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HYPOTHESIS AND COMMENTARY

The Promise of Stem Cell Research in Pigs and Other Ungulate Species

Bhanu Prakash V. L. Telugu · Toshihiko Ezashi · R. Michael Roberts

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Abstract Despite two decades of effort, establishment of pluripotent embryonic stem cells (ESC) from ungulates such as cattle and pigs has remained an elusive goal, with true ESC only successfully isolated from rodents and primates. The many reports describing ESC-like cultures from other "difficult" species has largely depended upon adopting strategies successful for mouse and human and have not yet produced a cell type that both proliferated continuously in culture without differentiation and demonstrated full pluripotent potential. These difficulties may have been exacerbated in ungulates by the lack of specific markers exclusive to inner cell mass (ICM) and its derivative the epiblast and by unique features of their preimplantation development. Especially important may have been the choice of culture condition, including growth factors, for establishing and sustaining the ESC. Recent modifications to culture medium, notably the inclusion of particular protein kinase inhibitors, have permitted ESC derivation from rat and previously "non-permissive" mouse strains. These conditions appear to stabilize the biochemical networks that sustain pluripotency and to render the cells dependent upon LIF signaling. In addition, the recent successful generation of induced pluripotent stem cells (iPSC) from pig by procedures that should be easily adapted to other species, is also likely to advance the area quickly. The pig is a particularly desirable species to create pluripotent cell lines because of its value as a biomedical model in transplantation at a time when there is mounting pressure to rush stem cells to the clinic before their safety has been adequately tested in animals.

Keywords Porcine · Bovine · Epiblast · Embryonic stem cells · Induced pluripotent stem cells

B. P. V. L. Telugu (✉) · T. Ezashi · R. M. Roberts
Division of Animal Sciences,
Christopher S. Bond Life Sciences Center, University of Missouri,
Columbia, MO 65211, USA
e-mail: telugub@missouri.edu

R. M. Roberts
Division of Animal Sciences and Department of Biochemistry,
Christopher S. Bond Life Sciences Center, University of Missouri,
Columbia, MO 65211, USA

Introduction

Embryonic stem cells (ESC) were first established from explant cultures of in vivo day (d) 3.5 mouse embryos almost three decades ago [1, 2]. Later, it was established that ESC could also be derived from totipotent blastomeres of earlier cleavage stage embryos, collected prior to when the inner cell mass (ICM) and trophectoderm (TE) emerged [3, 4]. Almost 15 years after the isolation of mouse (m) ESC, similar lines were successfully derived from monkey [5], and human blastocysts [6–8]. As discussed later, this success depended upon the development of a culture medium suited to the primate cells, which had different requirements from the murine ESC. Although cultures derived from dissociated sheep and pig epiblast were shown to differentiate into a range of different cell types, including some with the characteristics of beating heart muscle cells as early as 1985, the significance of these observations was not recognized by the authors [9]. Later attempts to isolate porcine ESC from ICM of blastocysts provided cultures that bore some superficial resemblance to murine ICM, but there was no evidence that the cells were either pluripotent or could sustain long term growth [10].

The derivation of ESC from blastocysts of primates in the late 1990s encouraged additional efforts to create similar lines from other species, including sheep [11], hamster [12], dog [13], cat [14], mink [15], rabbit [16], horse [17], cattle [18, 19] and pig [10, 20, 21]. However, in spite of a decade and a half's worth of further effort, authentic ESC have only been established from a second rodent species, the rat [22, 23]. The "ESC-like" or putative ESC from other species were either inadequately characterized or failed to meet all the usual criteria for "stemness".

Much of the excitement that surrounds ESC research is due to their two principle attributes: 1) the ability of such cells to proliferate indefinitely under defined culture conditions; 2) their pluripotency, which is defined by their ability to differentiate into cell types representing the three germ layers (mesoderm, ectoderm and endoderm) and ultimately all the primary cell types found in the body [24, 25]. The ability of ESC to survive long term in culture has made them a novel and robust model for studying pathways that drive differentiation and senescence [26]. The pluripotency of ESC on the other hand has elevated them as the foremost model system in regenerative medicine. In addition, mESC have revolutionized the field of transgenics. In the mouse, such cells have afforded a means for "knocking out" and "knocking in" genes by homologous recombination, in large part because of their ability to proliferate indefinitely, allowing the selection of the mutant cells to be achieved before senescence sets in. The cells bearing mutations can be introduced into recipient blastocysts to give rise to chimeric mice that, in turn, can pass on the mutated gene through their germline [27, 28].

