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In vitro gametogenesis from embryonic stem cells in livestock species: recent advances, opportunities, and challenges to overcome

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Abstract

Pluripotent stem cells (PSC) can be stabilized in vitro from pre-implantation stage embryos (embryonic stem cells, ESC) or by reprogramming adult somatic cells (induced pluripotent stem cells, iPSC). The last decade has seen significant advances in the livestock PSC field, particularly the development of robust methods for long-term culture of PSC from several livestock species. Along with this, considerable progress has been made in understanding the states of cellular pluripotency and what they mean for cell differentiation capacity, and significant efforts are ongoing to dissect the critical signaling pathways required for the maintenance of PSC in different species and distinct states of pluripotency. Among the cell types that can be generated from PSC, the germline holds special importance as they are the genetic link between generations; and devising methods to enable in vitro gametogenesis (IVG) and produce viable gametes could revolutionize animal agriculture, wildlife conservation, and human assisted reproduction alike. Within the last decade, many pivotal studies about IVG were published using rodent models, filling some critical knowledge gaps in the field. Most importantly, the entire female reproductive cycle was reproduced in vitro from mouse ESC. Although complete male gametogenesis in vitro has not yet been reported, significant advances were made showing the capacity of germline stem cell-like cells to generate healthy offspring. In this review, we provide an overview of PSC and advances in the establishment of livestock PSC; we present the breakthroughs made in rodents regarding IVG and the current progress towards livestock IVG, including the importance of a detailed understanding of fetal germline development. Finally, we discuss some key advances that will be critical to enable this technology at scale. Given the potential impact of IVG for animal agriculture, major efforts will likely continue to be employed by research institutions and industry towards the development of methods to achieve efficient generation of gametes in vitro.

Lay Summary

In this review, we summarize the current state of livestock embryonic stem cell establishment and the advances in production of sperm and eggs in vitro in rodents and livestock. We also discuss the potential and challenges of developing systems that support in vitro gametogenesis in livestock and the opportunities for this new technology in the reproductive field.

Introduction

Adequate reproductive performance associated with genomic selection is crucial to improve the frequency of desirable characteristics in livestock and enhance the efficiency of animal production systems. Elite animals can be selected to be gamete (oocyte or sperm) donors to maximize the gains in the next generation. Associated with donor animal selection, reproductive biotechnologies such as artificial insemination and in vitro embryo production (IVP) are powerful tools to accelerate genetic improvement. However, artificial insemination and IVP have limitations. Namely, these biotechnologies depend on the animal's reproductive life cycle, age, and a limited source of gametes, particularly oocytes.

Pluripotent stem cells (PSC) have two particular features: self-renewal and pluripotency. Self-renewal is the capacity to indefinitely generate new cells with the same characteristics as the original cell (enabled by high telomerase activity); pluripotency is the cell's ability to differentiate into all cell lineages (somatic and germline) found in the adult organism (Ying et al., 2008). PSC can be obtained from blastocysts via derivation of embryonic stem cells (ESC) or by reprogramming differentiated somatic cells into induced pluripotent stem cells (iPSC). Both ESC and iPSC have been receiving great attention due to their potential applications in areas such as in vitro gametogenesis (IVG), gene editing (for animal production or creating domestic animal models for human diseases), tissue regeneration, cellular agriculture, and more.

After years of concerted efforts by several groups, Hayashi and colleagues (Hikabe et al., 2016) demonstrated that the entire process of oogenesis could be recapitulated from mouse ESC in vitro, leading to generation of viable offspring. Since then, in vitro differentiation of germ cells has been explored in depth in rodents, enabling further protocol optimizations (Ishikura et al.2021, 2022; Yoshino et al., 2021).

Following the first report of stable bovine ESC (Bogliotti et al., 2018), Ross and colleagues (Goszczynski et al., 2019a) explored the potential to develop in vitro breeding schemes based on an embryo-stem cell-gamete cycle, including an intermediate genomic selection step to provide directional genetic progress. Such a scheme would significantly reduce the generational interval and allow for increased selection pressure leading to a projected ten fold acceleration in genetic progress compared to the reproductive technologies used currently (Goszczynski et al. 2019a, 2019b). By shortening the generational interval and enabling streamlined genomic testing to increase the accuracy of selection, IVG could be a powerful tool to decrease the environmental footprint of livestock production while increasing the efficiency of production of high-quality protein for a growing global population.

Here, we review the current advances in the establishment of livestock ESC, the understanding of in vivo germline development in livestock species, progress toward IVG in mice, humans, and livestock, and the potential and challenges to combine these advances to enable the generation of gametes from livestock PSC in vitro.

Overview of Embryonic Stem Cells

Martin Evans and Matthew Kaufman (1981) were the first to describe the successful establishment of mouse ESC (mESC) from the inner cell mass (ICM) of expanded blastocysts. In that same year, Martin (1981) derived mESC from ICM isolated by immunosurgery. After these first mESC reports, studies describing the establishment of livestock ESC were published in sheep (Handyside et al., 1987), pig (Strojek et al., 1990), cattle (Saito et al., 1992), and goat (Meinecke-Tillmann and Meinecke, 1996). All reports were based on standard conditions for mESC culture, including the isolation of ICM from in vivo-derived embryos, feeder cell support, and supplementation with growth factors such as insulin, epidermal growth factor (EGF), and leukemia inhibitory factor (LIF). However, low efficiency of derivation, inability to prevent cell differentiation, and lack of standardized culture conditions represented challenges that have taken several years to overcome.

The successful derivation of primate ESC was first described in rhesus macaque (Thomson et al., 1995) and after 3 yr, the first human ESC lines were established (Thomson et al., 1998). Those primate ESC were derived in distinct conditions from mESC; namely, they required supplementation of Activin A and FGF2 in the culture medium, whereas these factors were not necessary for the establishment or maintenance of mESC. Following the primate reports, groups working on livestock ESC incorporated FGF2 into the culture conditions (cattle: Wang et al., 2005; Yadav et al., 2005; Roach et al., 2006; Tian et al., 2006; and pigs: Miyoshi et al., 2000; Li et al. 2003, 2004). Most of the FGF-dependent livestock ESC lines reported self-renewal ability and pluripotency marker expression, however, these ESC lines demonstrated low efficiency to contribute to chimeric embryos.

The state of pluripotency of stem cells has been typically classified as naïve or primed, corresponding to the mouse ICM of the pre-implantation blastocyst and the epiblast of a post-implantation embryo, respectively (Nichols and Smith, 2009). Although ESC in both states express the core pluripotency markers POU5F1, SOX2, and NANOG, primed and naïve ESC are different in many characteristics such as signaling pathway dependence, gene expression, metabolism, epigenetic profile, and cell differentiation ability, among others (Nichols and Smith, 2009; Hanna et al., 2010). Specifically, naïve ESC are stabilized by LIF/Stat3 signaling and destabilized by FGF2/Erk, whereas primed ESC rely on FGF2/Erk signaling and do not respond to LIF/Stat3 (Brons et al., 2007; Tesar et al., 2007; Silva et al., 2008). Transforming growth factor β (TGF-β)/Smad signaling, which can be activated by Activin A/Nodal, is required for primed ESC establishment, whereas naïve ESC depend instead on bone morphogenic protein (BMP)/Smad signaling (Ying et al., 2003; James et al., 2005). Stimulation of canonical wingless-related integration site (WNT)/β-catenin signaling (e.g., by using the GSK3 inhibitor CHIR99021) promotes the derivation of naïve ESC (Mulas et al., 2019), however, it may lead to differentiation of primed ESC and therefore must be inhibited in those cells. IWR-1, a tankyrase inhibitor that stabilizes AXIN2, has been commonly used as a WNT inhibitor. (Sugimoto et al., 2015). Naïve ESC grow in dome-shaped colonies, spontaneously differentiate into teratomas with cells from all three embryonic germ layers, and are able to contribute to chimeras when injected into host embryos. Primed ESC grow in flat colonies and produce teratomas, but are not able to contribute to chimeric embryos. However, neither naïve nor primed ESC seem to efficiently undergo direct differentiation into the germline; instead, these cells must acquire a state of intermediate pluripotency between the naïve and primed states before efficient germline induction.

