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REVIEW ARTICLE

Perspectives of germ cell development *in vitro* in mammals

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ABSTRACT

Pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are able to differentiate into all cell lineages of the embryo proper, including germ cells. This pluripotent property has a huge impact on the fields of regenerative medicine, developmental biology and reproductive engineering. Establishing the germ cell lineage from ESCs/iPSCs is the key biological subject, since it would contribute not only to dissection of the biological processes of germ cell development but also to production of unlimited numbers of functional gametes *in vitro*. Toward this goal, we recently established a culture system that induces functional mouse primordial germ cells (PGCs), precursors of all germ cells, from mouse ESCs/iPSCs. The successful *in vitro* production of PGCs arose from the study of pluripotent cell state, the signals inducing PGCs and the technology of transplantation. However, there are many obstacles to be overcome for the robust generation of mature gametes or for application of the culture system to other species, including humans and livestock. In this review, we discuss the requirements for a culture system to generate the germ cell lineage from ESCs/iPSCs.

Key words: *gametogenesis, pluripotent stem cell, primordial germ cell.*

INTRODUCTION

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are invaluable sources for the production of gametes *in vitro*, as they can proliferate indefinitely while maintaining the ability to differentiate into both germ cells and somatic cells. A number of culture systems have so far been tested for use in the differentiation of ESCs/iPSCs into the germ cell lineage (Hubner *et al*. 2003; Toyooka *et al*. 2003; Geijsen *et al*. 2004; Nayernia *et al*. 2006). However, it has not been possible to establish a robust culture system that produces an unlimited number of germ cells. The methods tested so far are mostly based on random differentiation induced by either monolayer culture or embryoid bodies without factor(s) involved in pluripotency, followed by collection of a rare population of germ cells. However, such culture systems are not sufficient for mainly two reasons: the ideal *in vitro* system would induce a robust number of germ cells, and it would need to recapitulate the germ cell development *in vivo*. The latter criterion is particularly important, since such a culture system would serve as a useful platform to elucidate the mechanisms underlying germ cell development. Also, the more precise the *in vitro* recapitulation of germ cell development is, the more *in vitro*-derived germ cells will be identical to germ cells *in vivo*.

To establish the ideal culture system, several key points must be considered: (i) how to differentiate the germ cell lineage *in vivo*; (ii) what the state of pluripotent stem cells is; and (iii) how to produce and validate *in vitro*-derived germ cells. As described in detail below, germ cell development is highly orchestrated by a unique set of genetic and epigenetic regulations, many of which remain to be investigated (McLaren & Lawson 2005; Sasaki & Matsui 2008; Saitou & Yamaji 2010). Also, it has recently been revealed that pluripotent stem cells are not self-renewing in the sense that a parental cell divides into two identical daughter cells (Furusawa *et al*. 2004;

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Chambers *et al*. 2007; Hayashi *et al*. 2008; Toyooka *et al*. 2008). Rather, they are cell populations which fluctuate between different pluripotent states. The exact counterpart of pluripotent stem cells in the embryo *in vivo* should be considered to recapitulate precise differentiation. Regarding validation, the *in vitro*-derived germ cells should exhibit appropriate expression levels of germ cell markers and epigenetic reprogramming events. Most importantly, *in vitro*-derived germ cells need to demonstrate their potential to contribute to spermatogenesis, oogenesis and finally fertility to produce offspring. This article provides an overview of germ cell development *in vivo* and *in vitro* with perspectives on future development of the culture system and its application to other mammals.

