Mechanism and Reconstitution In Vitro of Germ Cell Development in Mammals

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The germ cell lineage creates new individuals, perpetuating/diversifying the genetic and epigenetic information across generations. Based on the knowledge obtained through investigations into the mechanisms of germ cell specification and development in mice, we have succeeded in precisely reconstituting the specification and subsequent development of germ cells in culture in both males and females: Embryonic stem cells (ESCs)/induced pluripotent stem cells (iPSCs) are induced into epiblast-like cells (EpiLCs) and then into primordial germ cell-like cells (PGCLCs), which robustly contribute to spermatogenesis and oogenesis and to fertile offspring. This in vitro mouse PGC specification/development system has led to the elucidation of signaling, transcriptional, and epigenetic regulation during germ cell development in a detailed fashion. More recently, based on this system, we and others have demonstrated the induction of human PGCLCs from human ESCs/iPSCs, creating an opportunity for understanding the mechanism of human germ cell development in vitro.

Germ cells are the only cell type that generates the full developmental potency to give rise to the next generations (totipotency). To carry out this fundamental role, primordial germ cells (PGCs), the precursors for the spermatozoa and the oocytes, undergo a number of unique events prior to their differentiation into spermatogonial stem cells (SSCs) or oocytes, which include repression of somatic programs, (re)acquisition of potential pluripotency, and reprogramming of epigenome information, for which, in the last decade, we and other groups have clarified at least a part of the molecular mechanisms (Surani and Hajkova 2010; Saitou and Yamaji 2012; Saitou et al. 2012). Based on these studies, we have successfully reconstituted the mouse germ cell specification pathway in culture: The embryonic stem cells (ESCs)/induced pluripotent stem cells (iPSCs) are induced into epiblast-like cells (EpiLCs), which are in turn induced into PGC-like cells (PGCLCs) with robust capacities both for spermatogenesis and oogenesis (Hayashi et al. 2011, 2012). The in vitro PGC specification/development system has been a basis for elucidation of the signaling, transcriptional, and epigenetic regulation associated with PGC specification and development (Aramaki et al. 2013; Nakaki et al. 2013; Kurimoto et al. 2015). Such knowledge would have been difficult to obtain using the in vivo materials, and thus the in vitro system will continue to be applied to answer many remaining questions in germ cell biology. More recently, based on this mouse system, we and others have achieved

the induction of human PGCLCs from human pluripotent stem cells (hPSCs) (Irie et al. 2015; Sasaki et al. 2015), which creates an unprecedented opportunity for understanding the mechanism for human germ cell development. In this manuscript, we summarize recent advances in elucidation of the mechanisms underlying germ cell development in both mice and humans, as well as in their reconstitution in vitro.

OUTLINE AND KEY MOLECULAR EVENTS OF PGC DEVELOPMENT IN MICE

The mammalian germline is set aside from somatic lineages in early postimplantation embryos. Mouse primordial germ cells (mPGCs) emerge from the epiblasts, a pluripotent epithelium that gives rise to all embryonic tissues. Upon the onset of gastrulation at around embryonic day (E) 6.25, the most proximal posterior epiblast cells receive a high dose of bone morphogenetic protein 4 (BMP4) signaling from the adjoining extraembryonic ectoderm and are induced into mPGCs, which form a cluster of alkaline phosphatase-positive cells within the extraembryonic mesoderm at ~E7.25 (Fig. 1; Ginsburg et al. 1990; Lawson et al. 1999; Saitou et al. 2002). PR domain-containing 1 (*Prdm1*; also referred to as *Blimp1*) is expressed in the precursors of mPGCs, and was identified as a critical determinant of the germ cell fate (Ohinata et al. 2005; Vincent et al. 2005). Molecular techniques, including single cell microarray analysis

Figure 1. Schematic representation of the character and key molecular events in specification of human and mouse germ cell fates. The peri-implantation development in primates and mice is represented with a schematic drawing made with reference to Luckett (1978) and Kaufman (1992). The inner cell mass, ectoderm, mesoderm, and endoderm are indicated with pale blue, purple, yellow, and pink, respectively. Primordial germ cells (PGCs) are represented with green circles. The cells derived from PSCs in vitro are represented with circles. PSCs, pluripotent stem cells; EpiLCs, epiblast-like cells; PGCLCs, primordial germ cell-like cells; TF, transcription factor; iMeLCs, incipient mesoderm-like cells.

