In Vitro Gametogenesis: A Research Timeline and Implications for the Future of Assisted Reproductive Technology

Nicole Buckley

A Senior Thesis submitted in partial fulfillment
of the requirements for graduation
in the Honors Program
Liberty University
Spring 2023

Acceptance of Senior Honors Thesis

This Senior Honors Thesis is accepted in partial
fulfillment of the requirements for graduation from the
Honors Program of Liberty University.

_____

Lindsey Stevenson, Ph.D.
Thesis Chair

_____

Jing Xu, Ph.D.
Thesis Reader

_____

Marilyn Gadomski Peyton, Ph.D.
Assistant Honors Director

_____

Date

Abstract

The novel reproductive technology, In Vitro Gametogenesis (IVG), includes the process of obtaining mature viable germ cells from pluripotent stem cells. To do so, embryonic stem cells and induced pluripotent stem cells are specified to primordial germ cells and differentiated to form gametes that undergo further maturation, which subsequently may undergo in vitro fertilization to form an embryo. With this capability, IVG holds the power to provide a novel treatment option for human infertility. As of now, research has been conducted on mice successfully, and with limited success in humans. Future research will likely focus on discovering the species-specific differences between mice and humans through studies with nonhuman primates. Research on large mammals such as the rhinoceros will also contribute to animal conservation efforts. While human IVG has not been fully developed yet, several concerns have arisen regarding the ethical and legal implications of this technology. While none of these concerns are tangible due to the current state of research findings and current International Society for Stem Cell Research guidelines, they may inform legislation in the future.

In Vitro Gametogenesis Background

In vitro gametogenesis (IVG) may become one of the most valuable new technologies to arise within the field of assisted reproductive technology. Gametogenesis (spermatogenesis or oogenesis) is the process by which germ cells are segregated from somatic cells through progressive differentiation via specific gene expression and cell signaling. This process has been sought to be replicated in vitro for decades and holds significance in multiple implications. Research enables a further understanding of IVG, including the mechanisms behind primordial germ cell specification and differentiation, along with gamete maturation. Additionally, with the successful development of IVG, this technology may hold the power to allow any individual access to reproduction (Palacios-Gonzalez et al., 2014). Later applications of IVG may also be utilized for animal conservation efforts (Goszczynski et al., 2019).

Through incremental discoveries within the development of IVG, the aim has been to both further understand the process of germ cell line differentiation, and additionally, work towards developing a sound procedure of IVG that can be utilized as a form of assisted reproductive technology, considering the complex nuances of germ cell differentiation. While IVG may be conducted through the extraction of various germ line precursor cells, the process towards developing IVG primarily includes the use of stem cells. Since 1981, scientists have been studying new ways to utilize stem cells. As characteristically undifferentiated, stem cells possess the capability and role of differentiating into various cell types (Zakrzewski et al., 2019). However, the number of different cells a stem cell may be capable of differentiating into is dictated by its potency. Embryonic stem cells (ESCs) have been known to be pluripotent in vivo, also possessing the capability to proliferate under certain culture conditions in vitro, while simultaneously enduring the ability to differentiate into any type of cell in an organism.

Pluripotent stem cells may be naturally obtained from embryos, as either ESCs or epiblast stem cells (EpiSCs; Hayashi et al., 2012). ESCs may be extracted from the inner cell mass of the blastocyst, and EpiSCs may be derived from the epiblast of post-implantation embryos. In addition, pluripotent stem cells may be derived by means of induction through culture. In 2006, researchers at Kyoto University discovered a process to reprogram adult somatic cells to possess the same pluripotency as ESCs or EpiSCs. The process requires nuclear programming, a technique that experimentally induces a change in the nucleus within a mature cell that is maintained and replicated as the cell undergoes division via mitosis (Takahashi & Yamanaka, 2006). First, extraction of mature somatic cells from an adult is performed, then genes that encode critical transcription factors are introduced. Transcription factors initiate gene expression to facilitate cell function. It was discovered that four transcription factors, Oct4, Sox2, Klf4, and c-Myc, are required to reprogram somatic cells to express genes and proteins that reflect ESCs. These factors induced and maintained the pluripotency of the induced pluripotent stem cells (iPSCs). Mice iPSCs were first reported in 2006, and human iPSCs were first reported the following year, in late 2007 (Takahashi et al., 2007). The significance behind the utilization of iPSCs lies in the capability to create patient-specific pluripotent stem cells, which in turn, may be differentiated into patient-specific germ cells. This would ultimately allow for clinical application of IVG. Given that replicating gametogenesis within an in vitro model is a complex process, a sufficient understanding of both male and female reproductive systems, spermatogenesis, oogenesis, and embryogenesis is critical.

Male Reproductive System

The male reproductive system consists of internal organs to include the testes, epididymis, vas deferens, prostate, and external organs to include the scrotum and penis (Gurung et al., 2022). Multiple ducts and glands are contributory to the formation, storage, and transport of sperm for the purpose of fertilization, and additionally the production of androgens contributory to male development. The two main types of somatic cells with roles in the testes include Leydig cells and Sertoli cells. Leydig cells are housed in the interstitial tissue of the testes, located adjacent to the seminiferous tubules. Their primary purpose is to produce testosterone. In contrast, Sertoli cells are housed along the periphery of the seminiferous tubules, the location where spermatogenesis begins. Sertoli cells are known to form what is termed a blood-testis barrier, which keeps germ cells contained within the seminiferous tubules. Within the process of spermatogenesis, germ cells are found to line the interior of the seminiferous tubules and move towards the lumen as they differentiate to maturation.

Spermatogenesis, the production of sperm in biologically male organisms, begins at puberty (Gurung et al., 2022). Germ cells are initially found in the basement membrane of the seminiferous tubules of the testes. Follicle-stimulating hormone produced by the anterior pituitary gland stimulates Sertoli cells for regulation of the process. The initial stage begins with mitosis of diploid spermatogonia also termed spermatogonial stem cells (SSCs), which ultimately allow the continuous proliferation of sperm replenishment. A portion of SSCs differentiate to primary spermatocytes, which then complete meiosis I to produce haploid secondary spermatocytes, which subsequently are subjected to meiosis II, yielding haploid spermatids. Spermatids then transform into immotile spermatozoa, which are then transported

through the tubules to the epididymis to undergo maturation for around 12 days, where they subsequently achieve motility and are stored in the epididymis to await ejaculation.

Female Reproductive System

The female reproductive organs include the ovaries, fallopian tubes, uterus, vagina, and vulva (Rosner et al., 2022). The ovaries house both germ cells termed oocytes, and also somatic cells, including granulosa, theca, and stromal cells that support follicle maturation. In utero, oogenesis begins when the oogonia undergo division and differentiation until primary oocytes are formed around the end of gestation. The number of primary oocytes then reduces significantly (Holesh et al., 2022). Oocytes are contained in primordial follicles that are housed in the ovaries. At the initial onset of puberty, the primary oocyte is subjected to maturation through the completion of meiosis I, yielding a secondary oocyte. The secondary oocytes are arrested at metaphase of meiosis II and are also known as MII oocytes. MII oocytes undergo the process of ovulation every uterine cycle, and upon fertilization, are termed mature ovum following the completion of meiosis II. The female reproductive system, differing from males, consists of the uterine or menstrual cycle, entailing the periodic preparation for pregnancy and fertilization by sperm, around every 28 days.