GERM CELL DEVELOPMENT IN MAMMALS

To acquire totipotency, the potential to differentiate into cells of any type, including placental cells, the germ cell lineage passes through a unique series of developmental processes. The process can be divided into at least three phases: primordial germ cell (PGC) specification, sex determination and gametogenesis. All germ cell lineages originate from PGCs, which are segregated from the somatic cell lineage at an early developmental stage. (McLaren & Lawson 2005; Sasaki & Matsui 2008; Saitou & Yamaji 2010) In mammals, it is thought that PGCs are induced from pluripotent cells in the post-implantation embryo by environmental cues such as extrinsic signaling molecules (Extavour & Akam 2003). Specifically, in mice, PGCs arise from the post-implantation epiblast at embryonic day (E) 6 in response to bone morphogenetic protein (BMP) 4 secreted from the neighboring extra-embryonic ectoderm. (Lawson *et al*. 1999) A characteristic gene expression program appending genome-wide epigenetic change is observed in epiblast cells heading to PGCs. First, they express somatic mesodermal genes such as *T*, *Hoxa1* and *Hoxb1*. However, around E6.25, the cells start to express a set of transcription factors involved in PGC specification, such as Blimp1/Prdm1, Prdm14 and Tfap2c. Blimp1 is a zinc finger transcriptional repressor that plays central roles in the cell fate decision of, for example, lymphocyte differentiation (Turner *et al*. 1994; Shapiro-Shelef *et al*. 2003; Kallies *et al*. 2006; Martins *et al*. 2006), sebaceous gland (Horsley *et al*. 2006) and intestinal maturation (Harper *et al*. 2011). In germ cell development, Blimp1 is a master regulator for PGC specification (Ohinata *et al*. 2005; Vincent *et al*. 2005). Genetic analysis showed that *Blimp1*-deficient PGCs fail to repress somatic cell genes, thereby resulting in severe disruption of PGC development at the early stage (Kurimoto *et al*. 2008). *Prdm14*-deficient embryos also showed defective PGC specification with impaired *Sox2* expression and aberrant histone modification at a genome-wide level, although a detail of Prdm14 function on PGC specification remains elusive (Yamaji *et al*. 2008). *Tfap2c*-deficient embryos lose PGCs by E8, possibly due to delayed expression of *Blimp1*, suggesting that Tfap2c is a positive regulator of *Blimp1*. Soon after specification, mouse PGCs start to migrate, while proliferating, along the hindgut endoderm toward the genital ridge that in turn forms either the ovaries or testes. (Sasaki & Matsui 2008; Ewen & Koopman 2010; Saitou & Yamaji 2010) While migrating, PGCs show extensive and dynamic change of epigenetic modifications on the genome. The methylation of CpG DNA decreases gradually from an initial level of 70% of CpGs to final levels of 14% and 7% of CpGs in E13.5 male gonocytes and female oogonia, respectively (Seisenberger *et al*. 2012). In keeping with the genome-wide demethylation, CpG methylation in the differentially methylated region of imprinting gene loci is also erased in both males and females by E13.5 (Hajkova *et al*. 2002; Sato *et al*. 2003; Kagiwada *et al*. 2013). Apart from the genome-wide DNA demethylation, global changes in histone modifications, such as a decrease in histone H3 lysine 9 dimethylation (H3K9me2) and an increase in histone H3 lysine 27 trimethylation (H3K27me3), also occur in the PGC genome (Seki *et al*. 2005, 2007; Hajkova *et al*. 2008). Although the biological significance of the global changes of histone modification remains unclear, it is likely that the changes are necessary for acquisition of totipotency at the terminal products.

When PGCs colonize in the genital ridge, they show their first sex-based difference: PGCs in females continue to proliferate to become oogonia and then enter into meiosis to become primary oocytes at around E13.5, whereas in males, after substantial proliferation PGCs enter into mitotic arrest to become gonocytes from around E15.5. (Hilscher *et al*. 1974; Speed 1982). A putative factor that triggers the onset of meiosis in oogonia is retinoic acid (RA), which is produced in the mesonephros adjacent to the gonad (Bowles *et al*. 2006; Koubova *et al*. 2006). Oogonia exposed to RA start to express meiosis-specific genes such as *Stra8* and then enter meiosis. Although both male and female mesonephros produce RA, male gonadal somatic cells express Cyp26b, the RA-metabolizing enzyme, which prevents meiotic induction in the gonocytes. Sex determination of somatic cells precedes that of germ cells, as male gonadal somatic cells begin to express the sex-determinant gene *Sry* at around E11.0 (Albrecht & Eicher 2001; Bullejos & Koopman 2001). Male gonadal somatic cells that had expressed *Sry* eventually differentiate into fetal Sertoli cell lineage, whereas their female counterparts differentiate into the granulosa cell lineage. These sex-specific granulosa and Sertoli cell lineages play an essential role in the

