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Mammalian Germ Cell Development: From Mechanism to *In Vitro* Reconstitution

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The germ cell lineage gives rise to totipotency and perpetuates and diversifies genetic as well as epigenetic information. Specifically, germ cells undergo epigenetic reprogramming/programming, replicate genetic information with high fidelity, and create genetic diversity through meiotic recombination. Driven by advances in our understanding of the mechanisms underlying germ cell development and stem cell/reproductive technologies, research over the past 2 decades has culminated in the *in vitro* reconstitution of mammalian germ cell development: mouse pluripotent stem cells (PSCs) can now be induced into primordial germ cell-like cells (PGCLCs) and then differentiated into fully functional oocytes and spermatogonia, and human PSCs can be induced into PGCLCs and into early oocytes and prospermatogonia with epigenetic reprogramming. Here, I provide my perspective on the key investigations that have led to the *in vitro* reconstitution of mammalian germ cell development, which will be instrumental in exploring salient themes in germ cell biology and, with further refinements/extensions, in developing innovative medical applications.

Germ Cells: An Enduring Link between Generations

Germ cells are a source of totipotency and an enduring link between generations, ensuring the transmission of genetic as well as epigenetic information to successive generations. This contrasts with somatic cells, which die away after differentiation and contribution to organismal physiology. The dichotomy between germ cells and somatic cells has naturally led to an assumption that germ cells bear key regulatory mechanisms that realize their unique and fundamental functions, and seminal experiments have created a conceptual framework explaining the basis for such capacities. In an early and prominent example, somatic cell nuclear transfer into oocytes in frogs revealed the remarkable capacity of oocytes to reprogram the somatic cell nucleus into totipotency and, at the same time, demonstrated that genetic information itself is preserved in somatic cells during organismal development (Gurdon, 1962). Two decades later, parental pronuclear transplantation experiments in mice established the concept of genome imprinting and predicted the reprogramming of imprinting during germ cell development (McGrath and Solter, 1984; Surani et al., 1984). These works, together with the measurement of DNA methylation dynamics during germ cell development, which suggested that germ cells erase and re-establish genome-wide DNA methylation during their development (Monk et al., 1987), provided some of the first evidence that germ cells undergo "epigenetic reprogramming." Meanwhile, genetic experiments revealed that germ cells exhibit an enhanced genetic integrity and a significantly lower frequency of spontaneous mutations compared with somatic cells (Ehling and Neuhauser, 1979; Murphey et al., 2013; Russell et al., 1979). On the other hand, it has been well established that germ cells are the only cells that are programmed to undergo meiotic recombination of parental chromosomes and generate sexually dimorphic gametes bearing a haploid genome for fertilization, thereby giving rise to enormous genetic diversity (Baudat et al., 2013).

Thus, to fulfill their salient functions, germ cells acquire at least three key capacities during their development: the capacity to reprogram epigenetic information into totipotency, the capacity to preserve genetic information with high fidelity, and the capacity to create genetic diversity through meiotic recombination and fertilization. Nonetheless, until relatively recently, little has been known regarding the molecular mechanisms that underpin these key functions in germ cells, particularly in mammals.

Elucidation of the Mechanism for Germ Cell Specification in Mice

One approach to elucidating the mechanisms underlying the capacities of germ cells would be to understand the mechanism and the consequences of germ cell specification. Two distinct modes have been identified for germ cell specification in metazoans: one is "epigenesis," which involves the induction of germ cell fate in pluripotent precursors, and the other is "preformation," in which blastomeres that inherit a preformed "germplasm" in oocytes take on the germ cell fate (Extavour and Akam, 2003). The former appears to be evolutionarily ancestral and is seen in organisms including mammals, whereas the latter has expanded by convergent evolution and is seen in many model organisms such as *C. elegans*, *D. melanogaster*, *Danio rerio*, and *X. laevis*. By the early 2000s, the mechanism for the latter had been relatively well investigated using genetics approaches in *D. melanogaster* and *C. elegans*, leading to the identification of key genes essential for their germ cell specification and to the concept that germ cell specification requires a transcriptional silencing imposed by the germplasm components (Seydoux and Strome, 1999). In contrast, the

mechanism for the former, though clearly distinct from preformation, was largely unclear at the start of the millennium, and its elucidation was a key challenge.