(Kurimoto et al. 2006), have demonstrated that *BLIMP1* robustly represses the somatic mesodermal program transiently activated upon mPGC specification (the program for “pattern specification,” “embryonic morphogenesis,” and “gastrulation,” etc.), allowing mPGCs to escape from the mortal somatic cell fate, and is thus essential for the establishment of all of the subsequent events in PGCs (Ohinata et al. 2005; Yabuta et al. 2006; Kurimoto et al. 2008).

Concomitant with repression of the somatic program, mPGCs regain a key transcription network for pluripotency, including *Oct3/4* (*Pou5f1*), *Sox2*, and *Nanog*, and indeed, mPGCs can dedifferentiate into PSCs under particular culture conditions (embryonic germ cells) (Matsui et al. 1992). Therefore, although mPGCs themselves are not pluripotent, they (re)acquire pluripotency in a hidden form (potential pluripotency).

After specification, at $\sim$ E7.25, mPGCs start to migrate into endoderm epithelium of hindgut toward future gonads, which will be formed in later developmental stages (at $\sim$ E10.5). The migratory mPGCs appear to arrest the cell cycle and commence epigenome reprogramming, including genome-wide DNA demethylation and reorganization of repressive histone modifications (Seki et al. 2005, 2007; Saitou et al. 2012). Defects in the epigenetic regulation lead to a variety of deficiencies in germ cell development, including loss of germ cells, impaired development of embryos and offspring, and inherited epigenetic abnormalities (Heard and Martienssen 2014), and therefore, epigenome reprogramming is considered to be a critical process in allowing mPGCs to establish a totipotent epigenome for the next generations.

Another PR domain-containing factor, *Prdm14*, was identified as a critical requirement for mPGC specifica-

tion with specific expression in mPGCs in response to BMP4. *Prdm14* deficiency led to ablation in the (re)acquisition of potential pluripotency and epigenome reprogramming (Yamaji et al. 2008). More recently, *Prdm14* was shown to play a critical role for the naïve pluripotency of mouse embryonic stem cells (mESCs) by antagonizing fibroblast growth factor (FGF) signaling and repressing de novo DNA methylation machinery (Ma et al. 2011; Yamaji et al. 2013). Therefore, among the key molecular events for mPGC specification, these studies showed that epigenome reprogramming and (re)acquisition of potential pluripotency are regulated by *Prdm14*, whereas all of the key PGC events including repression of somatic programs are governed by *Blimp1* (Fig. 1).

The migratory mPGCs exit from the hindgut, pass through the dorsal mesentery with highly active proliferation, and then enter the embryonic gonads, where they start sex-specific development (for males, mitotic arrest; for females, prophase I of meiosis). It is important to note that, prior to sex-specific development, mPGCs almost completely erase the genome-wide DNA methylation, including parental imprints, with the acquisition of unique histone modifications (Seisenberger et al. 2012; Lee et al. 2014).

