Submitted: December 12, 2013
Accepted: December 27, 2013
Advance Publication: December 28, 2013

Journal of REPRODUCTION ENGINEERING JRE

The article categories: Mini-review

Title: Prospects of female germ cell production *in vitro*

Page Heading: Egg generation from pluripotent stem cells

Author, Affiliation, Address:
Katsuhiko HAYASHI† and Mitinori SAITOU
Department of Anatomy and Cell Biology, Graduate School of Medicine, Kyoto University. Yoshida-Konoe-Cho,Sako-Ku, Kyoto 606-8501, Japan
†Correspondence: khayashi@anat2.med.kyoto-u.ac.jp

Abstract

Eggs are the only cell type harboring totipotency, and thus play a fundamental role in the creation of new individuals. There are multiple and complex regulations in the production of eggs, named oogenesis, and errors in this process can cause a number of clinical issues, such as infertility, ovarian cancer and developmental disorders. Thus the understanding and *in vitro* reconstitution of oogenesis are of biological and clinical importance. Recently, we produced fully potent eggs from pluripotent stem cells, i.e., embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). ESCs and iPSCs differentiate *in vitro* into primordial germ cells (PGCs), the precursor of egg cells, and are in turn matured to eggs by transplantation into adult female mice with fetal gonadal somatic cells. This review introduces the method and discusses applications of this technology to reproductive biology.

Key words: Primordial germ cells, pluripotent stem cells, oogenesis

Introduction

Eggs, the terminally differentiated cells of the female germ cell lineage, are established through a series of processes. All eggs (and sperm) originate from primordial germ cells (PGCs), the founder cell population of the germ cell lineage that is specified in mice from the pluripotent epiblast around embryonic day (E) 6.5 (Figure

1) [5, 26, 27]. Soon after specification, PGCs start to migrate, while proliferating, toward fetal gonads that are the future ovaries (or testes). Early differentiation of PGCs is accomplished in a sex-independent manner, but once the PGCs reach the fetal gonads they begin to differentiate in a sex-dependent manner. In females, PGCs in the gonads, thereafter called oogonia, proliferate to expand the pool of future oocytes by around E13.5 and then cease mitosis and enter meiosis. It is essential for PGCs to settle in the gonads to properly differentiate into oocytes, since ectopic PGCs, which are mainly caused by aberrant migration, would trigger apoptosis and eventually be eliminated from the embryo proper [24]. The gonadal environment is important not only in terms of survivability, as described above, but also for further differentiation of the oogonium; gonadal somatic cells contain precursors of granulosa and theca cells, both of which are essential compartments for ovarian follicles [3]. Based on morphological observation, the oogonia interact with surrounding somatic cells and then form primordial follicles, which are the most immature type of ovarian follicle. This process is thought to be controlled by a gonadotrophin-independent mechanism, mainly because null-mutation of gonadotrophin receptors does not abrogate the primordial follicle formation [2, 16]; however, aside from this detail it remains elusive how the differentiation of oogonia is regulated.

Apart from understanding the mechanism of oogenesis, an ultimate goal of reproductive biologists is to reconstitute oogenesis *in vitro*, so that an unlimited number of functional oocytes can be generated. Such *in vitro* reconstitution of oogenesis would open possibilities in basic biology as well as clinical application. Despite of making efforts to achieve oogenesis *in vitro*, yet there is no robust culture system that produces an unlimited number of functional oocytes. As a prerequisite to the establishment of oogenesis *in vitro*, we must first achieve a comprehensive understanding of the mechanisms underlying oogenesis *in vivo*.

Our recent studies have shown that the PGC specification can be reconstituted *in vitro* using mouse embryonic stem cells (ESCs) as well as induced pluripotent stem cells (iPSCs) [12]. ESC/iPSC-derived PGCs, called PGC-like cells (PGCLCs), are capable of differentiating into spermatozoa and oocytes that give rise to healthy individuals through *in vitro* fertilization [11, 12]. In this review, I describe the partial reconstitution of female germ cell development using ESC/iPSC-derived PGCLCs and discuss further issues to be resolved before the method can be applied to other species

and ultimately used to reconstitute the entire process of oogenesis *in vitro*.