The establishment of ESC from species other than rodents and primates, including domesticated ungulates, such as the pig, sheep, goat, cow, or horse, is of great interest both from the agricultural perspective and for biomedical applications. For example, large animal models, particularly a large monogastric species as the pig, are likely to provide models of human genetic diseases where rodent models are inappropriate [29]. The availability of continuously proliferating cell lines with a normal karyotype that can be used to knock in or knock out genes will have additional currency in creating such models and also for efficiently introducing or modifying genes that have production value in commercial herds and flocks. There may be an additional advantage of these cells in somatic cell nuclear transfer (SCNT) applications, in that the genetic material of such undifferentiated cells may be easier to "reprogram" when introduced into the oocyte cytoplasm than that from more differentiated cells, although this assumption is questionable [30].

Perhaps the greatest benefit from deriving the ungulate ESC will be in transplantation research. In order to explore the full potential of hESC or their alternatives, induced pluripotent stem cells (iPSC) discussed below, for transplantation, the safety and efficacy of such cells and the procedures to introduce them into recipients need to be tested thoroughly. As the mouse is probably not the most appropriate animal for such testing, other models are necessary. Species such as the pig or even the cow, whose body and organ size, anatomy, longevity and physiology much more resemble the human than the mouse, are probably far better models to employ in transplantation biology.

In the remainder of this review, we shall focus on the gains made in the field of ungulate stem cell research, the potential problems plaguing the area, and promising alternatives that could shape the field. Over the past two decades, a significant majority of research efforts to derive ESC from farm species has been dedicated to cattle and pig. Consequently, much of the discussion in this review will be confined to those two species.

ESC in Ungulate Species—A Progress Report

Putative porcine (p) ESC lines were first established by Evans et al. who employed d 7–9 expanded in vivo porcine blastocysts [20]. These putative pESC were maintained for more than a year on inactivated STO feeder cells and could form aggregate clusters, analogous to embryoid bodies, in vitro. However, both the pESC and the differentiated cells within the clusters were poorly defined. The pESC, for example, were characterized biochemically by alkaline phosphatase activity and their lack of vimentin, while the germ layers in the clusters were delineated by morphological characteristics alone [31]. About the same time, Piedrahita et al., also reported the derivation of pESC from the culture of ICM, in this case obtained by immunosurgery of d 7–8 in vivo blastocysts [21]. Several lines characterized as either "ESC-like" or "epithelial-like" were established. None of the ESC-like cells survived passage 10, but at least one of the epithelial-like lines survived up to passage 42. Interestingly, the epithelial-like cells formed vesicular aggregates in vitro comprising predominantly of polarized epithelial cells, indicating that they were probably not true embryoid bodies, whereas the ESC-like cells failed to differentiate. Following these initial publications several additional reports soon followed, employing embryos as early as the 4–6 cell stage, morulae [32], at d 5–6 [33], and from older embryos at d 9–12 [34, 35], as well as expanded blastocysts close to hatching, albeit with limited success. It is worth emphasizing that the ICM cells from d 6 to 7 pig blastocysts are capable of contributing to chimeras [31, 36, 37], and are, by this criterion, pluripotent and a potential source of ESC. Clearly, however, such once pluripotent cells can lose this potential when cultured for even a short period of time.

As with pigs, most attempts to isolate and culture ESC from cattle (bESC) used blastocysts [18, 38–43]. Additionally, d 12–14 embryos, which have a well developed embryonic disc, have also been utilized but with similar poor outcomes [44]. In addition, attempts to derive bESC from zygotes and early cleavage stage embryos have generally failed [19, 45], except for a single bovine embryonic cell line derived from a 2-cell embryo that was cultured for over 3 years [45] and one from a 16-cell stage embryo that survived continuous culture for more than 9 months [18, 19]. Cell lines from these sources demonstrated limited potential for differentiation as they could form structures resembling embryoid bodies, but failed to form teratomas.

