Challenges and prospects for the establishment of embryonic stem cell lines of domesticated ungulates

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Abstract

Embryonic stem (ES) cell lines provide an invaluable research tool for genetic engineering, developmental biology and disease models. These cells can be maintained indefinitely in culture and yet maintain competence to produce all the cells within a fetus. While mouse ES cell lines were first established over two decades ago and primate ES cells in the 1990s, validated ES cell lines have yet to be established in ungulates. Why competent, pluripotent ES cells can be established from certain strains of mice and from primates, and not from cows, sheep, goats or pigs is an on-going topic of interest to animal reproduction scientists. The identification of appropriate stem cell markers, functional cytokine pathways, and key pluripotency-maintaining factors along with the release of more comprehensive bovine and porcine genomes, provide encouragement for establishment of ungulate ES cell lines in the near future.

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1. Introduction

One of the most truly amazing displays of long-term cellular competence is that of embryonic stem (ES) cells. While these cells cannot be strictly considered as embryos, they do maintain the competence to contribute to the formation of an embryo upon combination with a host embryo (chimera production) and display pluripotency in that they can contribute to all tissues and organs of the chimeric mouse, including the germ cells. One of the big questions in regards to ES cells...
is: why it is so difficult to derive and maintain ES cells from embryos other than those from a few strains of mice?

A related question is to what extent does the competence of the starting material affect the process? In vitro-produced embryos have a lesser developmental competence than in vivo-derived embryos which is, in part, due to poorer inner cell mass (ICM) quality (Bavister, 2004). Because ES cells are derived from the ICM, the establishment of ES cells may be considered, in some aspects, a measure of the developmental competence of the ICM. But this does not explain the lack of success using in vivo-derived embryos, which should have a more developmentally competent ICM. Could inadequate culture conditions for sustaining embryonic development be disrupting the developmental competence of in vivo-derived embryonic stem cells? Furthermore, it is clear that in order to improve the prospects for establishment of ES cells in ungulates and other domestic animals, new approaches with improved criteria for defining ungulate embryonic stem cells are required. Continued propagation of poorly defined cell lines may mislead researchers in their search for appropriate culture conditions for true ES cell proliferation.

ES cells, pluripotent cells with the capacity for long-term propagation and broad differentiation plasticity, were first isolated from the ICM of mouse blastocysts over two decades ago (Evans and Kaufman, 1981). Because these cells are competent to form all cell types within the fetus, they are considered pluripotent depending on the particular cell line or environmental context. Whether or not mouse and primate ES cells can be considered truly totipotent, that is capable of forming all cell types including placental tissue, is debatable. Under certain limited conditions mouse ES cells can form trophectodermal cells in vitro (Ralston and Rossant, 2005) and in vivo (Beddington and Robertson, 1989), while human and marmoset ES cells can differentiate readily into trophectoderm cells (Thomson et al., 1995; Xu et al., 2002). However, strong ES cell contribution to the entire conceptus following chimera formation has not yet been demonstrated in either mice or non-human primates. In addition to their pluripotent/totipotent capabilities, ES cells can be clonally propagated and maintained in culture indefinitely. These characteristics have made them an invaluable research tool for genetic engineering, developmental biology and disease models.

The establishment of ES cell lines of domesticated ungulates, e.g., the pig, sheep, goat, cow or horse, is of interest for similar reasons to those for mouse ES cell lines. Several applied research initiatives await the establishment of ungulate ES cell lines. These include the creation of models of human genetic diseases and cell transplantation therapies. Domesticated ungulate ES cell lines would also be useful in the precise genetic engineering of these animals for improved production traits and products, for disease resistance and for biopharming. Ungulate ES cell lines could be used to accomplish these latter goals, either through chimera technology already well established in producing genetically modified mice (Bradley and Robertson, 1986; Bradley, 1987; Wells et al., 2003), or by improving the efficiency of somatic cell nuclear transfer (SCNT) technology that is currently used in the production of genetically engineered ungulates (Rideout et al., 2000; Keefer, 2004; Donovan et al., 2005; Wall et al., 2005). The immediate problem for these scientific goals is that no “proven” ungulate ES cell lines currently exist, despite the many peer-reviewed journal articles describing ungulate ES or ES-like cell lines over the past 15 years. Is this lack of success due to differences in competence of the starting material, i.e., the embryos themselves, or to the treatments used to initiate and maintain ES cells in vitro? In general, mouse ES cell lines are generated from in vivo-derived embryos. Given the low efficiencies of ES cell derivation even from in vivo-derived mouse embryos (McWhir et al., 1996), are researchers working with domestic animals hampered by their use of in vitro-produced embryos and/or by suboptimal
culture conditions? These and other factors affecting the derivation of ES cells from ungulate embryos will be discussed.

