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Specification of the germ cell lineage in mice

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1. ABSTRACT

Specification of the germ cell lineage is fundamental in development and heredity. In mice, and presumably in all mammals, germ cell fate is not an inherited trait from the egg, but is induced in pluripotent epiblast cells by signaling molecules. Recent studies are beginning to uncover the signaling requirements and key transcriptional regulators for the specification of the germ cell lineage in mice, as well as the distinct properties that the specified germ cells acquire uniquely. Accordingly, the evidence suggests that germ cell specification is an integration of the repression of the somatic program, re-acquisition of potential pluripotency, and ensuing genome-wide epigenetic reprogramming. The accumulated knowledge will be critical for the reconstitution of this key lineage *in vitro*, which may provide a useful foundation for reproductive and regenerative medicine.

2. INTRODUCTION

Specification of germ cell fate is fundamental in development and heredity. There are essentially two key pathways by which the specification is realized. One is through the inheritance of determinants, generally known as germ plasm, that are maternally deposited into specific blastomeres (preformation) and the other through the induction by specific signals from a pluripotent cell population that arises in the middle of embryonic development (epigenesis) (1, 2). Although the preformation mode is seen in many model organisms of modern biology, including *C. elegans* and *D. melanogaster*, recent studies have suggested that the epigenesis mode, which is seen in mice and probably all mammals, is more prevalent across and ancestral to the Metazoa (1). Despite the difference in the mode of germ cell specification, recent studies are beginning to show that both modes involve

common cellular events, most notably, the repression of the somatic program, although the mechanisms involved are markedly different among the organisms (3-6). Furthermore, it has also become increasingly evident that the specified germ cells utilize a number of common molecular pathways for their further development, reflecting conserved properties shared by this key lineage across essentially all the species (3). In this article, I will discuss the present knowledge on the mechanisms of germ cell specification in mice, which seems to involve an integration of the repression of somatic program, re-acquisition of potential cellular pluripotency, and ensuing genome-wide epigenetic reprogramming. These mechanisms may be related to those responsible for inducing pluripotency in somatic cells (7-12).

3. FROM FERTILIZATION TO THE SPECIFICATION OF THE NEW GERM CELL LINEAGE

The developmental program in the mouse is initiated upon the fusion of a highly specialized female gamete, the oocyte, with a male counterpart, the sperm. One of the first events to occur in the fertilized oocyte is the re-organization of the paternal genome, which includes replacement of the protamines with maternally deposited histones and subsequent apparent genome-wide DNA demethylation (13-16). These events take place prior to the S phase. It is notable that genome-wide DNA demethylation from the paternal genome is a conserved phenomenon across several mammalian species, which suggests the functional significance of this event (17). Apparently, this resetting of the epigenetic modifications is a well-programmed process in the initiation of embryonic development (18).

The resultant totipotent zygote, with key maternal factors, replicates haploid parental genomes and begins to undergo cleavage divisions, with the major zygotic transcription starting at the late 2-cell stage (19, 20). The first sign of cell fate specification seems to occur in the 8- to 16-cell stage, when the cells located outside start to show signs of differentiation towards trophectoderm (TE) cells and the cells located inside remain undifferentiated and maintain pluripotency (21). Although the precise mechanisms regulating this process are unknown, reciprocal inhibition of the key lineage determinants Oct4 and Cdx2 seems to play a role in the segregation of these two lineages (22, 23). More recently, a TEA DNA binding domain-containing transcription factor, Tead4, was shown to be critical for up-regulating Cdx2 specifically in the outside cells at the morulae stage embryos, which in turn is essential for TE differentiation (24, 25). Upon formation of the blastocoel cavity, the developing embryos are referred to as blastocysts. At around embryonic day (E) 3.5, the blastocysts consist of two clearly discernable cell types, the TE and the inner cell mass (ICM) cells. The ICM is the source of all the cells in the adult body, including germ cells, and is a pluripotent cell population (26, 27).

Following substantial genome-wide DNA demethylation in the preimplantation stages (28, 29), embryonic DNA methylation patterns start to be imposed through lineage-specific *de novo* methylation that begins in the ICM of a blastocyst (30, 31). Genome-wide DNA methylation levels increase rapidly, and this increase is mediated by the *de novo* DNA methyltransferases Dnmt3a and Dnmt3b (30). Another repressive modification, histone H3 Lysine-9 di-methylation (H3K9me2), increases after the two-cell stage, which thus precedes *de novo* DNA methylation (32).

The first lineage that differentiates from the ICM is the primitive endoderm (PE), which delineates the inner surface of the ICM at E4.5 (33). The undifferentiated cells in the ICM are now called the primitive ectoderm, which maintains pluripotency. Upon implantation, TE cells that are in direct contact with the ICM or the primitive ectoderm (polar TE) proliferate and grow into a thick column of extraembryonic ectoderm (ExE) cells. The primitive ectoderm cells then form, through a process of cavitation (34), a cup-shaped epithelial sheet, which is called the epiblast. It is from these epiblast cells that all the somatic cells as well as the germ cells in the adult body arise. The initial patterning of embryogenesis, including anterior-posterior polarity formation, the gastrulation that forms mesodermal and definitive endodermal cells, and germ cell specification is mediated through signaling molecules from the ExE and PE-derived visceral endoderm (VE) that cover the epiblast (33).

4. EXPERIMENTAL EMBRYOLOGY ON THE ORIGIN OF THE GERM CELL LINEAGE

In mammals, the origin of the germ cell lineage in embryogenesis had long been unknown due to the absence of the characteristic germ plasm in the egg as seen in other organisms such as *X. laevis* and *D. melanogaster*. It was in 1954 that Chiquoine found that the primordial germ cells (PGCs), the first population of the germ cell lineage that gives rise to both oocytes and sperm, can be identified as cells bearing high alkaline phosphatase (AP) activity in the endoderm of the yolk sac immediately beneath the primitive streak of a young 8-day embryo (35). These AP-positive PGCs migrate through the developing hindgut endoderm, eventually colonizing in the genital ridges after E9.5, where they begin to differentiate into functional gametes through highly complex developmental pathways. Much later, in 1990, the origin of the PGCs was traced back to a small cluster of AP-positive cells just posterior to the definitive primitive streak in the extraembryonic mesoderm (ExM), separated from the embryo by the amniotic fold, of E7.0-7.25 embryos (36) (Figure 1). It was suggested that PGCs are set aside as early as E7.0, possibly as one of the first “mesodermal” cell types to emerge. Subsequently, based on a clonal analysis of the fate of epiblast cells of E6.0 and E6.5 embryos, it was proposed that precursors of PGCs reside in the proximal epiblast close to the ExE in both stages of embryos and they are not lineage restricted while they are in the epiblast (37). This analysis also predicted that the founder population of PGCs, located in the extraembryonic mesoderm, consists of approximately 45 cells. Transplantation of distal epiblast cells of E6.5 embryos into

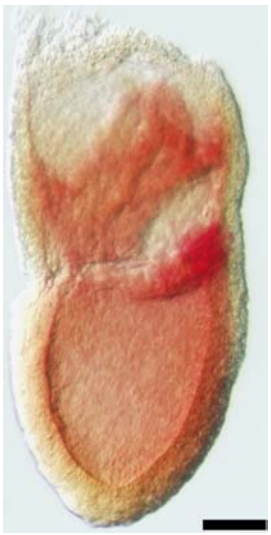

Figure 1. An early-bud (EB) (~E7.25) stage embryo stained for alkaline phosphatase (AP) activity. A cluster of AP-positive primordial germ cells (PGCs) locates just above the posterior proximal end of the epiblast. Anterior is to the left and posterior is to the right. Scale bar, 50μm.

the proximal region of the other embryos resulted in the contribution of the donor cells to the germ cell lineage, indicating that epiblast cells as late as E6.5 are pluripotent and can form germ cells when placed in an appropriate microenvironment (38).

