Embryo-derived Pluripotent stem cells from domesticated animals-Progress so far

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Abstract

Embryo-derived pluripotent stem cells or so-called Embryonic Stem (ES) cells are a unique kind of cells with an unlimited capacity of self-renewal and to differentiate into all the cells of the animal's body. These cells from domesticated or farm animals are of great importance in agriculture, bio-pharming, genetic engineering and many more. Even after close to four decades of ES cell derivation from mouse, it has yet not been possible to derive germline competent true ES cells from farm animals. This review discusses the efforts that have been made to date to derive ES or ES-like cells from domesticated ungulates and shed some light on the strategies to generate bonafide ES cells from these species.

Based on the observation in 1954 by Stevens and Little that males of the mouse strain 129 svj spontaneously generates teratocarcinoma (testicular tumor) having cells of all the three germ layers and subsequently by Solter et al. (1970, 1979, 1981) the formation of teratomas on transplantation of embryo on ectopic site of recipient mouse having same differentiation potential, it was thought that pluripotent cells must reside in embryo. These findings gave the initial idea to researchers, and the hunt for the isolation of embryonic stem cells began which finally led to their derivation by Evans and Kaufman in 1981 from the blastocyst stage of mouse embryo by growing the cells of the inner cell mass (ICM) on mouse embryonic fibroblasts (MEFs) as feeder layer. This discovery was followed by plethora of papers (For example Martin, 1981) showing the pluripotent nature of ES cells including the important finding that Mouse Embryonic Fibroblasts (MEFs) secrete cytokine Leukemia Inhibitory Factor (LIF) which maintains the ES cells in an undifferentiated state (Smith et al., 1992, 1998). The establishment of mouse ES cells opened the pathway to produce transgenic mice by gene knock out and knock in techniques to know the function of a gene and to generate mice of desirable traits. Additionally, these cells are an excellent model system to study embryonic development, drug screening, disease modeling and repairing a damaged organ by transplanting the ES cell-derived terminally differentiated specialized cells. These achievements arose excitement in the scientific fraternity and attempt to establish ES cells from higher mammals including human and farm animals started. Finally in 1998, after 17 years of mouse ES cell derivation, Thomson and group established the first human ES cell lines from the ICM of the blastocyst stage of human embryos on feeder layer of MEFs. In parallel, soon after the derivation of mouse ES cells, many research groups started their effort to derive pluripotent ES cells from domesticated ungulates as these cells can help in the creation of models of human genetic diseases, cell transplantation therapies, making transgenic animals for improved production traits and products, disease resistance, bio-pharming (Keefer et al., 2007) and as nucleus donor in cloning by somatic cell nuclear transfer (SCNT). ES cells from farm animals are of great interest for many reasons but mainly from the agricultural perspective and for biomedical applications. Large animals such as pig and cow can provide a model of human genetic disorder where mouse or rat models are not appropriate. Additionally, animals like pig, cow, and sheep whose body size as well as anatomy and physiology are much more like humans than rodents, are probably far better models to employ in transplantation biology (Telugu et al. 2010). Till date, numerous efforts have been made to derive ES cells from cow, pig, sheep, goat, buffalo, horse, etc., but it has not been possible yet to establish the true or bonafide germline-competent ES cell line from any of the domesticated animals. Even the most recent article described the derivation of a version of ES cells which are developmentally less competent and lacking chimera production with germline transmission (Bogliotti et al. 2018). We elaborate here the methods used by different groups for the generation of ES or ES-like cells, reasons for failure and future strategies which could lead to the successful establishment of ES cells in farm animals.

Early mammalian embryonic development at a glance

After fertilization, the cleavage division gives rise to a compact ball of cells called morula. After the morula stage, the first differentiation event in embryo occurs and the formation of blastocyst takes place. The blastocyst consists of an outer layer of trophectoderm, a fluid-filled cavity known as blastocoel and a mass of cells at one side called inner cell mass (ICM) which further differentiates into epiblast and hypoblast. Cells of the trophectoderm and hypoblast give rise to the placenta and extraembryonic tissue, and the epiblast leads to the formation of the embryo proper. As the gastrulation begins, the epiblast develops into three germ layer viz-ectoderm, mesoderm, and endoderm. These three layers give rise to all the cells of an animal body, and that is why epiblast considered as pluripotent. Cells of the epiblast in culture give rise to ES cells, so these cells retain pluripotent nature. The concept of ES cells is purely in vitro and holds true only in cultures where their commitment towards a particular lineage is arrested due to the culture conditions which maintain them in an undifferentiated state in the presence of growth factor. Usually, mouse and human embryo develop into blastocyst on day 3.5 and 7 respectively and in farm animals, it is somewhere between 7-10 days. It is at this stage that embryos are used for the derivation of ES cells.

