Overview of *In Vitro* Gametogenesis in Mice, Future Applications, and Related Social Changes and Ethics

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ABSTRACT: The purpose of this review was to introduce the *in vitro* gametogenesis and explore the ethical dilemmas that will face the international community when the technology becomes clinically useful. *In vitro* gametogenesis is a developing area of study that aims to generate gametes partially or fully in the laboratory setting. Establishing reliable IVG methods requires a strong knowledge of the genetic, epigenetic and environmental regulators and conditions that drive the gamete development, which to date, is still patchy. The author has therefore created a detailed outline of the gametogenic timeline in mice, complete with known regulatory genes and epigenetic states. Various culture systems have been created towards this aim with limited success; however, some significant milestones have been met and these are reviewed here. As research progresses, we get closer to the ultimate goal of clinical application. The author presents how IVG could help a specific subset of cancer patients who have no other fertility preservations options. More controversial applications of the technology, such as multiplex, solo, and same-sex parenting are discussed along with their ethical and social consequences.

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INTRODUCTION

Oh gamete! Oh, wondrous cell, life would not exist without thee! Gametes are unique for their totipotency, a complete differentiation potential towards any cell line, and ability to transmit the entire genetic code across generations. For years, pop science news articles have talked about a technology known as *in vitro* gametogenesis (IVG) which enables the derivation of egg and sperm from somatic cells, including skin cells, entirely in the laboratory. If valid, IVG would mean the end of infertility. It would also expand the possibility of biological parenthood to combinations of people that traditionally could never conceive together, such as same sex couples or couples in which the female partner is postmenopausal and enable solo or multiplex reproduction.

While most of the topics covered in this paper have been discussed by previous authors, this paper is unique in the breadth and detail of the information covered. In section one, we explore the current understanding of *in-vivo* gametogenesis in mouse. Section two will highlight some of the studies that show the greatest potential for successful *in vitro* gametogenesis. Finally, section three establishes some of the current and future applications of IVG for scientific and reproductive purposes. Here I suggest a possible future application for IVG in an overlooked population of pediatric cancer patients, and discuss some of the social and ethical ramifications of applied IVG. How are gametes made in the body? How do they maintain totipotency throughout embryonic development when most cells become more differentiated? Can *in vitro* gametogenesis be achieved in mice? Assuming *in* vitro gametogenesis were possible, what could be done with the technology? These are just some of the questions this paper will address.

DISCUSSION

1. *In Vivo* Gametogenesis in Mice

1a. Primordial Germ Cell Origins

Gametes originate from primordial germ cells found within the early embryo. It all starts from the moment of conception. A zygote is formed from the syngamy of the egg and sperm during fertilization. This single cell immediately enters a period of rapid cell proliferation. As the number of cells increases, the volume of the fertilized ovum remains constant, causing the growing cell mass to become more compacted. By 2-3 days post coitum (dpc) the structure contains sixteen cells and is known as a morula. The first level of cell differentiation is seen in the blastocyst within the first week of embryogenesis. The blastocyst is composed of two cell types that form an outer layer around a fluid filled cavity called the blastocoel. Trophoblasts comprise the thin, outermost layer of cells and provide nourishment to the developing embryo. These cells eventually become the placenta [1]. The second cell type, the inner cell mass (ICM), gives rise to epiblasts cells which in turn, become the embryo [2]. During implantation, the blastocyst becomes embedded in the wall of the uterus. Gastrulation is the next step during which the ICM further differentiates into the three germ layers (endoderm, mesoderm, and ectoderm) [1]. Primordial germ cell (PGC) precursors arise from the proximal epiblast cells of the ICM [3]. The germ layers ultimately differentiate into all the somatic cells that make up the new organism, including the gonads, while the PGCs are destined to become the totipotent gamete [2].

Epigenetic regulation plays a major role in germ cell differentiation. The term “epigenetic” refers to the modification of gene expression caused by factors other than changes in the DNA sequence. During its lifetime, the PGC undergoes heavy epigenetic

reprogramming, typically achieved via DNA demethylation or de novo DNA methylation and histone modifications. Genetic imprints are a type of epigenetic regulation in which a cluster of genes on either the maternal or paternal chromosome are inactivated, resulting in monoallelic expression at that locus. Immediately after fertilization the paternal imprint is removed from the zygote. Following the first cell cleavage, the maternal imprint is also gradually erased. During implantation, a *de novo*, individual-specific imprint is established in embryonic cells. This imprint is preserved in somatic cell lines throughout an organism's lifetime and helps with normal differentiation and development. Unlike somatic cells, germ cell precursors undergo complete erasure of genomic imprints along with other epigenetic modifications before initiating gametogenesis. Removal of the imprint is necessary for reconstituting totipotency in the gamete [4]. During gametogenesis a new imprint is established based on gender. [5].

The journey from PGC-precursor to mature gamete is very intricate and can be broken down into three stages: (1) specification of germ cell precursors from pluripotent epiblast cells, (2) migration of PGCs to the genital ridge with simultaneous cell proliferation, and (3) colonization of the GR followed by gametogenesis [2]. The next few sections will outline these stages in detail.

