—Mini Review—

Generation of Oocytes from Mouse ES/iPS Cells

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Abstract: One of the paramount goals in reproductive biology is to produce functional oocytes in culture through a series of differentiation processes that accurately mimic those *in vivo*. Such a culture system would provide a larger number of oocytes than those available *in vivo*, which would be of help in the elucidation of mechanisms underlying germ cell development. All germ cells originate from a small group of primordial germ cells (PGCs) that segregate from somatic cell lineages at an early developmental stage. Recently, we developed a culture system in which embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) differentiate into PGC-like cells (PGCLCs) through an epiblast-like cell population. PGCLCs are capable of differentiating into functional oocytes when transplanted into the ovarian bursa with E12.5 gonadal somatic cells. In this review, we introduce our differentiation method and discuss possible applications of this culture system.

Key words: ES cells, IPS cells, Primordial germ cells, Oogenesis

Introduction

The germ cell lineage is highly specialized for the transmission of genetic and epigenetic information to the next generation, and thus plays a major role in perpetuating species. To acquire totipotency, the germ cell lineage undergoes a unique series of developmental processes orchestrated by genetic and epigenetic regulations [1–3]. Accordingly, error (s) in the process can cause infertility and/or developmental disorders. Therefore, a rigorous understanding of how the germ cell lineage differentiates and finally acquires totipotency is of particular importance not only for reproductive and developmental biology, but also for clinical diagnosis and applications. The developmental process of the germ cell lineage has been well characterized in mice, and accumulated knowledge is mainly based on genetic, histological and physiological analyses, as described below.

Mouse PGCs arise from the proximal part of the pluripotent epiblast at around embryonic day (E) 6.5, which is the approximate time of the differentiation accompanying gastrulation (Fig. 1). The initial PGC specification is triggered by BMP4 secreted from the extraembryonic ectoderm adjacent to the epiblast [4]. During specification, a set of transcription factors, such as Blimp1/Prdm1, Prdm14, and Tfap2c, evoke a PGC-specific gene program and simultaneously stop the somatic cell program [5]. Thereafter, expression of genes involved in pluripotency, such as Pou5f1 and Nanog, is sustained in PGCs but not in somatic cell lineages. This is interesting in the sense that the germ cell lineage segregates from the somatic cell lineage while substantially retaining the pluripotency of the epiblast, which may conceivably illustrate that they are protected from irreversible differentiation in order to retain their capacity to reacquire totipotency.

After the specification, PGCs start to migrate, while proliferating, along the definitive endoderm toward the genital ridge at around E8 to E9.5 (Fig. 1). While migrating, PGCs undergo a unique epigenetic reorganization at a genome-wide level. Most of the methylation on the CpG dinucleotide is removed from the genome: the methylation levels drop from 70–80% to around 14% in males and 7% in females by E13.5 [6]. In conjunction with the genome-wide DNA demethylation, differential methylation patterns on genomic imprinting loci are erased by E13.5 [7]. In parallel, the pattern of histone modification also changes in a genome-wide manner during this period. Typical pattern changes include a decrease in dimethylated histone H3 lysine 9 (H3K9me2) and an in-