subsequent gametogenesis. In the perinatal period, the primary oocyte and simple squamous pre-granulosa cells form the primordial follicle (Edson *et al*. 2009). In mice, follicular development begins shortly after birth, while most of the primordial follicles are dormant until puberty. In contrast, male gonocytes attach to Sertoli cells and locate to the basement membrane of seminiferous tubules after birth and then resume the cell cycle to proliferate and become spermatogonia (Phillips *et al*. 2010). Some spermatogonia are set as spermatogonial stem cells that produce spermatozoa continuously throughout life. Sertoli cells establish a microenvironment for the maintenance of spermatogonial stem cells by secreting glial cell-derived growth factor (GDNF) (Parvinen & Ventela 1999).

Gametogenesis is the process that completes meiosis and morphological change, resulting in the production of fertile eggs and sperm. The details of gametogenesis – and the accumulating knowledge of the mechanisms underlying it – have been described elsewhere in a number of excellent articles.

DISTINCT PLURIPOTENT STATE OF ESCS CORRESPONDING TO DEVELOPMENTAL STAGE

Since it is evident that pluripotent stem cells, such as ESCs and iPSCs, are capable of differentiating into multiple cell lineages, including germ cells, it seems feasible that an optimal set of culture conditions could direct ESCs/iPSCs toward a germ cell lineage. As described above, in mice and possibly in other mammals, the germ cell lineage is derived from the pluripotent cell population in response to extrinsic signals. Therefore, cues from an optimal set of extrinsic signals are an important step in the induction of germ cell specification in ESCs/iPSCs. Evidence from genetic studies has uncovered the extrinsic signals essential for PGC specification, providing information on the extrinsic signals that should be used for the reconstitution. However, it is also evident that the optimal set of extrinsic signals is necessarily not sufficient for the reconstitution, as there are distinct types of pluripotent states with respect to the responsiveness to extrinsic signals; for example, BMP4, the central player in PGC specification, promotes self-renewal rather than differentiation into the germ cell lineage in mouse ESCs, which is in clear contrast to the pluripotent epiblast *in vivo*. Thus, reconstitution of a pluripotent state that can react properly to the signals is as essential as finding the optimal set of extrinsic signals. Recent studies have revealed the distinct pluripotent state and have shown progress in defining culture conditions that control the state *in vitro*.