PGC specification in vitro

ESCs and iPSCs are a valuable source for production of gametes *in vitro*, as they are capable of proliferating indefinitely while maintaining their ability to differentiate into all cell lineages of the embryo proper, including germ cells. A number of attempts have been made to generate germ cells *in vitro* from ESCs and iPSCs [8, 14, 19, 29]. Despite the intensive efforts, a culture system that produces a robust number of functional PGCs, the earliest step of germ cell development, has not been established. The reason for the unsuccessful attempts may be that it was not adequately determined whether the PGC specification in the culture system properly reconstituted that *in vivo*. Since PGC specification is a highly orchestrated process, it seems essential to properly reproduce it *in vitro*.

In mice, the manner of PGC specification is epigenesis; PGCs are specified from the pluripotent cell population in response to inductive signals triggered by intrinsic and extrinsic signals [6, 15]. Therefore, PGCs originate from cells that are poised to differentiate into various somatic cell lineages. The germ cell fate is induced in the proximal epiblast by direct BMP4 signals from the extraembryonic ectoderm [17, 22]. At E6.25, approximately 6 cells of the posterior proximal epiblast start to express *Prdm1/Blimp1*, a zinc finger transcriptional repressor, and these *Prdm1*-positive cells are lineage-restricted to become PGCs [23]. One day later, about 40 PGCs are located at the posterior end of the primitive streak, and begin to migrate into the allantois and along the developing hindgut toward the genital ridges [23, 25].

Recently we have developed a culture system in which PGC specification processes are reconstituted *in vitro* by using ESCs/iPSCs [11-13]. In this system, the ESCs/iPSCs first differentiate into a novel type of cells harboring the post-implantation epiblast status, called epiblast-like cells (EpiLCs), by inducing the transient differentiation under a defined condition with bFGF and activin (Figure 1). The EpiLCs differentiate robustly into PGCLCs in response to BMP4 (Figure 1). The manner of differentiation from ESCs/iPSCs to PGCLCs is highly similar, if not identical, to that from the inner cell mass (ICM) of the blastocyst to PGCs *in vivo*, based on the criteria of gene expression and epigenetic status. Importantly, PGCLCs are functional,

as they differentiate into fertile sperm upon transplantation into testis. Eggs fertilized with PGCLC-derived sperm give rise to healthy pups that eventually grow up to fertile adults, further demonstrating that PGCLCs are fully potent.

Oocyte production from pluripotent stem cells

Based on the results of gene expression analyses, it is likely that PGCLCs at day 6 of induction culture correspond to PGCs at E9.5 *in vivo*, at which time sexually dimorphic PGCs migrate in the hindgut toward the genital ridges. PGCLCs under the induction culture condition no longer differentiate or proliferate, but instead, eventually decrease in number for an unknown reason, suggesting another signal(s) is required for further differentiation of PGCLCs. Interestingly, PGCLCs, when aggregated with E12.5 female gonadal somatic cells, resume proliferation and differentiation [11]. Among several tissues, only gonadal somatic cells showed a robust effect on the proliferation and differentiation of PGCLCs. This suggests that gonadal somatic cells produce factor(s) essential for PGC proliferation and differentiation. It is noteworthy that the PGCLC induction condition includes stem cell factor (SCF), which is a factor known to promote PGC proliferation, indicating that gonadal somatic cells produce a crucial factor(s) other than SCF. PGCLCs with gonadal somatic cells showed several features observed in oogonia: expression of later germ cell-maker genes such as Mvh and Dazl, erasure of parental epigenetic status in imprinting gene loci, and entrance into the meiotic prophase. To validate oogenesis in PGC-gonadal somatic cell aggregation, hereafter called reconstituted ovary, we transferred them into the ovarian bursa of immunocompromised mice according to previous studies [10, 18]. At 4 weeks after transplantation, growing follicles containing PGCLC-derived oocytes were observed in the reconstituted ovary. Since follicles in reconstituted ovaries show almost no ovulation, the PGCLC-derived oocytes are collected by mechanically puncturing the follicles and then are subjected to an *in vitro* maturation culture. Most of the collected PGCLC-derived oocytes entered the fully-grown germinal vesicle (GV) stage and reached a diameter of approximately 70 μm. These GV oocytes were capable of resuming meiosis and of reaching the metaphase II (MII) stage, when oocytes are ready for fertilization. *In vitro* fertilization using sperm from wild-type males and PGCLC-derived oocytes yielded fertilized eggs. The resultant fertilized eggs gave rise to apparently healthy pups through transplantation into surrogate mothers,