5. SIGNALING FOR GERM CELL SPECIFICATION

The uncovering of signaling molecules and their signal transducers necessary for the specification of the germ cell lineage was accomplished by gene knockout studies (39, 40) (Table 1). Most notably, Bmp4, which is initially expressed in the ExE directly contacting the proximal epiblast from around E5.5, was the first to be demonstrated as essential for PGC specification (41). In the *Bmp4* mutants on a mixed genetic background, PGCs detectable by AP staining, as well as the allantoic mesoderm, were not formed. In *Bmp4* heterozygous mutants, the number of founder PGCs was reduced to less than half. Chimera analysis indicated that it is the Bmp4 expression in the ExE that is essential for the formation of PGCs. This study thus demonstrated for the first time that germ cell specification in mice depends on a secreted signal from the previously segregated, extraembryonic lineage. Subsequently, it was shown that Bmp4 expressed in the ExM is not required for the specification of PGCs but is necessary for the correct localization and survival of PGCs (42).

Bmp8b, which is expressed exclusively in the ExE from as early as E5.5, was also shown to be critical for PGC specification (43). On a predominantly C57BL/6 background, almost no PGCs or a reduced number of PGCs were formed in the homozygous and heterozygous mutants, respectively. Additionally, it was shown that embryos that are double heterozygotes for the *Bmp8b* and *Bmp4* mutations have similar defects in PGC numbers as *Bmp4* heterozygotes, indicating that the effects of the two Bmps are not additive.

Bmp2, expressed primarily in the VE of pregastrula and gastrula embryos, also plays a role in the establishment of the germ cell lineage (44). The number of PGCs is significantly reduced in *Bmp2* heterozygous and homozygous embryos on a largely C57BL/6 background. Notably, *Bmp2* and *Bmp4* have an additive effect on PGC generation, whereas *Bmp2* and *Bmp8b* do not. Thus, these findings indicate that at least three Bmps, Bmp4 and Bmp8b from ExE and Bmp2 from VE, are necessary for the proper establishment of PGCs *in vivo*.

Currently, it is unknown through which receptors these Bmps signal for PGC specification. A Bmp ligand initiates signaling by binding to and bringing together type I and type II receptor serine/threonine kinases on the cell surface. This allows receptor II to phosphorylate the receptor I kinase domain, which then propagates the signal through phosphorylation of the conserved C-terminal residues of the Smad1, 5, or 8 proteins, which, by forming heterodimers with Smad4, translocate to the nucleus and function as transcriptional regulators (45, 46). Bmps use three different type II receptors, Bmp type II receptor (Bmpr-II) and activin type II receptors (Actr-IIA and Actr-IIB), and three type I receptors, Activin receptor-like kinase (Alk) 3/Bmpr-IA, Alk6/Bmpr-IB, and Alk2. *BmprII*, which encodes a type II receptor for Bmp2 and Bmp4, is expressed uniformly in the embryonic and extraembryonic tissues at E6.5. Inactivation of *BmprII* results in early embryonic lethality, due to impaired growth of the epiblast and a failure to form mesoderm or gastrulate (47). *Alk3*, which encodes a type I receptor for most, if not all, Bmps, is expressed ubiquitously throughout development, including the epiblast at around E6.0. Embryos homozygous for *Alk3* show a reduced size of the epiblast as early as E6.5 and form no mesoderm and fail to gastrulate (48). The early and severe phenotypes of these mutants preclude the possibility of analyzing if it is these receptors that are involved in PGC specification. *Alk2*, which encodes a type I receptor for Bmp7 and possibly for Bmp2 and 4, is expressed specifically in the VE before gastrulation and later both in embryonic and extraembryonic cells during gastrulation (49, 50). *Alk2*-deficient embryos are morphologically normal before gastrulation but show severe gastrulation defects and fail to form proper mesoderm. This gastrulation defect can be rescued in chimeric embryos generated by injection of *Alk2* mutant ES cells into wild-type blastocysts, indicating that *Alk2* function in the VE is essential for the proper gastrulation (49, 50). Interestingly, there is a report showing that AP-positive PGCs are absent in *Alk2* mutants (51), although the mechanism through which Alk2 in the VE contributes to PGC specification remains unclear.

Consistent with the requirement of Bmps for PGC specification, several Smad proteins transducing Bmp signals are known to be required for PGC specification. *Smad1* and *Smad5*, which are expressed widely in the epiblast during gastrulation, are both necessary for PGC

Table 1. Mutants affecting PGC[1] specification and/or early PGC development

Genes	Expression in early embryos	Phenotypes relevant to PGC development	References
Bmp4	ICM[2], ExE[3] from E[4]5.5, ExM[5] during gastrulation.	Lack of PGCs and allantois in homozygous mutants; reduced number of PGCs in heterozygous mutants on mixed genetic backgrounds; early embryonic lethality in the C57Bl/6 background. Loss of Bmp4 in the ExM causes abnormal PGC localization and survival.	(41, 42, 178)
Bmp8b	ExE from E5.5.	Lack and reduced number of PGCs in homozygous and heterozygous mutants, respectively, on a largely C57Bl/6 background. A short allantois in homozygous mutants	(43, 179)
Bmp2	VE[6], stronger in the boundary between ExE and epiblast, at around E6.0-E6.75.	Significantly reduced numbers of PGCs both in the heterozygous and homozygous mutants at the N2 generation onto C57Bl/6 background; a short allantois in homozygous mutants.	(44, 180)
BmprII[7]	Uniform expression in embryonic and extraembryonic tissues during gastrulation.	Defects in gastrulation and lack of mesoderm formation.	(47)
Alk3[7]	Ubiquitous in gastrulation.	Defects in epiblast proliferation and lack of mesoderm formation.	(48)
Alk2	Primarily in the VE before gastrulation and later in both embryonic and extraembryonic cells during gastrulation.	Lack and reduced number of PGCs in homozygous and heterozygous mutants.	(49-51)
Smad1	Ubiquitous in the epiblast and strong in the nascent mesoderm during gastrulation.	Lack of PGCs in homozygous mutants.	(52, 54, 57)
Smad5	Ubiquitous in the epiblast and strong in the nascent mesoderm during gastrulation.	Lack and reduced number of PGCs in homozygous and heterozygous mutants.	(53, 55, 181)
Smad4	Ubiquitous during gastrulation and later in development.	Severely reduced number of PGCs in epiblast-specific *Smad4* mutants; defects in epiblast proliferation; failure to gastrulate and lack of mesoderm formation in simple *Smad4* mutants.	(56, 182, 183)
Smad2	Uniformly expressed throughout all tissue layers during gastrulation.	Abundant PGCs at E8.5 in a minority of surviving embryos. In most mutants, embryos become abnormal shortly after implantation and the entire epiblast adopts an extraembryonic mesodermal fate.	(52, 60, 61)
Blimp1	VE, lineage-restricted PGC precursors and PGCs, later many specific cell types in all three germ layers.	Early block in PGC specification.	(70, 76, 88)
Kit	PGCs after E7.25	Impaired PGC proliferation after E8.0 and eventual loss of PGCs.	(97, 111, 115, 122
SCF/Kitl	VE at E7.5 and along the migratory pathways of PGCs and in the genital ridges.	Impaired migration of PGCs to the gonad.	(112, 184, 185)
Oct4	Ubiquitous in the embryo proper until E7.5 and specific to PGCs after E7.5	Apoptosis of PGCs	(124)
Dnd1	PGCs after E6.75, apparently ubiquitous in embryos at E7.5.	Reduced number of PGCs as early as E8.0 and eventual loss of PGCs.	(97, 125-128, 130)
nanos3	PGCs after E7.25.	Reduced number of PGCs as early as E8.0 and eventual loss of PGCs.	(97, 134)
Tiar	Both in PGCs and their somatic neighbors at least until E8.25	Reduced number of PGCs in the genital ridges as early as E11.5 and eventual loss of PGCs.	(63, 97, 137)
Hif-2a	Ubiquitous in the embryo proper and the yolk sac at E7.5 and E8.5. Later in development, specific tissues such as the dorsal aorta and intersegmental arteries.	Reduced number of PGCs as early as E8.5 and eventual loss of PGCs.	(142, 186-189)