2. Ungulate ES cell lines

Over the past 15 years, many reports of porcine, bovine, caprine, ovine and equine ES cell lines, or what are often presented as “ES-like” cell lines, have been published. Similarly, a few ES-like cell lines from rodents other than the mouse (hamster, Doetschman et al., 1988; rat, Iannaccone et al., 1994; Vassilieva et al., 2000) and from rabbit (Schoonjans et al., 1996) have also appeared. None of these cell lines has been definitively proven to be ES cells, and, to our knowledge, none has been successfully used as a biological reagent in a manner similar to the use of human, monkey or mouse ES cells, i.e., directed pluripotent in vitro differentiation (Perrier et al., 2004; Takagi et al., 2005) or as a means of genetically engineering a mammal through embryonic chimera formation (Bradley, 1987).

ES cell lines were first isolated from explant cultures of in vivo-derived mouse blastocyst-stage embryos (Evans and Kaufman, 1981; Martin, 1981; Robertson, 1987). However, ES cell lines have also been derived from eight-cell and morula-stage embryos prior to the first embryonic differentiation events, i.e., formation of the ICM and trophectoderm of the blastocyst (Eistetter, 1989; Delhaise et al., 1996; Strelchenko et al., 2004). Generally, primate ES cell lines have been established from in vivo-derived blastocysts of monkeys (Thomson et al., 1995) and in vitro-fertilized (IVF) in vitro-cultured (IVC) blastocysts of humans (Thomson et al., 1998; Reubinoff et al., 2000; Lee et al., 2005a). Attempts to create ES cell lines of the pig, goat, sheep and horse have most often used in vivo blastocysts acquired from the reproductive tract at various stages, but generally from the early blastocyst stage to the later elongated or filamentous stage. Efficient and cost effective in vitro-produced (IVP) embryo culture systems are commonly used for cattle and, in this species, early blastocyst-stage embryos are the most frequent starting material for attempts at making ES cell lines. Production of these IVP embryos usually involves in vitro-maturation, IVF and culture to the morula or blastocyst stages. Although IVP blastocysts may be altered in terms of cell metabolism, epigenetic status and constituent cell numbers, it is probable that some will prove competent for the establishment of bovine ES cell lines. This conjecture seems reasonable since human ES cell lines have been derived from IVF/IVC embryos (although the oocytes were in vivo-matured), and because culture of IVP-derived bovine epiblast tissue displays normal differentiation and pluripotency in vitro (Talbot et al., 1995). Furthermore, IVP ungulate embryos are capable of establishing viable pregnancies following transfer into recipients.

Peer-reviewed reports of porcine ES, ES-like or ICM cell lines have been published by at least six groups, all of which used in vivo-derived blastocysts as their primary culture material (Notarianni et al., 1990, 1991; Piedrahita et al., 1990; Hochereau-de Reviers and Perreau, 1993; Wheeler, 1994; Chen et al., 1999a; Li et al., 2003, 2004a,b). Putative pig ES cell lines were also isolated from IVP pig embryos (Li et al., 2004a), as were several bovine ES or ES-like cell lines from IVP early blastocyst-staged embryos (Evans et al., 1990; Saito et al., 2002, 2003; Sims and First, 1994; Cibelli et al., 1998; Iwasaki et al., 2000; Mitalipova et al., 2001; Wang et al., 2005). Production of ovine, equine and caprine ES-like cell lines has also been reported (Notarianni et al., 1991; Keefer et al., 1996; Saito et al., 2002). Finally, although not the particular subject of this review, there are several reports of similar ES-like cell lines of the pig, goat and bovine that were derived from primordial germ cells found in the early genital ridge tissue, sometimes referred to as embryonic germ (EG) cell lines (Piedrahita et al., 1997; Shim et al., 1997; Mueller et al., 1999; Tsung et al., 2003; Rui et al., 2004). In the case of mouse and human EG cell lines, the cell
lines have been proven or are assumed to be functionally equivalent to ES cell lines (Matsui et al., 1992; Resnick et al., 1992; Shamblott et al., 1998).

While the above-mentioned studies have provided valuable information on ungulate embryonic cell culture, future studies need to focus more intensely on the critical characteristics that define true ES cell lines. The essential criteria include proof of continuous self-renewal (immortality), clear descriptions of morphology, use of cell-specific markers and demonstration of true pluripotency. Proofs of immortality over continuous culture, a key attribute of human and mouse ES cell lines (see below), require growth curves that demonstrate the rate of replication of the putative ES cell cultures. Long-term survival in culture is not sufficient proof because it does not demonstrate self-renewal. Furthermore, morphology of putative ungulate ES cells must be displayed through high quality images. Morphological and molecular marker comparisons to trophectoderm and visceral endoderm cells must be performed in order to prove that the cells are not derived from these cell lineages. Because trophectoderm and visceral endoderm of ungulates are easily cultivated on feeder cells, have well demonstrated morphologies, and have known cell-specific gene/protein expressions, these criteria can easily be satisfied and should not be overlooked (Flechon et al., 1995; Talbot et al., 2000a, 2005; Shimada et al., 2001; Miyazaki et al., 2002). Demonstrations of in vitro pluripotency supported by immunocytochemical evidence must include high resolution micrographs with adequate immunological and cell controls. Proofs of ES-like cell differentiation based on RT-PCR data also most include adequate controls and specificity. Samples tested should be free of feeder cells or provide proof that the differentiation markers are not produced by the feeder cells. Feeder cells may contain a mixture of cell types or have undefined gene expression profiles which can complicate interpretation of results (Talbot et al., 2004). Furthermore, demonstrations of embryoid body formation by ungulate ES-like cells must differentiate embryoid-like bodies from similar multicellular vesicle-like bodies that are commonly produced by the anchorage-independent growth of ungulate trophectoderm and visceral endoderm cells, or, for that matter, by other polarized, dome-forming epithelial cell lines (see Robertson (1987) for a clear description of mouse ES cell embryoid bodies).

