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Twenty Years of Embryonic Stem Cell Research in Farm Animals

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Contents

Notable distinctions between an embryonic stem cell (ESC) and somatic cell are that an ESC can maintain an undifferentiated state indefinitely, self-renew, and is pluripotent, meaning that the ESC can potentially generate cells representing all the three primordial germ layers and contribute to the terminally differentiated cells of a conceptus. These attributes make the ESC an ideal source for genome editing for both agricultural and biomedical applications. Although, ESC lines have been successfully established from rodents and primates, authentic ungulate stem cell lines on the contrary are still not available. Outstanding issues including but not limited to differences in pluripotency characteristics among the existing ESC lines, pre-implantation embryo development, pluripotency pathways, and culture conditions plague our efforts to establish authentic ESC lines from farm animals. In this review, we highlight some of these issues and discuss how the recent derivation of induced pluripotent stem cells (iPSCs) might augur the establishment of robust authentic ESC lines from farm animals.

Introduction: The Embryonic Stem Cell (ESC)

The establishment of ESC lines from explant cultures of the inner cell mass (ICM) obtained from *in vivo*-derived mouse (m) blastocysts was first reported over 31 years ago (Evans and Kaufman 1981; Martin 1981). Almost two decades passed before human (h) ESC was established from *in vitro*-derived embryos (Thomson et al. 1998). Since the establishment of these initial cell lines from blastocysts, additional lines have also been derived from human and mouse pre-blastocyst stage embryos including single blastomeres from cleavage stage embryos, morulas, and biopsied single blastomeres (Strelchenko et al. 2004; Klimanskaya et al. 2006; Eistetter 1989; Chung et al. 2006).

To be considered as genuine ESCs, the cells within the established lines have to possess distinct characteristics that distinguish them from tissue-specific stem cells and terminally differentiated somatic cell types. Firstly, the ESC must be capable of indefinite continuous self-renewal (Amit et al. 2000). Secondly, the ESC must be able to sustain an undifferentiated state indefinitely, although it is important to note that under sub-optimal conditions the ESC can undergo spontaneous differentiation (Nagy et al. 1993). Thirdly, the ESC must be capable of giving rise to all three primary embryonic germ layers including gametes (refer Talbot and Blomberg 2008). The following are the common methods to test the pluripotency in an ESC line: (i) their ability to form embryoid bodies *in vitro* and subsequent differentiation of those cells into specific cell types representing the three germ layers; (ii) the formation of teratomas upon transplantation into an immuno-compromised animal; (iii) derivation of chimeric offspring *in vivo* with a demonstration that the ESC contributed to tissues derived from the endoderm, mesoderm and ectoderm, in addition to the germline; and (iv) the most stringent of these tetraploid complementation, where the ESCs alone give rise to the embryo proper (Nagy et al. 1993). Curiously, the mESCs established from the pre-implantation embryos are the only cell types that met all these different measures of pluripotency.

Agricultural Importance of ESC Technology

The distinctive features of the ESC have elevated them as an invaluable resource for both agricultural and biomedical applications in livestock. For agricultural purposes, the ESC can serve as a valuable genetic engineering tool to improve the generation of livestock with advantageous genes that are important for economic and disease-resistant traits, or for the study of functional genomics in mammals. For biomedicine, the use of ungulate (u) ESCs is of interest towards the creation of human disease models where livestock could be a more accurate replica than the rodent (Whyte and Prather 2011). Their use as systems for xenotransplantation, enhanced pharmaceuticals/drug production/pharmacokinetic studies and regenerative studies, including the restoration of fertility, is also of significance. To remain within the context of the review, the current state of the technology will be discussed with the primary focus on ungulates.

A History and Status of ESC Technology in Ungulates

A little less than a decade after the first reports of the establishment of mESCs, reports of putative porcine (p) ESC from *in vivo*-derived early stage day (D) 7–9 blastocysts (Evans et al. 1990; Piedrahita et al. 1990) were published. However, the inability of these putative pESC to maintain an ESC-like state for more than a few passages coupled with their limited characterization has led to an overall conclusion that the cells were neither true ESC nor were they pluripotent. Subsequent reports of ESC/ESC-like lines from *in vivo*- or *in vitro*-derived embryos have been reported in bovine (Table 1), porcine (Table 2), caprine (Kumar et al. 2011) and ovine (Notarianni et al. 1991). Interestingly, a non-traditional approach, that is, the induction of demethylation to maintain bovine (b) ESC in an undifferentiated state (Lim et al. 2011), efficiently demonstrated the establishment of bESC lines and further enhanced their ability to form teratomas with cells representative of the germ layers.

