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= Mini Review =

Recent advance of germ cell production *in vitro* from pluripotent stem cells

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INTRODUCTION

Primordial germ cells (PGCs), precursors of the oocytes and the spermatozoa, accomplish a unique developmental program to finally acquire totipotency. During the program, there are a number of biologically significant processes such as expression of pluripotency-specific genes, epigenetic reprogramming and meiosis. A flaw in any of these processes can cause developmental disorders and infertility. Understanding the whole process of PGC development is thereby of particular importance for not only basic biology but also clinical issues. How PGCs execute the unique program has, however, remained unclear, mainly due to limitation of experimental materials; for example the number of nascent PGCs in an embryo is less than 50. To overcome the limitation, we recently developed a culture system that produces a robust number, nearly a million, of PGC-like cells, of which potential is almost equivalent to nascent PGCs in embryos. This review briefly introduces the significance of developmental processes of PGCs, summarizes the culture system, and discusses possible applications towards understanding a whole process of PGC development.

Key words: primordial germ cells, pluripotent stem cells, epiblast, BMP4, spermatogenesis

PGC development *in vivo*, its specification, reprogramming and meiosis

In the mouse, the major model organism of mammals, PGCs are specified in the post-implantation embryo by signaling molecules, in contrast to other experimental organisms, in which a germ cell determinant complex preexists in the ooplasm [7,14]. PGC fate is induced in the pluripotent epiblast by bone morphogenetic protein 4 (BMP4), a secreted molecule, from the adjacent extraembryonic ectoderm [17]. Around embryonic day (E) 6.25 about six cells of the posterior proximal epiblast start to express *Prdm1/Blimp1*, a zinc finger transcriptional repressor, and these *Prdm1/Blimp1*-positive cells are lineage-restricted to become PGCs [26]. Prdm1/Blimp1 is a master regulator for PGC specification, as PGCs in *Prdm1/Blimp1*-deficient embryos impair repression of somatic cell genes, thereby resulting in severe disruption of PGC development at the early stage. This study suggests that Prdm1/Blimp1 endows epiblast cells to PGCs by repressing somatic gene expression program; however, direct target(s) of the transcription repressor remain unclear. Following *Prdm1/Blimp1* expression, PGC-specific genes, such as *Prdm14*, *Nanos3*, *Dnd1*, *Tdrd5*, and *Stella* etc. start to be expressed in PGCs [16,41]. At the specification, PGCs are located at the posterior end of the primitive streak, and then start to migrate along the developing hindgut

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towards the genital ridges, the future testes or ovaries.

When epiblast cells give rise to PGCs, their cellular status transforms from a pluripotent epiblast state to a unipotent PGC state: it appears that PGCs soon after the specification commit to unipotent germ cell lineage that is designated to either the oocytes or the spermatozoa. It has been an enigma why PGCs keep unipotency despite of gene expression involved in pluripotency, such as *Oct4*, *Nanog* and *Sox2*. On the other hand, it is known that PGCs cultured under specific conditions reacquire pluripotency by transforming into pluripotent embryonic germ (EG) cells [22,28]. To address how PGCs switch their cell state, it seems important to reveal in detail the function of key pluripotent transcription factors, such as Oct4, Sox2 and Nanog, as well as germ cell specific transcription factors not expressed in the pluripotent cells.

While migrating towards the genital ridges, epigenetic marks, such as histone modification and DNA methylation, are extensively reorganized in the PGC genome (epigenetic reprogramming). Global changes in histone modifications, such as a decrease in histone 3 lysine 9 dimethylation (H3K9me2) and an increase in histone 3 lysine 27 trimethylation (H3K27me3), occur specifically in the PGC genome [9,32,33]. Although the biological significance of the global changes of histone modification is largely unclear, it is feasible that the changes are necessary for acquisition of totipotency at the terminal products. A clue is served from *Prdm14*-deficient embryos in which H3K9me2 levels remain high and H3K27me3 levels stay low [42]. Of interest, *Prdm14*-deficient PGCs are incapable of giving rise to pluripotent EG cells, suggesting that the global changes of histone modification (and maybe also Prdm14 itself) provide a permissive epigenetic environment enabling cells to maintain their potential pluripotency [42].