A common proof of the pluripotency of primate and mouse ES cell lines is that of teratoma formation in immunocompromised mice. This in vivo demonstration of pluripotency has been lacking for reports of putative ungulate ES cell lines, with the exception of the study by Hochereau-de Reviers and Perreau (1993), even though Anderson et al. (1996) demonstrated that it was feasible to obtain bovine and porcine teratomas in athymic mice using ICM cells. What is commonly reported is the use of the ES-like cell lines to produce chimeric animals by injection of the ES-like cells into blastocyst-stage ungulate embryos or by some related chimera-formation technique. In general, these efforts produce animals displaying minor chimera contribution from the ungulate ES-like cells, and, in all cases, cell controls were not done, i.e., the injection or combining of somatic cells, such as ungulate trophectoderm cells, were not performed to see what level of chimerism they would produce. This lack of somatic cell controls is not unique to tests of ungulate ES cells pluripotentiality, but is also missing in mouse studies, and presents an intriguing question as to the possible multipotentiality of other cell types. Nevertheless, these studies do demonstrate some degree of ES-like pluripotentiality of the ungulate ES-like cells. The possibility of cell fusion between the ES-like cells and the cells of the embryo, however, should also be considered. The incidence of cell fusion contributing to transdifferentiation of somatic stem cell following in vivo cell therapy is currently being debated (Herzog et al., 2003) and needs to be ruled out in cases of low chimeric contribution.

Some ungulate ES-like cell lines were used as nuclear donor cells to create cloned animals by nuclear transfer (Chen et al., 1999a; Saito et al., 2003). The use of the cells in nuclear cloning in
no way proves their ES cell character since numerous types of fully differentiated somatic cell nuclei have proven competent for the creation of cloned animals (Kato et al., 2000; Wakayama and Yanagimachi, 2001), and, in particular, nuclear cloned animals have also been created from trophectoderm cells (Tsunoda and Kato, 1998). The latter demonstration is significant because trophectoderm cells are a common cell contaminant in attempts to establish ungulate ES cell lines and are easily confused with epiblast cells (see below).

3. Definition of ES cell lines

ES cell lines are termed continuous cell lines and as such have the property of immortality, i.e., the cell cultures can be maintained indefinitely by continuous passages (Suda et al., 1987; Amit et al., 2000). This is in contrast to cell cultures that can be passaged for only limited periods of time and that can be referred to as finite cell lines (Freshney, 1994). The term “cell line” implies the maintenance of the cell culture’s original phenotype during continuous culture or passage, i.e., hundreds of population doublings, and also implies homogeneity of phenotype within the population of cells. For example, a muscle cell line should contain only myocytes and not myocytes and epithelial cells. For ES cell lines, this phenotypic definition is that of pluripotency, i.e., most of the cells should be capable of giving rise to somatic cells representative of the three primary embryonic germ layers (Evans and Kaufman, 1981; Martin, 1981). Furthermore, they should be able to differentiate into the germ cells, i.e., oocytes and spermatozoa (Bradley, 1987; Hubner et al., 2003; Geijsen et al., 2004). Therefore, similar to the pluripotent ICM cells of blastocysts, ES cells should be competent to contribute to all cells of the developing fetus. Also by definition, ES cell lines must be able to self-renew as stem cells; thereby maintaining their ability to differentiate into all cell types.

These definitive properties should not imply that ES cell lines must remain unchanged over time in continuous culture to be considered authentic ES cell lines. On the contrary, as with all cell lines the population of cells that comprise any ES cell line is subject to internal and external selective pressures. Stochastic events that are operating in each cell as it grows and divides will influence its comparative survival fitness within the population and within the given culture environment. So, by definition, as time in culture progresses, the various properties of the ES cell population will change; for example, the fastest growing cells will become a larger and larger proportion of the population of cells over time. Some stem cell traits are seemingly lost very early in passage, such as the ability to create live-born young that are completely ES-cell derived (Nagy et al., 1993). Over further passages, karyotypic abnormalities become more common within the population, and cell line competence for germ line chimera contribution can also be lost (Bradley, 1987; Robertson, 1987). However, lost properties definitive to ES cells can probably be restored in many, if not all, ES cell lines by recognizing that the cell line is a population of individuals and that each individual cell is phenotypically and genotypically different, albeit by sometimes extremely small measures. Differences even between two daughter cells have been described (Smith and Whitney, 1980). So, through single-cell cloning from the population of cells and screening the clonal populations for normal karyotypes, it has been possible to maintain the embryonic stem cell character of ES cell lines over extensive continuous culture (Suda et al., 1987).

