**CSIRO** PUBLISHING

*Reproduction, Fertility and Development*, 2021, **33**, 91–101
https://doi.org/10.1071/RD20265

# Artificially produced gametes in mice, humans and other species

*Katsuhiko Hayashi*[A,G], *Cesare Galli*[B,C], *Sebastian Diecke*[D] *and Thomas B. Hildebrandt*[E,F]

[A]Department of Stem Cell Biology and Medicine, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-0054, Japan.
[B]Avantea, Laboratory of Reproductive Technologies, 26100 Cremona, Italy.
[C]Fondazione Avantea, 26100 Cremona, Italy.
[D]Max-Delbrueck-Center for Molecular Medicine, 13092 Berlin, Germany.
[E]Leibniz Institute for Zoo and Wildlife Research, D-10315 Berlin, Germany.
[F]Freie Universität Berlin, D-14195 Berlin, Germany.
[G]Corresponding author. Email: hayashik@hgs.med.kyushu-u.ac.jp

**Abstract.** The production of gametes from pluripotent stem cells in culture, also known as *in vitro* gametogenesis, will make an important contribution to reproductive biology and regenerative medicine, both as a unique tool for understanding germ cell development and as an alternative source of gametes for reproduction. *In vitro* gametogenesis was developed using mouse pluripotent stem cells but is increasingly being applied in other mammalian species, including humans. In principle, the entire process of germ cell development is nearly reconstitutable in culture using mouse pluripotent stem cells, although the fidelity of differentiation processes and the quality of resultant gametes remain to be refined. The methodology in the mouse system is only partially applicable to other species, and thus it must be optimised for each species. In this review, we update the current status of *in vitro* gametogenesis in mice, humans and other animals, and discuss challenges for further development of this technology.

**Keywords:** germ cells, *in vitro*, pluripotent stem cells, reconstitution.

Published online 8 January 2021

## Outline of mammalian germ cell development *in vivo*

The germ cell lineage constitutes a cycle, in which a zygote becomes another zygote through several intermediates (Fig. 1). In mammalian development, the totipotent zygote first segregates into a pluripotent cell lineage and trophectoderm cell lineage, which become the embryo proper and the placental tissue respectively. The mammalian germ lineage emerges from the former cell lineage at an early stage of embryogenesis. Although several species differences exist, in principle the first sign of germ cell differentiation in mammals emerges upon gastrulation. In mice, primordial germ cells (PGCs) are induced in the pluripotent epiblast, a derivative of the inner cell mass of the blastocyst, at Embryonic Day (E) 6.5 in response to bone morphogenetic protein (BMP) 4 secreted from the adjacent extra-embryonic ectoderm (Lawson and Hage 1994; Tam and Zhou 1996; Lawson *et al.* 1999). PGC specification is governed by a set of transcription factors, including PR/SET Domain 1 (*Prdm1*), PR/SET Domain 14 (*Prdm14*), Transcription Factor AP-2 Gamma (*Tfap2c*) and Nanog homeobox (*Nanog*), that activate downstream germ cell genes, repress somatic genes and modulate genes involved in epigenetic modifications (Ohinata *et al.* 2005; Yamaji *et al.* 2008; Weber *et al.* 2010; Nakaki *et al.* 2013; Murakami *et al.* 2016). Once PGCs are specified, they undergo a unique series of differentiation events, including expression of germ cell-specific genes, such as Deleted In Azoospermia Like (*Dazl*) and (DEAD-Box Helicase 4 (*Ddx4*), a mouse homologue of Drosophila Vasa (*Vas*)), and sex-dependent differentiation (see below). Aside from the role played by BMP4, knockout studies have revealed that WNT signalling plays an essential role in the induction of the mesoderm and PGCs (Liu *et al.* 1999; Ben-Haim *et al.* 2006; Aramaki *et al.* 2013), yet the details of the cross-talk between BMP and WNT signalling remain elusive. Once specified in the posterior end of the embryo, PGCs start to migrate towards the embryonic gonads, the precursor of the testis or ovary, while proliferating. Proliferation of PGCs entails a genome-wide reorganisation of epigenetic modifications (epigenetic reprogramming) that is represented by massive DNA demethylation (Hajkova *et al.* 2002; Seisenberger *et al.* 2012) and conversion of histone H3 lysine 9 di- or tri-methylation (H3K9 me2/3) to histone H3 lysine 27 trimethylation (H3K27 me3) (Seki *et al.* 2005; Hajkova *et al.* 2008). This genomic reprograming is associated with

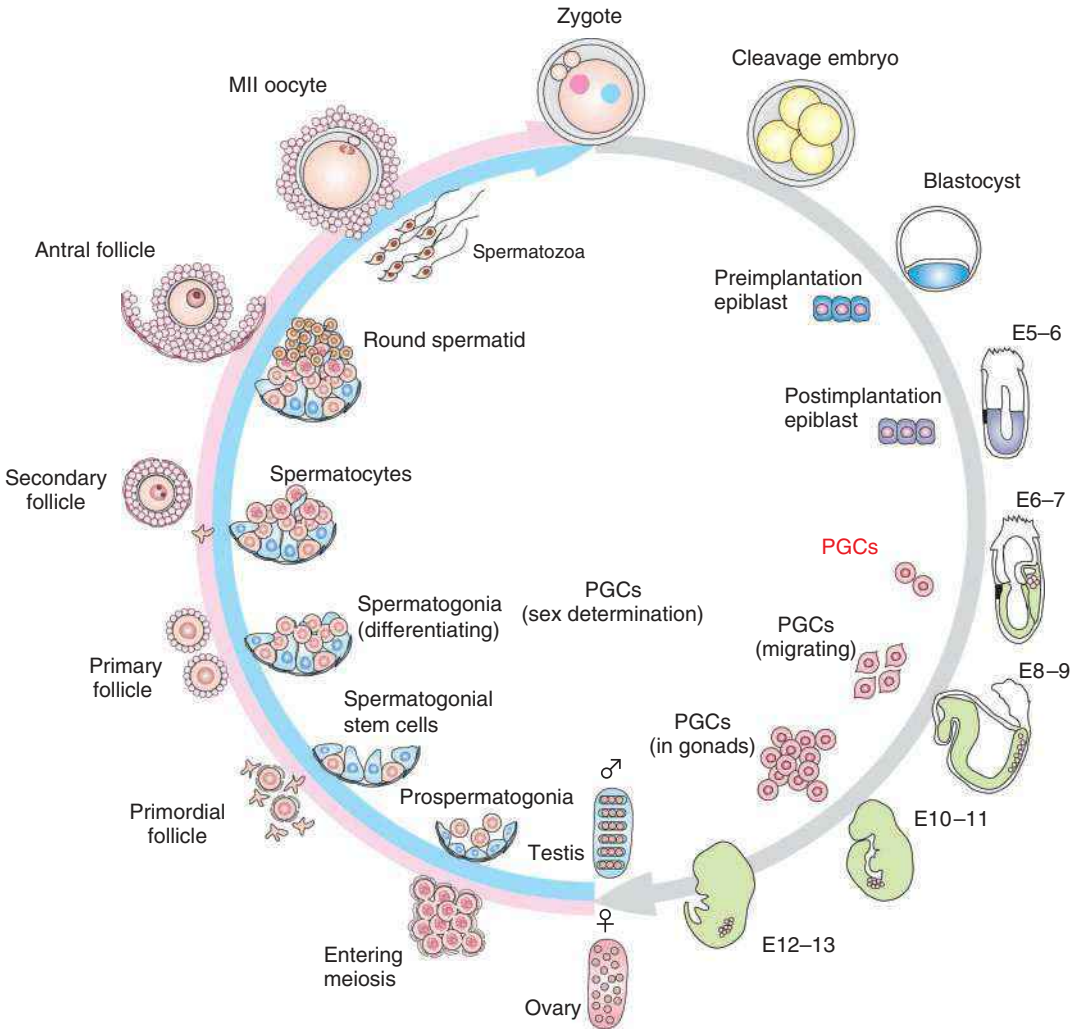


**Fig. 1.** Schematic representation of the germ cell cycle in mice. After fertilisation, followed by several cleavages, the egg forms the blastocyst including the pluripotent cell population, called the preimplantation epiblast. Primordial germ cells (PGCs) are specified from the postimplantation epiblast at around Embryonic Day (E) 6 and then start to migrate towards the gonads. In response to signals from the gonadal somatic cells, PGCs either enter meiosis in the female or differentiate into prespermatogonia in the male. After sex determination, oogenesis and spermatogenesis take place in the ovary and testis respectively. Finally, mature gametes (MII oocytes and spermatozoa) are fertilised, thereby completing the germ cell cycle.

subsequent germ cell differentiation and acquisition of developmental potency in gametes.

