Part 1 **Female gamete**

Chapter 1

Genetics of germline formation

Orkan Ilbay and Emre Seli

Introduction

Survival of sexually reproducing organisms in the course of evolution depends on their success in producing gametes. The male gamete, or sperm, and the female gamete, the egg or oocyte, and their precursors are referred to as germ cells. Gametes develop from primordial germ cells (PGCs) that are set-aside during early embryogenesis [1]. In most metazoans, PGCs have an extragonadal origin and migrate to reach the somatic gonad where they proliferate by mitosis to form oocytes or spermatoza [1]. Specification, migration, proliferation, and differentiation of PGCs are tightly regulated and share common features among evolutionarily distant species.

In this chapter, we will review molecular mechanisms that control germline formation through a complex cascade of gene activation (Figure 1.1). These mechanisms have significant implications for our understanding of reproductive disorders and for ongoing efforts in using stem cells to generate functional gametes.

Primordial germ-cell specification

In mammals, primordial germ cells (PGCs) are derived from the proximal epiblast during early embryogenesis. In humans, the first PGCs are detected in the yolk sac on day 24 of embryo development [2]. In mice embryos, at day E7.25, PGCs are distinguished as alkaline phosphatase-positive cells in the extra-embryonic mesoderm posterior to the primitive streak [3,4]. Specification of PGCs from pluripotent epiblast cells requires induction by extracellular signals, which results in the activation of PGC-specific genes and the suppression of somatic genes (Table 1.1).

BMP4

One of the signals that induce PGC precursors is bone morphogenetic protein 4 (BMP4), which belongs to the transforming growth factor-β (TGF-β) superfamily [5]. BMP4 is expressed by extra-embryonic ectoderm in pre-implantation mouse embryos at around E6.0. BMP4 diffuses into the epiblast, setting up a gradient, and induces expression of genes involved in PGC specification in epiblast cells that are adjacent to the extra-embryonic ectoderm. *Bmp4*-null mice embryos are devoid of PGCs [5]. They also lack allantois, which, like PGCs, is derived from precursor cells in the proximal epiblast.

BMP8B

Another protein that belongs to the BMP and TGF-β families and is required for PGC formation is bone morphogenetic protein 8B (BMP8B) [6]. *In situ* hybridization of embryo sections shows *Bmp8b* expression in the extra-embryonic ectoderm of pre-gastrula- and gastrula-stage embryos. Similar to *Bmp4*, *Bmp8b*-null mice lack PGCs. Further studies on BMP4/BMP8B signaling shows that the number of PGCs formed during in vitro culture of proximal epiblast cells obtained from E6.0–E6.25 mouse embryos markedly increases upon addition of BMP4- and BMP8B-expressing feeder cells [7]. Although other unidentified signals may be required for acquiring PCG competence before BMP4/BMP8B signaling, current data suggest that these proteins are necessary and sufficient for PGC formation from epiblast cells on day E6.0.

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Table 1.1 Regulatory proteins involved in primordial germ-cell specification.

Protein	Common Alias	Protein Function	Mutant Phenotype	Reference
BMP4		A TGF-Beta/Bmp family cytokine. Induces PGC formation.	Lack of PGCs and allantois.	Lawson *et al.* 1999 [5]
BMP8B		A TGF-Beta/Bmp family cytokine. Induces PGC formation.	Lack of PGCs (43%) or severe reduction in PGC number on a mixed genetic background. Lack PGCs on C57BL/6 background. Short or no allantois on both genetic backgrounds.	Ying *et al.* 2000 [6]
BMP2		A TGF-Beta/Bmp family cytokine. Structurally highly similar to Bmp4.	Reduced number of founding PGC. Short allantois. Additive effect with Bmp4 on PGC generation.	Ying *et al.* 2001 [9]
IFITM3	FRAGILIS	A trans-membrane protein implicated in cellular innate immunity to human pathogens (IFITM3). A PGC marker.	No effects on PGC formation and have no detectable effects on development of the germline.	Lange *et al.* 2008 [12]
DPPA3	STELLA	May play a role in maintaining cell pluripotentiality. A PGC marker.	No effect on PGC formation, but oocytes from mutant mice fail to develop normally beyond the four-cell stage.	Bortvin *et al.* 2004 [14]
PRDM1	BLIMP1	A PR domain-containing transcriptional repressor. Acts as a repressor of beta-interferon gene expression.	PGC-like cells at E7.5 that do not proliferate or migrate. Inconsistent repression of Hoxa1 and Hoxb1 genes.	Ohinata *et al.* 2005 [16]
PRDM14		A PR domain-containing transcriptional regulator. Necessary for PGC specification.	Lack of germ cells in adult ovaries and testes. Male and female infertility.	Yamaji *et al.* 2008 [18]