Table 1. Putative bovine ES cell lines

Embryonic stage	Time in culture	ESC characteristic	*In vitro* validation	*In vivo* validation	Citation
In vivo-derived					
Blastocyst ICM (frozen/thawed)	+20 weeks	Morphology, AP^+, $SSEA\text{-}1^+$, $STAT\text{-}3^+$, $OCT4^+$	EB formation and neural cell types	$Chimeras^+$	Saito et al. (2003)
In vitro-derived					
8–16 cell stage	+3 years	Morphology, $SSEA\text{-}1^+$, $\text{-}3^+$, $\text{-}4^+$	EB formation and various cell types	$Teratomas^-$	Mitalipova et al. (2001)
Morula and Blastocyst	+1 year	Morphology	EB formation and various cell types	ND	Stice et al. (1996)
Blastocyst D7	+1 year	Morphology, $vimentin^-$, AP^+		$Chimeras^+$	Cibelli et al. (1998)
Blastocyst D9	+13 weeks	ND	ND	$Chimeras^+$	Iwasaki et al. (2000)
Blastocyst – ICM	52 passages	Telomerase	ND	ND	Betts et al. (2001)
Blastocyst D9-10	Less than 100 days	Morphology	EB formation and various cell types	$Chimera^+$	Sims and First (1994)
Blastocyst intact and ICM D8	Intact 4–5 passages ICM 6–7 passages	Morphology, $OCT4^+$	–	–	Yadav et al. (2005)
Blastocyst D7 (5-azacytidine treatment)	20 passages	Morphology, AP^+, OCT4, $REX1^+$, $Nanog^+$, $SOX2^+$	EB	$Chimeras^+$/$teratomas^+$	Lim et al. (2011)

Gestational day D = ; Not detected = –; Detected = +; Not determined = ND.

Table 2. Putative porcine ES cell lines

Embryonic stage	Time in culture	ESC characteristic	*In vitro* validation	*In vivo* validation	Citation
Blastocyst D10	–	Morphology	–	–	Refer Brevini et al. (2007)
Blastocyst D7–8	Approximately 10th passage	Morphology, $Vimentin^-$	ND	Chimeras - teratomas -	Refer Brevini et al. (2007)
Blastocysts D7–9	+1 year	Morphology $Vimentin^-$	EB formation and various cell types	–	Refer Brevini et al. (2007)
Embryonic discs D10-11	8 weeks	Morphology	–	$Teratomas^+$	Refer Brevini et al. (2007)
Blastocyst D5.5–7.5	Approximately 44 passages	Morphology	EB formation and various cell types	$Chimeras^+$	Refer Brevini et al. (2007)
Blastocyst	35 passages	Morphology, AP^- and	Differentiated to	$Chimeras^+$	Refer Brevini et al. (2007)
D7–8	5–8 passages	AP^+\Morphology, AP^-	one cell type, AP-EB	–	
Late blastocyst			formation, AP +, multiple cell types		
Blastocyst D10.5–12		Morphology AP^-, $REX1^-$, $OCT4^+$, $SOX2^+$, $Nanog^+$, $Nodal^+$, $SSEA\text{-}1^+$, $SSEA\text{-}4^-$	EB, multiple cell types	–	Alberio et al. (2010)

Gestational day D = ; Not detected = –; Detected = +; Not determined = ND.

In contrast to mouse and primates, uESC have not been definitively proven to maintain self-renewal, remain in an undifferentiated state or differentiate into all cell types of the three germ layers either *in vitro* or *in vivo*. Although the formation of chimeras has been reported, tissue analysis was minimal or ESC-derived tissue types were limited (Table 1 and 2). Similarly, the origin of the differentiated teratoma tissue, though likely to be ESC-derived, has not been proven. Newer technologies such as laser capture microdissection provide a mechanism to definitively prove the source of the teratoma tissue.

Cells of the ICM in ungulates must be pluripotent to produce normal offspring but our limited knowledge about the biology of the ESC has contributed to an inability to optimally isolate and readily establish 'authentic' ESC lines. This problem is not unique to ungulates because success in rodents has also been largely defined by species and genetic background (Suzuki et al. 1999; Buehr et al. 2008). Focusing on livestock, several pertinent questions linger, (i) do the characteristics, morphological and/or physiological, that define the ESC, correlate with their pluripotency potential? (ii) What is the best timing for ICM or embryonic disc (ED) collection? (iii) What factors define pluripotency in the uESC? and finally, (iv) What is the appropriate *in vitro* culture system(s) to maintain pluripotent and self-renewing uESCs? These topics will be explored in some depth below.