Although never truly characterized as ESC, some of the cell lines derived in these early porcine and bovine studies did clearly possess the ability to proliferate for extensive periods in culture, and some could differentiate and organize themselves into structures resembling embryoid bodies and teratomas [33, 46]. Additionally, there has been a report of live chimera generation from pESC, although there was no contribution to the germ line [32]. A few porcine cell lines derived from primordial germ cells (embryonic germ cells) have also demonstrated the ability to become incorporated into chimeras, with similar lack of germ line transmission [47–50]. Even so, the general consensus is that none of these lines, including those from germ cells, were truly ESC and pluripotent [51, 52]. Something was being overlooked either in the manner in which they were first derived or, more likely, in the means whereby they were cultured [34, 51, 52].

Difficulties/Concerns for Derivation of Pluripotent Cells from Farm Species

Based on the available literature, it appears as though putative ESC-like colonies can be initially established from bovine and porcine embryos, but they cannot be sustained over long term in culture. Several factors, both extrinsic and intrinsic, likely contribute to the difficulties. These factors have been the focus of several recent reviews [34, 51, 52] and therefore will be discussed only briefly here.

Intrinsic Factors

Developmental Competence Even after implementing methods that had been successful in establishing mESC, Evans and his colleagues failed to derive authentic ESC from pig [10, 20]. On the same note, it has only been possible to establish ESC readily from a few of the many inbred strains of mouse, e.g. 129 and C57BL/6, [53]. Extending the technology to the majority of other inbred and all outbred strains has been much more difficult [54, 55]. Additionally, authentic ESC, until recently, had only been isolated from two other species, namely monkey and human, and those advances required a radical change in culture conditions [5, 6]. The ability to derive ESC not only appeared to be a capricious process, but the cells themselves were anomalous, not truly mimicking any known tissue type, with the exception of embryonal carcinoma cells (ECC) which exist in a pathological state. Indeed it seemed that murine ESC were laboratory artifacts, selected by chance through adaptation to the culture conditions to which they were exposed during early outgrowth. Although background genetics probably do undoubtedly contribute to the ease whereby ESC can be created from embryos, the recent derivation of authentic ESC from rat ICM, which is discussed in greater detail below [22], is beginning to provide some insights into how a pluripotent network can be sustained and protected, thereby providing renewed optimism that ESC might soon be available from other, previously “difficult” strains and species.

Differences in Pre-Implantation Developmental Biology Early embryonic development leading up to epiblast formation does not follow identical temporal paths in mice and ungulates. In mice, the first differentiation event, characterized by the formation of TE and ICM, takes place by d 3.5 within the uterine lumen. Between days 3.5 and 4.5, an additional layer of cells is formed at the base of the ICM and eventually lining the blastocoelic cavity called primitive or extra-embryonic endoderm. The events leading up to the formation of these three cell layers in embryo are short spanned in the mouse but emerge over a longer period in many other species. For example, in human [56], pig and cow [57–59] both blastocyst formation and hatching occur several days later than in the mouse. In mouse the cells of the ICM proliferate rapidly between days 5.5 and 6.5 and progress into a structure known as the ‘epiblast’ [60], the forerunner of the embryonic germ layers. The resultant epiblast persists for only a short duration until gastrulation is initiated on d 6.5. In ungulates, the ICM is quite small relative to TE before hatching, but epiblast begins to emerge by d 6 [61] and then persists over a much longer period than in the mouse. In addition, around d 10 in pig and d 12 in cow a thin sheet of polar TE cells overlying the epiblast called “Rauber’s layer” degenerates exposing the epiblast to the contents of uterine lumen [62, 63] and placing it in direct contact with the uterine epithelium. This loss of Rauber’s layer coincides with the initiation of a period of rapid growth of the conceptus. In the pig, for example, the greatly expanded blastocyst, which exists as a spherical vesicle of 8 to 10 mm in diameter at about d 10, elongates into a thread-like form up to a meter in length within just a few days [64, 65]. This growth and reorganization primarily involves the trophoblast and underlying extra-embryonic endoderm and not the epiblast and

probably reflects the need for the conceptus to increase its surface area and acquire large amounts of nutritional support from maternal uterine secretions. Finally, while mouse TE attaches and begins to invade the endometrium by ~d 4.5 post-coitus, the period of apposition is delayed until d 12–14 in ungulates, with full attachment not occurring until d 17–19 in pig and d 19–20 in cattle. In summary, in cattle and pigs, unlike the mouse or human, there is a long pre-apposition phase corresponding to an extensive expansion of trophoblast and delayed growth of the embryo proper. Therefore, the stage at which a pig or cow conceptus should be selected to establish ESC cultures is not easy to guess. Moreover, the fact that the epiblast is not enclosed in a protective layer of trophoblast may mean that its nutritional requirements differ from those of a mouse.