Abbreviations:[1] primordial germ cells, [2] inner cell mass, [3] extraembryonic ectoderm, [4] embryonic day, [5] extraembryonic mesoderm, [6] visceral endoderm, [7]The PGC phenotype has been unexplored due to profound earlier effects.

specification (52-54). Both *Smad1*-deficient embryos and *Smad5*-deficient embryos have severely reduced numbers of PGCs. *Smad1* and *Smad5* double heterozygous embryos show much smaller numbers of PGCs compared to that in either single heterozygote, indicating that Smad1 and Smad5 have an additive effect on PGC formation (55). Whether Smad1 and Smad5 have redundant functions or distinct roles in PGC specification remains to be clarified. Smad4, which functions by forming a heterodimer either with Smad1 or Smad5 for Bmp signal transduction, is also shown to be essential for PGC formation (56). Collectively, all the evidence to date demonstrates that both the Bmp signals emanating from ExE and those emanating from VE are essential for PGC specification. However, the molecular mechanisms by which these Bmp signals induce only a subset of proximal epiblast cells to commit to the PGC fate are still largely unexplored.

Interestingly, recent studies have shown that key signals for embryonic patterning converge, at least in some part, on Smad proteins (57-59). The Smad proteins consist of two conserved globular domains (MH1 and MH2 domains) connected by a linker region. The MH1 domain binds DNA, whereas the MH2 domain binds membrane receptors for activation by phosphorylation (as described above), nucleoporins for nuclear translocation, and other Smads and nuclear factors to form transcriptional complexes. It has been shown that MAPKs and GSK3 successively phosphorylate the conserved residues in the linker region, which are then recognized by Smurf ubiquitin ligase for degradation (58, 59). Therefore, signals through MAPKs and GSK3 negatively regulate and hence determine the duration of the Bmp signals. Notably, Aubin *et al.* generated two point mutants of Smad1: $Smad1^{C}$ that mutates the conserved receptor phosphorylation sites at the C-terminus and $Smad1^{L}$ that mutates the MAPK consensus sites in the linker region (57). The $Smad1^{C/C}$ mutants showed similar phenotypes to the Smad1 null mutants and exhibited severe defects in PGC specification. In contrast, the $Smad1^{L/L}$ mutants were viable and survived to adulthood with specific phenotypes in stomach homeostasis. However, interestingly, the $Smad1^{L/L}$ mutants also showed

defects in PGC specification, which were rescued by the functionally inactive $Smad1^{C}$ allele: the $Smad1^{L/C}$ mutants showed relatively normal PGC specification. These findings suggest that the tight balance of Bmp and MAPK signalings through Smad1 is critical for PGC specification.

Notably, in the mutants of Smad2, which transduces the signals of Tgfβ and Nodal, there appear many ectopic clusters of PGCs (52). This likely reflects the fact that all the epiblast cells of *Smad2* mutants seem to acquire a proximal posterior property (60, 61) at least in part due to the failure to form anterior visceral endoderm, a key signaling center secreting inhibitors of posteriorizing signals and conferring an anterior property to the epiblast (33, 62).

6. KEY MOLECULES AND EVENTS ASSOCIATED WITH GERM CELL SPECIFICATION

6.1. The founder PGCs repress the Hox genes

Despite these advances on the embryological origin of and signaling requirements for the PGCs, very little was known regarding the genes that determine the PGC fate and the intrinsic properties of PGCs until relatively recently. This was in part due to the difficulty of analyzing the founder population of PGCs, which is small in number and embedded deeply in somatic neighbors. However, single-cell gene expression analysis of the founder PGCs and their somatic neighbors revealed that the PGCs indeed show distinct properties (63). Most notably, PGCs that showed high levels of an interferon-inducible transmembrane protein fragilis/mil-1/ifitm3 (63-65) and exclusive expression of a small nuclear-cytoplasmic shuttling protein stella/Pgc7/Dppa3 (63, 66, 67) were found to specifically repress the Hox genes, including *Hoxb1* and *Hoxa1*, which are highly up-regulated in somatic neighbors (63). It has been proposed that the repression of the Hox genes reveals one of the mechanisms by which the PGCs escape from a somatic fate and retain their pluripotency. At the same time, it was identified that founder PGCs, similarly to their somatic neighbors, express the typical mesodermal markers *T* (*Brachyury*) and *Fgf8*, suggesting that the PGC fate is induced in a population of cells originally destined for a somatic mesodermal fate (63).

Since expression of stella and repression of the Hox genes are highly correlated in PGCs, it was postulated that stella may repress the expression of the Hox genes in PGCs. However, a gene knockout study showed that stella is dispensable in PGC specification (68). However, interestingly, it was found that stella-deficient oocytes lose their developmental potential (68), which was due to aberrant genome-wide DNA demethylation in a single-cell zygote (69). As discussed above, upon fertilization, the paternal haploid genome has been known to specifically remove its genome-wide DNA methylation (13, 14). Interestingly, in the absence of Pgc7/stella, genome-wide DNA demethylation occurs not only from the paternal genome but also from the maternal genome. Moreover, some of the specific methylations on the imprinted genes of the paternal genome, which are protected from genome-wide DNA demethylation in normal embryos, are also demethylated in Pgc7/stella (-/-) embryos. These findings indicate that Pgc7/stella functions to prevent DNA demethylation from the maternal allele and from imprinted genes on the paternal allele in normal development (69).

6.2. Blimp1 is a critical regulator for germ cell specification

Further single-cell analysis has identified *Blimp1*/*Prdm1* as a gene specifically expressed in founder PGCs (70). *Blimp1* encodes a potent transcriptional repressor with a N-terminal PR/SET domain, a proline-rich region, five C_2H_2 zinc fingers, and a C-terminal acidic domain, and was originally cloned as a gene specifically induced upon terminal differentiation of B-cells into plasma cells (B lymphocyte-induced maturation protein-1) (71). Blimp1 was later shown to be both necessary and sufficient for the terminal differentiation of B-cells (72, 73), leading to a notion that Blimp1 is a "master regulator" of plasma cell differentiation (74). Careful expression analysis has shown that *Blimp1* is first expressed in a few of the most proximal posterior epiblast cells at around E6.25 prior to the onset of gastrulation. *Blimp1*-positive cells increase in number and form a cluster of approximately 20 cells at E6.75 (mid-streak (MS) stage) and 40 cells with strong AP activity at E7.25 (early-bud (EB) stage) (Figure 2). Genetic lineage tracing showed that all the *Blimp1*-positive cells at early stages contribute almost invariantly to stella-positive PGCs. It was therefore concluded that *Blimp1*-positive cells appearing in the most proximal epiblast at E6.25 are lineage-restricted PGC precursors (70, 75).