RECONSTITUTION IN CULTURE OF THE MOUSE GERM CELL SPECIFICATION PATHWAY

The reconstruction of germ cell development in vitro has been considered an important challenge for the precise understanding of the molecular mechanisms of germ cell development and for the regeneration of reproductive capacities in a given species. Accordingly, in vitro differentiation of germ cell-like cells from ESCs has been attempted by exploiting random differentiation in embryoid bodies, but with limited success (Daley 2007). Ohinata et al. (2009) clarified a signaling principle for mPGC specification from the epiblasts and demonstrated that isolated mouse epiblasts are efficiently induced into *Blimp1*-positive and *Prdm14*-positive PGC-like cells in floating aggregates under a defined condition with stimulation by cytokines including BMP4, and that, remarkably, these cells successfully contribute to spermatogenesis upon transplantation into the testes of neonatal mice lacking endogenous germ cells (*W/W^v* mice). Therefore, the generation of an in vitro counterpart of the epiblasts was reasoned to be an essential step for in vitro induction of the germ cell fate from mPSCs. On the other hand, PSCs derived from the epiblasts, known as epiblast-stem cells (EpiSCs) (Brons et al. 2007; Tesar et al. 2007), exhibited a subpopulation expressing germ cell markers, and this population was increased by stimulation with BMP4, but only with limited efficiency (Hayashi and Surani 2009). Considering that the competence of the epiblasts to produce mPGCs is a transient property (E5.5–E6.25) (Ohinata et al. 2009), the low efficiency of EpiSCs for the germ cell fate was attributed to their becoming incompatible for efficient PGC induction during culture.

Given these findings, Hayashi et al. developed a strategy to induce pregastrulating EpiLCs from mPSCs in a ground-state pluripotency (Ying et al. 2008) by stimulation with activin A and basic fibroblast growth factor. Similar to the epiblasts at around E5.5–E6.25, EpiLCs exhibited an efficient competence to generate mPGC-like cells (mPGCLCs) in a floating culture in response to BMP4, and mPGCLCs, upon transplantation into the testes of neonatal *W/W^v* mice, showed a robust capability to produce spermatozoa, which contributed to healthy offspring (Hayashi et al. 2011). The competence of EpiLCs for mPGCLC generation reached an optimal level at around day 2 of EpiLC induction, and thereafter decreased, recapitulating the transient competence of the epiblasts to form PGC-like cells.

The transcriptomes of EpiLCs and mPGCLCs at day 6 of induction (d6 mPGCLCs) were very similar to those of E5.75 epiblast and E9.5 migratory mPGCs, respectively. The induction pathway of mPGCLCs well recapitulated that of mPGC specification in vivo; *Blimp1*-positive mPGCLCs transiently activated a mesoderm-like expression profile at day 2 of induction and robustly repressed it by day 6. d6 mPGCLCs did not show expression of late mPGC genes such as *Ddx4* and *Piwil2*. Importantly, this strategy was also valid for females: Female mPGCLCs were induced from female mPSCs through EpiLCs, and when aggregated with somatic cells of female embryonic gonads (the reconstituted ovaries), female mPGCLCs exhibited further differentiation to acquire the properties of late PGCs (i.e., E12.5 PGCs), and upon a longer culture, progressed into the prophase of the first meiotic division, demonstrating the capacity of mPGCLCs for the meiosis (Hayashi et al. 2012). Remarkably, when the reconstituted ovaries were transplanted under the ovarian bursa of immune-deficient mice, mPGCLCs matured into oocyte-like cells at the germinal vesicle stage with the formation of Graafian follicle-like structures, and some of the mPGCLC-derived oocytes, through in vitro maturation and fertilization, contributed to healthy offspring (Hayashi et al. 2012).

These findings demonstrated that the mPGCLC induction from mPSCs via EpiLCs is an in vitro counterpart of germ cell specification and development from the epiblasts in mice, providing a robust foundation for understanding and regulating the mechanism of mammalian germ cell development in both sexes in vitro.