Lack of True Pluripotent Cell Markers Compared to the mouse, much less is known about the early embryology of ungulate species and the molecular events that accompany lineage decisions. Perhaps not surprisingly, factors and cell surface antigens considered exclusive pluripotent markers in rodents and primates appear not to be as usefully diagnostic in ungulates. OCT4, for example, may continue to be expressed in TE for a longer period in ungulates than in the mouse [66–68]. NANOG expression has been observed in both epiblast and TE of cattle [39], and there are doubts as to whether, *SOX2* transcripts are localized exclusively to the pluripotent component of bovine and porcine conceptuses [69–71]. Expression of SOX2 and NANOG has been noted in at least one porcine trophoblast cell line [72]. In addition to the use of transcription factors, the presence or absence of certain cell surface antigens, often carbohydrate in nature, has held a prominent place in the lore of ESC and in identifying pluripotent cells. In cattle, however, the surface markers SSEA1, −3 and −4, TRA-1-60 and TRA-1-80 are expressed both in the ICM and TE [39]. SSEA1 and −4 are similarly non-specific in the goat and pig [68]. Together these data emphasize important differences in the pre-implantation biology of rodents and primates on the one hand, and ungulates on the other. Tools and technologies established for one species might be usefully adapted to another in some instances but not in all. What is clear is that it will be essential to use a broad panel of markers to define ESC from ungulates and that not all these will necessarily be consistent with those used for either mouse or human [42, 73].

Extrinsic Factors

Contaminating Cell Types Whole blastocyst explant cultures clearly have the potential to provide cells derived from TE and primitive endoderm, as well as the ICM. Even the germinal discs obtained by immunosurgery and designed to eliminate the outer TE layer are likely to carry endoderm and a trace population of TE. These contaminating cell types may have proliferation rates comparable or higher than ICM/epiblast cells under the culture conditions employed, so that the unwanted cells can come to dominate the mixed culture [42, 74–77]. As discussed above, this common problem is further exacerbated by the lack of markers exclusive to the pluripotent cells, making it very difficult to identify a "pure" population of potentially pluripotent cells as a founder group for establishing ESC lines.

Culture Conditions

a) *Autologous vs heterologous feeders:* Repeated failed attempts to derive ungulate embryonic stem cells (uESC) has sharpened the focus on whether heterologous feeder cells such as STO cells and mouse embryonic fibroblasts (MEF), the choice of feeders in most studies, are appropriate for the task [51, 78, 79]. Given the evolutionary distance between mouse and the farm species, the factors secreted by such cells might be anticipated to bind less well to ungulate receptors than to murine receptors and hence be less effective at supporting the growth of uESC than mESC. However, neither porcine embryonic fibroblasts [33, 80, 81] nor porcine uterine cells [35, 82] appear able to sustain putative pESC. Possibly other cells could offer better growth support, but the necessary studies have not been performed. Nonetheless, the fact that irradiated MEF provide adequate feeder support to porcine induced pluripotent stem cells[83–85] (discussed later), suggests that neither the kind of growth factor produced by the murine cells nor their binding affinity for the porcine receptors are limiting for maintaining pluripotent cells from pig and probably from other ungulates as well. It should also be emphasized that many of the papers describing attempts to produce uESC fail to describe the density of the feeder layers employed. An adequate number of feeder cells might be as critical to success as the choice of feeders themselves. If uESC are ever to be used routinely, there will be a need to determine what kind of cells work best and their optimal density. Ultimately, the growth factors and small molecules that promote and support pluripotency of uESC will need to be identified.

