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—Mini Review—

Epigenetic Reprogramming in Primordial Germ Cells in Mice

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Abstract: Germline cells are the sole source of the transmission of genetic and epigenetic information to the next generation. Epigenetic information is reprogrammed during germ cell development to reacquire cellular totipotency and prevent the accumulation of epimutations. In this review, we summarize epigenetic reprogramming, in particular, DNA demethylation in developing primordial germ cells (PGCs). The recent development of next-generation sequencing, and the discovery of 5-methylcytosine oxidation are major breakthroughs in the study of epigenetic reprogramming in PGCs. DNA methylation analysis with high-throughput sequencing has uncovered the dynamics of DNA methylation erasure at single-locus resolution, which has revealed the global loss of DNA methylation in migrating PGCs, and locus-specific DNA demethylation in gonadal PGCs. The disruption of ten-eleven translocation genes shows that they are required for DNA demethylation at germline-specific genes in gonadal PGCs. These findings indicate that passive and active demethylation pathways operate synergistically and/or in parallel to ensure efficient global demethylation in developing PGCs.

Key words: Primordial germ cells (PGCs), Epigenetic reprogramming, DNA demethylation, Histone methylation

Introduction

All cells in multicellular organisms contain identical genetic information, yet a variety of somatic cell types are generated with different gene expression programs. These programs are usually fixed to a stable cellular function through epigenetic mechanisms, including DNA methylation [1], histone tail modifications [2], and specific nuclear architecture [3]. 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 creation of the new generation.

There are essentially two modes, which are known as “preformation” and “epigenesis,” of specification of germ cell fate during the development of multicellular organisms [4]. In preformation, which is seen in model organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster*, localized determinants in oocytes, often referred to as germplasm, specify the new germline cells and segregate them from the somatic lineages at the onset of development. The germplasm set aside in precursor blastomeres for the germ cell lineage provides repressive mechanisms, including transcriptional and translational repression, to prevent the activation of the genetic programs for somatic differentiation from occurring in germline blastomeres [5]. In turn, specified germ cells in these animals establish chromatin-based silencing, e.g., low levels of histone H3 lysine-4 di-methylation, which ensures subsequent global transcriptional quiescence [6].

By contrast, during epigenesis, which is seen in mammals including mice, a potentially equivalent population of pluripotent cells at a relatively late stage of development is induced to form either germ cells or somatic mesoderm in response to signals from adjacent tissues [7, 8]. This implies that cells recruited for the germline may have to undergo “epigenetic reprogramming” from a somatic phenotype to a potentially totipotent germline phenotype and recent studies have demonstrated that this seems to be the case [9, 10]. Here, we summarize briefly, what is currently known about the epigenetic reprogramming of primordial germ cells (PGCs) in mouse embryos.

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Specification, migration, and arrival of PGCs

A new generation of the germ cell lineage is recruited from proximal epiblast cells as *Blimp1*(a potent transcriptional repressor with a PR domain and Zn fingers)-positive cells at embryonic day (E) 6.25 [8]. The PGCs, which form the first population of the germ cell lineage that is the source for oocytes and sperm, form a tight cluster of alkaline phosphatase-positive cells in the extra-embryonic mesoderm at E7.25 [11]. They migrate through the developing hindgut endoderm [12], eventually colonizing the genital ridges after E9.5 [13], where they begin to differentiate into functional gametes through highly complicated developmental pathways. The fully differentiated oocytes and sperms can now re-initiate and recapitulate all of these processes.