RECONSTITUTION OF MOUSE PGCLCs BY INDUCED TRANSCRIPTION FACTORS

To examine the intrinsic logic of mPGC specification, Nakaki et al. (2013) explored transcription factors (TFs) sufficient for mPGC specification, and demonstrated an efficient induction (>80%) of mPGCLCs by inducible, combinatorial overexpression of three TFs, *Blimp1*, *Prdm14*, and *Tfap2c*, in EpiLCs (TF-mPGCLCs). Among these three TFs, *Prdm14* was sufficient for induction of the germ cell state by itself, indicating the central role of this factor in conferring the germ cell fate upon EpiLCs. The TF-mPGCLCs reconstituted transcriptome and epi-

genetic reprogramming in mPGCs, and more notably, they contributed to functional spermatogenesis upon transplantation into the testes of neonatal *W/W^v* mice. Thus, these TFs, particularly *Prdm14*, are sufficient to create the genetic and epigenetic network for mPGCs (Nakaki et al. 2013).

Upon the induction of the TFs, EpiLCs swiftly acquired the germ cell fate, whereas the mesodermal genes, including *T*, *Hoxa1* and *Hoxb1*, were never up-regulated throughout the induction pathway, indicating that the mPGC specification can be carried out without the transient activation of the somatic mesoderm program, raising a question as to the role of the mesoderm program in mPGC specification (see below).

It is of note that the direct induction of the three TFs in mESCs led to the formation of distinct, teratogenic cells incapable of spermatogenesis. This demonstrated a critical requirement of the epigenome background or cellular context of EpiLCs for the TFs to act properly to create the germ cell fate. This study thus established a framework for the transcriptional logic of PGC specification by *Blimp1*, *Prdm14*, and *Tfap2c*, and for exploration of TF-based approaches to elucidate the mechanisms of further germ cell development. Consistent with this study, it was reported independently that the three TFs induce expression of mPGC markers in embryonal carcinoma cells (Magnusdottir et al. 2013).

MOLECULAR BASIS FOR THE COMPETENCE OF THE EPIBLAST FOR THE GERM CELL FATE

The molecular basis of the competence of the epiblasts and EpiLCs to take on the germ cell fate has been an open question. The expression of WNT3 starts in the pregastrulating epiblast and posterior visceral endoderm (Liu et al. 1999; Rivera-Perez and Magnuson 2005), and WNT3 has been identified as an essential cytokine for the epiblast to express *Blimp1* and *Prdm14* in response to BMP4, providing the epiblast cells with the competence for the germ cell fate (Ohinata et al. 2009; Tanaka et al. 2013).

By analyzing β -Catenin-knockout embryos/mESCs/EpiLCs and by overexpressing a constitutively active form of β -Catenin in *Wnt3*-knockout EpiLCs, Aramaki et al. (2013) demonstrated that the canonical β -CATENIN-mediated signaling pathway is a key downstream effector of WNT3 for the competence for mPGC specification. The exploration of downstream targets of BMP4 and WNT3/ β -CATENIN signaling identified *T* (*Brachyury*), an evolutionarily conserved TF important for posterior mesoderm formation (Herrmann et al. 1990), as a candidate for conferring the WNT3-mediated competence of the epiblasts. *T*-knockout embryos failed to form mPGCs, whereas overexpression of *T* in *Wnt3*-knockout EpiLCs robustly activated high-level expression of *Blimp1* and *Prdm14* without stimulation by WNT3 or BMP4. Indeed, *T* directly activated the expression of these PGC determinants by binding to their enhancers, indicating that *T* plays a primary and sufficient role as the downstream effector of WNT3 signaling.

T induces the PGC determinants in EpiLCs, independently from BMP4 signaling. This raises a question: What is the role of BMP4 in the PGC specification? When EpiLCs were preexposed to WNT3A, overexpression of *T* did not activate *Blimp1* and *Prdm14*, demonstrating that WNT signaling and/or its downstream effectors inhibit the function of *T* in PGC specification, and BMP4 plays a permissive role in allowing *T* to activate *Blimp1* and *Prdm14* (Aramaki et al. 2013). Thus, this study provided the molecular basis of the competence of the epiblast/EpiLCs for the germ cell fate and answered the question of why the mouse germ cell fate is determined at the onset of gastrulation and transiently activates the mesoderm program during the specification. As demonstrated by the TF overexpression experiments (Nakaki et al. 2013), once the mPGC fate is established, the mesodermal program including *T* is dispensable for the function of mPGCs.