b) *Growth Factor Requirement* (LIF *versus* FGF2): A particular source of confusion in ESC studies generally and for uESC, in particular, has been in the choice of growth factors required by the cells. In large part, this muddle has arisen because of the apparently disparate growth requirements of mESC and hESC, and the recent distinction in the mouse between ICM-derived and epiblast-derived stem cells [86, 87], which we discuss below. While murine ESC derived from ICM are dependent on LIF, human hESC obtained from blastocyst outgrowths require FGF2 (bFGF),

as well as a feeder layer to maintain proliferation and pluripotency. LIF has been ineffective in aiding derivation of uESC from pre-compacted, post-hatching and elongated embryos, although it may not be without effect altogether. For example, in cattle, human LIF was reported as detrimental to growth of putative ESC and pre-implantation embryos [88, 89], while murine LIF was essentially benign, raising the question as to whether the latter was capable of acting through the bovine LIF receptor [89]. In pig, on the other hand, human LIF promoted proliferation in d 10 embryo outgrowths when it was employed at higher than normal concentrations but failed to prevent differentiation of the putative pESC [81]. Regardless of whether or not LIF is an effective additive, it might be best avoided in any attempt to derive ESC from post-hatching and elongated embryos, since the transition from pluripotent ICM to pluripotent epiblast will have already begun and even have been completed. Importantly, even in the mouse, 'epiblast stem cells' require FGF2 for maintenance [86, 87], suggesting that most or all of the human lines so far derived are of the "epiblast" type. In this regard, porcine epiblasts from d 11 embryos have relatively high expression of the genes encoding *FGFR1* and *FGFR2,* and even *FGF2* itself, while demonstrating little evidence for transcribed *LIF* and *LIFR* [61]. On the other hand, FGF2 and several other promising growth factors have been no more effective than LIF in improving the derivation of uESC [52, 78]. Even in the mouse and human, gene expression networks that support pluripotency and ESC proliferation are still incompletely understood, although it is clear that two or more signal transduction pathways, probably acting in parallel must exist to account for the different pluripotent cell types. It seems likely that some version of these pathways will also support pluripotency in ungulate systems. If these pathways can be appropriately activated, a major step will have been taken to deriving uESC.

Future Directions

Emerging Technologies and Prospects for Advance

The derivation of ESC from rat embryos, a previously "difficult species", likely represents a major step forward for stem cell biology [22]. In that study, LIF-dependent rat ESC were derived from blastocysts by using a medium that included two protein kinase inhibitors. One, PD0325901 (PD), blocked the mitogen-activated protein kinase (ERK) pathway, while the second CHIR99021 (CH), targeted glycogen synthase kinase-3beta (GSK3B). The resulting colonies were validated as ESC-like both by in vitro and in vivo pluripotency tests, and their cells could contribute to germ-line competent chimeras.

Analogous approaches have been described by Hanna et al. [90], who derived LIF-dependent ESC from the non-obese diabetic mouse strain, NOD, previously considered "non-permissive" for ESC derivation. ESC production was achieved in two ways: by ectopic up-regulation of *c-MYC* and *KLF4* or by supplementing the mESC medium with small pharmaceutical compounds that activated pathways

