—Mini Review—

Embryonic Stem Cells: A Novel Attractive Research Tool for Germ Cell Development

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Abstract: *Despite recent astonishing advances in the treatment of infertility by assisted reproductive technology (ART), the fundamental approaches to solving complete infertility due to congenital absence of germ cells or impaired fertility arising from gonadotoxic therapies during prepubertal childhood still remain unclear. Current findings in stem cell biology have resulted in new possibilities for the treatment of reproductive diseases, i.e., germ cell formation may be inducible in vitro. The idea of generating artificial germ cells or gametes may lead to a solution for the treatment of infertility. In this article, we review the recent advances in research into formation of primordial germ cells (PGCs), which are the precursors of gametes, in vitro.*

Key words: *Embryonic stem cells, Germ cells, In vitro differentiation, Assisted reproductive technology*

Introduction

One of the most common causes of male reproductive defects such as azoospermia or oligospermias is abnormal germ cell differentiation. In females, one important cause of infertility is the quality and quantity of the oocyte reserve in the ovary. Basic research into germ cell development helps to define cytokines, genes and the molecular mechanisms responsible for the development, function and regulation of the male and female reproductive systems. Germ cells are exceedingly specialized cells, which are capable of generating an entirely new individual. The germ cells (gametes) are decisively different from somatic cells because they have a haploid number of chromosomes that are normally generated during the cellular division known as meiosis.

In mammals, germ cells develop in the embryo and become the cells that make up the reproductive system in males and females. Germ cells are first created in a 6.5-day embryo, and at this stage they are called primordial germ cells (PGCs) [1]. In the mouse, the PGCs are first clearly distinguishable as a very small pool of cells having high alkaline phosphatase expression at 7.25 days post-coitum (dpc), during the early post-implantation period, in the inner or mesodermal component of the wall of the secondary yolk sac [2]. By 8.5 dpc, these cells are located in the wall of the yolk sac close to the base of the allantois and at the caudal end of the primitive streak. The PGCs increase in number mitotically, and the majority of the PGCs reach the gonadal ridge by about 10.5–11.5 dpc [2, 3]. Then, in the genital ridges at 13.5 dpc, the number of PGCs may be as many as 25,000 and some start to differentiate into their own sex; i.e., female PGCs start meiotic cell division, while male PGCs continue mitotic cell division [4]. In the female at 13.5 dpc, the germ cells cluster and are uniformly distributed throughout the fetal ovary. At about this stage of ovarian development, evidence of primary meiosis is seen in the cells at the surface of the gonad. However the meiosis stops at the diplotene stage of the first meiotic cell division. In the male, at about this time, the PGCs are in the primary testicular cords and undergo mitotic proliferation forming prespermatogonia. At about 17–18 dpc, these cells are destined to give rise to spermatogonia.

The investigation of primordial germ cell (PGC) specification is the first important step in the process of elucidating the mechanisms of germ cell development. At present, two modes of the germ cell specification have been described. PGCs specification in Drosophila, amphibians or zebrafish appears to depend on inheritance of a specific asymmetrically localized germ plasm that

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contains several RNA molecules, such as *vasa* and *nanos*, which direct cells to the germ-line lineage [5–7]. On the other hand, in mammals, it is thought that germ cells, specification depends on functions of cytokines, such as those of the bone morphogenetic protein (BMP) family [8]. BMP signals are known to be essential for the specification and proliferation of primordial germ cells, and its functions have been confirmed *in vitro*. Research into extracellular factors, which are essential for germ cell specification, such as BMPs, or into more fundamental mechanisms of germ cell specification have important meaning since it could directly lead to the induction of germ cells from more primitive stem cells *in vitro*.

In vitro production of gametes by induction from the stem cells has become a very important aspect of the search for an alternative tool for infertility treatment, because elucidating the mechanisms of germ cell development in humans is very difficult, since many of the formative events are completed before birth. One of the most promising technologies aimed at directly addressing germ cell differentiation involves the use of pluripotent stem cells such as embryonic stem cells to generate germ cells *in vitro*.