GENOME-WIDE DNA DEMETHYLATION DURING GERM CELL DEVELOPMENT

One of the key aspects of epigenome reprogramming in mPGCs is the genome-wide DNA demethylation, for which two basic mechanisms had been proposed: one is replication-coupled, passive demethylation, with down-regulation of de novo and maintenance DNA methylation machineries upon PGC specification; and the other is enzyme-based active demethylation—that is, deamination of 5-methyl cytidine (5mC) by AID or APOBEC (Guo et al. 2011) and/or oxidation of 5mC by TET proteins (Kriaucionis and Heintz 2009; Tahiliani et al. 2009), followed by base-excision repair (BER) (Saitou et al. 2012; Lee et al. 2014). The active DNA demethylation was considered a predominant mechanism, based on several sets of findings—namely, the kinetic analysis of DNA demethylation of differentially methylated regions (DMRs) of several imprint genes (Hajkova et al. 2002); the apparent involvement of the cytidine deaminase, *Aid*, in DNA demethylation in mPGCs (Popp et al. 2010); and the apparent activation of the BER pathway and subsequent dramatic chromatin changes, including loss of linker histone H1 and of several histone modifications, perhaps through histone replacement, in mPGCs (Hajkova et al. 2010).

Kagiwada et al. (2013) performed quantitative analyses of the genome-wide gene expression profile, cell cycle, nucleus morphology, and DNA methylation dynamics of imprint DMRs. The transcriptome analyses of isolated mPGCs exhibited rapid down-regulation and stable low-level expression of *Uhrf1* (the recruiter of maintenance DNA methyltransferase, DNMT1, into the replication foci) (Sharif et al. 2007) and all of the de novo DNA methyltransferases, supporting the notion that mPGCs consistently possess little maintenance and de novo DNA methylation potential after specification. On the other hand, all of the genes proposed to be involved in active DNA demethylation, except *TET1* and *TDG*, were expressed at low or undetectable levels.

mPGCs appeared to exhibit a cell cycle arrest upon migration into the hindgut endoderm at ~E7.75–E8.75

and were released from this arrested state upon their exit from the hindgut to the dorsal mesentery at ~E9.5 (Seki et al. 2007; Kagiwada et al. 2013). Quantitative analyses of the cell cycle using a combination of BrdU incorporation and DNA content showed the restart of rapid and constant proliferation of mPGCs after E9.5, with an average doubling time of 12.6 h, indicating that mPGCs divide twice per day. In good agreement with the rapid proliferation and the loss of maintenance and de novo DNA methylation activity, bisulfite sequence analysis showed that DNA demethylation was already in progress at E9.5, and the kinetics of imprint erasure in mPGCs did not exceed the demethylation rate that was expected from the replication-coupled passive mechanism, with gene-dependent partial resistances to demethylation. Moreover, Kagiwada et al. (2013) did not detect drastic chromatin changes, such as the loss of linker histone H1 or the loss of several histone modifications, in mPGCs within the embryonic gonads. These findings indicated that, contrary to what had been proposed (Hajkova et al. 2010), the imprint erasure is initiated in early mPGCs and the demethylation can be explained largely by a replication-coupled passive mechanism. Consistent with this report, independent studies for the methylation states of the repetitive elements in mPGCs by high-throughput hairpin bisulfite sequencing demonstrated the prevalence of hemimethylated DNA in mPGCs, indicating that the replication-coupled passive demethylation is a major mechanism for genome-wide DNA demethylation in mPGCs (Seisenberger et al. 2012; Arand et al. 2015). Accordingly, mPGCs lacking TET1, an active DNA demethylase and the only TET protein expressed at a high level in mPGCs, undergo genome-wide DNA demethylation in an apparently normal fashion (Dawlaty et al. 2011). It is, however, important to note that *TET1*-deficient PGCs showed impairment for the DNA demethylation of several specific elements, including DMRs of a few imprinted genes and some genes critical for meiosis, resulting in impaired fertility both in males and females (Yamaguchi et al. 2012, 2013).