Gonad Development Within Embryogenesis

Within the process of embryogenesis of mice, the epiblast of the growing embryo contains cells which, within gastrulation and around embryonic day 6-6.5 (E6-6.5), give rise to primordial germ cells (PGCs), identified as a cluster of cells at the base of the allantois at E7.25 (Chuma et al., 2005). Through the development process, the number of PGCs continues to increase from 40 cells at E7.25 to around 25,000 cells at E13.5. These cells simultaneously migrate along the developing hindgut and mesentery to the genital ridge, beginning around

E10.5. The genital ridge is what ultimately develops into either the ovaries or testes, dictated by SRY expression in males establishing Sertoli cells, or lack thereof in females, establishing granulosa cells (Hayashi & Saitou, 2014). Within migration, PGCs undergo epigenetic reprogramming, where DNA is demethylated, and there are histone modifications including the decrease of H3K9me2 and increase of H3K27me3. At around E13.5, retinoic acid stimulates PGCs to express stimulated by retinoic acid gene 8 (Stra8) for entry into meiosis to produce oogonia in females. Oogonia subsequently differentiate into primary oocytes that are enveloped by gonadal somatic cells, forming what is termed the primordial follicle. It is critical to note that in addition, oocytes acquire the epigenetically reprogrammed genome that ultimately displaces totipotency to the zygote genome upon fertilization, contributing to necessary epigenetic events in offspring development. In contrast, around E15.5, PGCs in the male genital ridge enter mitotic arrest and transform into pro-spermatogonia, which are arrested until puberty (Chuma et al., 2005). This is primarily due to the mechanism of *Cyp26b1*, a retinoid degradation enzyme. Whereas retinoic acid stimulates female germ cells to initiate oogenesis in utero, in males *Cyp26b1* acts in an inhibitory fashion, to ensure low levels of retinoic acid in order to maintain the diploidy of spermatogonia, therefore preventing commencement of meiosis until puberty (Bowles et al., 2006; Maclean et al., 2007).

Within the process of human embryogenesis, it has been established that around week two of development, PGCs express transcription factors SRY-box 17 (*Sox17*), transcription factor AP-2 gamma (*Tfap2c*), and B lymphocyte-induced maturation protein-1/PR domain zinc finger protein 1 (*Blimp1*/*Prdm1*). Later, around week five, PGCs migrate and inhabit embryonic gonads to induce differentiation into oogonia or gonocytes that express deleted in AZoospermia gene (DAZL) and DEAD-box helicase 4 (DDX4). At this point, oogonia and gonocytes are very

similar in terms of genetic expression, epigenetic characteristics, and morphology (Yamashiro et al., 2018). However, around week 10 of development both undergo sexual differentiation, either into oocytes or spermatogonia. Also at week 10, germ cells undergo epigenetic reprogramming to include global genome DNA demethylation, imprint erasure, and X-inactivation.

Foundations of Male IVG Studies

IVG studies began through the utilization of mice as a mammalian model. In a foundational study of male IVG, Toyooka et al. (2003) prepared ESCs, and then aggregated them into 3D structures called embryoid bodies (EBs). EBs were utilized due to their capability to mimic the microenvironment of early embryos, thus possessing what were potentially essential transcription factors and other signaling molecules for ESC differentiation and PGC specification. Their protocol additionally yielded pre-mature germ cells that were transplanted into immature mouse testis for further germ cell differentiation. Allegedly, they observed mature spermatogonia formation, although no functional data was present. Geijsen et al. (2004) took these findings a step further, also utilizing ESC aggregation into EBs, but were able to extract mature spermatogonia and subsequently fertilize in vivo derived oocytes via intracytoplasmic sperm injection. Their successfully fertilized oocytes led to the formation of blastocytes that did not develop past blastulation. While these two research studies were able to confirm the derivation of sperm-like cells from ESCs, a study released in 2005 sought to determine the potential of germ line cells from earlier embryos to develop into SSCs, using extracted PGC precursor cells from pregnant C57BL/6 mice (Chuma et al., 2005). This study sought to, instead of beginning with the use of ESCs, observe the behavior of in vivo-derived PGC precursors as they differentiate into SSCs to elucidate shortcomings from the initial studies. Their procedure included the transfer of in vivo-derived epiblast cells or PGCs into testes of infertile *W/Wv* mice,

to examine their ability to repopulate the seminiferous tubules and undergo further differentiation. Their results showed that not only PGCs, but also epiblast cells, can differentiate into SSCs that subsequently induce spermatogenesis. This study provided foundational knowledge regarding the induction of PGC differentiation and confirmed that extracted precursor cells were able to differentiate in an environment apart from their origin. In addition, it was established that transplantation of PGCs is a viable mode for further research on subsequent PGC line differentiation. Meanwhile, research utilizing EBs continued, as Nayernia et al. (2006) sought to culture EBs with retinoic acid, a novel addition to the protocol. The team was able to yield two SSC lines, which were transplanted into testes for maturation. Spermatogonia were detected but were found to be immotile. Despite this finding, their spermatogonia were utilized to fertilize oocytes, and ultimately yielded offspring. However, their offspring were notably abnormally sized, and died prematurely. This may have been due to the failure to reproduce the epigenetic reprogramming that establishes the state of totipotency crucial for offspring development (Hayashi et al., 2014).

Foundations of Female IVG Studies

Simultaneously, initial research on female IVG was conducted, also using mice as a mammalian model. Hubner et al. (2003) became the first to utilize ESCs and successfully achieve the differentiation of ESCs to female germ cells. ESCs were spontaneously differentiated under a suspension condition that excluded LIF interleukin 6 family cytokine (LIF) or feeder cells. They observed that by d12 of culture, PGC colony formation had occurred. PGC clusters that were positive for GFP and VASA were cultured in new plates, and over the following 14 days, follicle-like structure formation occurred and expressed characteristics similar to the interactions between somatic cells and female germ cells in vivo, such as the production of

estradiol in culture. By d16 of culture, it was observed that PGCs had spontaneously differentiated to oogonia-like cells, which were subsequently able to enter meiosis and produce oocyte-like cells. By d26, it was observed that oocyte-like cells had departed from somatic cells. The isolated oocyte-like cells exhibited oocyte specific genetic markers, however, there was no evidence pointing to the commencement to oocyte maturation. Conclusions from this study and following studies, notably one by Qing et al. (2007) point to the lack of critical factors in their culture systems, to further support growth and differentiation of the oocyte-like cells in vitro.

Murine PGC Specification

While these protocols achieved limited success through spontaneous differentiation, a goal was established to employ a culture system that would regulate PGC specification and further differentiation. Returning to the discussion of embryogenesis, PGCs segregate from the somatic cell lineage relatively early within development, confirming PGC specification and subsequent germ cell formation as the first step within the establishment of a specific germ cell lineage from the proximal epiblast. Cells of the posterior proximal epiblast, around E6.25, express BLIMP1/PRDM1, a zinc finger transcriptional repressor, and PR-domain containing protein 14 (PRDM14), in response to the presence of bone morphogenetic protein (BMP4; Ohinata et al., 2005; Saitou et al., 2005). It has been known that cells positive for BLIMP1/PRDM1 are restricted to the cell lineage of PGCs, emphasizing its critical role in PGC specification. PRDM14 has been found to play roles in pluripotency restoration and epigenetic reprogramming, establishing characteristics required for the formation of PGCs, alongside the presence of NANOS3, NANOG, and SOX2 (Hayashi et al., 2012, Yamaji et al., 2008). At E7-7.25, it has been observed that around 40 PGCs additionally express developmental pluripotency-associated 3 (STELLA/DPPA3) at the posterior end of the primitive streak

