

# In vitro breeding: application of embryonic stem cells to animal production<sup>1</sup>

*Running title:* In vitro breeding

*Summary sentence:* In vitro breeding constitutes a fast and intense selection strategy to genetically improve livestock populations.

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## ABSTRACT

Embryonic stem cells (ESCs) are derived from the inner cell mass of preimplantation blastocysts. For decades, attempts to efficiently derive ESCs in animal livestock species have been unsuccessful, but this goal has recently been achieved in cattle. Together with the recent reconstitution of the germ cell differentiation processes from ESCs in mice, these achievements open new avenues for the development of promising technologies oriented toward improving health, animal production and the environment. In this article, we present a strategy that will notably accelerate genetic improvement in livestock populations by reducing the generational interval, namely *in vitro* breeding (IVB). IVB combines genomic selection (GS), a widely used strategy for genetically improving livestock, with ESC derivation and *in vitro* differentiation of germ cells from pluripotent stem cells. We also review the most recent findings in the fields on which IVB is based. Evidence suggests this strategy will be soon within reach.

## IN VITRO BREEDING (IVB)

The main goal of a breeding program is to improve a population by increasing the genetic merit of socioeconomically important traits. Important traits are mostly quantitative, and their genetic variance is explained by small effects provided by many quantitative trait loci (QTL). The advent of genomic technologies, such as SNP arrays and next generation sequencing, during the 2000's resulted in the discovery of thousands of QTLs identified in different livestock species that are now publicly available in databases like Animal QTLdb [1]. This, in turn, led to the implementation of genomic selection (GS) in the breeding programs of many livestock

species. GS aims at capturing the effect of QTLs through associations between phenotypes and DNA markers spanning the entire genome [2]. The first step in GS consists in estimating the effect exerted by each genomic marker within a reference population with known phenotypes and genotypes. It is important to mention that such reference populations need to be large enough and structurally accurate to allow for precise estimates [3]. These effects are then utilized to select the best candidates among the population and to calculate their genomic estimated breeding values (GEBVs) based on the estimations carried out in the reference. Thus, no phenotype information is needed. The GEBVs are calculated by summing up all the individual effects for each sample. By avoiding progeny testing and phenotypic measures, GS leads to an increase in genetic gain due to shorter generational intervals, as well as savings in cost. In this aspect, the rate of genetic gain through GS is doubled by using bulls at two years of age instead of five, with a decrease in cost up to 92% by avoiding progeny testing [4]. Thanks to these advantages, GS is now routinely implemented in advanced animal breeding programs of different livestock species such as cattle, pigs and sheep [5-7]. However, even though the generational intervals of mammalian livestock species have significantly decreased since the introduction of GS, some of these intervals remain constrained to years due to limiting factors, such as gestation and arrival to puberty. For instance, research utilizing the United States national dairy database has shown that the current generational intervals for Holstein cattle are ~2.5 years for sires and dams of bulls and ~4.5 and ~5 years for sires of cows and dams of cows, respectively [8].

During the next few years, genomic datasets are expected to comprise millions of individuals with sequence information [9], which will lead to a high number of QTLs. However, increasing the frequencies of all the favorable alleles of these QTLs through conventional

breeding methods, such as GS, may take a long time. This is mainly due to the low levels of recombination during meiosis that limits current breeding strategies. Therefore, the implementation of new technologies and strategies to breeding schemes might open new opportunities for animal production. In this article, we present and evaluate the benefits of a novel alternative to complement the GS methodology and increase genetic gain.

The efficient derivation of embryonic stem cells from bovine blastocysts [10] and the *in vitro* generation of germ cells from embryonic stem cells (ESCs) in mice [11], enable a large paradigm shift in the reproductive field, especially for livestock breeding. With these two valuable tools working together, science is only a few steps away from tracing the full path from embryo to functional gametes, which has already been accomplished in mice. Additionally, remarkable advancements have recently been achieved in germ cell differentiation of human pluripotent stem cells (PSC) [12]. In this context, we present a new strategy, *in vitro* breeding (IVB), which aims to combine these cutting-edge reproductive technologies with genomic selection to accelerate the genetic improvement of livestock populations (Figure 1).

Similar to GS, this method would begin with an estimation of genotypic values associated with productive traits of interest, which must be carried out in a proper reference population to achieve a high accuracy. Once these effects have been estimated, hundreds or thousands of embryos would be generated *in vitro* from high genetic merit males and females. Then, ESC cultures would be derived from the ICM of each blastocyst and genotyped to calculate estimated embryonic breeding values (EEBVs), which are based on the effects estimated in the reference population. It is worth mentioning that in cattle, the procedure for ESC derivation from blastocysts meets all the requirements for establishing high throughput *in vitro* schemes. After this calculation, each cell line genotype would have an EEBV for each trait, which would

determine the quality of each cell line in accordance with the breeding goal. Depending on the intensity of selection that breeders are willing to exert on their embryo population, tens or hundreds of cell lines with high genetic merit could be selected from the candidates. The next step would comprise the generation of functional gametes from the ESCs, which would be isolated to perform a new round of *in vitro* fertilization (IVF), ESC selection and germ cell differentiation. Although it is important to highlight the possibility of generating multiple embryos from the same cross, the main advantage of this strategy lies in the time it takes to carry out each breeding round. Assuming an IVF procedure followed by ESC derivation takes about four weeks in cattle, and germ cell differentiation takes about two or three months in mice, a breeding round through IVB could be completed in around three to four months. This would mean a huge reduction in the generational interval. Additionally, IVB might be complemented with other modern techniques to have a greater effect on genetic improvement in a reduced amount of time. The implementation of techniques such as genome editing [13] and gene drive [14] on selection programs have recently been proposed.