1b. Specification of PGCs

Precursor germ cells originate in the proximal epiblast adjacent to the extraembryonic ectoderm at embryonic day (E)6-6.5. During gastrulation they move through a structure, known as the primitive streak, into the extra-embryonic mesoderm where they complete their specification into competent PGCs. Specification depends partly on paracrine (neighbor) signaling from the extraembryonic ectoderm. Therefore, location (proximal epiblast region)

rather than cell type (proximal epiblast cells) is key to the induction of PGCs [6]. This was proven when, after transplanting distal epiblast cells, which normally do not give rise to PGCs, into the proximal epiblast region at E6.5, a small colony of PGCs was still formed [3]. Though the exact signaling pathways involved in PGC specification are not yet fully understood [5-6], researchers have identified some key players in the process.

Bone morphogenetic protein-4 (BMP-4) is a critical factor in PGC specification as it regulates somatic and germ cell fate in epiblast cells. BMP4 is one of the paracrine signals secreted from the extraembryonic ectoderm around E5.5 – E6.5. It stimulates proximal epiblast cells to express B-lymphocyte-induced maturation protein (Blimp1, also called PR-domain containing protein 1 or Prdm1) and Prdm14. Two regulators act as intermediates in this pathway. Wnt3 is secreted by the epiblast cells in response to BMP4 and activates a mesodermal (somatic) pathway transcription factor, T. T activates Blimp1 and Prdm14 towards the germline, but it also activates somatic genes. Blimp1, Prdm14, and BMP4 all suppress these somatic genes and PGC specification begins from there [7]. Studies have shown that Prdm14 expression alone is enough to induce PGC fate [8-9]. BMP-4 also activates the transcription factor interferon-induced transmembrane protein 1 (Ifitm-3; referred to as “Fragilis” throughout this paper) [10]. For somatic differentiation, BMP4 is inhibited by its antagonist, cerberus1 (CER1), which is secreted from a part of the embryonic endoderm known as the anterior visceral endoderm (AVE). If the AVE does not form, due to a mutation, all epiblast cells from E5.5. – E6.0 differentiate into PGCs in the presence of BMP4. Bmp8b also contributes to PGC specification by suppressing AVE development [7].

During gastrulation, Blimp1 and Fragilis are upregulated as the precursor germ cells move towards the extraembryonic mesoderm. Upregulation of these genes leads to

transcriptional modification of the epiblast cells which start to show increased expression of tissue nonspecific alkaline phosphatase (TNAP) and Stella (also called PGC-7 or Dppa3), important markers of pluripotency in PGCs, by E7.0-E7.5 [11]. Blimp1 also activates the transcription factor Tcfap2c which causes downregulation of genes in the Hox gene family including hoxa1 and hoxb1. These genes play a role in somatic development [12]. Inhibiting somatic differentiation at this early stage is important for maintaining pluripotency in germ cells so that they can later regain totipotency during gametogenesis [5-6, 11]. Gene knockout studies have shown that blimp1, Tcfap2c, and prdm14 are essential for PGC specification, and thus fertility, in mammals [13].

Compared to their somatic cell counterparts PGCs have high expression of pluripotency markers such as TNAP, Oct4, stage-specific embryonic antigen-1 (SSEA-1), Nanog, and Sox2. Initially these pluripotency markers are prevalent throughout the ICM and epiblasts, but with time their expression is downregulated in somatic cells and becomes concentrated in the growing PGC colony and some pluripotent somatic cells [14-16]. These genes are used to identify PGCs generated *in vitro* as discussed below.

A cell's stage throughout the process of specification can be identified by its gene expression profile at any given time. Precursor cells in the ICM and epiblast express Oct4, TNAP, SSEA-1, Nanog, Sox2, and the hox genes. During specification, expression of Nanog and Sox2 is repressed while BMP4, Wnt3, Prdm14, Blimp1, Fragilis, and Tcfap2c are activated sequentially. Oct4 and TNAP activity remains high throughout. In committed PGCs, Nanog and Sox2 expression is reactivated, and Stella is newly expressed, while the hox genes are downregulated [17]. PGCs in all stages of specification have a round

morphology [13]. The first forty specified PGCs are visible in the extraembryonic mesoderm by E7.5 [3] and are ready to move on to phase two: migration.

Table 1. Summary of Genes and Proteins Associated with PGC Specification.

Gene	Function
BMP-4	Initiates PGC competence in proximal epiblast cells.
Blimp1 / Prdm1	Suppresses hox genes and somatic differentiation, activates the germline
Prdm14	PGC specification
Wnt3	Involved in the BMP pathway
T	Activates both somatic and germ line regulators
Ifitm-3 / Fragilis	Expressed in
CER1	Suppresses BMP4 leading to somatic cell differentiation
Bmp8b	Suppreses AVE development leading to differentiation towards the germline
Stella	Marker of specified PGCs
Tcfap2c	Suppresses the hox genes and somatic differentiation
hoxa1	Stimulates somatic differentiation
hoxb1	Stimulates somatic differentiation
Oct4	Pluripotency gene expressed in PGCs and PGC precursors
SSEA-1	Marker of PGCs and PGC precursor cells
TNAP	Marker of pluripotency and PGCs

1c. PGC Migration and Proliferation

During migration PGCs follow a distinct path as they move from the extraembryonic mesoderm to the genital ridge within the embryo. PGCs reenter the embryo by passing through the allantois, an extraembryonic membranous outgrowth of the embryo's gut. They then gather into two clusters, on the right and left sides of the gut, and move bidirectionally via the newly formed hindgut (E8-E9.5), through the dorsal mesentery, a precursor of the digestive tract, and finally arrive at the genital ridge (GR). Proliferation occurs throughout the entire migratory process so that by E12 approximately 2500-5000 cells have arrived at the GR [1].