After reaching the gonads, the PGCs undergo a sex-dependent differentiation process (i.e. spermatogenesis in males and oogenesis in females). In the testis, PGCs continue to proliferate by around E15 and then arrest their cell cycle at $G_1$ to become prospermatogonia. During $G_1$ arrest, male-specific epigenetic modifications (i.e. *de novo* DNA methylation on the regulatory element controlling genomic imprinting) are accomplished in the spermatogonia (Seisenberger *et al.* 2012; Kobayashi *et al.* 2013; Kubo *et al.* 2015). In the ovaries, in contrast, PGCs cease proliferation and enter into meiosis, thereby becoming primary oocytes (McLaren and Southee 1997; Bowles *et al.* 2006; Koubova *et al.* 2006). Around birth, prospermatogonia locate on the basement membrane of the seminiferous tubule and resume mitosis, thereby becoming spermatogonia. Some of the spermatogonia become spermatogonial stem cells that sustain, for almost their entire lives, a capacity for both self-renewal and differentiation towards spermatozoa that is accomplished through meiosis and dynamic morphological changes, called spermiogenesis. In oogenesis, primary oocytes form a syncytium, in which each oocyte is interconnected via intercellular bridges. At the perinatal stage, the majority (up to 70%) of primary oocytes are eliminated by apoptosis accompanied by fragmentation of the syncytium (Pepling and Spradling 2001; Malki *et al.* 2014). The remaining oocytes form primordial follicles with the surrounding squamous pregranulosa cells. After puberty begins, some of the primordial follicles are periodically activated and the oocytes then undergo oocyte growth, which entails storage of a vast amount of maternal

proteins and RNAs in their cytoplasm and female-specific epigenetic modification (i.e. *de novo* DNA methylation on the regulatory element controlling genomic imprinting; Smallwood *et al.* 2011; Kobayashi *et al.* 2013; Shirane *et al.* 2013). During oocyte growth, oocytes have a characteristic large nucleus called a germinal vesicle (GV). When oocyte growth reaches a plateau, the oocytes resume meiosis, accompanied by GV breakdown (GVBD) followed by first polar body extrusion, and then arrest again at MII. Upon fertilisation, they become zygotes, thereby completing the germline cycle.

## Basic concept of *in vitro* gametogenesis using pluripotent stem cells

Given that pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are capable of differentiating into all tissues of the embryo proper, including the germ cell lineage, the cells would be expected to reproduce their germ cell differentiation process under appropriate conditions in culture. Indeed, reconstitution of the germ cell lineage using ESCs or iPSCs, a process known as *in vitro* gametogenesis, has been successful in mice and adapted to several mammalian species, including humans (see below). The entire process of *in vitro* gametogenesis can be largely divided into two phases: one is the production of PGCs, the precursors of gametes, which differentiate in a sex-independent manner, and the other is the production of oocytes and spermatozoa, which differentiate in a sex-dependent manner. Below are updates of recent findings on each process of *in vitro* gametogenesis.

## Reconstitution of PGC specification in mice

The first step in reconstituting germ cell differentiation is PGC specification from ESCs or iPSCs. In early studies, the production of PGCs from mouse (m) ESCs relied on spontaneous, or somewhat directional, differentiation in embryoid bodies (Hübner *et al.* 2003; Toyooka *et al.* 2003; Geijsen *et al.* 2004). However, the yield of PGCs using this method was insufficient in terms of both quality and quantity. Importantly, functionality, which is the most stringent criterion of their quality (i.e. whether the PGC-like cells can differentiate into fertile eggs and spermatozoa) was not tested. In addition, the time course of the germ cell differentiation from ESCs was not compatible with that *in vivo*, which suggests that these systems are insufficient to serve as a model of germ cell development.

With increased knowledge of PGC specification *in vivo* and the nature of the pluripotent state, reconstitution of PGC specification *in vitro* has become feasible. A key finding was derived from an *ex vivo* culture experiment that determined a set of growth factors sufficient for the induction of PGCs from the postimplantation epiblast around E6 (Ohinata *et al.* 2009): when cultured with BMP4, BMP8b, leukaemia inhibitory factor (LIF), epidermal growth factor (EGF) and Stem cell factor (SCF), epiblasts efficiently differentiated into PGCs that had the ability to give rise to functional spermatozoa upon transplantation into testes. Because ESCs did not differentiate to PGCs in response to this set of growth factors, the question arose as to how to confer the appropriate responsiveness to the growth factors, termed 'PGC competence' (Hayashi *et al.* 2011), to pluripotent stem cells. The solution came from the identification of two distinct pluripotent states, namely the naïve and primed pluripotent states. The former corresponds to the E4.5 epiblast and is maintained by culture with LIF and chemical inhibitors of glycogen synthase kinase (GSK) 3β and mitogen-activated protein kinase kinase (MEK), whereas the latter corresponds to the E6.5 or later epiblast and is maintained by culture with basic fibroblast growth factor (bFGF) and activin A (Brons *et al.* 2007; Tesar *et al.* 2007; Ying *et al.* 2008). These two states are interchangeable depending on the growth factors used in the culture (Bao *et al.* 2009; Guo *et al.* 2009). Importantly, although the typical primed pluripotent stem cells had little PGC competence, cells at the transient state between naïve and primed states had a robust PGC competence (Hayashi and Surani 2009; Hayashi *et al.* 2011). In particular, ESCs and iPSCs transformed swiftly into cells highly resembling E5.5 epiblast cells by culturing with bFGF and activin A for 2 days and efficiently differentiated into PGC-like cells (PGCLCs) in response to the above set of growth factors. Importantly, PGCLCs were also found to give rise to functional spermatozoa and oocytes upon transplantation into testes and ovaries respectively (Hayashi *et al.* 2011, 2012). This proof-of-concept paved a new path for the production of gametes from ESCs and iPSCs, in addition to providing a unique platform to improve our understanding of the molecular mechanisms underlying germ cell development. The latter is especially useful in understanding the nature of nascent PGCs, because the culture system can bypass the scarcity of the cells *in vivo*. Indeed, details of the transcriptional network and epigenetic landscape were comprehensively revealed using the culture system (Aramaki *et al.* 2013; Nakaki *et al.* 2013; Kurimoto *et al.* 2015; Murakami *et al.* 2016; Shirane *et al.* 2016).

## Reconstitution of oogenesis in mice

As described above, PGC differentiation is an asexual process that allocates a founder population of germ cells and erases the parental epigenetic modifications. For sexual differentiation of germ cells, signals from surrounding somatic cells are essential. Therefore, reconstitution of the somatic cell environment is required for further differentiation of PGCLCs. This was achieved by coculture of PGCLCs with embryonic gonadal somatic cells. In coculture with female gonadal somatic cells, collectively termed the 'reconstituted ovary' (rOvary), PGCLCs swiftly started to express marker genes of late PGCs, such as *Dazl* and *Ddx4*, and to proliferate with incomplete cytokinesis, thereby forming germline cysts as observed *in vivo* (Hayashi *et al.* 2012; Hikabe *et al.* 2016). Importantly, PGCLCs entered meiosis in rOvaries, thereby becoming primary oocytes. Oogenesis in the rOvary further proceeded under the culture conditions determined by the preceding studies of organ culture of fetal and neonatal ovaries (Eppig and O'Brien 1996; Morohaku *et al.* 2016). Briefly, rOvaries were placed onto a collagen-coated membrane and then cultured under three conditions, namely *in vitro* differentiation (IVDi), *in vitro* growth (IVG) and *in vitro* maturation (IVM), in which oogenesis proceeds to primary oocytes in secondary follicles, fully grown GV oocytes and MII oocytes respectively (Fig. 1). The resulting MII oocytes were capable of fertilisation with wild-type

spermatozoa and the fertilised eggs gave rise to pups. The pups were apparently healthy because they grew to adulthood with a frequency comparable to that of wild-type mice and were fertile (Hikabe *et al.* 2016). Of note, the system allowed us to produce functional oocytes from iPSCs reprogrammed from tail tip fibroblasts, making it theoretically possible to produce oocytes from any kind of somatic cell.

Despite the successful reconstitution of oogenesis, the quality of the *in vitro*-produced oocytes was far inferior to that of *in vivo* oocytes, as shown by the fact that the birth rate of pups from 2-cell embryos derived from *in vitro* oocytes was 20-fold lower than that from 2-cell embryos *in vivo*. This inferior potential was already observed in the low fertilisation rate (~50%) and blastocyst formation rate (~20% of fertilised eggs; Hikabe *et al.* 2016). This was attributable, at least in part, to the frequent mispairing of homologous chromosomes in the meiotic prophase I, the precocious resumption of meiosis and the aberrant gene expression during oocyte growth and maturation. These observations clearly indicate that the culture conditions of the current *in vitro* gametogenesis are suboptimal and will require further refinement. In addition, for the assessment of *in vitro*-produced oocytes, much attention should be paid to genetic and epigenetic mutations in *in vitro* oocytes. Although the pups derived from *in vitro* oocytes were apparently healthy, it is possible that they inherited an aberrant epigenome from the oocytes. In fact, this is likely, because even IVF, which has a much shorter, simpler manipulation process in culture than the culture series for *in vitro* gametogenesis, causes placental defects, possibly due to epimutation(s) (Bloise *et al.* 2012; de Waal *et al.* 2015). Indeed, the placentas derived from *in vitro*-produced oocytes were heavier than those from oocytes *in vivo* (Hikabe *et al.* 2016). Clearly, close attention should be paid when using iPSCs, because genetic and epigenetic mutations are carried into iPSCs from the parental somatic cells (Kim *et al.* 2010; Young *et al.* 2012). To standardise the process of *in vitro* gametogenesis, rigorous assessment of the resultant gametes and offspring will be essential in the future.