4. Cell culture properties of ES cell lines

ES cell lines have similar cell culture properties regardless of the species of origin or the tissue of origin, i.e., derivation from the ICM of the blastocyst-stage embryo or derivation from the
primordial germ cells of the embryonic genital ridge. Primary cultures of epiblast cells also share many culture characteristics and cellular features in common with ES cells.

ES cells have a distinct, although not unique, colony morphology. The cells of murine ES cells typically grow in compact colonial groups or ‘nests’ of cells that often have a convex, 3D shape and a distinct, glistening edge that meets with the flatter feeder cells on which ES cells

Fig. 1. Primary bovine ICM outgrowth on STO feeder cells showing (A) trophectoderm (t) and primitive endoderm (pe) monolayers, 200×, (B) primitive endoderm monolayer only, 100×, and (C) epiblast cell mass (e) surrounded by primitive endoderm cells (pe), 400×; arrowheads indicate boundary between epiblast mass and primitive endoderm. Note characteristic lipid droplets in trophectoderm cells and characteristic web-like network of robust microtubules in fully mature endoderm cells. (D and E) “ES-like” morphology of (D) pig trophectoderm cell line and (E) a pig epiblast-derived epithelial cell line (PICM-16). Note prominent lipid droplets in the trophectoderm cells. (F) Primary colony of pig epiblast cells after 24 h of culture on collagen type I (200×). Pure epiblast cells were isolated as described by Talbot et al. (1993b).
are often co-cultured (Robertson, 1987). The ES cells generally grow on top of or in between the feeder cells. Mouse ES cell colonies grow quickly to contain hundreds if not thousands of cells per colony, and the colonies will eventually fuse with one another to form monolayers if there are sufficient colonies in close proximity. If left undisturbed, i.e., not routinely passaged every several days, murine ES cells will begin to spontaneously differentiate at the periphery of the colony with the formation of flatter, larger and irregularly cuboidal visceral endoderm cells. With time, other somatic cell types may appear in or around the differentiating colony. Primate ES cell colony morphology is different from mouse ES cells in that the monkey or human ES cells are generally flatter in appearance and spontaneous differentiation tends to begin in the center of colonies if they are left undisturbed for a week or more without passage (Thomson et al., 1995, 1998; Thomson and Marshall, 1998). The primary colony morphology of ungulate epiblast cells is very similar to that of primate ES cells, and, likewise, spontaneous differentiation in ungulate epiblast cell colonies begins in the center of the colony (Talbot et al., 1993a,b, 1995).

Cell morphology of ES cells and primary cultures of epiblast cells are very similar across species (Fig. 1; Robertson, 1987; Talbot et al., 1993b; Thomson et al., 1995, 1998; Thomson and Marshall, 1998). The cells are generally uniform in size (10–15 μm in diameter) with a round to oval shape. Perhaps their most distinctive morphological feature when viewed by phase-contrast microscopy is their large nucleus surrounded by a narrow band of non-granular cytoplasm. Also, most nuclei are observed to contain one or two very large and distinct nucleoli. Transmission electron micrographs of mouse ES cells indicate that they usually lack or have minimal mature complex junctions/tight junctions between adjacent cells (Robertson, 1987; Hogan et al., 1994; Eshkind et al., 2002). Primate ES cells are similar to murine ES cells, but may display some complex epithelial junctions, particularly in the outer cells of multilayered colonies, and they appear to lack well-developed junction-associated tonal filaments (Park et al., 2004; Thomson and Marshall, 1998). An ultrastructural study of the ICM of in vivo pig blastocysts and of primary cultures of pig epiblast cells showed that, in contrast to primate and murine ES cells, the pig epiblast cells develop relatively robust complex junctions/tight junctions shortly after blastocyst formation (Talbot and Garrett, 2001). The cultured pig epiblast cells also have well-developed apical adhesion belt structure with associated actin filament bundles, typical of mature polarized epithelium (Talbot and Garrett, 2001). Given the above information, it seems probable that ungulate ES cell lines, once established, will be most similar to primate ES cells in colony and cell morphology.

5. Molecular markers of ES cells

A limitation to the establishment of ES cells in domestic species is the capability to identify truly pluripotent cells. In mice and primates, stem cell marker gene expression is generally limited to the ICM and is not observed in the trophectoderm (Henderson et al., 2002). There are considerable differences among mammalian species in regard to early embryonic development. While human and ungulates both form a germinal disk during early embryonic development (mice form an egg cyclinder), ungulates have an extended period prior to attachment (implantation) during which the trophectoderm undergoes extensive proliferation and elongation (Stroband et al., 1984; Gallicano, 2001). This extended period of proliferation may be reflected by altered patterns of gene expression, including those normally considered ICM-specific (van Eijk et al., 1999; Kirchhof et al., 2000; He et al., 2006). Therefore, identification of stem cell exclusive markers for domestic species must be scrutinized carefully.