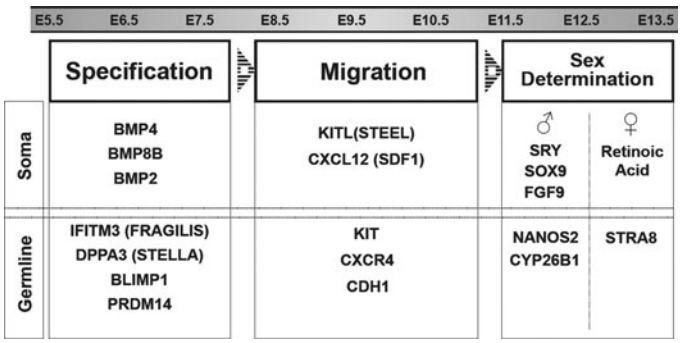

Figure 1.1 Germline formation: Bone morphogenetic proteins BMP4, BMP8B, and BMP2 induce PGC specification from the epiblast. BLIMP1 and PRDM14 are necessary for PGC specification. Nascent PGCs express FRAGILIS and STELLA, but these proteins are not necessary for PGC specification or germline formation. PGC specification is followed by migration. During migration PGCs express KIT while surrounding somatic cells express KITL. KITL is necessary for PGC motility as well as survival and proliferation of PGCs. CXCL12 is secreted from gonadal ridges and acts as a chemoattractant for PGCs. CXCR4 is a CXCL2 receptor expressed on the membranes of PGCs. E-CADHERIN expression on PGC membrane allows PGCs to interact with each other and migrate as a compact cluster. Migration ends when PGCs colonize the gonadal ridges and is followed by sex determination. FGF9 and retinoic acid (RA) from somatic cells regulate germ-cell sex determination in males and females, respectively. RA induces STRA8 expression and meiotic entry. CYP26B1 degrades RA protecting XY gonads from premature meiosis. FGF9 induces NANOS2 expression in germ cells, which in turn suppresses STRA8 expression.

BMP2

In addition to BMP4 and BMP8B expressed in the extra-embryonic ectoderm, expression of a third bone morphogenetic protein, BMP2, is detected in visceral endoderm at E6.0–E6.5 [8,9]. The highest BMP2 expression is observed in endoderm surrounding the posterior proximal epiblast, where BMP4/BMP8B signals act on PGC precursors. In *Bmp2*-null mice,

the size of the PGC founding population is markedly reduced. BMP2 has >90% amino acid sequence homology to BMP4, and PGC numbers in double heterozygous mutants show BMP2 and BMP4 have an additive effect on PGC generation [9].

FRAGILIS and STELLA

Once specified, PGCs are characterized by expression of specific genes. Comparison of single-cell gene expression profiles of nascent PGCs and their somatic neighbors reveals up-regulation of an interferon-inducible trans-membrane protein, FRAGILIS/IFITM3 in PGCs [10]. Expression of FRAGILIS is induced by BMP signaling from extra-embryonic ectoderm in a dose-dependent manner. FRAGILIS expression is absent in *Bmp4*-null mouse embryos, while it is significantly reduced in *Bmp4*-heterozygous mutants. Moreover, intimate contact with extra-embryonic ectoderm is sufficient to induce FRAGILIS expression in distal epiblast. Expression of two more members of the Fragilis family, FRAGILIS2 and FRAGILIS3, has also been detected in nascent PGCs [11]. Among FRAGILIS-positive cells, only the ones with the highest expression become committed to germ-cell fate. These committed cells start expressing STELLA (DPPA3) during PGC specification, and STELLA expression remains restricted to developing PGCs [10]. Interestingly, although both FRAGILIS and STELLA are differentially expressed in PGCs, neither appears to be essential for PGC specification [12–14].

BLIMP1/PRDM1

Homeobox (Hox) genes are turned on sequentially throughout the embryo during embryonic pattern formation and play an important role in metazoan embryonic development. However, PGC specification, which requires an escape from somatic cell fate, is marked by down-regulation of *Hox* genes. There are several findings suggesting that BLIMP1/PRDM1, a PR domain-containing transcriptional repressor, which has been shown to regulate plasma cell differentiation, plays a role in the suppression of the somatic program in PGC precursors (or nascent PGCs) [15,16]. In mice, four to eight BLIMP1-positive cells are observed among E6.25 epiblast cells that are always directly in contact with extra-embryonic ectoderm. At E6.5 BLIMP1 expression is observed in approximately 20 cells in the epiblast that are defined as lineage-restricted PGCs [16,17]. In *Blimp1*-deficient mice embryos at E7.5, the number of alkaline phosphatase-positive cells is markedly reduced. More importantly, although these PGC-like cells are alkaline phosphatase-positive, they do not proliferate or migrate as wild-type PGCs do. Comparison of transcripts from single PGC-like cells in *Blimp1*-deficient embryos with wild-type PGCs shows inconsistent repression of HOXA1 and HOXB1 [16].