Migration depends on at least three factors (none of which are well understood to date): molecular signaling, cell adhesion, and development of the hindgut. Somatic cells of the GR and migratory pathway secrete signal molecules known as attractant molecules by exocytosis and transmembrane transporters (like the ABC transporter protein family). G-protein coupled receptors (GPGCs), located on the cell surface of PGCs, pick up these signals and inform the cell about the concentration gradients of chemoattractant in their vicinity. In response, migratory PGCs extend polarized cytoskeletal projections known as filopodia that move the cell in the direction of the signal molecules.

In mice SDF-1 and steel factor are the two primary signal molecules involved in migration. Somatic cells of the GR secrete stromal derived factor 1 (SDF-1) a chemoattractant which is detected by GPCR chemokine (CXC motif) Receptor 4 (CXCR4). Somatic cells along the migratory pathway secrete steel factor, a ligand which binds to the c-kit receptor tyrosine kinase (RTK) on the PGC surface. The SDF-1/CXCR4 interaction controls the direction of PGC movement while c-kit RTK/steel factor binding is thought to enable movement and regulate migration rate.

Cell-cell adhesion of PGCs with themselves and with extracellular matrix proteins is also thought to contribute to PGC migration [13]. The previously mentioned filipodia form connections with other PGCs and somatic cells and move by “walking” along the cell surfaces and even penetrating cell monolayers [1]. Though the mechanisms are not well understood, several adhesion molecules have been identified as major players in PGC migration and proliferation. Epithelial cadherin (E-cad) is the adhesion molecule that regulates PGC-PGC adhesion. Prior to and following migration clusters of round PGCs are seen in the extraembryonic mesoderm and GR respectively. During migration however,

PCG-PCG adhesion is lessened [18], presumably to enable cells to migrate independently with the increased efficiency needed to transverse the numerous tissue types encountered on their journey to the GR [13]. Integin beta 1 [19] and fibronectin are also needed for migration, and PGCs lacking receptors for these molecules do not complete migration [1].

Though obvious, it is worth pointing out that since migration occurs through a pathway, it is dependent on correct development of these embryological structures. SRY-box17 (Sox17) is the transcription factor responsible for the expansion of the hindgut endoderm. PGCs in mice lacking this gene cannot reach the GR [13]. Failure to complete migration results in PGCs cell death, regulated by Nanog [20], and sterility caused by lack of gamete formation [13]. In addition to the gaps in our knowledge of the signaling pathways and molecules involved in migration, we have yet to identify the factors responsible for the initiation and loss of migratory ability in PGCs.

Migrating PGCs also have a unique gene expression. The murine vasa homolog (Mvh) protein helps PGCs maintain their cell function and assists with colonization of the GR. Many pluripotency genes such as *Oct-4*, *Nanog*, Stella, and *Sox2* are expressed throughout migration [6, 10]. Though PGCs do not produce any variation in cell product at this stage, the presence of pluripotency genes indicates the maintenance of an underlying pluripotent potential within germ cell precursors. Deleted in azoospermia-like (*DAZL*) initiates caspase (enzymes that trigger apoptosis) activity in stray PGCs to prevent formation of large tumors known as teratomas. *DAZL* is also important for sex differentiation of PGCs. *Mvh* and *DAZL* are both markers of premeiotic PGCs [5-6]. Mature germ cells at the end of migration also express *Ddx4* [16].

Migration is also the time when the epigenetic imprint is removed from germ cells [21]. Most migratory PGCs at E7.75-E8.75 are arrested in G2 of the cell cycle and RNA polymerase II dependent global transcription is temporarily inactivated. Instead of transcribing genes the cell uses this time for genome wide histone modifications such as a reduction in di-methylated Histone H3 Lysine 9 (H3K9me2) heterochromatin, and an increase in trimethylation of H3K27me3 which begin at E7.75 and E8.25 respectively [16]. Prdm14 contributes to these epigenetic changes by downregulating Euchromatic Histone Lysine N-Methyltransferases 1 and 2 (Ehmt1/2) [8]. As the PGCs move through the hindgut ectoderm, at E8.5-E9.5, they undergo demethylation of their promoter CpG DNA [16] under the control of the TET1, TET2, and TET3 genes [22]. Histone H, associated with linker DNA, is also lost during this process [21].