(Hayashi et al., 2012). Therefore, BLIMP1/PRDM1, PRDM14, and STELLA/DPPA3 were established as markers in early protocols. As previously mentioned, BMP4 has additionally been named as a key factor, identified to be present prior to gastrulation via expression in the extra-embryonic ectoderm (Lawson et al., 1999). Upon its release, it has been found to be contributory to the formation of PGC precursors, as PGCs differentiate from the epiblast. This finding was especially valuable in that BMP4 was established to contribute to proper mouse PGC specification and formation, which ultimately establishes the germ cell line that gives rise to mature spermatogonia and oogonia. BMP4 activity is enabled by Wnt signaling via WNT3 (Hayashi et al., 2012). Ohinata et al. (2009) were able to reconstitute PGC specification in vitro using a mouse model with a culture system that carefully supported the differentiation process leading to mature germ cells, presenting data showing that they were able to specify mouse primordial germ cell-like cells (PGCLCs) that displayed germ cell specific markers, after culture with BMP4. However, their data demonstrated poor growth of mouse PGCLCs. After 132 hours in culture, the epiblast derived mouse PGCLCs grew to a count of approximately 400 cells. The corresponding stage in vivo typically comes out to a count of around 5,000-10,000 PGCs. Thus, their culture yielded a low count of PGCLCs for the natural progression of development. In response, they added bone morphogenic protein 8b (BMP8b*)* and epidermal growth factor (EGF*)* with BMP4, LIF, and KIT ligand (KITLG), which subsequently yielded around 3,000 mouse PGCLCs.

In response, Hayashi et al. (2011) sought to further develop a culture model for mouse PGCLC specification, and ultimately became the first to develop epiblast-like cells (EpiLCs) in mice. Rather than utilizing preexisting epiblasts, the team hypothesized that there is a window of time in which cells acquire PGC competence within the transitory process between ESCs to

EpiSCs. Therefore, Hayashi's team cultured ESCs under EpiSC conditions in hopes of deriving PGC competence in their PGCLCS. ESCs were prepared with activin A and fibroblast growth factor 2 (FGF2). ESCs were then cultured with fibroblast growth factor inhibitor, CHIR99021, which is a glycogen synthase kinase 3a/b inhibitor, and LIF. ESCs cultured for two days subsequently demonstrated PGC competence and differentiated into PGC precursors, termed EpiLCs. Based on preliminary findings emphasizing BLIMP1/PRDM1 and STELLA/DPPA3 as key players in PGC specification, Hayashi's team established BVSC (Blimp1-mVenus/Stella-ECFP) as a key marker in evaluation of successful PGCLC specification from EpiLCs. The critical necessity of BMP4 was further confirmed as EpiLCs were subjected to culture with BMP4 alone, BMP4 and LIF, and lastly, Klk1b15-ps, BMP4, BMP8b, LIF, KITLG, and EGF. Further confirming evidence from Ohinata's study, it was observed that BMP4 stimulated BLIMP1/PRDM1 expression in EpiLCs. In addition, the combinatorial relationship of BMP8b, EGF, BMP4, LIF, and KITLG from Ohinata's study was regarded in synthesizing the final culture system of their trials, however, with the addition of Klk1b15-ps. It was determined that the third and final culture system was optimal, after observing that day two EpiLCs induced BVSC-positive cells, mainly from BMP4, while LIF, KITLG, BMP8b, and EGF supported maintenance and proliferation of BVSC-positive cells in PGCLCs. Gene expression profile analysis of PGCLCs demonstrated mechanisms like those of PGC specification in vivo.

Murine PGCLC Differentiation to Male Gametes

Following successful PGCLC specification from EpiLCs induced from both ESCs and iPSCs, Hayashi et al. (2011) next sought to examine whether their PGCLCs underwent proper spermatogenesis via transplantation into the seminiferous tubules of W/Wv neonatal mice, who lacked endogenous germ cells. It was demonstrated that three out of six testes transplanted with

BVSC+ cells contained seminiferous tubules that displayed proper spermatogenesis, observing thick tubules that contained abundant spermatozoa with normal morphology. The PGCLC-derived spermatozoa were then injected into in vivo-derived oocytes via intracytoplasmic sperm injection, and the resulting zygotes were observed to develop properly and were implanted into surrogate mothers. Upon birth, these offspring were healthy and developed properly. While this protocol yielded a robust number of spermatozoa that were able to contribute to viable offspring, the team concluded that a more comprehensive understanding of the specific biochemical mechanisms of the proteins involved in PGC differentiation was still needed.

The team established their goal to continue to develop their culture system and ultimately, reconstitute the entire process of spermatogenesis in vitro to produce fertile spermatozoa. In 2016, Zhou et al. (2016) built upon this protocol to differentiate PGCLCs from EpiLCs, however, with the utilization of a new medium model named N2B27. N2B27 characteristically contains retinoic acid precursor Vitamin A and insulin. They found that their medium successfully supported PGCLC formation that were subsequently able to undergo meiosis. To do so, they ensured that an environment with low CYP26B1, to maintain retinoic acid in high levels, allowing meiosis to occur, in contrast to the process in vivo. Subsequently, they subjected PGCLCs to culture with activin A, bone morphogenic protein 2 (BMP2), BMP4, bone morphogenic protein (BMP7), and retinoic acid, which led to the silencing of BLIMP1/PRDM1 and STELLA/DPPA3, and additionally the upregulation of STRA8, enabling the initiation of meiosis. Furthermore, they observed that under their culture system, PGCLCs failed to differentiate into self-renewing SSCs in vitro that would allow for proliferation of spermatogonia, with suspicion that this occurred due to the lack of critical growth factors of the testicular microenvironment of the basement membrane in vivo, such as glial cell line-derived

neurotrophic factor (GDNF), FGF2, and EGF in their culture system. Despite this setback, their PGCLCs entered meiosis and gave rise to spermatid-like cells when co-cultured with neonatal testicular cells. Spermatid-like cells were subsequently injected into oocytes, producing viable offspring. The Zhou research team therefore established retinoic acid as a critical factor in stimulating spermatogenesis in an in vitro system.

Ultimately, the reconstitution of in vitro spermatogenesis was accomplished. In a novel protocol, Ishikura et al. (2021) utilized ESCs to obtain PGCLCs, which were subsequently subjected to culture and aggregated with embryonic testicular somatic cells within a reaggregation cultured named reconstituted testis. PGCLCs were then differentiated into spermatogonium-like cells. These cells were ultimately differentiated into germline stem cell-like cells (GSCLCs). This was performed with the aim to replicate the function of SSCs, which allow for the proliferation and replenishment of sperm characteristically present in male mammals. To examine this, GSCLCs were transplanted into the testes of adult mice. The data indicated that 80% of the population achieved spermatogenesis. The results demonstrated that the obtained GSCLCs displayed a robust capacity to induce spermatogenesis. GSCLC-derived spermatozoa were then injected into mature oocytes, and yielded embryos that were implanted into surrogate mothers, and born as healthy, viable offspring.

