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RESEARCH HIGHLIGHT

Gametogenesis in a dish

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Recent progress in the induced pluripotent stem cell (iPSC) field as well as the establishment of germline stem cell isolation and culture methodologies may provide an *in vitro* platform for the study of physiological and pathological human gamete development and open new avenues for cell replacement-based personalized treatment of infertility.

Human infertility affects many couples worldwide. Based on a report from the World Health Organization (WHO), about 10%-15% of the reproductive aged population is infertile and around 25% of married women of reproductive age in developed countries suffer from infertility. Human infertility is frequently linked to defective gamete (oocyte or sperm) development, which can be triggered by genetic mutations or by environmental factors. In addition, high dose anti-cancer treatments also lead to gamete damage, and consequently leave patients with transient or permanent infertility. For patients with reduced oocyte or sperm quality, gamete donation, a process that is accompanied by a series of ethical, personal and legal concerns, is the only available option. Recent exciting achievements in gamete *in vitro* formation from either gonadal stem cells or pluripotent stem cells, including embryonic stem cells (ESCs) and iPSCs, have not only broadened our knowledge of human gamete development and related disorders, but also may hold great promise for the development of alternative infertility treatments.

In males, continual spermatogenesis during adult life is dependent on an adult germline stem cell population known as spermatogonial stem cells (SSCs). These cells reside at the base of seminiferous tubules of the testes, can be identified by their ability to generate a colony through spermatogenesis after transplantation into the testes of germ cell-deficient recipients [1], and can be isolated from testes and maintained and cultured *in vitro* [2]. Contrary to this proven experimental observation, the existence of female germline stem cells has remained controversial for many years. Indeed, it has been a longstanding belief that female mammals lose the capability of restoring their oocyte population after birth. This assumption was greatly challenged by Johnson *et al*. [3] in 2004, when they identified oogonial stem cells (OSCs) that sustain oocyte generation in both juvenile and adult mouse ovaries. Subsequent studies by Zou *et al*. [4] showed that these female germline stem cells could be isolated from adult mouse ovaries by immunomagnetic bead sorting and expanded long term *in vitro*. However, the identification, isolation and *in vitro* culture conditions of human OSCs remained to be fully elucidated. Recently, White *et al*. [5] reported the isolation of OSCs from both mouse ovaries and human ovarian cortical tissue of reproductive-aged women. They used an antibody against the surface variant of the DEAD box polypeptide 4 (Ddx4), a germ cell-specific RNA helicase, to detect the putative OSCs in the dissociated ovarian tissue. Utilizing the externally exposed epitope of Ddx4, they purified the viable OSCs via a fluorescence-activated cell sorting (FACS)-based protocol. Compared to the previous study that used magnetic bead sorting to isolate mouse OSCs [4], this FACS-based protocol decreased the possibility of contamination with non-targeted cells and dead or damaged cells, and also allowed for the measurement of other cellular properties of the sorted OSCs. This study also demonstrated that the isolated OSCs are actively dividing and can be expanded for months in a culture dish. More importantly, they can spontaneously generate oocytes under appropriate *in vitro* and *in vivo* conditions. This work strongly supports earlier studies [3, 4], which indicated that the ovaries of female mammals maintain the ability to produce oocytes during adulthood. Altogether, these recent observations may have revolutionary implications for human health, including infertility treatments and fertility preservation