These observations were unexpected in light of the initial studies by clonal analysis of the fate of epiblast cells, which indicated that germ cell restriction is not evident in the early epiblast cells (37). It is possible that the clonal analysis may have failed to label one of the very few early *Blimp1*-positive epiblast cells. The epiblast clones contributing to PGCs in the clonal analysis might be the ones that were initially negative for *Blimp1*, but following division of these cells, some of them may have become *Blimp1*-positive and given rise to PGCs while the others gave rise to somatic descendants. It will be necessary to precisely clarify the mechanism that is responsible for the initial increase in the numbers of *Blimp1*-positive cells to resolve this issue fully. It is currently unknown how long the *Blimp1*-positive germ cell precursors continue to be recruited from the epiblast cells, and how the cell cycle of the *Blimp1*-positive cells is subsequently regulated, which together might be the key to understanding the properties of these PGC precursors.

Blimp1 function is essential for PGC specification (70, 76, 77). In *Blimp1* mutants, PGC specification seems to be halted at a very early stage, with only about 20 AP-positive PGC-like cells formed (70). These PGC-like cells do not subsequently increase in number and fail to show migration. Gene expression analysis of *Blimp1* mutant PGC-like cells indicates that these cells fail to consistently repress the Hox genes, with inadequate acquisition of some of the genes specific for PGCs, including stella (70). Although further analysis will

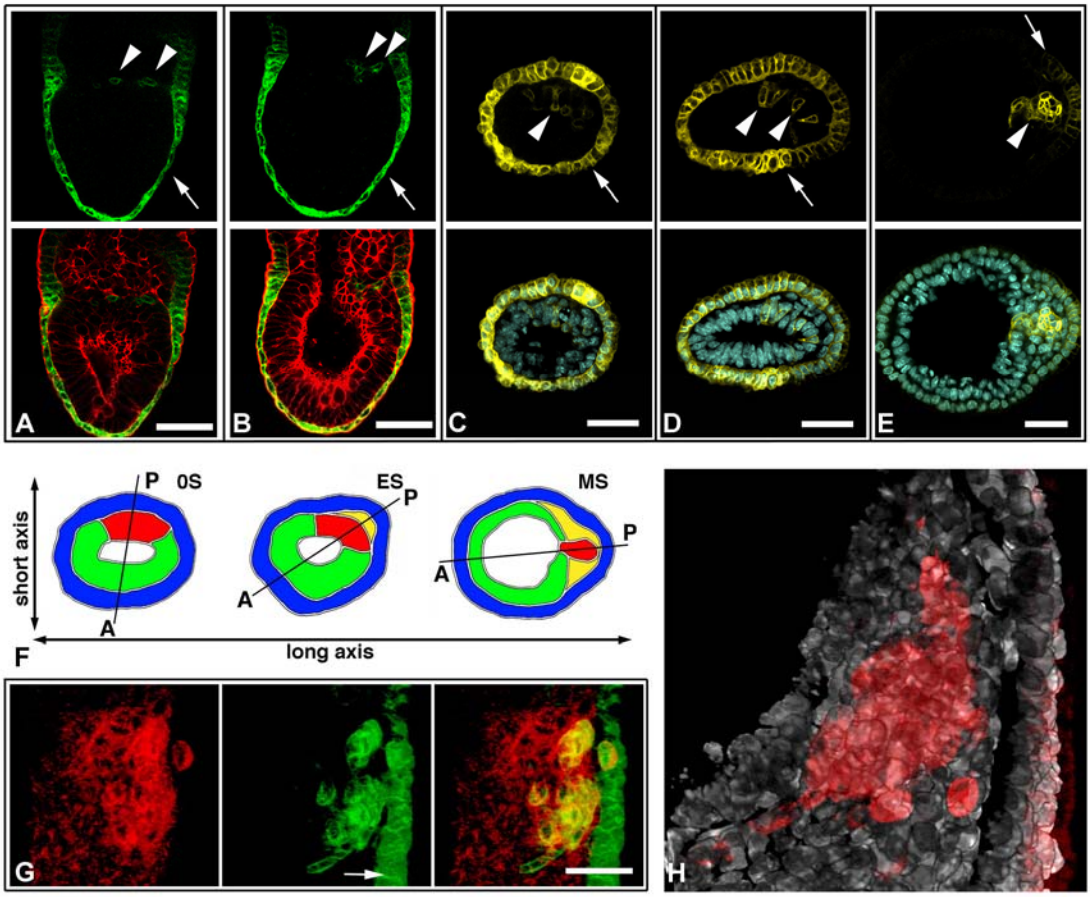

Figure 2. Origin of the *Blimp1*-positive germ cell lineage. A, B, Lateral views of pre/no-streak (P/0S, ~E6.25) (A) and early-streak (ES, ~E6.5) (B) stage embryos bearing a transgene expressing membrane targeted EGFP under the control of *Blimp1* regulatory elements (*Blimp1*-mEGFP) counterstained with phalloidine (red, bottom panels) (70). Arrowheads indicate *Blimp1*-positive lineage restricted primordial germ cell (PGC) precursors in the epiblast and arrows indicate *Blimp1* expression in the visceral endoderm (VE). C-E, Transverse views of P/0S (C), ES (D), and mid-streak (MS, ~E6.75) (E) stage *Blimp1*-mEGFP (pseudo-colored in yellow) embryos counterstained with DAPI (cyan, bottom panels). Arrowheads indicate *Blimp1*-positive lineage restricted PGC precursors in the epiblast (C, D) or in the nascent mesoderm (E) and arrows indicate *Blimp1* expression in the VE. F, Rotation of the embryonic axis and distribution of *Blimp1*-expressing cells during early gastrulation. *Blimp1*-positive cells (red), *Blimp1*-negative epiblast cells (green), nascent mesoderm (yellow) and VE (blue) are shown. A, anterior; P, posterior. G, Base of allantois region of an early-bud (EB) stage embryo stained for AP (left) and *Blimp1*-mEGFP (middle). A merged image is shown in the right. Arrows indicate *Blimp1* expression in the VE. H, Projected three-dimensional image of confocal sections of *Blimp1* expression (red) at the EB stage, counterstained with DAPI (white). Scale bars, 50μm.

be required for a more precise understanding of the function of Blimp1 in PGC specification, the findings described above indicate that Blimp1 plays a central role in PGC specification.

Studies in other contexts have shown that Blimp1 functions as a transcriptional repressor by recruiting a repressor complex of Groucho family proteins (78) and HDAC2 (79) through its proline-rich region and Zinc fingers, and the histone methyltransferase G9a for histone H3 lysine9 di-methylation (80), for which the Blimp1 zinc fingers are essential to generate the appropriate complex (81). Blimp1 can also activate the expression of some genes via its carboxyl terminal acidic domain (82). Therefore, the Blimp1 protein has a modular structure that can potentially perform diverse functions in a context-dependent manner.