Received: May 14, 2014
Accepted: July 3, 2014
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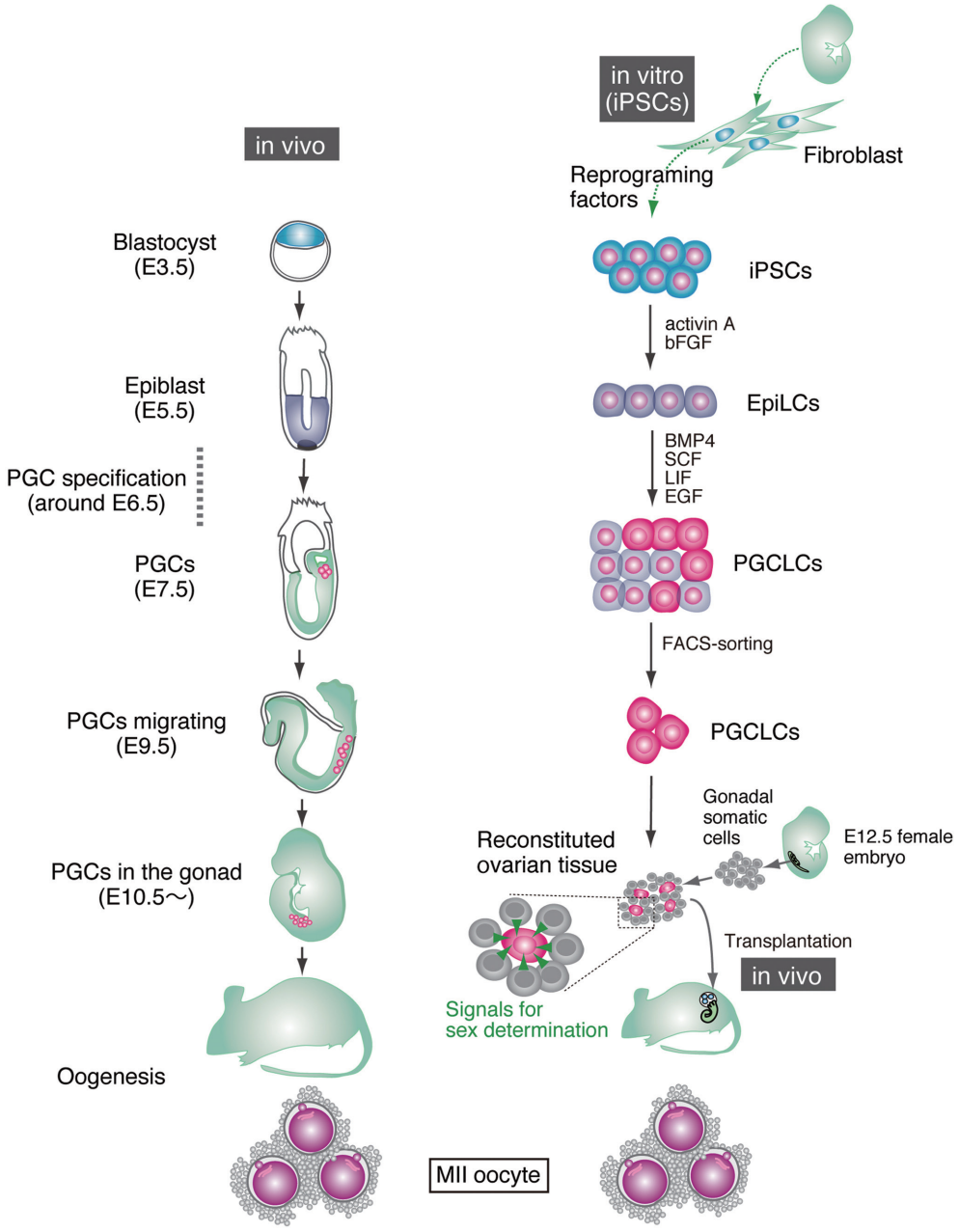

Fig. 1. Differentiation process of the germ cell lineage *in vivo* and *in vitro*
PGCs originate from the pluripotent epiblast, which is a descendant of pluripotent cells in the ICM of the blastocyst. PGCs migrate and settle in the gonads, the future ovary or testis, where sex-dependent differentiation is accomplished. In the ovary, oocytes are produced through a serial process of differentiation (oogenesis). The differentiation process from pluripotent cells to PGCs can be reconstituted *in vitro*. With an appropriate set of cytokines, iPSCs derived from fetal fibroblasts differentiate into EpiLCs and then PGCLCs. PGCLCs (depicted as pink cells) are aggregated with gonadal somatic cells, forming a structure called the reconstituted ovarian tissue, to support sex determination and subsequent oogenesis in recipient females. In the reconstituted ovarian tissue, oogenesis commences and produces mature oocytes, although a percentage of the oocytes show defects.

crease in trimethylated histone H3 lysine 27 (H3K27me3) [8]. Genome-wide DNA demethylation and histone modification change appear to contribute to the acquisition of totipotency. However, the biological significance of these epigenetic reorganizations remains elusive.