Methods to induce naïve mESC into a transient intermediary state of pluripotency (named epiblast-like cells, or EpiLC) needed for in vitro germline competence were first described by Hayashi and colleagues (2011). However, these cells could not be stably maintained long-term. In 2015, a new type of PSC was reported when Wu et al. (2015) described the isolation of a stem cell type with unique spatial characteristics and distinct molecular and functional features, designated as region-selective epiblast PSC. These cells demonstrated pluripotent characteristics and were efficient in generating intra- and interspecies chimeric embryos. More recently, PSC in a formative or "intermediate" state were successfully derived from mice, horses, and humans (Kinoshita et al., 2021a; Wang et al., 2021; Yu et al., 2021). These cells could contribute to chimeric embryos and more efficiently differentiate into germ cells, when compared with naïve or primed PSC.

Advances of Embryonic Stem Cell Establishment in Livestock

In 2018, Ross and colleagues described the successful derivation and stable maintenance of bovine ESC (bESC) using FGF2 and IWR-1 supplementation of a custom base medium similar to mTeSR (named CTFR) in inactivated mouse embryonic fibroblast (MEF) feeder cells (Bogliotti et al., 2018). In this report, bESC were derived from whole blastocysts or isolated ICM via microdissection or immunosurgery from

embryos produced by IVP or somatic cell nuclear transfer, with derivation efficiencies around 50% regardless of the embryo source or method for ICM isolation. The authors went on to demonstrate that bESC could be used as nuclear donors for subsequent somatic cell nuclear transfer. The success of CTFR to derive bESC opened new perspectives in the livestock ESC field, and the same culture system was later used to derive ESC from sheep embryos (Vilarino et al., 2020). In both reports, the resulting ESC showed features of primed pluripotency.

The Activin A/Nodal pathway plays a significant role in the maintenance of the primed pluripotency state (Hanna et al., 2010), but feeder-dependent culture systems do not require Activin-A supplementation since it is secreted by the MEFs. Looking to replace the feeder system with a feeder-free condition, Soto et al. (2021) added Activin A to a chemically defined base medium (N2B27 base medium supplemented with BSA, FGF2, and IWR-1; named NBFR) to culture bESC on a vitronectin substrate. Moreover, they demonstrated the dependence of bESC on Activin A, since supplementation of FGF2 and IWR-1 without Activin A was not sufficient to sustain pluripotency of bESC in feeder-free conditions.

Performing a screening of serum replacement and signaling molecules, Choi et al. (2019) developed a culture medium for deriving and maintaining porcine ESC from blastocysts. Interestingly, the small molecule CHIR99021, which allows the activation of the WNT/β-catenin signaling pathway by inhibiting GSK3β, was added in combination with the canonical WNT inhibitor IWR-1, in addition to FGF2 and Activin A. However, the absence of Activin A and CHIR99021 did not significantly influence the expression of pluripotency markers in pig ESC. These results confirmed the key actions of FGF2 and modulation of canonical WNT signaling for maintenance of pluripotency of ESC from ungulates.

After over 400 tested conditions, a combination of inhibitors for GSK3, SRC, and Tankyrases and the supplements vitamin C, LIF, and Activin A was used to culture porcine and human stem cells (iPSC and ESC) known as expanded potential stem cells (EPSC; Gao et al., 2019). The resulting porcine EPSC contributed to chimeric embryos and the human EPSC demonstrated robust potential to differentiate into trophoblast-like cells. By revising those cocktails (same small molecule inhibitors and cytokine combinations, but in different concentrations), Zhao and colleagues (2021) demonstrated the establishment of bovine ESC with an expanded potential for differentiation, including the ability to contribute to chimeric embryos (Zhao et al., 2021).

Under the stimulation of FGF2 and Activin A and inhibition of the WNT pathway by XAV939 in a culture system called AFX, PSC were derived from embryos of three livestock species: pig, sheep, and cattle (Kinoshita et al., 2021b). The AFX culture system was developed in feeder-free conditions on a combination of laminin and fibronectin as substrates and captured cells with bilaminar disc epiblast profile resembling a primed pluripotent state. Using medium containing FGF and TGFβ1 supplemented with IWP2 and XAV939 (two WNT inhibitors), Kobayashi et al. (2021) derived primed rabbit PSC.

In summary, the recent findings and protocols developed demonstrate the dependence of FGF signaling and WNT pathway inhibition to derive and maintain livestock ESC, resulting in the establishment of cells in the primed state of pluripotency (Figure 1). These reports also underscore the critical role of signaling pathway modulation to achieve subtle differences in pluripotency states. Importantly, capturing and maintaining livestock ESC in a naïve pluripotent state continues to be a challenge and more studies are needed to fill this gap. However, it must be noted that the generation of
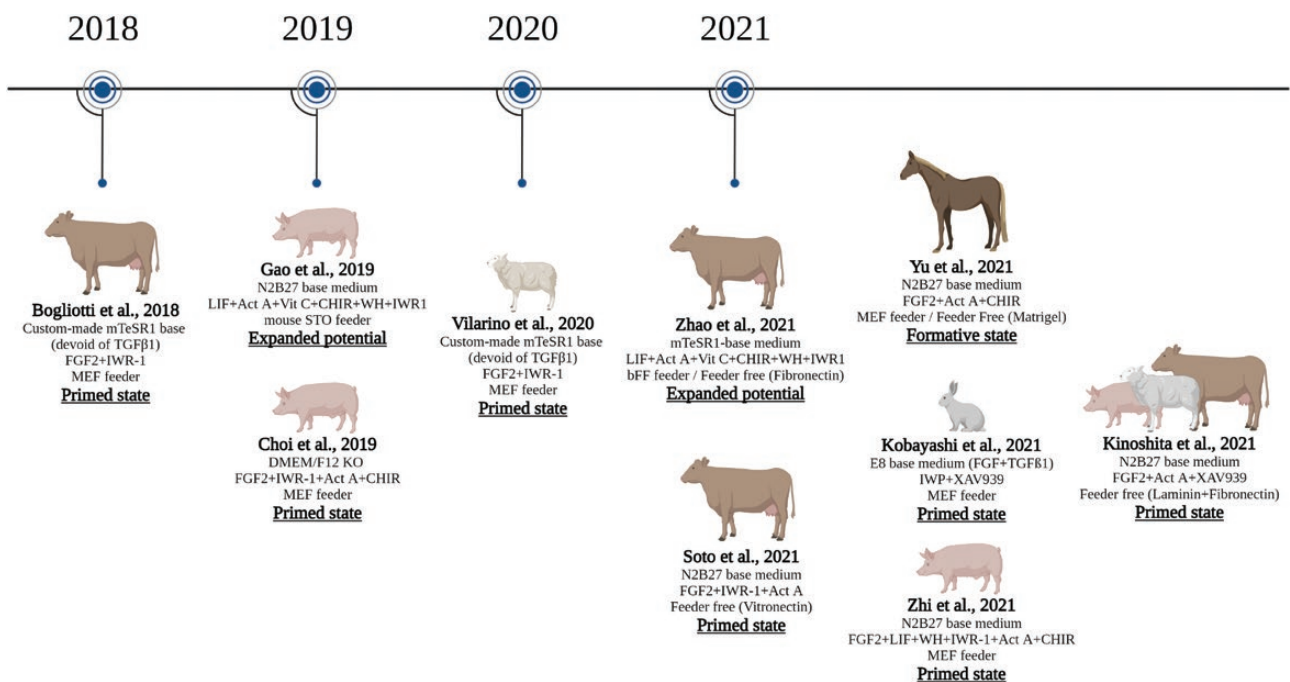
Figure 1. Reports of embryonic stem cell establishment and characterization in different livestock species. The main media components utilized, substrate, and pluripotent state of the cells (if described by authors) are mentioned under each development. Act-A, Activin A; bFF, bovine fetal fibroblast; CHIR, CHIR99021; IWP, IWP2; MEF, inactivated mouse embryonic fibroblast; STO, SIM (Sandos Inbred Mice) mouse embryonic fibroblast; Vit C, vitamin C; WH, WH-4-023.