## Reconstitution of spermatogenesis in mice

Similar to the reconstitution of oogenesis in rOvaries, spermatogenesis is also partially reconstituted by coculture with male gonadal somatic cells, generating the so-called 'reconstituted testis' (rTestis; Ishikura *et al.* 2016). In the rTestis, the seminiferous tubules are self-organised while incorporating PGCLCs. In the reconstituted seminiferous tubules, ESC-derived PGCLCs differentiated into prospermatogonia and then eventually into spermatogonia that expressed promyelocytic leukaemia zinc-finger (PLZF), a marker of spermatogonial stem cells. Along with the differentiation of spermatogonia, the Sertoli cells also positioned themselves properly in the seminiferous tubules. However, despite the histological normality, spermatogenesis was arrested by spermatocytes and none of them completed meiosis in rTestes. Although the arrest of spermatogenesis was not resolved, the potential of PGCLC-derived spermatogonia was made evident by the successful derivation of germline stem cells (GSCs), a primary spermatogonial stem cell line that has the capacity for self-renewal and spermatogenesis (Kanatsu-Shinohara *et al.* 2003). PGCLC-derived germline stem-like cells (GSCLCs) propagated indefinitely and had the capacity to differentiate into spermatozoa upon transplantation into the testes. The resultant spermatozoa were used for fertilisation by intracytoplasmic sperm injection (ICSI) and the fertilised eggs developed to grossly normal pups, thus demonstrating that ESCs differentiated into functional GSCLCs in the rTestis system. Conversely, at least some of the GSCLCs were far inferior to the GSCs derived from spermatogonia *in vivo*. Of note, GSCLCs bore hypermethylation in regulatory regions driving genes involved in meiosis and spermatogenesis and in the differentially methylated region (DMR) of maternally imprinted genes (Ishikura *et al.* 2016). These aberrant DNA methylations may have been due to insufficient DNA demethylation in PGCLCs and excessive *de novo* DNA methylation during GSCLC derivation. Both are likely, because genome-wide DNA demethylation in PGCLCs was not completed at the stage before integration into the rTestis (Shirane *et al.* 2016; Ohta *et al.* 2017) and genome-wide *de novo* methylation occurring in prospermatogonia (Seisenberger *et al.* 2012; Kobayashi *et al.* 2013; Kubo *et al.* 2015) was susceptible to *in vitro* culture (Lee *et al.* 2009). Therefore, with the criteria set above, further refinement of the culture conditions will be essential in order to complete the reconstitution.

Another ground-breaking work in the reconstitution of spermatogenesis was reported in mice (Zhou *et al.* 2016). In that study, ESC-derived PGCLCs were reaggregated with *c-kit* mutant (*W*/*Wv*) neonatal testicular cells and cultured with cytokines and hormones. Under the culture conditions used, PGCLCs differentiated into spermatid-like cells within 2 weeks of culture. By round spermatid injection (ROSI), the resultant spermatid-like cells contributed to zygotes that developed to 2-cell embryos at a high rate (85–92%). The 2-cell embryos fully developed to term, although the developmental rate was lower (2–5%) than the control using round spermatids *in vivo* (9.5%). Although no follow-up study has been reported, it appears that the differentiation period was shorter than the equivalent differentiation process *in vivo*, which takes at least 4 weeks. Therefore, in addition to the reproducibility of the culture system, rigorous assessment of the process of spermatogenesis, especially meiosis, during the 2 weeks would provide valuable information to standardise this method. Nevertheless, there is no study that has completed the entire process of spermatogenesis. Particularly in spermiogenesis, dynamic morphological changes are highly dependent on Sertoli cells, which, for example, phagocytose residual bodies shed from the cytoplasmic portion of the elongating spermatid (Kerr and de Kretser 1974; Pineau *et al.* 1991; Maeda *et al.* 2002). In addition, sustainable spermatogenesis is supported by the flow of seminal fluid in the seminiferous tubules (Fawcett and Hoffer 1979; Abe *et al.* 1982), which was not reproduced in the closed structure of the rTestis. To complete spermatogenesis in culture, such environmental factors may need to be considered.

## Reconstitution of PGC specification in humans

In early studies using human (h) ESCs, the germ cell lineage could be induced from hESCs by spontaneous differentiation or

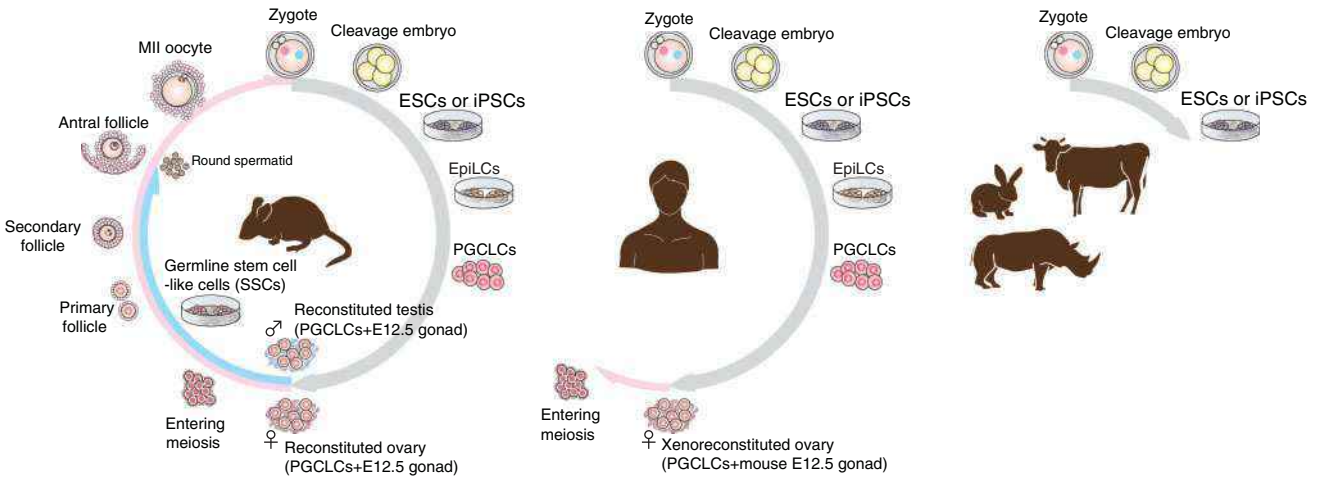


**Fig. 2.** Reconstitution of the germ cell cycle in culture. Schematic representations show the proceedings of *in vitro* gametogenesis. In mice, oogenesis can be fully reconstituted from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), but spermatogenesis has not been entirely reconstituted. In humans, primordial germ cell-like cells (PGCLCs) can be induced from human ESCs or human iPSCs. In the xenoreconstituted ovary system, human PGCLCs differentiate into oogonia, some of which proceed to primary oocytes. In several other mammalian species, such as the bovine, rabbit and southern white rhinoceros, ESCs or iPSCs are established and ready to use for *in vitro* gametogenesis. E12.5, Embryonic Day 12.5; SSCs, spermatogonial stem cells.

somewhat directional differentiation in response to BMP4 (Clark *et al.* 2004; Kee *et al.* 2006; Park *et al.* 2009). Although these induced germ cells were not well characterised, these studies suggested that germ cells could be directly derived from self-renewing hESCs in response to BMP4, suggesting that, unlike mESCs, hESCs somehow have PGC competence. Meanwhile, in other studies, the culture conditions were refined to place hESCs and hiPSCs in a naïve pluripotent state (Chan *et al.* 2013; Gafni *et al.* 2013; Takashima *et al.* 2014; Theunissen *et al.* 2014). Using some of these culture conditions, a robust differentiation of PGCLCs from hESCs and hiPSCs was achieved. When hESCs and hiPSCs were maintained under a '4i' condition that included inhibitors for GSK3β, MEK, c-Jun N-terminal kinase (JNK) and p38, hESCs and hiPSCs enhanced PGC competence immediately or after culturing with transforming growth factor-β (or activin A) and bFGF for 2 days (Gafni *et al.* 2013; Irie *et al.* 2015). Under these culture conditions, hESCs and hiPSCs efficiently differentiated into hPGCLCs in response to BMP4, SCF, LIF and EGF, which is the same as the set of growth factors used for the induction of mPGCLCs. hPGCLCs shared similar profiles of gene expression and epigenetic genome modification with hPGCs *in vivo* (Tang *et al.* 2015). In another method, hiPSCs that were maintained under a conventional condition with bFGF developed enhanced PGC competence by culturing with activin A and a GSK3β inhibitor for 2 days (Sasaki *et al.* 2015). The resulting cells, called incipient mesodermal cells (iMeLCs), efficiently differentiated into hPGCLCs in response to BMP4, SCF, LIF and EGF (Fig. 2*a*). Much as in the iMeLC method, the hESCs and hiPSCs differentiated into mesodermal-like cells with activin, bFGF and a low (5 ng $mL^{-1}$) concentration of BMP4, and then hPGCLCs were induced from the mesodermal-like cells with a high (100 ng $mL^{-1}$) concentration of BMP4 (Sugawa *et al.* 2015). The requirement for the preinduction culture period suggests that PGC competence is conferred to hESCs and hiPSCs at a specific pluripotent state different from the naïve or primed state. Supporting this idea, transcriptome analysis indicates that hESCs and hiPSCs under the 4i condition, which can differentiate directly to PGCs in response to the cytokines, have a distinct pluripotent state from the naïve pluripotent state (Nakamura *et al.* 2016). The PGC-competent state could be equivalent to the formative state that is currently perceived as an intermediate state between the naïve and primed pluripotent states, in which pluripotent cells acquire competence for multilineage induction entailing a distinct responsiveness to external cues (Kalkan and Smith 2014; Kalkan *et al.* 2017).