1d. Colonization of the Genital Ridge and Sex-specific Gametogenesis

By the time PGCs arrive at the genital ridge, gonadal sex differentiation has already been initiated, and in this environment, the PGCs begin their specification into oocytes or spermatogonia. Colonization happens around E11.5 – E12.5 and there are now 24,000 PGCs in the mouse genital ridge [5-6]. PGCs acquire their gametogenic competence at the GR and commit towards male or female sex cell fate in response to somatic signals from the differentiated gonad [23]. Gonadal differentiation is controlled by the SRY (sex determining region of the Y) gene associated with the Y chromosome. Expression begins around E10.5 in mice and stimulates formation of the male reproductive system. In the absence of SRY, the female reproductive tract automatically develops after E 11.4 [24-25]. In the gonad, supporting cells nourish the developing gamete and steroidogenic cells regulate gamete development through the production of steroid hormones. Sertoli (male) and granulosa

(female) cells are types of supporting cells. Leydig (male) and theca (female) cells are the primary steroidogenic cells [25].

In males, PGCs go through a couple rounds of mitosis and then arrest in G0 of the cell cycle until shortly after birth when gametogenesis finally happens. Beginning on E14.5 prospermatogonia spend this period of mitotic arrest involved in extensive epigenetic reprogramming including reestablishment of a de novo paternal imprint. Most prospermatogonia initiate meiosis by five days after birth and haploid spermatids are present by three weeks post-partum. Some prospermatogonia differentiate into spermatogonial stem cells (SSCs) which have the usual properties of stem cells, namely the ability to simultaneously self-replicate and develop into a more differentiated cell type- the primary spermatocyte. Starting in puberty, SSCs differentiate into spermatogonia which complete meiosis to provide haploid spermatids. Sertoli cells add a flagellum and sperm head and the spermatid becomes a mature spermatozoon ready to fertilize an egg [26]. There are many regulatory genes involved in spermatogenesis, and I will mention just a few here. Nanos2 is a gene noteworthy for its role in differentiation of prospermatogonia. Plzf is a transcription factor that maintains SSCs [25]. TEKT1 is a marker of mature haploid gametes. Pluripotency genes like Stella and Nanog are downregulated in prospermatocytes [6].

Upon colonizing the GR, PGCs undergoes further genome wide DNA demethylation and erasure of the parental imprint. Loss of heterochromatin markers continues with the removal of H3K9me3, and H3K64me3 by E11.5 and E12.5 respectively [21]. At E13.5 the parental imprint has been fully erased and in females the second X chromosome (that was inactivated during gastrulation) is reactivated [27]. All the epigenetic reprograming throughout migration and colonization ensures that the maternal and paternal imprinting

states are essentially equivalent prior to meiosis so that germ cell development can proceed down either the female or male pathway in response to environmental signals [28].

In males a de novo paternal imprint is established when prospermatogonia are in mitotic arrest between E14.5 and birth while in females the maternal imprint forms in the period of oocyte growth mid-meiosis long after birth. De novo DNA methyltransferase (DNMT3a and DNMT3b) genes are responsible for creating the imprints in both oogenesis and spermatogenesis. This new imprint protects the integrity of the genome and ensures proper development of the zygote conceived from that gamete. Following fertilization, the oocyte dictates the epigenetics for the growing zygote. For example, high expression of TET3 is seen in oocytes and regulates demethylation of the paternal genome in zygotes, a step that happens immediately after fertilization [16]. However, the mechanisms for this are not well understood. [21].

Oogenesis in females follows a different timeline. Following several rounds of cell division, female PGCs (now called primary oocytes) immediately begin meiosis, progressing through the diplotene stage of prophase I and arrest here until birth. In the embryo there are approximately 3 million primary oocytes, but over time many of these cells undergo apoptosis so that this number is highly reduced by the onset of puberty. At birth granulosa cells form an initial thin layer around primary oocytes to make primordial follicles. Activin A (ActA), a member of the transforming growth factor β (TGF-β) super-family, promotes germ cell proliferation during the formation of primordial follicles [29]. During puberty, hormonal signals regulate the maturation of primordial follicles. Granulosa and theca cell proliferate to provide more supportive layers around the oocyte in the primary, secondary and antral follicles. The oocyte also acquires its de novo maternal imprint pattern during this growth

period. The full-sized oocyte resumes meiosis. The first polar body is extruded at the completion of meiosis I and the haploid cells are called secondary oocytes. Meiosis arrests again in metaphase II. If Fertilization occurs, the oocyte completes meiosis and the cycle of gametogenesis renews in the zygote [30].

Different regulatory genes control spermatogenesis and oogenesis and timing of meiotic entry. In female embryos retinoic acid (RA), produced by somatic mesodermal cells, activates stimulated by retinoic acid gene 8 (Stra8) which initiates meiosis. Polycombcomplex1 (PCR1) is expressed until E13.5 and inhibits Stra8 to prevent early entry into meiosis. Males also produce RA, but it is degraded by CYP26B1. In this way, prospermatogonia are blocked from entering meiosis. CYP26B1 expression is reduced in females and RA levels remain high for the initiation of meiosis. [25]. Meiotic markers SCP1, SCP2, and SCP3 are seen in prophase and indicate genetic recombination. Uhrf1 is essential for male meiosis and a recent study revealed that organisms fail to complete meiosis and are sterile without this factor [22]. Two post-meiotic markers of mature haploid gametes are TEKT1 in males and GDF9 in females [5]. Nanog is downregulated in both prospermatocytes and primary oocytes [6]. Stella, on the other hand, is downregulated only in prospermatocytes, and maintained in the developing and grown oocyte [5].