Is an ESC an ESC?

A major distinction in the initial establishment of mESC and hESC lines is the population of embryos from which they were derived. Because of ethical reasons, the majority of hESCs have only been derived from the epiblast of surplused *in vitro* produced embryos, whereas many of the mESC lines have been generated from *in vivo*-derived embryos. Recently, additional mESC lines were created from post-implantation stage embryos following the transformation of epiblast to the egg cylinder or subsequent embryonic disc (mEDS) (Brons et al. 2007; Tesar et al. 2007). While the expression of three core pluripotency markers [Oct4 (aka Pou5f1),

Sox2 and Nanog] within these and other established mESC and hESC lines are common, there are also striking differences (Table S1), which underscores intra- and inter-species differences.

So far, mESCs have been the only stem cell lines that fulfilled all the four criteria/tests of pluripotency. The mESCs therefore represent a more 'naïve' stemness state and hence are dubbed as 'naïve' ESC (Nichols and Smith 2009). The mEDS cells in contrast represent an advanced differentiated state primarily because of their inability to establish robust germ-line chimeras, therefore referred to as 'primed' ESCs (Nichols and Smith 2009). As a consequence of the strong similarities between the mEDS and hESCs, additional hypotheses arose including the possibility that hESC lines may have been established from an advanced epiblast stage and are therefore equivalent to the 'primed' state ESC. These questions remain unanswered and may be very relevant with respect to uESCs as well.

Divergence in the Development of the Embryo

Historically, the pre-implantation embryo has been the source for the derivation of ESC lines. Advancement to the morula stage is similar between human, mouse and ungulates occurring between D3.5 and 4. Although the ICM is not evident at this stage (Eisetter 1989; Strelchenko et al. 2004), the fate of distinct blastomeres that give rise to a specific region of the embryo or extra-embryonic tissue has already been set (Plusa et al. 2005). A lingering question is whether ESCs are derived from specific blastomeres already destined to become epiblast tissue.

Beyond the morula stage, development of the embryo is more divergent between species. Human and mouse embryos reach the early blastocyst stage between D4 and 5, whereas in cattle and swine, it is approximately D6–7. The early blastocyst is comprised of three primary defined cell types: (i) the ICM (embryo proper) that will give rise to the epiblast, (ii) the trophectoderm and (iii) the primitive endoderm; the latter two contributing to extraembryonic membranes (refer Talbot and Blomberg 2008). In human and mouse, the epiblast develops *in vivo* following implantation but ungulates have an extended peri-implantation period following hatching during which (i) the epiblast is formed (pig, D6-D12; cow, D8-D12; Vejlsted et al. 2006), (ii) except for equine, the extraembryonic membranes expand (Geisert et al. 1982), (iii) gastrulation begins and (iv) the Rauber's Layer is shed, exposing the epiblast to the uterine luminal fluid (Fléchon et al. 2004). These phenomena that are distinct from mouse or human suggest that ungulate embryos are likely exposed to unique developmental cues and raise questions regarding the optimal time to establish uESC lines.

Molecular Cues Regulating Stemness

Divergence in extrinsic factors

Dramatic differences in the timing of development and morphology between mouse, human and ungulate embryos are most likely associated with species-specific alterations in temporal and spatial gene expression. Mouse and human ESCs both require co-culture with feeders, suggesting paracrine factors are needed for their renewal and maintenance of pluripotency (Peiffer et al. 2008; Talbot and Blomberg 2008). However, the profile of extrinsic factors required to maintain stemness distinguishes mouse from human and naïve from primed ESCs. For example, mESCs are LIF dependent, whereas hESCs are FGF2 dependent in feeder-free culture (refer Talbot and Blomberg 2008). The LIF receptor (LIFR) and signal transducer GP130 are both present in mESC, and their activation modulates MYC via STAT3 to inhibit differentiation and promote self-renewal. Signalling through BMP4, and subsequent activation of SMAD transcription factors, is also of importance (Talbot and Blomberg 2008). In the mESC, BMP4 prevents mESC differentiation in feeder-free cultures but the effect is opposing in hESC. BMP4 must be inhibited by antagonists, activin A and noggin, in concert with FGF2 to prevent spontaneous differentiation of hESC. Interestingly, primed mEDS cells can be retained under the same culture conditions as hESC, and upon exposure to BMP4, differentiate like their human counterparts (Brons et al. 2007; Tesar et al. 2007).