naïve bovine induced pluripotent stem cells (iPSC) has been reported (Kawaguchi et al., 2015; Pillai et al., 2021; Su et al., 2021).

Gametes Produced In Vitro From Pluripotent Stem Cells

Since the first reports of the ability of stem cells to contribute to the germline and produce healthy progeny, much progress has been made in developing this technology in rodents (Figure 2). The first reports in mice showing that PSC could be differentiated into primordial germ cell-like cells (PGC-LC) and further develop into functional male and female gametes in vivo were published in 2011 and 2012 (Hayashi et al. 2011, 2012). The protocol consisted of transitioning naïve ESC into EpiLC, followed by PGC-LC induction using cells bearing a fluorescent construct to detect PRDM1 expression. After differentiation, sorted PGC-LC were transplanted to the seminiferous tubule (male) or aggregated with fetal ovarian somatic cells and transplanted under the ovarian bursa (female) to complete growth. Although these were breakthrough findings for the reproductive and stem cell biology fields, those early studies needed to transplant the aggregates into live animals to complete gamete maturation and had very low efficiency in producing offspring.

The reconstitution of the entire female gametogenic cycle in vitro was achieved for the first time in 2016 by Hikabe and colleagues. After differentiation, PGC-LC were aggregated with fetal ovarian somatic cells to create a reconstituted ovary (rOvary) and were cultured initially in the presence of retinoic acid, followed by a small molecule antagonist of the estrogen receptor. This strategy produced secondary follicle-like structures that were dissociated from the rOvary in order to continue development into fully-grown oocytes. At this point, follicle growth becomes dependent on gonadotropins, particularly FSH (Edson et al., 2009). An additional culture step in the presence of FSH, BMP15, and GDF9 resulted in the formation of cumulus-oocyte complexes that were subjected to in vitro maturation and showed cumulus expansion and extrusion of the first polar body. These in vitro-produced cumulus-oocyte complexes were used for IVP, and the resulting embryos transferred to surrogate females to generate pups. This system represented another groundbreaking advance, since transplantation of PGC-LC into a recipient for final oocyte growth and maturation was eliminated and the entire cycle could be done in vitro. Additionally, the induction system has proven to work with different cell lines of mESC and iPSC.

Male germline development has not been fully recapitulated in vitro due to difficulties in replicating the final step of spermiogenesis, whereby spermatids become motile spermatozoa. Zhou and colleagues (2016) reported the successful generation of spermatocyte-like cells from mESC. The first step here again involved induction of EpiLC from mESC,
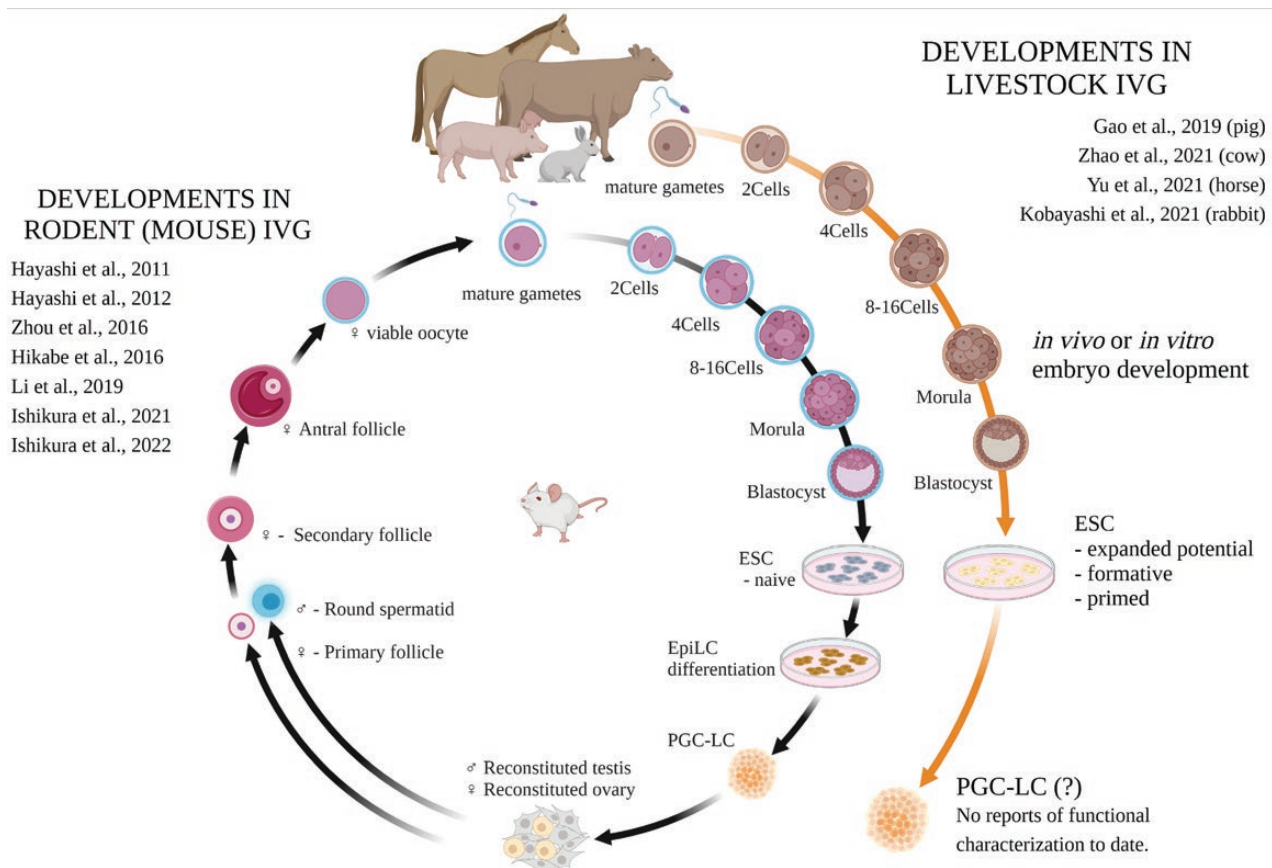
Figure 2. Overview of in vitro gametogenesis (IVG) developments in mice and the current state of IVG in livestock species. Although the differentiation of PGC-LC has been reported in pigs, cows, horses, and rabbits based on gene and protein expression analyses, no functional experiments have yet demonstrated the ability of these cells to generate differentiated gametes, pregnancies, or offspring. EpiLC, epiblast-like stem cells; ESC, embryonic stem cells; PGC-LC, primordial germ cell-like cells.

