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INVITED REVIEW

Germ Cell Biology

# Insights into female germ cell biology: from *in vivo* development to *in vitro* derivations

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**Understanding the mechanisms of human germ cell biology is important for developing infertility treatments. However, little is known about the mechanisms that regulate human gametogenesis due to the difficulties in collecting samples, especially germ cells during fetal development. In contrast to the mitotic arrest of spermatogonia stem cells in the fetal testis, female germ cells proceed into meiosis and began folliculogenesis in fetal ovaries. Regulations of these developmental events, including the initiation of meiosis and the endowment of primordial follicles, remain an enigma. Studying the molecular mechanisms of female germ cell biology in the human ovary has been mostly limited to spatiotemporal characterizations of genes or proteins. Recent efforts in utilizing *in vitro* differentiation system of stem cells to derive germ cells have allowed researchers to begin studying molecular mechanisms during human germ cell development. Meanwhile, the possibility of isolating female germline stem cells in adult ovaries also excites researchers and generates many debates. This review will mainly focus on presenting and discussing recent *in vivo* and *in vitro* studies on female germ cell biology in human. The topics will highlight the progress made in understanding the three main stages of germ cell developments: namely, primordial germ cell formation, meiotic initiation, and folliculogenesis.**
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## INTRODUCTION

Although sperms and oocytes are derived from primordial germ cells (PGCs) formed in human fetuses, the key steps of their developments differ considerably after the formation of spermatogonia and oogonia. Male germ cells are arrested at mitotic stage in the fetal testis until puberty. When spermatogonial stem cells start to differentiate and enter meiosis, the human testis can produce hundred millions of sperm daily. In contrast, female germ cells begin with an endowment of about 5 million oogonia and then proceed to meiosis in the fetal ovary. However, the female ovary only produces one mature oocyte per menstrual period, and the total number of oocytes ovulated is about 456 if an individual starts to ovulate at age 12 and menopause at age 50. Therefore, the depletion of oogonia has been thought to be caused by a lack of female germline stem cells (GSCs) in the adult ovary.

Our understanding of female germ cell biology is mostly extrapolated from model organisms such as mice. A human system will give us a more precise understanding of human female germ cell development since there are many differences between animal and human germ cells. *In vitro* production of germ cells from pluripotent stem cells, including human embryonic stem cells (hESCs) and induced pluripotent stem cells, has provided an unprecedented opportunity to explore the cellular and molecular mechanisms of human germ cell biology.[1–7] Pluripotent stem cells are cells that can give rise to all three somatic lineages and the germ cell lineage. If these cells can be directed to differentiate into germ cell lineages, the process of germ cell biology can be studied from the early stages to the mature sperm or oocyte. On the other hand, developing and setting up an *in vitro* system that can mimic the *in vivo* developmental process require some basic knowledge of female germ cell biology. In this review, we first examined what we have learned from *in vivo* studies and compared them to the progress made by using *in vitro* systems.

### *Primordial germ cell development*

Primordial germ cells are the embryonic precursors of the gametes.[8,9] These cells are first identified in the proximal epiblast around the third week of human gestation.[8] Then, the PGC population gradually proliferates during PGC migration to gonadal ridges at 4–5 weeks.[10,11] The transition of PGCs into gonocytes starts at 8 weeks of gestation, followed by sex-specific differentiation.[12] In the female gonads, oogonia generally enter meiosis and remain quiescent in the first meiotic prophase during embryonic development around week 12, whereas in the male, spermatogonia arrest in G0/G1 of mitosis and do not enter meiosis until puberty.[13–15]

#### *Primordial germ cells specification*

Several studies have proposed the extragonadal location of PGCs in human embryos.[8,16,17] Despite the first observations on the extragonadal location of PGCs, the exact mechanisms of signal interaction that controls the specification of PGCs in human is poorly understood. The mechanisms underlying PGC specification in mammals are relatively conserved.[18–21] Evidence from mice studies has demonstrated that bone morphogenetic protein 4 (BMP4) and BMP8b released from the extraembryonic ectoderm and BMP2 from the proximal visceral endoderm induce the formation of nascent PGCs.[21] Similar to these studies in mice, Childs *et al.*[20] demonstrated expression of genes