Although functional assessment of hPGCLCs is not easy, the culture system provides a unique platform that greatly facilitates the genetic analysis of PGC specification. Knockout studies using hESCs and hiPSCs identified genes involved in PGC specification in humans. For example, it was found that *PRDM1*, which is essential for mouse PGC specification, was also essential for hPGCLC differentiation (Irie *et al.* 2015; Kojima *et al.* 2017); during hPGCLC differentiation, *PRDM1*-knockout hESC- and hiPSC-derivatives failed to repress somatic genes and to upregulate germ cell genes (Irie *et al.* 2015; Kojima *et al.* 2017). This phenotype is similar to that of *Prdm1*-knockout mouse embryos (Kurimoto *et al.* 2008), suggesting that the function of this gene is conserved between mice and humans. Conversely, it appeared that SRY (sex determining region Y)-box 17 (*SOX17*), which is dispensable for mouse PGC specification, was essential for hPGCLC differentiation; *SOX17*-knockout hESCs and hiPSCs failed to differentiate into hPGCLCs (Irie *et al.* 2015; Kojima *et al.* 2017). Conversely, enforced expression of *SOX17* and *PRDM1* was sufficient for hPGCLC differentiation (Kobayashi *et al.* 2017), reinforcing the importance of these transcription factors for human PGC specification. In addition, Transcription factor AP-2 gamma (*TFAP2C*) and Eomesodermin (*EOMES*) were essential for hPGCLC differentiation (Kojima *et al.* 2017); the former is also

required for mouse PGC specification, whereas the latter is not. *EOMES* is a target of WNT signalling during hPGCLC differentiation and triggers *SOX17* expression. In mice, Brachyury, T-box transcription factor T (*T*) is a target of WNT signalling during mPGCLC differentiation and triggers *Prdm1* and *Prdm14*, which are essential for mouse PGC specification. In contrast, *T* is dispensable for hPGCLC differentiation (Kojima *et al.* 2017). *EOMES* and *T* are transcription factors belonging to the T-box gene family, which has diverse roles during embryogenesis (Showell *et al.* 2004). The functional redundancy of these genes on PGC specification is evident from the fact that enforced expression of *Eomes* transactivated *Prdm1* during mPGCLC differentiation, although the magnitude of the transactivation was lower than that for *T* (Aramaki *et al.* 2013). These findings indicate that the gene responsible for WNT signalling is altered during evolution. Perhaps, such an alteration may contribute to speciation in mammalian evolution, because WNT signalling is critical factor for PGC specification, the origin of germ cell development. Conversely, two key external signals, namely BMP and WNT, for PGC specification are well conserved between mice and humans, suggesting that the first priority for PGC specification in embryogenesis is to establish an environmental cue that provides BMP and WNT signalling to a pluripotent cell population. Supporting this idea, porcine PGCs are specified in the posterior region of the epiblast in response to BMP4 and WNT3 (Kobayashi *et al.* 2017). Cynomolgus monkey PGCs were specified in the nascent amnion, which expresses pluripotent marker genes, and is exposed to BMP and WNT3A (Sasaki *et al.* 2016). Collectively, these findings indicate that the location of PGC specification in the embryo of each species depends on the differential expression pattern of BMP and WNT.

## Reconstitution of oogenesis in humans

Sex-dependent differentiation of germ cells essentially requires a proper interaction with the surrounding somatic cells. Based on studies on *in vitro* gametogenesis in mice, fetal gonadal somatic cells have a greater potential to support oogenesis than adult gonadal somatic cells (K. Hayashi, unpubl. data). This requirement of fetal gonadal somatic cells has hampered the development of *in vitro* gametogenesis in humans. One possible solution would be to replace the human fetal gonadal somatic cells with potentially equivalent cells of other species. Such xenoreconstituted ovaries have been tested using hPGCLCs and mouse fetal gonadal somatic cells (Yamashiro *et al.* 2018). In the xenoreconstituted ovaries, the hPGCLCs were intermingled with mouse fetal gonadal somatic cells and survived for at least 120 days. The profiles of the transcriptome and DNA methylome in the hPGCLCs were similar to those in hPGCs *in vivo*. Strikingly, some PGCLCs entered meiosis, demonstrating that hiPSCs were transformed into primary oocytes in the xenoreconstitution system. Although the culture period for their differentiation was slightly longer than that *in vivo*, the culture system provides a useful tool for evaluating gene function in human germ cell development beyond the PGC stage. However, from the viewpoint of oocyte production, a remaining question is whether the xenoreconstitution system is capable of completely supporting human oogenesis. To assess this capability, it will be necessary to develop a culture system that fully supports oocyte maturation using primary oocytes *in vivo*. One such culture system could consist of the production of MII oocytes from unilaminar follicles in the ovarian tissue (McLaughlin *et al.* 2018). In addition, there have been several attempts to generate mature oocytes from secondary follicles (Yu *et al.* 2009; Telfer and Zelinski 2013; Skory *et al.* 2015; Yin *et al.* 2016). These ongoing efforts are expected to provide clues to *in vitro* gametogenesis using hESCs and iPSCs.

## Prospects of *in vitro* gametogenesis in livestock and wild or endangered animals

A possible use of *in vitro* gametogenesis would be for accelerating the genomic selection in livestock breeding. Genomic selection is the single nucleotide polymorphism-based prediction of the potential of livestock animals, such as with regard to their breeding value and generation intervals. Since it was first proposed (Meuwissen *et al.* 2001), genomic selection has been coupled with the rapidly developing sequencing technologies, resulting in a wide implementation in livestock, including cattle (Wiggans *et al.* 2017), swine (Lillehammer *et al.* 2013) and chickens (Sitzenstock *et al.* 2013). Indeed, in recent decades genetic selection has revolutionised the pursuit of genetic improvements in animal breeding. Conversely, a bottleneck of genomic selection is that the generation intervals are still reliant on the mating of individual animals, and thus on the amount of time required for the individuals to reach puberty. In this context, *in vitro* gametogenesis is expected to get rid of the bottleneck and accelerate further genomic selection (Hou *et al.* 2018; Goszczynski *et al.* 2019). In mice, alteration of generations was achieved in culture without animal breeding: ESC-derived oocytes were fertilised with spermatozoa and developed to blastocysts, and then gave rise to the second-generation of ESCs (Hikabe *et al.* 2016). Therefore, in theory, *in vitro* gametogenesis using livestock ESCs and iPSCs would make it possible to alter generations in culture without waiting for the growth of individuals. So far, much effort has been made to establish livestock ESCs and iPSCs that stably and indefinitely propagate in culture while maintaining their pluripotency. Of note, bovine ESCs have recently been established and well characterised as pluripotent stem cells (Bogliotti *et al.* 2018). Because gene selection has been well implemented in cattle, bovine ESCs may one day provide the first example of a genetic improvement without animal breeding. Apart from the bovine, efforts to establish pluripotent stem cells harbouring a stable pluripotent state continue in other livestock animals, including swine, chicken, goat and horse, therefore raising the possibility that the novel breeding system penetrates to the livestock industry in future. It is of note that, apart from rodents, primates and bovine, both ESCs and iPSCs are established and well characterised in the rabbit (Fang *et al.* 2006; Wang *et al.* 2007; Honda *et al.* 2010). On the basis of early embryo development, the rabbit is counted among Rauber's layer species that also includes bovine, swine and horse (van Leeuwen *et al.* 2020). Considering the similar manner of early embryo development closely associated with PGC specification, using rabbit ESCs and iPSCs would provide a valuable model of *in vitro* gametogenesis for these livestock animals.