PRDM14

More recently, expression of another PR-domain-containing transcriptional regulator, PRDM14, has been detected in BLIMP1/PRDM1-positive PGCs starting on E6.75 [18]. When both BLIMP1/PRDM1 and PRDM14 expression are examined in whole embryos, PRDM14 expression is more restricted to PGCs than BLIMP1/PRDM1. Germline-specific expression of PRDM14 is supported by the *Prdm14*-null phenotype. When crossed, $Prdm14^{+/-}$ mice deliver $Prdm14^{-/-}$ mice in the expected Mendelian ratio and with no apparent defects, suggesting that PRDM14 is not critical for embryonic development. However, adult *Prdm14*-null females and males are sterile and they lack germ cells, indicating that PRDM14 is essential for germ-cell development.

PGC migration

Once specified in the proximal epiblast, at around E7.5, PGCs start moving through the primitive streak into the adjacent endoderm [19] (Figure 1.1). At E8.0 PGCs migrate along the endoderm, while the endoderm gives rise to hindgut. PGCs then become incorporated into the hindgut and move along with the hindgut epithelium. Between E9.0–E9.5, PGCs exit from the hindgut, split into two populations, and migrate towards the dorsal body wall. PGCs reach and colonize the nascent gonadal ridges between E11.5 and E12.5 [20]. In humans, PGC migration and colonization of gonads take place between the 4th and 6th weeks of gestation [21].

In general, migration of PGCs from primitive streak to genital ridges is believed to be governed by chemotactic cytokines, cell surface receptors, and cell adhesion factors. In addition, PGCs seem to migrate passively during embryonic morphogenetic

Table 1.2 Regulatory proteins involved in primordial germ-cell migration.

Protein	Common Alias	Protein Function	Mutant Phenotype	Reference
KITLG *Kitl (mouse)*	*STEEL, STEEL FACTOR*	A cytokine that binds to the Kit receptor. Can exist both as a trans-membrane protein and a soluble protein. Facilitates PGC motility and essential for survival and proliferation of PGCs.	Marked decrease in PGC motility from the site of specification till they colonize the genital ridges.	Mahakali Zama *et al.* 2005 [23] Gu *et al.* 2009 [25]
KIT	*C-KIT*	Trans-membrane receptor for Kitl. Facilitates PGC motility and essential for survival and proliferation of PGCs.	Severe defects in PGC migration, number of PGC colonize gonadal ridges severely reduced.	Buehr *et al.* 1993 [88]
CXCL12		A cytokine secreted by genital ridges and acts as a chemoattractant for PGCs. Also produced by activated monocytes and neutrophils and expressed at sites of inflammation.	Reduced number of PGCs in the gonads.	Ara *et al.* 2003 [27]
CXCR4		A G-protein-coupled receptor, specific for Cxcl12.	Defects in PGC migration and survival; number of PGCs in the gonads is reduced.	Molyneaux *et al.* 2003 [26]
CDH1	*CD324*	(E-cadherin) a calcium-dependent cell–cell adhesion glycoprotein.	Chd1-null embryos die around the time of implantation. Perturbed PGC compaction in gonads when E-cadherin is blocked in embryo slice cultures.	Larue *et al.* 1994 [89] Bendel-Stenzel *et al.* 2000 [28]

movements and expansion of the hindgut endoderm. Although molecular mechanisms behind PGC migration are not well established there are several proteins that seem to be involved in this process (Table 1.2).

Expression of FRAGILIS and STELLA persist in PGCs during migration. Together with other interferon-induced trans-membrane (IFTM) family proteins, IFTM1 was thought to regulate initiation of PGC migration in mice [22]. However, targeted deletion of the *Iftm* gene cluster including *Iftm1* did not disturb initiation or completion of PGC migration [12].

KITL and c-KIT

KITL (Steel Factor) and its receptor KIT (c-KIT) are implicated in the regulation of PGC migration in mice, especially during the migration along the endoderm for about two days starting on E7.5 [23–25]. As a surface receptor, c-KIT is expressed on PGCs throughout all stages of their migration, while all the somatic cells surrounding PGCs express KITL. KITL expression in the surrounding somatic cells seems to facilitate PGC motility rather than acting as a chemotactic stimulus. Hence, PGCs in *Kitl*-deficient mice embryos move in the proper direction but at a reduced rate, which is described as 50% reduction in average velocity. In addition to facilitating migration, KITL emerges as a niche factor that is essential for survival and proliferation of PGCs.