In the gonad, PGCs keep proliferating and then start to differentiate in a sex-dependent manner from E12.5 onward. The sex-dependent differentiation depends on the gonadal somatic cells, since male PGCs that are artificially aggregated with female gonadal somatic cells differentiate in a female manner and *vice versa* [9, 10]. In the female gonad, PGCs cease proliferating and enter meiosis, becoming primary oocytes, in response to factors such as retinoic acid [11, 12]. At this time, the primary oocytes form a germ cell nest, in which the oocytes connect to each other through intercellular bridges, and are surrounded with pregranulosa cells [13]. The number of primary oocytes peaks at this stage, since shortly after this, in the perinatal stage, there is a massive loss of primary oocytes to the formation of primordial follicles, in which single primary oocytes are surrounded by simple squamous granulosa cells. The primordial follicles are the source of oogenesis throughout life. Although quantitative control by cell death at the perinatal stage is thought to be involved in subsequent oogenesis [14, 15], it is not clear whether there is qualitative control at this stage, such as the elimination of primary oocytes with low potency from the pool. A portion of the primordial follicles begins oogenesis by largely undefined mechanisms. But, recent findings suggest that PTEN-related signaling and functional inhibition of Foxo3a are involved in the initiation of oogenesis from primordial follicles [16, 17]. Following oogenesis, the details of which have been described elsewhere in other excellent reviews [18, 19], fully grown oocytes resume meiosis to the metaphase II (MII) stage and are ovulated from the follicle.

As described above, numerous aspects of the developmental process of germ cell lineages remain to be elucidated. Reconstitution *in vitro* of germ cell development, especially the early period of development, would contribute to a better understanding of their biological significance of this period. This review describes an established *in vitro* method of PGC differentiation from mouse ESCs/iPSCs, the production of oocytes from the PGCs, and future perspectives of practical applications.

Reconstitution *in Vitro* of PGC Specification

As described above, although early PGC specification presumably includes a process for establishing totipotency, the molecular mechanisms are hardly accessible, mainly due to a limited number of PGCs *in vivo*. Therefore, the reconstitution of PGC specification *in vitro* using pluripotent stem cells, such as ESCs and iPSCs, would overcome this issue, making it feasible to address the nature of totipotency. It is also necessary that the manner of PGC specification is reconstituted properly, as otherwise the model would not be useful either in research or for the provision of functional gametes. With careful consideration of the manner of PGC specification *in vivo*, we recently established a culture system in which ESCs/iPSCs differentiate first into epiblast-like cells (EpiLCs) in the presence of activin A and bFGF, and then into PGC-like cells (PGCLCs) in a culture with BMP4, SCF, LIF and EGF [20] (Fig. 1). Differentiation from ESCs to EpiLCs, and from EpiLCs to PGCLCs requires 2 days, and 2 to 6 days of culture, respectively, which largely corresponds to the time course of the developmental process from the inner cell mass (ICM) of the blastocyst to PGCs via the epiblast. Gene expression changes during the differentiation process *in vitro* are surprisingly similar to those occurring in the differentiation from ICM to PGCs *in vivo*. Consistently, PGCLCs undergo epigenetic reorganization in a manner similar to PGCs *in vivo* demonstrating a low level of H3K9me2, a high level of H3K27me3 and genome-wide DNA demethylation with partial erasure of imprint status. Based on transcriptome analysis, PGCLCs appear to be equivalent to PGCs at E9.5 [20]. However, it became evident that the developmental state of PGCLCs did not proceed beyond this time point even when the culture term was extended. Rather, PGCLCs eventually died in the extended culture, suggesting a lack of factor (s) required for further differentiation and/or survival in culture. As to the context of PGCs *in vivo* at E9.5, they are migrating along the hindgut and preparing to enter the genital ridge, the future ovary or testis. Consistent with the situation of PGCs *in vivo*, PGCLCs, when cultured with gonads collected from E12.5 female embryos, showed further differentiation as indicated by the appearance of later markers such as *Mvh* and *Dazl* transcripts and meiosis entry [21], and transcriptome analyses indicate that the gene expression pattern of PGCLCs with the gonads is similar to that of E12.5 PGCs.