Murine PGCLC Differentiation to Female Gametes

After successful PGCLC differentiation in male mice in 2011, just one year later, in 2012, Hayashi's team developed a protocol of oocyte derivation from PGCLCs, in vitro. They first obtained BVSC positive female ESCs that were subjected to differentiation into mouse EpiLCs (Hayashi et al., 2012). EpiLCs were subjected to culture to induce PGCLC specification, making note of observations displaying similarities to PGCLC specification in males in their

previous study, which was somewhat unexpected. Subsequently, PGCLCs were subjected to culture in what is termed a reconstituted ovary (rOvaries) in order to maintain the environment of ovaries in vivo. To do so, PGCLCs were FACS sorted and reaggregated with E12.5 gonadal somatic cells to replicate the in vivo primordial follicle (Hayashi et al., 2012). To further explore PGCLC potential for oogenesis, d3 PGCLCs, d6 PGCLCs, and in vivo female E12.5 mouse PGCs were utilized for formation of reconstituted ovaries for comparative purposes and then transplanted into mice ovarian bursa. It was observed that d3 PGCLCs displayed substantial growth and STELLA/DPPA3 fluorescence, indicating the formation of oocyte-like cells. D3 PGCLC-derived oocyte-like cells were subsequently subjected to in vitro fertilization (IVF), which yielded embryos that were transferred to surrogate mothers. Ultimately, healthy, fertile offspring were born, confirming the protocol's success. Despite their success, the team made note of the inefficiency of the process to yield offspring from PGCLCs as compared to PGCs, observing that upon fertilization, at the pronuclear stage, around half of the mouse PGCLC-derived zygotes displayed an abnormal phenotype of three pronuclei. This abnormality ultimately contributed to a low birth rate of PGCLC-derived offspring. In the same study, the potential for female iPSCs to replicate this protocol was tested. iPSCs were obtained from embryonic fibroblast cell lines and differentiated into PGCLCs. Following the same protocol, iPSC derived PGCLCs were subjected to in vitro maturation (IVM) and IVF and yielded three viable offspring. The team concluded that female ESCs and iPSCs were thus capable of PGCLC induction, and ultimately, the production of mature oocytes capable of yielding viable offspring. While this study yielded mature oocytes, the transfer of PGCLCs to an ovarian bursa resulted in abnormal follicular formation. Therefore, a protocol that would eliminate the need for grafting through the creation of a culture system supporting follicle maturation was required.

In response, Kyushu University researchers were finally able to reconstitute the entire process of mouse oogenesis in vitro. Dividing their protocol into three phases, in vitro differentiation (IVDi), in vitro growth, and IVM, the team utilized ESCs in order to obtain PGCLCs (Hikabe et al., 2016). They proceeded with aggregation of PGCLCs with E12.5 female gonadal somatic cells to form rOvaries. Around 5,000 PGCLCs were aggregated with 50,000 gonadal somatic cells per rOvary. Within the stage of IVDi, five days post the beginning of culture, clusters of PGCLCs were observed. At 11 days into culture, follicle formation occurred. To confirm the supportive function of gonadal somatic cells surrounding oocytes, forkhead box L2 (FOXL2), a granulosa cell marker, was utilized. By day 21 of culture *Foxl2* expression was detected. A note was made of limited chromosome pairing accuracy, as compared to the in vivo model. Despite this observation, an abundance of secondary follicle-like structures containing primary oocytes were yielded from IVDi. However, they observed that granulosa cell proliferation was limited, possibly due to the limiting space of the rOvary. In response, to proceed with maturation, individual secondary follicle-like structures were extracted and subjected to the same culture. From this, it was observed that granulosa cells were able to proliferate. Subsequently, it was observed that primary oocytes underwent proper maturation as germinal vesicle oocytes. The data indicated that an average of 55 fully grown oocytes were extracted per rOvary. Upon IVM, meiosis was initiated, which yielded MII oocytes, with data indicating that 77.8% of these contained the correct number of chromosomes. The term MII, or metaphase II of meiosis II, indicates the maturation status of oocytes. Therefore, this protocol yielded oocytes that displayed morphological traits and genetic markers replicate of oocytes at the point where in vivo, they are arrested at metaphase II, and await fertilization via sperm to commence with the completion of meiosis II. Upon evaluation of oogenesis, it was found 424

genes were differentially expressed, indicating that the gene expression of the in vitro-derived oocytes contrasted with the in vivo model. Therefore, the team concluded that oocyte growth within the IVG and IVM cultures may have been jeopardized in some way. Despite these observations, the team subjected MII oocytes to IVF with in vivo-derived sperm to form two cell embryos that were implanted into pseudopregnant female mice. Overall, the data demonstrated that 3.5% of 2-cell embryos achieved full-term development, and it was noted that embryogenesis was delayed at the cleavage stage, along with early and late gestation. However, the mice pups that were born developed normally, and none were observed to die prematurely. Ultimately, the results of this study demonstrated the successful reproduction of an entire cycle of the female germ line in vitro. It was emphasized that in this protocol gonadal somatic cells were obtained from embryos, and if clinical application were to occur in humans, a protocol for differentiating PSCs into gonadal somatic cells must be established.

Eventually, this feat was achieved in mice in 2021. The protocol called for the same procedure, however, with the exception of the induction of fetal ovarian somatic cell-like cells (FOSLCs; Yoshino et al., 2021). The aim of this procedure was to replace the utilization of in vivo-derived somatic cells with in vitro-derived cells that replicate the supportive role of gonadal somatic cells in follicle development. Through a novel protocol, ESCs were differentiated into EpiLCs utilizing activin A and FGF2. The EpiLCs were then cultured with a variety of transcription factors to upregulate specific genes that expressed in gonadal somatic cell precursors. Th team utilized Nr5a1 gene as a key genetic marker for differentiation to gonadal somatic cells. They were subsequently able to isolate cells that differentiated from the EpiLCs that expressed Nr5a1 and were morphologically similar to in vivo gonadal somatic cells at E12.5, indicating successful differentiation into FOSLCs. These FOSLCs were then reaggregated with

PGCLCs to form what was termed rOvaroids and subjected to IVDi for 21 days. Subsequently, follicles were isolated from the rOvaroid, and subjected to culture for 12 days, and then IVM for 16 hours. Overall, this protocol was able to yield MII oocytes, that subsequently contributed to viable offspring. This protocol holds special significance, as it established a protocol for the reconstitution of the entire ovarian follicle.

Human PGC Specification

Like murine research, studies on human IVG sought to achieve PGC specification from ESCs and iPSCs. Protocols primarily began with ESC culture in EBs. Clark et al. (2004) was the first to isolate early PGCLCs from ESCs through spontaneous differentiation. Prior to this study, it was unknown whether human ESC lines were capable of differentiating into germ cell lines in vitro, as had already been displayed in mice. Before further experimentation, the team formulated a genetic profile of expected germ cell specific genes to serve as genetic markers in their study. Their genetic profile included *GDF3*, *STELLA*, *NANOG*, *NANOS1*, *DAZL*, *VASA*, *cKIT*, *PUM1*, *PUM2*, *SCP1*, *GDF9*, and *TEKT1*, that inspired genetic markers in later studies. Early on, VASA was established as a key marker for successful PGCLC specification from PSCs. After EB formation, it was observed that VASA expression began on day three, however, there was no observable data pointing to the formation of haploid germ cells. A later protocol also achieved spontaneous differentiation of pre-meiotic diploid PGCLCs from ESCs (Tilgner et al., 2008). A subsequent study performed this protocol through co-culture with fetal gonadal cells, that showed limited success with a presentation of immature pre-meiotic PGCLCs from ESCs and iPSCs, using SSEA1, cKIT, and PLAP as genetic markers (Park et al., 2009). Subsequently, it was established that similar to mice, human PGC specification was stimulated by BMPs. BMP4 was shown to increase the expression of VASA, a germ-cell specific marker

within the process of EB differentiation from ESCs (Kee et al., 2009). Additionally, it was found that BMP7 and BMP8b are critical for PGC specification, and that BMP7 and BMP8b in combination with BMP4 displayed additive impacts on germ cell induction (Kee et al., 2006). The significance of BMP4 was later confirmed by Geens et al. (2011), although their procedure could not elucidate the combinatorial effects of BMP7 and BMP8. A later study in 2015 implemented a two-step specification culture system utilizing ESCs and iPSCs. With an emphasis on specifying pre-migratory PGCLCs, their culture system implemented activin A + BMP4 + FGF2 → BMP4 + LIF (Sugawa et al., 2015). Their protocol yielded a substantial amount of pre-meiotic PGCLCs. Of note, upon genetic analysis of their sample, the team was able to unveil genetic expression during the early phase of PGC development, which was previously unclear. Their findings identified that similar to mice, human PGCs demonstrate expression of PRDM14-regulated genes and undergo global epigenetic reprogramming. A novel protocol for PGCLC specification was released in 2016, utilizing a stepwise process with activin A + CHIR → BMP4 + LIF + KITLG + EGF, to induce iPSC differentiation to incipient mesoderm-like cells that were subsequently able to robustly produce PGCLCs (Sasaki et al., 2015). They observed that PGC specific gene expression was upregulated during the transitory period of incipient mesoderm-like cells to PGCLC, including *TFAP2C*, *BLIMP1*/*PRDM1*, *SOX17*, *SOX15*, and *cKIT*.