In the mammalian embryo, pluripotency is established from the epiblast in the inner cell mass (ICM) of the preimplantation blastocyst (hereafter called the ICM to distinguish it from the post-implantation epiblast) (Selwood & Johnson 2006; Surani *et al*. 2007). This is a transient state, since the ICM soon transforms into epithelial cells of the post-implantation epiblast (hereafter simply the epiblast) that are ready to differentiate into various somatic cells or germ cells. ESCs established from the ICM remain in a pluripotent state while proliferating indefinitely (Evans & Kaufman 1981; Martin 1981), meaning that ESCs are not completely equivalent to the ICM. What, then, is the nearest *in vivo* counterpart of ESCs? In previous studies employing classic culture conditions, mouse ESCs were maintained with fetal calf serum (FCS) and leukemia inhibitory factor (LIF) on a feeder layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) (Smith *et al*. 1988; Williams *et al*. 1988). Under these conditions, interestingly, mouse ESCs are composed of heterogeneous cell populations: mouse ESCs express heterogeneously pluripotent-associated genes such as *Stella* (or *Dppa3*/*Pgc7*), *Zfp42*, *Pecam1*, *Nanog* and *Fgf5* (Hayashi *et al*. 2008). The *Stella*-positive cell population is enriched in *Zfp42*, *Pecam1* and *Nanog* transcripts, whereas *Fgf5* expression is mutually exclusive to the other genes. It is known that these genes are developmentally regulated in pluripotent cells during early development: *Stella*, *Zfp42*, *Pecam1* and *Nanog* are preferentially expressed in the ICM, whereas *Fgf5* is expressed in the epiblast. Transcriptome analysis has shown that the *Stella*-positive cell population is enriched in ICM-associated genes, whereas the *Stella*-negative cell population is enriched in epiblast-associated genes. Interestingly, under culture conditions supporting the self-renewal of mESCs, these two sub-populations – the ICM-like and epiblast-like populations – were mutually interchangeable, suggesting that mESCs are in a metastable state that fluctuates between the ICM and epiblast. The metastable ESC state is affected by environmental cues, as almost all of the cells are pushed into an ICM-like state under chemically defined culture conditions with LIF and small-molecule inhibitors of the fibroblast growth factor 4 (FGF4)/mitogen-activated protein kinase (MAPK) pathway and of glycogen synthase kinase 3 (GSK3) (Ying *et al*. 2008). Under these conditions, known as 2i+LIF, FGF4/MAPK signaling is completely blocked, so that ESCs are unable to convert into an epiblast-like state from an ICM-like state. Moreover, LIF is not required for the self-renewal of ES cells in medium containing 2i and an FGF receptor inhibitor, a set of conditions termed 3i (Ying *et al*. 2008). Based on the dispensability of extrinsic cytokine signals, there is the ground state of ESCs that is maintained by an intrinsic program of self-renewal (Nichols & Smith 2009). Evidence that almost all of the E4.5 ICM can give rise to mESCs under the 2i+LIF conditions suggests that the properties of the ground state of mouse ESCs are closest to those of the E4.5 ICM (Nichols *et al*.

2009). This state has been referred to as the naïve pluripotent state.

Recently, a different type of pluripotent stem cells has been obtained by culturing the E5.5-E6.5 epiblast in medium containing activin A and basic fibroblast growth factor (bFGF) (Brons *et al*. 2007; Tesar *et al*. 2007). The post-implantation epiblast-derived pluripotent stem cells, called epiblast stem cells (EpiSCs), have significantly different properties, compared even to the epiblast-like population of mouse ESCs. EpiSCs seldom contribute to chimeras following blastocyst injection, although EpiSCs are capable of multi-lineage differentiation, as demonstrated by their ability to form teratomas when grafted into adult mice. EpiSCs have characteristic gene expression and epigenetic profiles, which are different from those in ESCs (Tesar *et al*. 2007; Hayashi & Surani 2009a). Despite expression of the pluripotency factors, EpiSCs are prone to differentiate under conditions that have been reported to sustain self-renewal (Brons *et al*. 2007; Tesar *et al*. 2007). For example, EpiSCs show a high degree of cellular heterogeneity and spontaneously differentiate into PGCs and somatic cell lineages such as endoderm (Hayashi & Surani 2009b). These properties of EpiSCs are partially reminiscent of the post-implantation epiblast. At E5.5-E6.5, epiblast cells are exposed to various differentiation-inducing factors that are secreted from surrounding tissues, such as the extra-embryonic ectoderm and visceral endoderm (Pfister *et al*. 2007). The pluripotent cell state with such a propensity to differentiate has been referred to as the primed pluripotent state (Nichols & Smith 2009).