CXCL12 (SDF1), CXCR4, and E-cadherin

A chemotactic cytokine and its receptor regulate homing of PGCs to gonadal ridges after they leave the hindgut at E9.5. Chemokine (C-X-C motif) ligand 12 (CXCL12, also called SDF1), secreted by genital ridges and the surrounding mesenchyme, acts as a chemoattractant for PGCs. A SDF1-specific receptor, chemokine (C-X-C motif) receptor 4 (CXCR4), expressed by migrating germ cells, responds to the SDF1 signal from genital ridges [26,27]. In both $Sdf1^{-/-}$ and $Cxcr4^{-/-}$ mice, PGC colonization of the gonads is impaired, while the number of PGCs reaching the mesentery of the hindgut at E9.5 is not affected. Moreover, ubiquitous expression of SDF1 in cultured embryo slices perturbs homing of PGCs to genital ridges, and ectopic SDF1 expression causes PGCs to migrate to designated locations [26]. In addition to homing by SDF1/CXCR4, migration of PGCs from

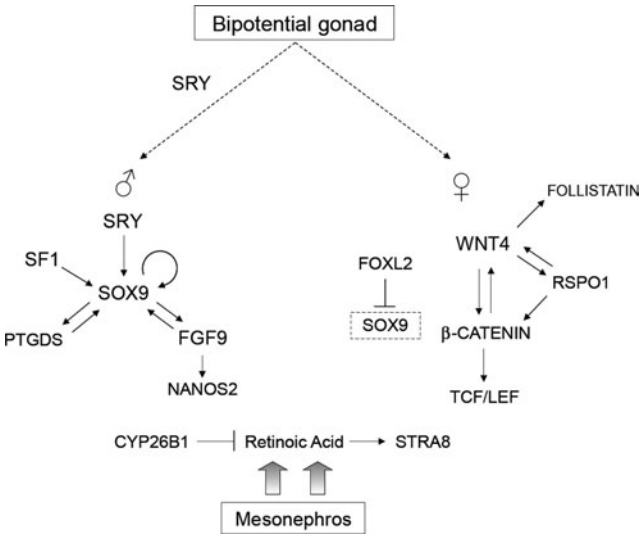

Figure 1.2 Sex determination: Sex determination in gonads precedes germ-cell sex determination. In XY gonads, transient SRY expression up-regulates SOX9 expression. SOX9 induces up-regulation of PTDGS and FGF9 expression. SOX9 itself, FGF9, and PGD2 (which is produced by PTGDS) induce SOX9 expression. Therefore, once induced by SRY, SOX9 expression is maintained. SF1 plays a role in synergistic activation of SOX9 by SRY and SOX9 itself. FGF9 up-regulates NANOS2 expression in germ cells. Together with CYP26B1, which degrades retinoic acid (RA), NANOS2 plays a role in preventing premature meiotic entry in XY germ cells. In XX gonads, RSPO1 up-regulates WNT4 expression and β-CATENIN activity. WNT4 up-regulates RSPO1, Follistatin, and β-catenin activity. β-CATENIN signaling, in turn, up-regulates WNT4 expression and regulates TCF/LEF-mediated transcriptional activation. FOXL2 suppresses SOX9 expression, hence male sex differentiation. RA signaling induces STRA8 transcription in germ cells, which leads to meiosis.

the hindgut to genital ridges is accompanied by up-regulation of a cell–cell adhesion glycoprotein, E-cadherin, which allows PGCs to interact or recognize each other to form a compact cluster and condense into the genital ridges [28].

Sex determination

Until the colonization of the genital ridges, XX and XY PGCs are indistinguishable in terms of morphology and behavior [29]. At the end of their migratory pathway, at around E12.0 in mice, PGCs colonize the genital ridges and are thereafter referred to as germ cells (GCs) or gonocytes. After entry into the gonadal ridges, expression of germline-specific gene products GCNA1 (germ cell nuclear antigen 1), DDX4 (DEAD (Asp-Glu-Ala-Asp) box polypeptide 4), DAZL (deleted in azoospermia-like) commences in both XX and XY germ cells [30–32]. Germ-cell sex determination starts in gonadal ridges and is largely regulated by the niche. Both XX and XY germ cells have the potential to be committed to spermatogenesis or oogenesis, when/if exposed to the somatic cells of a developing testis or ovary, respectively. In mice, male germ cells become committed to spermatogenesis between E11.5 and E12.5 and female germ cells become committed to oogenesis between E12.5 and E13.5 [33].

In mammals, genital ridges, or gonadal primordia, are derived from the coelomic epithelium of the mesonephros, and become visible at E10.0 in mice and at day 32 of pregnancy in humans. Proliferation and differentiation of epithelial cells give rise to gonadal primordia, which express the same gonadal-specific gene products (SF1, WT1, M33/CBX12, EMX2) in both XX and XY tissues [34]. Hence, early gonadal ridges are bipotential, which means they can follow either a testicular or an ovarian fate (Figure 1.2). At this point, the expression of sex-determining region Y (SRY) encoded by a gene on the Y chromosome, triggers a cascade of molecular events that strongly dictate initiation of differentiation of bipotential genital ridges into testes [35,36]. Female sex determination and development of gonadal ridges into ovaries also requires the onset of female-specific gene expression. However, molecular mechanisms establishing the female fate can be superseded by SRY expression in XX mice, which results in testes formation [37]. Moreover, absence of a functional *Sry* gene is enough for XY genital ridges to develop into ovaries and XY GCs to develop as oocytes [38]. In brief, mammalian sex determination seems to require a precise switch mechanism between ovary and testis formation, which is regulated by a single gene on the Y chromosome. SRY, and other genes involved in sex determination are listed in Table 1.3.