Molecular signaling and the concept of naïve and primed pluripotency

It's a well-established fact that only a few inbred mouse strains which frequently develop teratocarcinoma such as 129 svj and C57BL/6 (Stevens and Hummel, 1957) can give rise to ES cells while from other strains it is difficult without genetic modification and manipulating culture conditions. So129 svj and C57BL/6 are called permissive and others non-permissive. This strain specificity is restricted to mice only as ES cells from other species have no preference towards the genetic background. The exact reason behind this is not known, but it is sure that some genetic factor is involved. Recent studies have reported that embryo-derived pluripotent stem cells exist in one

of the two forms. The first is a naïve state which represents full pluripotent or ground state. Mouse ES cells (mESC) come under this category. The second primed state is of limited pluripotency within which mouse epiblast stem cells (mEpiSC) derived from post-implantation blastocyst and human ES cells (hESC) can be placed (Nichols and Smith, 2009). The establishment of the naïve state is species dependent, and the permissive species can maintain a naïve state during stabilization into ES cells from naïve epiblast during pre-implantation embryo development. In contrast, non-permissive species do not maintain naïve state and instead are stabilized into a primed state from naïve epiblasts during the establishment of ES cells. There are many differences between naïve and primed ES cells. Naïve ES cells form round or dome-shaped colonies which are small in size, their pluripotency is maintained by LIF and BMP4 (Ying et al., 2003), both X-chromosome remain activated in female ES cells, Oct4 expression is controlled by the distal enhancer, they can grow after trypsin-EDTA mediated dissociation into single cells and they give rise to germline competent chimera. In contrast, primed ES cells form flattened monolayer-like large colony, their pluripotency is maintained by FGF and Activin/Nodal signaling (Levenstein et al., 2006; Beattie et al., 2005; Vallier et al., 2009), one X-chromosome is inactivated, Oct4 expression is controlled by proximal enhancer, they can't survive when dissociated into single cells and can be passaged mainly by mechanical dissection, they are unable to generate chimera with germ line transmission. LIF and Activin/Nodal signal immortalize stem cell population at distinct developmental stages, namely early and late epiblast respectively. Mouse EpiSCs are derived from pre-gastrulation stage (5.5-6.5 dpc). They express Oct4, Nanog, and Sox2 but not Rex1, Tbx3 which are mES cell marker. They are also capable of forming teratoma. Human ES cells are usually generated from the late epiblast stage of the pre-implantation embryo. Mouse EpiSC and hES cells have a common mechanism of epigenetic regulation of transcription which is distinct from mES cells. Genes associated with germline, including Stella, Piwil2, Stra8, and Dazl are expressed by mES cells but not by mEpiSc and hES cells. The expression of specific genes suggests that mESCs and EpiSCs are two distinct pluripotent states representing cells of the pre-implantation embryo and late epiblast cells.

How ES cells are identified

There are certain pre-requisites before we can tag a particular cell line as ES cells. They grow in discrete colonies, should be Alkaline Phosphatase positive, presence of cell surface marker such as SSEA-1(mouse & pig) and SSEA-3, SSEA- 4, TRA-1-60, TRA-1-81(human and ungulates) , presence of nuclear marker Oct4, Sox2, Nanog and Rex1, High telomerase activity, capable to differentiate in vitro into cells of all three germ layers, form teratoma (disorganized array of cells which are derivatives of all the three germ layers) when injected into immunocompromised (SCID) mice, give high frequency chimera with germline transmission i.e. ES cells contribution to gamete formation should be confirmed.

ES cells in domesticated ungulates

Though till date only mouse and rat ES cells are capable of colonizing the germline and producing functional germ cells, over the last 30 years, many reports of ES or ES-like cells from farm animals have also been published.