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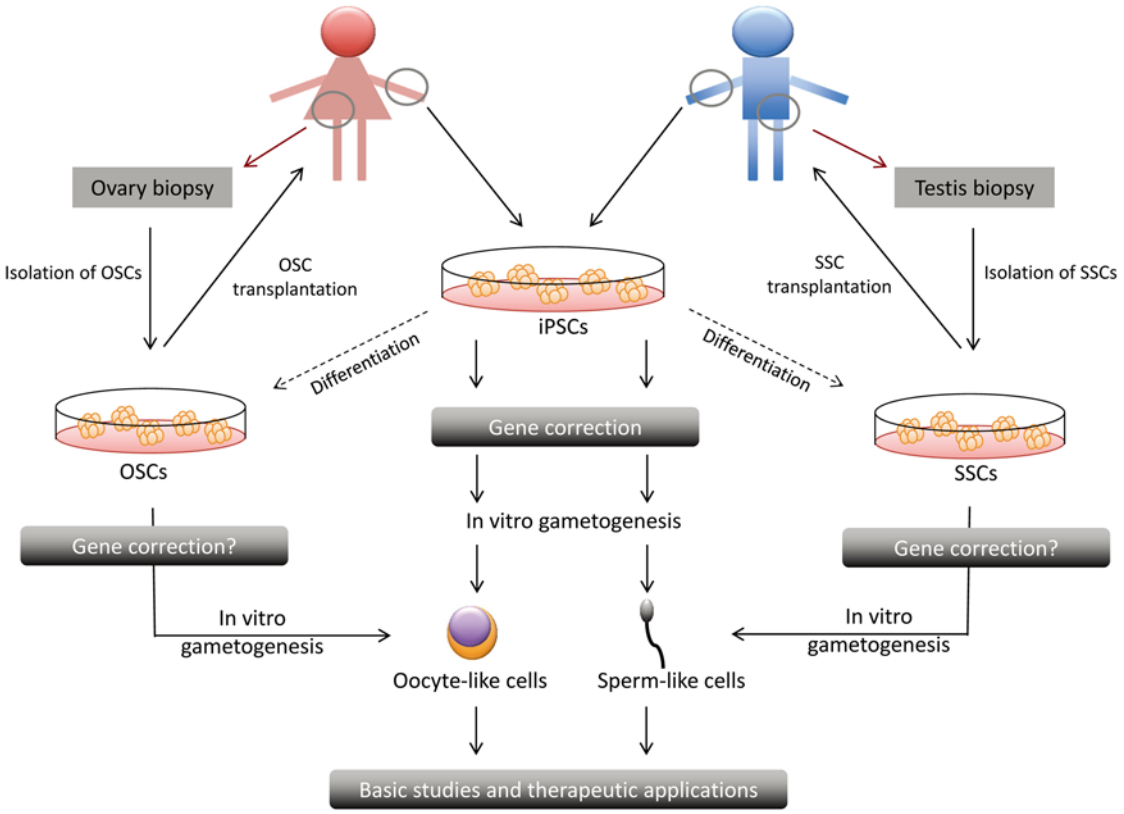

Figure 1 Proposed model for *in vitro* gametogenesis from iPSCs or germline stem cells. The germline stem cells (SSCs from males, or OSC from females) could be isolated, cultured, and transplanted back to patients to restore fertility. Meanwhile, *in vitro* gametogenesis could be induced from both germline stem cells and patients-derived iPSCs to generate oocytes and sperm for both research and therapeutic purposes. Gene editing could be applied in cultured iPSCs, and germline stem cells to correct disease-related mutations. iPSCs: induced pluripotent stem cells. SSCs: spermatogonial stem cells. OSCs: oogonial stem cells.

for female patients receiving cancer treatments. Animal models, especially the mouse, together with *in vitro* approaches, including research on ESCs and iPSCs, have and will provide the conceptual and practical knowledge necessary for these expectations to be realized.

For instance, for patients with defects in early germ cell development, research on ESC and iPSCs opens a hopeful alternative for gamete regeneration. A number of labs have reported the *in vitro* derivation of germ cell lineages from murine ESCs [6-10]. For female gamete generation, Hubner *et al*. [6] differentiated mouse ESCs containing a GFP reporter under the control of a truncated, germ cell-specific promoter for Oct4 in an adherent culture system, and enriched the GFP-positive aggregates for further culture for several weeks. They subsequently observed the formation of multicellular structures resembling ovarian follicles, as well as the extrusion of oocyte-like cells from these structures. Although the oocytes generated in their culture system bear parthenogenesis activity, there was no evidence indicating that these oocytes could be fertilized. In contrast to the 2D culture method employed for derivation of female gametes, two other groups [7, 8] created male germ cells from mouse ESCs through 3-dimensional embryoid body (EB) culture. The male germ cells generated from the EB culture could either express spermatozoid markers when transplanted into the testis of male mice [7] or fertilize oocytes by intracytoplasmic injection [8], raising the possibility that these *in vitro*-derived male germ cells may be functional to generate offspring. Indeed, the work from Nayernia *et al*. [9] showed that the mouse ESC-derived male gametes could generate live offspring, but the pups had incorrect imprinting profiles, and most of them died in the first month, suggesting that the imprinting status and global demethylation and re-methylation events should be carefully analyzed in the ESC-derived gametes. In this regard, Hayashi *et al*. [10]developed a method to reprogram mouse ESCs into germ cells *in vitro*, in a way that represents the key molecular and genetic events that occur

during *in vivo* germ cell development. Given that germ cells are formed in the embryonic epiblast, they first converted mouse ESCs into epiblast-like cells, and then further to germ cells. Analysis of the global transcription profiles, epigenetic profiles, and the cellular dynamics during germ cell induction revealed that the generated germ cells are closely related to those in the mouse embryo [10]. Additionally, these ESC-derived germ cells undergo functional spermatogenesis when transplanted into germ cell-deficient mouse testis. Sperm derived from these transplanted germ cells are capable of fertilizing oocytes *in vitro*, and further generating viable offspring that are developmentally normal and fertile [10]. More interestingly, a very recent study showed that androgenetic haploid mouse ESCs can be derived and genetically manipulated *in vitro* [11]. Interestingly, these cells, when injected into oocytes, can give rise to fertile adult mice, suggesting that these sperm-like ESCs can be used as a genetically tractable alternative for *in vitro* fertilization [11]. Together, all these mouse studies not only offer *in vitro* models for basic research of germ cell development, but also raise the exciting possibility of whether such methods could be used to derive gametes from human stem cells.