To demonstrate the benefits of this strategy, we conducted a simulated test [15] comparing a GS-based breeding program to an IVB-based breeding program over 25 years of selection. The population founders (50 males and 50 females) were simulated based on parameters estimated from real genotypes (58,990 SNPs) for milk yield in a Holstein cattle population. In each generation, the top ten males and females were selected as parents for the next generation based on their phenotypes. The selected parents were randomly mated to produce 500 males and 500 females each generation and the cumulative selection response was calculated using the phenotypic mean of the animals. The difference in the number of generations bred over 25 years is very clear, 100 generations for IVB against ten for GS (Figure 2). It takes the GS

program 2.5 years to obtain its first generation, while IVB would allow 10 generations of mating and selection in this same period. This expedited progress would revolutionize animal agriculture by allowing substantial improvements in production efficiency in a short amount time, resulting in fewer animals needed to provide larger amounts of animal products, and in turn, decrease the animal agriculture footprint on the environment.

This type of combined strategy has been previously proposed in the early 1990s, prior to the implementation of the genomic technologies, which made possible the discovery of thousands of QTLs [16]. Such is the case of velogenesis, which initially proposed to grow, mature and fertilize prepubertal oocytes *in vitro* with sperm from selected progeny-tested bulls and to transfer them into postpubertal animals. The combination of velogenesis with marker-assisted selection gave rise to a new concept, velogenetics. This consisted of incorporating favorable mutations into a new genetic background by repeated backcrosses carried out by velogenesis. By the time the idea was conceived, the number of mapped QTLs was very limited, and markers were not available to explain a large part of the genetic variation for quantitative production traits. For this reason, the concept was initially conceived to be applied in cases such as disease resistance. Our strategy makes use of high throughput technologies like SNP arrays to determine hundreds of thousands of genotypes that might explain most of the genetic variance in production traits. It is also worth mentioning that the term “*in vitro* breeding” has been used for decades to refer to a number of *in vitro* plant breeding techniques, such as micropropagation, *in vitro* flowering, and *in vitro* pollination, among others [17]. Although these plant biotechnology techniques are grouped under the same name as our proposed strategy, the concept is considerably different.

A recently proposed approach makes use of fibroblast cell cultures established from *in vitro*-produced embryos to genotype and select the candidates, which are later used as donor cells for somatic cell nuclear transfer (SCNT) cloning and subsequent embryo transfer (ET) [18]. Although such strategies provide some benefits by increasing the intensity of selection, the clones would still have to develop into sexually mature animals in order to contribute to the next generation. In IVB, continuous cloning and transfers are not necessary since germ cells are generated in the same cycle, which allows for a large reduction in the generation interval.

Although IVB looks promising, more studies are needed to define responsible guidelines and to optimize its practical and sustainable implementation in real breeding schemes. The tempting possibility of obtaining the maximum genetic potential in just a few embryos and using them to breed future generations could generate significant losses in genetic variance within populations. Potentially, one of the most limiting factors could be the requirement for re-estimation of marker effects, since the accuracy of the prediction decreases after a few rounds of selection as a consequence of linkage disequilibrium break-down. This happens because the current version of GS relies on the use of SNP markers rather than causal mutations. In the future, costs of sequencing are expected to decrease enough to permit the sequencing of the reference populations and thus, retain higher accuracy across multiple generations.

Additional concerns include potential epigenetic changes that may result as a consequence of continuous cell and embryo culture. The determination of proper procedures to ensure efficient epigenetic reprogramming of the embryos is worthy of attention. In large domestic animals, *in vitro* embryo production as well as *in vitro* culture of *in vivo*-derived embryos have been associated with detrimental fetoplacental development such as lower pregnancy rates, early embryonic loss, prolonged gestation and fetal overgrowth, among

others[19]. Although many of these problems have been solved by reducing the concentration of serum in the culture medium and eliminating co-culture systems, some issues remain unanswered and need further study. In general, no adverse neonatal or adult health outcomes have been reported by the bovine industry as consequence of *in vitro* embryo production [20]; however, long-term monitoring is complicated because animals are often sold as calves or young adults, used for a few years and culled.