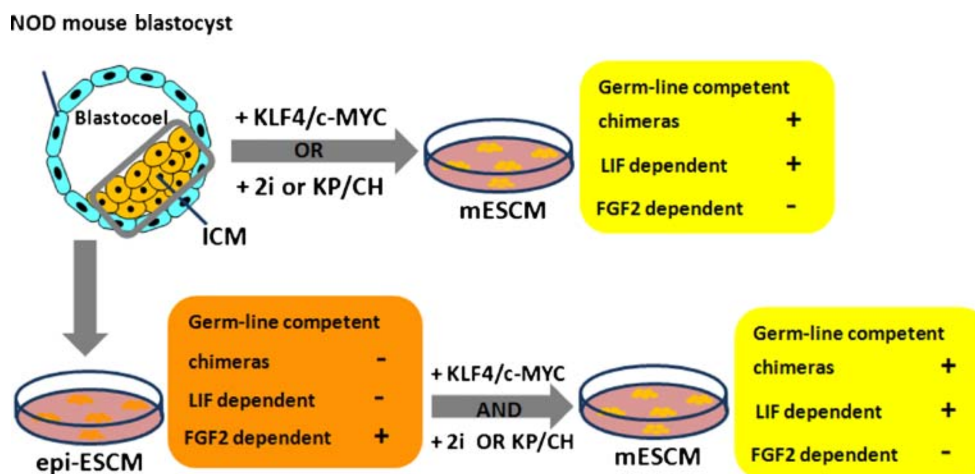

Fig. 1 Strategies for derivation of LIF dependent ESC. The figure outlines two possible means for derivation of LIF dependent ESC. When ICM cells from non-obese diabetic (*NOD*) mice were either infected with retroviruses to provide upregulation of *c-MYC* and *KLF4* or cultured in the mESC medium (*mESCM*) supplemented with one of the two possible combinations of small compounds, PD/CH (2i) or KP/CH, LIF dependent mESC, were obtained. Additionally, when ICM cells were cultured in the presence of epiblast stem cell medium (*epi-ESCM*), which was supplemented with FGF2 rather than LIF and lacking the small compounds, 'flattened' colonies, similar to epiblast stem cells (*epi-ESC*) or hESC were obtained. However, epi-ESC could be coaxed into LIF dependent or authentic ESC either by upregulation of *KLF4/c-MYC* or by transient upregulation of *KLF4/c-MYC* followed by culturing in modified medium with chemical inhibitors. [Adopted from Hanna et al. [90]]

directed by c-MYC and KLF4 [90] (Fig. 1). The actions of c-MYC were seemingly mimicked by up-regulating the Wnt signaling pathway [91], achieved in this instance by inhibiting GSK3B with CH, a component of 2i-medium described above for rat ESC. A second compound, kenpaullone (KP), which is a selective inhibitor of GSK3B and cyclin B/CDK1 [92], appeared to substitute for KLF4 function [92]. Hanna et al. [90] established LIF/Stat dependent ESC from NOD mice by providing the ESC precursor cells with two different formulations of small compounds: PD/CH and KP/CH (Figs. 1 and 2). The resulting NOD-ESC closely resembled counterpart ESC from the 129 mouse strain and were capable of giving rise to chimeras. Several additional low molecular compounds either promote or stabilize pluripotency/self renewal. For more detailed information, readers are directed to an excellent review by Feng et al. [93]. Such refinements of existing methods, which are moving the technology forward to a less empirical state, may provide the key to establishing stem cell lines from ungulates and other species that have resisted traditional approaches. It seems likely that the ICM and epiblast cells of ungulates and possibly many other species have low levels of endogenous c-MYC and KLF4 expression compared to "permissive" mouse strains such as 129 (Fig. 2). Unless concentrations of these transcription factors can be raised, either ESC cannot emerge at all (as in rats and probably ungulates) or the resulting outgrowths, if they do form at all, will be of the "epiblast type", which in humans are dependent on FGF2/Activin/Nodal and not on LIF signaling (Fig. 2). The hope is that by, augmenting the endogenous concentrations of c-MYC /KLF4 in the founder cell population through use of an appropriate combination of small molecules, such as the 2i (inhibitors) and KP/CH, the LIF/Stat signaling pathway will be up-regulated and stabilized, thereby allowing the outgrowth of LIF-dependent ESC (Fig. 2). Further support for this hypothesis has been afforded by the recent discovery that KLF4 is a signaling intermediate downstream of the LIF/Stat pathway in mESC [94]. We predict that such approaches or close variations thereof will yield ungulate ESC in the near future, possibly before this article is published.

Induced Pluripotent Stem Cells (iPSC) an Alternative to Stem Cells

Even if the modified culture conditions described above do not yield uESC, iPSC provide a useful alternative to the embryo-derived cells. It is barely three years since Takahashi and Yamanaka reported the derivation of mouse induced pluripotent stem cells (miPSC) [95] by re-programming somatic cells to ground state pluripotency through expression of a combination of four transcription factors *OCT4, SOX2, KLF4* and *c-MYC* (OSKM) delivered to the cell by retroviral transduction. Progress since then has been rapid. The technology has been modified in a variety of ways to make it potentially more efficient