Pig (Porcine)

Due to its resemblance in larger morphology, organ size and physiology to human, pig is useful for the transplantation therapies and for the study of human genetic diseases. Therefore, the isolation of ES cells from porcine encourages new development in animal production as well as in biomedical research. Over the past 30 years many studies on the isolation of porcine ES cells from pre-implantation embryos have been reported. Early attempts to isolate porcine ESC from in vivo developed blastocysts were reported in the late 1980s as well as in 1990s (Piedrahita et al., 1988; Evans et al., 1990; Piedrahita et al., 1990a,b; Strojek et al., 1990; Notarianni et al., 1990, 1991; Hochereau-de Reviers and Perreau,1993; Talbot et al., 1993a,b; Anderson et al., 1994; Wheeler, 1994; Gerfen and Wheeler, 1995; Moore and Piedrahita, 1996,1997; Wianny et al., 1997; Chen et al., 1999), albeit with less success More recently various attempts have been made to establish porcine ES cells from blastocysts produced in vitro or in vivo (Ock et al., 2005; Brevini et al., 2007a, b; Keefer et al., 2007; Vackova et al., 2007; Hall et al., 2008; Alberio et al., 2010; Brevini et al., 2010a; Telugu et al., 2010, 2011; Haraguchi et al., 2012; Park et al., 2013; Hou et al., 2016). These cells usually grow in vitro as flattened, polygonal epithelial like cells (Piedrahita et al., 1988; Strojek et al., 1990; Anderson et al., 1994; Chen et al., 1999; Alberio et al.. 2010), could be maintained in *in vitro* culture for extended periods of time and form embryoid bodies, but differentiated only into a limited number of cell types (Talbot et al., 1993b; Chen et al., 1999). They were also found positive for alkaline phosphatase (AP) staining (Talbot et al., 1993a; Chen et al., 1999; Li et al., 2003, 2004a; Shiue et al., 2006). Some of the studies showed expression of nuclear and cell surface markers including OCT4, NANOG, SOX2 and SSEA-1(Wianny et al., 1997;

Vackova and Madrova, 2006; Bloomberg et al., 2008; Alberio et al., 2010; Vassiliev et al., 2010a, 2010b).Chimera formation was also shown in studies but no germline transmission was reported (Wheeler, 1994; Chen et al., 1999; Vassiliev et al., 2010a). Attempts towards the establishment of porcine ESC lines also entailed embryos from in vitro production (Miyoshi et al., 2000; Li et al., 2004b; Ock et al., 2005; Kim et al., 2007; Brevini et al., 2010b; Vassiliev et al., 2010a, 2010b), parthenogenetic activation (Brevini et al., 2005, 2010b; Ock et al., 2005; Kim et al., 2007) and somatic cell nuclear transfer (SCNT; Kim et al., 2007, 2010b). LIF was not essential for culture of putative porcine ESCs nor did it contribute to improved culture conditions in initial reports. However, ES cells isolated by Telugu et al., 2011; was LIF dependent and more like naïve mouse ES cells but chimera formation was not reported. The forced overexpression of inducible human (h) KLF4 or constitutively expressed hOCT4 and hKLF4 in cells from the ICM resulted in stable AP positive colonies expressing OCT4, SOX2, and SSEA1. The resulting colonies resembled mouse ESCs not only morphologically but also at the transcriptome profile level. Park et al., 2013 and Hou et al., 2016 reported the derivation of FGF and Activin/Nodal-dependent primed ES cells. Haraguchi et al., 2012 generated self-renewing ES-like cells from ICM using small molecule inhibitors CH99021 and PD184352.

Cow (Bovine)