The best approach will be to use a panel of markers because any one marker may not be truly definitive. In the past, researchers have depended on only a few markers, e.g., OCT4 (POU5F1),
a single stage-specific embryonic antigen (e.g., SSEA1) or alkaline phosphatase (AP), without a clear understanding of the expression of these gene markers in embryos of different species. Recent work has indicated that these markers, used alone, may not be sufficient as they are not specific to the ICM in ungulates. For example, several different laboratories have now shown that

![Image](https://via.placeholder.com/150)

**Fig. 2. OCT4 mRNA and protein in extraembryonic tissue of in vivo-derived pig elongating blastocysts (11 days post-fertilization).** Immunohistochemical analysis of cell nuclei from embryo trophectoderm (A) and porcine PICM-19 cell line (Talbot et al., 2002) (B) labeled with rat monoclonal anti-human-OCT4 antibody (R&D Systems, Minneapolis, MN) were OCT4 positive and negative, respectively; corresponding trophectoderm (C) and PICM-19 (D) nuclear areas stained with DAPI (4′,6-diamidino-2-phenylindole; Sigma Chem. Co., St. Louis, MO), 400×. RT-PCR analysis for the expression of OCT4 by extraembryonic trophectoderm from gestational day 11 ovoid (∼9 mm) and tubular (>30 mm) embryos (E) with lane L, molecular marker ladder; lane 1, negative control (ovoid RNA, no reverse transcriptase in RT); lane 2, OCT4 transcript from ovoid embryo trophectoderm; lane 3, negative control (tubular RNA, no reverse transcriptase in RT) and lane 4, OCT4 transcript from tubular trophectoderm. RT-PCR analysis for the expression of OCT4 by pig visceral endoderm (PE) cell lines (F), PE-1 (lanes 1 and 2) and PE-2 (lanes 3 and 4) at passages 12 and 11, respectively; lane 5, STO cell negative control. Total RNA from embryos or cell lines was isolated and processed for RT-PCR assay as previously described (Blomberg et al., 2005). Forward and reverse primer sets (5′-GCAAACGATCAAGCAGTGAG-3′ and 5′-GGTGACAGACACCGAGGAA-3′, respectively) define a 200 bp OCT4 amplicon (GenBank accession # TC205936).
OCT4 protein is expressed in both the ICM and trophectoderm of ungulates, i.e., pigs, cattle and goats (van Eijk et al., 1999; Kirchhof et al., 2000; He et al., 2004). Furthermore, recent tests in our laboratories have detected OCT4 gene expression by RT-PCR in porcine endoderm cell lines (Fig. 2) and caprine embryonic cell cultures, and, by immunocytochemistry, in the trophectoderm of day 11 porcine ovoid blastocysts (Fig. 2) and expanded caprine blastocysts. We have also shown that SSEA1 and SSEA4 genes are expressed by both ICM and trophectoderm of caprine embryos (He et al., 2006). Similarly, the AP gene appears to be expressed at different times by either epiblast or trophectoderm (Talbot et al., 1995; Vejlsted et al., 2005). Therefore, these markers should be considered characteristic of ES cells, but may not be used as definitive markers.

Researchers working with farm animals now have new resources (e.g., gene banks, BAC libraries, microarrays and quantitative real-time PCR) which, along with rapid advances being made in mouse and human stem cell biology, should aid in identifying specific markers of pluripotency. Several studies have been published in which sequential analysis of gene expression (SAGE), expressed sequence tags (EST) and/or microarrays have been used to compare and identify unique expression patterns of transcripts found in human and mouse stem cells. Depending on the study, comparisons were made between either differentiated versus undifferentiated ES cells, mouse versus human ES cells, or adult compared with embryonic stem cells (Bhattacharya et al., 2004; Ginis et al., 2004; Wei et al., 2005). Through such studies, a list of potential “stemness-related genes” have been identified. This global gene survey approach is very useful for the identification of candidate genes and pathways for further study. The genes and pathways that appear to be related to “stemness” in mouse and primate ES cell lines are good candidates for study in embryo-derived ungulate cell lines. For example, Nanog and other newly identified mouse and human ES cell-specific genes should aid in identifying ES cells in ungulates. The expression pattern(s) of these genes in ungulate embryos during early development, however, must be determined first. NANOG does appear to be a specific marker of pluripotency for ruminants because NANOG mRNA and protein are found in the ICM and strongly down-regulated in the trophectoderm of caprine blastocysts (He et al., 2005, 2006). Whether other apparently ES cell-specific genes, such as ZFP42 (Rex1), TDGF1 (Cripto) and Esg1 (Dppa5) may prove useful has yet to be confirmed for ungulates (Bhattacharya et al., 2004).

Accepted genetic nomenclature uses italics to distinguish the gene (DNA and mRNA) from the protein and capitals to distinguish analogous genes (orthologs) in humans, non-human primates and domestic species from those identified in mice. Hence, the mouse gene is Nanog and the bovine gene is NANOG.