DNA demethylation dynamics in PGCs

Approximately 25 years ago, global DNA methylation levels were shown to decrease from epiblast (E6.5) to gonadal PGCs (E12.5), which suggested the existence of DNA methylation reprogramming during PGC development [14]. Key technical developments for the detection of DNA methylation, such as the bisulfite sequencing method, has allowed us to monitor the DNA demethylation dynamics in particular genomic regions, including imprinted loci, in PGCs [15]. Analysis of the X-linked enzyme HPRT showed that X-chromosome reactivation also occurs in gonadal female PGCs [16]. The temporal window for the erasure of the genomic imprint and X-chromosome reactivation is consistent with the timing in which PGCs enter into the genital ridges, which raised the possibility that a specific signal from the genital ridges induces genome-wide DNA demethylation in gonadal PGCs [15, 17]. Previously, we monitored global DNA methylation levels in PGCs during their specification, migration, and gonadal periods using whole-mount immunofluorescence analysis of transgenic embryos in which PGCs are visualized by EGFP with an anti-5-methylcytosine (5mC) antibody [9]. Contrary to the established theory that epigenetic reprogramming in PGCs commences with the entry of PGCs into the genital ridges [15, 17], global DNA methylation levels had already declined in PGCs by E8.0, and a further reduction of global DNA methylation levels occurred in gonadal PGCs. The recent development of methods for the detection of DNA methylation (MeDIP-Seq and BS-Seq) has facilitated the analysis of the global DNA methylation status of migrating and gonadal PGCs. Consistent with whole-mount immunofluorescence studies, global analysis using massive deep sequencing has provided evidence that the erasure of DNA methylation in PGCs occurs during the migration phase (approximately E8.5) and the gonadal stage (after E10.5) [18]. Genome-wide methylation levels decline in PGCs during the migration phase, while DNA methylation in a number of regions, e.g., the imprinting control region and the promoters of germline-specific genes, are resistant to global DNA demethylation in migrating PGCs. DNA methylation of these resistant regions during the first demethylation phase in migrating PGCs is lost completely in the second demethylation phase in gonadal PGCs. Such locus-dependent differences of demethylation kinetics suggest that more than two distinct molecular mechanisms for DNA demethylation take place during PGC development.

Mechanisms of DNA demethylation during PGC development

DNA methylation can be erased through two pathways, replication-dependent passive and replication-independent active mechanisms. UHRF1 recognizes the hemimethylated CpG created by DNA replication, and mediates the loading of DNMT1, which is involved in the maintenance of DNA methylation during cell division, to replication regions. Therefore, when the maintenance of DNA methylation by the UHRF1/DNMT1 complex stalls, replication-dependent passive dilution of DNA methylation takes place. Recently, the discovery of the conversion of 5mC to 5-hydroxymethylcytosine (5hmC) by ten-eleven translocation [19] proteins has provided new insights into the mechanisms behind active DNA demethylation. The deamination of 5hmC by activation-induced cytidine deaminase [20] produces 5-hydroxymethyluridine, which can serve as a substrate for the base excision repair (BER) pathway during cytosine regeneration [21]. Alternatively, 5hmC is further oxidized to 5-formylcytosine and 5-carboxylcytosine, which are repaired by thymine DNA glycosylase (TDG) to produce unmodified cytosines [22, 23].

The classical method for calculating the PGC number, alkaline phosphatase (AP) staining, indciates that the doubling time of PGCs is constant at approximately 16 h during their development from E8.5 to E13.5 [24]. Furthermore, it has been suggested that DNA demethylation commences at a specific period, i.e., the entry of PGCs into the genital ridges [15]. These findings suggested that replication-independent active mechanisms control the erasure of genome-wide DNA methylation in gonadal PGCs. However, the identification of molecular markers of early PGCs has allowed us to count the precise number of early PGCs [10]. In contrast to the number of PGCs calculated by AP staining, determination of the number of PGCs using *Blimp1*-mVENUS and *Stella*-EGFP transgenic embryos has clearly shown that the expansion