Derivation of Germ Cells from Mouse ESCs

To date, the most used source of germ cell formation *in vitro* is embryonic stem cells (ESCs), which are established from the inner cell mass of preimplantation blastocysts [9–12]. Especially, it is well established that mouse embryonic stem cells can be differentiated *in vitro* into primordial germinal cells. The initial reports of the induction of PGCs from mouse ESCs were in 2003 when Toyooka *et al*., described the production of mouse male germ cells, and Hubner *et al*., reported successfully obtaining oocyte-like structure *in vitro* [13, 14].

Derivation of male germ cells in vitro

Toyooka *et al.* first reported male germ cells from mouse ESCs [13]. They produced germ cell−specific Mvh-GFP and LacZ knock-in mouse ESCs, induced embryoid body (EB) formation and isolated PGC-like cells using fluorescence activated cell sorting (FACS). These cells possessed the potential to form sperm-like cells when transplanted into infertile mouse testes. Geijsen *et al*. also reported EBs contain PGC-like cells [15]. In long-term culture of EBs, meiosis-specific FE-J1−positive cells appeared in EBs, and one third of these cells were haploid. When these haploid cells were injected into MII oocytes, 20% of the oocytes developed into blastocysts. These two reports indicate that ESCs can spontaneously differentiate into male germ cells possessing the haploid genome.

In 2006, Nayerinia *et al.* first succeeded in producing offspring using artificial germ cells induced from ESCs [16]. They used mouse ESCs containing meiosis-specific Stra8-GFP and sperm-specific Protamin-DsRed. In their experiment, they first induced GFP-positive cells by culturing the ESCs in retinoic acid containing medium. By repeating this procedure, they finally obtained DsRed positive sperm-like cells. When these sperm-like cells were injected into oocytes by intracytoplasmic sperm injection (ICSI), 65 oocytes developed into blastocysts and 12 offspring were born. This is the only report that has demonstrated that gametes derived from ESCs *in vitro* have the potential to produce offspring. Unfortunately, the newborn mice died within five months because of unidentified abnormalities.

Derivation of female germ cells in vitro

Female germ cell differentiation from ESCs was first demonstrated by Hubner *et al* [14]. They introduced an Oct4ΔPE-GFP transgene that enabled germ cell-specific GFP observation of mouse ESCs, and detected PGC-like cells as GFP−positive cells. Interestingly, in their observation, the GFP-positive primitive germ cells spontaneously detached from the adhering differentiated ESC-derivatives, and the dethatched cells formed ovarian follicle-like structures that contained oocyte-like cells in subsequent *in vitro* culture in the medium used for *in vitro* maturation of ovarian immature oocytes. Surprisingly some of the oocyte-like cells could be parthenogenetically activated and developed into blastocysts. This report of Hubner *et al*. indicates that mouse ESCs can spontaneously differentiate into germ cell lineage in adherent conditions, and if conditions are suitable, these germ cells can further differentiate to the gamete, which can activate and initiate early development. Lacham-Kaplan *et al.* reported a follicle-like structure was formed by mouse ESCs when newborn mouse testis-conditioned medium was used as a differentiation inducer [17]. Their strategy enabled more efficient production of the germ cells, since about 80% of the EBs contained follicle-like structures. Qing *et al.* succeeded in obtaining the germ cell markers, Mvh and GDF9-expressing colonized germ cells, which contain oocyte-like cells, by co-culturing EBs with cumulus cells [18]. They described the importance of interaction with surrounding cells, which can convert the stem cells into germ cells, and this was clearly demonstrated in the study of Nicolas *et al.,* who successfully produced putative female germ cells not only by co-culturing with mouse fetal gonads, but also by directly transplanting

the stem cells into *in vivo* ovaries [19]. Unfortunately, the germ cells induced in their experiment were immature in growth, and only reached a size of approximately 30–40 *μ*m even though their morphology and gene expression were similar to normal oocytes. Most importantly, there are as yet no reports of female gametes possessing the capacities of fertilization and development to offspring, unlike *in vitro* germ cell differentiation into male cells.