Male PGCLC Differentiation

While trials were underway to further enhance human PGCLC specification from ESCs and iPSCs, protocols were also under development to achieve PGCLC differentiation. In 2009, a research study called for smaller ESC clusters to be subjected to culture with FGF2 (Bucay et al., 2009). The culture system yielded PGCLCs that were subsequently subjected to culture with

laminin, and induction of Sertoli cells was observed. A novel culture system that utilized FGF2 and additionally, a co-culture with mouse embryonic fibroblasts eventually yielded meiotic cells, confirmed by the expression of meiotic proteins, synaptonemal complex protein 3 (SYCP3) and MutL homolog 1 (MLH1), in 90% of their cells (West et al., 2008). Additionally in 2009, a study aggregated ESCs into EBs, cultured this time with retinoic acid. It has been known that the presence of retinoic acid induces IVG in mice. Their system yielded a small population of spermatid-like cells, displaying a post-meiotic phenotype (Aflatoonian et al., 2009). A later protocol also implemented the use of retinoic acid in their EB culture system, and observed the expression of spermatid specific gene *TEKT1*, in their PGCLCs (Xuemei et al., 2013). PGCLC differentiation protocols eventually transitioned to the utilization of a stepwise introduction of signaling mediators to regulate the differentiation process in a stage-dependent manner. Two studies in 2011 first tested this approach. West et al. (2011) developed a process utilizing FGF2 + KSR → FGF2 + FCS with ESCs, and Eguizabal et al. (2011) implemented retinoic acid → FGF2 + LIF + forsokolin + CYP26 inhibitor with ESCs and iPSCs. These systems both found success in yielding male post-meiotic germ cells that resembled spermatogonia. Of note, the latter protocol was successful in the induction of male post-meiotic germ cells from iPSCs, drawing one step closer to potentially achieving patient specific IVG (Eguizabal et al., 2011).

Female PGCLC Differentiation

Despite success in female murine studies, reconstituting human ovaries posed a greater challenge. However, in 2018, a novel protocol for further PGCLC differentiation was introduced. The procedure sought to further develop PGCLCs in vitro through the utilization of xenogeneic reconstituted ovaries (xrOvaries) that additionally contained mouse embryonic ovarian somatic cells (Yamashiro et al., 2018). They implemented a previous protocol to induce iPSCs into

incipient mesoderm-like cells, and subsequently into PGCLCs that displayed a measure of epigenetic reprogramming. With the previous success of mouse PGCLC differentiation within rOvaries, the team produced xrOvaries to replicate a similar environment. The protocol yielded oogonia. Returning to the discussion on oogenesis, it is known that oogonia are subjected to mitosis to form primary oocytes and undergo subsequent maturation to yield mature ovum. Therefore, this study yielded limited success. A challenge remains presently in how to create a culture system that supports oocyte maturation.

IVG Research on Non-Human Primates and Large Mammals

Of late, research on IVG has ventured into various other animal models, with special significance placed on the monkey as a non-human primate model, and additionally, the rhinoceros as a large mammal model. To begin, the monkey as an IVG model is utilized to elucidate further discoveries that may be contributory to a further understanding of the mechanisms behind PGC specification and PGC differentiation in humans. While previously discussed human IVG protocols have established limited success, they identified species-specific differences within PGC specific gene expression, epigenetic reprogramming, and PSC properties between humans and previously utilized murine models. Therefore, to overcome the limitations that stand in the way of replicating murine studies for human IVG, studies on monkeys are especially useful. In addition, research on monkeys overcomes the ethical and legal barriers of stem cell research utilizing human specimens. Of note, monkey ESCs and iPSCs were utilized in a novel protocol performed in 2019 (Sakai et al., 2020). The protocol achieved differentiation to PGCLCs and additionally to endoderm and extraembryonic mesenchyme-like cells. PGCLCs demonstrated specificity via identification of SOX17, TFAP2C, and BLIMP1/PRDM1 expression. The study emphasized that the genetic profile of monkey PGCLC induction and

human PGCLC induction was largely similar, but greatly differed from the genetic profile of mouse PGCLC induction. Of note, it was also confirmed that SOX17 was a critical factor in PGC specification. As previously discussed, SOX17 was found to serve a critical function in human PGC specification and the induction of PGC differentiation. However, SOX17 possesses no significant role in murine studies. These findings suggest intriguing possibilities for how the regulation of certain cell signaling pathways and gene expression may have been conserved in some species, but not in others (Saitou & Hayashi, 2021). To conclude, this foundational study utilizing monkeys as a non-human primate model established a protocol for PSC derivation from ESCs and iPSCs, and PGC specification and differentiation. This study also confirmed similarities between humans and monkeys regarding cell signaling and gene expression profiles, establishing the monkey as a viable model for human IVG. Later studies utilizing the monkey will ultimately contribute to research that may reveal a deeper understanding into human IVG.

In contrast, research on rhinoceros possesses a focus on animal conservation efforts. Most recently, Hayashi's team embarked on a research project on northern white rhinoceros (NWR), a species categorized as extinct in the wild. The team utilized a close relative of NWR, the southern white rhinoceros (SWR), and developed a culture system to reconstitute PGC specification utilizing ESCs (Hayashi et al., 2022). Of note, they observed that the optimal timing for WNT inhibition is at the onset of mesoderm differentiation, and that BMP signaling was only required for initial differentiation of PGCLCs, but not at the latter end of the process. They concluded that future studies should, by principle, express specificity in protocol in regard to the timing and duration of WNT and BMP signaling. This study also determined that SOX17 played a critical role in the differentiation of SWR PGCLCs, confirming a common theme among other mammal models, such as the monkey and human. Upon refining the present

protocol, the team also utilized NWR iPSCs for PGCLC production. Despite multiple limitations in the way of reproducing the entire process of IVG in the present study, their successful procedure of PGCLC specification induction was the first step towards reconstituting the entire process in vitro. Given that in the future, the process is completely reconstituted, this technology could be utilized for animal conservation efforts, such as for the NWR species.

Ethical and Legal Concerns

IVG has been shown to promote multiple beneficial applications. Research has already revealed a plethora of new information regarding the mechanisms behind murine gametogenesis, which has opened the door to a further understanding of human gametogenesis. However, with the potential for human reproductive utilization, many questions arise as to how and when this technology will be introduced into the clinical setting, and if it is ethical to do so.