Accordingly, *Blimp1* has been shown to be expressed in many cell types during development and subsequently in the adults in several vertebrate species, and in at least some of these species it appears to play a critical role during the establishment of the identity of diverse cells. For example, in the zebrafish, Blimp1 is required for the specification of at least three cell types, i.e., slow-twitch muscle fibers (83) and the common progenitors of the neural crest and sensory neurons (84, 85), the latter two of which, interestingly enough, also require Bmp signaling. In the Xenopus and zebrafish, *Blimp1* is expressed in the anterior mesendoderm and prechordal plate, where it is

required for regulating various functions of these tissues (86, 87). Notably, germ cell specification in *Xenopus* and zebrafish is predetermined by the inheritance of germ plasm, and consequently, no defects in germ cell formation have been reported in these organisms in the absence of *Blimp1*. In mice, *Blimp1* is expressed in the analogous anterior visceral endoderm (AVE) and anterior definitive endoderm (ADE)/prechordal plate, and then widely in specific cell types derived from all three germ layers (76, 77, 88). *Blimp1*-deficient embryos die at around E10.5, but the early axis formation, anterior patterning and neural crest formation proceed normally in these animals. *Blimp1* deficiency instead disrupts morphogenesis of the caudal branchial arch, which leads to widespread blood leakage, tissue apoptosis and failure to correctly elaborate the labyrinthine layer of the placenta (76). Furthermore, Blimp1 was shown to play critical roles in multipotent progenitor cell populations in the posterior forelimb, caudal pharyngeal arches, secondary heart field and sensory vibrissae and maintains key signaling centers at these diverse tissue sites (77). In the adult, Blimp1 was shown to define a progenitor population that governs cellular input to the sebaceous gland (89) and to be a critical regulator of keratinocyte transition from the granular to the cornified layer (90). Blimp1 also plays a critical role in controlling T-lymphocyte homeostasis (91, 92).

Interestingly, a recent study has provided evidence that Blimp1 forms a novel complex with an arginine-specific methyltransferase, Prmt5 (93). Prmt5 belongs to the protein arginine methyltransferase family and functions by conferring ω-N^G, N'^G-symmetric di-methylation to arginine residues in diverse target proteins, which include histone H4, H2A, and H3, and spliceosomal proteins SmD1, SmD3, and SmB/B' (94). In cultured 293T and P19 embryonal carcinoma cells, transiently overexpressed Blimp1 co-immunoprecipitates Prmt5. Blimp1 and Prmt5 show apparent co-localization in the nuclei of PGCs, at least from E8.5 to E10.5, and the levels of symmetrical di-methylation of histone H4 arginine3 (H4R3me2) in the PGC nuclei seem to be elevated in this period. Notably, at around E11.5, both Blimp1 and Prmt5 translocate from the nucleus to the cytoplasm in PGCs, which coincides with the down-regulation of H4R3me2 in the PGC nuclei. Thus, the Blimp1/Prmt5 complex may play an essential role in maintaining the germ cell lineage during its migration period (93). Whether the Blimp1/Prmt5 complex may play a role in PGC specification remains to be determined.

Remarkably, studies in *Drosophila* show that the homologue of *Prmt5*, *Dart5*/*Capsuleen*, is essential for the maturation of spermatocytes in males and, notably, for germ cell specification in females (95, 96). As described above, the germ cell fate in *Drosophila* is determined by "preformation" but not by "epigenesis" as in mammals. Accordingly, embryonic blastomeres that acquire posteriorly localized pole plasm, which contains maternally deposited germ cell determinants, take on the germ cell fate (3, 6). In *Dart5*/*Capsuleen* mutants, assembly of the pole plasm, especially the localization of an essential pole plasm component Tudor, is critically impaired (95, 96). The arginine methyltransferase activity of Dart5/Capsuleen seems essential for this process. The spliceosomal Sm proteins are identified as *in vivo* substrates of symmetric arginine di-methylation by Dart5/Capsuleen. However, the localization of Tudor in the pole plasm and the methylation of the Sm proteins appear to be separable processes (96). Further studies are needed to clarify the mechanism by which Dart5/Capsuleen functions in germ cell specification in *Drosophila*.

6.3. A molecular program for germ cell specification

The identification of *Blimp1* as a gene that marks the lineage-restricted PGC precursors as early as E6.25 and the development of a method for a more quantitative amplification of mRNAs expressed in single cells enabled the measurement of expression dynamics of key genes associated with PGC specification (97). Importantly, it was shown that *Blimp1*-positive PGC precursors at E6.75 show an expression profile similar to that in their *Blimp1*-negative somatic neighbors. At E7.25, although the expression of *Oct4*, a key regulator of pluripotency (7, 98-101), is similar between *Blimp1*-positive PGCs and their somatic neighbors, *Blimp1*-positive PGCs specifically express stella and regain expression of *Sox2*, another essential gene associated with pluripotency (7, 102, 103). This specific re-acquisition of Sox2 would enable the exclusive formation of Oct4-Sox2 complex in PGCs and may herald the regaining of the potential pluripotency in PGCs, which is manifested by their exclusive potential to derive pluripotent embryonic germ (EG) cells in culture (104, 105) (see section 7.3). The Hox genes were found to be repressed as early as E7.25 and the genes such as *T* (*Brachyury*) and *Fgf8* are repressed later than E7.75 in *Blimp1*-positive PGCs. Therefore, the transitions from *Blimp1*-positive PGC precursors to stella-positive PGCs and to more advanced migrating PGCs seem to involve a highly dynamic, stage-dependent transcriptional orchestration that begins with the regaining of the pluripotency-associated gene network, followed by stepwise activation of PGC-specific genes, differential repression of the somatic mesodermal program, as well as potential modulation of signal transduction capacities and unique control of epigenetic regulators (see section 7.4). Future studies involving genome-wide analysis of gene expression dynamics during germ cell specification will provide a more comprehensive view of the events associated with this process.

7. KEY MOLECULES AND EVENTS IN PGCS AFTER THEIR SPECIFICATION

7.1. Migration and proliferation of PGCs

The specified PGCs that form a tight cluster at the base of allantois initiate their migration individually toward future genital ridges at around E7.5 (106, 107). They actively migrate out of the initial cluster into the endoderm epithelium and are incorporated into the forming hindgut diverticulum and embedded in the hindgut epithelium by E8.5. They are highly motile in the hindgut and apparently very rapidly exit it at around after E9.0 to reach the mesentery (108, 109). During the E10.0-E10.5 period, they migrate directionally from the dorsal body wall

into the genital ridges (109). A number of mutations have been identified that affect the viability or behavior of the PGCs during their migration period (see section 7.2). However, the precise molecular mechanisms governing the complex PGC migration process remain to be determined.

Classical studies have suggested that, once specified, PGCs proliferate constantly with a doubling time of 16 hours during their migration and colonization of the genital ridges (37, 106). However, a recent study involving a more accurate counting of the PGC number by PGC reporter mice and fluorescent activated cell sorting analysis of the cell cycle of the PGCs showed that from at around E8.0 to E9.0 when PGCs are in the hindgut epithelium, a majority of them (~60%) are arrested at the G2-phase of the cell cycle and proliferate relatively slowly (110) (see also section 7.4.1). Therefore, it appears that *Blimp1*- and stella-positive PGCs increase their number relatively constantly up to approximately 100 by E8.0, after which most of them enter the G2 arrest of the cell cycle. When PGCs exit from the hindgut epithelium after E9.0, they are released from the G2 arrest and resume rapid proliferation.

7.2. Molecules essential for early PGC development

The mutations of the signaling molecules and Blimp1 directly affect the germ cell specification process itself. There are a number of genes that are known to play critical roles in the early phase of PGC development, presumably after the PGC fate is specified (Table 1).

Classical genetic studies have shown that the *Steel* and *W* loci are critical for the migration, proliferation and/or survival of PGCs (111, 112). The *Steel* locus encodes a secreted ligand, the stem cell factor/Kit ligand (SCF/Kitl), and the *W* locus encodes its cell surface tyrosine kinase receptor, Kit (113, 114). Detailed studies have shown that in *Kit* mutants, PGC specification seems apparently normal but the numbers of PGCs do not increase after E8.5 in their migration period (111, 115). The molecular mechanisms through which the SCF-Kit signaling pathway controls early PGC development are still obscure, although *in vitro* culture experiments and *in vivo* studies have shown that SCF prevents apoptosis of PGCs (116-119) through the AKT/mTOR/Bax pathway (119-121). Since *Kit* is expressed in PGCs as early as E7.25 (97, 122), the SCF-Kit signaling may play a role in PGC specification.