followed by PGC-LC induction. Then, PGC-LC were sorted based on expression of integrin β3 and SSEA1 and aggregated with testicular somatic cells. This system resulted in spermatocyte-like cells that produced viable and fertile offspring following intracytoplasmic sperm injection (ICSI).

In 2019, the differentiation of spermatid-like cells from mESC using cytokine induction with no dependence on fetal gonadal somatic cells was reported (Li et al., 2019). The process included the initial step of differentiation into EpiLC followed by PGC-LC induction, and differentiation into spermatogonia stem cell-like cells, which could then be transplanted into the seminiferous tubules of fertility-compromised mice or reseeded with feeder cells for meiosis induction using retinoic acid. Although this protocol had a low success rate with only around 10% of cells reaching the final stage of differentiation and rare live births, it represented a critical advance in the field of male IVG.

Recently, Ishikura and colleagues reported the in vitro differentiation of mESC to male germline stem cell-like cells via PGC-LC induction. Differentiation from ESC to PGC-LC was performed following the same protocol used for females. Then, PGC-LC were sorted based on transgenic expression of PRDM1 and cultured with a cocktail designed to reduce genome-wide CpG methylation into a precursory state for sex differentiation. After this crucial step of epigenetic reprogramming, PGC-LC were aggregated with fetal testicular somatic cells and cultured to reach a spermatogonium-like state. Sorted spermatogonium-like cells were cultured for up to 1.5 mo until forming new germline stem cell-like cell colonies (Ishikura et al. 2021, 2022). The germline stem cell-like cells were able to differentiate into spermatozoa upon transplantation into adult testes or round spermatids when transplanted into in vitro cultured testis explants, and both methods were capable of generating healthy offspring.

Despite intense research efforts by several groups, it took 10 yr from the first description of the complete differentiation of PSC into functional gametes in mice to accomplish the same in another rodent species, the rat (Oikawa et al., 2022). Interestingly, the protocols used to successfully accomplish IVG in the rat were overall very similar to the mouse. Sorting of PGC-LC was based on transgenic expression of NANOS3, and the resulting cells were aggregated with male or female fetal gonad somatic cells. Curiously, female gonadal somatic cells seemed more efficient than male somatic cells in supporting male PGC-LC development, as indicated by the greater expression of the germ cell markers DDX4 and NANOS3 when female gonadal somatic cells were used. PGC-LC differentiated from rat EpiLC and PGC-LC cultured in aggregates with female gonadal somatic cells were sorted again based on expression of NANOS3 and transplanted into neonatal seminiferous tubules to complete development, after which they were able to generate healthy pups upon artificial insemination of oocytes through round spermatid injection (ROSI) and testicular sperm extraction with intracytoplasmic sperm injection (TESE-ICSI). Interestingly, the authors reported the obtention of spermatid-like cells from both PGC-LC cultured alone and PGC-LC aggregated with female somatic cells, indicating that the use of somatic fetal cells could possibly be omitted.

Recent advances are starting to overcome the previous bottleneck of maintaining viable PGC and PGC-LC in culture for extended periods. Successful long-term culture of human PGC-LC induced from iPSC has been recently reported

(Kobayashi et al., 2022). These PGC-LC were first cocultured with fetal mouse fibroblast feeder cells, and later transitioned to a feeder-free system in Matrigel and feeder-conditioned medium. This system was able to sustain long term expansion (up to 160 d) without compromising PGC signature.

The above reports paved the way for development of methods to differentiate gamete precursor cells from PSC in different species, and although each species has its particularities, many cellular mechanisms seem to be conserved. Recent progress has been made in PGC-LC differentiation from PSC in nonrodent species including rabbits, cows, pigs, horses, sheep, and goats (Gao et al., 2019; Kobayashi et al., 2021; Yu et al., 2021; Zhao et al., 2021) as will be discussed in the next section. However, despite the demonstration of successful PGC-LC fate induction based on gene and protein expression of germ cell markers, to date no studies have been published that demonstrate the functionality of these PGC-LC in any nonrodent species. Similarly, to our knowledge, there are no published reports describing successful long-term culture of livestock PGC-LC yet.

Progress in Understanding In Vivo Germline Development in Domestic Animals

An enhanced understanding of the events and regulatory factors guiding germline development in vivo is critical for the establishment and optimization of conditions that support these processes in vitro. Until recently, little was known about the transcriptional regulation of germline development in livestock and therefore it had to be inferred from other models. The majority of studies analyzing mammalian germline specification and development in vivo have been done in mice. Characterization of human primordial germ cells (PGC) and in vitro-generated PGC-LC indicate many similarities with mice in the signals that command specification, but also some key differences. For example, SOX17 is a critical factor for human PGC specification but does not play a role in the mouse (Irie et al., 2015; Kojima et al., 2017). In addition, SOX2 expression is maintained in mouse PGC, yet repressed in human cells, while PRDM14 plays a secondary role in germ cell development in humans compared to mice (Campolo et al., 2013; Tang et al., 2015; Sybirna et al., 2020). These differences are not surprising, considering that the mouse embryo develops as an egg cylinder, whereas the epiblast of humans and other mammalian species develops as a bilaminar disc (Alberio et al., 2021); moreover, these discrepancies show that the methods of induction and characterization of in vitro PGC-LC might have to be considered on a species-to-species basis in order to efficiently yield functional germ cell precursors.

In the last few years, studies with domestic animals including pigs, cattle, and rabbits have shown evolutionary conservation in the transcriptional network regulating PGC development, and many similarities to humans in these processes. Alberio, Surani, and colleagues demonstrated in 2017 that porcine PGC are specified in the posterior epiblast of early primitive streak stage embryos with sequential expression of SOX17 and PRDM1. By exposing isolated epiblast disks of various stages to cytokines including BMP2/4, they found that preprimitive streak epiblasts are competent for PGC induction, but that this competency quickly wanes following primitive streak formation and onset of gastrulation (Kobayashi et al., 2017). Importantly, this suggests that for

the induction of PGC-LC from PSC, cells in a pluripotent state similar to the preprimitive streak epiblast would likely be the best candidates, at least in the pig. The subsequent steps of porcine PGC development, including the mechanisms and timing of DNA demethylation, chromatin remodeling, and X chromosome reactivation in migratory PGC were described by the same group (Zhu et al., 2021). Again, the authors found many similarities to these events from studies in human PGC and PGC-LC. This knowledge will be crucial for stabilizing PGC-LC in vitro and supporting their continued development toward gametes.