After selection, one possible scenario could be to clone embryos via SCNT[18], given that ESCs are undifferentiated and PSCs have been shown to possess high reprogramming efficiency when used as nuclei donors for SCNT cloning [21, 22]. However, there are epigenetic mechanisms affecting the efficiency of this procedure that need to be considered. It is known that H3K9 methylation is implicated as an important barrier affecting SCNT reprogramming [23-25]. For instance, H3K9 and DNA are often hypermethylated in cloned cattle embryos [26]. Although epigenetic reprogramming after SCNT is not always fully achieved, multiple strategies have been proposed in different animal species to overcome this issue. These include the treatment with inhibitors for methyltransferases or histone deacetylases, which has shown promising results in mice and pigs [27, 28]. In cattle, the inhibition of H3K9 methyltransferases and the injection of H3K9 demethylases in nuclear transfer embryos have shown improved blastocyst rates [29, 30]. After cloning, the embryos could be transferred into recipient cows and raised until puberty, when they would produce semen to introduce their genetics into other populations.

## EMBRYONIC STEM CELLS

ESCs are derived from the inner cell mass (ICM) of preimplantation blastocysts. Due to their pluripotency, they are capable of self-renewal and differentiating into any cell type of the three primary germ layers. Because of these features, ESCs are perhaps the most studied type of pluripotent stem cells (PSCs), and their derivatives have become one of the main cell sources for modern cell therapies, pharmaceutical testing procedures and other biotechnological applications, such as genetic engineering.

Since pluripotency encompasses several stages of development, different types of ESCs can be obtained in culture from cells isolated in different embryonic stages [31]. In general, ESCs can be classified as naïve and primed. Mouse ESCs reside in the so-called naïve pluripotent state, which is characterized by presenting domed colonies, have increased single-cell survival, are dependent on JAK/STAT signaling, can efficiently contribute to chimeras when introduced into blastocysts, contain reactivated X-chromosomes in females and show high homogeneity in terms of pluripotency [32-35]. Although the derivation efficiency varies among strains, mouse ESCs are normally derived in medium containing leukemia inhibitory factor (LIF) and ERK/MAPK signaling inhibitors such as CHIR99021 and PD0325901 (also known as 2i conditions) over mitotically inactivated feeder layers [36-38]. Human ESCs, on the other hand, are generally derived in a primed pluripotency state, similar to stem cells derived from the post-implantation epiblast in mouse (EpiSCs, Epiblast Stem Cells) [39, 40]. Human ESCs display flat colony morphology, have low single-cell clonogenicity, are dependent on TGF-beta/activin/nodal signaling and display a lower efficiency to contribute to chimeras.

Since naïve pluripotency is desired in culture, different strategies have been reported to induce this state in human ESCs, either from pre-existing primed hESCs or by direct derivation from the blastocyst stage. These methods include the use of naïve human stem cell medium (NHSM) [41], the reverse-toggle protocol [42], 5i/L/F/A medium [43], the reset of ESCs through ectopic expression of master regulatory genes followed by culturing in medium without basic fibroblast growth factor (bFGF) [44], and the use of naïve conversion medium (NCM) [45]. However, the resulting naïve ESCs have failed to differentiate towards functional cell types when differentiation protocols, previously shown to work on the primed ESCs, were applied [46]. This might suggest that such protocols need further optimization or that naïve ESCs need priming before undergoing differentiation. Despite the differences between murine and human ESCs, stable long-term ESC lines have been successfully established for both species over the years. In many domestic mammals, however, ESC derivation remains a challenge.

Deriving bovine ESCs has been a complicated goal for many years due to the high tendency for spontaneous differentiation and cell death [47, 48]. Several studies have evaluated the use of different culture media and/or combinations of LIF and bFGF [49-52]; DNA methylation inhibitors such as 5-azacytidine [53]; different feeder layers [49, 50]; 2i conditions [54, 55]; micro-drop culture strategies [56]; and the knock-down of trophectoderm-driving genes like caudal type homeobox 2 (*CDX2*) [57], but they resulted either in limited cell proliferation, low derivation efficiency or loss of pluripotency marker expression after a number of passages. Additionally, many of the reported ESC-like lines did not pass both standard tests for pluripotency, i.e., *in vivo* teratoma assay and chimera formation. However, the first case of the stable and efficient derivation of ESCs from bovine blastocysts has been recently reported [10]. These ESCs showed a stable morphology, as well as a similar transcriptome, karyotype,

population-doubling time, pluripotency marker gene expression and epigenetic features as previously described in mouse/human ESCs. This procedure was efficient (up to 100% of efficiency in optimal conditions), quick (3-4 weeks) and easy (passage by trypsinization), all of which are essential characteristics for establishing high throughput methods. Cell culture was carried out under the same conditions used for culturing human “region-selective” PSCs, a primed-type of stem cells that share molecular features with gastrula-stage epiblasts [58]: custom TeSR1 base medium lacking TGF-beta supplemented with bFGF and WNT Antagonist I (IWR1). The latter is an inhibitor of the canonical WNT- $\beta$ -catenin signaling pathway, which regulates many stem cell pluripotency and cell fate decisions during development.