demonstrating the PGCLC-derived oocytes are potent, though not as fully potent as oocytes *in vivo* (see below for details). These pups grow up and show reproductive ability with normally sized litters.

Problems to be solved

Although the production of functional oocytes appeared to be successful, several problems remain to be solved. In principle, it is clear that, compared to production from oocytes *in vivo*, successful production of pups from PGCLC-derived oocytes is less efficient [11]. Among the several possible reasons for the low productivity, we found that PGCLC-derived oocytes frequently exhibited aberrant fertilization; half of the fertilized eggs possessed three pronuclei composed of two maternal pronuclei and a paternal pronucleus. This suggests that PGCLC-derived oocytes have a limited ability to extrude the second polar body upon fertilization. The detailed reasons for the deficient second polar body extrusion remain to be clarified. In addition to this deficiency, we found embryos absorbed at a later stage of pregnancy in the uteri of surrogate mothers, suggesting that the totipotency of PGCLC-derived oocytes is limited. We consider that the reason for these deaths was an error in epigenetic reprogramming during oogenesis.

Since reconstituted ovaries were transferred to produce the oocytes, the environmental cues that organize oogenesis in the reconstituted ovary remain essentially unclear (Figure 1). To reconstitute the entire process of oogenesis using pluripotent stem cells, the next step would be to develop a culture system in which oocytes mature within the reconstituted ovary. This is challenging work, as no report has so far succeeded in the production of functional oocytes from PGCs, except by using nuclear transfer; Obata et al. succeeded in generating pups by transferring the nuclei of oocytes that had matured in an *in vitro* culture from PGCs, into enucleated fully grown oocytes from adult ovaries, followed by *in vitro* fertilization [21]. Another study showed that, despite the low efficiency, the neonatal primordial follicles matured *in vitro* to functional oocytes [4, 20]. Collectively, these results underscore the necessity of developing a culture system in which PGCLCs in the reconstituted ovary differentiate into primary oocytes of a stage consistent with neonatal primary oocytes *in vivo*.

Nevertheless, the successful production of oocytes from ESCs/iPSCs via transplantation arouses interest for its potential application to other animals, especially

humans. Needless to say, there are both ethical and technical steps that have to be overcome. Focusing on the technical steps, first it should be taken into consideration that the features of ESCs/iPSCs are different between humans and mice [9]. Recent studies have focused on identifying culture conditions under which human ESCs might become similar to mouse ESCs [7]. The development of such culture conditions might ultimately allow us to apply PGCLC production using mice to that using human ESCs. However, the functional validation of mouse PGCLCs by transplantation into ovaries is not readily applicable to human PGCLCs. An alternative may be to use non-human primate ESCs for both the production and functional validation of PGCLCs.

Concluding remarks

Here I introduced a culture system that produces functional oocytes from ESC/iPSC-derived PGCLCs. As described above, the culture system reconstitutes *in vitro* early PGC specification and subsequent early differentiation of oogonia. The latter process requires female gonadal somatic cells. From a biological point of view, it is of particular interest to know interaction between the PGCLCs and somatic cells at a molecular level. Since several factors, such as Wnt4 and R-Spondin, have been identified as determinants for ovary differentiation [1, 28, 30], it is worth testing whether these factors can compensate for the function of gonadal somatic cells in the differentiation of PGCLCs. If not, this would suggest that the differentiation of oogonia require other factors, which must be produced by gonadal somatic cells. Identification of the factors and reconstitution of oogonia differentiation under defined conditions are of practical importance, particularly with respect to human application, since it is impossible to obtain a sufficient number of gonadal somatic cells from human embryos. Nevertheless, we have reported for the first time that ESC/iPSC-derived PGCLCs are capable of differentiating into functional oocytes that can give rise to fertile individuals. This is a first step towards the establishment of a culture system that entirely reconstitutes oogenesis *in vitro*.