In the same year, Soto and Ross used a combination of gene and protein expression analysis approaches to trace germline development in cattle between days 40 and 90 of fetal development (Soto and Ross, 2021). Single-cell RNA-sequencing of bovine fetal ovaries at approximately 50 d of fetal age showed that PGC were still in the early stages of differentiation, with a subset of cells transitioning toward meiotic competence. Moreover, similarities in the transcriptomic profile between bovine and human PGC highlighted the conservation of germline development between the two species. Importantly, this work identified some of the surface markers of bovine PGC, which will be of great value for sorting PGC-LC from cell aggregates after germ lineage induction (Soto and Ross, 2021). Also, in 2021, Kobayashi and colleagues reported PGC specification in rabbits, showing that these cells were also specified in the posterior epiblast of the bilaminar disc and had transcriptomic similarities with other bilaminar disc species including pigs, cows, and humans (Kobayashi et al., 2021). This area of research will be strengthened by continued studies of different time points of germline development in vivo. Excitingly, the similarities in the timing and regulation of these processes to humans suggest that, compared to the mouse, advancements toward establishing human IVG systems will be more directly applicable to this process in livestock, and vice versa.

Steps Toward Enabling IVG in Livestock

Studies of PGC-LC induction from PSC in rodents and humans have highlighted the role that the pluripotent state has toward differentiation capacity and the limitations that both the naïve and primed states have for direct germline

lineage induction (Hayashi et al., 2011; Sasaki et al., 2015). The first reports of conditions that support stable livestock PSC lines have facilitated further research into alternative conditions that stabilize distinct pluripotent states. Namely, expanded potential culture systems have been established for porcine and bovine PSC (Gao et al., 2019; Zhao et al., 2021). Both reports showed that the resulting EPSC had the capacity to differentiate into putative PGC-LC following embryoid body formation after transcription factor-mediated induction using the SOX17 transgene. However, aside from the expression of several early PGC genes, further characterization of these cells and assessment of their developmental competence have yet to be demonstrated. Moreover, future experiments assessing the capacity of these cells to give rise to the germ lineage without transgenic intervention, or an alternative culture condition that would permit this, will be important for applying IVG to livestock.

A recent report established horse ESC in a formative state of pluripotency (Yu et al., 2021) and demonstrated that these cells were directly amenable to induction of a PGC-LC fate using the cytokine cocktail reported for mESC (Hayashi and Saitou, 2013), putting into evidence that despite some differences in the regulatory pathways, the predominant signaling molecules initiating PGC specification may be conserved. The first report of methods for PGC-LC induction from rabbit ESC was published in 2021 (Kobayashi et al., 2021). Interestingly, direct PGC-LC induction from primed rabbit ESC using a similar cytokine cocktail was more efficient compared to the use of a mesendodermal pre-induction step (as used for PGC-LC induction from primed PSC) and contrary to what was shown in primed human PSC (Sasaki et al., 2015; Kobayashi et al., 2021). This demonstrates that, beyond the pluripotent state of the PSC, species-specific signaling differences may need to be considered when developing the best methods for PGC-LC induction. The progress with PGC-LC induction in rabbits and horses is especially valuable toward developing these methods in domestic ungulates including cows, pigs, and sheep as, unlike humans, these species have more commonalities in embryonic development around the time of PGC specification, including the presence of the Rauber's layer and initiation of gastrulation before implantation (Artus et al., 2020; van Leeuwen et al., 2020). The methods described to date to induce PGC-LC from PSC from pigs,

Table 1. Summary of the methods and cytokine cocktails used to induce PGC-LC from livestock ESC

Species	Pig	Cow	Horse	Rabbit
Report	Gao et al., 2019	Zhao et al., 2021	Yu et al., 2021	Kobayashi et al., 2021
Pluripotent state of ESC	Expanded potential	Expanded potential	Formative	Primed
Pre-induction treatment	SOX17 transgene	SOX17 transgene; Activin A predifferentiation	N/A	N/A
# Cells per aggregate	5,000–6,000	5,000–6,000	3,000	2,000
Base medium	Advanced RPMI 1640	Advanced RPMI 1640	GMEM	Advanced DMEM/F12
Supplement or KSR[1], %	B27, 1%	B27, 1%	KSR, 15%	B27, 1%
BMP2 or BMP4, [ng/mL]	BMP2 [500]	BMP2 [500]	BMP4 [200]	BMP4 [100]
[LIF], ng/mL	10	10	1000 U/mL	10
[SCF], ng/mL	100	100	100	100
[EGF], ng/mL	50	50	50	50
Duration	3–4 d	3–4 d	3 d	2–3 d

[1]KSR, knockout serum replacement

cows, horses, and rabbits are summarized in Table 1; the progress of IVG of these species in relation to mice is depicted in Figure 2. Further research focused on optimizing PGC-LC induction protocols on a species-to-species basis will facilitate continued improvements for individual systems.

Producing a Scalable System to Apply IVG Technology to Livestock Reproduction

Beyond the challenge of devising a system that can support the specification and development of germ cells in vitro, improving the efficiency of this system would be crucial before IVG could be applied at scale to the livestock industry. Recent progress has been made with the establishment of systems that support higher efficiency and lower cost production of human PGC-LC, in addition to conditions that allow the maintenance and expansion of PGC-LC. In 2019, Wang et al. reported a modified system in which the PSC aggregates for PGC-LC induction were cocultured with methylcellulose and maintained as groups in larger wells. Coculture with 0.35% methylcellulose supported the maintenance of larger embryoid bodies in low-cell binding 6-well rather than U-bottom 96-well plates, increasing the efficiency of human PGC-LC induction about 8 to 10 times for the same consumption level of medium, without affecting the gene and protein expression of the resulting PGC-LC (Wang et al., 2019). In that same year, Gell et al. developed a two-dimensional extended culture system that supported the survival and proliferation of human PGC-LC for 3 wk. These cells maintained the transcriptional identity of a germ cell without reverting into a pluripotent state, which has been seen for ex vivo culture of human PGC. Moreover, extended culture of human PGC-LC supported recapitulation of the earliest stages of epigenetic reprogramming (Gell et al., 2020). Recently, Kobayashi and colleagues described conditions that supported extended culture of stable, homogenous human PGC-LC populations without the need for serum or feeder cells for at least 160 d (Kobayashi et al., 2022). This was the first report that maintained PGC-LC long-term without the need for fluorescence-activated cell sorting (FACS) to maintain a pure cell population over the extended culture period, as was necessary previously (Murase et al., 2020). This culture system will be useful for studying the later stages of epigenetic remodeling of PGC, including global DNA demethylation, which is required for developmental progression from early to late PGC identity. Further, this method could be applied to domestic animals for the maintenance and expansion of newly specified PGC-LC from three-dimensional induction systems before moving into the later steps of IVG, which could help increase the scale of the system.

