MOLECULAR BIOLOGY & GENETICS

Special Topic: Stem Cell Research in China

Gamete differentiation from pluripotent stem cells

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INTRODUCTION

The germ cells are the only cells capable of transmitting genetic and epigenetic information to the next generation and germ cell development *in vivo* undergoes complex processes to achieve this goal. An effective gamete differentiation model *in vitro* from pluripotent stem cells has benefits for elucidating the mechanism of the germline cell development and holds promising prospects in treating infertility. Here, we summarize the great progress in gametogenesis from mouse pluripotent stem cells and the achievements in human gamete differentiation research, potential clinical applications and several challenges that exist in current research are covered in this perspective as well.

Deriving from an early stage in embryonic development, primordial germ cells undergo sophisticated processes to form mature gametes, the spermatozoa and the oocytes, which unite at fertilization to generate a new individual. Since the germ cells specifically undertake the duty of transmitting genetic and epigenetic information to the next generation, many researchers have paid much attention to elucidating the mechanism of germline cell development. Clarity of this developmental process may in turn promote the understanding of the life circle, genetic diversity and evolution. Remarkably, great progress in the reconstitution of germ-cell development *in vitro* has been made using mouse and human pluripotent stem cells (PSCs). The success of obtaining functional male or female gametes in mice may help us to reconstitute complete human gametogenesis in the near future, which not only facilitates the understanding of the development of human germ cells, but also may provide us with new therapies for infertility.

GAMETE DIFFERENTIATION FROM PSCS IN MICE

The induction of mPGCLCs from mPSCs *in vitro*

The primordial germ cells (PGCs) is an essential stage during gamete development *in vivo*, which means that the induction of mouse primordial germ-cell-like cells (mPGCLCs) *in vitro* is becoming a core step that needs to be achieved foremost in research on gamete differentiation *in vitro*. Recent research showed that the epiblast-like cells (EpiLCs) stage—a cellular stage similar to pregastrulating epiblast—acted as a transition in the induction of mPGCLCs from mPSCs [1], which simulates normal PGCs development. Following cytosines were first used in the induction of mPGCLCs from EpiLCs, including BMPs, SCF, LIF and EGF. Further research also confirmed that the overexpression of three key transcription factors Blimp1 (also known as Prdm1), Prdm14 and Tfap2c (also known as AP2γ) could direct EpiLCs into a PGC state efficiently [2]. To validate the function of mPGCLCs, different strategies were employed in males and females, respectively. In males, the transplantation of the induced mPGCLCs into the neonatal testes of W/W^{v} mice can obtain the functional spermatozoa [1]. In females, correspondingly, mPGCLCs transplanted under mouse ovarian bursa successfully matured into germinal vesicle-stage oocytes [3]. These results successfully validate the function of mPGCLCs, which paved the way for further gamete differentiation research.

Functional mouse male gametes from mES-derived mPGCLCs

After the success of the induction of mPGCLCs, another problem in gamete differentiation that needs to be resolved is the induction of mature gametes from mPGCLCs *in vitro*. Zhou *et al.* adopt a co-culture method, including the aggregation of mPGCLCs with neonatal testicular somatic cells and sequential exposure to morphogens and sex hormones including FSH, BPE and testosterone, which unprecedently produced functional round spermatids that can be used to obtain healthy offspring through intracytoplasmic sperm injection (ICSI) (Fig. 1) [4]. However, round spermatids may still have several flaws, resulting in the low survival rate of transplanted embryos. Though several problems still existed, this milestone research completed meiosis *in vitro* and obtained functional male gametes, which provides a platform to investigate meiotic mechanisms and the generation of human haploid spermatids.