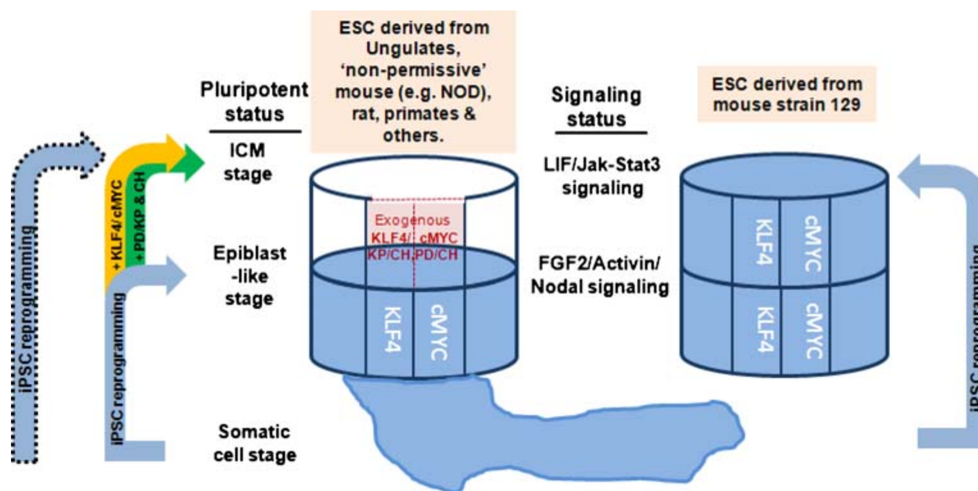

Fig. 2 Hypothetical model for pluripotent states across various genetic backgrounds. The schematic figure outlines various stages of embryo development, associated pluripotent potential, and the networks that sustain them. ICM cells from 'permissive' mouse strain (129) exhibit high endogenous levels of c-MYC and KLF-4 (*represented by full barrel*), and therefore can give rise to authentic LIF-dependent ESC with relative ease. On the other hand, NOD, a non-permissive mouse strain, and other species including rat, human and ungulates may have low endogenous levels of *c-MYC* and *KLF4* in their ICM (*emptying half-level barrel*) and are only capable of giving rise to Epi-ESC. However, when endogenous levels of KLF4/c-MYC are augmented either by means of exogenous supplementation (*brown arrow*) or by modified medium (*green arrow*), the cells regain the potential to give rise to authentic ESC (full-level barrel represents 'ICM-like ESC'). Similarly, somatic cells can be reprogrammed to ground state pluripotency by ectopic expression of *OSKM* factors, but unless c-MYC and KLF4 levels are sustained (green or brown arrow), their phenotype will be that of Epi-ESC (*blue arrow on left hand side*) and not ICM-like ESC (*broken arrow on left*). [Adopted from Hanna et al. [90]]

through use of different gene combinations and potentially safer for future transplant studies by altering the delivery of the reprogramming factors to the cells. It has been applied to adult as well as fetal somatic cells and to differentiated cells other than fibroblasts [95–106]. Finally, iPSC have been generated from monkey [107], rat [108], and more recently pig [83–85], an advance we discuss below. The recent reports that miPSC can provide an entire ICM and give rise to pups wholly derived from them is the ultimate validation that iPSC lie very close to ESC in their pluripotent properties [109, 110].

Induced PSC, like ESC, have great promise in biomedical and agricultural research. They should increase the efficiency whereby select genes can be deleted or added to a genome, contribute to germ-line chimeras, and possibly behave as efficient donors for SCNT [30]. In addition, there is likely to be a major advantage of iPSC over ESC in human transplantation applications, specifically the ability to produce 'patient specific' stem cells. Somatic cells, such as skin keratinocytes, could be quickly cultured from a skin biopsy and within a few weeks converted into iPSC. The iPSC could then be directed towards a specific tissue lineage, for example, cardiomyocytes or a particular kind of neuronal precursor, and transplanted into the same donor from which the progenitor cells were first isolated, thereby minimizing the risk of immune-rejection. However, utilizing transplants derived from ESC and especially iPSC is unlikely to be without risk to the patient. There must be reasonable assurance that the transplanted cells will not give rise to tumors, including teratomas, or cause a massive immune response. There must also be an efficient means of delivering the transplant specifically and efficiently to a particular site, and a reasonable assurance that the transplant will function appropriately. In our view, thorough animal testing must be required before stem cell technologies are brought into the clinic. We also argue that the mouse cannot substitute for routinely testing technologies in a species whose size, anatomy, immunology, physiology and longevity resemble the human. For cost reasons alone, such a need is unlikely to be met through the use of non-human primates. We argue, therefore, that the pig is the species of choice. The near simultaneous publication of three reports that iPSC can be derived from pigs (piPSC) [83–85] may be a significant milestone in attempts to develop the pig as a model system in transplantation biology [30].