CHROMATIN REMODELING DURING GERM CELL DEVELOPMENT

Concomitant with the progress of genome-wide DNA demethylation, mPGCs reprogram repressive chromatin marks in an apparently genome-wide manner: They down-regulate histone H3 lysine 9 dimethylation (H3K9me2) and simultaneously up-regulate H3K27me3 (Seki et al. 2005), but the scale and specificity of these regulations had been elusive. Kurimoto et al. (2015) clarified the dynamics of the genome-wide remodeling of key histone modifications, H3K9me2 (repression by G9A/GLP), H3K27me3 (repression by Polycomb repressive complex 2 [PRC2]), H3K4me3 (active promoters), and H3K27ac (acetylation) (active enhancers), and the genome-wide binding sites of key TFs, T, and BLIMP1, during in vitro mPGC specification/development.

The chromatin immunoprecipitation sequencing (ChIP-seq) analysis revealed that, consistent with the ob-

servations from immunofluorescence analyses of mPGCs in vivo (Seki et al. 2005, 2007), the repressive mark H3K9me2 exhibited a progressive, genome-wide reduction from day 2 (d2 mPGCLCs, representing mPGC precursors expressing key PGC genes [*Blimp1*, *Prdm14*, *Tfap2c*] as well as mesodermal genes) to day 6 of induction (d6 mPGCLCs, representing E9.5 mPGCs). Concomitant with the H3K9me2 reduction, DAPI-dense heterochromatin and LAMIN B1 around the nuclear periphery in d6 mPGCLCs were diminished, indicating that mPGC(LC)s alter their nuclear architecture.

In contrast, H3K27me3 exhibited large-scale reorganization during transition from mESCs to EpiLCs and from EpiLCs to mPGCLCs. The H3K27me3 level was the highest in mESCs and decreased and was converted to a more flat, lawn-type distribution in EpiLCs. During induction of mPGCLCs from EpiLCs, the H3K27me3 further decreased, reaching the lowest genome-wide level in d2 mPGCLCs. At this stage, however, H3K27me3 exhibited punctate enrichment around a set of genes, including genes for embryonic development such as *Hox* clusters. In accordance with the immunofluorescence analyses of mPGCs in vivo (Seki et al. 2005, 2007), the genome-wide H3K27me3 level was then regained in d6 mPGCLCs, with stronger enrichment around developmental regulators. These findings indicate that genes regulating embryonic development were more strongly repressed by PRC2 in mPGCLCs than in mESCs and EpiLCs.

In general, promoters show reciprocal distribution of repressive H3K27me3 and active H3K4me3 modifications, and the promoters with significant levels of both marks, the “bivalent” promoters, have been proposed to represent a poised transcriptional state (Voigt et al. 2013). During in vitro mPGC specification, EpiLCs exhibited the highest numbers of bivalent promoters, and such promoters were enriched in genes for developmental regulators, especially for neuron differentiation. Notably, such promoters in EpiLCs showed lower enrichment levels (i.e., closer to the threshold level of the definition of the enrichment) of both H3K27me3 and H3K4me3, suggesting that EpiLCs may represent a bona fide primed state for immediate differentiation into the three germ layers. The bivalent genes were reduced in number in d2 mPGCLCs because of up- and down-regulation of H3K27me3 and H3K4me3, respectively, suggesting that some of the developmental regulators may lose transcriptional potential in d2 mPGCLCs. The numbers of the bivalent genes were then regained in d6 mPGCLCs with concomitant up-regulation of H3K27me3 and H3K4me3 levels, indicating that d6 mPGCLCs acquire a more permissive chromatin state around many developmental regulators with more strong PRC2-mediated repression.