Among all domesticated animals, cow is the most common and of great economic importance in the livestock species being a source of significant nutrition for a large proportion of the world population. Therefore, successful establishment of bovine ES cells would not only be of economic importance, but would allow targeted modification of the bovine genome and thus would be highly valuable for biomedical and agronomical applications (Maruotti et al., 2012). The first attempt to generate bovine ES cells date back in 1991 with a brief report of primary culture of ICM cells. The isolated cells maintained a normal karyotype for four passages (Strelchenko et al., 1991). Subsequently, these cells were tested for in vitro differentiation potential but chromosomal abnormality was observed even at early passages. After that numerous attempts to isolate and culture ES or ES-like cells from the blastocyst have been reported in bovine (Saito et al., 1992; Sims and First, 1994; Van Stekelenburg- Hamers et al 1995; Talbot et al., 1995; Stice et al., 1996; Cibelli et al., 1998; Iwasaki et al., 2000; Mitalipova et al., 2001; Talbot et al., 2002; Saito et al., 2003; Wang et al., 2005; Gjorret et al., 2005; Yadav et al., 2006; Talbot et al., 2007; Munoz et al., 2008b; Pant and Keefer, 2009; Cao et al., 2009; Kwon et al., 2009; Gong et al., 2010; Pashaiasl et al., 2010; Jin et al., 2012; Wu et al., 2016; Bogliotti et al., 2018). In most of the cases day 7-9 blastocysts were used for the isolation of bovine ESC. The embryos used for derivation of putative ESC lines have been produced by different methods,including in vitro fertilization (Talbot et al., 1995, 2007; Van Stekelenburg-Hamers et al., 1995; Stice et al., 1996; Iwasaki et al., 2000; Wang et al., 2005; Munoz et al., 2008b; Pant and Keefer, 2009; Gong et al., 2010; Jin et al., 2012; Maruotti et al., 2012), superovulation and in vivo embryo production (Saito et al., 1992, 2003; Talbot et al., 1995, 2007; Stice et al., 1996; Cao et al., 2009), parthenogenesis (Talbot et al., 2007; Pashaiasl et al., 2010) and SCNT (Wang et al., 2005; Kwon et al.,2009). In most of the cases bovine ES-like cell lines showed high AP activity (Talbot et al., 1995; Saito et al., 2003; Wang et al., 2005; Cao et al., 2009; Kwon et al., 2009; Gong et al., 2010; Pashaiasl et al., 2010; Lim et al., 2011). Characterization of putative ESCs showed the expression of pluripotency markers, including OCT4, NANOG, SSEA-1, SSEA-4, TRA-1-60 and TRA-1-81(Saito et al., 2003; Wang et al., 2005; Munoz et al., 2008b; Cao et al., 2009). Exposure of bovine blastocysts with high concentration of 5-azazytidine, an epigenetic modifier that reduces DNA methylation resulted in up to 18 times more efficient isolation of pluripotent cells than traditional methods (71.4% vs 4.0%; P < 0.001). These putative bovine ESCs expressed OCT4, REX1 mRNA and SSEA-1 and SSEA-4 proteins; and were able to form embryoid bodies in vitro and teratomas when injected in Severe Combined Immuno Deficient (SCID) mice (Lim et al., 2011). Jin et al., 2012 described a well-defined culture conditions for the derivation of putative bovine ESCs where they mentioned that mouse embryonic fibroblasts are better than bovine embryonic fibroblasts as feeder layer and mechanical passaging yields more colonies than enzymatic treatment. They also showed that use of LIF and stem cell factor (SCF) is a better option as compared to LIF alone. Several genes characteristic of ICM (for example, NANOG, SOX2, and STAT3) and TE (ELF5, GATA3, and KRT18) in mouse and human showed similar patterns in bovine (Ozawa et al., 2012). Inhibition of MAP2K and GSK3 signaling promotes bovine blastocyst development and epiblast-associated expression of pluripotency factors and it may prime bovine epiblast for subsequent derivation of pluripotent stem cell cultures (Harris et al., 2013). Canonical WNT signaling regulates development of bovine embryos to the blastocyst stage and can facilitate ESCs derivation in this species (Denicol et al., 2013). Verma et al., 2013 showed that dual Kinase Inhibition Promotes Pluripotency in Finite Bovine Embryonic Cell Lines. Thiazovivin, a Rho kinase inhibitor, improves stemness maintenance of embryo-derived stem-like cells under chemically defined culture conditions in cattle (Park et al., 2015). Kim et al., 2015 established and maintained somatic cell nuclear transfer embryo-derived stem-like cells (SCNT-eSLCs) from the traditional Korean beef cattle species, HanWoo

(*Bos taurus coreanae*). Microarray data showed that TGFβ, WNT, and LIF signaling were related to the maintenance of pluripotency. In contrast, some differentially expressed genes related to the LIF pathway were down-regulated, suggesting that reactivation of the pathway may be required for the establishment of true bovine embryonic stem cells (ESCs). Interestingly, oncogenes were co-down-regulated, while tumor suppressor genes were co-up-regulated in eSLCs, implying that this pattern may induce abnormal teratomas (Kim et al., 2017). Employing a culture system containing fibroblast growth factor 2 and an inhibitor of the canonical Wnt-signaling pathway, pluripotent primed bovine ESCs (bESCs) with stable morphology, transcriptome, karyotype, population-doubling time, pluripotency marker gene expression, and epigenetic features were derived. Under this condition, bESC lines were efficiently derived (100% in optimal conditions), were established quickly (3–4 wk), and were simple to propagate (by trypsin treatment). When used as donors for nuclear transfer, bESCs produced normal blastocyst rates, thereby opening the possibility for genomic selection, genome editing, and production of cattle with high genetic value (Bogliotti et al., 2018).