Generation of oocytes from PGCLCs

There are several relevant reports on the generation of oocytes from PGCs. In these reports, the gonads were dissociated, reaggregated, and then transferred into either the ovarian bursa or kidney capsule [22, 23]. The reaggregations yielded mature oocytes that could be fertilized and gave rise to healthy individuals. According to

these reports, the supposition that the PGCLCs give rise to functional oocytes has been validated by transplantation into the ovarian bursa with gonadal somatic cells [21]. In brief, PGCLCs derived from female ESCs/iPSCs were aggregated with dissociated E12.5 female gonadal somatic cells, from which residual PGCs were removed using anti-SSEA1 antibody conjugated magnetic beads. The aggregations, thereafter called reconstituted ovarian tissue, were transplanted into the ovarian bursa of immunocompromised female mice. The reconstituted ovarian tissue yielded a number of growing or fully grown germinal vesicle (GV) oocytes at 4 weeks after the transplantation (Fig. 2, middle). GV oocytes from the reconstituted ovarian tissue resumed meiosis and reached MII in maturation culture *in vitro*(Fig. 2, bottom). MII oocytes derived from PGCLCs become zygotes after *in vitro* fertilization (IVF), and in turn give rise to offspring through transplantation into surrogate mothers. The resultant individuals were apparently healthy, since they grew as quickly as wild-type mice and became fertile adults with a normal litter size. Based on the successful production of offspring, it is evident that oocytes derived from PGCLCs are fully functional. However, based on the following observations, it would also appear that the oocytes from PGCLCs are not as potent as those *in vivo*. First, IVF using PGCLC-derived oocytes resulted in a number of zygotes harboring three pronuclei [21]. Immunofluorescence analyses clearly showed that almost all the zygotes with three pronuclei were digynic. Consistent with this finding, there was no second polar body extrusion among the zygotes with three pronuclei. Although the reason for the preferential digynic fertilization remains unclear, oocyte aging may play a role, since in some cases this phenomenon is known to cause digynic zygotes [24], and it would be interesting to investigate whether pluripotent stem cell-derived oocytes age prematurely. Second, fewer offspring are obtained from embryos transplanted into surrogate mothers: zygotes from PGCLCs are 3–5 times less efficient than those from PGCs [21]. This might be attributable to contamination of parthenogenic oocytes with two maternal pronuclei that are indistinguishable from normal zygotes. Another possible cause might be that PGCLC-derived oocytes possess defects in epigenetic regulation. It is known that the maternal imprint is established according to oocyte growth. In our study, we sometimes observed embryos being aborted in the uterus, which may have been due to an aberrant imprint status in the oocyte. These possibilities remain to be evaluated in future studies.