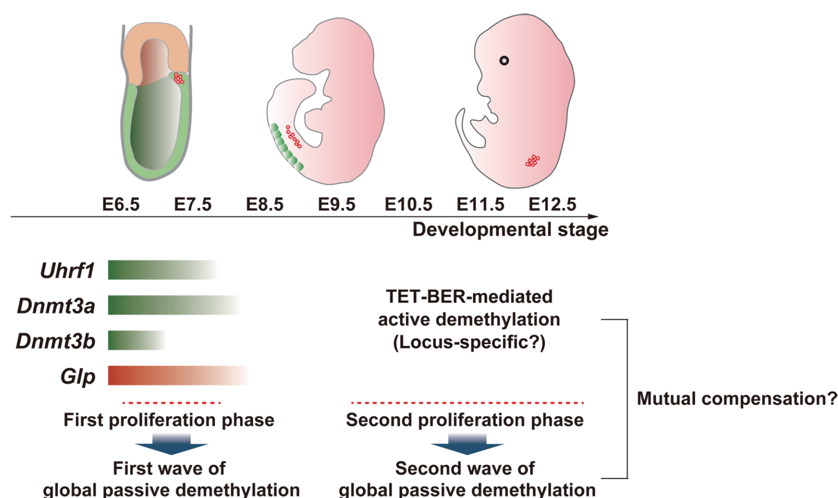

Fig. 1. A schematic diagram mechanistic links between passive and active DNA demethylation in PGCs
Nascent PGCs actively repress four major modifiers (*Dnmt3a*, *Dnmt3b*, *Uhrf1*, *Glp*) to maintain DNA methylation during DNA replication. The distinct proliferation phase of PGC with different background expression levels of the four factors might trigger passive demethylation at distinct genome regions in nascent and gonadal PGCs. TET-BER-mediated active demethylation and passive demethylation might be mutually compensated to robustly erase global DNA demethylation in PGC development.

of PGCs is not constant, and the majority of migrating PGCs enter into G2 arrest of the cell cycle from E7.75 to E9.25. This provides evidence that developing PGCs have two distinct proliferation phases: the first proliferation phase from E6.5 to E7.5 and the second proliferation phase at E9.5 to E13.5. Interestingly, the two-step temporal window of DNA demethylation in developing PGCs is consistent with the proliferation phases of PGCs, and nascent PGCs actively repress the transcription of the major components involved in the regulation of DNA methylation, *Dnmt3a*, *Dnmt3b* and *Uhrf1* (Fig. 1) [9, 25, 26]. Recent evidence suggests that passive demethylation takes places in PGCs [18, 27]. The localization of DNMT1 at replication foci is impaired in rapid cycling gonadal PGCs, and is associated with the transcriptional repression of *Uhrf1* [18, 27]. Furthermore, the frequency of hemimethylated CpG sites, which are produced by the impairment of DNA methylation, on long interspersed element-1 (LINE-1) sites increases in gonadal PGCs [18]. In contrast, the components of TET-BER-mediated active DNA demethylation, including *Tet1*, *Tet2*, *Aid*, and *Tdg*, contribute to the erasure of DNA methylation in developing PGCs (Fig. 1) [28–30]. Loss-of-function approaches have provided evidence that *Tet1* and *Tet2* are required for the locus-specific DNA demethylation of gonadal PGCs. DNA methylation at the promoter regions of meiotic genes is erased and followed by the upregulation of meiotic genes in gonadal female PGCs, which suggests that the removal of DNA methylation from the promoter regions of meiotic genes is essential for meiotic entry in these cells. *Tet1*-deficient female mice show subfertility associated with the failure of meiotic entry in gonadal PGCs [31]. *Tet1* deficiency also leads to defective DNA demethylation and the decreased expression of early meiotic genes. Furthermore, progeny derived from *Tet1*/*Tet2* double knockout mice have abnormally high methylation patterns at some imprinted loci [30], which might reflect the failure of imprinting erasure in *Tet1*/*Tet2* double knockout PGCs.

Loss-of-function approaches have raised the possibility that TET-BER-mediated active demethylation controls locus-specific but not global DNA demethylation in gonadal PGCs, because abnormal DNA demethylation is observed at specific loci, including germline-specific genes and imprinted loci [30, 31]. Interestingly, the removal of DNA methylation from LINE-1 sites depends on replication-dependent passive demethylation and TET-mediated active demethylation in PGCs. This suggests that if active demethylation mechanisms are stalled, passive demethylation can compensate for the impairment of DNA demethylation in gonadal PGCs (Fig. 1).