In ungulate species, the need of LIF for ESC maintenance is not evident. The *LIFR* expression in *in vivo*-derived porcine ICM is inconsistent (Blomberg et al. 2008; Hall et al. 2009). In putative pESCs, LIFR has either been undetected (Vackova et al. 2007) or detected but shown not be important for the maintenance of pESC phenotype (Alberio et al. 2010). In the bovine, GP130 and LIFR expression is evident in *in vitro*-derived ICM prior and during *in vitro* culture (Pant and Keefer 2009), but disparity of expression between *in vitro* (present) versus *in vivo* (absent) produced blastocysts raises the question if potential alterations are a consequence of *in vitro* derivation (Eckert and Niemann 1998). The negative impact of LIF supplementation on bovine embryo development during *in vitro* culture (Rodríguez et al. 2007) and its inability to influence pluripotency status of pESCs leads one to surmise that it is not an essential factor for stemness.

An examination of BMP4 expression in *in vivo*-derived pig ICM and cultured epiblast did not detect BMP4 until spontaneous differentiation was evident in the epiblast, and expression was maintained in epiblast-derived somatic cell lines (Blomberg et al. 2008). Furthermore, putative inhibition of BMP4 expression by the addition of BMP4 antagonists, noggin or activin A, does not effectively block differentiation of the porcine epiblast (Talbot and Blomberg 2008), but BMP4 does induce pESCs to differentiate (Alberio et al. 2010). In contrast to pig, receptors for BMP4 have been found within the undifferentiated bovine ICM (Pant and Keefer 2009). To date, the role of BMP4 is unclear in the bovine; however, in swine, BMP4 does appear to stimulate differentiation but the mechanism inhibiting BMP4 may be distinct from hESC.

Intrinsic factors associated with pluripotency

Many of the factors considered to be pluripotency markers are confined to the ICM of the mouse or human

embryo, including the core molecules, POU5F1, SOX2 and NANOG. Additional factors present in both mESC and hESCs include alkaline phosphatase, MYC and REX1 (Talbot and Blomberg 2008). While POU5F1, initially thought to be the master regulator of stemness in the mESC cannot maintain pluripotency alone (Niwa et al. 2000), in concert with SOX2 and KLF4 it can reprogram somatic cells to ESC-like cells (Nakagawa et al. 2008). In addition to expression, tight control of the expression level of pluripotency factors is also apparent. For example, over-expression or inhibition of *Pou5f1* in mESCs results in their differentiation to either endoderm/mesoderm or trophectoderm, respectively (Niwa et al. 2000).

Analysis of recently established putative bESC (Lim et al. 2011; Table 1) and pESC (Alberio et al. 2010; Table 2) show that they also express POU5F1, SOX2 and NANOG; however, only the bESCs were AP and REX1 positive. It is noteworthy to point out that both AP and REX1 expression were observed in the initial culture of the porcine epiblasts but were lost during cell line establishment (Alberio et al. 2010). Although the three core pluripotency factors were present, the expression of other crucial factors may be compromised with *in vitro* culture.

Persisting Challenges

Timing and isolation of primary cultures

One of the basic perplexities in establishing ESC lines in large animal species is identifying the appropriate stage of the embryo for isolation. To date, the early to mid-stage blastocyst has been predominantly utilized (Tables 1 and 2). However, the recent report on the derivation of pESC lines from D10.5 to 12 embryos suggests an alternative stage for consideration (Alberio et al. 2010). Nevertheless, instead of the epiblast stage of the embryo which will invariably give rise to the primed ESCs, attempts should also be made to capture ESCs from earlier stage embryos that harbour 'naïve' blastomeres. This has proven to be a much more demanding task in ungulates with emerging reports pointing to differences in the gene repertoire between the mouse and pig embryos at a comparable developmental stage (Bauer et al. 2010; Telugu et al. 2011). Therefore, it is reasonable to assume that the so-called naïve ESCs cannot be established from the farm animals without understanding and manipulating gene networks.

Recognition of authentic ESC

This represents the classic 'chicken-egg' conundrum in ungulates. With no uESC that have been validated beyond a reasonable standard, and with numerous inconsistencies in the putative markers that have been reported so far, the identification of 'true' uESCs continues to be a confounding issue for the stem cell biologists in the livestock field. These apparent irregularities in the markers also extend to piPSC that have recently been published (refer Roberts et al. 2009). Rigorous efforts including proteomic and phage display screens should be directed to systematically assess the markers at the embryo level.