On the other hand, histological studies revealed that, in mice, primordial germ cells (mouse PGCs: mPGCs), the founders of the germ cell lineage, become discernable as alkaline phosphatase-positive cells at around embryonic day (E) 7.25 at the base of the incipient allantois in the extraembryonic mesoderm (Chiquoine, 1954; Ginsburg et al., 1990). Pioneering clonal analyses indicated that the mPGC precursors reside within the epiblast cells most proximal to the extraembryonic ectoderm at E6.0; at E6.5 these precursors move posteriorly, and at around E7.25, the mPGCs are lineage restricted from their close somatic relatives, including the allantoic mesoderm cells (Lawson and Hage, 1994). Remarkably, analyses of embryos deficient for bone morphogenetic protein 4 (BMP4), which shows a specific expression in the extraembryonic ectoderm from around E5.5, revealed that BMP4 is an essential signal for mPGC specification (Lawson et al., 1999). Because not only mPGCs but also their somatic relatives, including the allantois, are absent in BMP4-deficient embryos, it was suggested that BMP4 may play a dose-dependent function in the lineage specification of mPGCs and their somatic relatives, or there may exist an additional signal that specifically determines the germ cell fate (two-signal model) (Lawson et al., 1999; McLaren, 1999).

Building on this foundation, single-cell cDNA differential screening between putative mPGCs and their somatic neighbors defined by the expression of several marker genes was performed, and this screening identified genes specifically or highly expressed in mPGCs, including *Stella*/*Dppa3*, *Fragilis*/Ifitm3, *Blimp1*/*Prdm1*, and *Prdm14*, creating the basis for a systematic analysis of the mechanism of mPGC specification (Ohinata et al., 2005; Saitou et al., 2002; Yamaji et al., 2008). Notably, it was shown that *Blimp1* begins to be expressed in the most proximal epiblast cells from around E6.25, *Blimp1*-expressing cells contribute specifically to mPGCs, and *Blimp1* is essential for mPGC specification, but not for specification of the somatic neighbors, including the allantois, unequivocally defining the origin of the germ cell lineage in mice (Ohinata et al., 2005). *Blimp1* was shown to function as a master regulator for the terminal differentiation of B cells into plasma cells by "extinguishing" the B cell gene-expression program (Keller and Maniatis, 1991; Shaffer et al., 2002; Shapiro-Shelef et al., 2003). The finding that a master regulator for somatic cell terminal differentiation also specifies the germ cell fate was surprising, and the known function of BLIMP1 as a robust transcriptional repressor was reminiscent of transcriptional silencing for germ cell specification in *D. melanogaster* and *C. elegans* (Nakamura and Seydoux, 2008; Seydoux and Strome, 1999). Subsequently, the original single-cell cDNA amplification method was improved for quantitative single-cell cDNA microarray analyses (Kurimoto et al., 2006), which were applied to the exploration of global gene-expression dynamics during mPGC specification, revealing that mPGC specification consists of at least three key events, i.e., repression of the somatic program, reacquisition of the pluripotency network, and ensuing epigenetic reprogramming (Kurimoto et al., 2008) (see below), and that *Blimp1* is essential for all three events and *Prdm14* for at least the last two (Kurimoto et al., 2008; Yamaji et al., 2008). The single-cell cDNA microarray procedure was later extended into the first single-cell RNA sequence technology (Tang et al., 2009).