To date, the only species in which full in vitro recapitulation of the reproductive cycle has been accomplished is mice (Hikabe et al., 2016; Zhou et al., 2016). These reports represent a major step in understanding gamete development; however, the efficiency of obtaining offspring from ESC-derived gametes was extremely low in the initial reports. Following the transfer of two-cell embryos into pseudopregnant recipients, only 3.5% gave rise to viable offspring compared to 61.7% of embryos from in vivo-generated oocytes (Hikabe et al., 2016). For male IVG, haploid spermatid-like cells were obtained, however, intracytoplasmic sperm injection was required to generate offspring. Compared to conventional in vitro fertilization, intracytoplasmic sperm injection is labor-intensive

and requires trained skills, which makes it less likely to be adopted at scale by the livestock industry. Therefore, developing conditions that support the process of spermiogenesis, enabling in vitro fertilization, will be important for male IVG to be scalable. Further, the success of obtaining pups from transferred two-cell embryos produced from ESC-derived spermatid-like cells was 2.8% compared to 9.5% for those from control spermatids (Zhou et al., 2016). In order for these approaches to be feasible for livestock, extensive optimization and characterization of ESC-derived gametes would be needed to select the best candidates, considering the effort and cost associated with embryo transfer.

Perspectives/Conclusion

Concerted efforts within the last 10 yr by independent research groups around the world have resulted in significant progress towards the accomplishment of IVG. Advances in this field are happening at large steps, but to this point, the vast majority have been made in mouse models. The inconsistency of findings regarding the state of pluripotency that favors the differentiation of the germ line will likely be solved as the knowledge about livestock PSC improves and as we learn more about the regulatory pathways at play on a species-by-species basis. Moreover, a deeper understanding of the early events in fetal PGC specification and development within the gonad will provide the clues that are currently missing before any major breakthroughs are possible. Once consistent differentiation of PGC-LC from PSC in livestock species is achieved, new systems will be necessary to take these PGC-LC through oogenesis and spermatogenesis to yield competent gametes. Another critical point of consideration which was not addressed in detail in this review is the potential need of somatic cells to support gamete development in vitro. Finally, once proof-of-concept has been established, methods for scalability of IVG to enable commercial exploitation of this technology by the livestock industry will likely be among the major scientific efforts in this field.

Acknowledgments

Conflict of Interest Statement

The authors declare no real or perceived conflicts of interest.

Literature Cited

Alberio, R., T. Kobayashi, and M. A. Surani. 2021. Conserved features of non-primate bilaminar disc embryos and the germline. *Stem Cell Rep*. 16:1078–1092. doi:10.1016/j.stemcr.2021.03.011

Artus, J., I. Hue, and H. Acloque. 2020. Preimplantation development in ungulates: a 'ménage à quatre' scenario. *Reproduction*. 159:R151–R172. doi:10.1530/REP-19-0348

Bogliotti, Y. S., J. Wu, M. Vilarino, D. Okamura, D. A. Soto, C. Zhong, M. Sakurai, R. V. Sampaio, K. Suzuki, J. C. I. Belmonte, et al. 2018. Efficient derivation of stable primed pluripotent embryonic stem cells from bovine blastocysts. *Proc. Natl. Acad. Sci. U.S.A.* 115:2090–2095. doi:10.1073/pnas.1716161115

Brons, I. G. M., L. E. Smithers, M. W. B. Trotter, P. Rugg-Gunn, B. Sun, S. M. Chuva De Sousa Lopes, S. K. Howlett, A. Clarkson, L. Ahrlund-Richter, R. A. Pedersen, et al. 2007. Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature*. 448:191–195. doi:10.1038/nature05950

Campolo, F., M. Gori, R. Favaro, S. Nicolis, M. Pellegrini, F. Botti, P. Rossi, E. A. Jannini, and S. Dolci. 2013. Essential role of Sox2 for the establishment and maintenance of the germ cell line. *Stem Cells*. 31:1408–1421. doi:10.1002/stem.1392

Choi, K. H., D. K. Lee, S. W. Kim, S. H. Woo, D. Y. Kim, and C. K. Lee. 2019. Chemically defined media can maintain pig pluripotency network in vitro. *Stem Cell Rep*. 13:221–234. doi:10.1016/j.stemcr.2019.05.028

Edson, M. A., A. K. Nagaraja, and M. M. Matzuk. 2009. The mammalian ovary from genesis to revelation. *Endocr Rev*. 30:624–712. doi:10.1210/er.2009-0012.

Evans, M. J., and M. H. Kaufman. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature*. 292:154–156. doi:10.1038/292154a0

Gao, X., M. Nowak-Imialek, X. Chen, D. Chen, D. Herrmann, D. Ruan, A. C. H. Chen, M. A. Eckersley-Maslin, S. Ahmad, Y. L. Lee, et al. 2019. Establishment of porcine and human expanded potential stem cells. *Nat. Cell Biol*. 21:687–699. doi:10.1038/s41556-019-0333-2

Gell, J. J., W. Liu, E. Sosa, A. Chialastri, G. Hancock, Y. Tao, S. E. Wamaitha, G. Bower, S. S. Dey, and A. T. Clark. 2020. An extended culture system that supports human primordial germ cell-like cell survival and initiation of DNA methylation erasure. *Stem Cell Rep*. 14:433–446. doi:10.1016/j.stemcr.2020.01.009

Goszczynski, D. E., H. Cheng, S. Demyda-Peyrás, J. F. Medrano, J. Wu, and P. J. Ross. 2019a. In vitro breeding: application of embryonic stem cells to animal production. *Biol. Reprod*. 100:885–895. doi:10.1093/biolre/ioy256

Goszczynski, D. E., A. C. Denicol, and P. J. Ross. 2019b. Gametes from stem cells: status and applications in animal reproduction. *Reprod. Domest. Anim*. 54:22–31. doi:10.1111/rda.13503

Handyside, A., M. L. Hooper, M. H. Kaufman, and I. Wilmut. 1987. Towards the isolation of embryonal stem cell lines from the sheep. *Roux Arch. Dev. Biol*. 196:185–190. doi:10.1007/bf00376313

Hanna, J. H., K. Saha, and R. Jaenisch. 2010. Pluripotency and cellular reprogramming: facts, hypotheses, unresolved issues. *Cell*. 143:508–525. doi:10.1016/j.cell.2010.10.008

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

Hayashi, K., H. Ohta, K. Kurimoto, S. Aramaki, and M. Saitou. 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., and M. Saitou. 2013. Generation of eggs from mouse embryonic stem cells and induced pluripotent stem cells. *Nat. Protoc*. 8:1513–1524. doi:10.1038/nprot.2013.090

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

Irie, N., L. Weinberger, W. W. C. Tang, T. Kobayashi, S. Viukov, Y. S. Manor, S. Dietmann, J. H. Hanna, and M. A. Surani. 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., H. Ohta, M. Nagano, and M. Saitou. 2022. Optimized protocol to derive germline stem-cell-like cells from mouse pluripotent stem cells. *STAR Protoc*. 3:101544. doi:10.1016/j.xpro.2022.101544

Ishikura, Y., H. Ohta, T. Sato, Y. Murase, Y. Yabuta, Y. Kojima, C. Yamashiro, T. Nakamura, T. Yamamoto, T. Ogawa, and M. Saitou. 2021. In vitro reconstitution of the whole male germ-cell

development from mouse pluripotent stem cells. *Cell Stem Cell*. 28:2167–2179.e9.e9. doi:10.1016/j.stem.2021.08.005