Sustaining pluripotency and propagation in culture

Another major challenge is identifying the right set of conditions to propagate and sustain uESCs in culture. This has been a rather arduous task, because most experiments to replicate the culture conditions that are successful with mouse or human have failed to yield self-sustaining ESC lines. For many years, this has led investigators on an unsuccessful path of trial and error experiments to identify the 'missing' factor(s) (Talbot and Blomberg 2008). Second, to the choice of the growth factor(s), another consideration is the concentration of the supplement; this is because the commercially available mouse or human ligands do not necessarily carry similar potency in farm animals. Third unresolved issue is the choice of cell lines for use as feeders, as well as their density in culture. Again, progress in iPSC technology will probably serve as a beacon for these endeavours.

Lessons From iPSC

Over the last two decades, the goal of establishing naïve uESCs has remained elusive. In this regard, the recent derivation of iPSC, especially from various farm animals holds considerable promise. In the initial landmark publication, Takahashi and Yamanaka (2006) reported the reprogramming of mouse somatic cells to iPSC through the forced expression of four transcription factors *Pou5f1, Sox2, Klf4* and *c-Myc*. The miPSC that was reported in this and subsequent studies had common properties of the ESC including their ability to form teratomas, generate germ-line chimeras, and even live offspring (Kang et al. 2009; Zhao et al. 2009). The technology has since been adopted to successfully establish iPSC from several other species. It is beyond the scope of the current report to discuss all of the developments that have taken place in this field, including the choice of factors, pharmacological compounds, delivery mechanisms, cell types, etc., and therefore the discussion will be limited to the establishment of iPSC from pig, the farm animal in which steady progress has been made in the recent years.

In 2009, three near simultaneous publications reported the derivation of piPSC (Esteban et al. 2009; Ezashi et al. 2009; Wu et al. 2009). The results from these and another that followed (West et al. 2010) showed that the piPSC resemble the human rather than the mouse ESC, thereby indicating that the primed pluripotent state represents the default or ground state of pluripotency in pigs. With the exception of few permissible mouse strains, this remains to be the case for all other mammalian species as well. In this regard, the recent reports on the creation of naïve state, LIF-dependent piPSC from porcine embryonic and somatic cells are proving to be particularly insightful (Telugu et al. 2010, 2011). The altered pluripotent stemness state in these stem cells has been achieved by blocking the FGF signalling cascade, promoting pluripotency-associated WNT signalling pathways, culturing in the presence of LIF-based medium and upregulation of candidate pluripotent genes. It should be noted that the advent of small molecules that can be used to

target-specific signalling pathways and the delineation of reprogramming genes that could be selectively applied to the target cells have created a basis for the creation of these novel naïve piPSC. Although, the full scope or potential of these established lines are still to be explored, these studies bear evidence to the growing convergence of the iPSC and ESC technologies.

Although the iPSC hold great promise, especially those that could contribute to the germ-line chimeras, it also has its share of concerns. The salient among them are the predominant use of integrating retroviral vectors leading in some, possibly all cases, to the continued expression of the transgenes in the reprogrammed cells, with the lone exception of the recently reported ovine iPSC lines (Liu et al. 2012), which showed evidence of silencing. In addition to the risk of mutagenesis, continued expression of pluripotent genes may limit the differentiation potential of the iPSC as well. For iPSC to have better utility, the inserted transgenes will have to be either deleted or effectively silenced after the cells have been reprogrammed. Alternatively, reprogramming must be achieved through the use of non-integrating vectors (Okita et al. 2008; Yu et al. 2009), introduction of 'stemness' proteins rather than genes (Zhou et al. 2009), or pharmaceutically with a suitable combination of small molecules.

In summary, notwithstanding the potential pitfalls, the iPSC technology is creating a rational basis for the establishment and improvement of uESC lines. With a growing list of molecules shown to either improve or supplant reprogramming factors to make iPSC, it can be envisioned that the progress in iPSC technology coupled with the identification of mechanisms for integration-free manipulation of gene products will pave way for the development of safer, more robust ESC lines from farm animals.

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Conflicts of interest

None of the authors have a conflicts of interest to declare.

Author contributions

Le Ann Blomberg and Bhanu Telugu worked jointly on the outline of the review article and writing of the content.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Divergent characteristics of naïve and primed ES cells.

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