2. *In Vitro* Gametogenesis

After reviewing the process of gametogenesis as it transpires within an organism, we now look at how researchers are applying and building upon this knowledge within the *in vitro* setting. In this section we will explore some of the more influential studies of *in vitro* gametogenesis, that have succeeded in producing offspring, to establish where the technology stands today. For a more comprehensive analysis of IVG attempts throughout

history refer to [2,6,5,16,26,31]. We will look at studies that attempt to derive gametes from pluripotent and induced pluripotent stem cells, obtained from both embryonic and adult somatic and germline cells. Here again we will primarily focus on research using mice, with brief mention of attempts at IVG with human cells. In the next section the discussion becomes more theoretical as we move into future uses for IVG and their impact on society.

2a. IVG In Mice

Murine iPSCs/ESCs to PGCLCs in vitro:

The first direction in IVG was to reprogram pluripotent somatic stem cells into germline precursor cells known as primordial germ cell-like cells, or PGCLCs. There are two types of pluripotent stem cells: epiblast stem cells (EpiSCs) and embryonic stem cells (ESCs). EpiSCs are derived from the post implantation epiblast at E5.5 - E6.5 and have not yet been successfully reprogrammed towards germline fate [17]. ESCs however, can be reprogramed. Cells are obtained from the ICM preimplantation blastocyst around E 3.5- E4.5 and converted into epiblast-like cells (EpiLCs). EpiLCs are then cultured to form PGCLCs that can complete gametogenesis under certain conditions, as we will see later [32].

To establish PGCLCs in culture, ESCs must replicate three key features of *in vivo* PGC specification. These are 1) repression of the somatic program, 2) reestablishment of pluripotent potential, and 3) erasure of the epigenetic program [17]. Hayashi et al. developed a protocol that successfully achieves this end. The same results have been replicated by many groups [33-35] and their and their method has since been patented [9].

Hayashi et al. method and Results:

First, ESCs taken from the E3.5 blastocyst were transformed with vectors containing fluorescent tags for two PGC specific genes, blimp-1 and Stella (from here on this tag will be

referred to as BVSC which stands for Blimp1-mVenus and stella-ECFP [36]). Next, ESCs were cultured with ActA, basic fibroblast growth factor (bFGF), and 1% concentrated knockout serum replacement (KSR). (KSR was the original ingredient that was missing in previous studies that yielded poorer results. Concentrations greater and lower than 1% were proven ineffective for PGCLC formation). These ESCs differentiated into EpiLCs complete with the markers and flat morphology seen in the post-implantation E5.75 epiblast. To produce the PGCLCs, EpiLCs were cultured in a GK15 solution with added BMP4. PGCLCs were derived from these EpiLCs on day two of the culture and expressed characteristic PGC markers by day six in culture [32].

This process resembles many aspects of *in vivo* PGC specification. BMP4, the factor shown to be essential for germline fate in epiblast cells [7] is also needed *in vitro*. Additionally, in the same way epiblast cells have a limited time window during which they can differentiate into PGCs, EpiLCs, must be removed from their GK15-solution-with-BMP4 culture on exactly the second day for successful induction of PGCLCs. Previously it was noted that Wnt3 and T are required for Blimp1 activation that induces PGC fate. Notably EpiLCs express Wnt3 and T, and Blimp1 expression is noted after two days in culture. Unlike in the *in vivo* process, *in vitro* EpiLCs proceeded directly to PGCLC fate without first settling the extraembryonic mesoderm as seen with PGC precursor cells. By day six in culture, PGCLCs showed a similar genetic (i.e. expression of Blimp1, Prdm14, T, Stella, CXCR4, Integrin-beta3, Integrin-alphaV, KIT, N-Cadherin, and SSEA-1 among others, and repression of the hox genes) and epigenetic (i.e. reduced H3K9me2, low expression of Dnmt3, elevated H3K27me2, and global demethylation of cytosine 5mC) profile to specified PGCs in early migration. Parental imprints Snrpn and kcnq1ot1 (maternal imprint genes) and

Igf2r and H19 (paternal imprint genes) were still methylated in PGCLCs, and most cells were arrested in G2. Again, these are both characteristics of migratory PGCs [7,32]. Induced pluripotent stem cells (iPSCs) can be converted into EpiLCs and then differentiated into PGCLCs in both male [32] and female [35] mouse models.

To confirm the reproductive integrity, these PGCLCs were then transplanted into gonadal tissue where they completed gametogenesis *in vivo* and those gametes were fertilized. The process of synthesizing these semi *in vitro* gametes was essentially the same for male and female gametes, with the main differences being the genotype of the ESC (XY/XX) and transplantation into either ovarian or testicular tissue.