The identification of *Blimp1* as a gene that defines the origin of the germ cell lineage promoted understanding of the signaling mechanism for germ cell specification. Accordingly, it was shown that essentially all the epiblast cells from ~E5.75 to ~E6.25, but not those from earlier or later, are competent to generate mPGCs in response to BMP4 in a dose-dependent manner, and in developing embryos, the allocation of the germ cell fate is constrained by the balance between the inducing, i.e., BMP4, and the antagonizing signals that the epiblast cells receive from surrounding extraembryonic tissues (Ohinata et al., 2009). Importantly, as originally demonstrated for mPGCs *in vivo* (Chuma et al., 2005), PGCs induced from the epiblast by BMP4 *ex vivo* contributed to spermatogenesis upon transplantation into testes of neonatal mice, demonstrating their functional potential as *bone fide* germ cells (Ohinata et al., 2009). Thus, a basic framework for elucidating the mechanism for germ cell specification in mice at the signaling and transcriptional levels was established.

Epigenetic Reprogramming

Until the 1990s, the major epigenetic modifications known to play key roles in gene regulation were DNA methylation (Li et al., 1992) and histone acetylation (Brownell et al., 1996). In the early 2000s, there was dramatic progress in our understanding of the epigenetic regulation of gene expression, with the identification and functional characterization of histone methyltransferases bearing the SET (Suv39/Enhancer of zeste/Trithorax) domains and the proposition of the histone code hypothesis for gene expression/cellular identity and memory, which was based on the relatively stable nature of methylation compared with other modifications, such as acetylation and phosphorylation (Jenuwein and Allis, 2001; Rea et al., 2000). The enzymes for histone demethylases and DNA demethylases (see below) were subsequently identified (Shi et al., 2004; Tahiliani et al., 2009), creating a basis for investigating the epigenetic regulation at a systems level (Klose et al., 2006; Wu and Zhang, 2014). On the other hand, it was a

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Mitinori Saitou has received several awards, such as the Takeda Prize for Medicine, the Academic Award of the Mochida Memorial Foundation, the Asahi Prize, the Uehara Prize, the Imperial Prize and Japan Academy Prize, and the ISSCR 2020 Momentum Award. He is an associate member of the European Molecular Biology Organisation.

surprising discovery that the epigenetic states of somatic cells are reprogrammed to an embryonic pluripotent state through the expression of only a few defined factors to generate induced pluripotent stem cells (iPSCs) in both mice and humans (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). It is also of note that the first draft of the human genome sequence was completed in early 2000 (Lander et al., 2001), and those of many other key organisms, including mice, followed soon thereafter (Waterston et al., 2002), accelerating the discovery of genes with key functions and the establishment of genome-wide analysis technologies/platforms, including RNA sequencing (Wang et al., 2009), chromatin immunoprecipitation sequencing (Barski et al., 2007; Johnson et al., 2007; Mikkelsen et al., 2007; Robertson et al., 2007), and whole-genome bisulfite sequencing (WGBS) (Cokus et al., 2008; Hayatsu and Shiragami, 1979; Lister et al., 2008).

Coincident with the above research, epigenetic dynamics during germ cell development in mice were also investigated using various technologies. Key findings in this line of investigation include the apparent significant upregulation of genome-wide histone H3 lysine 27 trimethylation (H3K27me3) levels and downregulation of H3K9me2 levels in germ cells that colonize embryonic gonads (oogonia/gonocytes) (at ~E12.5) (Seki et al., 2005, 2007). Most notably, consistent with classical studies involving Southern blot analysis and more locus-specific analyses such as those analyzing imprint genes (Hajkova et al., 2002; Kafri et al., 1992; Monk et al., 1987), the WGBS analysis revealed that gonadal germ cells indeed erase nearly all DNA methylation in their genome, and unlike typical somatic cells and embryonic cells, including the epiblast cells that bear ~80% of genome-wide CpG methylation (5mC) levels, gonadal germ cells show only ~5% of 5mC and the remaining methylations are enriched in sequences such as evolutionary young endogenous retroviruses (Popp et al., 2010). These findings establish the concept that germ cells nearly completely erase their parental epigenetic memory based on DNA methylation relatively early during their development (during the mPGC stage) and perhaps reprogram genome-wide histone modifications so that they ensure the epigenetic integrity of the DNA methylation-free epigenome. The remaining DNA methylation can serve as a basis for transgenerational epigenetic inheritance (Lane et al., 2003; Popp et al., 2010).