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encoding BMP ligands with an increase in the expression of BMP2 in the human fetal ovary. Human *in vitro* studies has also suggested that BMP4 along with BMP7 and BMP8b are necessary for human germ cell differentiation.[18] WNT signaling is also involved in PGC specification.[19] It has recently been reported that WNT3 and BMPs signaling pathway both contributed to activate BLIMP1 and PRDM14, which leads to the specification of PGCs.[22] The expression of these factors may indicate conserved roles in PGC specification. Consistent with the mouse studies, BLIMP1 is expressed in human fetal gonads and hESCs-derived germline cells.[23,24] Recent work suggested that BLIMP1 is co-expressed with OCT4 in human PGC precursors, but not with the late germ cell marker, VASA.[25] Additionally, BLIMP1 may modulate the induction of germ cell specification by turning off SOX2 during early human development.[23] These findings suggest that BLIMP1 might act in a molecular switch to regulate the germline fates determination during early human development.

***Migration***

Primordial germ cells exit from the wall of the hindgut to incorporate into the extracellular matrix (ECM) of the dorsal mesentery.[26,27] These cells are able to move actively through the ECM substrates to reach the gonadal crest. Several hypotheses explaining PGC migration to the gonad have been suggested. Migratory PGCs seem to interact with mesenchymal cells through ECM molecules, such as glycosaminoglycan and fibronectin.[26] These different types of proteoglycans may facilitate the migration of the PGCs. Chemoattractants, stem cell factor (SCF or c-Kit ligand) and stromal-derived factor-1, are also implicated in guiding PGCs to the gonad.[28,29] Support for this hypothesis was provided by c-Kit receptor staining, which revealed that the receptor of c-Kit ligand is expressed in human PGCs.[29] A recent study has shown that human PGCs move together with autonomic nerve fibers and Schwann cells, and it is proposed that these nerve fibers may support the PGC migration.[30] During migration, human PGCs proliferate by mitotic divisions and they enter into a process of cell differentiation after colonization, and are known as oogonia and spermatogonia.[31,32]

***Sex determination and transition from primordial germ cells to oogonia***

Sex determination in humans is not well understood at the molecular level. Mammalian sex determination is dependent on genetic control primarily induced by the chromosomal set with the presence or absence of a particular gene on the Y chromosome, SRY.[33] The malfunction of SRY is most likely responsible for some human XY disorders of sexual development.[34–36] For example, the reduction of WT1, a potential regulator of SRY, is associated with XY sex reversal (a case of Frasier syndrome).[36] Mouse studies showed that the expression of SRY in the genital ridges initiates testis formation by directing precursors of supporting cells to Sertoli cells rather than granulosa cells.[33,37,38] In addition, *Foxl2* and *Rspo1* seem to play important roles in female sex determination.[39–41] In experimental models, both XX *Rspo1*$^{-/-}$ gonads and XX *Foxl2*$^{-/-}$ gonads are associated with female-to-male sex reversal.[40–42] The regulation of germ cell proliferation is associated with forming of the functional unit of the ovary termed primordial follicle.[43,44] Activin A, a member of the transforming growth factor-β family, has been shown to increase transiently the number of PGCs during the developmental period leading up to primordial follicle formation in human.[44] Hereafter, oogonia in the primordial follicle generally enter prophase of the first meiotic division, while spermatogonia do not enter meiosis until puberty.[45,46]

***In vitro induction of human primordial germ cells***

Human embryonic stem-based systems that can recapitulate basic mechanisms of PGC specifications will provide a valuable platform to study human germ cell development.[1,3,5–7] The *in vitro* cell culture system of differentiation also gives us new chances to test extrinsic factors essential during human germ cell development, including growth factors that may induce germ cell formation or specifications. Studies in mice have demonstrated that the formation and specification of the mouse PGC precursor population are dependent on BMP2, BMP4 and BMP8b.[47–49] Additionally, mouse ESCs bearing BLIMP1 and *Stella* transgenes were induced into PGC-like cells by BMP signals and underwent oogenesis in reconstituted ovaries.[50] Using hESCs, researchers found that the signaling pathway of BMPs also functions to induce human PGCs. One study shows that BMP4, BMP7, BMP8b can promote PGC formation from hESCs *in vitro*.[18] In other studies, stimulation with other factors such as fibroblast growth factor 2 (FGF2), SCF or WNT3A also can enrich the germ cell-like population.[7,51,52]