Importantly, EpiSCs hardly differentiate into PGCs in response to BMP4 added into culture media, whereas epiblast cells *in vivo* do. Considering the fact that only the epiblast cells around E6 possess the ability to differentiate into PGCs in response to BMP4, an ability called PGC-competence, it is likely that the state of EpiSCs is more differentiated than the E6 epiblast. Indeed, it has been reported that EpiSCs have a gene expression pattern similar to that of the ectoderm cells of the late-gastrula-stage embryo, which no longer possess PGC-competence (Han *et al*. 2010; Kojima *et al*. 2014). As described above, BMP4 has no impact on PGC derivation from mESCs. Collectively, these observations suggest that the pluripotent state of the E6 epiblast with PGC-competence is between the naïve and primed pluripotent states in mice. Interestingly, recent reports have demonstrated that the naïve and primed pluripotent states are interchangeable; under the naïve pluripotent state mouse ESCs under EpiSC culture conditions are converted to primed pluripotent state EpiSCs (Guo *et al*. 2009), while primed pluripotent state EpiSCs revert to naïve pluripotent state mouse ESCs by the enforced expression of naïve pluripotent factors, Klf4 or Prdm14 and Klf2 (Guo *et al*. 2009; Gillich *et al*. 2012), or simply by culturing under mESC conditions (Bao *et al*. 2009). Given that the differentiation process mimics differentiation *in vivo* from ICM to epiblast, it is likely that ESCs acquire PGC-competence during conversion from the naïve to primed pluripotent state.

RECONSTITUTION OF PGC SPECIFICATION *IN VITRO*

Recently we developed a culture system in which the PGC specification processes are reconstituted *in vitro* by using mouse ESCs/iPSCs (Hayashi *et al*. 2011). In the development of this culture system, we focused on how to convert ESCs/iPSCs in the naïve state to an epiblast-like state with PGC-competence. For this purpose, we induced the transient differentiation of naïve ESCs/iPSCs under a defined set of conditions, including bFGF and activin A. Under these culture conditions, the mouse ESCs/iPSCs exhibited a rapid change in cell morphology; round colonies became flat, assuming a more epithelium-like structure. At various periods of culture, cells were dissociated and reaggregated with or without BMP4. This culture experiment brought a clear result that mouse ESCs/iPSCs at day 2 of culture with bFGF and activin A differentiate efficiently into PGC-like cells (PGCLCs) in response to BMP4. In the experiment, PGCLC differentiation was detected by either the expression of PGC-specific reporter genes, such as *Blimp1-mVenus* and *stella-CFP*, or the expression of PGC-specific surface proteins, such as SSEA1 and Itgβ3. The surface proteins facilitate the detection of PGCLCs without the reporter gene construct, and thereby render the culture system applicable to any type of mouse ESCs and perhaps to other mammals. The differentiation capacity changes with time, as cells at day 1 of culture did not differentiate into PGCLCs, and those at day 3 showed an attenuated capacity to do so. This is consistent with the fact that only the E6 epiblast possesses high PGC competence. We called a novel type of cell harboring PGC-competence epiblast-like cells (EpiLCs). The manner of differentiation of naïve mouse ESCs/iPSCs to EpiLCs is highly similar, if not identical, to that from the ICM to epiblast *in vivo*, based on the criteria of gene expression and epigenetic status. Interestingly, the gene expression pattern of EpiLCs is indeed distinct from that of EpiSCs, illustrating the substantial difference in PGC competence between the pluripotent cell states.

The manner of differentiation from EpiLCs to PGCLCs is also highly similar to that in PGC specification *in vivo*; the differentiating cells start to express a critical set of PGC-specific genes that includes *Blimp1*, *Prdm14* and *Tfap2c*, and repress somatic genes such as *Hoxa1* and *Hoxb1*. In addition to having a similar pattern of gene expression, the genome-wide reorganization of epigenetic modification in PGCLCs

occurs in a manner similar to that of PGCs *in vivo*, as a decrease in H3K9me2 and an increase in H3K27me3 were observed in the PGCLC genome. Collectively, these results demonstrated that the manner of differentiation from naïve mouse ESCs/iPSCs to PGCLCs via EpiLCs well recapitulated that from the ICM to PGCs *in vivo*, which should contribute to dissection of the mechanisms underlying PGC specification, which have been inaccessible so far due to the limited number of early embryonic materials, such as epiblast and nascent PGCs.