First, it is critical to obtain an understanding of the current regulations surrounding IVG research. In 2021, the International Society for Stem Cell Research established an update to the ISSCR Guidelines for Stem Cell Research and Clinical Translation in response to the progress made in research regarding human IVG. The guidelines were last revised in 2016, however, the 2021 updates introduce further specification into activities that are permitted or not permitted. The regulations are separated into respective categories, with specifics regarding whether the research activity is permitted, if so, what measures must be taken before proceeding, or if the activity is prohibited. Category 1A establishes research that is permitted upon review under preexisting mandates and committees (Clark et al., 2021). Under Category 1A, research utilizing human PSC lines is permitted within cell culture or routine research practices including in vitro differentiation. Next, Category 1B outlines activity that is to be reported to the oversight process and subject to further review if determined by committee or local policies. Under Category 1B,

research that includes formation of human stem-cell based embryo models in vitro without subsequent development into an entire embryo is permitted. In addition, research on IVG utilizing cells such as genetically modified PSCs is permitted, given that there are no fertilization attempts or further development of embryos. Category 2 entails research utilizing embryos or embryo models that are permitted only upon receiving approval from a specialized review conducted by a scientific ethics committee. Examples of Category 2 activities include in vitro culture of human embryos that are kept in culture until primitive streak formation around 14 days, production of stem-cell based embryo models, and research that generates human gametes in vitro from stem cells. Category 3A entails research that is currently not permitted due to unresolved ethical dilemmas or safety concerns. Category 3A activities include the use of in vitro-derived gametes for the purpose of fertilization or human reproduction, and research where embryos have undergone genome modification and are subsequently transplanted into a human uterus. Lastly, Category 3B entails activities that are prohibited in research and are deemed unethical under international ethical standards. Of note, these activities include transferring in vitro-derived human embryos into the uterus of a human subject or animal subject.

While current research initiatives are pushing towards developing a protocol to further increase safety, efficacy, and success of the technology as applied to humans, the ISSCR's new guidelines prohibit research activity that would enable the clinical use of human IVG. Despite the current scientific limitations of IVG and ISSCR guidelines, commentators are already discussing the novel possibilities of solving female infertility, same-sex reproduction, multiplex parenting, and solo reproduction (Palacios-González et al., 2014).

Multiple lawyers and bioethicists have put forth concerns of the implementation of IVG as a means of assisted reproductive technology in the clinical setting. The continued use of

human ESCs in further research and the potential to yield a multitude of embryos in vitro that die prematurely or undergo other complications has been discussed, with commentary on the possible devaluation of human life (Cohen et al., 2017). Additionally, conversations regarding the potential use of genetic screening of viable embryos obtained in vitro has raised concerns, as it opens the door to genetic editing of embryos through technologies such as CRISPR. Lastly, there are questions raised about the potential use of IVG for same-sex reproduction or solo reproduction, or theoretical multiplex genetic parenting through mitochondrial DNA replacement (Palacios-González et al., 2014). While none of these hypothetical situations of IVG have been achieved in the laboratory setting, there are present concerns regarding a societal shift in the view of family, inbreeding through solo reproduction, and implementation of the technology that does not align with a therapeutic need due to infertility. Additionally, some inquire whether this seemingly boundless ability to reproduce may be detrimental to adoption rates. Regardless of these concerns, the current state of human IVG research has not been furthered to the point of tangible concerns. However, it is critical to note that preliminary concerns should continue to be discussed for the purpose of implementing legislation that carefully regulates the utilization of human IVG once it has been developed to the point of clinical use.

There is already work in the United States to bring IVG into the world of reproductive medicine, accessible to those who struggle with infertility or couples who cannot conceive naturally for other reasons. Currently, there are three start-up companies making headway towards providing this access, including Conception, Ivy Natal, and Gameto. These companies market their aim to provide an opportunity for women to have children into their 40’s and 50’s, eliminate the barriers of infertility, and allow same-sex male couples to have their own biological children. They additionally assert another goal, in that they would like to utilize their technology

to serve as a platform for genetic screening and genetic editing of embryos to reduce the risk of diseases such as Alzheimer's, heart disease, and different forms of cancer. One may question if the development of these start-up companies is premature or inappropriate, especially with the recent update in ISSCR guidelines as of 2021. While IVG holds many promising benefits, there is an absolute necessity for a strong and comprehensive evidence-based foundation for clinical application, which is only possible with competent research under ISSCR guidelines.

Conclusion

Overall, this review detailed a timeline of IVG research. A thorough discussion of gametogenesis included oogenesis and spermatogenesis, embryogenesis, and the fundamental characteristics of somatic cells, stem cells, and germ cells. Foundationally, gaining an understanding of the mechanisms behind the process of PGC specification and differentiation in mice was the first step. Key transcription factors and cell specific gene markers were identified in order to understand the progression of germ cell line progression and eventually isolate PGCLCs. Various studies emphasized the utilization of different signaling mediators, and eventually, stepwise protocols were implemented to induce a stage-dependent process of differentiation in vitro. A variety of culture systems and protocols were implemented, and ultimately led to the successful reconstitution of mature gametes in mice. Simultaneously, research on human IVG established similar goals, and utilized findings from successful mice studies to inform their protocol development. Researchers questioned whether the same signaling mediators possessed roles in PGC specification and differentiation. It was ultimately observed that between murine models and humans, there are species-specific differences in multiple facets of germ cell line differentiation. Research on non-human primates may alleviate this gap in knowledge, and recent studies have confirmed the monkey as a viable model of human IVG.

Additionally, more recently research on large mammals such as the rhinoceros has begun, with an aim towards animal conservation. Lastly, despite the current standing of human IVG, there are already concerns among bioethics and law professionals, pointing to the devaluation of human life, the ethics of genetic editing in embryos, and the hypothetical boundless opportunity for same-sex couples, multiplex genetic parents, or individuals to reproduce. Despite these concerns, the current state of research and ISSCR restrictions does not tangibly display any indications of concern for now. However, it is important to note that these discussions may inform future legislation over human IVG and its implementation into the clinical setting.

Appendix

Blimp1/Prdm1 – PR/SET domain 1
BMP2 – bone morphogenic protein 2
BMP4 – bone morphogenetic protein 4
BMP7 – bone morphogenic protein 7
BMP8b – bone morphogenic protein 8b
c-Myc – MYC proto-oncogene, bHLH transcription factor
cKIT – KIT proto-oncogene, receptor tyrosine kinase
CYP26A1 – Cytochrome P450 26 subfamily A member 1
Cyp26b1 – Cytochrome P450 Family 26 Subfamily B Member 1
DAZL – deleted in azoospermia like
DDX4 – DEAD-box helicase 4
EB – embryoid body
EGF – epidermal growth factor
EpiLC – epiblast-like cell
EpiSC – epiblast stem cell
ESC – embryonic stem cell
FGF2 – fibroblast growth factor 2
FOSLC – fetal ovarian somatic cell-like cell
FOXL2 – forkhead box L2
GDF3 – Growth differentiation factor 3
GDF9 – growth differentiation factor 9
GDNF – glial cell derived neurotrophic factor
GSCLCs – germline stem cell-like cells
GSK3a – glycogen synthase kinase 3 alpha
GSK3b – glycogen synthase kinase 3 beta
H3K27me3 – histone H3 trimethyl Lys27
H3K9me2 – histone H3 dimethyl Lys9
iPSC – induced pluripotent stem cell
IVF – in vitro fertilization
IVG – in vitro gametogenesis
IVM – in vitro maturation
KITLG – KIT ligand
Klf4 – KLF transcription factor 4
Klk1b15-ps – kallikrein 1-related peptidase b15, pseudogene
LIF – LIF interleukin 6 family cytokine
MII – metaphase II
MLH1 – MutL homolog 1
NANOG – Nanog homeobox
NANOS1 – nanos C2HC-type zinc finger 1
NANOS3 – Nanos C2HC-Type Zinc Finger 3
NWR – northern white rhinoceros
Oct4 – POU class 5 homeobox 1
PGC – primordial germ cell
PRDM14 – PR/SET domain 14