Male germline formation

In mice, male germ cells become committed to spermatogenesis between E11.5 and E12.5 [33]. Germ cells develop into spermatogonia and enter mitotic arrest, which takes place between E12.5 and E14.5 in mice. Mitotic arrest lasts until a few days after birth in mice

Table 1.3 Regulatory proteins involved in male and female germline formation.

Protein	Common Alias	Protein Function	Mutant Phenotype	Reference
SRY		A HMG-box-containing transcription factor that initiates male sex determination.	XY-to-female sex reversal.	Lovell-Badge *et al.* 1990 [38]
SOX9		A HMG-box-containing transcription factor activated by Sry.	Complete XY-to-female sex reversal.	Chaboissier *et al.* 2004 [46] Barrionuevo *et al.* 2006 [47]
FGF9		A fibroblast growth factor family protein. Involved in maintenance of Sox9 and germ line sex determination in males.	Disturbed Sertoli cell differentiation. Male-to-female sex reversal.	Colvin *et al.* 2001 [51]
PTGDS		Catalyzes the conversion of prostaglandin H_2 (PGH_2) to prostaglandin D_2 (PGD_2). PGD_2 is involved in regulation of Sox9 expression.	Reduced Sox9 expression in XY gonads but normal testis formation.	Moniot *et al.* 2009 [55]
NANOS2		Plays a role in suppression of meiosis, promotes male fate in germ cells.	Premature meiosis in XY germ cells.	Suzuki *et al.* 2008 [59]
CYP26B1		Involved in specific inactivation of retinoic acid.	Increased retinoic acid level in fetal testis. Premature meiotic entry in XY germ cells (at E13.5) and increased apoptosis after E13.5. Lack of germ cells in male neonates.	MacLean *et al.* 2007 [61]
WNT4		A secreted signaling protein involved in female fetal genital development and suppression of male fate. Involved in activation of β-catenin.	Formation of coelomic vessel and presence of steroidogenic cells in XX gonads.	Vainio *et al.* 1999 [65]
FST		Binds to activin and functions as an activin antagonist. Specifically inhibits follicle-stimulating hormone release. Co-expressed with Wnt4 in female gonads.	Coelomic vessel formation and presence of steroidogenic cells in XX gonads. Increased germ-cell apoptosis, almost no germ cells at birth. No defect in XY testes formation.	Yao *et al.* 2004 [67]
RSPO1		A secreted activator protein of R-spondin family. Activator of the beta-catenin signaling cascade, leading to TCF-dependent gene activation	Differentiation of seminiferous tubules and coelomic vessel formation in XX gonads.	Chassot *et al.* 2008 [70]
CTNNB1		(β-catenin) a key component of the canonical Wnt signaling pathway. Regulates TCF/LEF-mediated transcriptional activation.	Partial sex reversal in females. Formation of steroidogenic cells and a coelomic vessel in XX gonads. Down-regulated Wnt4 expression.	Liu *et al.* 2009 [72]
FOXL2		A fork-head DNA-binding domain-containing transcriptional regulator. Critical factor essential for ovary differentiation and maintenance, and repression of the genetic program for somatic testis determination.	Post-natal sex reversal in XX gonads. Transdifferentiation of granulosa cells into Sertoli-like cells.	Uhlenhaut *et al.* 2009 [77] Ottolenghi *et al.* 2005 [78]
NR0B1	DAX1, AHCH	A transcriptional regulator. Functions as an anti-testis gene by acting antagonistically to Sry.	No effect on female development. XY-to-female sex reversal depending on the mouse strain.	Yu *et al.* 1998 [84] Meeks *et al.* 2003 [85] Bouma *et al.* 2005 [86]
STRA8		A retinoic acid-responsive protein involved in the regulation of meiotic initiation in both spermatogenesis and oogenesis.	Failure in undergoing pre-meiotic DNA replication in germ cells. No oocytes or follicles in ovaries of 8-week-old mice.	Baltus *et al.* 2006 [90]

and until puberty in men. During adult life, spermatogonial cells proliferate while maintaining their identity, hence producing a pluripotent spermatogonia population. They also differentiate into spermatocytes. Spermatocytes enter meiosis to give rise to spermatids, which will mature into spermatozoa. Mitotic arrest in fetal testes, and each step in spermatogenesis in adult life, is regulated by signals from the somatic environment.