SPERM AND OOCYTE PRODUCTION FROM PLURIPOTENT STEM CELLS

Needless to say, the most rigorous functional validation of PGCLCs is to test their potential to contribute to gametogenesis and fertility of offspring. So far no study has reported healthy and fertile offspring derived from mouse ESC/iPSC-derived PGCs. Whether PGCLCs have the potential to undergo spermatogenesis can be determined by transplantation into seminiferous tubules, based on a previous study showing that E8.5 PGCs transplanted into seminiferous tubules contribute to spermatogenesis. According to that study, PGCLCs derived from male mouse ESCs were transplanted into the seminiferous tubules of neonatal *W/Wv* males who do not have their own germ cells, resulting in the contribution of PGCLCs to spermatogenesis (Hayashi *et al*. 2011). Mature spermatozoa yielded from the testes were functional, as fertilized eggs with the spermatozoa developed fully to healthy offspring with normal-size placentas. The offspring, both male and female, grew normally and had the ability to bear the next generation.

Similar to PGCLCs derived from ESCs, those from iPSCs are also capable of differentiating into fully functional spermatozoa that eventually give rise to healthy and fertile offspring. However, it appears that PGCLCs from two out of three iPSC lines, for which the germline transmission through chimera mice has been proven, were aberrant, as they did not contribute to spermatogenesis, but rather formed teratomas in the transplanted testes. In contrast, all three ESC lines tested gave rise to fully potent PGCLCs. This suggests that the capacity of iPSCs for differentiating into PGCLCs is different in each iPS line, consistent with previous reports showing different properties of each iPSC line (Miura *et al*. 2009). It is unclear whether the limited capability for PGCLC differentiation is caused by genetic or epigenetic alteration. Nevertheless, it is important on a practical level to choose iPSCs that have a good propensity to differentiate into PGCLCs.

As described above, PGCLCs are potent to contribute properly to spermatogenesis. It is also demonstrated that PGCLCs derived from female mouse ESCs/iPSCs give rise to oocytes by reaggregation with E12.5 female gonadal somatic cells followed by transplantation into the ovarian bursa of immunocompromised adult mice (Hayashi *et al*. 2012). Oocytes derived from PGCLCs give rise to healthy and fertile offspring after *in vitro* maturation and *in vitro* fertilization, followed by transplantation to surrogate mothers. This result is well consistent with a previous study showing that E12.5 gonads that were dissociated, reaggregated and transplanted into the kidney capsule produced functional oocytes. However, PGCLC-derived oocytes were not fully equivalent to PGC-derived oocytes, as the percentages of normal fertilization and of full-term development were lower in PGCLC-derived oocytes than in PGC-derived oocytes. Specifically, it was evident that almost half of fertilized eggs possess three pronuclei. In almost all cases, two out of three pronuclei were of maternal origin, suggesting that PGCLC-derived oocytes have a defect in extrusion of the second polar body (Hayashi *et al*. 2012).

TOWARD GAMETOGENESIS *IN VITRO*

Although PGCLCs fulfill the gold standard of germ cells in both males and females, one goal in developing the culture system is to reconstitute *in vitro* the entire process of germ cell development. So far, successful differentiation of PGCLCs into spermatozoa and oocytes depends on environmental cues from the testis and ovary, respectively, *in vivo*. Reconstitution *in vitro* of gametogenes has been an attractive issue for many decades. There have been many attempts to reconstitute spermatogenesis in organ culture or cell culture with specific devices (Steinberger *et al*. 1964; Rassoulzadegan *et al*. 1993; Staub *et al*. 2000; Feng *et al*. 2002). Despite a long and concerted effort, no robust and reproducible culture system has been developed for producing functional spermatozoa. Recently, Ogawa and colleagues established an *ex vivo* culture system, in which the entire process of spermatogenesis could be reproduced in a piece of neonatal testis cultured on an agarose block (Sato *et al*. 2011a). Mature spermatozoa obtained by the *ex vivo* culture method were functional, and could fertilize eggs that developed normally into healthy offspring. Furthermore, the group showed that germline stem cells, spermatogonial stem cells that proliferate indefinitely *in vitro* while maintaining their spermatogenic potential, gave rise to haploid cells by transplantation into *W/Wv* or busulfan-treated testis, followed by *ex vivo* culture (Sato *et al*. 2011b). The *ex vivo* culture is therefore a possible pathway through which PGCLCs give rise to mature spermatozoa in culture.