James, D., A. J. Levine, D. Besser, and A. Hemmati-Brivanlou. 2005. TGFβ/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development*. 132:1273–1282. doi:10.1242/dev.01706

Kawaguchi, T., T. Tsukiyama, K. Kimura, S. Matsuyama, N. Minami, M. Yamada, and H. Imai. 2015. Generation of naïve bovine induced pluripotent stem cells using PiggyBac transposition of doxycycline-inducible transcription factors. *PLoS One*. 10:e01354031–e01354018. doi:10.1371/journal.pone.0135403

Kinoshita, M., M. Barber, W. Mansfield, Y. Cui, D. Spindlow, G. G. Stirparo, S. Dietmann, J. Nichols, and A. Smith. 2021a. Capture of mouse and human stem cells with features of formative pluripotency. *Cell Stem Cell*. 28:453–471.e8.e8. doi:10.1016/j.stem.2020.11.005

Kinoshita, M., T. Kobayashi, B. Planells, D. Klisch, D. Spindlow, H. Masaki, S. Bornelöv, G. G. Stirparo, H. Matsunari, A. Uchikura, et al. 2021b. Pluripotent stem cells related to embryonic disc exhibit common self-renewal requirements in diverse livestock species. *Development*. 148:dev199901. doi:10.1242/dev.199901

Kobayashi, T., A. Castillo-Venzor, C. A. Penfold, M. Morgan, N. Mizuno, W. W. C. Tang, Y. Osada, M. Hirao, F. Yoshida, H. Sato, et al. 2021. Tracing the emergence of primordial germ cells from bilaminar disc rabbit embryos and pluripotent stem cells. *Cell Rep*. 37:109812. doi:10.1016/j.celrep.2021.109812

Kobayashi, M., K. Misato, J. Odajima, K. Shioda, Y. S. Hwang, K. Sasaki, P. Chatterjee, C. Kramme, R. E. Kohman, G. M. Church, et al. 2022. Expanding homogeneous culture of human primordial germ cell-like cells maintaining germline features without serum or feeder layers. *Stem Cell Rep*. 17:507–521. doi:10.1016/j.stemcr.2022.01.012

Kobayashi, T., H. Zhang, W. W. C. Tang, N. Irie, S. Withey, D. Klisch, A. Sybirna, S. Dietmann, D. A. Contreras, R. Webb, et al. 2017. Principles of early human development and germ cell program from conserved model systems. *Nature*. 546:416–420. doi:10.1038/nature22812

Kojima, Y., K. Sasaki, S. Yokobayashi, Y. Sakai, T. Nakamura, Y. Yabuta, F. Nakaki, S. Nagaoka, K. Woltjen, A. Hotta, et al. 2017. Evolutionarily distinctive transcriptional and signaling programs drive human germ cell lineage specification from pluripotent stem cells. *Cell Stem Cell*. 21:517–532.e5.e5. doi:10.1016/j.stem.2017.09.005

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

Li, M., Y. H. Li, Y. Hou, X. F. Sun, Q. Sun, and W. H. Wang. 2004. Isolation and culture of pluripotent cells from in vitro produced porcine embryos. *Zygote*. 12:43–48. doi:10.1017/s0967199404002679

Li, N., W. Ma, Q. Shen, M. Zhang, Z. Du, C. Wu, B. Niu, W. Liu, and J. Hua. 2019. Reconstitution of male germline cell specification from mouse embryonic stem cells using defined factors in vitro. *Cell Death Differ*. 26:2115–2124. doi:10.1038/s41418-019-0280-2

Li, M., D. Zhang, Y. Hou, L. Jiao, X. Zheng, and W. H. Wang. 2003. Isolation and culture of embryonic stem cells from porcine blastocysts. *Mol. Reprod. Dev*. 65:429–434. doi:10.1002/mrd.10301

Martin, G. R. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U.S.A*. 78:7634–7638. doi:10.1073/pnas.78.12.7634

Meinecke-Tillmann, S., and B. Meinecke. 1996. Isolation of ES-like cell lines from ovine and caprine preimplantation embryos. *J. Anim. Breed. Genet*. 113:413–426. doi:10.1111/j.1439-0388.1996.tb00632.x

Miyoshi, K., Y. Taguchi, Y. Sendai, H. Hoshi, and E. Sato. 2000. Establishment of a porcine cell line from in vitro-produced blastocysts and transfer of the cells into enucleated oocytes. *Biol. Reprod*. 62:1640–1646. doi:10.1095/biolreprod62.6.1640

Mulas, C., T. Kalkan, F. von Meyenn, H. G. Leitch, J. Nichols, and A. Smith. 2019. Defined conditions for propagation and manipulation of mouse embryonic stem cells. *Development*. 146:178970. doi:10.1242/DEV.173146

Murase, Y., Y. Yabuta, H. Ohta, C. Yamashiro, T. Nakamura, T. Yamamoto, and M. Saitou. 2020. Long-term expansion with germline potential of human primordial germ cell-like cells in vitro. *EMBO J*. 39. doi:10.15252/embj.2020104929. Available from: https://onlinelibrary.wiley.com/doi/10.15252/embj.2020104929.

Nichols, J., and A. Smith. 2009. Naive and primed pluripotent states. *Cell Stem Cell*. 4:487–492. doi:10.1016/j.stem.2009.05.015

Oikawa, M., H. Kobayashi, M. Sanbo, N. Mizuno, K. Iwatsuki, T. Takashima, K. Yamauchi, F. Yoshida, T. Yamamoto, T. Shinohara, et al. 2022. Functional primordial germ cell-like cells from pluripotent stem cells in rats. *Science*. 376:176–179. doi:10.1126/science.abl4412

Pillai, V. V., P. P. Koganti, T. G. Kei, S. Gurung, W. R. Butler, and V. Selvaraj. 2021. Efficient induction and sustenance of pluripotent stem cells from bovine somatic cells. *Biol. Open*. 10:bio058756. doi:10.1242/bio.058756

Roach, M., L. Wang, X. Yang, and X. C. Tian. 2006. Bovine embryonic stem cells. *Methods Enzymol*. 418:21–37. doi:10.1016/S0076-6879(06)18002-7

Saito, S., N. Strelchenko, and H. Niemann. 1992. Bovine embryonic stem cell-like cell lines cultured over several passages. *Roux Arch. Dev. Biol*. 201:134–141. doi:10.1007/BF00188711

Sasaki, K., S. Yokobayashi, T. Nakamura, I. Okamoto, Y. Yabuta, K. Kurimoto, H. Ohta, Y. Moritoki, C. Iwatani, H. Tsuchiya, et al. 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

Silva, J., O. Barrandon, J. Nichols, J. Kawaguchi, T. W. Theunissen, and A. Smith. 2008. Promotion of reprogramming to ground state pluripotency by signal inhibition. *PLoS Biol*. 6:e2532237–e2532247. doi:10.1371/journal.pbio.0060253

Soto, D. A., M. Navarro, C. Zheng, M. M. Halstead, C. Zhou, C. Guiltinan, J. Wu, and P. J. Ross. 2021. Simplification of culture conditions and feeder-free expansion of bovine embryonic stem cells. *Sci. Rep*. 11:1–15. doi:10.1038/s41598-021-90422-0

Soto, D. A., and P. J. Ross. 2021. Similarities between bovine and human germline development revealed by single-cell RNA sequencing. *Reproduction*. 161:239–253. doi:10.1530/REP-20-0313

Strojek, R. M., M. A. Reed, J. L. Hoover, and T. E. Wagner. 1990. A method for cultivating morphologically undifferentiated embryonic stem cells from porcine blastocysts. *Theriogenology*. 33:901–913. doi:10.1016/0093-691x(90)90825-e

Su, Y., L. Wang, Z. Fan, Y. Liu, J. Zhu, D. Kaback, J. Oudiz, T. Patrick, S. P. Yee, X. C. Tian, I. Polejaeva, Y. and Tang. 2021. Establishment of Bovine-Induced Pluripotent Stem Cells. Int J Mol Sci. 22(19):10489. doi:10.3390/ijms221910489.