Another anticipated application of *in vitro* gametogenesis is for the production of gametes from iPSCs of endangered species. Indeed, iPSCs have been established from several wild animals, including drills (*Mandrillus leucophaeus*; Ben-Nun *et al.* 2011), northern white rhinoceroses (*Ceratotherium simum cottoni*; Ben-Nun *et al.* 2011; Korody *et al.* 2017), olive baboons (*Papio anubis*; Navara *et al.* 2013), snow leopards (*Panthera uncia*; Verma *et al.* 2012), Ryukyu spiny rats (*Tokudaia osimensis*; Honda *et al.* 2017) and others (Stanton *et al.* 2019). However, the road to achieving *in vitro* gametogenesis in wild animals will be long and bumpy, because information on gene expression and embryology is much more limited than in mice and humans. This is true even for the first step, which is validation of iPSCs. Although iPSCs in these reports expressed pluripotent marker genes and showed the ability to differentiate into the three germ layers in *in vitro* differentiation experiments or teratoma analysis, this does not mean that the cells had the potential for germ cell differentiation. Indeed, an early study on mouse iPSCs showed that germline transmission was not entirely parallel to pluripotent gene expression and the ability to differentiate into three germ layers observed in the teratoma (Takahashi and Yamanaka 2006). Therefore, it would be more helpful if the quality of iPSCs could be compared with bona fide pluripotent stem cells, such as ESCs. In this context, the successful establishment of ESCs from the southern white rhinoceros (*Ceratotherium simum simum*), which is the closest species to the northern white rhinoceros, is noteworthy; two ESCs have been derived from blastocysts obtained by the collection of oocytes from female southern white rhinoceroses followed by ICSI using frozen spermatozoa from a male southern white rhinoceros (Hildebrandt *et al.* 2018). These ESCs uniformly propagated while expressing pluripotent marker genes and exhibited an ability to differentiate into three germ layers in the *in vitro* differentiation culture. Not only are rhinoceros ESCs useful to validate rhinoceros iPSCs, but they would also be useful to determine the culture conditions for PGCLC differentiation in the future. The second step for *in vitro* gametogenesis in wild animals would be derivation of PGCLCs. Based on knowledge of *in vitro* gametogenesis in mice and humans, key signalling molecules for PGC specification should be conserved in a wide range of mammalian species, which should be taken into consideration to determine a culture condition for PGCLC differentiation. The third step would be generation of gametes from the PGCLCs obtained. However, as discussed above in the context of *in vitro* gametogenesis in humans, the limited sources of fetal gonadal somatic cells is a crucial problem to be solved. It is nearly impossible (even more difficult than in humans) to obtain fetal gonadal somatic cells from wild animals. Therefore, as discussed above, the xenorreconstitution system would be one option to bypass this obstacle. Alternatively, derivation of gonadal somatic cells from pluripotent stem cells would be another option.

## Concluding remarks

Here, we summarised the current status of *in vitro* gametogenesis and discussed the future perspectives of the technology. The development of *in vitro* gametogenesis was pioneered by research on mice, but the technology is now increasingly being applied to other mammalian species, including humans. However, even in mice, the quality of gametes produced in the culture system is inferior to that of gametes *in vivo*, so further refinements will be needed. For this purpose, a better understanding of *in vivo* gametogenesis would provide a basis for the refinement of *in vitro* gametogenesis. Moreover, we should consolidate the commonalities and diversities in gametogenesis among species. For example, although the shape of oocytes or spermatozoa is largely similar among mammalian species, requirements during the differentiation process are quite diverse. In the technical development of *in vitro* gametogenesis so far, such species-specific requirements have been empirically achieved through labour-intensive trial and error. Therefore, the development of a method to predict the requirements could greatly accelerate the application of *in vitro* gametogenesis. To generalise *in vitro* gametogenesis to the fields of reproductive biology and regenerative medicine, multiple insights and much effort will be required.

## Conflicts of interest

The authors declare no conflicts of interest.

## Acknowledgements

The authors' work reported herein was supported, in part, by KAKENHI Grants-in-Aid from MEXT, Japan (18H05544 and 18H05545), the Takeda Science Foundation, the Luca Bella Foundation and a Grant-in-Aid from The Open Philanthropy Project (to Katsuhiko Hayashi), as well as by the Bio-Rescue Initiative of the Federal Ministry of Education and Research, Germany (to Thomas B. Hildebrandt and Sebastian Diecke).

## References

Abe, K., Takano, H., and Ito, T. (1982). Response of the epididymal duct in the corpus epididymidis to efferent or epididymal duct ligation in the mouse. *J. Reprod. Fertil.* **64**, 69–72. doi:10.1530/JRF.0.0640069

Aramaki, S., Hayashi, K., Kurimoto, K., Ohta, H., Yabuta, Y., Iwanari, H., Mochizuki, Y., Hamakubo, T., Kato, Y., Shirahige, K., and Saitou, M. (2013). A mesodermal factor, T, specifies mouse germ cell fate by directly activating germline determinants. *Dev. Cell* **27**, 516–529. doi:10.1016/J.DEVCEL.2013.11.001

Bao, S., Tang, F., Li, X., Hayashi, K., Gillich, A., Lao, K., and Surani, M. A. (2009). Epigenetic reversion of post-implantation epiblast to pluripotent embryonic stem cells. *Nature* **461**, 1292–1295. doi:10.1038/NATURE08534

Ben-Haim, N., Lu, C., Guzman-Ayala, M., Pescatore, L., Mesnard, D., Bischofberger, M., Naef, F., Robertson, E. J., and Constam, D. B. (2006). The nodal precursor acting via activin receptors induces mesoderm by maintaining a source of its convertases and BMP4. *Dev. Cell* **11**, 313–323. doi:10.1016/J.DEVCEL.2006.07.005

Ben-Nun, I. F., Montague, S. C., Houck, M. L., Tran, H. T., Garitaonandia, I., Leonardo, T. R., Wang, Y. C., Charter, S. J., Laurent, L. C., Ryder, O. A., and Loring, J. F. (2011). Induced pluripotent stem cells from highly endangered species. *Nat. Methods* **8**, 829–831. doi:10.1038/NMETH.1706

Bloise, E., Lin, W., Liu, X., Simbulan, R., Kolahi, K. S., Petraglia, F., Maltepe, E., Donjacour, A., and Rinaudo, P. (2012). Impaired placental nutrient transport in mice generated by *in vitro* fertilization. *Endocrinology* **153**, 3457–3467. doi:10.1210/EN.2011-1921

Bogliotti, Y. S., Wu, J., Vilarino, M., Okamura, D., Soto, D. A., Zhong, C., Sakurai, M., Sampaio, R. V., Suzuki, K., Izpisua Belmonte, J. C., and

Ross, P. J. (2018). Efficient derivation of stable primed pluripotent embryonic stem cells from bovine blastocysts. *Proc. Natl Acad. Sci. USA* **115**, 2090–2095. doi:10.1073/PNAS.1716161115

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

Brons, I. G., Smithers, L. E., Trotter, M. W., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S. M., Howlett, S. K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R. A., and Vallier, L. (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* **448**, 191–195. doi:10.1038/NATURE05950

Chan, Y. S., Goke, J., Ng, J. H., Lu, X., Gonzales, K. A., Tan, C. P., Tng, W. Q., Hong, Z. Z., Lim, Y. S., and Ng, H. H. (2013). Induction of a human pluripotent state with distinct regulatory circuitry that resembles preimplantation epiblast. *Cell Stem Cell* **13**, 663–675. doi:10.1016/J.STEM.2013.11.015

Clark, A. T., Rodriguez, R. T., Bodnar, M. S., Abeyta, M. J., Cedars, M. I., Turek, P. J., Firpo, M. T., and Reijo Pera, R. A. (2004). Human STELLAR, NANOG, and GDF3 genes are expressed in pluripotent cells and map to chromosome 12p13, a hotspot for teratocarcinoma. *Stem Cells* **22**, 169–179. doi:10.1634/STEMCELLS.22-2-169

de Waal, E., Vrooman, L. A., Fischer, E., Ord, T., Mainigi, M. A., Coutifaris, C., Schultz, R. M., and Bartolomei, M. S. (2015). The cumulative effect of assisted reproduction procedures on placental development and epigenetic perturbations in a mouse model. *Hum. Mol. Genet.* **24**, 6975–6985. doi:10.1093/HMG/DDV400

Eppig, J. J., and O'Brien, M. J. (1996). Development *in vitro* of mouse oocytes from primordial follicles. *Biol. Reprod.* **54**, 197–207. doi:10.1095/BIOLREPROD54.1.197

Fang, Z. F., Gai, H., Huang, Y. Z., Li, S. G., Chen, X. J., Shi, J. J., Wu, L., Liu, A., Xu, P., and Sheng, H. Z. (2006). Rabbit embryonic stem cell lines derived from fertilized, parthenogenetic or somatic cell nuclear transfer embryos. *Exp. Cell Res.* **312**, 3669–3682. doi:10.1016/J.YEXCR.2006.08.013