Induction of PGCs in differentiated hESCs opens the door to study human PGC *in vitro*, but it is necessary to isolate the PGC populations from a mixed population of different cell lineages. A reporter system, in which the promoter of a germ-cell specific gene, VASA (or DDX), is connected to a fluorescent protein, was constructed to accomplish this goal.[3] Under BMP induction, about 5% of the differentiated population expressed green fluorescent protein (GFP) and also express other PGC markers. These *in vitro* derived human PGCs were hypomethylated and proliferated slowly on inactivated mouse embryonic fibroblasts. Using this system, the researchers determined the genetic requirement of PGC formation and found that human DAZL was required for PGC formation. Another study showed that ectopic expression of VASA in human ESCs promoted PGCs formation and progression through meiosis.[53]

***Meiotic progression***

***From late primordial germ cells to initiation of meiosis***

Two independent groups reported that retinoic acid (RA) plays an important role in initiating meiosis in mice.[54,55] RA, which is produced by the mesonephros of both sexes, may play a role in the initiation of meiosis.[56] RA induces Stra8, the meiotic gatekeeper gene, in ovarian germ cells, while in the testicular germ cells this is prevented by the RA-degrading enzyme CYP26B1. However, a recent study with Raldh2$^{-/-}$ mice lacking RA activity has shown that Stra8 and meiosis markers, SYCP3 and γH2AX, were detectable in the fetal ovary.[57] The authors suggested that CYP26B1 might inhibit the meiosis initiation by degrading an unknown inducer of Stra8 other than RA in the testis. Although meiosis seems to be a prerequisite event of folliculogenesis, a recent study in mice using Stra8 knockout mice indicated that the folliculogenesis can proceed in the absence of the meiosis initiation.[58] The principal mechanisms and potential inducers other than RA that govern meiotic initiation and folliculogenesis remain to be elucidated.

Only a few studies have reported meiotic initiation in human fetal gonad.[32,46,59] In contrast to mice, the developmental stages of the germ cells in the human fetal ovary are much less synchronized. Recent findings in the fetal human gonad suggested that RA is a possible intrinsic factor involved in meiosis entry in the human ovary.[46,59] Subsequently, STRA8 was up-regulated in response to RA, followed by the induction of SYCP3 and DMC in the fetal human gonad. Surprisingly, the expression of CYP26B1 was not significantly different between ovaries and testes. This suggested that the role of CYP26B1 in the regulation of meiosis may not be the major mechanism of meiosis inhibition in the human fetal testis. One previous study investigated the expression pattern of DMRT1 in the human fetal ovaries.[32] These results suggested that there is a transient up-regulation of DMRT1 in human fetal ovary prior to initiation of meiosis. Taken together, these

findings hint that the detailed mechanisms of meiosis in humans might be different from that previously described in mice.

***Meiotic recombination***

Once meiosis is initiated, meiotic recombination is promoted by meiosis-specific regulatory networks. Genes involved in the formation of a chromosomal scaffold and recombination, such as REC8, SPO11, DMC1, SYCP1, SYCP2, SYCP3, and MLH1 are essential for meiotic progression.[32,60,61] During the first meiotic division, double-strand breaks (DSBs) in DNA can induce crossing over between homologous chromosomes, followed by the recruitment of meiotic-specific proteins to DSBs sites that are involved in meiotic recombination.[62] PRDM9 has recently been identified as the major determinant in specifying meiotic recombination hot spots in mouse and human.[63,64] Further study showed that the variation in the zinc finger domain of PRDM9 affects DSB loci distribution and outcomes of meiotic recombinations in human sperm.[65] Zheng *et al.*[66] analyzed the global functional network by integrating diverse genomic datasets to understand the meiotic initiation and progression in the human fetal ovary. The authors found that meiosis-specific genes are highly clustered with themselves compared to low clustering of meiosis-nonspecific genes. Interestingly, DAZL was identified as a novel meiotic-initiation gene in this study, in accordance with its role in mice. Very recently, DAZL and BOULE were found to show distinct spatio-temporal expression patterns during meiosis in human fetal ovaries, in contrast to their co-expression pattern in mouse fetal ovaries.[67] Again, these recent studies found a common principal of meiotic progression in mouse and human ovary, but subtle differences were also significant.

***In vitro induction of meiosis***

Until date, a few studies reported successful *in vitro* induction of meiotic progression from undifferentiated human stem cells. Clark *et al.*[1] observed an increase in transcriptional expression of meiotic makers when hESCs were induced to differentiate by fetal bovine serum, but failed to detect clear synaptonemal complex formation and MLH1 foci. Meiotic initiation marked by γH2AX and meiotic prophase indicated by elongated SYCP3 staining were detected in an independent study when DAZL, BOULE or DAZ was overexpressed in hESCs.[3] Moreover, a small percentage of haploid spermatids could be isolated in the differentiated cultures, suggesting a completion of meiosis in the male cell lines. In the same study, about 20% of female hESCs were induced to enter into meiotic prophase, but no follicle-like cells was detected.