Buffalo (Bubaline)

Buffalo is the most important farm animal in Asia; half of the total population exists in India and buffalo ESCs would be useful for improvement of genetic modification and for facilitated studies on gene regulation (Sharma et al. 2011). The first attempt to isolate Buffalo ES cells were made by authors in 2002 (unpublished report) from the morula stage embryos. The blastomeres were mechanically removed and plated on the mitomycin-c treated mouse as well as goat embryonic fibroblasts. The cells got attached and expanded for a week after which they petered out. Following this, few reports about the derivation of Buffalo ES cells were published (Chauhan et al., 2006; Verma et al., 2007; Sritanaudomachi et al., 2007; Huang et al., 2010b; Anand et al., 2011; Sharma et al., 2011; George et al., 2011; Sharma et al., 2012; Kumar et al., 2011, 2012; Muzaffar et al., 2012; Singh et al., 2013). Buffalo ES-like cells were established from 6–8-day-old embryos produced either by in vitro fertilisation (Verma et al., 2007; Huang et al., 2010b; George et al., 2011; Sharma et al., 2011, 2012; Muzaffar et al., 2012), after parthenogenetic activation (Sritanaudomchai et al., 2007; Muzaffar et al., 2012) or by hand-made cloning (George et al., 2011; Muzaffar et al., 2012). Hatched blastocysts and mechanical isolation allowed higher primary colony formation than the use of zona pellucida-intact blastocysts (Verma et al., 2007; Anand et al., 2011). Buffalo ES-like cells displayed a round or dome shaped, compact morphology with well-defined edges (Sharma et al., 2011) and expressed ES pluripotency markers, including AP, SSEA-4, TRA-1-60, TRA-1-81, OCT4 and SOX2 (Verma et al., 2007; Huang et al., 2010b; Anand et al., 2011; Muzaffar et al., 2012; Sharma et al., 2012). Buffalo ES-like cells were karyotypically normal (Verma et al., 2007; Anand et al., 2011; Sharma et al., 2011; Muzaffar et al., 2012) and could be maintained in vitro over 135 passages in culture medium supplemented with both basic fibroblast growth factor (bFGF) and LIF (Sharma et al., 2011). They formed embryoid bodies and differentiated into various cell types (George et al., 2011; Sharma et al., 2011; Muzaffar et al., 2012). However, the ability to form teratomas and to contribute to chimera formation could not yet be determined. Rock inhibitor Y-27632 improves survival of buffalo ES cells under unfavourable conditions such as enzymatic dissociation to single cells or antibiotic-assisted selection after transfection, without compromising their pluripotency (Sharma et al., 2013).

Sheep (Ovine)

Sheep ESCs would provide a valuable research tool for genetic breeding and the production of transgenic animals (Zhao et al., 2011). . Ovine ES-like cells have been isolated from late morulae or early in vivo blastocysts from Days 6–7 (Handyside et al., 1987; Piedrahita et al., 1990a; Notarianni et al., 1991; Wells et al., 1997) and grew slowly either as cystic structures (Handyside et al., 1987) or with an epithelial-like morphology (Piedrahita et al., 1990b). As in the other domestic species, in vitro produced blastocysts from Days 6–8 were used for isolation of ovine ES-like cells (Dattena et al., 2006; Zhu et al., 2007; Zhao et al., 2011). Ovine ES-like cells showed the typical morphology of mouse ESCs such as the dome-shaped structure, smooth surface and strong refraction (Zhao et al., 2011). These cells could be maintained for >30 passages without feeders and in semi-defined medium containing N2, B27, GSK3 inhibitor (CHIR99021) and bFGF (Zhao et al., 2011). Ovine ES-like cells stained positive for alkaline phosphatase (Talbot et al., 1993a; Dattena et al., 2006; Zhu et al., 2007; Zhao et al., 2011) and expressed specific pluripotency markers such as OCT4, SOX2 and NANOG (Sanna et al., 2009; Zhao et al., 2011) and the stage-specific embryonic markers SSEA-1, SSEA-3 and SSEA-4 (Dattena et al., 2006). In the absence of a feeder layer and human LIF, ovine ES-like cells formed embryoid bodies (Dattena et al., 2006; Zhao et al. 2011) and differentiated into derivatives of the three germ layers (Dattena et al., 2006). After injection into immuno-deficient mice they formed teratomas containing a variety of different tissues but contribution to the germline was not observed (Zhao et al., 2011).