Fawcett, D. W., and Hoffer, A. P. (1979). Failure of exogenous androgen to prevent regression of the initial segments of the rat epididymis after efferent duct ligation or orchidectomy. *Biol. Reprod.* **20**, 162–181. doi:10.1095/BIOLREPROD20.2.162

Gafni, O., Weinberger, L., Mansour, A. A., Manor, Y. S., Chomsky, E., Ben-Yosef, D., Kalma, Y., Viukov, S., Maza, I., Zviran, A., Rais, Y., Shipony, Z., Mukamel, Z., Krupalnik, V., Zerbib, M., Geula, S., Caspi, I., Schneir, D., Shwartz, T., Gilad, S., Amann-Zalcenstein, D., Benjamin, S., Amit, I., Tanay, A., Massarwa, R., Novershtern, N., and Hanna, J. H. (2013). Derivation of novel human ground state naive pluripotent stem cells. *Nature* **504**, 282–286. doi:10.1038/NATURE12745

Geijsen, N., Horoschak, M., Kim, K., Gribnau, J., Eggan, K., and Daley, G. Q. (2004). Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature* **427**, 148–154. doi:10.1038/NATURE02247

Goszczynski, D. E., Cheng, H., Demyda-Peyras, S., Medrano, J. F., Wu, J., and Ross, P. J. (2019). *In vitro* breeding: application of embryonic stem cells to animal production. *Biol. Reprod.* **100**, 885–895. doi:10.1093/BIOLRE/IOY256

Guo, G., Yang, J., Nichols, J., Hall, J. S., Eyres, I., Mansfield, W., and Smith, A. (2009). Klf4 reverts developmentally programmed restriction of ground state pluripotency. *Development* **136**, 1063–1069. doi:10.1242/DEV.030957

Hajkova, P., Erhardt, S., Lane, N., Haaf, T., El-Maarri, O., Reik, W., Walter, J., and Surani, M. A. (2002). Epigenetic reprogramming in mouse primordial germ cells. *Mech. Dev.* **117**, 15–23. doi:10.1016/S0925-4773(02)00181-8

Hajkova, P., Ancelin, K., Waldmann, T., Lacoste, N., Lange, U. C., Cesari, F., Lee, C., Almouzni, G., Schneider, R., and Surani, M. A. (2008). Chromatin dynamics during epigenetic reprogramming in the mouse germ line. *Nature* **452**, 877–881. doi:10.1038/NATURE06714

Hayashi, K., and Surani, M. A. (2009). Self-renewing epiblast stem cells exhibit continual delineation of germ cells with epigenetic reprogramming *in vitro*. *Development* **136**, 3549–3556. doi:10.1242/DEV.037747

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

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

Hikabe, O., Hamazaki, N., Nagamatsu, G., Obata, Y., Hirao, Y., Hamada, N., Shimamoto, S., Imamura, T., Nakashima, K., Saitou, M., and Hayashi, K. (2016). Reconstitution *in vitro* of the entire cycle of the mouse female germ line. *Nature* **539**, 299–303. doi:10.1038/NATURE20104

Hildebrandt, T. B., Hermes, R., Colleoni, S., Diecke, S., Holtze, S., Renfree, M. B., Stejskal, J., Hayashi, K., Drukker, M., Loi, P., Goritz, F., Lazzari, G., and Galli, C. (2018). Embryos and embryonic stem cells from the white rhinoceros. *Nat. Commun.* **9**, 2589. doi:10.1038/S41467-018-04959-2

Honda, A., Hirose, M., Hatori, M., Matoba, S., Miyoshi, H., Inoue, K., and Ogura, A. (2010). Generation of induced pluripotent stem cells in rabbits: potential experimental models for human regenerative medicine. *J. Biol. Chem.* **285**, 31362–31369. doi:10.1074/JBC.M110.150540

Honda, A., Choijookhuu, N., Izu, H., Kawano, Y., Inokuchi, M., Honsho, K., Lee, A. R., Nabekura, H., Ohta, H., Tsukiyama, T., Ohinata, Y., Kuroiwa, A., Hishikawa, Y., Saitou, M., Jogahara, T., and Koshimoto, C. (2017). Flexible adaptation of male germ cells from female iPSCs of endangered *Tokudaia osimensis*. *Sci. Adv.* **3**, e1602179. doi:10.1126/SCIADV.1602179

Hou, Z., An, L., Han, J., Yuan, Y., Chen, D., and Tian, J. (2018). Revolutionize livestock breeding in the future: an animal embryo-stem cell breeding system in a dish. *J. Anim. Sci. Biotechnol.* **9**, 90. doi:10.1186/S40104-018-0304-7

Hübner, K., Fuhrmann, G., Christenson, L. K., Kehler, J., Reinbold, R., De La Fuente, R., Wood, J., Strauss, J. F., 3rd, Boiani, M., and Scholer, H. R. (2003). Derivation of oocytes from mouse embryonic stem cells. *Science* **300**, 1251–1256. doi:10.1126/SCIENCE.1083452

Irie, N., Weinberger, L., Tang, W. W., Kobayashi, T., Viukov, S., Manor, Y. S., Dietmann, S., Hanna, J. H., and Surani, M. A. (2015). SOX17 is a critical specifier of human primordial germ cell fate. *Cell* **160**, 253–268. doi:10.1016/J.CELL.2014.12.013

Ishikura, Y., Yabuta, Y., Ohta, H., Hayashi, K., Nakamura, T., Okamoto, I., Yamamoto, T., Kurimoto, K., Shirane, K., Sasaki, H., and Saitou, M. (2016). *In vitro* derivation and propagation of spermatogonial stem cell activity from mouse pluripotent stem cells. *Cell Rep.* **17**, 2789–2804. doi:10.1016/J.CELREP.2016.11.026

Kalkan, T., and Smith, A. (2014). Mapping the route from naive pluripotency to lineage specification. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **369**, 20130540. doi:10.1098/RSTB.2013.0540

Kalkan, T., Olova, N., Roode, M., Mulas, C., Lee, H. J., Nett, I., Marks, H., Walker, R., Stunnenberg, H. G., Lilley, K. S., Nichols, J., Reik, W., Bertone, P., and Smith, A. (2017). Tracking the embryonic stem cell transition from ground state pluripotency. *Development* **144**, 1221–1234. doi:10.1242/DEV.142711

Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Miki, H., Ogura, A., Toyokuni, S., and Shinohara, T. (2003). Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biol. Reprod.* **69**, 612–616. doi:10.1095/BIOLREPROD.103.017012

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

Kerr, J. B., and de Kretser, D. M. (1974). Proceedings: the role of the Sertoli cell in phagocytosis of the residual bodies of spermatids. *J. Reprod. Fertil.* **36**, 439–440. doi:10.1530/JRF.0.0360439

Kim, K., Doi, A., Wen, B., Ng, K., Zhao, R., Cahan, P., Kim, J., Aryee, M. J., Ji, H., Ehrlich, L. I., Yabuuchi, A., Takeuchi, A., Cunniff, K. C., Hongguang, H., McKinney-Freeman, S., Naveiras, O., Yoon, T. J., Irizarry, R. A., Jung, N., Seita, J., Hanna, J., Murakami, P., Jaenisch, R., Weissleder, R., Orkin, S. H., Weissman, I. L., Feinberg, A. P., and Daley, G. Q. (2010). Epigenetic memory in induced pluripotent stem cells. *Nature* **467**, 285–290. doi:10.1038/NATURE09342

Kobayashi, H., Sakurai, T., Miura, F., Imai, M., Mochiduki, K., Yanagisawa, E., Sakashita, A., Wakai, T., Suzuki, Y., Ito, T., Matsui, Y., and Kono, T. (2013). High-resolution DNA methylome analysis of primordial germ cells identifies gender-specific reprogramming in mice. *Genome Res.* **23**, 616–627. doi:10.1101/GR.148023.112

Kobayashi, T., Zhang, H., Tang, W. W. C., Irie, N., Withey, S., Klisch, D., Sybirna, A., Dietmann, S., Contreras, D. A., Webb, R., Allegrucci, C., Alberio, R., and Surani, M. A. (2017). Principles of early human development and germ cell program from conserved model systems. *Nature* **546**, 416–420. doi:10.1038/NATURE22812

Kojima, Y., Sasaki, K., Yokobayashi, S., Sakai, Y., Nakamura, T., Yabuta, Y., Nakaki, F., Nagaoka, S., Woltjen, K., Hotta, A., Yamamoto, T., and Saitou, M. (2017). Evolutionarily distinctive transcriptional and signaling programs drive human germ cell lineage specification from pluripotent stem cells. *Cell Stem Cell* **21**, 517–532e515. doi:10.1016/J.STEM.2017.09.005

Korody, M. L., Pivaroff, C., Nguyen, T. D., Peterson, S. E., Ryder, O. A., and Loring, J. F. (2017). Four new induced pluripotent stem cell lines produced from northern white rhinoceros with non-integrating reprogramming factors. *bioRxiv* 202499.