The POU domain transcription factor *Oct4*, the first and the most representative gene shown to be associated with pluripotency (7, 98-101, 123), has been demonstrated to be essential for the survival of PGCs: The germ-cell-specific deletion of *Oct4* results in the apoptosis of PGCs (124). This is in contrast to the *Oct4* deficiency in pre-implantation embryos, which leads to the loss of the pluripotency in the ICM cells: The *Oct4*-deficient ICM cells are restricted to differentiation towards TE cells (100). Therefore, the loss of Oct4 function at different developmental stages and in different cell types leads to different biological effects. How precisely Oct4 deficiency leads to apoptosis in PGCs remains to be determined. It is also critical to explore the function of Sox2 in PGC development.

Another classical mutant that is known to affect early PGC development is the *ter* mutation (125-127). The *ter*/*ter* mutation causes germ cell deficiency and a high incidence of congenital testicular teratomas on a 129/SV background (125-127). However, on C57BL/6 and LTXBJ backgrounds, the *ter*/*ter* mutation causes only germ cell loss but not germ cell tumors, indicating that the *ter* gene causes germ cell deficiency singly in both sexes and elevates the incidence of congenital testicular teratomas on a 129/Sv-*ter* male background (128). Recently, the *ter* mutation was identified as a point mutation that introduces a termination codon in the mouse orthologue (*Dnd1*) of the zebrafish *dead end* gene (129, 130). Dnd1 has an RNA recognition motif and is most similar to the apobec complementation factor, a component of the cytidine to uridine RNA-editing complex (131), and shows expression in PGCs as early as E6.75 (97). In *ter*/*ter* mutants, the PGC number decreases as early as E8.0, indicating an essential role of Dnd1 in early PGC development. It is of note that a conserved protein is essential for PGC development both in the zebrafish and the mouse, whose germ cell specification depends on preformation and epigenesis modes, respectively. The precise role of this gene product in PGC development remains to be investigated.

nanos3 is a mouse homologue of an evolutionarily conserved *Nanos* gene, which encodes an RNA-binding protein and plays a critical role in germ cell development and abdominal patterning in *Drosophila* (132-134). In the absence of maternal Nanos, PGCs fail to migrate into the gonad and do not become functional germ cells (135, 136). In mice, specific expression of *nanos3* in PGCs begins as early as E7.25 (97). In the absence of *nanos3*, PGC specification seems to occur normally but the number of PGCs is reduced after E8.0, and eventually all the PGCs are lost (134). The mechanism of nanos3 function also remains to be elucidated.

The T-cell-restricted intracellular antigen1 (TIA-1)-related protein, TIAR, has also been shown to be critical for the survival of migrating PGCs (137). TIA-1 and TIAR belong to the RNA recognition motif (RRM)/ribonucleoprotein family of RNA-binding proteins (138-140). Under conditions of cellular stress, TIA-1 and TIAR are postulated to function as translational repressors by associating with eIF1, eIF3, and the 40S ribosomal subunit and forming a translationally inactive noncanonical preinitiation complex (140, 141). TIA-1 and TIAR have self-aggregating properties and facilitate the accumulation of the transcriptionally inactive preinitiation complexes into discrete cytoplasmic foci called stress granules (140, 141). However, why and how *Tiar* mutation specifically leads to the loss of PGCs remains to be clarified.

A recent interesting report has shown that Hypoxia-inducible factor-2α (Hif-2α) plays a critical role for PGC specification and/or early PGC development, presumably by regulating *Oct4* expression in PGCs (142). Hifs are the primary transcriptional regulators of both cellular and systemic hypoxic adaptation in mammals (143, 144). Hifs are heterodimers consisting of a regulated subunit (Hifα) and a constitutive subunit (Hifβ, also known

as Arnt (aryl hydrocarbon nuclear translocator)) and regulate the expression of at least 180 genes involved in metabolism, cell survival, erythropoiesis, and vascular remodeling (143). In *Hif-2α* mutant mice, the AP-positive PGC number seems severely reduced as early as E8.0 (142). In the future, it will be important to explore the precise role of Hif-2α in PGC specification.

7.3. PGCs can de-differentiate into pluripotent EG cells *in vitro*

Classically, it has been shown that PGCs are the origin of teratocarcinomas, which contain a range of differentiated cell types derived from three germ layers and a population of undifferentiated embryonic cells, known as embryonal carcinoma (EC) cells (145). It is also important to note that, in the presence of leukemia inhibitory factor (LIF), stem cell factor (SCF), and basic fibroblast growth factor (bFGF), the specified PGCs (from E8.0 to E13.5) can 'de-differentiate' into ES cell-like pluripotent embryonic germ (EG) cells in culture (104, 105). EG cells can differentiate into multiple cell types *in vitro* and *in vivo*, and contribute both to the somatic and the germ cell lineage when they are introduced into blastocysts. These findings have led to the notion that PGCs are potentially pluripotent, although PGCs themselves do not contribute to any tissues when they are transferred into blastocysts (105, 146). It is of interest to explore the mechanism by which PGCs de-differentiate into a pluripotent state *in vitro* and under disease conditions (see also section 8), and conversely, the mechanism by which they normally prevent themselves from reverting to an overtly pluripotent state. In this regard, recent studies have shown that constitutive activation of the PI3 kinase/Akt signaling pathway in PGCs results in teratoma formation *in vivo* and enhances EG cell derivation *in vitro* (147, 148). p53 has been shown to be one of the crucial downstream targets of this signaling pathway in PGCs (148).

7.4. Epigenetic reprogramming in migrating PGCs

7.4.1. Genome-wide epigenetic reprogramming in migrating PGCs

In development, a single zygote generates a myriad of diverse cell types with different gene expression programs. These programs are usually fixed in a stable cellular function through epigenetic mechanisms, including DNA methylation (149), histone tail modifications (150-152) and specific nuclear architecture (153). Thus, each somatic cell type acquires a specific and stable epigenetic signature, referred to as "cellular memory," which is often mitotically heritable. In contrast, the genome of the germ cell lineage, which is the sole pathway to the next generation, must be maintained in an epigenetically reprogrammable state for the new generation to continue to be created.

However, as discussed so far, germ cell fate in mice is induced by "epigenesis" in proximal epiblast cells that are otherwise destined toward somatic mesodermal fates. This implies that cells recruited for the germline may have to undergo "epigenetic reprogramming" from a somatic to a potentially totipotent germline phenotype. Indeed, it has long been known that PGCs undergo profound epigenetic reprogramming, which includes genome-wide DNA demethylation (28, 154), erasure of parental imprints (155, 156), and re-activation of the inactive X-chromosome (157), after they colonize the genital ridges. Recent studies, however, are beginning to reveal that epigenetic reprogramming is an integral part of germ cell specification (110, 158, 159) (Figure 3).

It has been shown that *Blimp1*-positive lineage restricted PGC precursors at around E6.75 bear genome-wide epigenetic modifications indistinguishable from their somatic mesodermal neighbors, some of which should share common precursors with the germ cell lineage (110). These modifications include both active and repressive modifications of histone H3—i.e., di- and tri-methylation of H3 lysine4 (H3K4me2 and me3) and acetylation of H3 lysine9 (H3K9ac) (active modifications), mono-, di-, and tri-methylation of H3 lysine9 (H3K9me1, me2, and me3) (repressive modifications), and di- and tri-methylation of H3 lysine 27 (H3K27me2 and me3) (repressive modifications) (110). Therefore, PGCs may not possess specific epigenetic signatures at their outset, although there could be yet-to-be-discovered histone and chromatin modifications that are unique to the PGC precursors.