An accessible and reliable hallmark of epigenetic reprogramming is X-chromosome reactivation in female PGCs. In general, one of two X-chromosomes in the female embryo is inactivated, so that amount of gene transcript from X-chromosome is equivalent to the male embryo. The exception is PGCs, in which the inactive X-chromosome starts to reactivate [4,5,35]. The non-coding RNA *Xist*, an functional marker of X-chromosome inactivation, is downregulated in some PGC precursors as early as E7.0, but only at E10.5 have the majority of PGCs lost *Xist* [35]. Disappearance of *Xist* is followed by a gradual loss of H3K27me3 enrichment on the inactive X-chromosome [5], and instead an increase in a global level of H3K27me3. Molecular mechanisms underlying X chromosome reactivation are largely unclear. Recent reports raise the possibility that X-chromosome reactivation is possibly a consequence of the expression of pluripotency genes. For example, *Xist* is repressed by Oct4, Sox2 and Nanog in embryonic stem (ES) cells [23], indicating that *Xist* is downregulated in PGCs by the transcription factors expressed.

Besides the global change of the histone modification, genome wide DNA demethylation also occurs specifically in PGCs. Along the genome-wide DNA demethylation, parental state of genomic imprinting is erased. It is apparent that DNA demethylation, specifically erasure of genomic imprinting, is required for acquisition of totipotency, as incomplete erasure of genomic imprinting causes developmental disorders [1]. Much attention has been paid to understand how such genome wide DNA demethylation is achieved. In general, there are two pathways of DNA demethylation, passive and active pathways. The former is a replication-dependent manner, in which 5-methylcytosine (5mC) is diluted in the absence or under the prevention of maintenance DNA methyltransferase (Dnmt1) or/and its associated molecules, such as Uhrf1 [34,39]. The latter involves enzymes that directly modify and/or remove 5mC and of which the mechanism has not been fully elucidated [29]. Recently it has been proposed that DNA demethylation is an active process occurring through base-excision repair, which may in turn trigger the extensive chromatin changes of PGCs in the genital ridge [9,10]. This study also suggests the possibility that Tet-family proteins, which catalyze the conversion of 5-methylcytosine to 5-hydroxymethylcytosine, play a role in genome-wide DNA demethylation in PGCs. This study, however, does not exclude a possibility that passive DNA demethylation is involved in the genome wide demethylation. Further analysis will provide insights into the molecular mechanism.

At E12.5, PGCs differentiate in a sex-dependent manner. PGCs in the female embryo undergo meiosis, whereas those in the male embryo start to arrest cell cycle at G1. The sex-dependent differentiation of PGCs is triggered by somatic cells of mesonephros and gonad. In female embryos, somatic cells in mesonephros

produce retinoic acid that induces meiosis in PGCs through a specific gene expression such as *Stra8* [2,15]. PGCs entering meiosis, then pass through leptotene, zygotene and pachytene, and finally arrest at diplotene at the birth. Some primordial follicles start to grow at puberty. In male embryos, PGCs arrest at G1 phase, called gonocytes, from about E13.5 to birth. After birth, gonocytes attach to the basement membrane of seminiferous tubules and then resume cell cycle to proliferate [27]. Some gonocytes differentiate into spermatogonial stem cells that produce spermatozoa throughout life.

Reconstitution of PGC specification *in vitro* development *in vivo*

As described above, there are a number of issues in PGC development that remain to be addressed. The main reason for this is the limited number of nascent PGCs, where the specification and the early process of epigenetic reprogramming occur. To address the biological significance of the PGC-specific gene expression signature and epigenetic reprogramming, a culture system to generate a large number of PGCs *in vitro* would be required for molecular and biochemical analysis. So far, several attempts have been made to generate germ cells from mouse ES cells [8,13,24,36]. These methods are mostly based on undirected differentiation of ES cells as monolayer culture or embryoid bodies and selection of rare germ cell-like cells. However, the ideal *in vitro* system would induce germ cell fate rather than select for randomly differentiated cells and it would recapitulate the earliest stages of PGC specification in an ordered manner. Most importantly, PGCs produced *in vitro* must be functional, which would otherwise essentially remain unclear whether PGC specification and epigenetic reprogramming *in vitro* recapture properly those *in vivo*. The gold standard to evaluate the function of germ cells is whether they give rise to healthy offspring. The studies mentioned above did not succeed in generating such fertile gamete [8,13,24,36].