Derivation of Germ Cells from Human ESCs

Our understanding of the molecular mechanisms of germ cell development in mammals has significantly progressed due to the utility of the mouse. However, the molecular regulation of human germ cell development is almost unknown, and it is very difficult to investigate due to the lack of a good model. Therefore, ESC models may contribute to understanding the early germ cell differentiation that begins at the embryonic stage. Reports of germ cell differentiation from primate ESCs are fewer than those for mouse ESCs. Clark *et al.* first reported germ cell differentiation from human ESCs in 2004 [20]. Their experimental strategy was based on the observation of the spontaneous differentiation from ESCs to PGCs, however the efficiency of the germ cell differentiation was very low. To date, more advanced results have been reported. In 2009, Kee *et al.* succeeded in producing haploid germ cell-like cells from human ESCs by forced expression of DAZ family genes in the human ESCs and cultured with BMP4 [21]. Now, it has been confirmed that BMP4 plays a crucial role in the development of PGC *in vivo* and the derivation of PGCs from ESCs in primate [22]. In the latter study, they showed that DAZL was important for initiating PGC development in human ESCs, and BOULE and DAZ have promotion effects on the progression of meiosis. More recently, West *et al.* established germ-like cell lines expressing VASA and OCT4 from human ESCs [23]. These cells possess the property of stable self-renewal on feeder cells, and very interestingly, these cells spontaneously differentiated to haploid cells by ceasing the passage. Above two studies suggest that haploid cells can be obtained also from human ESCs, but properties of fertilization and further development, including the ability to produce offspring, have yet to be demonstrated because of ethical constraints.

Important Challenges in Germ Cell Differentiation *In Vitro*

Although many studies on the derivation of PGCs from ESCs have been performed, there is very little evidence demonstrating their competence i.e., ability to generate offspring, and some results are difficult to reproduce [24]. One important potential cause of the inconsistency of the results is that heterogeneity of ESC-characteristics possibly affects the efficacy of germ cell differentiation. At present, the idea that variation of differentiation characteristics between cell-lines strongly influences the result of *in vitro* differentiation has been broadly accepted. Moreover, even in the same cell-line, and even in a single colony, heterogeneous states between individual single cells have been demonstrated [25–27]. In addition to the above, culture conditions, batch of culture medium, supplement, or technique of individual researchers may also be important factors behind the poor reproducibility of results.

Furthermore, genetic normality of germ cells derived *in vitro* is also an important issue. Nayernia *et al.* suggested that *in vitro* derived germ cells have some epigenetic abnormalities which may result in short-lived offspring. On the other hand, Novak *et al.* suggested that germ cells spontaneously derived from ESCs do not progress to meiosis because of abnormality in the synaptonemal complexes [28]. During germ cell specification and maturation, epigenetic alteration broadly occurs. X chromosome reactivation [29], exchange of H3K9 dimethylation to H3K27 trimethylation [30] and stripping of epigenetic marks on the developmentally very important genetic modifications, such as parental imprints, ensure parental origin-specific monoallelic expression of some specific genes in the next generation and establishes new marks that are different in male and female gametes, and they are performed with very rigorous control [31]. Many of these epigenetic changes are associated with acquisition of the capacity to support post-fertilization development. Therefore, fundamentally strict epigenetic control during *in vitro* germ cell induction is essential for obtaining fully functional germ cells from ESCs. Thus, it will be vital to elucidate the detailed mechanisms of the epigenetic controls.