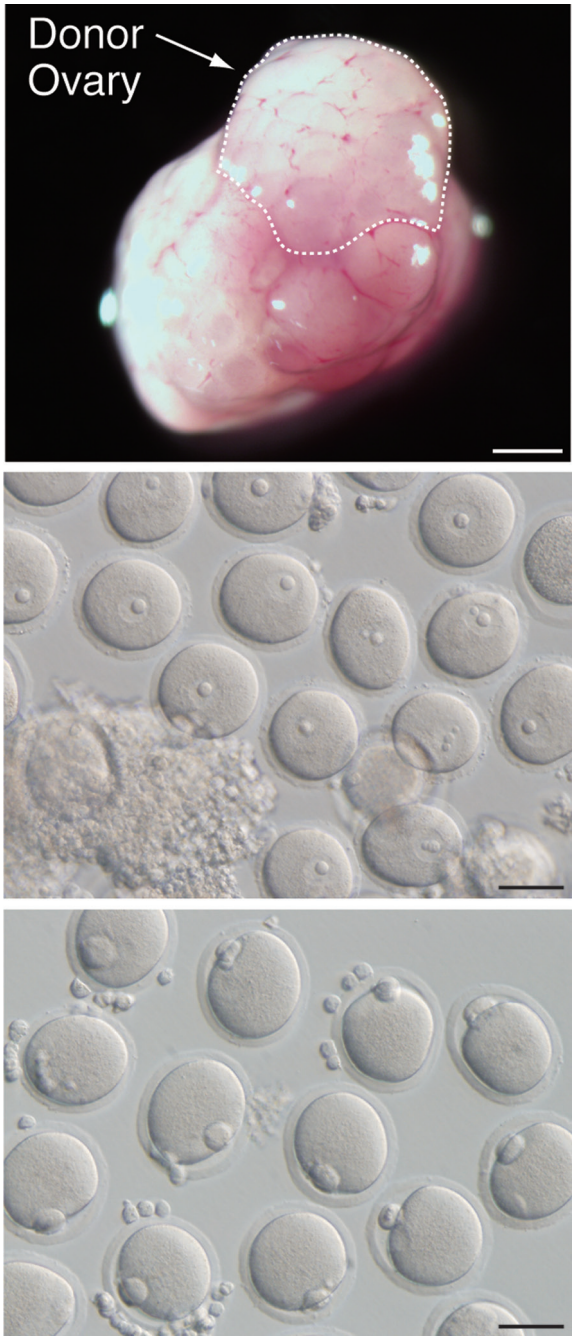

Fig. 2. Oocytes from iPS cells
The reconstituted ovarian tissue (dashed white lines) contains a number of follicles with angiogenesis (Top). Scale bar, 500 μm. From follicles in the reconstituted ovarian tissue, fully grown GV oocytes are obtained (Middle), and are matured into MII oocytes in maturation culture *in vitro*(Bottom). Scale bar, 50 μm.

Differentiation of gonadal Somatic Cells into primordial Follicles

It is also of note that oocyte growth in the reconstituted ovarian tissue proceeds synchronously, and consequently no primordial follicle remains [21]. This is not only the case in PGCLCs, but also in E12.5 PGCs, which show synchronous oocyte growth in the reconstituted ovarian tissue. In contrast, 2-week-old ovaries transferred into adult females clearly retained primordial follicles. This suggests that fetal oocytes and/or gonadal somatic cells have not yet acquired the capacity of functional primordial follicles to sustain dormancy under the environmental conditions of the adult female. Although the molecular mechanisms sustaining primordial follicles remain unclear, it is clear from a histological point of view that primary oocytes and the surrounding somatic cells are reorganized during the perinatal stage [13]. In midgestation, primary oocytes form a germ cell nest, also known as a germ cell cyst. Germ cell nests are surrounded with pregranulosa cells. At the perinatal stage, more than half of the primary oocytes undergo cell death, and the single primary oocytes become enclosed by granulosa cells, forming a cell complex known as a primordial follicle. It is possible that primordial follicles at the perinatal stages acquire the ability to sustain dormancy, whereas the fetal germ cell nest-pregranulosa complex is too sensitive to pause oocyte growth. Indeed, the reconstituted ovarian tissue composed of PGCLCs and E12.5 gonadal somatic cells harbor a number of multi-oocyte follicles (MOFs) at 4 weeks of transplantation, suggesting that oocyte growth commences directly from the germ cell nest without formation of primordial follicles. Recent studies have revealed the molecular mechanisms related to defective primordial follicle formation accompanying with MOFs. For example, gene-disruption and *ex vivo* culture analyses have revealed that inhibition of Notch signaling leads to the defective formation of primordial follicles and MOFs [25]. More relevantly, it has been reported that high levels of estrogen and progesterone lead to defective primordial follicle formation with MOFs [26, 27], leading to the idea that continuous exposure to high doses of steroid hormones may result in the creation of MOFs in the reconstituted ovarian tissues. It is likely that the reconstituted ovarian tissue is exposed to high doses of steroid hormones in the uterus and recipient adult females, whereas the ovaries in normal development pass through the perinatal stage with its accompanying decline in steroid hormones. Interestingly, cell death of primary oocytes during the perinatal stage seems necessary to modulate the number of follicles that mature in the juvenile stage. Evidence suggests that an excess number of follicles, some of which include MOFs, cause abnormal oocyte growth resulting in aberrant fertilization [14, 15]. Therefore, it seems that synchronous growth of an excess number of PGCLC-derived oocytes in the reconstituted ovarian tissue in recipient adult females causes their low potency. However, considering the fact that the potency of PGC-derived oocytes in the reconstituted ovarian tissue is not as low as that of PGCLC-derived oocytes [21], there may be an additional reason. Namely, there may be a gap between the PGCLCs, which correspond to E9.5 PGCs, and E12.5 gonadal somatic cells, based on the evidence that such gap attenuates oogenesis [28]. Collectively, these results suggest that there are likely several complex interacting causes of the low potency of PGCLC-derived oocytes.