Attempts to derive gametes from human stem cells have attracted the attention of numerous researchers. In 2004, Clark *et al*. [12] demonstrated that human ESCs can spontaneously differentiate into germ cells in EB cultures, but at a low frequency. Subsequent studies have tried different strategies to increase the efficiency of germ cell formation, including the addition of growth factors that are known to be critical for germ cell development in mice, the enrichment of germ cell-like populations using germ cell-specific surface markers, and co-culture with human fetal gonadal cells. However, the cells generated using these methods were still limited to the early stages of the germ cell lineage before entry into meiosis. Recently, Kee *et al*. [13] reported that overexpression of members of the *DAZ* (*Deleted in AZoospermia*) gene family, *DAZ*, *DAZL*, and *BOULE*, could promote human ESCs to generate post-meiosis haploid cells, providing exciting evidence that human ESCs can also generate post-meiosis gametes in culture. Along with new developments in the iPSC field, current efforts in many labs are aimed at deriving gametes from human iPSCs. In 2011, Panula *et al.* [14] reported their successful derivation of male gametes from human fetal- and adult-derived iPSCs. Meanwhile, Eguizabal *et al.* [15] obtained haploid cells from both cord blood-derived human iPSCs and keratinocyte-derived human iPSCs. These studies undoubtedly bring us a step closer to the generation of personalized gametes.

However, given the inefficiency of terminal gametogenesis from human stem cells (germline stem cells, ESCs and iPSCs) *in vitro*, and the lack of a feasible functional assay, using human stem cell-derived gametes to treat infertility is still more hypothetical than a reality. Future studies will be focused on the establishment of robust and reproducible methods to induce terminal gametogenesis. In addition, several key scientific issues still remain to be determined. i) Are the epigenetic marks of the derived germ cells completely reset to a status equivalent to those of their physiological counterparts? In particular, most of the current studies in humans focus on verifying only one known paternal imprinted gene (*H19*) in their germline derivatives [13, 15], which cannot represent all of the important imprinted loci required for maintaining a functional germline identity. In this regard, genome-wide epigenetic analyses, including DNA methylation and histone modifications, should be included when characterizing these converted cells. ii) Will the genetic integrity be compromised during reprogramming, meitosis, or long-term *in vitro* culture? Indeed, chromatin abnormalities and genomic mutations caused by reprogramming/programming and *in vitro* serial passaging have been found in both pluripotent stem cells and adult stem cells, which hold the risk of carcinogenesis. iii) Are the cell surface antigens expressed on the isolated or converted germline stem cells totally compatible to that required for transplantation? It has been reported that even isogenic iPSC lines can cause immuno-rejection in an autologous transplantation experiment [16]. Thus, a more careful probing and characterization of human germline cell-specific hallmarks is necessary before the consideration of clinical applications. iv) Can the mouse-human species difference affect the translation of the current knowledge derived from work in mouse to human studies? For example, mouse iPSCs demonstrate a "native" pluripotent status, whereas human iPSCs are more close to an epiblast state. Since many properties including signaling system and X-chromatin reactivation differ between the two species, in principle, the methodologies established from mouse studies will need to be adjusted for humans. v) Can human germline stem cells be induced into functional gametes *in vitro*? Given that both SSCs and OSCs can be isolated and cultured [2, 5], and that the complete spermatogenesis can be induced from murine SSCs in an *in vitro* organ culture system [17], it is possible that human germline stem cells may offer a more efficient way to generate personalized gametes. vi) Could one envision methodologies for the direct conversion from human somatic cells to a germline-like cell status? These might be a better cell replacement since the potential safety concerns associated with induced pluripotency could be bypassed.

Despite these and other obstacles, the recent discoveries open up a new era in

reproductive biology and regenerative medicine (Figure 1). Gametogenesis in a dish may provide a useful model for the screening of drugs that improve gamete activity, or toxins that are potentially harmful to human fertility. In addition, the *in vitro* culture conditions employed in these studies, together with recently published methods in gene editing [18], make it possible to correct genes in patient-derived iPSCs or even germline stem cells, thus enabling the establishment of disease models for infertility caused by genetic mutations, or more optimistically, to generate gametes free of disease-related mutations for future therapy.

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