Due to their importance in animal production and biomedical research, pigs are another mammalian livestock species under intense study. Similar to cattle, there have been many attempts to establish ESCs by culturing outgrowths from the ICM or epiblast of embryos generated by normal conception, IVF, parthenogenetic activation and SCNT cloning [59-70]. In general, results have been similar to those from early bovine studies, i.e., limited capability to survive after extended passages and unintended differentiation. Porcine ES-like cells with some ESC features have been obtained by initially culturing ICM-derived cells on mouse embryonic fibroblast (MEF) feeder layers with LIF and bFGF and then transferring them to 2i conditions [62]. These cells showed signs of pluripotency, such as alkaline phosphatase activity and expression of octamer-binding transcription factor 4 (OCT4; also known as POU5F1) and the homeobox protein NANOG, LIF-dependency and phosphorylation of signal transducer and activator of transcription 3 (STAT3). They also showed long-term culture survival, by surviving more than 100 passages without exhibiting changes in their morphology. Similar signs have been observed in ES-like cell lines derived from SCNT-embryos grown on MEF feeder layers with

medium containing bFGF supplemented with 20% knockout serum replacement (KOSR) [64], or with bFGF, LIF, 10% knockout-serum and 5% fetal bovine serum (FBS) [71]. Using these approaches, cells survived more than 45 passages without losing their pluripotency signs. Although porcine ESCs exhibiting all signs of pluripotency have yet to be obtained, evidence suggests this goal is soon to be achieved.

Until now, research in ovine ESC derivation has allowed the generation of ES-like cells with dome-shaped colonies, bFGF-dependency and survival for at least 30 passages without any obvious changes or differentiation [72]. This has been done by supplementing a basal N2/B27 medium with bFGF and a GSK3B inhibitor (CHIR99021) in absence of feeder layers and passaging colonies with TrypLE. Cells formed teratomas containing a variety of different tissues including cartilage and neural tissue when injected into kidney capsules of severe combined immunodeficiency (SCID) mice but failed to contribute to embryonic development upon blastocyst transplantation. In an earlier study, ovine ES-like cells derived in DMEM medium supplemented with LIF on mouse fibroblast feeders exhibited colonies with atypical morphologies, limited pluripotency and poor growth rate, which culminated in complete differentiation at very early passages [73].

Among the three species (cattle, pig and sheep), cattle is the only species with established and stable ESC lines exhibiting all the features of true ESCs to date. For this reason, IVB has been targeted towards using cattle as the pilot species.

## GERM CELL DIFFERENTIATION FROM EMBRYONIC STEM CELLS

One of the most interesting aspects about ESCs is their potential to produce modified animals and transmit the modified genome to the next generation through the germ line. In mammals, germ cells are founded by primordial germ cells (PGCs), which are initially specified in the early post-implantation embryo through coordinated secretion of Wnt Family Member 3 (WNT3) and Bone Morphogenic Proteins (BMPs) by different components of the embryo [74-76]. PGCs then migrate to the developing gonad, where they ultimately undergo meiosis and generate gametes. The complete progression of germ cell differentiation from ESCs has been recently reconstituted in mice for both females and males after many years of research [11, 77, 78].

The first attempts to derive PGCs from ESCs were carried out through embryoid body formation [79-82]. However, the induction efficiency was low and the need for new strategies became evident. Since PGCs originate from the epiblast, researchers evaluated the derivation of primordial germ cell-like cells (PGCLCs) from EpiSCs, pluripotent stem cells derived from the epiblast [83]. A small subpopulation of EpiSCs expressing PR/SET Domain 1 (PRDM1; also known as BLIMP1) and Developmental Pluripotency Associated 3 (DPPA3; also known as STELLA), markers for established PGCs, were observed as a result. However, the efficiency was very low (~1.5 %), indicating that EpiSCs had probably acquired properties that were incompatible with efficient PGC derivation in culture. This led to the exploration of a two-step procedure to induce PGCLCs through the differentiation of ESCs into an intermediate state referred to as EpiSC-like cells (EpiLCs) [84] and then into PGCLCs (Figure 3). EpiLCs are

similar to pre-gastrulating epiblast cells but distinct from EpiSCs. Such differentiation was carried out upon stimulation with activin A and bFGF. Then, EpiLCs were differentiated into primordial germ cell-like cells (PGCLCs) by stimulation with BMP4, LIF, stem cell factor (SCF) and epidermal growth factor (EGF). The efficiency of this method was 30 % higher than the one observed in EpiSCs and the global transcription profiles, epigenetic reprogramming and cellular dynamics of the induction were concordant with PGC specification *in vivo* [85-87]. That work suggested that PGCLC induction likely occurred during the transition from ESCs to EpiSCs.

PGCLCs have also been established from EpiSCs through a different strategy: ectopic expression of master regulatory genes involved in PGC specification [88]. Such genes include *BLIMP1*, *TFAP2C* and *PRDM14*. Additionally, the overexpression of the *PRDM14* gene alone has been sufficient to achieve an efficient derivation of PGCLCs, however this strategy has only worked in EpiSCs [88]. It has also been observed that the induced expression of NANOG stimulates the induction of putative PGCLCs independently of BMP signaling in EpiLCs [89].