References

1. **Chassot AA, Ranc F, Gregoire EP, Roepers-Gajadien HL, Taketo MM, Camerino G, de Rooij DG, Schedl A, Chaboissier MC.** Activation of beta-catenin signaling by Rspo1 controls differentiation of the mammalian ovary. Hum. Mol. Genet. 2008; 17: 1264-1277.
2. **Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P.** Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proc. Natl. Acad. Sci. U S A 1998; 95: 13612-13617.
3. **Edson MA, Nagaraja AK, Matzuk MM.** The mammalian ovary from genesis to revelation. Endocr. Rev. 2009; 30: 624-712.
4. **Eppig JJ, O'Brien MJ.** Development in vitro of mouse oocytes from primordial follicles. Biol. Reprod. 1996; 54: 197-207.
5. **Ewen KA, Koopman P.** Mouse germ cell development: from specification to sex determination. Mol. Cell Endocrinol. 2010; 323: 76-93.
6. **Extavour CG, Akam M.** Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. Development 2003; 130: 5869-5884.
7. **Gafni O, Weinberger L, Mansour AA, Manor YS, Chomsky E, Ben-Yosef D, Kalma Y, Viukov S, Maza I, Zviran A, Rais Y, Shipony Z, Mukamel Z, Krupalnik V, Zerbib M, Geula S, Caspi I, Schneir D, Shwartz T, Gilad S, Amann-Zalcenstein D, Benjamin S, Amit I, Tanay A, Massarwa R, Novershtern N, Hanna JH.** Derivation of novel human ground state naive pluripotent stem cells. Nature 2013.
8. **Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K, Daley GQ.** Derivation of embryonic germ cells and male gametes from embryonic stem cells. Nature 2004; 427: 148-154.
9. **Hanna JH, Saha K, Jaenisch R.** Pluripotency and cellular reprogramming: facts, hypotheses, unresolved issues. Cell 2010; 143: 508-525.
10. **Hashimoto K, Noguchi M, Nakatsuji N.** Mouse offspring derived from fetal ovaries or reaggregates which were cultured and transplanted into adult females. Dev Growth & Differ. 1992; 34: 233-238.
11. **Hayashi K, Ogushi S, Kurimoto K, Shimamoto S, Ohta H, Saitou M.** Offspring from oocytes derived from in vitro primordial germ cell-like cells in

mice. Science 2012; 338: 971-975.