When transplanted into the seminiferous tubules of W/W^v mice (white spotting variant mice that are sterile due to a point mutation in the c-kit gene which prevents gamete formation [37]), XY genotype PGCLCs successfully underwent spermatogenesis that resulted in mature spermatozoa. Using intracytoplasmic sperm injection (ICSI), an *in vitro* fertilization technology in which mature sperm are injected directly into the ovum [38], these gametes contributed to the formation of healthy zygotes. The zygotes developed normally, displayed proper imprinting patterns, and were themselves fertile as adults [32].

A similar process was used in XX genotype PGCLCs. To mimic the *in vivo* genital ridge environment and induce female gamete development, PGCLCs were aggregated with female E12.5 gonadal tissue to form reconstituted ovaries *in vitro*. Here PGCLCs performed meiosis and expressed synaptonemal complex protein 3 (SCYP3), indicating proper genetic recombination, after arresting in Prophase I. When transplanted into mouse ovaries, the PGCLCs developed into oocyte like cells (OLCs) and showed high Stella expression. They continued to undergo Meiosis, stopping at Metaphase II, were fertilized using *in vitro*

fertilization, and healthy embryos were placed in a surrogate. Healthy pups with normal imprinting and fertility were born. Of note, the efficiency of the semi *in vitro* generated oocytes was low and 53% of the embryos created through IVF showed three pronuclei (PN) instead of two, as seen in normal fertilization. Further analysis showed that these oocytes had not extruded a second polar body with the completion of meiosis and most commonly exhibited an XXY genotype. The pups born through this method all originated from normal, 2PN embryos [35]. Despite the reduced efficiency, this experiment demonstrates successful acquisition of reproductive function in XX PGCLC.

Induced pluripotent stem cells (iPSCs) can be created from embryonic or adult mouse skin cells and used to generate PGCLCs *in vitro*. Tail tip fibroblasts taken from mice were transduced with pluripotency genes Oct4 and Sox2, and somatic cell reprogramming genes Klf4, and c-Myc. After some time, they developed into embryonic stem cell-like cells [39-40]. iPSCs can be converted into EpiLCs and then differentiated into PGCLCs in both male [32] and female [35] mouse models.

From PGCLC to gamete: In vitro oogenesis:

Hikabe et al. developed a method in which mature oocytes were derived from PGCLCs entirely *in vitro*. iPSCs taken from adult and embryonic murine fibroblasts were differentiated into PGCLCs using the previously described methods. A reconstituted ovary organ culture was made with the PGCLCs and somatic tissue taken from the E12.5 early ovary. Primary oocytes developed followed by secondary follicles, then antral follicles with fully grown oocytes, and these oocytes proceeded to metaphase II *in vitro*. The synaptonemal complex formed in 46.2% of the primary oocytes in prophase I, which indicates reduced meiotic efficiency during *in vitro* gametogenesis compared with the *in vivo* rates.

Additionally, the oocytes that made it to metaphase II (MII) showed aberrant gene expression, but similar maternal epigenetic imprinting patterns, as compared with *in vivo* derived oocytes. Nonetheless, when these MII oocytes were fertilized by IVF they gave rise to healthy pups. However, the fertilization success rate was also low at just 3.5% [41]. Clearly, the technique is not yet perfect and, to make the process 100% *in vitro*, gonadal tissue for the organ culture would have to be generated from PSCs instead of transplanted from an existing embryo. Still, the fact that mature oocytes were generated *in vitro* is promising for the future of IVG.

From PGCLC to gamete: In vitro spermatogenesis:

Spermatogenesis was more difficult to achieve in culture than oogenesis because of two main obstacles. In endogenous spermatogenesis, prospermatocytes differentiate into both spermatogonial stem cells (SSCs) and spermatids. Researchers have struggled for years to derive SSCs *in vitro*. Additionally, researchers could not figure out how to get past the meiotic checkpoint in prophase I that removes meiotic cells with DNA strand breaks and misaligned chromosomes which is more robust in spermatogenesis than in oogenesis [26].

A major goal in reproductive biology is to constitute SSC-like cells (SSCLCs) *in vitro*. SSCs differ from PGCs in that they can colonize adult testes. Ideally, *in vitro* derived SSCs could be transplanted into adult testes to activate spermatogenesis *in vivo*. A group of researchers successfully induced SSCLCs from ESCs that were able to complete gametogenesis and give rise to healthy, reproductively competent offspring. However, these cells displayed aberrant DNA methylation patterns and showed low efficiency when it came to fertilization. Therefore, more work needs to be done before this technique can be utilized [42]. A different study developed an organ culture system from neonatal mouse testes

containing SSCs/prospermatogonia and KSR, which supported gametogenesis *in vitro* [43]. A combination of these two studies would enable conversion of ESC to spermatozoa entirely *in vitro* (with borrowed gonadal tissue for the organ culture) but has yet to be tested.