The steps of germ cell differentiation, which occur after PGC specification, diverge between males and females. However, both pathways share one main aspect: they need co-culture with somatic gonadal cells to initiate meiosis. The potential of PGCLCs to undergo spermatogenesis has been evaluated both *in vivo* and *in vitro*. The former approach consisted of transplanting *BLIMP1* and *STELLA*-positive PGCLCs into seminiferous tubules of neonatal mice lacking endogenous germ cells. As consequence, PGCLCs underwent spermatogenesis and gave rise to spermatozoa with normal morphology. This PGCLC-derived sperm was later used to fertilize oocytes via intracytoplasmic sperm injection (ICSI). The blastocysts resulting from this fertilization gave rise to viable offspring with normal sex-specific methylation status [84]. Regarding *in vitro* assays, there are at least two main approaches that have been studied. The first

consisted of differentiating PGCLCs into spermatogonia-like cells (SLCs) by aggregating them with somatic testicular cells from E12.5 ICR mice (reconstituted testes) [90]. After two days of floating culture and 21 days of gas-liquid culture, cells expressing DEAD-Box Helicase 4 (DDX4; also known as VASA), a marker for gonadal germ cells, and Zinc Finger and BTB Domain Containing 16 (ZBTB16; also known as PLZF), a spermatogonial stem cell (SSC) marker that initiates expression in pro-spermatogonia, were identified and cultured. These cells gave rise to germ line stem cells (GSCs), the primary cell line that colonizes adult testes, contributes to spermatogenesis and gives rise to fertile offspring. The second important *in vitro* study consisted in co-culturing established PGCLCs with early postnatal testicular cells of  $KIT^W/KIT^{W-V}$  mice, which are devoid of PGCs [78]. Cells were cultured under retinoic acid (RA), a BMP mixture (2-4-7), and activin A. After six days of exposure, testis somatic cells actively migrated toward PGCLCs, forming aggregated colonies with cells positive for the meiosis markers, STRA8 and DMC1, and the germ cell markers, DDX4 and NANOS3, but negative for BLIMP1, STELLA, SSEA1 and OCT4, suggesting differentiation from the PGC/SSC state. On day 7, RA, BMPs and activin were withdrawn and replaced by follicle-stimulating hormone (FSH), bovine pituitary extract (BPE), and testosterone (T). Three days later (day 10), cells started expressing post-meiotic Protamin 1 (PRM1), with haploid spermatid markers, Tp1, Prm1, acrosin, and haprin, became upregulated by day 14. When injected into wild-type oocytes, these spermatid-like cells gave rise to fertile offspring.

The reconstitution of oogenesis *in vitro* has followed a similar co-culture strategy but making use of reconstituted ovaries (rOvaries) instead [91]. For this, PGCLCs were first co-cultured with E12.5 gonadal somatic cells from ICR female mice for two days and implanted into the ovaries of four-week-old immunodeficient KSN females. This led to the generation of

secondary follicle-like structures that were collected from the ovaries after four weeks and fertilized to produce viable offspring. This procedure, however, exhibited a very low efficiency (3.9%), since zygotes derived from PGCLCs were unable to extrude second polar bodies, resulting in digynic triploid (maternal-maternal-paternal) or diploid (maternal-maternal) phenotypes with failed fertilization. This marked the need of modifications to achieve better results and to avoid dependency on grafting. In this context, the implementation of an estrogen-receptor antagonist (ICI182780) during co-culture meant a significant advance, since it helped avoid the formation of follicles with abnormal external layers and multiple oocytes [77]. The culture of rOvaries under such an estrogen inhibitor led to the generation of 237 secondary follicle-like structures per rOvary after three weeks of culture [11]. Follicles were then manually separated from each other to receive equal signaling and cultured for 11 days *in vitro* growth (IVG) medium containing FSH, which allowed oocytes to reach the germinal vesicle (GV) stage. On average, 55 fully grown oocytes were obtained per rOvary [11]. After the oocytes were transferred into *in vitro* maturation (IVM) conditions, 28.9% of the GV oocytes extruded a first polar body. In general, these new MII oocytes showed normal morphology compared to *in vivo* derived oocytes and almost complete methylation patterns at the evaluated maternal imprinted genes (*H19* and *IGF2R*), but showed a higher frequency of aneuploidy and a lower rate of fertilization. The process of oogenesis *in vitro* was validated through RNA-Seq, showing close resemblance with the differentiation process *in vivo*. Following IVF, the newly generated oocytes developed into 2-cell embryos, which after transferred into pseudo-pregnant albino females, gave rise to seemingly normal fertile pups with colored eyes [11, 77]. Lastly, new ESC lines were successfully derived from the blastocysts of *in vitro*-generated oocytes.

The approach that worked in mice, however, did not confer germ line competence to human ESCs [92]. This might be due to differences in the regulation of pluripotency and early post-implantation development between species. Numerous marked differences have been identified between mouse and other species such as pigs, humans and monkeys [93, 94]. For instance, it has been shown that the expression of BLIMP1, the first and main regulator of PGCs in mice, is downstream of SOX17 in human PGCs [92]. The role of BLIMP1 in human PGCs is related to the repression of endodermal genes [95]. The expression of SOX17, in turn, depends on the duration and dosage of WNT signaling, which induces the expression of EOMES to activate SOX17 [96]. Additionally, unlike in murine cells, NANOG alone cannot induce human PGC formation; and PRDM14 expression is rather low and cytoplasmic in porcine PGCs [94]. Nonetheless, despite the indicated differences, efficient derivation of human early PGCs *in vitro* has been achieved using at least two independent methods.