Similar to the case of spermatogenesis, attempts to reconstitute oogenesis *in vitro* have been made for some time. A milestone study reported that primordial follicles of the neonatal ovary could be successfully

grown to mature oocytes. The *in vitro* growth oocytes are fertile and the resultant fertilized eggs developed to healthy offspring (Eppig & O'Brien 1996; O'Brien *et al*. 2003), although the low efficiency of offspring generation indicates that refinement of the culture conditions might be required. Several reports have identified potential growth factors involved in the growth and survival of primordial follicles, which might be used as culture additives for the *in vitro* growth of follicles (Parrott & Skinner 1999; Kezele *et al*. 2002; Nilsson & Skinner 2003; Spears *et al*. 2003; Lee *et al*. 2004). In addition, genetic analyses have revealed intrinsic and extrinsic factors important for proper follicle growth (Edson *et al*. 2009). In light of these findings, it will be important to rigorously refine the *in vitro* culture conditions that produce a robust number of mature oocytes from primordial follicles. To date, no report has succeeded in the production of functional oocytes from PGCs, except through the use of a nuclear transfer: Obata *et al*. succeeded in generating pups by transferring the nuclei of oocytes that were grown from PGCs by *in vitro* culture, into enucleated, fully grown oocytes from adult ovaries, followed by *in vitro* fertilization (Obata *et al*. 2002). How PGCs differentiate into primary oocytes and form primordial follicles with surrounding somatic cells is largely unknown. Therefore, basic analysis of the mechanisms underlying primordial follicle formation is prerequisite for establishment of a culture system using PGCLCs.

GERM CELL PRODUCTION *IN VITRO* IN OTHER MAMMALS

In the case of mice, the necessary conditions for successful production for functional PGCLCs from pluripotent stem cells can be consolidated as follows: (i) establishment of the ground state; (ii) knowledge of early germ cell development *in vivo*; (iii) growth factors for inducing EpiLCs and PGCs; and (iv) sophisticated transplantation methods to validate the functionality of PGCLCs. To apply the mouse PGCLC culture system to other mammals, the necessary conditions above should be taken into consideration.

First, it has been thought that human ESCs (hESCs) are in a primed pluripotent state, since they are similar to mouse EpiSCs with respect to culture conditions, colony morphology and vulnerability to single cell dissociation. Therefore, much effort has been expended to determine culture conditions under which hESCs can be maintained in naïve state. Two recent reports individually found a combination of small molecule inhibitors and growth factors that provides naïve state in hESCs (Chan *et al*. 2013; Gafni *et al*. 2013). Although the reported culture conditions were different, hESCs under any of the conditions were similar to those in naïve mESCs with respect to the colony morphology and the gene expression. hESCs under the defined culture condition may be considered as a starting material corresponding to naïve mouse ESCs in a PGCLC culture system.

Second, compared to the process of early embryogenesis in mice, the molecular mechanisms underlying early human embryogenesis, including PGC specification, have been less known, meaning that it is less informative to determine culture conditions for inducing human EpiLCs and PGCLCs. Seeking useful information, it was recently reported that Blimp1 and BMP-mediated signaling might be conserved in rabbit PGC specification. The morphological manner of rabbit early embryogenesis seems to resemble that of human embryogenesis: like human embryos, rabbit early post-implantation embryos have an embryonic disc, which is in contrast to the cup-shaped mouse epiblast. Moreover, the colony morphology of rabbit ESCs is similar to that of hESCs under traditional culture conditions. Rabbit embryogenesis may be a fruitful source for determining a method of PGCLC induction that can be adopted for use in other mammals.