6. Challenges in the establishment of ungulate ES cell lines

6.1. Recognition of ES cells

An initial problem in the isolation and culture of ungulate ES cells is in recognizing contaminating cell types in the primary culture of blastocysts or ICMs that may be mistaken for ES cells. When the entire blastocyst is used to initiate a primary culture, trophectoderm, endoderm and epiblast cells may all survive and grow in the culture. However, of the three cell types present, only epiblast cells are the source of ES cell lines (Brook and Gardner, 1997). The blastocyst’s outer trophectoderm cells can be eliminated by immunodissection, but the isolated ICM is composed of both epiblast tissue and a visceral endoderm cell layer (Solter and Knowles, 1975; Talbot et al., 1993b). Furthermore, some trophectoderm cells can often survive the procedure (unpublished observations). Similarly, physical dissection methods for isolating the ICM should be assumed
to always leave viable trophectoderm cells attached to the ICM. So, as with mouse and human primary blastocyst or ICM cell cultures, trophectoderm and endoderm cells may be mistaken for ES cells and are in one sense “weeds” in the primary culture (Robertson, 1987; Talbot et al., 1993b). This is particularly true with ungulate cells since their trophectoderm and visceral endoderm are very resilient epithelial cells that grow rapidly in feeder-cell co-cultures (Talbot et al., 1993b, 1995, 2000a, 2005; Flechon et al., 1995; Shimada et al., 2001; Miyazaki et al., 2002). Although the cell morphology of ungulate trophectoderm and endoderm is distinctly different from that of primary ungulate epiblast cells (Fig. 1), and from each other, to the inexperienced eye they can appear similar. Therefore, as an essential control, it is important that putative ES cells of ungulates be tested for markers of trophectoderm or visceral endoderm cells. For example, a definitive marker of bovine, caprine and ovine trophectoderm is the expression of interferon-tau (Talbot et al., 2000a; Shimada et al., 2001; Miyazaki et al., 2002). For visceral endoderm, a specific marker (with the exception of hepatocytes) is the presence of serum proteins such as alpha-fetoprotein and transferrin (Talbot et al., 2000a). Furthermore, it is important to appreciate the fact that these two cell types, like other polarized or “dome-forming” epithelial cells, will make “embryoid-like” bodies if grown without attachment to a solid cell culture substrate. Therefore, these and other properties of ungulate trophectoderm and endoderm should be carefully evaluated. Investigators attempting to derive ES cells of ungulates should be thoroughly familiar with the in vitro morphology and gene expression of these extraembryonic tissues and should provide proofs that the cells they are claiming to be ES cells are in fact not trophectoderm or endoderm.

Other contaminating epithelial cells that might be confused with ES cells can also occur. The spontaneous differentiation of the primary epiblast cells to epithelial cell types is rapid and common (Talbot et al., 1993b, 1995). These differentiation events may go unnoticed by the inexperienced investigator because the epiblast cells are usually underneath the primary trophectoderm and endoderm outgrowths and are obscured from view or go unrecognized as epiblast cells. Many epithelial cells grown on fibroblast feeder-cell monolayers can look “ES-like”, especially shortly after passage, e.g., fetal kidney epithelial cells are often confused with primordial germ cells for this reason and also because kidney epithelial cells express the AP gene to a greater extent (Gibson-D’Ambrosio et al., 1987; unpublished observations). This ES-like morphology is even more pronounced if the feeder cells are prepared at a relatively greater density. As shown in Fig. 1, a pig epiblast-derived epithelial cell line exhibits an ES-like morphology shortly after passage. In some cases these epithelial cells may also be AP positive (Talbot et al., 1993a,b, 1995).

Finally, the feeder cells themselves may be a source of confusion in the identification and proof of ungulate ES cell lines. STO feeder cells, for example, are very pleomorphic and may resemble various cell types. Most notably, it is common for STO feeder cells to adopt the morphology of oligodendrocytes, astrocytes or neurons, particularly if they are exposed to various members of the fibroblast growth factor family (unpublished observations). The use of rodent or ungulate primary fetal fibroblasts as feeder cells necessarily introduces many different types of cells into the ES cell derivation culture system. For example, macrophages can comprise as much as 50% of the “fibroblast” population in the early passage mouse fibroblast cultures that are routinely used for preparing feeder cells (Talbot et al., 2004). Therefore, neurons, myocytes, endothelial cells and somatic stem cells, such as hematopoetic stem cells, may be present among the fibroblasts that comprise a primary or early secondary culture used for making feeder cells. While these cells are rendered non-dividing by treatment with mitomycin C or gamma irradiation, the very real possibility of their presence must be considered in the interpretation of sensitive RT-PCR assays that are so commonly performed as proofs of stem cell differentiation potency.
7. Timing of isolation and initiation of primary cultures