Zhou et al. developed a method that overcame the meiotic barrier and achieved complete spermatogenesis *in vitro*. PGCLCs were created using the Hayashi et al. method. These cells expressed all the genetic and epigenetic markers of PGCs as seen *in vivo*. PGCLCs were cultured with RA, BMP2, BMP4, BMP7, and ActA to stimulate meiosis. After six days PGCLCs had completed prophase I. Hormonal regulators of meiosis including follicle stimulating hormone (FSH), bovine pituitary extract (BPE), and testosterone were added to the culture between days 6-14. By day 14 meiosis was completed and the haploid spermatid-like cells (SLCs) gave rise to fertile offspring following ICSI and IVF, and these pups displayed normal epigenetic imprint and karyotyping [34].

This study was the first to overcome the meiotic checkpoint hurdle. In 2014 Handel et al. wrote an article in which they put together a gold standard for meiotic integrity which could be applied to *in vitro* gametogenesis. The SLCs produced by Zhou et al. complete all the outlined requirements including epigenetic reprogramming, chromosomal synapsis and DNA recombination, and the formation of haploid cells at the end of meiosis [34,44]. It is surprising that imprint patterns appear normal in these SLCs, as the cells entirely skipped the quiescence and epigenetic reprogramming period normally seen in male mice from E13.5-10 days. [17,26]. If these results are validated, this method promises to be a complete, all-in-one tool for *in vitro* spermatogenesis.

2b. IVG In Humans

Our understanding of early embryonic and gamete development in the human is even more limited than in the mouse. Because of that, attempts at IVG in humans have not had much success to date. A couple groups were able to generate PGCLCs from human ESCs [45] using a similar method to Hayashi et al. Current research efforts are focused on developing a systematic culture system for human IVG. Meiosis is still a major barrier that has prevented the generation of mature gamete *in vitro* in humans [46]. Still, studies with human PGCLCs will help us learn more about gametogenesis in humans and over time, as we saw in the mouse, scientists continue to improve the technique so that one day (not too far in the future) it will become available for a myriad of applications,

3. Current and Future Applications of IVG:

While IVG technology is not currently advanced enough for safe use in humans, it has already proven to be an invaluable research tool for scientists today. By stimulating steps of gametogenesis *in vitro*, scientists can learn about the epigenetic and genetic regulation in male and female gametogenesis. For example, the intermediate genes, Wnt3 and T, of the BMP pathway, imperative for PGC competence, were discovered from experiments attempting to derive PGCs *in vitro* [47**]**. Researchers can manipulate genes or environmental conditions around ESCs to identify specific gene functions. One such gene knockout study, using *in vitro* generated PGCs, demonstrated the role of Tcfap2c in downregulating somatic line hox genes [12,43] discovered the usefulness of knockout serum replacement (KSR) for deriving gametes in organ culture through pure trial and error while manipulating the *in vitro* culture environment [48].

Once the technique is perfected, IVG will provide scientists with an endless supply of gametes that are equivalent to the endogenous egg and sperm. These gametes can be studied on their own, under different conditions as described above, or fertilized to provide embryos for stem cell and early development research. As we continue to accrue more information, we get closer to making IVG a viable technology for human fertility.

Fifteen percent of couples worldwide suffer from infertility with the underlying cause for twenty-eight percent of that population being problems with gametogenesis. Acute ovarian failure leads to early menopause and is a common cause of infertility in women. In men, azoospermia is a lack of sperm in the semen. Morphological aberrations in spermatozoa can also cause infertility [46].

At the end of 2018, I organized a Medical Ethics Society conference in Yeshiva University where reproductive endocrinologist and fertility specialist, Bat-Sheva Maslow, spoke about the unique challenges for fertility preservation in survivors of childhood cancers. Certain cancer treatments almost always lead to complete loss of fertility in the patient, she explained. These include bone marrow transplants, fully body radiation, high dose radiation to the brain (affects the hypothalamus which regulates reproductive hormones), and high doses of chemotherapy. According to the National Cancer Institute Surveys of 5 Year Relative Survival, survival rates for children diagnosed with malignancies before age fourteen rose from 58% in 1975-1977 to 84% between 2008-2014 [49]. The growing population of pediatric cancer survivors entering adulthood has necessitated a greater focus on fertility options for pediatric patients. Currently cryopreservation of gametes prior to treatment is considered the gold standard. Upon recovery, the gametes can be fertilized. Unfortunately, cryopreservation is not a viable option for everyone. Harvesting the gametes

usually takes about 2-4 weeks. This is time that a leukemia patient does not have. Prepubescent children, who lack mature gametes, also do not qualify. Alternative treatments, such as gonadal suppression (drugs are administered to shut off the reproductive system in order to minimize chemo's effect on the gonads) and modifications to cancer medications, that make them less gonadotoxic, have had limited success. A newer technique involves cryopreservation of prepubertal ovarian and testicular tissue followed by transplantation back into the gonad with return of normal function and development. However, few pregnancies, relative to the number of attempts, have been achieved to date using this method [51].

in vitro gametogenesis would solve the problem. Building on the tissue cryopreservation method introduced to me by Dr. Maslow, I see two potential uses for IVG in this population. At the time of cancer diagnosis, a small sample of either testicular or ovarian tissue would be removed and cryopreserved. Once the patient has recovered, and is ready to have children, this tissue provides a source for SSCs or primary oocytes that are present soon after birth. These gamete precursors could be matured *in vitro* into haploid gametes that can be fertilized with ICSI or IVF. This technique was already achieved with low efficiency in mice [41,43] and its application in humans may not be far off. Alternately, if the cryopreserved tissues lacked gamete precursors cells or the cells were damaged, iPSCs derived from somatic cells such as skin, could be selectively differentiated into egg or sperm and used for reproduction.