The mechanism for the genome-wide DNA demethylation in germ cells has been a subject of intensive investigation, and two relevant mechanisms have been proposed: one is enzyme-based active DNA demethylation and the other is replication-coupled, passive DNA demethylation (Hajkova et al., 2002). The identification of DNA demethylases, first in plants (DEMETER: DNA glycosylase) (Gehring et al., 2006) and then in mammals (TET1/2/3 [Ten-eleven translocation]: 5mC dioxygenase that converts 5mC into 5-hydroxymethylcytosine [5hmC]) (Tahiliani et al., 2009), was an exciting advance, and many investigations have since been performed to explore the role of TET enzymes in physiological contexts (Saitou et al., 2012; Wu and Zhang, 2014). A key conclusion, however, has been that although TET enzymes indeed play pivotal roles in gene regulation by binding to CpG-rich promoters through their CXXC motifs and regulating their DNA methylation fidelity in various contexts (Saitou et al., 2012; Williams et al., 2011; Wu and Zhang, 2014), they have only a minor, if any, role in genome-wide DNA demethylation in mPGCs

(Dawlaty et al., 2011; Hill et al., 2018; Yamaguchi et al., 2012, 2013). The level of 5hmC in mPGCs is very low (~0.1%) throughout their development, and the conversion of 5mC into 5hmC did not account for the dramatic reduction of the 5mC level in mPGCs (Hill et al., 2018). Consistent with this finding, germ cells deficient for *Tet1*, a dominant paralog expressed in mPGCs, underwent genome-wide DNA demethylation normally, and both *Tet1*-deficient males and females were fertile (Dawlaty et al., 2011; Yamaguchi et al., 2012, 2013). Nonetheless, it was found that, after genome-wide DNA demethylation, TET1 plays a role in safeguarding a demethylated state of promoters of key germline genes, including those for meiosis, during oocyte development, thereby contributing to a robust production of oocytes (Hill et al., 2018; Yamaguchi et al., 2012, 2013).

In contrast, evidence that genome-wide DNA demethylation in mPGCs occurs through replication-coupled, passive DNA demethylation has been accumulating. Upon mPGC specification, *de novo* DNA methyltransferases *DNMT3a* and *3b*, as well as a key machinery for maintenance DNA methylation, *Uhrf1*, are transcriptionally repressed, and thus mPGCs bear little DNA methyltransferase activity (Kagiwada et al., 2013; Kurimoto et al., 2008; Ohno et al., 2013; Seki et al., 2005). A measurement of the 5mC levels of the differentially methylated regions of the imprint genes (Kagiwada et al., 2013), of genome-wide 5mC levels/profiles (Seisenberger et al., 2012), and of strand-specific hemimethylation profiles (Arand et al., 2015) during a critical period of germ cell development provided a result consistent with passive demethylation during a rapid doubling of germ cells. As discussed below, the system that reconstitutes germ cell development *in vitro* also provided evidence consistent with a replication-coupled, passive DNA demethylation as the mechanism for the genome-wide DNA demethylation in germ cells (Ohta et al., 2017; Shirane et al., 2016). Further investigations are warranted as to the mechanism and consequence of the epigenetic reprogramming, including studies on histone modification reprogramming and nuclear architecture dynamics, which creates a basis for programming the sexually dimorphic epigenome during subsequent male or female germ cell development, which together give rise to a totipotent epigenome (Sasaki and Matsui, 2008).

In Vitro Reconstitution of Mouse Germ Cell Development

The *in vitro* reconstitution of germ cell development using PSCs as starting materials has been a key goal in developmental biology, with a robust potential for reproductive biology and medicine (Daley, 2007). In particular, induction of a homogeneous germ cell population at relevant developmental stages would provide abundant experimental materials for promoting mechanistic understanding of critical pathways in germ cell development. The elucidation of the mechanism for germ cell specification as described above and the development of a method for culturing mouse embryonic stem cells (mESCs) in a uniform state similar to that of epiblast cells at around E4.5 with the use of chemical inhibitors for key signaling pathways (referred to as a ground state of "naive" pluripotency) (Ying et al., 2008) served as two critical foundations toward the realization of this goal.