Functional mouse female gametes from mPSCs-derived mPGCLCs *in vitro*

Before 2016, female gametes could only be obtained through transplanting mPGCLCs under mouse ovarian bursa, which

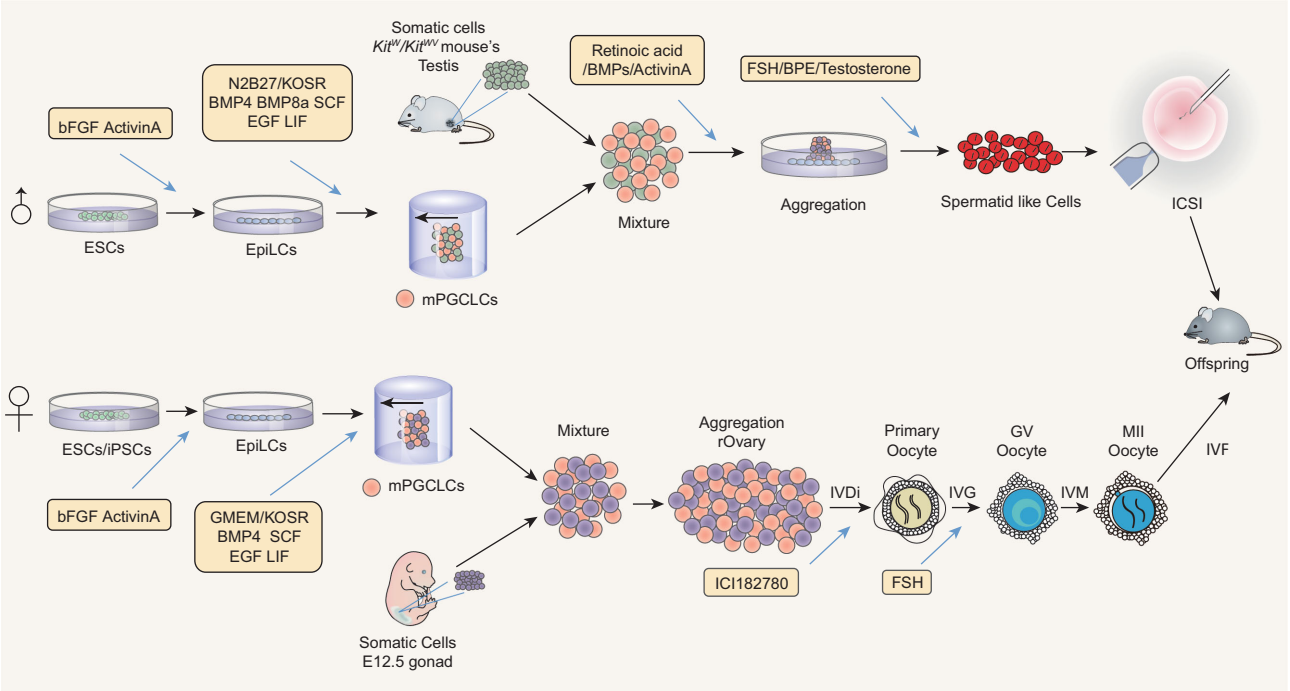

Figure 1. The process of male/female gamete induction from mESCs/iPSCs. Male: Aggregated with testis somatic cells from Kit^{W}/Kit^{WV} mouse, mPGCLCs initialized from ESCs will complete the meiosis process, under assistance of cytosines and hormones, to gain the functional spermatid like cells. Female: mPGCLCs derived from ESCs/iPSCs can differentiate to MII oocytes experiencing the aggregation with the somatic cells from E12.5 gonads and three steps after aggregation, including IVDi, IVG and IVM.

indicated that the induction of female gametes still needed an environment *in vivo* to finish the meiosis [3]. But a recent breakthrough based on a system [5] that can fully reproduce mammalian oogenesis from mouse fetal PGCs *in vitro* successfully finished the whole female-gamete-induction process from embryonic stem cells (ESCs) and iPSCs *in vitro* (Fig. 1) [6]. The whole oogenesis process was divided into three sections, including *in vitro* differentiation (IVDi), *in vitro* growth (IVG) and *in vitro* maturation (IVM). By aggregating primordial germ cell-like cells (PGCLCs) and the somatic cells from E12.5 gonads, scientists created several reconstituted ovaries. These reconstituted ovaries were first placed on Transwell with α MEM-based medium and the medium was replaced four days later with StemPro34-based medium containing ICI182780 to prevent the formation of multiple oocyte follicles in IVDi. In IVG process, individual secondary follicle-like structures (2FLs) from the rOvaries were separated and cultured using IVG medium containing follicle-stimulating hormone in a dish to generate germinal vesicle oocytes. After culture with IVM medium, functional MII oocytes were finally obtained, indicating that a brand new platform for producing oocytes and investigating the molecular mechanisms of totipotency had been constructed.