Given that PGCLCs are induced from other mammalian ESCs, whether the PGCs are fully functional has to be tested. At the moment, transplantation is the only method to validate the functionality of PGCLCs. There are two methods of transplantation to obtain mature sperm: one is transplantation of testicular cells into the seminiferous tubules, and the other is xenotransplantation of testicular tissue. The former can be further divided into two categories: transplantation into homo/allogenetic seminiferous tubules or xenogenetic seminiferous tubules. Transplantation into homo/allogenetic seminiferous tubules has been successfully done in various species, such as mouse (Brinster & Avarbock 1994; Brinster & Zimmermann 1994), pig (Honaramooz *et al*. 2002), goat (Honaramooz *et al*. 2003), cattle (Izadyar *et al*. 2003) and monkey (Schlatt *et al*. 1999, 2002). Although sustainable spermatogenesis has not been rigorously tested in some species, these studies showed that colonization of donor-derived spermatogonial cells and subsequent spermatogenesis occurred in recipient seminiferous tubules. Successful transplantation of testicular cells into xenogenetic seminiferous tubules was reported in rodents: rat testicular cells completed spermatogenesis and produced functional mature sperm in mouse seminiferous tubules (Clouthier *et al*. 1996; Shinohara *et al*. 2006). However, application of the xenogenetic transplantation seems limited, as spermatogonial cells from other species, such as hamster, rabbit, pig, bull, primate and human, failed to complete spermatogenesis in mouse seminiferous tubules, although each species showed different degrees of incomplete spermatogenesis (Dobrinski *et al*. 1999, 2000; Ogawa *et al*. 1999;

Nagano *et al*. 2001, 2002). The incomplete spermatogenesis is thought to be caused by physiological incompatibility between germ cells and supporting cells. Collectively, transplantation of PGCLCs into homo/allogenetic seminiferous tubules, and perhaps a restricted type of xenogenetic seminiferous tubules, may be an option to validate PGCLCs of mammals other than mice. Alternatively, transplantation of a piece of testicular tissue into immunocompromised mice is a considerable option to validate the functionality of PGCLCs, since such transplantations have been successfully done in several species (Rodriguez-Sosa & Dobrinski 2009). However, it is necessary to prepare not only PGCLCs but also testicular somatic tissues that support spermatogenesis in the transplanted mouse. Such testicular somatic tissues can be prepared from embryonic or adult tissues or can be substituted, especially in the case of human, by *in vitro* differentiation from pluripotent stem cells. Recently it was reported that Sertoli cells were differentiated directly from fibroblasts by defined factors (Buganim *et al*. 2012). Accumulating knowledge about the differentiation of testicular tissues may make it possible to reconstitute testicular somatic cells sufficiently to support the spermatogenesis of PGCLCs.

Validation of the functionality of female PGCLCs in mammals other than mice seems more difficult than that of males. The methodology of transplantation has made less progress in female reproductive organs. There has been no significant report on the transplantation of PGCs or primary oocytes alone into ovarian tissue: such transplantations essentially require supporting somatic tissue that eventually differentiates into granulosa and theca calls. Regarding xenotransplantation, several reports have attempted to transplant ovarian tissues from non-rodents into immunocompromised mice (Bols *et al*. 2010), although further improvements will be needed for a robust system to produce oocytes by transplantation. Basic studies on the methodology for producing oocytes would seem to be required to achieve this goal. Alternatively, it would be feasible to establish an *in vitro* culture system that reconstitutes the entire process of oogenesis. As described above, it is demonstrated that the neonatal primordial follicles matured *in vitro* to functional oocytes (Eppig & O'Brien 1996; O'Brien *et al*. 2003). Therefore, according to accumulation of basic studies, it may be possible to establish a culture system that produces oocytes from immature types of cells, such as primary oocytes and perhaps PGCs.

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