As part of this effort, mESCs were induced into epiblast-like cells (EpiLCs) bearing properties similar to those of the epiblast at ~E6.0, and then, in response to BMP4, EpiLCs were induced into mPGC-like cells (mPGCLCs) with properties similar to those of mPGCs at ~E9.5 (Hayashi et al., 2011). In contrast, epiblast stem cells (EpiSCs), which bear a primed pluripotency and resemble the epiblast cells after E6.5 (Brons et al., 2007; Kojima et al., 2014; Tesar, 2016; Tesar et al., 2007), showed little, if any, competence to give rise to mPGCLCs (Hayashi et al., 2011). Importantly, male mPGCLCs contributed to spermatogenesis when transplanted into testes of neonatal mice lacking endogenous germ cells, and female mPGCLCs contributed to oogenesis upon aggregation with embryonic ovarian somatic cells followed by transplantation under the ovarian bursa of immunodeficient mice, and the resultant spermatozoa and oocytes gave rise to fertile offspring, demonstrating that mPGCLCs are a *bona fide in vitro* counterpart to mPGCs (Hayashi et al., 2011, 2012). The induction of EpiLCs from mESCs is a straightforward and relatively uniform process, and mPGCLCs are induced from EpiLCs at a maximum induction efficiency of ~40% (Hayashi et al., 2011), creating an opportunity for a systematic understanding of the mechanism and consequences of mPGC specification *in vitro*. Indeed, the signaling, transcriptional, epigenetic, and metabolic mechanisms of mPGC specification have been studied based on this *in vitro* system (Aramaki et al., 2013; Hackett et al., 2018; Kurimoto et al., 2015; Murakami et al., 2016; Nakaki et al., 2013; Shirane et al., 2016; Tischler et al., 2019; Zhang et al., 2018), and more studies will be needed to further delineate the mechanism for germ cell specification in mice.

Coincident with the development of the mPGCLC induction system, significant progress has been made in *ex vivo* cultures of immature gonads, including the maturation of mPGCs into fully functional oocytes by *ex vivo* cultures of embryonic ovaries and the maturation of spermatogonia into spermatids by *ex vivo* cultures of neonatal/perinatal testes (Komeya et al., 2016; Morohaku et al., 2016; Sato et al., 2011). Such progress prompted efforts to explore whether mPGCLCs mature into oocytes or spermatozoa by coculturing them with embryonic

gonadal somatic cells. For *in vitro* oogenesis, mPGCLCs aggregated with embryonic ovarian somatic cells (reconstituted ovaries: rOvaries) were cultured under an air-liquid interphase condition, under which mPGCLCs differentiated successfully into primary oocytes at the secondary follicle stage; such oocytes/follicles were further matured into fully grown oocytes under similar conditions with gonadotropins, and remarkably, the resultant oocytes, through *in vitro* maturation and fertilization, contributed to fertile offspring (Hikabe et al., 2016). Similarly, for *in vitro* spermatogenesis, mPGCLCs aggregated with embryonic testicular somatic cells (reconstituted testes: rTestes) were cultured under air-liquid interphase conditions, under which mPGCLCs differentiated into spermatogonia-like cells (but not into spermatozoa), which were then propagated in culture as germline stem cell-like cells (see below) with potential to contribute to spermatogenesis in adult testes lacking endogenous spermatogenesis (note that mPGC(LC)s contribute to spermatogenesis only when transplanted into neonatal testes; Ishikura et al., 2016; Ohta et al., 2004) (Ishikura et al., 2016). These studies provided the proof of principle for the induction of fully functional oocytes and spermatogonia from PSCs *in vitro*. It is nonetheless critical to note that oocytes and spermatogonia induced *in vitro* exhibited genetic and epigenetic abnormalities to various and significant degrees (Hikabe et al., 2016; Ishikura et al., 2016), and the culture systems would require substantial improvements, with a key focus on appropriate regulation of epigenetic reprogramming and programming.