Recently, we developed a robust culture system using ES cells to produce a large number of PGC-like cells (PGCLCs), of which the potential is almost equivalent to nascent PGCs *in vivo* [12]. Notably, the culture system recaptures the developmental process *in vivo*, as ES cells differentiate, by culturing with Activin A and basic fibroblast growth factor (bFGF), into epiblast-like cells (EpiLCs) and then EpiLCs differentiate into PGCLCs in response to BMP4. Based on the criterion of gene expression, EpiLCs mirror epiblast cells *in vivo*. Considering that ES cells mimic to some extent the inner cell mass (ICM) of preimplantaion blastocysts, it can be interpreted that the culture system recapitulates *in vitro* the differentiation process from ICM to postimplantation epiblast. Under a culture condition with a set of cytokines, which can produce functional PGCs from epiblast *ex vivo*, PGCLCs were induced at an efficient rate (more than 40%). This PGCLC induction strictly depends on BMP4, as withdrawal of BMP4 from the culture totally abolished the induction, faithfully recapitulating PGC specification *in vivo*. Characteristics of PGCLCs are indistinguishable from those of PGCs *in vivo*, based on criteria of gene expression, epigenetic status and potential to differentiate into fertile sperm. Microarray analyses showed that the transcriptome in PGCLCs mirrors that of early PGCs *in vivo*. Interestingly, PGCLCs express properly genes characteristic of early PGC development, but not marker genes expressed at late PGC development, such as *Mvh* and *Dazl*, as well as at meiotic-specific genes, such as *Stra8* and *Sycp3*. This indicates that under the culture condition PGCLCs arrest their development at a stage corresponding to E8–9.5 PGCs *in vivo*. At E8–9.5, PGCs *in vivo* arrest cell cycle at G2 phase and hardly proliferate while migrating in hind gut endoderm. Consistently, PGCLCs arrest their cell cycle at G2 phase and the number of PGCLCs is only slightly increased. These results indicate that additional (and still uncharacterized) growth factor(s) are required for exit of the G2 arrest and for further proliferation. Epigenetic reprogramming in PGCLCs occurs at a similar manner of that in PGCs *in vivo*, as a decrease in histone 3 lysine 9 dimethylation (H3K9me2) and an increase in histone 3 lysine 27 trimethylation (H3K27me3) were observed in the PGCLC genome. Most importantly, when PGCLCs were transferred into testis of germ cell-less W/Wv males, PGCLCs gave rise to fertile spermatozoa. Fertilized eggs with the spermatozoa developed fully to healthy offspring with the normal size of placentas. The offspring, both male and female, grew normally and had the ability to bear the next generation. These results clearly demonstrate that PGCLCs are properly derived from EpiLCs in the culture system. Taken together, the culture system reconstitutes

developmental processes from ICM to PGCs, which will contribute to address many issues that remain unclear due to the limitation of the material.

Questions to be addressed by the *in vitro* culture system

The culture system allows us to address several key questions to understand nature of PGC development. One would be molecular mechanism(s) of how BMP4 induces PGC fate in the epiblast. BMP signaling is transduced by Smad proteins. Once BMP binds to its receptors, Smad1, 5 and 8, which belong to receptor-regulated Smads (R-Smads), are activated through their serine-phosphorylation, bind to Smad4, the common mediator Smad (co-Smad), localize into nuclei and then act as a transcriptional factor. Consistently, it has been shown that disruption of *Smad1* and *5*, but not *Smad8*, genes result in attenuation of PGC specification [3,11,37]. Therefore, it would be very useful to identify the target genes of the R-Smad proteins to understand the molecular mechanism of BMP4 signaling on PGC specification. EpiLCs are suitable for ChIP analysis for isolating the target genes, since millions of EpiLCs can be easily prepared. Likewise, isolating target gene(s) of *Prdm1/Blimp1* is also of particular interest. Prdm1/Blimp1 is known as a master transcription repressor that directs terminally differentiated B-lymphocytes to antibody-secreting plasma cells [38]. It is feasible that this transcription repressor orchestrates PGC differentiation from pluripotent epiblast. Therefore, to reveal target genes of Prdm1/Blimp1 will be important to illustrate the gene expression network controlling PGC specification. Apart from Smad1 and Prdm1/Blimp1, other key transcription factors such as Oct4, Nanog and Sox2 are also worth investigating further to understand why PGCs are unipotent despite of pluripotent gene expression. Furthermore, to draw an accurate epigenome landscape of PGCs will be important to broaden our understanding of the contribution of a chracteristic pattern of histone modifications to PGC development. Specifically, comparison of the landscape between PGCs and pluripotent ES or/and EG cells will help to understand epigenetic nature of unipotency with potential pluripotency, which is observed in developing PGCs.