GAMETE DIFFERENTIATION FROM PSCS IN HUMANS

The induction of hPGCLCs from hPSCs *in vitro*

Though the induction of human germ cells, including haploid cells, from hPSCs has been reported in several studies, the induction efficiency in these studies was usually very low and the characterization of the induced cells was not specific [7,8]. The lack of a careful comparison between the process of differentiation *in vivo* and induction *in vitro* is another conspicuous flaw of this research. Nevertheless, the effective induction of male or female gametes from mPSCs, through mPGCLCs, provides valuable information for inducing human germ-cell fate. Considering hPSCs cultured under conventional conditions represent a primed pluripotency of post-gastrulating epiblasts, Irie *et al.* (2015) used the 4i hESCs/hiPSCs, cultured with the medium containing four inhibitors (MAPKi, GSK3i, p38i and JNKi) (Fig. 2). Following the similar procedure of mPGCLC induction, the 4i hPSCs were induced into hPGCLCs directly with high efficiency (up to 45.5%), whereas the conventional hPSCs in the primed state of pluripotency only formed hPGCLCs insufficiently (0–5%) [9]. The hPGCLCs induced though 4i hPSCs possess similar gene expression to that of week 7 hPGCs and are negative for genes such as DDX4 and DAZL. An astonishing finding was that SOX17, previously considered as a key factor in endoderm development in both mice and humans, was identified as a critical specifier of hPGCs and acted upstream of BLIMP1. On the other hand, Sasaki *et al.* (2015) showed human-induced pluripotent stem cells (hiPSCs) with primed pluripotency cultured under a feeder-free, defined condition could be directly induced into

hPGCLC using the same method as the mPGCLC induction [10]. Remarkably, the induction of hPGCLCs could be more robust if they experience an intermediate stage of incipient mesoderm-like cells (iMeLCs) (Fig. 2). The authors showed evidence that the hPGCLCs obtained through iMeLCs exhibited a similar gene expression pattern to that of gonadal PGCs of cynomolgus monkeys as well as of the hPGCLCs induced by Irie *et al.* (2015). Though further experiments will be required to demonstrate whether hPGCLCs obtained from the two studies above are reliable counterparts of hPGCs *in vivo*, the researchers may try to obtain human gametes based on current results.

Challenges for deriving gametes from hPSCs

Although the progress of gamete differentiation is optimistic, several challenges still exist. Foremost to be considered is that hPGCLCs induced from hPSCs only correspond to the early stage hPGCs. Therefore, identifying key cytokines promoting the maturation of hPGCLCs becomes urgent. Limitation of material is another problem that needs to be solved. The somatic cells of neonatal testes or embryonic gonads play important roles in mouse gamete differentiation *in vitro*, but may not be viable in aggregating with hPGCLCs considering the species differences and the difficulty of gathering the counterparts in humans or non-human primates. Induction of gonadal somatic cells from PSCs or establishment of a method independent of somatic cells could be alternative choices.

Potential applications and ethical considerations

Success of *in vitro* gamete differentiation from hPSCs will not only promote understanding of human germ-cell development and the causes of infertility, but also bring hope to infertile people. With the assistance of a combination of ICSI and *in vitro* gamete differentiation techniques, these infertile people may probably achieve their dreams—raising their own baby. An *in vitro* induction system also offers a good platform to screen potential drugs for diseases relating to the anomalies in germ-cell development. In consideration of using gametes derived from hPSCs, several methods need to be employed to assess the risk and validity of this technology, including large animal experiments, especially on non-human primates, and the creation of human embryos *in vitro*. Furthermore, the genetic and epigenetic profiles of the gametes or embryos should be examined carefully to avoid the birth of abnormal infants. Even the system of generating artificial gametes may mature in the future; the ethical and sociological concerns should not be ignored either. For instance, the definition of whether the embryo can be regarded as an individual varies in different regions of the world. *In vitro*, a human embryo culture until the 14th day is usually regarded as the ethically permitted maximum period to evaluate the developmental potential of gametes. To select the 'best' embryo suitable for transplantation, many embryos will be produced to meet the demand. However, the criterion of the 'best' embryo remains obscure, since different aspects, such as genome and growth of the embryo, will influence the setting of the criterion. In conclusion, to promote the development and application of gamete differentiation research, further discussion on the ethical problem among society will be essential.