Subsequently, however, from around E8.0 onwards, PGCs that have started their migration begin to show the genome-wide reduction of the two major repressive modifications, DNA methylation and H3K9me2 (110, 158). The global reduction of H3K9me2 in migrating PGCs seems to occur in a progressive, cell-by-cell manner and by E8.75, nearly all the PGCs show low H3K9me2 levels. Cell cycle analysis of the migrating PGCs indicated that a majority of them (~60%) are in the G2 phase from around E8.0 to around E9.0 (110). Interestingly, concomitant with this period, PGCs seem to transiently pause their global transcription by RNA polymerase II (RNAPII) (110). These observations suggest that the genome-wide reduction of the repressive modifications progresses when the PGCs are arrested in the G2 phase, during which they simultaneously shut off their RNAPII-dependent transcription. Therefore, genome-wide reductions of DNA methylation and H3K9me2 in migrating PGCs are considered to involve active processes.

Gene and protein expression analysis has shown that PGCs repress critical de novo DNA methyltransferases, Dnmt3a and 3b, as early as E7.25 (97, 158), but continue to express a maintenance methyltransferase, Dnmt1, at least at the mRNA level. They also repress, at around E7.75, the histone lysine methyltransferase Glp, which is essential for conferring genome-wide H3K9me1 and me2 in embryonic development (110, 160). Single-cell gene expression analysis of the JmjC domain-protein family, which consists of ~30 proteins across the genome and can demethylate all the methylation states (mono-, di-, and tri-) of H3K4, H3K9, H3K27, and H3K36 (161, 162), has shown that *Jhdm2a* (*Jmjd1a*), which encodes H3K9me1 and 2 demethylase, is indeed expressed in migrating PGCs (110). However, its expression is similarly seen in somatic neighbors. These findings suggest that the specific repression of Glp in PGCs may lead to the loss of H3K9 methyltransferase activity in

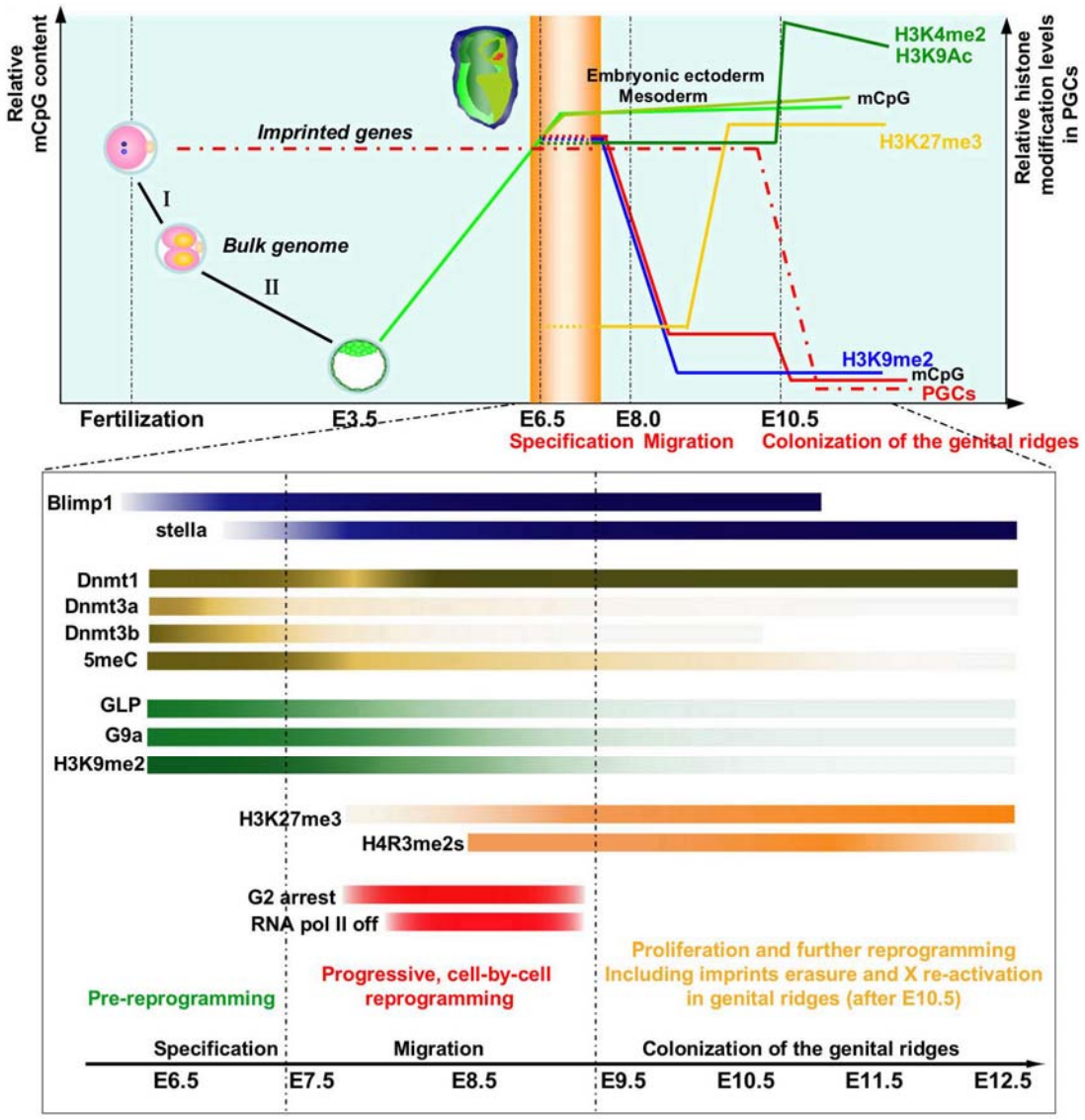

Figure 3. Genome-wide epigenetic reprogramming in migrating primordial germ cells (PGCs). Genome-wide epigenetic reprogramming in PGCs is shown in combination with that during the period up to germ cell specification. Only DNA methylation behavior is shown for the early stages. I, active genome-wide DNA demethylation from the paternal genome (13,14); II, passive replication-dependent demethylation of both the paternal and maternal genomes after the two-cell stage (29). See text for details.

PGCs, which triggers the genome-wide reduction of H3K9me2, either through a turnover of methyl groups or a replacement of the entire H3 molecule with an unmodified H3 molecule. Consistently, a report involving a mass spectrometry analysis of H3K9 methyl groups differentially pulse-labeled by a heavy isotope demonstrated that H3K9me2 turns over without replication (163). Furthermore, it was also reported that chromatin-associated histones and non-chromatin-associated histones are continually exchanged in *Xenopus* oocytes, and that the maintenance of H3K9me2 at a specific site requires the continual presence of an H3K9 histone methyltransferase (164). Thus, repression of an essential enzyme may shift the equilibrium between methylation and demethylation toward demethylation, leading to the erasure of H3K9me2 in migrating PGCs.

Recently, a loss-of-function experiment for *Jhdm2a* has been reported (165). *Jhdm2a* is shown to be essential for spermatogenesis. Jhdm2a functions to remove H3K9me1/2 on the promoters of the genes for transition nuclear protein 1 and protamine 1, which is essential for their proper expression and in turn for packaging and condensation of sperm chromatin. Whether or not Jhdm2a plays a role in the genome-wide reduction of H3K9me2 in PGCs remains to be investigated.