PSC – pluripotent stem cell
PUM1 – Pumilio RNA binding family member 1
PUM2 – Pumilio RNA binding family member 2
SOX15 – SRY-box transcription factor 15
Sox17 – SRY-box transcription factor 17
Sox2 – SRY-box transcription factor 2
SSC – spermatogonial stem cell
STELLA/DPPA3 – developmental pluripotency associated 3
Stra8 – stimulated by retinoic acid 8
SWR – southern white rhinoceros
SYCP1 – Synaptonemal complex protein 1
SYCP3 – synaptonemal complex protein 3
TEKT1 – Tektin 1
Tfap2c – transcription factor AP-2 gamma
WNT3 – Wnt family member 3

References

Aflatoonian, B., Ruban, L., Jones, M., Aflatoonian, R., Fazeli, A., & Moore, H. D. (2009). In vitro post-meiotic germ cell development from human embryonic stem cells. *Human Reproduction*, *24*(12), 3150–3159. https://doi.org/10.1093/humrep/dep334

Bowles, J., Knight, D., Smith, C., Wilhelm, D., Richman, J., Mamiya, S., Yashiro, K., Chawengsaksophak, K., Wilson, M. J., Rossant, J., Hamada, H., & Koopman, P. (2006). Retinoid signaling determines germ cell fate in mice. *Science*, *312*(5773), 596–600. https://doi.org/10.1126/science.1125691

Bucay, N., Yebra, M., Cirulli, V., Afrikanova, I., Kaido, T., Hayek, A., & Montgomery, A. M. P. (2009). A novel approach for the derivation of putative primordial germ cells and Sertoli cells from human embryonic stem cells. Stem Cells, 27(1), 68–77. https://doi.org/10.1634/stemcells.2007-1018

Chuma, S., Kanatsu-Shinohara, M., Inoue, K., Ogonuki, N., Miki, H., Toyokuni, S., Hosokawa, M., Nakatsuji, N., Ogura, A., & Shinohara, T. (2005). Spermatogenesis from epiblast and primordial germ cells following transplantation into postnatal mouse testis. Development (Cambridge, England), 132(1), 117–122. https://doi.org/10.1242/dev.01555

Clark, A. T. (2004). Spontaneous differentiation of germ cells from human embryonic stem cells in vitro. Human Molecular Genetics, 13(7), 727–739. https://doi.org/10.1093/hmg/ddh088

Clark, A. T., Brivanlou, A., Fu, J., Kato, K., Mathews, D., Niakan, K. K., Rivron, N., Saitou, M., Surani, A., Tang, F., & Rossant, J. (2021). Human embryo research, stem cell-derived embryo models and in vitro gametogenesis: Considerations leading to the revised ISSCR guidelines. Stem cell reports, 16(6), 1416–1424. https://doi.org/10.1016/j.stemcr.2021.05.008

Cohen, I. G., Daley, G. Q., & Adashi, E. Y. (2017). Disruptive reproductive technologies. Science translational medicine, 9(372), eaag2959. https://doi.org/10.1126/scitranslmed.aag2959

Eguizabal, C., Montserrat, N., Vassena, R., Barragan, M., Garreta, E., Garcia-Quevedo, L., Vidal, F., Giorgetti, A., Veiga, A., & Belmonte, J. C. (2011). Complete meiosis from human induced pluripotent stem cells. Stem Cells, 29(8), 1186–1195. https://doi.org/10.1002/stem.672

Geens, M., Sermon, K. D., Van de Velde, H., & Tournaye, H. (2011). Sertoli cell-conditioned medium induces germ cell differentiation in human embryonic stem cells. Journal of Assisted Reproduction and Genetics, 28(5), 471–480. https://doi.org/10.1007/s10815-011-9541-9

Goszczynski, D. E., Denicol, A. C., & Ross, P. J. (2019). Gametes from stem cells: Status and applications in animal reproduction. Reproduction in domestic animals = Zuchthygiene, 54 Suppl 4, 22–31. https://doi.org/10.1111/rda.13503

Gurung, P., Yetiskul, E., & Jialal, I. (2022). Physiology, male reproductive system. StatPearls Publishing LLC.

Hayashi, K., Ogushi, S., Kurimoto, K., Shimamoto, S., Ohta, H., & Saitou, M. (2012). Offspring from oocytes derived from in vitro primordial germ cell–like cells in mice. Science, 338(6109), 971–975. https://doi.org/10.1126/science.1226889

Hayashi, K., Ohta, H., Kurimoto, K., Aramaki, S., & Saitou, M. (2011). Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. Cell, 146(4), 519–532. https://doi.org/10.1016/j.cell.2011.06.052

Hayashi, K., & Saitou, M. (2014). Perspectives of germ cell development in vitro in mammals. Animal science journal = Nihon chikusan Gakkaiho, 85(6), 617–626. https://doi.org/10.1111/asj.12199

Hayashi, M., Zywitza, V., Naitou, Y., Hamazaki, N., Goeritz, F., Hermes, R., Holtze, S., Lazzari, G., Galli, C., Stejskal, J., Diecke, S., Hildebrandt, T. B., & Hayashi, K. (2022). Robust induction of primordial germ cells of white rhinoceros on the brink of extinction. Science Advances, 8(49). https://doi.org/10.1126/sciadv.abp9683

Hikabe, O., Hamazaki, N., Nagamatsu, G., Obata, Y., Hirao, Y., Hamada, N., Shimamoto, S., Imamura, T., Nakashima, K., Saitou, M., & Hayashi, K. (2016). Reconstitution in vitro of the entire cycle of the mouse female germ line. Nature, 539(7628), 299–303. https://doi.org/10.1038/nature20104

Holesh, J.E., Bass, A.N., & Lord, M. (2022). Physiology, ovulation. StatPearls Publishing LLC.

Hubner, K., Fuhrmann, G., Christenson, L. K., Kehler, J., Reinbold, R., De La Fuente, R., Wood, J., Strauss, J. F., 3rd, Boiani, M., & Schöler, H. R. (2003). Derivation of oocytes from mouse embryonic stem cells. Science (New York, N.Y.), 300(5623), 1251–1256. https://doi.org/10.1126/science.1083452

Ishikura, Y., Ohta, H., Sato, T., Murase, Y., Yabuta, Y., Kojima, Y., Yamashiro, C., Nakamura, T., Yamamoto, T., Ogawa, T., & Saitou, M. (2021). In vitro reconstitution of the whole male germ-cell development from mouse pluripotent stem cells. Cell Stem Cell, 28(12). https://doi.org/10.1016/j.stem.2021.08.005

Kee, K., Gonsalves, J. M., Clark, A. T., & Pera, R. A. (2006). Bone morphogenetic proteins induce germ cell differentiation from human embryonic stem cells. Stem Cells and Development, 15(6), 831–837. https://doi.org/10.1089/scd.2006.15.831

Lawson, K. A., Dunn, N. R., Roelen, B. A. J., Zeinstra, L. M., Davis, A. M., Wright, C. V. E., Korving, J. P. W. F. M., & Hogan, B. L. M. (1999). BMP4 is required for the generation of primordial germ cells in the mouse embryo. Genes & Development, 13(4), 424–436. https://doi.org/10.1101/gad.13.4.424

MacLean, G., Li, H., Metzger, D., Chambon, P., & Petkovich, M. (2007). Apoptotic extinction of germ cells in testes of CYP26B1 knockout mice. Endocrinology, 148(10), 4560–4567. https://doi.org/10.1210/en.2007-0492