Dynamics of histone methylation during PGC development

Whole-mount immunofluorescence analysis has shown that PGCs have reduced stable repressive marks, e.g., histone H3 lysine 9 dimethylation (H3K9me2), and acquire plastic repressive marks, e.g., histone H3 lysine 27 trimethylation, suggesting that PGCs convert stable repressive marks to plastic repressive marks to acquire cellular totipotency [9, 10]. The G9a/GLP heterodimer is essential for conferring H3K9me2 in early embryos and embryonic stem cells (ESCs) [32, 33], and *Glp* transcription is actively repressed in nascent PGCs [34], suggesting that the active repression of *Glp* expression triggers the removal of genome-wide H3K9me2 in developing PGCs. Recently, repressor screening of germ cell characteristics in ESCs by high-throughput RNA interference assays has shown that Max comprehensively represses germline-specific genes, in a process which is mediated by the recruitment of G9a to the promoter regions of these genes. Furthermore, the transcription of Max and *Glp* is actively repressed in nascent PGCs, indicating that these cells regulate the activity of H3K9 methyltransferase at two distinct levels, i.e., expression and recruitment to target loci, to ensure the genome-wide hypomethylation of H3K9me2.

Contrary to the erasure of DNA methylation and H3K9me2, PGCs acquire H3K27me3 during their development. The enrichment of H3K27me3 is observed at the promoter region of *Stra8*, which is required for meiotic initiation, in gonadal PGCs. Recently, an experiment involving conditional *Ring1*/*Rnf2* double-deficient mice, which are deficient in the two central components of polycomb repressive complex 1 that recognizes H3K27me3 in developing PGCs, show the premature entry of female gonadal PGCs into meiosis, indicating that the enrichment of H3K27me3 at the promoter region of *Stra8* acts as gatekeeper for the proper timing of meiotic entry in these cells [35]. Cumulative findings suggest that developing PGCs establish a primed state for the transcription of germline-specific genes that is characterized by the hypomethylation of DNA methylation and H3K9me2 and the hypermethylation of H3K27me3 at the promoter regions of these genes. This condition allows gonadal PGCs to activate these genes fully in response to transcriptional activators.

Conclusion

In this review, we have summarized the epigenetic reprogramming of mouse PGCs. During the past 5 years, some of the molecular mechanisms underlying the epigenetic reprogramming of developing PGCs have been uncovered. However, the limited number of PGCs *in vivo* has restricted the number of methods that can be used to elucidate the detailed molecular mechanisms of epigenetic reprogramming. Saitou's group exploited an *in vitro* differentiation system of functional PGCs (PGC-like cells: PGCLCs) derived from ESCs and induced pluripotent stem cells (iPSCs) [36]. This system allows us to address the detailed molecular mechanisms of epigenetic reprogramming in developing PGCs. Because independent distinct molecular pathways regulate epigenetic reprogramming in developing PGCs, it is possible that loss-of-function experiments in one molecular pathway will not uncover the molecular mechanisms due to compensation by another pathway. Therefore, the combination of disruption of molecules regulating the epigenetic reprogramming of PGCs in PGCLCs and the recapitulation of molecular pathways regulating the epigenetic reprogramming of PGCs in epiblast-like cells is needed to clarify the molecular mechanisms of epigenetic reprogramming in developing PGCs. Epigenetic reprogramming takes place during not only PGC development but also during the derivation of iPSCs from fully differentiated somatic cells. Furthermore, the aberrant DNA methylation patterns of iPSCs, including the hypermethylation of imprinted loci and the retention of the original DNA methylation patterns of somatic cells, compromise the efficient differentiation capacity of iPSCs to the equivalent of that of ESCs [37, 38]. We consider that the elucidation of the molecular mechanisms underlying the epigenetic reprogramming of PGCs will facilitate the improvement of the quality and efficiency of iPSC derivation.

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