***Folliculogenesis***

The primary oocytes that are arrested at the prophase stage of the first meiotic division synthesize and accumulate RNAs and proteins for oocyte growth and maturation.[68,69] Byskov *et al.* found that a group of sterols, termed meiosis activating sterols, were able to induce oocyte maturation *in vitro*.[70,71] These sterols are most likely acting downstream of follicle-stimulating hormone (FSH) signaling pathway to induce oocyte maturation. In addition, communication between oocytes and their surrounding granulosa cells are crucial at all stages of folliculogenesis, both for oocyte maturation and for granulosa cells differentiation.[72,73] Oogonial nests surrounded by somatic cells and those cells may have consequences for the activating cues in primordial follicle formation.[44] Communication between oocyte and surrounding granulosa cells through autocrine, endocrine, and paracrine manners coordinate further development of ovarian follicle.[74–76] Subsequently, primordial follicles are activated and further develop into primary follicle or more mature follicles.[77]

During the transitions from primordial to antral follicles, many molecular mechanisms must be tightly controlled in order to produce mature and functional oocytes.[78–80] The phosphoinositide 3 kinase (PI3K)-protein kinase B (Akt) signaling pathway leads to primordial follicle survival and growth, whereas PTEN is a negative regulator of the PI3K pathway.[79,80] In a mouse model, a deficiency in NOBOX, an ovary-specific gene, leads to decreased expression of many major oocyte transcripts, such as OCT4, FGF8, Zar1, growth differentiation factor 9 (GDF9), Bmp15, and H1foo.[69,81] Consistent with the mouse studies, misregulation of NOBOX is associated with premature ovarian failure (POF) in human.[82] Thus, both PTEN/PI3K signaling pathway and NOBOX are crucial in the regulation of early oogenesis and folliculogenesis. Until date, only a few transcriptional factors, such as FIGLA and NOBOX, have been associated with oocyte development.[83,84] It has been reported that TATA-binding protein 2 (TBP2) is involved in mouse oocyte development.[85,86] TBP2-deficient mice show a decreased number of secondary follicles and reveal an altered expression of GDF9, Bmp15, ZP3, and H1foo. Although the role of TBP2 has not yet been elucidated in human, enrichment of TBP2 in the ovary is conserved in vertebrates. Altogether, the above studies suggest a potential role for TBP2 in the human folliculogenesis.

***Bidirectional communications of oocyte and granulosa cells***

The bidirectional communication between oocytes and its surrounding somatic cells is essential for the differentiation and maturation of ovarian follicle compartments.[72,73] The complex intraovarian mechanisms have been implicated in this process as ovarian paracrine or autocrine regulators. In particular, GDF9 and BMP15 are principal participants of follicular development and fertility.[87–89] In humans, missense mutations in GDF9 and a point mutation in BMP15 have been associated with POF.[89–91] Extensive studies of GDF9 and BMP15 have been carried out in mutant animal models. GDF9-deficient mice exhibit growth arrest at the primary follicle stage similar to the human phenotype;[92] in contrast, Bmp15 knockout mice are subfertile, but patients carrying mutations of BMP15 exhibit severe reproductive defects.[89,93] The differences in mutants phenotype of mice versus phenotypes associated with human BMP15 mutations might be owing to specie-specific differences between monoovulatory human and polyovulatory mice.[94] Granulosa cell proliferation and antral cavity accumulation within preantral follicles ultimately give rise to antral follicles. Unlike in earlier stages, growing follicles in their late stages express functional FSH and luteinizing hormone (LH) receptors which are able to respond to gonadotropins.[95] The surge of gonadotropins, FSH and LH, initiate a cascade of events leading to follicular rupture and oocyte release.[96]

***In vitro derivations of oocytes***

Although *in vitro* derivation of human oocytes or follicles from stem cells is a much anticipated advance in human reproduction, there has been no successful report so far. Even in mouse studies, only a few studies have shown progress in deriving oocytes from mouse stem cells. The first exciting report was done by Hübner *et al.*[97] in 2003, in which follicle-like aggregates were found in the suspension of a spontaneously differentiated culture. These aggregates expressed GDF9 mRNA transcript and estradiols. The isolated oocyte-like cells also expressed zona pellucida proteins ZP2 and ZP3; however, no offspring was reported in this study. The most recent report by Hayashi *et al.* showed the generation of fertilizable oocytes from PGC-like cells derived from mouse ES cells.[50] This was achieved by isolating PGC-like cells induced from mouse epiblast stem cells, followed by their aggregation with fetal ovaries and transplantation into mouse ovarian bursa. Moreover, offspring were produced by *in vitro* fertilization of the derived oocytes.