The first approach converted primed (traditional) ESCs into naïve ESCs under a four-inhibitor (4i) condition [92]. Culture conditions were composed of LIF, bFGF, TGF-beta1, CHIR99021 (GSK3 inhibitor), PD0325901 (MEK inhibitor) SB203580 (MAPK inhibitor) and SP600125 (JNK inhibitor) [41]. These naïve ESCs were then induced into EpiLCs by culture under bFGF and TGFβ1 for two days; and lastly, into PGCLCs. This last step was carried out under BMP4, LIF, SCF and EGF in low adhesion dishes (~5 days), resulting in about 30% efficiency. In comparison to conventional human ESCs, which have poor germ line competence, these 4i-cultured ESCs exhibited an upregulation of early-mesodermal genes (e.g., *T*, *MIXL1*, *RUNX1*, *PDGFRA*), which may provide competence for germ cell fate. Some studies have suggested that human PGCs may originate at the onset of gastrulation from mesodermal precursors, and not from the pre-gastrulation epiblast [93, 97]. The transcription profile of

PGCLCs obtained under this condition was very similar to the one of *in vivo* PGCs [92].

Additionally, their imprinting status indicates they are likely pre-migratory PGCs, given that DNA methylation and imprint erasure had recently been initiated [92, 95].

In a different strategy, human ESCs were first converted into incipient mesoderm-like cells (iMeLCs) by exposure to activin A and GSK3B inhibitor [93, 96]. These iMeLCs were then differentiated into PGCLCs under the same conditions used in the first approach [92]. As result, aggregates containing 30% of BLIMP1 and TFAP2C-positive PGCLCs were generated after four days. The transcription profiles of these PGCLCs were similar to the ones from PGCLCs generated through the first method. Robust PGC induction has also been reported using a similar method in a RB27 medium [94].

Recently, human oogonia-like cells have been generated from induced PSC [12]. For this, male and female induced PSCs were induced into PGCLCs via iMeLCs [93] and cultured in xenogeneic reconstituted ovaries with mouse embryonic ovarian somatic cells. After 4 months, cells downregulated early germ-cell and core/naïve pluripotency genes and upregulated STRA8, a gene essential for meiosis initiation, and SYCP3, but not  $\gamma$ H2AX, DMC1 and SYCP1, indicating that they had not yet initiated meiotic recombination [12]. The gene expression properties and epigenetic marks of these cells were similar to those of week 7-9 oogonia/gonocytes. Nonetheless, reactivation of the X-chromosome was incomplete, and further research is still needed. Lastly, it has also been shown that human PGCs can be induced into spermatogonial stem cell-like cells (SSCLCs), enter meiosis and differentiate into haploid spermatogenic cells by overexpressing *DAZL* and other genes or by adding factors, such as retinoic acid, to the culture medium [98-101].

Although the number of reports pertaining to germ cell differentiation in domesticated mammal species is not as high as in mouse and human, there are some interesting studies that are worth remarking [102]. One of these studies has shown that porcine PGCs originate from the posterior pre-primitive-streak competent epiblast by sequential upregulation of SOX17 and BLIMP1 in response to WNT and BMP signaling [94]. Unlike murine PGCs, porcine PGCs express PRDM14 weakly and the expression is apparently cytoplasmic, whereas the expression of SOX2 is undetectable [94].

A well-defined culture system based on the EpiLC differentiation approach [84, 103] has been proposed to generate porcine PGCLCs from induced PSCs [104]. Essentially, porcine PSCs were induced from porcine embryonic fibroblasts and converted into EpiLCs by culture under Activin A and bFGF for two days. These cells were then disaggregated and cultured in medium supplemented with BMP4, BMP8a, LIF, SCF and EGF, which led to their differentiation into PGCLCs. PGCLCs proliferated robustly and formed tight and large aggregates during the first four days of differentiation, showing a decrease in proliferation after day 5. PGCLC identity was supported by PGC markers (BLIMP1, PRDM14, STELLA), high OCT4 and SOX2 expression levels, *in vivo* PGC-like epigenetic status and by transcriptome and gene ontology analyzes that reflected a gamete production scheme. PGCLCs were then differentiated into SSCLCs, which had the potential to enter meiosis and undergo spermatogenesis *in vivo*, after being exposed to RA, glial cell line-derived neurotrophic factor and testosterone *in vitro*. The identity of these SSCLCs was determined by the expression of DAZL and STRA8.

The number of comprehensive studies focused on germ cell differentiation in cattle is lower than in other species. Nonetheless, some studies have supported the importance of retinoic acid and/or BMPs during germ cell differentiation, either from induced PSCs [105] or from

ovarian stem cells [106]. The importance of retinoic acid in gametogenesis and induction of meiosis has also been reported in buffalo [107, 108].

Although no clear procedures for deriving PGCLCs from ovine PSCs have been reported to date, some studies have shown the beneficial roles of melatonin in oocyte maturation [109] and *in vitro* generation of functional sperm from SSCs [110]. This *in vitro*-generated sperm successfully fertilized oocytes and zygotes developed up to the blastula stage. Undoubtedly, the discovery of the complete progression of germ cell differentiation in mice has set an invaluable precedent for reproductive sciences and fueled the enthusiasm of researchers and industries working on agricultural species to pursue similar approaches. It is only a matter of weeks, months or a few years at most until science achieves this goal.