Goat (Caprine)

The relatively limited availability of goat ovaries from slaughterhouse hampered in vitro production of embryos and hence isolation of ES cells from this species. Putative caprine ESCs were isolated from in vitro fertilised (Meinecke-Tillmann et al., 1996; De et al., 2008; Pawar et al., 2009; Garg et al., 2012) or cloned embryos (Dutta et al., 2011). Primary colony formation was higher when ICMs were mechanically isolated from hatched blastocysts (De et al., 2008). These cells could be maintained for up to nine passages on a goat fetal feeder layer and expressed both AP and the OCT4 gene. Caprine ES-like cells could be cultured in vitro on goat feeder cells and in the presence LIF for 22 passages, had a normal karyotype and expressed AP, OCT4, SOX2, NANOG, TRA-1-60, TRA-1-81 and SSEA-4, (Garg et al., 2012). Moreover, these cell lines formed embryoid bodies and could be differentiated into rhythmically beating cells that stained positive for cardiac-specific markers including α-actinin, C-troponin and α-myosin heavy chain (Garg et al., 2012). Putative ES-like cells were derived from blastocysts cloned from fibroblasts, ES-like cells and lymphocytes (Dutta et al., 2011). These ES-like cells stained positive for AP, OCT4, SOX2, NANOG, TRA-1-60 and TRA-1-81 and formed embryoid bodies with derivatives from the three germ layers. Goat ESC-like cells have been derived from embryonic disks cultured in vitro for 12–14 days and maintained on goat fetal fibroblast feeder supplemented with LIF. These cells demonstrated long-term proliferative capacity (>120 passages) and gave rise to well-differentiated tumors containing representative tissue from the three main germ layers (Behboodi et al., 2011).

Horse (Equine)

Isolation of equine ESCs is hampered by the shortage of oocytes and embryos and the deficient in vitro embryo production systems in this species. Attempts to isolate ESC lines of the horse were almost exclusively conducted with in vivo blastocysts (Saito et al., 2002; Li et al., 2006a; Guest and Allen 2007; Desmarais et al., 2011); only one study used cloned and parthenogenetically activated blastocysts (Desmarais et al., 2011). The first attempts to isolate horse ES-like cells were reported more than a decade ago (Saito et al., 2002). Two ES-like cell lines composed of tightly-packed compacted cells were established from in vivo derived equine blastocysts recovered on Days 6–7 after fertilisation. These lines proliferated over 38 and 56 passages; maintained a normal diploid karyotype and expressed AP, SSEA-1, STAT3 and OCT4. They had the capacity to differentiate in vitro into hematopoietic and endothelial cell lineages. In a subsequent study, two equine ES-like cell lines, isolated from in vivo-derived Day 7–8 equine blastocysts, closely resembled mouse ESC morphology with a small cytoplasmic to nuclear ratio and prominent nucleoli (Li et al., 2006a). The cells proliferated over 15–28 passages on inactivated mouse or equine fetal fibroblast layers supplemented with LIF and expressed stem-cell markers such as AP, SSEA-1, TRA-1-60, TRA-1-81 and OCT4, which is a primary pluripotency gene across differents species. In vitro differentiation into derivatives of the three germ layer cells (endoderm, mesoderm and ectoderm) was observed, whereas no in vivo differentiation potential was found as indicated by the lack of teratoma fomation (Li et al., 2006a).

It is important to mention here that all the above mentioned ES cell lines derived from different farm animals fulfill some of the criteria of being ES cells, like cell surface marker, expression of pluripotency specific genes and in some cases formation of teratoma was also shown. Even in the case of Telugu et al., 2011 derivation of naïve ES cells were claimed but in none of the instances, high-frequency chimerism with germ line transmission was reported. So the establishment of true ES cells and ES cell-mediated transgenesis is still a challenge in these animals.

Why is it so Difficult to Derive ES Cells in Domestic Ungulates?

