State of the Science for Generating Gametes from Pluripotent Stem Cells

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Introduction

The generation of gametes (eggs and sperm) in the laboratory from pluripotent stem cells (PSCs) holds tremendous promise as a basic research tool to understand the cell and molecular foundations of fertility and also causes of infertility. However, a byproduct of this research is the generation of gametes that could be used for reproductive purposes, which raises considerable ethical and policy considerations. In 2008 The Hinxton Group (an International Consortium on Stem Cells, Ethics and Law) created a consensus statement on the Science, Ethics and Policy Challenges of Pluripotent Stem Cell (PSC) derived gametes. At that time the state-of-the science for generating gametes was mostly descriptive, with evidence that immature progenitor gametes referred to as primordial germ cells (PGCs) could be differentiated from PSCs *in vitro*. In the seven years since this policy statement was published, tremendous progress has been made on the production of gametes from PSCs, particularly in the mouse model. Specifically, the field has now shown that male and female gametes generated from PSCs are functional following fertilization resulting in the birth of healthy fertile offspring [1,2]. Given the pace of discovery in the mouse model, the production of gametes from human PSCs is now on the horizon.

The germline and gametogenesis

The term "germline" refers to the lineage of cells in the body responsible for making eggs in the ovary and sperm in the testis. "Gametogenesis" is a term that refers to the final stages

of germline differentiation involving the complex process of meiosis creating the haploid egg and sperm cells. Although eggs and sperm are normally used for reproductive purposes only after puberty, the genesis of germline cells begins during prenatal life with the specification of PGCs from a primitive embryonic layer called the epiblast. PGCs then proliferate and undergo a complex series of differentiation events before becoming quiescent as spermatogonia in boys, or immature oocytes in girls. These male and female germline cells remain quiescent until puberty where gametogenesis begins in the testis or ovary respectively to create the gametes. Therefore, gametogenesis represents the final "finishing" event in germline differentiation with a considerable amount of germline development occurring during prenatal life.

Scientists understand very little about the earliest events in human germline differentiation, especially during the prenatal period. In mouse, the timing of PGC specification has been narrowed down to a window of embryonic development corresponding to embryonic (E) day 6.0-6.25 post fertilization, which is soon after the blastocyst implants into the uterus. Induction of PGCs from the epiblast in the mouse embryo requires inductive signaling from the extra embryonic ectoderm. The major signaling pathway that drives mouse PGC specification from the epiblast is bone morphogenetic protein 4 (BMP4) [3].

Initial evidence that PGCs can be induced from the epiblast came from elegant studies with isolation and culture of epiblasts from time-mated mouse embryos. In these studies, competence to generate PGCs was acquired by the cultured epiblasts between E6.0-E6.5

following contact with the extra embryonic ectoderm, a known source of BMP4 [4]. Building on this work, it was shown that BMP4 was sufficient to induce PGC like cells (PGCLCs) from E6.0 epiblasts in a floating culture system. PGCLCs differentiated in this manner are diploid and incapable of fertilization, instead requiring additional differentiation as well as gametogenesis to become sperm[5]. This was achieved by transplanting the male PGCLCs directly into the seminiferous tubules of a neonatal mouse testis, or by transplanting as a "reconstructed gonad" (a mixture of male PGCLCs with male embryonic testicular somatic cells) under the capsule of a mouse adult testis. Remarkably PGCLC-derived sperm could be isolated from these transplants and used to fertilize oocytes resulting in the birth of live healthy young.

Generation of functional gametes using mouse PSCs

Once the signaling pathways were discovered for differentiating PGCLCs from epiblast *in vitro*, the same principles were applied to differentiate PGCLCs from male and female PSCs. Specifically, the first step involved differentiating the PSCs into an Epiblast like cell (EpiLC) reminiscent of the successful epiblast studies described above [1,5]. Using this approach both male and female PGCLCs have now been differentiated from male and female ESC lines respectively through an EpiLC intermediate followed by PGCLC differentiation in a floating aggregate [1,2]. Not every cell type in a floating aggregate is a PGCLC, instead PGCLCs must be harvested from the aggregate using either genetically modified ESC lines that contain transgenes distinguishing PGCLCs from non-PGCs, or by the use of surface receptors such as State Specific Embryonic Antigen 1 (SSEA1) and Integrin-β3. PGCs have also been differentiated *in vitro* using SSEA1 and cKIT surface receptors following spontaneous

differentiation [6,7]. Thus, the identification of PGCLCs *in vitro* does not require the genetic modification of ESC lines.

Although, the generation of PGCLCs *in vitro* from mouse PSCs is a robust protocol, the cells are still diploid, immature, and not yet capable of gametogenesis. In order for the PGCLCs to differentiate and undergo gametogenesis testicular or ovarian somatic cells are required. With regard to the generation of sperm, one study revealed that the isolation and transplantation of PGCLCs into the seminiferous tubules of newborn mice promoted gametogenesis and sperm production similar to the studies with purified epiblast [1,5]. Intracytoplasmic sperm injection of the PGCLC-derived sperm into wild type oocytes resulted in generation of zygotes and blastocysts that when transferred into foster mothers resulted in the birth of healthy offspring with normal placentas and patterns of imprinted genes. These offspring developed into healthy and fertile adults [1].