12. **Hayashi K, Ohta H, Kurimoto K, Aramaki S, Saitou M.** Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. Cell 2011; 146: 519-532.
13. **Hayashi K, Saitou M.** Generation of eggs from mouse embryonic stem cells and induced pluripotent stem cells. Nat. Protoc. 2013; 8: 1513-1524.
14. **Hubner K, Fuhrmann G, Christenson LK, Kehler J, Reinbold R, De La Fuente R, Wood J, Strauss JF, 3rd, Boiani M, Scholer HR.** Derivation of oocytes from mouse embryonic stem cells. Science 2003; 300: 1251-1256.
15. **Johnson AD, Drum M, Bachvarova RF, Masi T, White ME, Crother BI.** Evolution of predetermined germ cells in vertebrate embryos: implications for macroevolution. Evol. Dev. 2003; 5: 414-431.
16. **Kumar TR, Wang Y, Lu N, Matzuk MM.** Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat. Genet. 1997; 15: 201-204.
17. **Lawson KA, Dunn NR, Roelen BA, Zeinstra LM, Davis AM, Wright CV, Korving JP, Hogan BL.** Bmp4 is required for the generation of primordial germ cells in the mouse embryo. Genes Dev. 1999; 13: 424-436.
18. **Matoba S, Ogura A.** Generation of functional oocytes and spermatids from fetal primordial germ cells after ectopic transplantation in adult mice. Biol. Reprod. 2011; 84: 631-638.
19. **Nayernia K, Nolte J, Michelmann HW, Lee JH, Rathsack K, Drusenheimer N, Dev A, Wulf G, Ehrmann IE, Elliott DJ, Okpanyi V, Zechner U, Haaf T, Meinhardt A, Engel W.** In vitro-differentiated embryonic stem cells give rise to male gametes that can generate offspring mice. Dev. Cell 2006; 11: 125-132.
20. **O'Brien MJ, Pendola JK, Eppig JJ.** A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. Biol. Reprod. 2003; 68: 1682-1686.
21. **Obata Y, Kono T, Hatada I.** Gene silencing: maturation of mouse fetal germ cells in vitro. Nature 2002; 418: 497.
22. **Ohinata Y, Ohta H, Shigeta M, Yamanaka K, Wakayama T, Saitou M.** A signaling principle for the specification of the germ cell lineage in mice. Cell 2009; 137: 571-584.

23. **Ohinata Y, Payer B, O'Carroll D, Ancelin K, Ono Y, Sano M, Barton SC, Obukhanych T, Nussenzweig M, Tarakhovsky A, Saitou M, Surani MA.** Blimp1 is a critical determinant of the germ cell lineage in mice. Nature 2005; 436: 207-213.

24. **Runyan C, Schaible K, Molyneaux K, Wang Z, Levin L, Wylie C.** Steel factor controls midline cell death of primordial germ cells and is essential for their normal proliferation and migration. Development 2006; 133: 4861-4869.

25. **Saitou M, Barton SC, Surani MA.** A molecular programme for the specification of germ cell fate in mice. Nature 2002; 418: 293-300.

26. **Saitou M, Yamaji M.** Germ cell specification in mice: signaling, transcription regulation, and epigenetic consequences. Reproduction 2010; 139: 931-942.

27. **Sasaki H, Matsui Y.** Epigenetic events in mammalian germ-cell development: reprogramming and beyond. Nat. Rev. Genet. 2008; 9: 129-140.

28. **Tomizuka K, Horikoshi K, Kitada R, Sugawara Y, Iba Y, Kojima A, Yoshitome A, Yamawaki K, Amagai M, Inoue A, Oshima T, Kakitani M.** R-spondin1 plays an essential role in ovarian development through positively regulating Wnt-4 signaling. Hum. Mol. Genet. 2008; 17: 1278-1291.

29. **Toyooka Y, Tsunekawa N, Akasu R, Noce T.** Embryonic stem cells can form germ cells in vitro. Proc. Natl. Acad. Sci. U S A 2003; 100: 11457-11462.

