et al. 2014). Meanwhile, cell cycle inhibitors p21Cip1 and p57Kip2 are downregulated and cell cycle activators CyclinE1 and E2 are upregulated (Fig. 2), suggesting that PGD2 signaling is involved in the control of cell cycle genes in fetal testes, contributing to the arrest of mitotic process. Moreover, at late embryonic stages, the ectopic expression of pluripotency markers Pou5f1 (Oct4), Sox2, and Nanog was detected in L/H-Pgds−/− testes and the male germ cell marker Nanos2 is downregulated in mutant testes suggesting that PGD2 has a role in the germ cell differentiation in the embryonic testis. Somatic factors, Notch1 (Garcia et al. 2013) and Cyp26B1, an RA-metabolizing enzyme of the cytochrome P450 family that is produced by the Sertoli cells and that protects germ cells from RA (Bowles et al. 2006), were significantly reduced in E13.5 mutant gonads (Fig. 2), suggesting that PGD2 produced by Sertoli cells influences the differentiation of the embryonic germ cells (Moniot et al. 2014). Finally, the DP2 receptor is responsive to the effects of PGD2 in the male germline, because Dp2−/− testes have the same phenotype as that of the L/H-Pgds−/− testes (Fig. 2; Moniot et al. 2014). PGD2 signaling is thus an early pathway acting in both paracrine and autocrine manners (Fig. 2), contributing to the proper differentiation of male fetal germ cells.

Conclusions, perspectives

The development and maturation of the reproductive organs are complex and highly regulated biological
mechanisms, in which numerous factors and signaling pathways are involved. In this review, we addressed the advancement of knowledge on PGD_2 signaling in female and male reproduction, particularly in the formation of embryonic gonads and the maturation of adult reproductive organs. Whereas PGD_2 signaling through both PGDSs is involved in the differentiation of the embryonic tests at the somatic and germ cell levels, its roles in steroidogenesis and spermatogenesis in adults are still under debate. The dual roles of the L-PGDS enzyme suggest that this protein plays a role in both the development and maturation of sperm and spermatogenesis. Seminal L-PGDS, an important carrier of bile pigments, retinoids, thyroid hormones, and essential fatty acids, would contribute to providing, beyond the blood–testes barrier, thyroid hormones, and retinoids to the developing germ cells in the seminiferous tubules and the maturing spermatozoa in the epididymis. Both PGDSs are indeed expressed in testes of patients with impaired spermatogenesis, suggesting their involvement in fertility (Leone et al. 2002).

The increasing incidence of disorders of the reproductive organs in men, such as cryptorchidism, hypospadias, decreased semen quality, and testosterone concentration, or testicular cancers, has been observed in recent decades (Toppari et al. 1996, Skakkebaek et al. 2001). The use of NSAIDS drugs during the second trimester of pregnancy is associated with an increased risk of cryptorchidism in humans (Jensen et al. 2010) and in rats (Kristensen et al. 2011a). As PGD_2 is a potential target for endocrine disruptors and NSAIDs, our findings thus open new perspectives for future investigations into how germ cell development can be perturbed by the external environment. Germ cells that are not controlled appropriately during fetal life can later transform into carcinoma in situ (CIS), the pluripotent precursor cells for testicular germ cell tumors (Kristensen et al. 2008). Indeed, PGD_2/DP_2 signaling is involved in the control of key regulators of the G1/S phase checkpoint and in the repression of pluripotent markers’ expression in the male embryonic germline; its ablation resulting in CIS-like phenotype in the mice gonad. Further work will determine whether the double L/H-Pgds mutation can lead to a high incidence of germ-line tumors in the 129sv background. PGD_2 signaling through L-PGDS and SOX9 expression suppresses NT2/D1 cell migration and invasion, suggesting an important role for PGD_2 in cancer cell suppression in the tests (Wu et al. 2012).

L-PGDS is abnormally expressed in ovarian tumors (Su et al. 2003, Malki et al. 2007). The antiproliferative effect of PGD_2 has been highlighted in human ovarian cancer cell lines (Kikuchi et al. 1986, Su et al. 2003); stimulation of the PGD_2/DP_2 signal transduction pathway upregulates SOX9 expression leading to the inhibition of cancer cells growth (Malki et al. 2007).

The recent findings, showing that endocrine disruptors and NSAIDS influence the PGD_2 production in the testes and that PGD_2 signaling is involved in multiples steps of the embryonic tests differentiation, might introduce this pathway in the etiology of the reproduction diseases. As the pharmacology of the PGD_2 signaling is well documented, either activators of this pathway or DP_1/DP_2 agonists may be useful as new therapeutic agents.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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