This later approach opens the parenthood door not only to those with underlying biological reproductive impairments, but also to those who are infertile due to social realities. With gene manipulation, it may be possible to reprogram the genotype of a stem cell in the future. This would enable the development of female XX ESCs into spermatids and male XY

ESC into oocytes using IVG. If this were the case, same sex couples could produce biological children that share approximately 50% of their genetic code with each parent as is seen with the progeny of heterosexual couples. Solo reproduction, in which the sperm and egg are created from the same individual, would also be a possibility. This specific application may be unadvisable however, since it increases the risk of recessive disease inheritance in the zygote from the parental heterozygous mutations. IVG also enables the formation of a child using genetic material from more than two individuals. For example, let us say that four people wanted to have a biological child together. This could be achieved through two rounds of IVG. First, two embryos would be created from two pairs of egg and sperm (with one gamete derived from each parent). PGCs from each embryo would then undergo another round of IVG. These egg and sperm would contribute to the final child, and the four parents would be its biological grandparents. Theoretically, this can be done with any number of parents using many rounds of IVG. On a more relatable note, this technology would also enable postmenopausal women to reproduce. Combined with surrogacy or, perhaps, artificial wombs in the future, IVG could be the first step towards the end of infertility. It will be interesting to see how the combination of assisted reproductive technologies (ARTs) could lead to a reproductive reality, drastically different from what we know to be true.

The consequences of applied IVG will be enormous and because of this, it is necessary to consider some of the ethical challenges before the technology is clinically useable. Imagine yourself sitting on the subway next to a famous model, athlete, or scholar. With just one hair or nail clipping, you have access to their reproductive material. It is easy

to see how this technology could get out of hand when the materials are so accessible. A person could unknowingly be made a parent without any active participation or consent.

At the same medical ethics conference, David Hoffman, a health care lawyer and clinical ethicist, introduced a number of ethical considerations. Though he was speaking in the context of a different futuristic assisted reproductive technology, I believe many of those principles can be applied to IVG. An indirect consequence of IVG is that it eliminates the biological clock which means there is less urgency for reproduction. While the timeline for having children is extended, the human lifespan has not increased significantly. This will increase the age gap between parents and children which changes the family dynamic and puts more strain on the children. On the other hand, IVG provides an inexhaustible supply of gametes and allows people to have more control over family size.

Where the technology is right now, there is significant concern about the health of the gamete products; however, if it turns out that IVG gametes end up being a healthier than those produced endogenously, society may adopt this as a common form of conception. Given the expense of assisted reproductive technologies, and out of respect for the so-far-functional status quo, this is unlikely, but it is still something to consider. The high cost of assisted reproductive technologies, likely including IVG, is something else to consider and prepare for on an institutional and government level. Fee assistance programs would need to be set up to provide across the board access for individuals occupying all socioeconomic levels. This would be difficult to achieve in practice, if IVG were indeed to produce healthier offspring, as the demand would be very high. In that case, we would need to consider the possibility of creating a genetically superior population of people from those who can access the technology. As with all new medical technologies, especially those in the field of

reproduction, when IVG comes to the marker, people are likely to respond with some skepticism and concern. Will clinicians have the right to refuse IVG treatment, especially in the more extreme applications such as multiplex and same-sex parenthood, if again, these techniques are proven clinically safe [51]?

CONCLUSION

In this paper we have followed the journey of the primordial germ cell through specification, migration, colonization and meiosis. We have identified many of the major genes associated with PGC development as well as the typical epigenetic changes that occur throughout its lifetime. We looked at studies which attempted partial or total *in vitro* gametogenesis in mice. Hayashi et al. and Zhou et al. being two of the most significant studies which were able to reconstitute PGCLCs and mature spermatozoa respectively. Despite meeting these milestones, *in vitro* gametogenesis in mice remains a work in progress, largely due to gaps in our understanding of the genetic, epigenetic, and environmental factors that stimulate *in vivo* gametogenesis. Our knowledge of the human *in vivo* processes is even weaker, and we have not yet been able to generate reproductively competent gametes in a consistent manner. The possibilities for applied IVG are enormous. Currently continued experimentation using this technique is helping scientists learn more about all aspects of gametogenesis in both animal and human models. Once perfected, IVG will help restore fertility in patients who cannot produce functional gametes. I have shown how this is especially promising for survivors of pediatric cancers for whom there are no other options, currently available, that would enable transmission of their genetic code to the next generation. IVG would also extend biological parenthood for same sex couples, solo reproduction, and multiplex parenting. With these great social changes come a lot of ethical

challenges that will need to be addressed before the technology comes to clinical use. Considering how far IVG has already progressed, this may be a lot sooner than we think. David Hoffman: “So this is your warning. Now is the time to start [thinking]. It’s a lot like climate change, you can ignore it at your peril.”

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