The ChIP-seq analyses of the genome-wide binding sites for T and BLIMP1 revealed that in response to BMP4, T recruits H3K27ac to activate a set of mesodermal genes as well as the mPGC determinants (*Blimp1* and *Prdm14*), and then BLIMP1 represses a broad range of developmental regulators, possibly by recruiting PRC2 and spreading H3K27me3. Collectively, the results of this study provided the first evidence for the dynamic

remodeling of chromatin states for transcriptional regulation during germ cell specification and development, and demonstrated that T and BLIMP1 function successively to create active and repressive chromatin states, respectively, around their key targets for establishment of the germline epigenome.

INDUCTION OF HUMAN PGCLCs

The induction of human germ cell fate from human PSCs had been considered difficult, because unlike mPSCs, which bear a naïve pluripotency (Nichols and Smith 2009), hPSCs exhibit a primed pluripotency (Rosant 2008; Nichols and Smith 2009) and bear a property more similar to mEpiSCs (Brons et al. 2007; Tesar et al. 2007), which possess little, if any, potential to take on the germ cell fate (Hayashi and Surani 2009). Furthermore, there has been essentially no knowledge as to the mechanism for hPGC specification because of the difficulty in studying early human embryos (Fig. 1).

Recently, however, based on the concept of mPGCLC induction, two groups independently reported the induction of human PGC-like cells from hPSCs maintained on feeders (Irie et al. 2015) or in MEF-conditioned medium (Sugawa et al. 2015). In the study by Irie et al., *SOX17* was shown to be critically required for induction of human PGC-like cells, and a “distinctive” pluripotency (suggested to correspond to the naïve pluripotency in mice) (Gafni et al. 2013) was shown to play a key role in the high competence of hESCs for the germ cell fate (Irie et al. 2015).

Sasaki et al. (2015) demonstrated the robust induction of human PGC-like cells from human iPSCs (hiPSCs) in a primed pluripotent state maintained under a defined, feeder-free condition. Sasaki et al. exploited hiPSCs bearing fluorescent reporters for *BLIMP1* (*BLIMP1-2A-tdTomato*: BT) and *TFAP2C* (*TFAP2C-2A-EGFP*: AG) and induced hiPSCs first into a state similar to the incipient mesoderm (incipient mesoderm-like cells: iMeLCs) by stimulation with activin A and a WNT-signaling agonist (CHIR99021) for ~2 d. iMeLCs showed expression of a number of early mesodermal markers such as *EOMES*, *T*, and *MIXL1* at moderate levels and retained a high level of the key pluripotency factors *POU5F1*, *SOX2*, and *NANOG*. When cultured as floating aggregates under conditions essentially identical to those for mPGCLC induction, iMeLCs exhibited robust (~30%–40%) activation of BT and AG in response to BMP4, and the BTAG-positive cells remained at least until day 8–10 of induction.

The BTAG-positive cells exhibited a transcriptome similar to those of the gonadal PGCs of cynomolgus monkeys (roughly corresponding to E10.5–E13.5 mPGCs), except for the expression of late PGC genes such as *Ddx4* and *Piwil2*, and were thus defined as cells representing early hPGCs (hPGCLCs). The hPGCLCs induced by Sasaki et al. (2015) also showed a transcriptome similar to those of human gonadal PGCs (except late PGC genes), and the TNAP/NANOS3-positive cells reported by Irie et al. (2015). The cells defined by Sugawa et al.

(2015), on the other hand, exhibited remarkably different gene expression profiles from those described by Sasaki et al. (2015) and Irie et al. (2015), presumably because of the differences in the induction and/or sorting strategies.