As described earlier that naïve ES cells are truly germline competent cells but attaining naïve state seems difficult in ungulates and primates including human. The question here is that what makes rodents so different from all other mammalian species when it comes to the derivation of ES cells. Two aspects of early embryonic development are typical of rodents: the formation of the so-called egg cylinder and the possibility for early embryos to enter a quiescent state called diapause.

After blastocyst formation in mammals, the epiblast and hypoblast together form a flat multilayer structure known as the embryonic disc. Rodent embryos instead form the egg cylinder which required thorough re-organization of the epiblast that assumes a cup shape surrounded by hypoblast (Coucouvanis and Martin 1999). This peculiar shape and correlated morphological changes may substantially delay the transition from the naïve to primed epiblast making the former the default state when cells are isolated from pre-implantation blastocysts. In contrast, the flattened shape of the embryonic disc may favor such transition making it practically impossible to catch the naïve state before it evolves directly to the primed state establishing EpiESC lines (Gandolfi et al., 2012). A second consideration that may mitigate ES cell derivation from farm animals or primates is diapause. This is the state of

arrested embryonic development that occurs naturally in rodents when suckling mother becomes pregnant. This state can be induced experimentally by lowering blood estrogen level to prevent the uterus from becoming receptive for implantation. In diapause, embryo arrest synchronously in the late blastocyst after segregation of epiblast and hypoblast. Cell division at this stage is significantly reduced, and the naïve epiblast has an intrinsic capacity of self-renewal in these species. LIF/Stat3 signaling is required in the epiblast during diapause but not for the development without diapause (Nichols   and Smith 2009). So the capacity of epiblast self-renewal in diapause provides the biological foundation for in vitro propagation of ground epiblast. That's why ES cell derivation is problematic in ungulates which do not exhibit diapause.

Apart from these many extrinsic and intrinsic factors are involved which make the ungulates ES cell derivation a challenge. An initial problem in the isolation and culture of ungulates ES cells is in recognizing contaminating cell types such as trophectodermal (TE) and visceral endoderm cells in the primary culture of ICMs that may be mistaken for ES cells (Keefer et al., 2007). Putative ES cells of ungulates should be tested for markers for trophectoderm and visceral endoderm (Talbot et al., 2000a; Shimada et al., 2001; Miyazaki et al., 2002). What point in the pre-implantation development of ungulate blastocysts is best for the isolation and establishment of ES cell lines is also not known. Another challenge is the ability to dissociate epiblast cells from one another effectively. This sensitivity to cell to cell dissociation is more pronounced in the epiblast cells of ungulates. Primary culture of Alkaline Phosphatase positive undifferentiated cells is susceptible to lysis by either physical manipulation, withdrawal of calcium or exposure to trypsin-EDTA (Talbot et al., 1993b, 1995; Talbot and Garrett, 2001). Perhaps the most significant problem in the establishment of ungulates ES cell lines is the spontaneous differentiation. Primary culture of farm animal epiblasts plated on STO feeder cells spontaneously differentiate into multiple cell types (Talbot et al., 1993a, b, 1994, 1995, 1996, 2002). Larger initial colonies of epiblast cells tend to differentiate sooner and the smallest, consisting of 20 or fewer cells slowly die off. Cytokines like LIF and bFGF which stops differentiation in mouse and primates are not found useful in ungulates ICM (Moore and Piedrahita, 1997; Talbot et al., 1993b, 1995). Another problem is the lack of true pluripotent cells marker in ungulates because markers exclusive to mouse and human ES cells may not be useful in recognizing ungulates ES cells. For example, Oct4 is found to be expressed in TE of ungulates far longer than in the mouse (Van Eijk et al., 1999, Kirchoff et al., 2000, He et al., 2006). Nanog is expressed both in the epiblast, and TE of cattle (Munoz et al., 2008b) and expression of Sox2 and Nanog has been noted in at least one porcine TE cell line (Bloomberg et al., 2008). In cattle, cell surface marker SSEA-1, 3, 4 and TRA-60 & TRA-1-81 are expressed both in ICM and TE (Munoz et al., 2008b). So it is essential to use a broad panel of markers to define ES cells form ungulates and that not all these will necessarily be matched with either of mouse or human.