Differences between ESCs and iPSCs in oocyte Production

Although both ESCs and iPSCs give rise to oocytes through PGCLC induction, there may be a substantial difference between the embryonic and induced pluripotent stem cells. Experiments employing two ESC and three iPSC lines showed that all the ESC lines successfully gave rise to mature oocytes, whereas only one out of the three iPSC lines did. The other two iPSC lines seemed to differentiate normally into EpiLCs, and then PGCLCs, but the PGCLCs formed teratomas in the recipient ovaries (data not shown). Distinct differentiation properties were also observed in males: PGCLCs from all ESC lines tested showed spermatogenesis in the recipient testes, whereas only one out of 3 iPSC lines did, and the two other iPSC lines formed teratomas in the recipient testes. Although there are too few examples to reach any definitive conclusions on this matter, the previous reports suggest that the propensity for differentiation somehow depends on the individual iPSC line and/or the origin of iPSCs [29]. Therefore, it will be necessary to sort the germ cell-potent iPSC lines from the others. Since there are so far no defined criteria for identifying germ cell-potent iPSC lines, it might be useful to compare the gene expression and epigenetic status of PGCLC-competent and –incompetent iPSC lines.

Application of the PGCLC System

Although there are several issues to be addressed, the PGCLC culture system nevertheless provides a platform from which a number of germ cell precursors can be obtained in culture from ESCs/iPSCs. This fact has led re-

searchers, and perhaps some fertility clinicians and patients with infertility, to the idea that the system might be applicable to other mammals, including humans. There are, however, many steps remaining before such applications can be developed, and these are discussed from a technical point of view below.

Differences in stem Cell Properties

It is widely accepted that mouse and other mammalian pluripotent stem cells possess distinct properties with respect to their optimal culture conditions, cell cycle progression and differentiation propensity. Moreover, such differences are not simply related to difference of species, since recent studies have established the concept of two metastable pluripotent states, a naïve state and a primed state, that are interchangeable depending on culture conditions [30]. The former is thought to mimic the pluripotent states in early embryogenesis, whereas the latter mimics those in relatively later stages of embryogenesis. Therefore, despite the interchangeability of these two states, the conversion from the naïve to the primed state, which follows a developmental manner *in vivo*, is much easier than the other way around [31–33]. In mice, representative criteria of the naïve and primed pluripotent states are clearly defined by the characteristics of ESCs and epiblast stem cells (EpiSCs), respectively. Specifically, passage in a single cell suspension, dome-shaped colony formation in culture, gene expression corresponding to ICM of the blastocyst, and the presence of two transcriptionally active X chromosomes (in females) are representative characteristics of ESCs and therefore of the naïve pluripotent state, whereas passage in small cell clumps, flat colony formation, gene expression corresponding to the post-implantation epiblast, and a heterogenic active state of the X chromosome are representative characteristics of EpiSCs and therefore of the primed pluripotent state. Based on these criteria, human ESCs are thought to be in a primed pluripotent state. Considering that PGCLCs are differentiated robustly from EpiLCs, but not from ESCs or EpiSCs [20, 34], reconstitution of a state equivalent to EpiLCs in human ESCs would seem to be required. The PGCLC-competent state of EpiLCs is a transitional state between the naïve and primed states. Therefore, as a first step toward their reconstitution, it will be necessary to develop a culture system that stabilizes the naïve state in human ESCs. Recent studies have achieved the production of naïve state human ESCs [35–37]. However, the culture conditions of these studies differed, and the conditions most suitable for the induction of a PGCLC-competent state remain to be determined. Based on the limited information available, it seems that the function of BMP4 in PGC derivation is conserved among mammalian species [38, 39]. Therefore, it is feasible that BMP4 is a key growth factor in the culture system for PGC derivation from PGCLC-competent EpiLCs in other mammals.