Koubova, J., Menke, D. B., Zhou, Q., Capel, B., Griswold, M. D., and Page, D. C. (2006). Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proc. Natl Acad. Sci. USA* **103**, 2474–2479. doi:10.1073/PNAS.0510813103

Kubo, N., Toh, H., Shirane, K., Shirakawa, T., Kobayashi, H., Sato, T., Sone, H., Sato, Y., Tomizawa, S., Tsurusaki, Y., Shibata, H., Saitsu, H., Suzuki, Y., Matsumoto, N., Suyama, M., Kono, T., Ohbo, K., and Sasaki, H. (2015). DNA methylation and gene expression dynamics during spermatogonial stem cell differentiation in the early postnatal mouse testis. *BMC Genomics* **16**, 624. doi:10.1186/S12864-015-1833-5

Kurimoto, K., Yabuta, Y., Ohinata, Y., Shigeta, M., Yamanaka, K., and Saitou, M. (2008). Complex genome-wide transcription dynamics orchestrated by Blimp1 for the specification of the germ cell lineage in mice. *Genes Dev.* **22**, 1617–1635. doi:10.1101/GAD.1649908

Kurimoto, K., Yabuta, Y., Hayashi, K., Ohta, H., Kiyonari, H., Mitani, T., Moritoki, Y., Kohri, K., Kimura, H., Yamamoto, T., Katou, Y., Shirahige, K., and Saitou, M. (2015). Quantitative dynamics of chromatin remodeling during germ cell specification from mouse embryonic stem cells. *Cell Stem Cell* **16**, 517–532. doi:10.1016/J.STEM.2015.03.002

Lawson, K. A., and Hage, W. J. (1994). Clonal analysis of the origin of primordial germ cells in the mouse. *Ciba Found. Symp.* **182**, 68–84. doi:10.1002/9780470514573.CH5

Lawson, K. A., Dunn, N. R., Roelen, B. A., Zeinstra, L. M., Davis, A. M., Wright, C. V., Korving, J. P., and Hogan, B. L. (1999). Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes Dev.* **13**, 424–436. doi:10.1101/GAD.13.4.424

Lee, J., Kanatsu-Shinohara, M., Ogonuki, N., Miki, H., Inoue, K., Morimoto, T., Morimoto, H., Ogura, A., and Shinohara, T. (2009). Heritable imprinting defect caused by epigenetic abnormalities in mouse spermatogonial stem cells. *Biol. Reprod.* **80**, 518–527. doi:10.1095/BIOLREPROD.108.072330

Lillehammer, M., Meuwissen, T. H., and Sonesson, A. K. (2013). Genomic selection for two traits in a maternal pig breeding scheme. *J. Anim. Sci.* **91**, 3079–3087. doi:10.2527/JAS.2012-5113

Liu, P., Wakamiya, M., Shea, M. J., Albrecht, U., Behringer, R. R., and Bradley, A. (1999). Requirement for Wnt3 in vertebrate axis formation. *Nat. Genet.* **22**, 361–365. doi:10.1038/11932

Maeda, Y., Shiratsuchi, A., Namiki, M., and Nakanishi, Y. (2002). Inhibition of sperm production in mice by annexin V microinjected into seminiferous tubules: possible etiology of phagocytic clearance of apoptotic spermatogenic cells and male infertility. *Cell Death Differ.* **9**, 742–749. doi:10.1038/SJ.CDD.4401046

Malki, S., van der Heijden, G. W., O'Donnell, K. A., Martin, S. L., and Bortvin, A. (2014). A role for retrotransposon LINE-1 in fetal oocyte attrition in mice. *Dev. Cell* **29**, 521–533. doi:10.1016/J.DEVCEL.2014.04.027

McLaren, A., and Southee, D. (1997). Entry of mouse embryonic germ cells into meiosis. *Dev. Biol.* **187**, 107–113. doi:10.1006/DBIO.1997.8584

McLaughlin, M., Albertini, D. F., Wallace, W. H. B., Anderson, R. A., and Telfer, E. E. (2018). Metaphase II oocytes from human unilaminar follicles grown in a multi-step culture system. *Mol. Hum. Reprod.* **24**, 135–142. doi:10.1093/MOLEHR/GAY002

Meuwissen, T. H., Hayes, B. J., and Goddard, M. E. (2001). Prediction of total genetic value using genome-wide dense marker maps. *Genetics* **157**, 1819–1829.

Morohaku, K., Tanimoto, R., Sasaki, K., Kawahara-Miki, R., Kono, T., Hayashi, K., Hirao, Y., and Obata, Y. (2016). Complete *in vitro* generation of fertile oocytes from mouse primordial germ cells. *Proc. Natl Acad. Sci. USA* **113**, 9021–9026. doi:10.1073/PNAS.1603817113

Murakami, K., Gunesdogan, U., Zylicz, J. J., Tang, W. W. C., Sengupta, R., Kobayashi, T., Kim, S., Butler, R., Dietmann, S., and Surani, M. A. (2016). NANOG alone induces germ cells in primed epiblast *in vitro* by activation of enhancers. *Nature* **529**, 403–407. doi:10.1038/NATURE16480

Nakaki, F., Hayashi, K., Ohta, H., Kurimoto, K., Yabuta, Y., and Saitou, M. (2013). Induction of mouse germ-cell fate by transcription factors *in vitro*. *Nature* **501**, 222–226. doi:10.1038/NATURE12417

Nakamura, T., Okamoto, I., Sasaki, K., Yabuta, Y., Iwatani, C., Tsuchiya, H., Seita, Y., Nakamura, S., Yamamoto, T., and Saitou, M. (2016). A developmental coordinate of pluripotency among mice, monkeys and humans. *Nature* **537**, 57–62. doi:10.1038/NATURE19096

Navara, C. S., Hornecker, J., Grow, D., Chaudhari, S., Hornsby, P. J., Ichida, J. K., Eggan, K., and McCarrey, J. R. (2013). Derivation of induced pluripotent stem cells from the baboon: a nonhuman primate model for preclinical testing of stem cell therapies. *Cell. Reprogram.* **15**, 495–502. doi:10.1089/CELL.2012.0093

Ohinata, Y., Payer, B., O'Carroll, D., Ancelin, K., Ono, Y., Sano, M., Barton, S. C., Obukhanych, T., Nussenzweig, M., Tarakhovsky, A., Saitou, M., and Surani, M. A. (2005). Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature* **436**, 207–213. doi:10.1038/NATURE03813

Ohinata, Y., Ohta, H., Shigeta, M., Yamanaka, K., Wakayama, T., and Saitou, M. (2009). A signaling principle for the specification of the germ cell lineage in mice. *Cell* **137**, 571–584. doi:10.1016/J.CELL.2009.03.014

Ohta, H., Kurimoto, K., Okamoto, I., Nakamura, T., Yabuta, Y., Miyauchi, H., Yamamoto, T., Okuno, Y., Hagiwara, M., Shirane, K., Sasaki, H., and Saitou, M. (2017). *In vitro* expansion of mouse primordial germ cell-like cells recapitulates an epigenetic blank slate. *EMBO J.* **36**, 1888–1907. doi:10.15252/EMBJ.201695862

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., and 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**, 783–795. doi:10.1002/STEM.13

Pepling, M. E., and Spradling, A. C. (2001). Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. *Dev. Biol.* **234**, 339–351. doi:10.1006/DBIO.2001.0269

Pineau, C., Le Magueresse, B., Courtens, J. L., and Jegou, B. (1991). Study *in vitro* of the phagocytic function of Sertoli cells in the rat. *Cell Tissue Res.* **264**, 589–598. doi:10.1007/BF00319048

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., Yamanaka, S., and Saitou, M. (2015). Robust *in vitro* induction of human germ cell fate from pluripotent stem cells. *Cell Stem Cell* **17**, 178–194. doi:10.1016/J.STEM.2015.06.014

Sasaki, K., Nakamura, T., Okamoto, I., Yabuta, Y., Iwatani, C., Tsuchiya, H., Seita, Y., Nakamura, S., Shiraki, N., Takakuwa, T., Yamamoto, T., and Saitou, M. (2016). The germ cell fate of cynomolgus monkeys is specified in the nascent amnion. *Dev. Cell* **39**, 169–185. doi:10.1016/J.DEVCEL.2016.09.007

Seisenberger, S., Andrews, S., Krueger, F., Arand, J., Walter, J., Santos, F., Popp, C., Thienpont, B., Dean, W., and Reik, W. (2012). The dynamics of genome-wide DNA methylation reprogramming in mouse primordial germ cells. *Mol. Cell* **48**, 849–862. doi:10.1016/J.MOLCEL.2012.11.001

Seki, Y., Hayashi, K., Itoh, K., Mizugaki, M., Saitou, M., and Matsui, Y. (2005). Extensive and orderly reprogramming of genome-wide chromatin modifications associated with specification and early development of germ cells in mice. *Dev. Biol.* **278**, 440–458. doi:10.1016/J.YDBIO.2004.11.025