Sugimoto, M., M. Kondo, Y. Koga, H. Shiura, R. Ikeda, M. Hirose, A. Ogura, A. Murakami, A. Yoshiki, S. M. C. de Sousa Lopes, et al. 2015. A simple and robust method for establishing homogeneous mouse epiblast stem cell lines by Wnt inhibition. *Stem Cell Rep*. 4:744–757. doi:10.1016/j.stemcr.2015.02.014

Sybirna, A., W. W. C. Tang, M. Pierson Smela, S. Dietmann, W. H. Gruhn, R. Brosh, and M. A. Surani. 2020. A critical role of PRDM14 in human primordial germ cell fate revealed by inducible degrons. *Nat. Commun*. 11:1282. doi:10.1038/s41467-020-15042-0

Tang, W. W. C., S. Dietmann, N. Irie, H. G. Leitch, V. I. Floros, C. R. Bradshaw, J. A. Hackett, P. F. Chinnery, and M. A. Surani. 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

Tesar, P. J., J. G. Chenoweth, F. A. Brook, T. J. Davies, E. P. Evans, D. L. Mack, R. L. Gardner, and R. D. G. McKay. 2007. New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature*. 448:196–199. doi:10.1038/nature05972

Thomson, J. A., J. Itskovitz-Eldor, S. S. Shapiro, M. A. Waknitz, J. J. Swiergiel, V. S. Marshall, and J. M. Jones. 1998. Embryonic stem cell lines derived from human blastocysts. *Science*. 282:1145–1147. doi:10.1126/science.282.5391.1145

Thomson, J. A., J. Kalishman, T. G. Golos, M. Durning, C. P. Harris, R. A. Becker, and J. P. Hearn. 1995. Isolation of a primate embryonic stem cell line. *Proc. Natl. Acad. Sci. U.S.A*. 92:7844–7848. doi:10.1073/pnas.92.17.7844

Tian, H. B., H. Wang, H. Y. Sha, X. J. Xu, M. Zhu, Y. B. Wu, S. H. Cheng, J. Q. Chen, Y. X. Shi, Z. L. Bai, et al. 2006. Factors derived from mouse embryonic stem cells promote self-renewal of goat embryonic stem-like cells. *Cell Biol. Int*. 30:452–458. doi:10.1016/j.cellbi.2006.02.006

Vilarino, M., D. Alba Soto, Y. Soledad Bogliotti, L. Yu, Y. Zhang, C. Wang, E. Paulson, C. Zhong, M. Jin, J. Carlos Izpisua Belmonte, et al. 2020. Derivation of sheep embryonic stem cells under optimized conditions. *Reproduction*. 160:761–772. doi:10.1530/REP-19-0606

Wang, L., E. Duan, L. Y. Sung, B. S. Jeong, X. Yang, and X. C. Tian. 2005. Generation and characterization of pluripotent stem cells from cloned bovine embryos. *Biol. Reprod*. 73:149–155. doi:10.1095/biolreprod.104.037150

Wang, X., T. Liao, C. Wan, X. Yang, J. Zhao, R. Fu, Z. Yao, Y. Huang, Y. Shi, G. Chang, et al. 2019. Efficient generation of human primordial germ cell-like cells from pluripotent stem cells in a methylcellulose-based 3D system at large scale. *PeerJ*. 6:e6143. doi:10.7717/peerj.6143

Wang, X., Y. Xiang, Y. Yu, R. Wang, Y. Zhang, Q. Xu, H. Sun, Z. A. Zhao, X. Jiang, W. Xiaoqing, et al. 2021. Formative pluripotent stem cells show features of epiblast cells poised for gastrulation. *Cell Res*. 31:526–541. doi:10.1038/s41422-021-00477-x

Wu, J., D. Okamura, M. Li, K. Suzuki, C. Luo, L. Ma, Y. He, Z. Li, C. Benner, I. Tamura, et al. 2015. An alternative pluripotent state confers interspecies chimaeric competency. *Nature*. 521:316–321. doi:10.1038/nature14413

Yadav, P. S., W. A. Kues, D. Herrmann, J. W. Carnwath, and H. Niemann. 2005. Bovine ICM derived cells express the Oct4 ortholog. *Mol. Reprod. Dev*. 72:182–190. doi:10.1002/mrd.20343

Ying, Q.-L., J. Nichols, I. Chambers, and A. Smith. 2003. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell*. 115:281–292. doi:10.1016/s0092-8674(03)00847-x

Ying, Q.-L. L., J. Wray, J. Nichols, L. Batlle-Morera, B. Doble, J. Woodgett, P. Cohen, and A. Smith. 2008. The ground state of embryonic stem cell self-renewal. *Nature*. 453:519–523. doi:10.1038/nature06968

Yoshino, T., T. Suzuki, G. Nagamatsu, H. Yabukami, M. Ikegaya, M. Kishima, H. Kita, T. Imamura, K. Nakashima, R. Nishinakamura, et al. 2021. Generation of ovarian follicles from mouse pluripotent stem cells. *Science*. 373:eabe0237. doi:10.1126/science.abe0237

Yu, L., Y. Wei, H. X. Sun, A. K. Mahdi, C. A. P. Arteaga, M. Sakurai, D. A. Schmitz, C. Zheng, E. D. Ballard, J. Li, et al. 2021. Derivation of intermediate pluripotent stem cells amenable to primordial germ cell specification. *Cell Stem Cell*. 28:1–18. doi:10.1016/j.stem.2020.11.003

Zhao, L., X. Gao, Y. Zheng, Z. Wang, G. Zhao, J. Ren, J. Zhang, J. Wu, B. Wu, Y. Chen, et al. 2021. Establishment of bovine expanded potential stem cells. *Proc. Natl. Acad. Sci. U.S.A*. 118:1–9. doi:10.1073/pnas.2018505118

Zhou, Q., M. Wang, Y. Yuan, X. Wang, R. Fu, H. Wan, M. Xie, M. Liu, X. Guo, Y. Zheng, et al. 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

Zhu, Q., F. Sang, S. Withey, W. Tang, S. Dietmann, D. Klisch, P. Ramos-Ibeas, H. Zhang, C. E. Requena, P. Hajkova, et al. 2021. Specification and epigenomic resetting of the pig germline exhibit conservation with the human lineage. *Cell Rep*. 34:108735. doi:10.1016/j.celrep.2021.108735