With regard to the generation of oocytes, female PGCLCs differentiated from EpiLCs are capable of generating immature oocytes after combination with embryonic ovarian somatic cells in a reconstructed ovary [2]. Similar to the reconstructed testis, this reconstructed ovary does not develop *in vitro,* instead it is placed under the gonad capsule (in this case the ovarian capsule) of a recipient animal to promote PGCLC gametogenesis into immature oocytes. In order to complete gametogenesis, the PGCLC-derived oocytes are harvested from the reconstructed ovary and subject to *in vitro* maturation followed by *in vitro* fertilization with wild type sperm [2]. Remarkably some of the fertilized oocytes become blastocysts resulting in the birth of healthy offspring following transfer into foster mothers.

However, many of the fertilized oocytes do not progress beyond the zygote stage due to failure in meiotic chromosome segregation. Therefore, more research is required to understand why oocytes derived from female PGCLCs exhibit increased risk for meiotic errors, and whether this same phenomenon will occur with oocytes derived from human PSCs.

Differentiation of gametes from human PSCs

Based on the successful studies with mouse PSCs, the search for conditions to differentiate human PSCs into gametes has been invigorated. Spontaneous differentiation in the presence of serum results in low-efficiency PGCLC differentiation (<1%) [8]. However three recent approaches using directed differentiation in the presence of cytokines have reported robust high-efficiency production of PGCLCs from human PSCs [9-11]. The three approaches are similar in that they are all based on the signaling principles developed in the mouse PSC differentiation protocol. A common theme in all three protocols involved the differentiation of human PGCLCs in floating aggregate cultures containing BMP4. A second common discovery was that PGCLCs differentiated from cells expressing brachyury [9-11]. Despite the similarities with mouse, an important discovery was that PGCLC differentiation from human PSCs required the transcription factor SOX17, whereas Sox17 is not required to generate mouse PGCs [10]. This discovery points to the importance of studying human germline development using human cells, and presents an excellent example illustrating that although the major signaling principles for PGC fate are similar between mouse and human, the transcription factor network downstream is different. Therefore, additional research using human PSCs to uncover conserved and divergent approaches to human

germline differentiation are required. This will provide a more comprehensive understanding of the cell and molecular foundation of human fertility, and the utility of translating findings in mouse models to specific human reproduction research questions.

Given the publication of robust PGCLC differentiation protocols from human PSCs, the next step involves promoting the differentiation of human PGCLCs into gametes. Lessons from the mouse revealed that this is achieved by combining PGCLCs with embryonic gonadal somatic cells followed by transplantation of reconstructed gonads into host recipients [1,2]. This poses two significant challenges for the generation of human eggs and sperm from PSCs. The first is the source of embryonic/fetal human supporting cells. The most obvious source will be the *in vitro* differentiation of testicular and ovarian supporting cells from PSCs. However, it is unclear which of the supporting cells in the testis and ovary will be sufficient to recreate a reconstructed gonad. An alternate source will be to use de-identified fetal tissue following elected terminations. However, this source poses logistical challenges with the inability to time *in vitro* PGCLC differentiation with the random acquisition of precious fetal gonadal tissue.

The second major challenge is the recipient animal of the reconstructed human gonad. Mouse studies indicate that differentiation of reconstructed gonads and gametogenesis of PGCLCs requires transplantation into either an adult testis or adult ovary. Could a large animal model be used to support this experiment? Finally, if spermatozoa or oocytes are achieved, how will the function of these cells be tested? Could embryos be created for research purposes to study the pre-implantation stages of human development with no

intention of transfer? These experiments are permitted in the United Kingdom with a research license from the Human Fertilization and Embryology Authority. However, in the United States these experiments are not allowed using research funds or laboratory space supported by the National Institute of Health. Another consideration are whether these experiments are premature without first repeating the entire process of PGCLC differentiation and production of gametes using a species closely related to humans such as non-human primates? Alternatively, should non-human primates or other species be used to create interspecies chimeras with human PSCs as a viable approach for generating human gametes? Does the field need to go back to the mouse model to figure out if it is possible to promote gametogenesis entirely *in vitro* without the need for supporting cells or transplantation? Given that germ cell differentiation has never been achieved from progenitor stage human germline cells isolated from the body, the possibility of achieving gametogenesis entirely *in vitro* seems unlikely.

Conclusions

Since the 2008 Hinxton Group meeting to discuss the science, ethics and policy concerns of PSC-derived gametes, substantial scientific progress has been made necessitating the revisiting of the position statement. Today the field has generated the critical evidence that mouse PSCs can be differentiated into functional gametes capable of supporting the birth of offspring that are healthy and fertile. Furthermore, the earliest stages of human germline differentiation can now be achieved efficiently using directed differentiation. However, despite this tremendous progress significant hurdles still remain for coaxing immature human PGCLCs into fertilization ready gametes. Navigating these complex challenges,

some of which may involve policy changes will require ongoing conversations with key stakeholders on the importance of human fertility and infertility research. Only then can this line of basic research towards a functional human gamete for research follow a prudent path forward while adhering to the highest standards of quality and integrity.

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