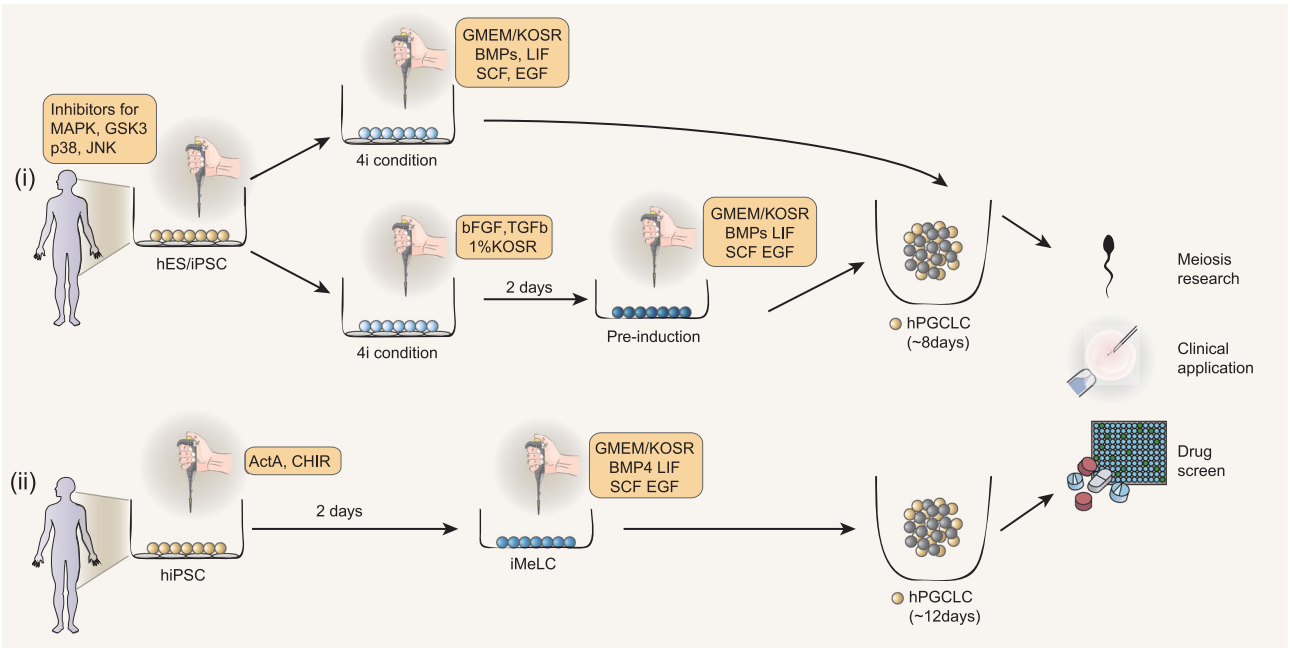

Figure 2. Two different strategies are employed in the induction of hPGCLCs. (i) naïve hES/iPSC under the 4i condition can generate hPGCLCs directly or through pre-induction transition stage. (ii) primed hiPSC is capable to form hPGCLCs with a transition stage, iMeLCs. Remarkably, BMPs, LIF, SCF and EGF are necessary in the induction strategies discussed above, which is identical to the process in mice.

CONCLUSIONS

Recent years have witnessed great progress in gamete differentiation *in vitro* using PSCs, especially in the mouse model, the success of which is a milestone in reproductive biology and regenerative medicine. It is anticipated to obtain functional male or female gametes in humans by utilizing the successful experiences in mice, though there are still some obstacles to be overcome. Furthermore, we may have better understanding of human germ-cell development and the causes of germ-cell abnormalities in infertility through the induction platform, which may promote us in finding viable ways to solve these common problems worldwide.

ACKNOWLEDGEMENTS

We thank all members of the Group of Stem Cell and Regenerative Medicine for discussion and help. Several other articles could not be quoted due to limited context, and we apologize for this inconvenience. This work was supported by the National Natural Science Foundation Project (31371506 and 31322036).

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National Science Review
4: 525–528, 2017
doi: 10.1093/nsr/nwx070
Advance access publication 24 June 2017