Sources of supporting Somatic Cells

Given that PGCs can be derived from human ESCs, it will still be necessary to prepare the gonadal somatic cells that play essential roles in support of oogenesis. To produce reconstituted ovarian tissues by our method, a 10:1 ratio of gonadal somatic cells to PGCLCs was required [21]. This strategy is practically possible only for mice, not in other mammals. Therefore, gonadal somatic cells would have to be substituted for some other available source. In theory, there are three possible ways of bypassing this requirement. The first would be to amplify a small number of gonadal somatic cells obtainable from rare embryos. The second would be to make pluripotent stem cells differentiate into gonadal somatic cells, or functionally equivalent cells. The third would be to substitute the function of gonadal somatic cells with defined factors.

How to reconstitute the entire Process of oogenesis

In our study oogenesis in the reconstituted ovarian tissue took place in the transplanted recipient female, under totally undefined conditions [21]. Unfortunately, due to immunorejection and the general scarcity of recipient animals, transplantation is not easily achieved in mammals other than mice. One possible solution may be xenotransplantation, and indeed, application of this procedure has already been attempted using several different approaches [40, 41]. The transplantation of human ovarian tissue to immunocompromised female mice has demonstrated that the donor ovarian tissue resumes oogenesis, but most of the follicles degenerated, suggesting that the environmental cues in xenotransplantation are insufficient or incompatible for the completion of oogenesis [42–44]. There are ways of improving this situation, such as genetic manipulation to optimize, i.e., humanize, mice for transplantation, and temporal xenotransplantation to obtain immature oocytes before degeneration commences. Another possible solution is to simply produce immunocompromised animals of the species, even though greater cost and effort are required to maintain such animals. Technically speaking, a novel

genetic manipulation strategy, the zinc-finger nuclease and Cas9/CRSPR system, has made possible the generation of immunocompromised animals, including non-human primates [45]. For human oocyte production, immunocompromised non-human primates could be more suitable recipient than mice. Finally, it would be ideal to reconstitute the entire process of oogenesis *in vitro*. Despite intensive efforts, only a few studies have succeeded in generating offspring from mice using oocytes from neonatal primordial follicles that were matured in culture [46, 47]. Moreover, even in those studies, the culture system was not sufficiently robust, since the success rate of functional oocyte production was fairly low [47]. The successful differentiation of fully mature oocytes from PGCs in culture has not yet reported, with the exception of a study in which functional oocytes were generated by transferring the nucleus from PGC-derived oocytes into enucleated mature oocytes [48]. The same study also showed that PGCs could be grown to GV-oocytes of approximately 50μm diameter in organ culture of the gonad, indicating that PGCs mature under these conditions. In mammalian species other than mice, there have also been a number of attempts to produce fertile oocytes from immature oocytes at various stages. In large animals such as cattle, it is possible to generate functional MII oocytes from immature growing oocytes [49]. Likewise, in primates, it has been reported that immature growing oocytes reached the MII oocyte stage, though whether they are capable of developing to full term remains to be tested [50–52]. Further refinement of culture conditions based on a basic understanding of oogenesis will make it possible to develop a culture system that reconstitutes the entire process of oogenesis.

Acknowledgements

We apologize to researchers whose work has not been cited due to space limitations. This study was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; by JST-PRESTO/CREST/ERATO.

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