SRY

Mammalian male sex determination is initiated by SRY (sex-determining region Y) expression in XY genital ridges, which triggers Sertoli cell differentiation in supporting cell precursors. SRY is encoded by a single exon gene located on the Y chromosome and is the founding member of the SOX (SRY-like box) gene family, which comprises 20 genes in mice and humans. SRY protein (204 amino acids) is a chromatin-remodeling transcription factor and a member of the high mobility group (HMG)-box family of DNA-binding proteins. SRY, through its HMG domain, binds DNA sequences that contain the (A/T)ACAA(T/A) motif and bends the DNA [39]. SRY expression in mouse XY genital ridges starts at E10.5, reaches a peak at E11.5, and ceases by E12.5 [35]. Although it has a transient pattern, any delay (even 6 hours) in initiation of SRY expression results in a failure in testis development, suggesting that SRY expression must start or reach a threshold in a specific time window [40,41]. This transient expression of SRY acts as a trigger and initiates the cascade of molecular events that induce Sertoli cell differentiation and testis formation. A crucial part of the downstream events is activation of SOX9 expression.

SOX9

SOX9 (SRY-like box 9) is a HMG-box-containing transcription factor encoded by a member of the *SOX* gene family. In mice, low levels of SOX9 expression is observed in both XX and XY genital ridges at E10. However, in females, SOX9 expression is repressed and becomes undetectable in genital ridges by E11.5, whereas in XY genital ridges SOX9 expression is up-regulated soon after the onset of SRY expression [42,43]. Up-regulated SOX9 expression is restricted to Sertoli cell lineage and persists after E12.5 [42]. In humans, heterozygous *SOX9* mutations can result in partial or complete male-to-female sex reversal [44], and duplication of *SOX9* gene has been reported in a female-to-male sex reversed case [45]. Mice lacking *SOX9* fail to perform male-specific differentiation and undergo complete male-to-female sex reversal [46,47]. Conversely, transgenic XX mice failing to repress SOX9 expression undergo sex reversal [48]. Therefore, current evidences suggest that while SRY expression in a specific time window is necessary for the onset of up-regulation of SOX9, SOX9 is necessary and sufficient for male sex determination.

SRY directly binds to TESCO (testis-specific enhancer of *SOX9* core), which is an enhancer element located upstream of SOX9, and activates SOX9 expression [49]. SOX9 itself binds to the TESCO element and creates a positive-feedback loop, which provides an insight to continuous SOX9 expression in Sertoli cell lineage. In both cases, together with SRY or SOX9, a transcriptional activator SF1 (steroidogenic factor 1), also called NR5A1, binds to and is involved in the synergistic activation of TESCO [49].

FGF9 and retinoic acid

In addition to the auto-regulatory positive-feedback loop involving SOX9 described above, FGF9 (fibroblast growth factor 9) seems to be required for maintenance of SOX9 expression and a positive feed-forward loop between FGF9 and SOX9 has been postulated [50]. FGF9 is a signaling molecule secreted by the Sertoli cells and induces differentiation in supporting cell precursors by up-regulating SOX9 expression. Targeted deletion of *Fgf9* or its receptor *Fgfr2* (fibroblast growth factor receptor 2) in mice disrupts Sertoli cell differentiation and leads to male-to-female sex reversal [51,52]. Prostaglandin D_2 (PGD_2) is another paracrine factor implicated in regulation of SOX9 expression [53]. SOX9, in turn, activates the expression of prostaglandin D synthase (PTGDS), which is the enzyme that produces PGD_2, forming a positive-feedback loop [54]. PGD_2 is able to induce SOX9 transcription in XX gonads in culture. In *Ptgds*-null mice, the SOX9 transcript level in XY gonads is reduced, but sex determination or testis formation is not affected [55].

FGF9 and retinoic acid (RA) are expressed at the gonadal niche and act as key determinants of sexual fate in XY and XX germ cells, respectively. Evidence suggests that FGF9 and RA have opposing effects on germ cells in terms of meiotic entry decision and sex determination. First, FGF9 promotes

germ-cell survival specifically in XY gonads [56]. In *Fgf9*-null male mice, but not in females, the number of germ cells declines after E11.5 [57]. More importantly, FGF9 alone can markedly increase the survival of purified E11.5 XY germ cells in culture, proving that its effect is direct. Second, it has been shown that FGF9 acts to inhibit meiosis in XY germ cells, and also in XX germ cells [57,58]. The effect of RA on cultured E11.5 germ cells, which is determined by the expression level of an RA-activated gene (STRA8), is attenuated when FGF9 is included in the media [57]. In control of meiotic fate decision, FGF9 up-regulates NANOS2 (nanos homolog 2 (Drosophila)) expression in germ cells, which in turn prevents STRA8 expression [58]. NANOS2 expression is restricted to germ cells in male gonads and in *Nanos2*-null mice fetal germ cells enter meiosis and undergo apoptosis at around E15.5 [59]. Lastly, a P450 enzyme, CYP26B1, is expressed in somatic cells of the embryonic testes by E12.5, which effectively degrades pro-meiotic RA [60]. In *Cyp26b1-null* mice RA levels are increased in E12.5 testes and germ cells prematurely enter meiosis at E13.5, which is followed by apoptosis [61].