Expansion of a progenitor or a stem cell population *in vitro* has been a key goal in stem cell biology, and in the case of the germ cell lineage, this was achieved for spermatogonial stem cells in several rodents, which propagate as germline stem cells with a robust self-renewal and spermatogenic potential (Kanatsu-Shinohara et al., 2003; Kanatsu-Shinohara and Shinohara, 2013). Attempts were made to explore whether mPGCLCs can be propagated, leading to the finding that in the presence of stem cell factor and chemicals that stimulate cAMP signaling, mPGCLCs exhibit a limited but substantial propagation (~50-fold for a week) with retention of the capacity to contribute to spermatogenesis upon transplantation (Ohta et al., 2017). The reason mPGCLCs show only a limited expansion remains unknown and poses an interesting theme to explore. Notably, it was shown that, upon propagation and while retaining their transcriptome, mPGCLCs progressively erase their genome-wide DNA methylation in a manner consistent with a replication-coupled, passive demethylation, and acquire 5mC levels and profiles very similar to those of gonadal germ cells, demonstrating that mPGCLCs bear an intrinsic capacity for epigenetic reprogramming, for which embryonic gonads are not required (Ohta et al., 2017). Accordingly, the mPGCLC expansion system provided an experimental framework to investigate the mechanism of sex determination of germ cells in a constructive fashion, leading to the finding that BMP signaling and its downstream effector ZGLP1, an evolutionarily conserved transcriptional regulator with GATA-like zinc fingers, but not retinoic acid signaling and its downstream effector STRA8 (Baltus et al., 2006; Bowles et al., 2006; Koubova et al., 2006), induces the female germ cell fate and determines the oogenic fate, revising a prevailing view to provide an integrated scenario for the female sex determination mechanisms of germ cells in mice (Miyauchi et al., 2017; Nagaoka et al., 2020). Both the reconstituted gonad culture system and the mPGCLC expansion system will continue to serve as useful platforms to investigate the mechanism for mouse germ cell development.

Non-Human Primates as Models for Human Development

From the early 2000s onward, soon after the derivation of human ESCs (hESCs) (Thomson et al., 1998), there has been a pursuit of the induction of the human germ cell fate from hESCs (Clark et al., 2004). The early years might have been premature in terms of directly targeting such a goal, since the mechanism for human germ cell specification and the properties of human germ cells were unclear. The advances in understanding of the mechanism of mouse germ cell specification and its *in vitro* reconstitution suggested that such knowledge could be translated to humans. On the other hand, it was becoming gradually evident that some of the key mechanisms for early mammalian development, e.g., the signaling and transcriptional mechanisms, are substantially divergent among mammals and that, in fact, mice are not humans (Rossant, 2011, 2015). A prominent example involves the significant differences in the properties of "ESCs" among mammals: ESCs were originally derived from the 129 strain of mice (Evans and Kaufman, 1981; Martin, 1981), but they were not readily derived even from closely related rodents, such as rats, until the development of a sophisticated culture system using chemical inhibitors for key signaling pathways (Buehr et al., 2008; Ying et al., 2008). Moreover, hESCs were derived under conditions substantially different from those for mESCs, showed properties clearly distinct from those of mESCs, and, later, were found to be closer to mouse EpiSCs bearing a "primed" pluripotency (Nichols and Smith, 2009; Rossant, 2015). Thus, for aiming at the induction of human germ cell fate from hESCs, these notions stressed the importance of investigating the mechanism of early development, including germ cell development, in humans or in closely related primates.