Notably, analyses of the dynamics of the transcriptome during hPGCLC induction revealed that hPGCLC specification occurred without the prominent activation of somatic programs. This is in contrast to the transient but eminent activation of the somatic mesodermal program—that is, the program for “pattern specification,” “embryonic morphogenesis,” and “gastrulation,” etc., followed by robust repression during induction of mPGCLCs (Hayashi et al. 2011; Kurimoto et al. 2015). Instead, upon hPGCLC induction, the program related to “neuron differentiation” was transiently up-regulated and then repressed. Consistently, analyses of the hPGCLC induction from *BLIMP1*-knockout hiPSCs revealed that while *BLIMP1* played critical roles in the specification and the maintenance of hPGCLCs (Irie et al. 2015; Sasaki et al. 2015), the role of *BLIMP1* for the repression of somatic programs was variable (e.g., *BLIMP1*-deficient cells failed to maintain expression of *T* and *MIXL1*, whereas they failed to repress *EVX1* and *SP5*, and had essentially no effects on *EOMES* and *MSX2*). On the other hand, *BLIMP1* was critical to repress genes associated with “neuron differentiation” and to activate genes potentially important for hPGCs and pluripotency, such as *NANOS3*, *KLF4*, *TFCP2L1*, and *TCL1B*, indicating that *BLIMP1* functions not only as a repressor but also as an activator for the transcriptional network for the hPGCLC fate. These findings highlight the difference in the molecular mechanisms of germ cell fate specification between humans and mice.

Collectively, the study by Sasaki et al. (2015) demonstrated that the human primed pluripotent state possesses robust competence for the germ cell fate and would require revision of the spectrum of developmental potential of human pluripotent states. Accordingly, gene expression analyses have suggested that hPSCs may bear a property somewhat between EpiLCs and EpiSCs.

PERSPECTIVE

In vitro reconstitution of mouse germ cell development from mPSCs provided a robust foundation for understanding the mechanism of germ cell development, including signaling pathways, transcriptional networks, and epigenetic regulation. There remain many open questions: for example, how and to what extent do the mPGCLCs undergo DNA methylation reprogramming, and especially, what is the relationship between DNA methylation reprogramming and the histone modification states? Does the mechanism for DNA methylation reprogramming during mPGCLC induction resemble those during preimplantation development and/or during naïve mESC induction, as has been proposed previously (Seisenberger et al. 2012; Ficiz et al. 2013; Leitch et al. 2013)? Is it possible to differentiate mPGCLCs into cells corresponding to mPGCs at late stages (~E12.5) without the use of an aggregation culture with gonadal somatic cells

and to induce mitotic arrest or meiosis by defined factors? Is it possible to differentiate mPGCLCs into SSCs or oocytes in vitro?

The mechanism for human germ cell development has largely been elusive because of inherent difficulties and/or limitations in the analysis of human embryos. The in vitro induction of hPGCLCs creates a unique opportunity for understanding the mechanism of human germ cell development, including the exploration of signaling pathways for and identification of the TFs essential for hPGC specification. On the other hand, for a more precise understanding of the mechanism of hPGC specification, it is critical to investigate the mechanism of PGC specification using a model organism that exhibits a mode of PGC specification similar to humans, such as nonhuman primates. Such investigations could clarify key issues such as the source for signaling molecules essential for PGC specification or the precise degree of similarity between the pathways for hPGCLC induction and hPGC specification in vivo.

Recently, three groups independently reported genome-wide DNA methylation and transcription profiles of hPGCs obtained from aborted embryos at the stages of arrival of the hPGCs to—and proliferation/differentiation within—the genital ridges (corresponding to ~E9.5–E13.5 in mice) (Gkountela et al. 2015; Guo et al. 2015; Tang et al. 2015), and revealed fundamental similarities in epigenome reprogramming between humans and mice. These discoveries have posed several critical challenges for the future. Namely, it will be important to explore whether hPGCLCs are further differentiated into cells corresponding to late-stage hPGCs in vitro, to examine the epigenetic reprogramming in such cells, and to determine whether such cells may have the potential to further differentiate into either spermatogonia or oocytes.

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