All three of the publications [83–85], including one from our laboratory [84], describing the derivation of piPSC from fibroblasts took the same approach, namely employing integrating retroviral vectors to drive expression of the OSKM combination of transcription factors. The resulting piPSC did not have identical phenotypes, at least in terms of surface markers (Table 1), but did appear to resemble human rather than mouse ESC. In particular, they were dependent on FGF2 and a feeder layer of irradiated MEF to maintain their stemness properties. As with human ESC and iPSC, it would appear that factors produced by MEF, including activin and nodal, in combination with the supplied FGF2 are needed to maintain pluripotency and prevent differentiation. This laboratory is presently testing the hypothesis that if porcine iPSC derivation is attempted in the presence of LIF in combination with select protein kinase inhibitors, it will be possible to obtain LIF-dependent cells that can contribute to germ-line competent chimeras. Our preliminary results (unpublished data) suggest that the hypothesis is correct and that LIF-dependent porcine iPSC can be readily obtained by using this approach.

Additionally, taking into account the recent demonstrations that p53-mediated senescence and the existence of

Table 1 A brief overview of piPSC reported by Esteban et al, Ezashi et al. and Wu et al.

		Esteban et al. [83]	Ezashi et al. [84]	Wu et al. [85]
Dependency		bFGF	bFGF	bFGF
Pluripotent markers	*OCT4*	?	+	+
	SOX2	+	+	+
	NANOG	+	+	+
	Lin-28	+	+	+
	REX1	+	+	+
	CDH1	?	+	+
Surface markers	SSEA1	?	+	−
	SSEA3	?	−	+
	SSEA4	+	±*	+
	TRA-1-60	?	−	+
	TRA-1-81	?	−	+
Tests of pluripotency	Teratoma	+	+	+
	Embryoid body	?	+	+

All three groups registered expression of pluripotent markers in piPSC, however, major inconsistencies were evident in cell surface marker repertoire. Ezashi et al. reported expression of SSEA1 and weak SSEA4 expression was above the background, but lower than that of hESC, whereas Wu et al. reported expressions of SSEA3, −4, TRA-1-60 and TRA-1-81

efficient apoptotic pathways counteract reprogramming and the establishment of a pluripotent state in somatic cells [111–116]. It seems likely that lowering the endogenous levels of p53 by either RNAi or pharmacological inhibitors may be exploited as a means to generate ungulate iPSC more efficiently. The likely downside of this approach is that by interfering with p53, the gatekeeper for DNA damage, there will be a greater chance of accumulating mutations and chromosomal damage. Of note, iPSC in a p53-null background were unable to maintain their ESC-like morphology past passage 5 as a result of genomic instability. The cells also gave rise to tumors and eventual death in chimeric mice [112]. As an alternative to reducing p53 concentrations by genetic means, it has been possible either to utilize primary cell populations such as keratinocytes with low endogenous levels of active p53 [113], or, to employ cells with a high endogenous proliferation potential such as somatic stem and progenitor cells, for example neural stem cells [117] and cord blood derived mesenchymal stem cells [118, 119] for efficient iPSC derivation. In these instances, *OCT4* alone or in combination with *SOX2* were sufficient to drive reprogramming without any requirement for ectopic *c-MYC* and *KLF4* [113, 117, 119].

Although the iPSC technology has great promise, it also has its share of concerns. Until recently, the majority of iPSC were generated by using integrating retroviral vectors leading in some, possibly all cases to the continued expression of the transgenes in the reprogrammed cells [98, 102, 120–123], which is disturbing as cMYC is a *bona fide* oncogene and raises the risk of cancer if it is not silenced [105]. Additionally, the continued expression of pluripotent genes may limit the differentiation potential of the iPSC [124]. For iPSC to have utility, these 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 [125, 126], introduction of "stemness" proteins rather than genes [127, 128], or pharmaceutically with a suitable combination of small molecules.

Conclusion

The recent spate of improved technologies for deriving ESC from embryos and for reprogramming somatic cells will inevitably lead to the isolation of pluripotent cells from a wide range of mammals, including cattle, dog, and other domesticated species. The pig has already yielded iPSC, albeit through use of the less desirable retroviral approach, and, as stressed earlier, this species is a particularly desirable target for study because of its value as a biomedical model in transplantation research.

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Conflict of Interest All authors claim no conflict of interest.

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