Accordingly, using cynomolgus monkeys (*Macaca fascicularis*) as a human model, investigations were performed to characterize the transcriptome properties/dynamics of key embryonic cell types, particularly pluripotent epiblast cells, at a single-cell level in both the pre- and the early postimplantation stages (Nakamura et al., 2016), and to explore the origin of the germ cell lineage (Sasaki et al., 2016). The former study revealed that the epiblasts in cynomolgus monkeys are specified in the blastocysts at ~E7 and, upon implantation at ~E9, change their transcriptomes substantially and, thereafter, while generating gastrulating cells from ~E12, maintain their transcriptome in a relatively stable manner for a week or more (until ~E17). Notably, it was shown that cynomolgus monkey ESCs, which are derived from preimplantation blastocysts and bear properties, including key transcriptomes, highly similar to those of hESCs, exhibit a strikingly similar transcriptome to those of the postimplantation epiblast cells at E16/17 and then to those at E13/14 (Nakamura et al., 2016). The latter study showed that, unexpectedly, the cells that express key markers (SOX17 and TFAP2C) of the human germ cell lineage (see below) originate in the amnion, an extraembryonic epithelium that segregates from the epiblast upon implantation: $SOX17^{+}/TFAP2C^{+}$ cynomolgus monkey PGCs first emerge in the dorsal amnion as early as ~E11 (a few cells are first detectable); appear to migrate down the posterior midline of the amnion, with a recruitment of newly SOX17/TFAP2C-expressing cells, toward an area underneath the posterior epiblast; and expand their number in this region by E17. Of note, the amnion, and particularly the posterior amnion, expressed OCT4 and NANOG, but downregulated SOX2, and expressed *BMP4*; the immediate effectors of *BMP4*, such as *IDs* and *MSXs*; and, importantly, key mesodermal markers such as *T* and *EOMES*, fulfilling a requirement as a precursor for PGCs and raising the notion that the properties of the early amnion in primates are similar to those of the proximal-posterior epiblasts in mice (Sasaki et al., 2016).

These studies created a framework for understanding the mechanism of early postimplantation development, including germ cell specification, in primates, which, while involving largely conserved principles across mammals, bears a key divergence from that of mice, and such divergence requires critical consideration when attempting to reconstitute development of the relevant lineages from primate PSCs. More recently, several works involving the culture of preimplantation human/monkey blastocysts for a prolonged period to mimic their early postimplantation development have been reported (Chen et al., 2019; Deglincerti et al., 2016; Ma et al., 2019; Niu et al., 2019; Shahbazi et al., 2016; Xiang et al., 2020; Zhou et al., 2019). Although such efforts are very important, the success of these cultures can be evaluated only through rigorous comparison of the culture outcomes with *in vivo* development. Appropriate investigations into the mechanism of primate development *in vivo* will continue to be critical for advancing our understanding of human development.

In Vitro Reconstitution of Human Germ Cell Development

The overall advances in germ cell biology described above, with rapid progress in genome editing technologies after the early 2010s (Joung and Sander, 2013; Zhang et al., 2014), and the evolving sophistication of hPSC culture conditions have all made their contributions to the induction of the human germ cell fate from hPSCs. It was shown that hPSCs cultured under a distinct condition with a set of chemical inhibitors of key signaling pathways, or hPSCs after a transient induction into an incipient mesoderm-like state, are induced into human PGCLCs (hPGCLCs) in response to BMP4 under conditions essentially identical to those for mPGCLC induction from EpiLCs (Irie et al., 2015; Sasaki et al., 2015). Importantly, the hPGCLC induction system revealed that there are critical differences between the transcriptional regulation of hPGC(LC) specification and that of mPGC specification: *SOX17*, which is dispensable for mPGC specification, functions upstream of *BLIMP1* for hPGC(LC) specification, and both the transcription factors themselves and their hierarchy of actions are different between human and mouse germ cell specification (Chen et al., 2018; Irie et al., 2015; Kojima et al., 2017; Pierson Smela et al., 2019; Sasaki et al., 2015; Sybirna et al., 2020). More recently, an hPGCLC expansion system was developed, and experiments using this system revealed that, in stark contrast to mPGCLCs, hPGCLCs propagate $\sim 10^{6}$-fold over a period of 4 months and maintain not only their transcriptome, but also their epigenome during the expansion (Murase et al., 2020). Thus, unlike the specification and propagation of mPGC(LC)s, hPGC(LC) specification and propagation are genetically dissociable from epigenetic reprogramming.