The optimal time for the initiation of blastocyst cell cultures for establishing ungulate ES cell lines is not known. Compared to mice and humans, the blastocysts of ungulates have an extended period of preimplantation development. The blastocysts of the pig, sheep and cow first develop at approximately 6–7 days post-fertilization. The blastocyst then increases in size relatively slowly over the next few days of development as a spherical form that increases in diameter. During this time there is probably only a modest increase in the number of epiblast cells in the ICM of the blastocyst compared with the increase in trophoderm and visceral endoderm cells. At 11–12 days post-fertilization, depending on the species, the blastocyst elongates by the rapid growth of the trophoderm and visceral endoderm to form a long, thin, filamentous blastocyst greater than 100 mm in length (Chang, 1952; Carlson, 1981). The epiblast is exposed to the uterine environment during this preimplantation development by the loss of the overlaying layer of trophoderm cells, or Rauber’s layer, and the ICM is thereafter referred to as the embryonic or germinal disc (Stroband et al., 1984). Gastrulation, marked by the formation of the primitive streak in the embryonic disc, begins during this elongation phase and at this point mesoderm differentiation and migration from the epiblast is well underway (Talbot et al., 2000b; Guillomot et al., 2004). What point in the preimplantation development of ungulate blastocysts is best for the isolation and establishment of ES cell lines? One report examined this question using whole blastocyst culture of in vivo-produced pig embryos that were judged to be early blastocysts, having just emerged from the zona pellucida or blastocysts that had “hatched” from the zona pellucida some hours earlier (Chen et al., 1999b). Early hatched blastocysts had many fewer trophoderm cells and a less flattened ICM. The success rate for the establishment of pig ES-like cell cultures was decidedly greater (12 cultures (21%) versus none) from recently hatched blastocysts than from late-hatched blastocysts (Chen et al., 1999b). Three of the resulting cultures were composed of AP positive cells, a marker of the undifferentiated state, and were purported to differentiate into multiple cell types in vitro, although no supporting evidence of this was shown. Another report used in vivo pig blastocysts from days 5–6 to 10–11 of gestation and found that days 10–11 blastocysts yielded ES-like cell cultures. Few or none were propagated from days 5 to 6 embryos or day 11 blastocysts that had elongated (Hochereau-de Reviers and Perreau, 1993). In our experience, AP positive, pluripotent epiblast cell cultures can be obtained from either the early pig blastocyst stage (7–8 days post-coitus) or from later stage embryonic discs (12–14 days post-coitus) when gastrulation has begun. No difference in the failure to inhibit spontaneous differentiation of the epiblast cells or in the inability to propagate the epiblast cells was noted across these time points (Talbot et al., 1993b; unpublished observations).

STO feeder cells were required for the survival of pig and bovine epiblast cells in primary culture (Talbot et al., 1993b, 1995). Without feeder-cell support, cultures of primary pig epiblast cells failed to grow, and instead, senesced and died over a 10–14 days period (unpublished observations). Similar results were reported with feeder-free, short-term, primary cultures of pig ICMs, with or without the addition of leukemia inhibitory factor (LIF) to the medium (Moore and Piedrahita, 1997). It is probable that ungulate ES cell line establishment will therefore require feeder cells, at least in their initial culture, as has been true for the establishment of most mouse and primate ES cell lines. Although for both mouse and human ES cell derivation STO feeder cells have been used successfully (Martin, 1981; Park et al., 2003), the use of primary or early secondary fetus-derived feeder cells is often thought to be an advantage. Primary feeder-cell populations presumably supply different kinds and amounts of factors for the maintenance and growth of ES cells compared with STO feeder cells. Moreover, the use of homologous primary feeder
cells, e.g., bovine fibroblasts for bovine epiblast culture, would presumably remove the potential problem of species specificity that exists with some cell ligand/cell receptor systems. However, primary fetal fibroblasts of the cow, which would be expected to produce bovine LIF and basic fibroblast growth factor (bFGF), and most likely several other growth factors or cytokines, did not maintain bovine epiblast cells in the undifferentiated state in our experience (unpublished observation). Likewise, caprine fetal fibroblasts could in some cases maintain caprine ES-like cells during short-term culture but usually stimulated these cells to differentiate rapidly (unpublished observations). It should be hoped that through further research it might be possible to replace feeder-dependent culture conditions for establishment of ungulate ES cell lines as has been done in mouse and human (Pease et al., 1990; Xu et al., 2001, 2005; Wang et al., 2005).

8. Dissociation and clonal propagation

Another challenge for the establishment of ungulate ES cells is the ability to effectively dissociate epiblast cells from one another. Typically, cells are separated from one another by treatment with enzymes (trypsin, collagenase, pronase) or in combination with chemical agents such as ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(β-aminoethyl ether)-N,N′,N′,N′-tetraacetie acid (EGTA), citrate or Ca²⁺/Mg²⁺-free phosphate buffered saline (PBS). By these treatments, a suspension of individual cells is created so that the culture can be subdivided for continued growth, and growth inhibition due to crowding can be reduced. Perhaps most importantly, the dissociation into single cells breaks down the cell-to-cell signaling that fosters stem cell differentiation. Thus, the routine passage of the cell population helps maintain the pluripotency of the cell population over time. Species differences appear to exist in the ease with which ES cells can be separated into a single-cell or near single-cell suspension.