As described above, PGCLCs arrest their cell cycle at G2 phase, indicating that the growth factors in the culture medium are not sufficient to support further proliferation and differentiation. Considering PGC development *in vivo*, PGCs exit the G2 arrest after E9 and enter into genital ridge while proliferating. It is possible that somatic cells surrounding PGCs produce factor(s) supporting PGC proliferation and differentiation. Alternatively, biological activity of the recombinant proteins used in the culture is not as potent as those *in vivo*. Stem cell factor (SCF), a ligand of c-kit, is known to support proliferation of PGCs. However, the biological activity of SCF depends on its form; membrane-bound SCF has higher activity in supporting PGC proliferation than a secreted form of SCF [19,21]. Since soluble SCF is used in the culture system, its activity might not be enough to support proliferation of PGCLCs. As described above, PGCLCs do not express later PGC marker genes, such as *Mvh* and *Dazl*, and meiotic marker, *Sycp3*. It is reported that these genes start to be expressed according to DNA demethylation in PGCs [18]. These observations indicate that PGCLCs do not complete DNA demethylation, a hallmark of epigenetic reprogramming. To find factor(s) or condition(s) supporting proliferation and differentiation it is important to develop a culture system that supports the exit of G2 arrest, later marker gene expression, epigenetic reprogramming and meiosis entry.

Gametogenesis in vitro from PGCLCs

A goal of developing the culture system is to reconstitute *in vitro* the entire process of germ cell development. Although PGCLCs are fully potent, successful differentiation into spermatozoa still depends on environmental cues from the testis *in vivo*. Although it seems impossible to reconstitute all the environmental cues *in vitro*, a recent study presented an *ex vivo* culture system, in which the entire process of spermatogenesis was reproduced in a piece of neonatal testis cultured on an agarose block [30]. Mature spermatozoa obtained by this *ex vivo* culture method were functional, and could fertilize eggs that developed normally into healthy offspring. Furthermore, it was shown that germline stem cells, spermatogonial stem cells that proliferate indefinitely *in vitro* while keeping spermatogenic potential, gave rise to haploid cells by transplantation into W/Wv or busulfan-treated testis, followed by the *ex vivo* culture [31]. The *ex vivo* culture is therefore a bypass through which PGCLCs give rise to mature

spermatozoa in culture. This is a first step towards reconstituting the entire process of germ cell development.

Besides spermatogenesis, reconstitution of oogenesis *in vitro* from PGCLCs is also of particular importance. To date there is no defined stem cell population in oogenesis. However, it has been reported that reconstituted ovaries, which are reaggregates of dissociated fetal gonads containing PGCs, are able to produce fertile oocytes, when transplanted into the kidney capsule [20]. This suggests in theory that PGCLCs could possibly give rise to fertile oocytes in reconstituted ovaries as well. Although apart from nuclear transfer transplantation is the only method so far to obtain mature oocytes from PGCs, it is known that neonatal oocytes in primordial follicles can differentiate into mature oocytes under certain culture conditions *in vitro* [6,25]. In this culture system, however, efficiency of successful maturation to fully potent oocytes is relatively low, suggesting that the culture conditions are suboptimal even for isolated primordial follicles. Refinement of culture conditions using primordial follicles may precede establishment of a culture system using PGCLCs.

Application of the *in vitro* culture system to human ES cells

Successful production of functional PGCs from pluripotent stem cells may lead to an idea of applications to human reproductive medicine. Without mentioning the ethical issues, there are, however, technical obstacles to the direct application of our developed culture system to human PGC production from human ES cells. First of all, human and mouse ES cells are essentially different in responsiveness to growth factors. For example, bFGF promotes self-renewal of human ES cells, whereas it promotes differentiation of mouse ES cells to EpiLCs. Likewise, ActivinA/Nodal also promotes self-renewal of human ESCs through upregulation of *Nanog* expression [40], whereas that signal promotes differentiation of ESCs into EpiLCs with downregulation of *Nanog*. Furthermore, mouse ES cells can self-renew stably under a serum- and feeder-free condition, whereas this condition has not been firmly established in human ES cell culture. It is known that mouse ES cells cultured with pharmacological inhibitors of MEK and GSK3β remain in the ground state [43], which allows the ES cells to differentiate homogenously in response to a set of growth factors. In contrast, a culture condition placing human ES cells in the ground state has not yet been identified. Finding such a culture condition may be necessary for producing a large number of PGCLCs from human ES cells. Most importantly, there is no tool to evaluate whether human PGCLCs from human ES cells are functional. This obstacle may be overcome in part by using primate ES cells.

Concluding remarks

Here, we discussed several aspects of PGC development *in vivo* and *in vitro*, as well as possible future applications. PGCs are the sole lineage that acquires totipotency, by definition self-contained entities that can give rise to the whole organism. Totipotency is essentially different from pluripotency in the sense that pluripotency is a capability of differentiating into all cell lineages that compose the body, but do not support the formation whole organism formation (including the placenta). How germ cells acquire totipotency is the everlasting question for researchers concerning reproductive biology. We think that the culture system that we have successfully developed will contribute to provide new theoretical and empirical *insights* into the nature of totipotency.

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