Female germline formation

In mice, female germ cells become committed to oogenesis between E12.5 and E13.5 [33]. Unlike the mitotically arrested spermatogonia in male fetal testis, female germ cells in fetal ovaries (oogonia) initiate meiosis and give rise to primary oocytes by E13.5 in mice, and between the 8th and the 13th week of gestation in humans [62]. Primary oocytes become arrested in the prophase of the first meiotic division and remain arrested until puberty. After puberty, with each menstrual cycle a number of primary oocytes are released from the prophase I arrest, continue meiosis and become arrested again in the metaphase of the second meiotic division; this second meiotic arrest is maintained until fertilization. As a crucial step in female germ-cell sex determination, cues to initiate meiosis are provided by female fetal ovaries.

Due to the fact that the presence or absence of male sex-determining factor, SRY, can greatly affect sex determination in mammalian gonads, differentiation of XX gonadal ridges into ovaries is often described as the "passive" or the "default" developmental path. In addition, molecular mechanisms establishing the female fate may be redundant, as implied by the fact that SRY-negative female-to-male sex reversal is very rare in humans and even in transgenic mice. Nevertheless, identification of SRY-negative female-to-male sex reversal cases in two siblings in three families, has led researchers to postulate that there is a recessive gene (termed the Z gene) that is responsible for repressing male development and/or activating female development [63]. While the Z gene is yet to be discovered, transcriptome analysis of mouse gonads during sex determination (between E10.5 and E13.5) shows that 1223 genes in females (and 1083 genes in males) are up-regulated [64]. Some of these genes have well-studied implications in female sex determination.

WNT4

WNT (wingless-related MMTV integration site) genes play key roles as intercellular signaling molecules in development that are conserved between *C. elegans* and vertebrates. In the canonical WNT signaling pathway, WNT ligands bind to cell surface receptors of the FRIZZLED family, leading to stabilization of cytoplasmic β-catenin. In turn, β-catenin transduces the signals to the nucleus and activates TCF/LEF (T-cell factor/lymphoid enhancer factor) family transcription factors to promote specific gene expression. There are 19 WNT and 10 FRIZZLED genes in mouse and human genomes.

WNT4 (wingless-related MMTV integration site 4) is a secreted signaling protein, which is implicated in control of female sexual development and the prevention of testes formation. During embryogenesis, between E9.5 and E10.5, WNT4 is expressed in both males and females, in the mesenchyme of the mesonephros and müllerian duct, and is essential for müllerian duct formation [65]. As sex-specific differentiation commences, WNT4 expression is repressed in the male gonad, while it is maintained in the female gonad. *Wnt4* deficiency has no effect in male sexual development, whereas it results in the presence of steroidogenic cells and formation of coelomic vessels in XX gonads. A loss-of-function mutation in human *WNT4* resulting in a phenotype similar to that observed in *Wnt4*-null mice, including regression of müllerian ducts, has been reported [66]. Downstream of WNT4, an activin-binding protein encoded by *Fst* gene, FOLLISTATIN, is employed [67]. FOLLISTATIN expression co-localizes with WNT4 in the female

gonad, whereas it is absent in *Wnt4*-null mice. Moreover, *Fst*-deficient female mice fail to repress coelomic vessel formation and greatly mimic the *Wnt4*-null phenotype.

RSPO1

RSPO1 (R-sponsin 1) mutations have been associated with complete female-to-male sex reversal in humans [68]. In mouse embryos, RSPO1 expression is observed in the urogenital ridge, starting by E10.5, and in various other tissues, including the dorsal neural tube (E10–12), developing dermis (E12.5) and kidneys (E11.5) [68,69]. The sex-specific expression pattern is observed at E12.5 as a marked increase in RSPO1 expression in XX gonads. By E14.5, RSPO1 expression in XX gonads is fivefold higher than XY gonads [68]. Ablation of *Rspo1* in mice induces differentiation of seminiferous tubules in XX gonads but does not result in sex reversal as expected [70]. In addition, WNT4 expression is down-regulated in *Rspo1*-null gonads at E11.5 and E12.5, implying that RSPO1 is required for activation of WNT4 expression. Moreover, RSPO1 is a potent activator of WNT/β-catenin signaling and has been shown to inhibit internalization of LRP6 (low density lipoprotein receptor 6), which acts as a co-receptor with FRIZZLED in WNT signaling [71]. Hence, *Rspo1* deficiency results in a marked reduction in β-catenin activity [70].