Following the genome-wide loss of DNA methylation and H3K9me2, genome-wide H3K27me3, another repressive modification mediated by the polycomb repressive complex 2 (PRC2), becomes up-regulated in migrating PGCs at around E8.25 onwards (110, 158). Both the percentage of PGCs with high levels of H3K27me3 and the degree of H3K27me3 up-regulation in individual PGCs increase as the development proceeds, indicating that, as in the case for the demethylation of H3K9me2, H3K27me3 up-regulation proceeds progressively, in a cell-by-cell manner.

PRC2 consists of three core components, Ezh2, Eed and Suz12 (166, 167). Single-cell gene expression analysis has shown that genes for these three core components are expressed at similar levels both in the PGCs and their somatic neighbors by at least E7.75 (97). Considering the fact that PGCs globally shut off RNAP II-dependent transcription after around E8.0, the up-regulation of genome-wide H3K27me3 in PGCs after E8.25 may not depend on the *de novo* transcription of some specific factors. It was reported that in *Suv39h*-deficient cells, which essentially lack H3K9me3 at the pericentromeric heterochromatin, H3K27me3 is ectopically up-regulated in this location, which suggests that there is "crosstalk" between H3K27 methylation and H3K9 methylation, which rescues the de-regulated chromatin structure (168). A similar system may operate in migrating PGCs, in which hypo-methylated regions for H3K9me2 might be "sensed and rescued" by H3K27me3.

7.4.2. Potential significance of epigenetic reprogramming in migrating PGCs

What could be the potential biological significance of epigenetic reprogramming in migrating PGCs? Essentially, migrating PGCs convert their genome-wide repressive modifications from DNA methylation and H3K9me2 to H3K27me3 (Figure 3). It has been reported that DNA methylation and H3K9me2 are critical for the stable maintenance of the repressed genes (169, 170). In contrast, PRC2-mediated H3K27me3 is, for example, enriched in pluripotent ES cells and functions to repress developmentally regulated lineage-specific genes (171, 172). Many of these developmentally regulated genes bear bi-valent modifications with H3K27me3 and H3K4me3, which ensure that these repressed genes can be expressed quickly upon the induction of differentiation.

DNA methylation and H3K9me2 are therefore considered to play critical roles in stably maintaining the repressed state of unused genes during cell fate specification, whereas H3K27me3 may participate in a more plastic repression of the lineage-specific genes in pluripotent cells. Since PGCs bear significant levels of genome-wide H3K4me3, specific up-regulation of H3K27me3 in PGCs may contribute to the creation of an ES cell-like genome organization. Indeed, it is possible to derive pluripotent EG cells from PGCs (104, 105) (see section 7.3). This fact has been the basis for the notion that PGCs possess potential pluripotency. Epigenetic reprogramming and specific expression/re-acquisition of the key pluripotency genes *Oct3/4*, *Sox2*, and *Nanog* in PGCs (see section 6.4) may thus be critical for their potential pluripotency (97, 173). It might be the case that genes such as *Blimp1* are important to prevent PGCs from overtly reverting to a pluripotent state *in vivo*. To understand the significance of genome-wide epigenetic reprogramming in migrating PGCs, it is critical to determine the target sequences from which DNA methylation and H3K9me2 are removed and upon which H3K27me3 is imposed.

Soon after PGCs enter the genital ridges, they undergo further DNA demethylation, including the erasure of parental imprints between E9.5 and E12.5 (156). Repetitive elements such as IAP (intracisternal A particle elements, classified as LTRs, ~ 1,000 copies per mouse genome) and LINE1 (the autonomous long interspersed nucleotide element-like element, ~10,000-100,000 copies per genome) are highly methylated in migrating PGCs and somatic cells. Post-migrating PGCs demethylate the CpG methylation on LINE1 elements, while most of the CpG cites on the IAP elements remain highly methylated (156, 174). The female PGCs, which initially undergo random X-chromosome inactivation as in the somatic cells, reactivate the inactive X (157), which most likely reflects the global epigenetic reprogramming process in gonadal PGCs. A recent report showed that X reactivation may start in PGCs as early as E7.75 (159). The precise analysis of genome-wide epigenetic reprogramming in migrating PGCs will be important for understanding the events occurring in PGCs in the genital ridges.

8. PGC SPECIFICATION/DEVELOPMENT AND INDUCED PLURIPOTENCY IN SOMATIC CELLS

Remarkably, a series of recent studies has shown that retroviral transduction of four transcription factors, Oct4, Sox2, Klf4, and c-Myc, can reprogram adult mouse fibroblast cells to an undifferentiated state similar to the state of ES cells, albeit at a low frequency (~$1/10^4$-~$1/10^5$ per transduced cells), and these cells have been termed induced pluripotent stem (iPS) cells (7-9). Subsequently, human iPS cells were also generated using two different sets of transcription factors (OCT4, SOX2, KLF4 with or without c-MYC, or OCT4, SOX2, NANOG, LIN28) (10-12, 175), creating an unprecedented opportunity to produce patient-specific stem cells for the study of the diseased state in culture. Moreover, iPS cells were shown to be generated by direct reprogramming of lineage-committed somatic cells (hepatocytes and gastric epithelial cells), and retroviral integration into specific sites was not required (176).

As has been discussed, PGC specification involves re-activation of Sox2 and maintains Oct4 and Nanog. Consequently, the specified PGCs are the only cells that express all three of the key pluripotency regulators Oct4, Sox2 and Nanog after gastrulation, and they also express Klfs and N-Myc (M.S., unpublished observation), although they repress c-Myc upon their specification (97). Accordingly, PGCs, which are basically unipotent *in vivo*, can be reprogrammed into pluripotent EG cells in the presence of LIF, SCF, and bFGF at a ratio of

approximately one EG cell colony generated from one out of ~50 PGCs ((104, 105, 148) and M.S., unpublished observation). This ratio is clearly higher than the ratio of iPS cell induction from somatic cells, although this depends considerably on their developmental stages (104, 105, 148).

These two lines of evidences raise a question as to how a cell that expresses these key genes becomes a pluripotent stem cell. Although the precise mechanisms of iPS cell induction, which appear to require at least several cell division cycles, are currently unknown, it is conceivable that the epigenetic state of a cell regulates the phenotype conferred by the transcriptional activity of these potential pluripotency inducing factors. Under normal ES cell conditions, Oct4, Sox2, and Nanog co-occupy many target genes, many of which are developmentally important transcriptional regulators (172, 177). The genome-wide epigenetic reprogramming that seems integral to and ensues PGC specification may thus create a more permissive state for the generation of pluripotent stem cells under an appropriate condition. Further studies on germ cell development and the mechanisms of iPS cell induction would lead to a more integrated understanding of the potency of a cell in general.

9. PERSPECTIVE

In the last decade, our understanding of the mechanism for the specification of the germ cell lineage in mice has considerably increased at the molecular level. However, there are still many gaps in our knowledge. Specifically, for example, do the signaling molecules for PGC specification directly up-regulate the expression of *Blimp1*? Why does only a subset of proximal epiblast cells acquire germ cell fate? What is the mechanism of Bmp8b function? Are there any other key transcriptional regulators critical for PGC specification? How precisely do PGCs undergo genome-wide epigenetic reprogramming? Does epigenetic reprogramming in migrating PGCs have any implications in further reprogramming of PGCs in the genital ridges, including erasure of parental imprints? Studies specifically targeting each of these questions will be needed for a conceptually more integrated and a more comprehensive understanding of the PGC specification in mice. Such endeavors will lead to a more precise reconstruction of germ cell fate *in vitro*, which will have a profound impact on reproductive and regenerative medicine.

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