Shirane, K., Toh, H., Kobayashi, H., Miura, F., Chiba, H., Ito, T., Kono, T., and Sasaki, H. (2013). Mouse oocyte methylomes at base resolution reveal genome-wide accumulation of non-CpG methylation and role of DNA methyltransferases. *PLoS Genet.* **9**, e1003439. doi:10.1371/JOURNAL.PGEN.1003439

Shirane, K., Kurimoto, K., Yabuta, Y., Yamaji, M., Satoh, J., Ito, S., Watanabe, A., Hayashi, K., Saitou, M., and Sasaki, H. (2016). Global landscape and regulatory principles of DNA methylation reprogramming for germ cell specification by mouse pluripotent stem cells. *Dev. Cell* **39**, 87–103. doi:10.1016/J.DEVCEL.2016.08.008

Showell, C., Binder, O., and Conlon, F. L. (2004). T-box genes in early embryogenesis. *Dev. Dyn.* **229**, 201–218. doi:10.1002/DVDY.10480

Sitzenstock, F., Ytournel, F., Sharifi, A. R., Cavero, D., Taubert, H., Preisinger, R., and Simianer, H. (2013). Efficiency of genomic selection in an established commercial layer breeding program. *Genet. Sel. Evol.* **45**, 29. doi:10.1186/1297-9686-45-29

Skory, R. M., Xu, Y., Shea, L. D., and Woodruff, T. K. (2015). Engineering the ovarian cycle using *in vitro* follicle culture. *Hum. Reprod.* **30**, 1386–1395. doi:10.1093/HUMREP/DEV052

Smallwood, S. A., Tomizawa, S., Krueger, F., Ruf, N., Carli, N., Segonds-Pichon, A., Sato, S., Hata, K., Andrews, S. R., and Kelsey, G. (2011). Dynamic CpG island methylation landscape in oocytes and preimplantation embryos. *Nat. Genet.* **43**, 811–814. doi:10.1038/NG.864

Stanton, M. M., Tzatzalos, E., Donne, M., Kolundzic, N., Helgason, I., and Ilic, D. (2019). Prospects for the use of induced pluripotent stem cells in animal conservation and environmental protection. *Stem Cells Transl. Med.* **8**, 7–13. doi:10.1002/SCTM.18-0047

Sugawa, F., Arauzo-Bravo, M. J., Yoon, J., Kim, K. P., Aramaki, S., Wu, G., Stehling, M., Psathaki, O. E., Hubner, K., and Scholer, H. R. (2015). Human primordial germ cell commitment *in vitro* associates with a unique PRDM14 expression profile. *EMBO J.* **34**, 1009–1024. doi:10.15252/EMBJ.201488049

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–676. doi:10.1016/J.CELL.2006.07.024

Takashima, Y., Guo, G., Loos, R., Nichols, J., Ficz, G., Krueger, F., Oxley, D., Santos, F., Clarke, J., Mansfield, W., Reik, W., Bertone, P., and Smith, A. (2014). Resetting transcription factor control circuitry toward ground-state pluripotency in human. *Cell* **158**, 1254–1269. doi:10.1016/J.CELL.2014.08.029

Tam, P. P., and Zhou, S. X. (1996). The allocation of epiblast cells to ectodermal and germ-line lineages is influenced by the position of the cells in the gastrulating mouse embryo. *Dev. Biol.* **178**, 124–132. doi:10.1006/DBIO.1996.0203

Tang, W. W., Dietmann, S., Irie, N., Leitch, H. G., Floros, V. I., Bradshaw, C. R., Hackett, J. A., Chinnery, P. F., and Surani, M. A. (2015). A unique gene regulatory network resets the human germline epigenome for development. *Cell* **161**, 1453–1467. doi:10.1016/J.CELL.2015.04.053

Telfer, E. E., and Zelinski, M. B. (2013). Ovarian follicle culture: advances and challenges for human and nonhuman primates. *Fertil. Steril.* **99**, 1523–1533. doi:10.1016/J.FERTNSTERT.2013.03.043

Tesar, P. J., Chenoweth, J. G., Brook, F. A., Davies, T. J., Evans, E. P., Mack, D. L., Gardner, R. L., and McKay, R. D. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* **448**, 196–199. doi:10.1038/NATURE05972

Theunissen, T. W., Powell, B. E., Wang, H., Mitalipova, M., Faddah, D. A., Reddy, J., Fan, Z. P., Maetzel, D., Ganz, K., Shi, L., Lungjangwa, T., Imsoonthornruksa, S., Stelzer, Y., Rangarajan, S., D'Alessio, A., Zhang, J., Gao, Q., Dawlaty, M. M., Young, R. A., Gray, N. S., and Jaenisch, R. (2014). Systematic identification of culture conditions for induction and maintenance of naive human pluripotency. *Cell Stem Cell* **15**, 471–487. doi:10.1016/J.STEM.2014.07.002

Toyooka, Y., Tsunekawa, N., Akasu, R., and Noce, T. (2003). Embryonic stem cells can form germ cells *in vitro*. *Proc. Natl Acad. Sci. USA* **100**, 11457–11462. doi:10.1073/PNAS.1932826100

van Leeuwen, J., Rawson, P., Berg, D. K., Wells, D. N., and Pfeffer, P. L. (2020). On the enigmatic disappearance of Rauber's layer. *Proc. Natl Acad. Sci. USA* **117**, 16409–16417. doi:10.1073/PNAS.2002008117

Verma, R., Holland, M. K., Temple-Smith, P., and Verma, P. J. (2012). Inducing pluripotency in somatic cells from the snow leopard (*Panthera uncia*), an endangered felid. *Theriogenology* **77**, 220–228.e2. doi:10.1016/J.THERIOGENOLOGY.2011.09.022

Wang, S., Tang, X., Niu, Y., Chen, H., Li, B., Li, T., Zhang, X., Hu, Z., Zhou, Q., and Ji, W. (2007). Generation and characterization of rabbit embryonic stem cells. *Stem Cells* **25**, 481–489. doi:10.1634/STEMCELLS.2006-0226

Weber, S., Eckert, D., Nettersheim, D., Gillis, A. J., Schafer, S., Kuckenberg, P., Ehlermann, J., Werling, U., Biermann, K., Looijenga, L. H., and Schorle, H. (2010). Critical function of AP-2 gamma/TCFAP2C in mouse embryonic germ cell maintenance. *Biol. Reprod.* **82**, 214–223. doi:10.1095/BIOLREPROD.109.078717

Wiggans, G. R., Cole, J. B., Hubbard, S. M., and Sonstegard, T. S. (2017). Genomic selection in dairy cattle: the USDA experience. *Annu. Rev. Anim. Biosci.* **5**, 309–327. doi:10.1146/ANNUREV-ANIMAL-021815-111422

Yamaji, M., Seki, Y., Kurimoto, K., Yabuta, Y., Yuasa, M., Shigeta, M., Yamanaka, K., Ohinata, Y., and Saitou, M. (2008). Critical function of Prdm14 for the establishment of the germ cell lineage in mice. *Nat. Genet.* **40**, 1016–1022. doi: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., and Saitou, M. (2018). Generation of human oogonia from induced pluripotent stem cells *in vitro*. *Science* **362**, 356–360. doi:10.1126/SCIENCE.AAT1674

Yin, H., Kristensen, S. G., Jiang, H., Rasmussen, A., and Andersen, C. Y. (2016). Survival and growth of isolated pre-antral follicles from human

ovarian medulla tissue during long-term 3D culture. *Hum. Reprod.* **31**, 1531–1539. doi:10.1093/HUMREP/DEW049

Ying, Q. L., Wray, J., Nichols, J., Batlle-Morera, L., Doble, B., Woodgett, J., Cohen, P., and Smith, A. (2008). The ground state of embryonic stem cell self-renewal. *Nature* **453**, 519–523. doi:10.1038/NATURE06968

Young, M. A., Larson, D. E., Sun, C. W., George, D. R., Ding, L., Miller, C. A., Lin, L., Pawlik, K. M., Chen, K., Fan, X., Schmidt, H., Kalicki-Veizer, J., Cook, L. L., Swift, G. W., Demeter, R. T., Wendl, M. C., Sands, M. S., Mardis, E. R., Wilson, R. K., Townes, T. M., and Ley, T. J. (2012). Background mutations in parental cells account for most of the genetic heterogeneity of induced pluripotent stem cells. *Cell Stem Cell* **10**, 570–582. doi:10.1016/J.STEM.2012.03.002

Yu, R. R., Cheng, A. T., Lagenaur, L. A., Huang, W., Weiss, D. E., Treece, J., Sanders-Beer, B. E., Hamer, D. H., Lee, P. P., Xu, Q., and Liu, Y. (2009). A Chinese rhesus macaque (*Macaca mulatta*) model for vaginal *Lactobacillus* colonization and live microbicide development. *J. Med. Primatol.* **38**, 125–136. doi:10.1111/J.1600-0684.2008.00316.X

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., and Zhou, Q. (2016). Complete meiosis from embryonic stem cell-derived germ cells *in vitro*. *Cell Stem Cell* **18**, 330–340. doi:10.1016/J.STEM.2016.01.017