β-catenin

As the key intracellular component of WNT signaling, β-catenin (CTNNB1*)* is required during female development. Binding of extracellular WNT ligands to FRIZZLED cell surface receptors leads to stabilization of cytoplasmic β-catenin, which is otherwise subject to phosphorylation and degradation. Ultimately, β-catenin accumulates and translocates to the nucleus to regulate TCF/LEF-mediated transcriptional activation. Employing a *LacZ*/target-gene fusion reporter, the active β-catenin-signaling pathway is detected in somatic cells of XX gonads starting by E12.5, but not in XY gonads [70]. Both WNT4 and RSPO1 contribute to stabilization of β-catenin in female gonads. *Wnt4*-null and *Rspo1*-null mice show only partial XX sex reversal and have similar ovarian phenotypes. Conditional ablation of *Ctnnb1* in SF1-positive somatic cells in female gonads results in a phenotype very similar to *Wnt4*-null and *Rspo1*-null mice [72]. Moreover, WNT4 expression is down-regulated in β-catenin-deficient female gonads, while RSPO1 expression is not affected. This may suggest a positive-feedback loop between WNT4 and β-catenin, while RSPO1 functions upstream of β-catenin. Interestingly, while overexpression of WNT4 has not been successful in causing male-to-female sex reversal [73,74], ectopic expression of a stable β-catenin in XY gonads has been sufficient to disrupt the male pathway and promote ovarian development [75].

FOXL2

Forkhead box L2 (FOXL2) is a transcriptional regulator containing a fork-head DNA-binding domain. In mice, FOXL2 expression is activated in XX gonads at around E12.5. FOXL2 is required for granulosa cell differentiation and ovary maintenance [76]. Moreover, it has been shown that FOXL2 can bind and repress TESCO (an enhancer element of SOX9) [77]. Hence, FOXL2 deficiency results in SOX9 up-regulation in XX ovaries, but only in perinatal and adult mice [77, 78]. In addition, conditional deletion of *Foxl2* in 8-week-old mice results in transdifferentiation of granulosa cells into Sertoli-like cells, which is described as post-natal sex reversal in the XX gonad [77]. A similar phenotype, transdifferentiation and post-natal sex reversal, is reported in mice lacking both *Esr1* (estrogen receptor 1) and *Esr2* (estrogen receptor 2) [79].

DAX1

DAX1, also called NR0B1 (nuclear receptor subfamily 0, group B, member 1), is encoded by an X-linked gene and has the potential to interfere with sex determination in a dose-dependent manner. DAX1 is first expressed in the bipotential genital ridges of both XX and XY mice embryos at E11.5 [80]. Between E12 and E12.5 DAX1 expression ceases to exist in males while it persists at least until E15.5 in female gonads. Duplications of a *DAX1*-containing region of Xp21 in XY individuals are associated with male-to-female sex reversal [81,82]. Also in mice, extra copies of the *Dax1* gene can cause complete male-to-female sex reversal [83]. Unexpectedly, DAX1 deficiency does not affect sex determination in XX mice, which demonstrates that DAX1 is not required for female development [84]. Moreover, targeted deletion

of *Dax1* can cause complete male-to-female sex reversal in some mouse strains in a *Sry*-dependent manner [85,86].

Retinoic acid

Sex determination in XX germ cells is regulated by retinoic acid (RA) signaling, which triggers meiosis to initiate oogenesis. RA is produced not by gonads but by mesonephros of both sexes by E10 [60]. In females, RA signal induces up-regulation of the premeiotic marker STRA8 (stimulated by retinoic acid gene 8) at around E12.5 [86], while it is degraded by CYP26B1 in developing male testes. Still, high levels of RA in fetal testis can induce STRA8 up-regulation and meiosis in XY germ cells, as in *Cyp26b1*-null mice [60,61].

Summary

In mammals, germline is established early in embryogenesis. Primordial germ cells (PGCs) are derived from the distal epiblast, under the control of extracellular signals. Specifically, BMP4 and BMP8B are essential for PGC formation. Critical regulators for PGC specification like BLIMP1 and PRDM14, as well as novel PGC markers like FRAGILIS and STELLA have been identified by comparison of single-cell gene expression profiles of early PGCs with surrounding somatic cells. PGC specification is followed by migration of PGCs to gonadal ridges. During migration, cell surface receptors on PGCs, secreted ligands from surrounding somatic cells, and chemotactic cues from gonadal ridges play important roles.

Germ-cell colonization of the gonads is followed by sex determination. Expression of sex-specific genes in somatic tissues initiates molecular events that lead to testis or ovary development. Particularly, expression of Y-linked SRY, which activates SOX9 expression promotes testis differentiation. As for female sex determination, although a major player has not been identified, WNT/β-catenin signaling seems to plays a key role. FGF9 and retinoic acid signaling from differentiated somatic cells to germ cells controls meiotic entry and germ-cell sex differentiation in developing testes and ovaries, respectively.

In recent years, elegant studies in a multitude of model organisms have improved our understanding of the genetic regulation of germline formation. Further studies will enable us to better understand the biology of this complex yet extremely important process and reinforce the framework for studies directed at generation of gametes from stem cells.

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