Hence, the later report required an aggregation and transplantation step that would not be feasible if this were to be extended to human oocyte derivations.

Despite reported studies performed with human ES cells, human oocyte-like cells have a limited capability to generate mature and functional oocytes. For instance, Liu *et al*. suggested that pluripotent human amniotic fluid cells (HuAFCs) have the potential to differentiate into oocyte-like cells *in vitro*.[98] Furthermore, the oocyte-like cells from HuAFCs express DMC1, SCP3, DAZL, ZP3 and GDF9. Many attempts need to be carried out to achieve *in vitro* derivations of functional oocyte or follicles.

### *Existence of female germline stem cells in adult ovary*

Recently, the central dogma of female reproductive biology has been challenged by some surprising observations. Female GSCs exist in lower species, such as invertebrates, fish, and birds, and possess the capacity to provide a reservoir of oogonia to generate new oocytes in the adult ovaries;[99,100] however, no GSCs have been found in the mammalian adult ovary. In 2004, Johnson *et al*.[101] concluded that female GSCs existed in the postnatal mammalian ovary and that this population can give rise to functional oocytes under certain conditions. A study was done in mice and is based on the morphological appearance and immunological detection of the germline-specific marker (mouse VASA homolog, also known as VASA homolog) to isolate a fraction of GSCs from dispersed ovaries. Follow-up studies documented rare mitotically active cells with germline characteristics in post-menopausal woman that spontaneously form oocyte-like cells *in vitro* and *in vivo*.[102] Zou *et al*.[103] offered additional evidence for the existence of GSCs in the adult mammalian females, by allowing a retrovirus to carry the expression of GFP in female GSCs prior to transplantation. Another follow-up study showed that GFP-expressing oocytes were observed in chemo-ablated mice transplanted with female GSCs;[104] and the authors concluded that the oocytes came from circulating GSCs from bone marrow. However, these results raised doubts and generated debates on whether the methods used to examine the existence of GSCs were reliable.[105] Following these initial reports supporting the existence of GSCs in postnatal ovaries, many researchers attempted to verify the results but found opposite conclusions. First, Eggan *et al*.[106] found no circulating ovulated oocytes from circulating cells of bone marrow by using the parabiotic mouse model approach. Two other studies looking for mitotically active GSCs in mice also clearly showed that there are no postnatal GSCs in mouse ovaries.[107,108] Byskov *et al*. described that oogonia and some diplotene oocytes exists in the first-trimester human ovaries.[109] In perinatal ovaries, a few oogonia, numerous diplotene oocytes, and follicles formation were observed. However, the researchers found no oogonia in postnatal human ovaries older than 2 years. Therefore, the existence of GSCs in postnatal ovary remains ambiguous.

## FUTURE PERSPECTIVE

Studies of *in vitro* derived germ cells, using different methodologies, have made possible to delineate some functional signatures indicative of human female germ cell development.[2,110–120] However, there are technical challenges to overcome and to develop in the *in vitro* differentiation system. For instance, the efficiency of derivation of PGCs from hESC and completion of meiosis are still relatively low in *in vitro* systems. Finally, somatic cells surrounding germ cells in gonads may be needed for *in vitro* maturation of human gametes. Stepwise construction of an *in vitro* differentiation system consisting of germ cells and somatic cells may yield the most fruitful progress to mimic *in vivo* development of human germ cells. Indeed, a more detailed understanding of the mechanisms of human germ cell development and its interaction with the gonadal niche is central to develop more effective differentiation systems. Meanwhile, studies using model organisms such as mice and conventional studies of human reproductive biology may help us to build the *in vitro* system. Even though the advance of *in vitro* systems for human reproduction is still limited, efforts to improve the efficiency should be continued because of the unprecedented opportunity we can explore to understand human germ cell biology, as well as for the therapeutic potential in regenerative medicine.

## AUTHOR CONTRIBUTIONS

DJ and KK designed and wrote the manuscript.

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