Morohaku, K., Tanimoto, R., Sasaki, K., Kawahara-Miki, R., Kono, T., Hayashi, K., Hirao, Y., & Obata, Y. (2016). Complete in vitro generation of fertile oocytes from mouse primordial germ cells. Proceedings of the National Academy of Sciences, 113(32), 9021–9026. https://doi.org/10.1073/pnas.1603817113

Nayernia, K., Nolte, J., Michelmann, H. W., Lee, J. H., Rathsack, K., Drusenheimer, N., Dev, A., Wulf, G., Ehrmann, I. E., Elliott, D. J., Okpanyi, V., Zechner, U., Haaf, T., Meinhardt, A., & Engel, W. (2006). In vitro-differentiated embryonic stem cells give rise to male gametes that can generate offspring mice. Developmental Cell, 11(1), 125–132. https://doi.org/10.1016/j.devcel.2006.05.010

Ohinata, Y., Payer, B., O'Carroll, D., Ancelin, K., Ono, Y., Sano, M., Barton, S. C., Obukhanych, T., Nussenzweig, M., Tarakhovsky, A., Saitou, M., & Surani, M. A. (2005). BLIMP1 is a critical determinant of the germ cell lineage in mice. Nature, 436(7048), 207–213. https://doi.org/10.1038/nature03813

Palacios-González, C., Harris, J., & Testa, G. (2014). Multiplex parenting: IVG and the generations to come. Journal of Medical Ethics, 40(11), 752–758. https://doi.org/10.1136/medethics-2013-101810

Park, T. S., Galic, Z., Conway, A. E., Lindgren, A., van Handel, B. J., Magnusson, M., Richter, L., Teitell, M. A., Mikkola, H. K., Lowry, W. E., Plath, K., & Clark, A. T. (2009). Derivation of primordial germ cells from human embryonic and induced pluripotent stem cells is significantly improved by Coculture with human fetal gonadal cells. Stem Cells, 27(4), 783–795. https://doi.org/10.1002/stem.13

Qing, T., Shi, Y., Qin, H., Ye, X., Wei, W., Liu, H., Ding, M., & Deng, H. (2007). Induction of oocyte-like cells from mouse embryonic stem cells by co-culture with ovarian granulosa cells. Differentiation, 75(10), 902–911. https://doi.org/10.1111/j.1432-0436.2007.00181.x

Rosner, J., Samardzic, T., & Sarao M.S. (2022). Physiology, female reproduction. StatPearls Publishing LLC.

Sakai, Y., Nakamura, T., Okamoto, I., Gyobu-Motani, S., Ohta, H., Yabuta, Y., Tsukiyama, T., Iwatani, C., Tsuchiya, H., Ema, M., Morizane, A., Takahashi, J., Yamamoto, T., & Saitou, M. (2020). Induction of the germ cell fate from pluripotent stem cells in cynomolgus monkeys. Biology of reproduction, 102(3), 620–638. https://doi.org/10.1093/biolre/ioz205

Saitou, M., & Hayashi, K. (2021). Mammalian in vitro gametogenesis. Science, 374(6563). https://doi.org/10.1126/science.aaz6830

Saitou, M., Payer, B., O'Carroll, D., Ohinata, Y., & Surani, M. A. (2005). Blimp1 and the emergence of the germ line during development in the mouse. Cell Cycle, 4(12), 1736–1740. https://doi.org/10.4161/cc.4.12.2209

Sasaki, K., Yokobayashi, S., Nakamura, T., Okamoto, I., Yabuta, Y., Kurimoto, K., Ohta, H., Moritoki, Y., Iwatani, C., Tsuchiya, H., Nakamura, S., Sekiguchi, K., Sakuma, T., Yamamoto, T., Mori, T., Woltjen, K., Nakagawa, M., Yamamoto, T., Takahashi, K., … Saitou, M. (2015). Robust in vitro induction of human germ cell fate from pluripotent stem cells. Cell Stem Cell, 17(2), 178–194. https://doi.org/10.1016/j.stem.2015.06.014

Sugawa, F., Araúzo-Bravo, M. J., Yoon, J., Kim, K. P., Aramaki, S., Wu, G., Stehling, M., Psathaki, O. E., Hübner, K., & Schöler, H. R. (2015). Human primordial germ cell commitment in vitro associates with a unique PRDM14 expression profile. The EMBO Journal, 34(8), 1009–1024. https://doi.org/10.15252/embj.201488049

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., & Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell, 131(5), 861–872. https://doi.org/10.1016/j.cell.2007.11.019

Takahashi, K., & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell, 126(4), 663–676. https://doi.org/10.1016/j.cell.2006.07.024

Tilgner, K., Atkinson, S. P., Golebiewska, A., Stojković, M., Lako, M., & Armstrong, L. (2008). Isolation of primordial germ cells from differentiating human embryonic stem cells. Stem Cells, 26(12), 3075–3085. https://doi.org/10.1634/stemcells.2008-0289

West, F. D., Machacek, D. W., Boyd, N. L., Pandiyan, K., Robbins, K. R., & Stice, S. L. (2008). Enrichment and differentiation of human germ-like cells mediated by feeder cells and basic fibroblast growth factor signaling. Stem Cells, 26(11), 2768–2776. https://doi.org/10.1634/stemcells.2008-0124

West, F. D., Mumaw, J. L., Gallegos-Cardenas, A., Young, A., & Stice, S. L. (2011). Human haploid cells differentiated from meiotic competent clonal germ cell lines that originated from embryonic stem cells. Stem Cells and Development, 20(6), 1079–1088. https://doi.org/10.1089/scd.2010.0255

Xuemei, L., Jing, Y., Bei, X., Juan, H., Xinling, R., Qun, L., & Guijin, Z. (2013). Retinoic acid improves germ cell differentiation from human embryonic stem cells. Iranian journal of reproductive medicine, 11(11), 905–912.

Yamaji, M., Seki, Y., Kurimoto, K., Yabuta, Y., Yuasa, M., Shigeta, M., Yamanaka, K., Ohinata, Y., & Saitou, M. (2008). Critical function of PRDM14 for the establishment of the germ cell lineage in mice. Nature Genetics, 40(8), 1016–1022. https://doi.org/10.1038/ng.186

Yamashiro, C., Sasaki, K., Yabuta, Y., Kojima, Y., Nakamura, T., Okamoto, I., Yokobayashi, S., Murase, Y., Ishikura, Y., Shirane, K., Sasaki, H., Yamamoto, T., & Saitou, M. (2018). Generation of human oogonia from induced pluripotent stem cells in vitro. Science (New York, N.Y.), 362(6412), 356–360. https://doi.org/10.1126/science.aat1674

Yoshino, T., Suzuki, T., Nagamatsu, G., Yabukami, H., Ikegaya, M., Kishima, M., Kita, H., Imamura, T., Nakashima, K., Nishinakamura, R., Tachibana, M., Inoue, M., Shima, Y., Morohashi, K. I., & Hayashi, K. (2021). Generation of ovarian follicles from mouse pluripotent stem cells. Science (New York, N.Y.), 373(6552), eabe0237. https://doi.org/10.1126/science.abe0237

Zakrzewski, W., Dobrzyński, M., Szymonowicz, M., & Rybak, Z. (2019). Stem cells: Past, present, and future. Stem cell research & therapy, 10(1), 68. https://doi.org/10.1186/s13287-019-1165-5

Zhou, Q., Wang, M., Yuan, Y., Wang, X., Fu, R., Wan, H., Xie, M., Liu, M., Guo, X., Zheng, Y., Feng, G., Shi, Q., Zhao, X.-Y., Sha, J., & Zhou, Q. (2016). Complete meiosis from embryonic stem cell-derived germ cells in vitro. Cell Stem Cell, 18(3), 330–340. https://doi.org/10.1016/j.stem.2016.01.017