Given the fundamental role of germ cells in the transmission of genetic information, such differences in the mechanism of germ cell specification between humans and mice might be counterintuitive. On the other hand, the mechanism for germ cell specification has been broadly divergent among metazoans ("epigenesis" and "preformation") (Extavour and Akam, 2003), and the structures of early postimplantation embryos are substantially different between humans and mice (Rossant, 2015). It is therefore rather reasonable for humans and, more generally, primates to adopt a strategy for germ cell specification that reflects their embryonic structures at a relevant stage. Indeed, the structures of early postimplantation embryos are divergent among mammals (Hopf et al., 2011;

Kobayashi et al., 2017), and although mice have been used as a representative model for mammalian development, their early postimplantation embryos exhibit a unique egg-cylinder structure, which is clearly unlike the disc-shaped structure of the early postimplantation embryos in other mammals.

Concurrent with the studies described above, significant efforts were made to explore and characterize the transcriptomic and epigenetic properties of human fetal germ cells (FGCs), encompassing a wide developmental period (from about week 5 to about week 24 of human development). These investigations revealed that human germ cells exhibit conserved as well as unique transcriptional profiles across their development and, as in mice, show epigenetic reprogramming, including a comprehensive genome-wide DNA demethylation, with human-specific demethylation "escapees," including evolutionarily young transposable elements (Gkountela et al., 2015; Guo et al., 2015; Li et al., 2017; Tang et al., 2015). In an attempt to explore whether hPGCLCs, which showed properties similar to those of hPGCs just after their specification and prior to epigenetic reprogramming, differentiate further as human germ cells, an aggregation culture of hPGCLCs with mouse embryonic ovarian somatic cells was performed (xenogeneic rOvaries). Notably, the results showed that, over a period of ~3–4 months, hPGCLCs undergo genome-wide DNA demethylation, differentiate into oogonia, and then differentiate into retinoic acid-responsive FGCs, an immediately precursory state for meiotic oocytes that appears after approximately week 12 of human development (Yamashiro et al., 2018). This study demonstrated the capacity of hPSCs and hPGCLCs for human germ cell development, establishing a framework for reconstituting human germ cell development *in vitro*. Accordingly, a recent study has shown that an aggregation culture of hPGCLCs with mouse embryonic testicular somatic cells (xenogeneic rTestes) differentiates hPGCLCs into gonocytes and then into prospermatogonia (Hwang et al., 2020).

Perspective

During the past 2 decades, the mechanisms that regulate mammalian germ cell development have been clarified to a great extent, first mainly through molecular genetics approaches using the mouse as a model organism, and then through powerful genomics and epigenomics approaches in both mice and humans. As outlined here, the knowledge gleaned from such studies, combined with the advances in stem cell and reproductive technologies, have realized an *in vitro* reconstitution of mouse germ cell development, demonstrating the proof of principle for the induction of fully functional oocytes and spermatogonia from mPSCs *in vitro* and, more recently, creating a framework for the *in vitro* reconstitution of human germ cell development. These novel approaches and their future extensions, which will provide scalable experimental materials in a relatively uniform and readily accessible manner, should play decisive roles in further elucidating the mechanisms underpinning salient functions of the germ cell lineage, including those for epigenetic reprogramming and programming for totipotency, the faithful preservation of genetic information, and the creation of genetic diversity through meiotic recombination. This collective knowledge base will serve as a foundation for stem cell-based reproductive medicine and preservation of species diversity.

AUTHOR CONTRIBUTIONS

M.S. conceived this work and wrote the manuscript.

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CONFLICTS OF INTEREST

M.S. is a founder of Houjou, Inc., and is an inventor on patent applications relating to the induction of germ cells from PSCs filed by Kyoto University.

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