## CONCLUDING REMARKS

In the current context of a growing world population, the demand for food is expected to be substantially higher by 2050 [111]. The IVB strategy proposed in this article may be of help to address this issue, as the genetic gain achieved in a short time could be translated into more and better food to satisfy such demands. However, IVB largely depends on advancements in the field of *in vitro* germ cell differentiation, which has shown significant advances in mice.

Although differences in the PGC specification pathways exist between mice and other mammals, the idea behind IVB remains practical and as the simulation performed in this work clearly shows, the benefits of its implementation would be very significant. As soon as reliable protocols for germ cell differentiation become available for livestock species, IVB could become an integral part of breeding programs for a broad range of productive traits.

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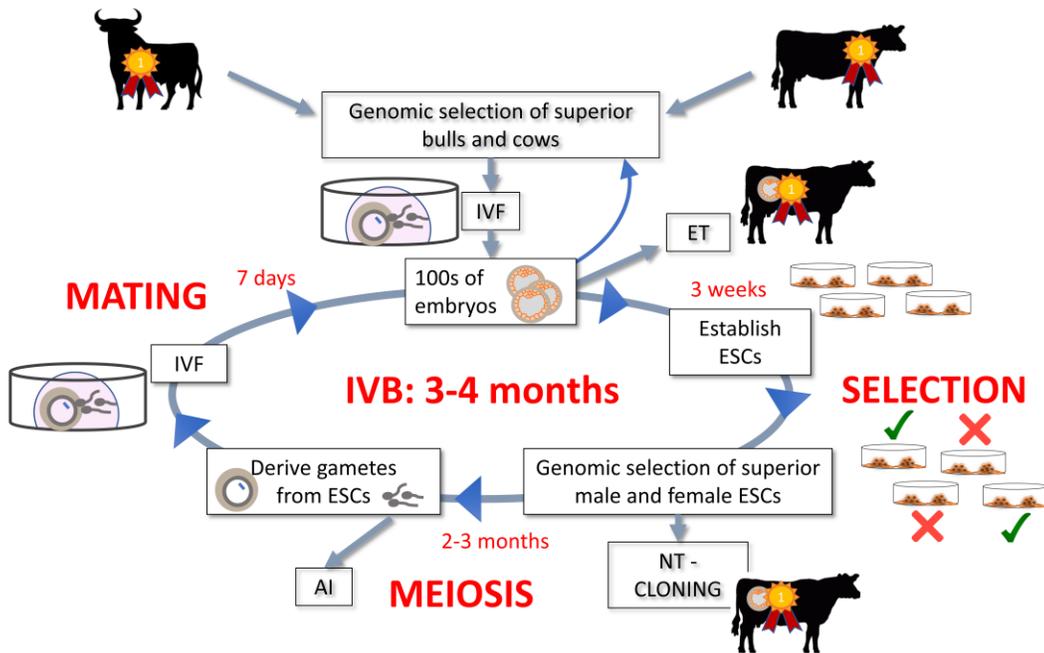
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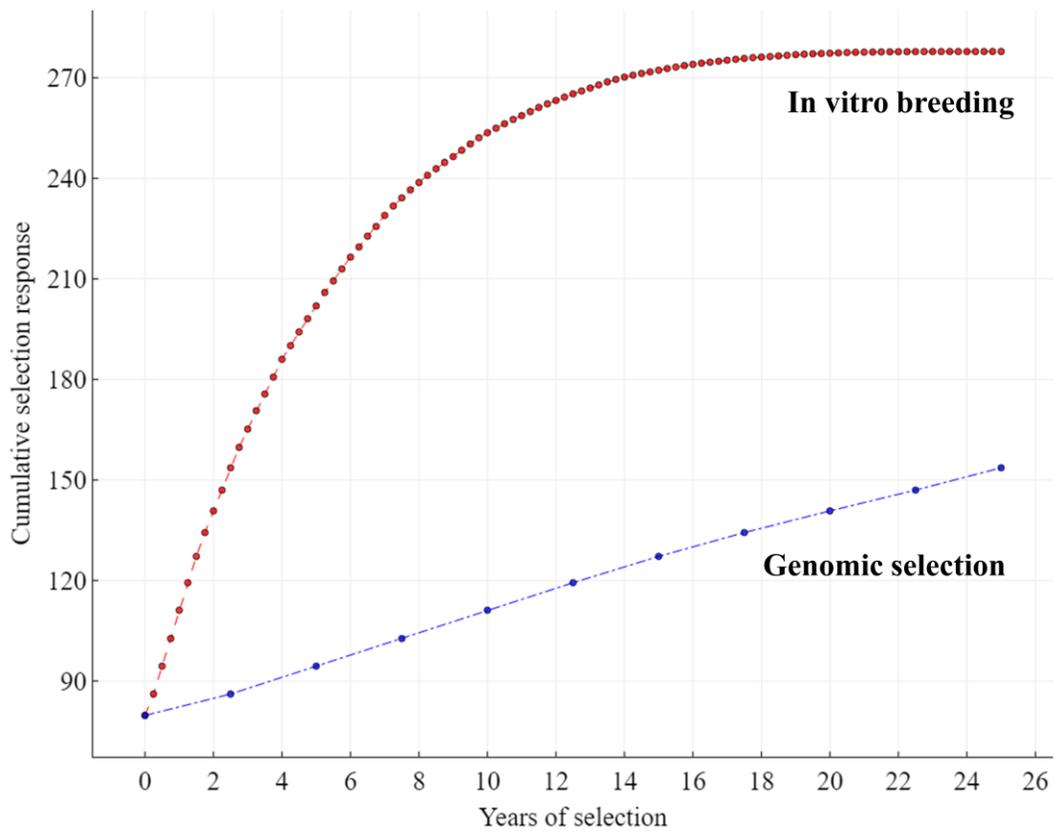
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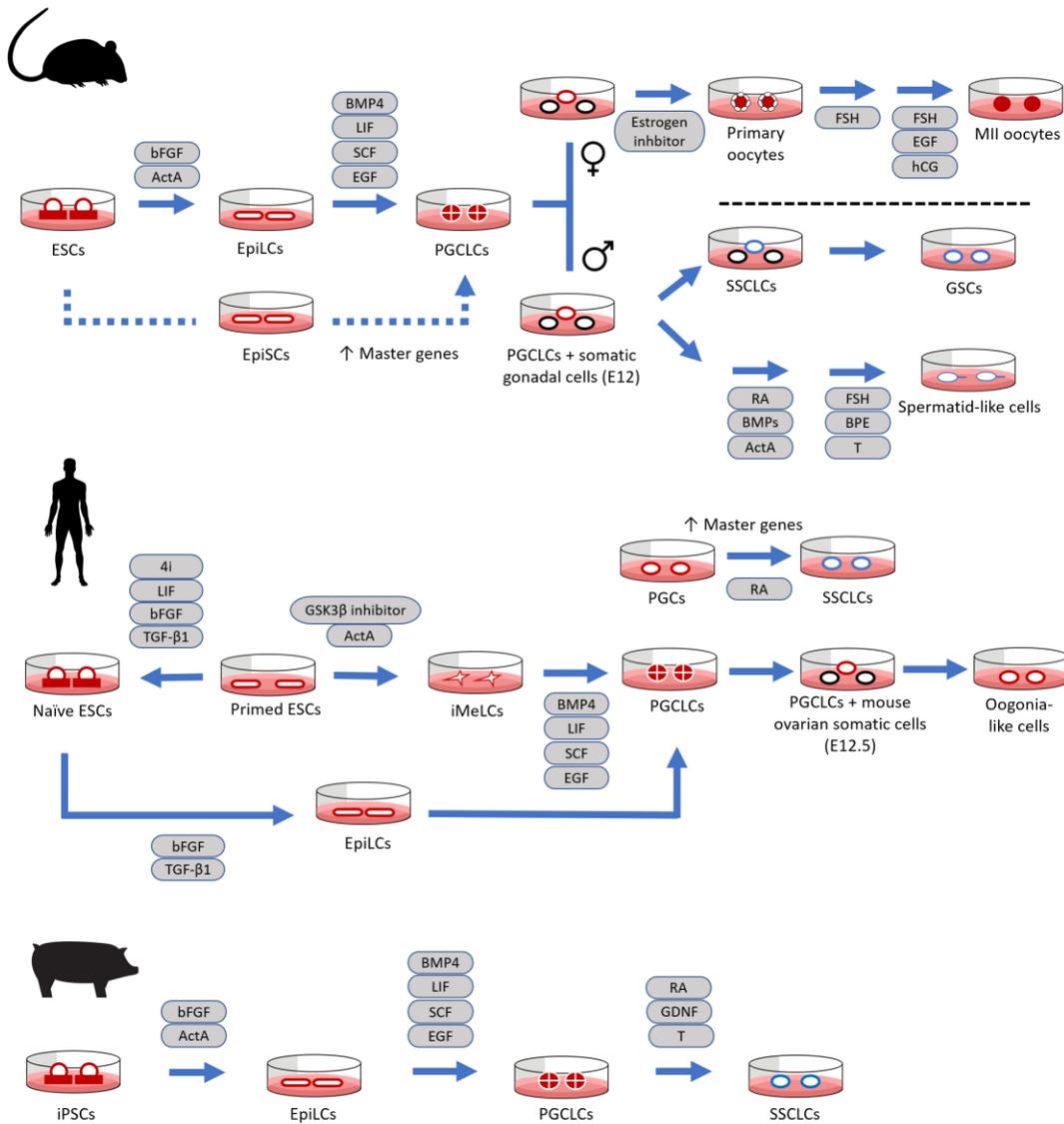
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**Figure 1.** In Vitro Breeding (IVB). Diagram of the strategy, estimated times and possible alternatives for its implementation in animal production systems. NT: nuclear transfer.



**Figure 2.** Benefits of IVB in comparison with conventional GS. Estimation of the cumulative selection response over 25 years of selection.



**Figure 3.** In vitro differentiation of germ cells from PSCs in mouse, human and pig over the last few years.