Strategies for the establishment of farm animal's naïve ES cells

Understanding of the molecular mechanism underlying maintenance of pluripotency and antagonistic signals that operate for differentiation of stem cells have given some clue to derive true ES cells from non-permissive mouse strain such as Non-Obese Diabetic (NOD) mouse as well as primates and farm animals by manipulating culture conditions and ectopic expression of pluripotency genes. The naïve and primed state are interconvertible. Mouse ES cell can be converted into EpiSCs by adding bFGF and activinA instead of LIF (Guo et al., 2009) in the culture medium while the reverse is possible only by genetic manipulation, i.e., overexpression of transcription factor Klf4, Klf2, Nanog or Nr5a (Guo et al., 2009; Guo and Smith, 2010; Silva et al.,2009) or small molecule inhibitors. It has been seen that the regulation of Klf4 transcriptional circuitry is important in naïve pluripotency. Wnt and ERK1/2 signaling pathways also play important roles. Wnt and ERK pathway work antagonistically where induction of Wnt pathway maintains pluripotency, but ERK causes differentiation. So NOD ES cells have been derived either by overexpression of Klf4 or c-myc and alternatively by activation of the Wnt pathway (using GSK3b inhibitor PD0325901) and inhibition of ERK pathway (ERK inhibitor CHIR99021). The latter combination of PD and CH is called as 2i medium (Hanna et al., 2009). PD can be substituted by Kenpaullone (KP) which is a GSK3b and CDK1/Cyclin B inhibitor. Using the same strategy Rat ES cells derivation has been reported. Alternatively, Rat ES cells can also be derived by overexpression by Oct4, Sox2, Nanog, and Lin28. Primed human ES cells can be converted into naïve by doxycycline-induced Klf4 or C-myc expression plus medium having PD/CH/LIF. Forskolin (FK) is a protein kinase A agonist which can induce Klf4 and Klf2 expression. So primed hES cells can also be converted into naïve by adding PD/CH/LIF/FK in the culture medium. FGF4 signaling through the MEK/ERK pathway drives ES cells to commitment. Genetic impairment or selective chemical blockade of this pathway can sustain self-renewal of ES cells even in the absence of LIF signaling. One of the potent inhibitors of FGFR is SU5402. SU+PD+CH is called 3i medium and using this, mES cell from non-permissive strains have been derived (Ying et al., 2008). All these cultures were LIF dependent. Similar strategies have been used to derive naïve ES cells from mouse, Rat, human, and pig.

Naïve pluripotency

Species	Culture conditions	Authors
Mouse (permissive)	LIF/BMP4 or 3i	Ying et al, 2008
Mouse (non-permissive)	LIF + 2i	Hanna et al, 2009
Rat	LIF + 2i or 3i	Buehr et al, 2008
Rat	3i	Li et al, 2008
Human	LIF + 5 factors (Oct4, Sox2, Kf4, C-Myc, Nanog)	Hanna et al., 2010
Human	LIF + 2i + 4 factors (Oct4, Sox2, Nanog, Lin28)	Buecker et al., 2010
Pig	LIF + 2 factors (Klf4, Oct4)	Telugu et al., 2011

Apart from these, naïve human ES cells were generated either by converting primed ES cells or directly from the embryo. For example, Ware et al. (2014) converted primed hES cells into naïve by adding histone deacetylase inhibitors butyrate and suberoylanilide hydroxamic acid followed by culture in 2i and FGF2. The same group isolated naïve hES cells by growing the ICM in the presence of 2i and FGF2. So like the mouse, rat, and human, it is possible to derive naïve ES cells from farm animals also. However, we need to define culture conditions better to be confident that a line retains full developmental competence. Once culture conditions are perfected, which requires a tighter definition of small molecule inhibitors and optimal growth factor use, naive lines that may best correlate to the in vivo ICM will likely be derived de novo from preimplantation ungulates embryo.

Conclusion

Stem cell research has the potential to expand our knowledge of basic and system biology, and using this technology will allow the veterinary field to translate stem cell research into treatment for many chronic diseases. Naive pluripotent ES cells from ungulates could be used for the generation of biomedical models, and it will help in accelerating animal breeding and transgenesis. Still, we do not have those naive state pluripotent stem cells but are much closer than ever before. The use of the ectopic expression of pluripotency genes or small molecules inhibitors and histone deacetylase inhibitors is recent and not much parallel works have been done in case of farm animals. But there is enough evidence that using the strategies mentioned above it is very likely that true ES cells from ungulates can be established as these animals closely resemble human in many ways.

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