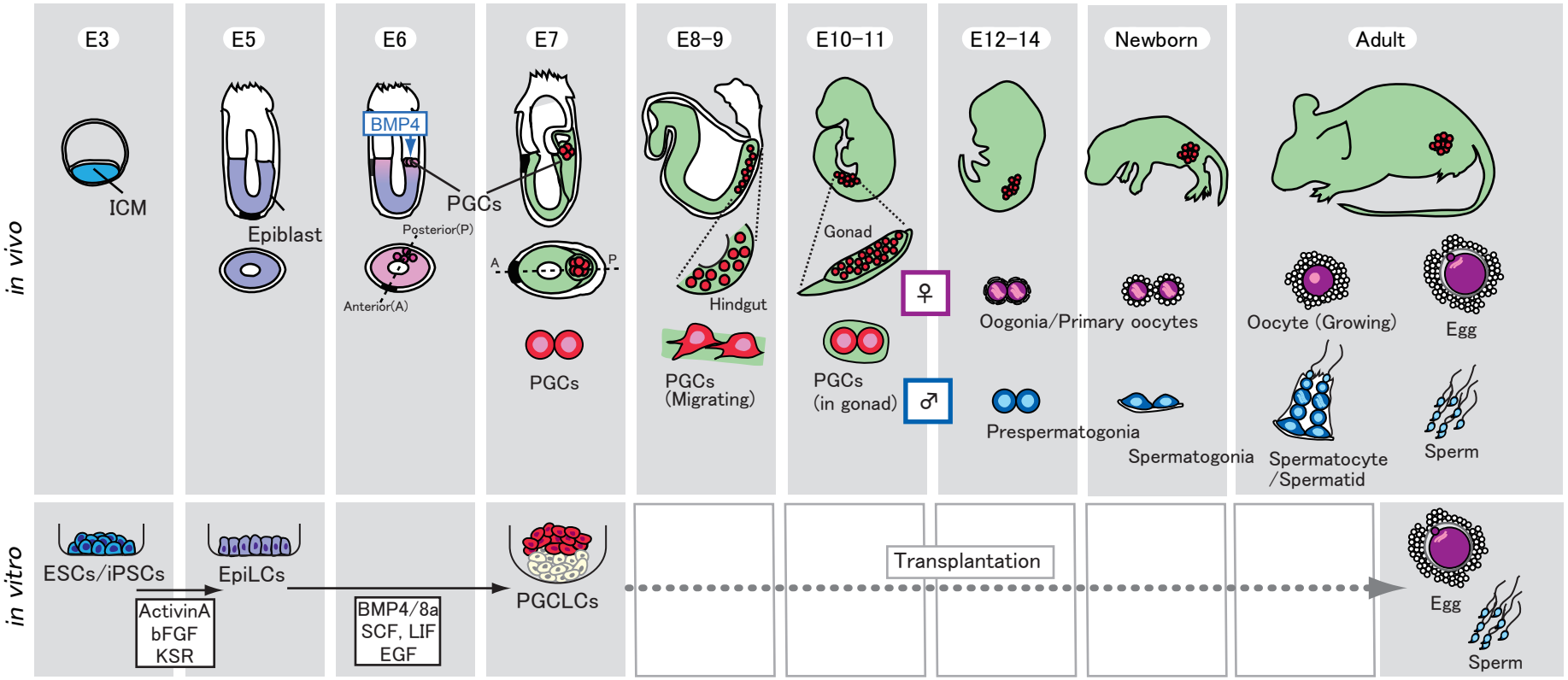
30. **Vainio S, Heikkila M, Kispert A, Chin N, McMahon AP.** Female development in mammals is regulated by Wnt-4 signalling. Nature 1999; 397: 405-409.

Figure Legend

Figure 1. Germ cell development in mice and reconstitution *in vitro*

Primordial germ cells (PGCs) are specified from pluripotent epiblast in response to BMP4, and then start to migrate along hindgut toward genital ridges. After reaching genital ridges, PGCs differentiate either oogonia or prespermatogonia depending on the sex of the embryo. Using pluripotent stem cells, we recently developed a culture system that reconstitutes PGC specification *in vitro*. Although PGCs *in vitro* (PGC-like cells: PGCLC) are capable of differentiating into functional sperm and oocyte, differentiation process after reaching genital ridges are accomplished *in vivo* where PGCLCs are transplanted. Therefore, reconstitution *in vitro* of the process would be the next step for establishing a culture system that reconstitutes an entire process of germ cell development *in vitro*.

Figure 1


in vivo
E3
ICM
E5
Epiblast
E6
BMP4
PGCs
Posterior(P)
Anterior(A)
E7
A
P
PGCs
E8–9
Hindgut
PGCs
(Migrating)
E10–11
Gonad
♀
PGCs
(in gonad)
♂
E12–14
Oogonia/Primary oocytes
Prespermatogonia
Newborn
Spermatogonia
Adult
Oocyte (Growing)
Egg
Spermatocyte
/Spermatid
Sperm
in vitro
ESCs/iPSCs
ActivinA
bFGF
KSR
EpiLCs
BMP4/8a
SCF, LIF
EGF
PGCLCs
Transplantation
Egg
Sperm