A Novel Alternative Source for Germ Cell Generation

Although ESCs may be a great research tool for germ cell development, the use of these cells creates some problems: ethical problems associated with the destruction of embryos in the establishment of new cell-lines, immunological rejection when transplanted to patients, and the fact that ESCs cannot be established from severe infertile patients. To address these problems, somatic cell nuclear transfer (SCNT) is being considered as an

alternative method for creating ESCs. SCNT enables the generation of embryonic stem cells (ESCs) that genetically match the donor making it possible to use them in the treatment of refractory diseases through cell replacement therapies or to donate patient-specific germ cells via *in vitro* differentiation. Surprisingly, Mizutani *et al.* actually achieved the generation of offspring from infertile mice by SCNT-ESC techniques. However, this technique cannot avoid ethical limitations because the cell line was established using embryos [32].

Induced pluripotent stem cells (iPSCs) have also attracted a great deal of attention as an ethically unproblematic alternative to ESCs [33], since iPSCs can be produced from somatic cells of patients, such as skin or blood cells, by the routine introduction of three or four genes that reprogram these somatic cells to an embryonic-like state, from which they are able to turn into any type of cell, like ESCs. Importantly, iPSCs raise the possibility that they may not only be a novel source of infertile patient-specific gametes, but also of differentiating derivatives for *in vitro* models recreating a disease, which could be used in research to elucidate mechanisms of disease development or drug discovery and development applications. Currently, a number of studies have reported successfully generating patient-specific iPSC-lines from individuals with different diseases. In order to model the disease, reliable and efficacious protocols are required to control the differentiation to the desired cell type. To date, some research into the production of germ cells from iPSCs *in vitro* has been initiated, and the successful production of the haploid cells has already been reported [34]. Very recently, Hayashi *et al.* reported the successful derivation of PGCs from mouse ESCs and iPSCs *in vitro.* These PGCs could develop into fertile sperms when transplanted into *in vivo* testes, and Hayashi *et al.* were the first to succeed in obtaining newborns from stem cell-derived sperm [35]. On the other hand, some problems have also been suggested. Park *et al.* observed that the genomic imprints of the iPS-derived PGC were incomplete [36]. They suggested that errors in epigenetic modification may occur during the reprogramming for the iPS-derivation, which may result in the failure of the PGCs. Furthermore, it has also been elucidated that the characteristics of the iPSCs are markedly diverse between each cell-line [37]. This can directly influence the property of germ cell differentiation and is a serious risk factor, reducing the reproducibility and reliability of the experiment or treatment. In light of the problems with iPSCs, and the current difficulties with germ cell derivation from stem cells, more impairments may become visible when the technology is applied to more advanced animals like humans; for example, it may bring about higher function disorders, which is difficult to detect in the rodent models. Thus, much more detailed and careful elucidation of the stem cell and germ cell characteristics including epigenetic modifications, as well as accumulation of knowledge using non-human primates are essential.

Conclusion

We are just beginning the study of methods for generating germ cells *in vitro* and many key questions remain. Nevertheless, the potential advantages of stem cells in reproductive biology and medicine are apparent. Unlimited supplies of the ESCs and/or iPSCs and their potential to derive multiple lineages will have an enormous impact on future medicine including ART. Furthermore, stem cell-derived gametes could open a new field of technology. If these oocytes can reprogram somatic cell nuclei and successfully progress early development, patient-specific ESCs resulting from SCNT could be created without much attention to the source.

Owing to recent advances in stem cell biology, various types of stem cells are available, and we can easily access enormous amounts of information on molecular signaling related to stemness or control of differentiation programs. Although, knowledge of germ cell development is continuously accumulating. The process of germ cell formation is complicated. In normal gametogenesis, both sperm development and oocyte growth and maturation require support structures such as Sertoli cells, theca or granulosa cells. Understanding of the support niche for germ cell formation *in vivo* is essential for obtaining “functional” germ cells at “more advanced stages”. The recent remarkable achievement of Sato *et al.* in recreating complete spermatogenesis *in vitro* from pre-meiotic spermatocyte in *in vitro* organ culture has been reported [38]. Feedback of the knowledge and methodology of these studies will advance the path to “more advanced” and “functional” germ cell derivation from stem cells. Although many challenges remain ahead, the resulting generation of gametes using these approaches may improve the future of reproductive biology and medicine.

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