The dissociation of primate ES cells into single-cell suspensions is a complicating factor in the culture of these cell lines. Enzymatic and chemical dissociation of human or monkey ES cells typically give re-plating efficiencies of less than 1% (Thomson and Marshall, 1998; J.A. Thomson, personal communication). In contrast, mouse ES cells are commonly dissociated by treatment with trypsin–EDTA for routine passage and maintenance of pluripotency, and their plating efficiencies are usually 20% or greater (Robertson, 1987). This also makes mouse ES cells more amenable to techniques fundamental to their use in creating genetically engineered mice, such as efficient colony-cloning and blastocyst injection (Bradley, 1987). Dissociation of the primary blastocyst or ICM culture used to establish monkey or human ES cell lines can be particularly troublesome in terms of cell survival and a careful physical dissociation (microdissection and micropipette aspiration) of the primary colony into small groups of cells is often performed (Thomson et al., 1995; Thomson and Marshall, 1998). Again, in contrast, primary colony outgrowths of mouse ICMs or blastocysts are usually dissociated with trypsin–EDTA treatment in combination with mechanical manipulation to initiate the secondary passage and establishment of ES cell lines (Robertson, 1987; C.L. Stewart, personal communication).

This sensitivity to cell-to-cell dissociation is apparently even more pronounced in the epiblast cells of ungulates. Primary cultures of AP positive, undifferentiated, ungulate epiblast cells prepared by the successive immunodissection, culture and physical dissection method are extremely sensitive to lysis by either physical manipulation, withdrawal of calcium, or exposure to trypsin–EDTA (Talbot et al., 1993b, 1995; Talbot and Garrett, 2001). Primary cultures of pig epiblast cells, for example, will rupture and lyse after only 5 min exposure to Ca²⁺/Mg²⁺-free PBS with the cells completely disintegrating in 30–60 min. This inability to dissociate the ungulate epiblast cells from one another is a critical problem for the two reasons outlined above. First, the
subdivision and expansion of the culture is rendered difficult, and second, but just as importantly, it is not possible to disrupt the cell-to-cell communication that helps precipitate the differentiation of ES cells. In stark contrast, colonial outgrowths of ungulate trophoderm or visceral endoderm tissue are very resistant to dissociation by PBS or trypsin–EDTA and are not prone to the rapid and catastrophic lysis that occurs with ungulate epiblast cells (Talbot et al., 2000a). A further examination of this problem will probably be necessary for the successful establishment and manipulation of ungulate ES cells. For example, we have noted that pig epiblast cells can be viably dissociated from each other if saline is used instead of PBS and if a rapid reattachment of the dissociated cells to a solid substrate is fostered (unpublished observations). It can only be hoped that the yet undiscovered cell culture factors that will enable the growth and maintenance of pluripotent ungulate ES cells will, in addition, render the cells more resilient to subculture and passaging.

9. Spontaneous differentiation

Perhaps the most significant problem hindering the establishment of ungulate ES cell lines is the inability to control the spontaneous differentiation of ungulate epiblast cells in culture (Talbot et al., 1993b, 1995). Primary cultures of pure pig, sheep and cow epiblast cells plated on STO feeder cells “spontaneously” differentiate into multiple cell types when left undisturbed in culture (Talbot et al., 1993a,b, 1994, 1995, 1996, 2002). Microscopic observations and the simultaneous loss of AP activity indicate that differentiation events begin 48–96 h post-plating of the epiblast cell mass in culture (Talbot et al., 1993a,b, 1995). Larger initial colonies of epiblast cells tend to differentiate sooner and the smallest, consisting of 20 or fewer cells, usually become senescent and slowly die off. Concurrent with the differentiation, many of the resulting cell types will then tolerate disaggregation by trypsin–EDTA (Talbot et al., 1993a, 1995). Cytokines and growth factors that inhibit spontaneous differentiation in mouse and primate ES cell lines, including LIF and bFGF, do not inhibit this differentiation in ungulate ICM or epiblast primary cultures (Moore and Piedrahita, 1997; Talbot et al., 1993b, 1995). However, further tests in alternative cell culture environments containing other growth factors, species homologous growth factors, or specific chemical inhibitors of differentiation signal pathways might yet be found to maintain the pluripotency of ungulate ES cells (Piedrahita et al., 1997; Spotter et al., 2001).

10. Induced differentiation

As discussed above for mouse and primate ES cell lines, the necessary proof for any claim of the establishment of ungulate ES cell lines must be the demonstration of their pluripotency either by differentiation into defined cell types in vitro or by teratoma formation in vivo. These proofs have been common practice with both mouse and primate ES cell lines since the first reports in the literature (Martin, 1981; Thomson et al., 1995, 1998). Bovine and porcine embryos have been shown to produce teratomas when injected into immunocompromised mice, and so it should be with any putative ungulate ES cell lines (Anderson et al., 1996).

Ideally, to qualify as pluripotent, the defined cell types resulting from the in vitro or in vivo differentiation of ungulate ES cells should be derivatives of neuroectoderm, mesoderm and definitive endoderm. Immunocytochemical proofs of specific protein markers such as cellular intermediate filaments should be of high quality and of sufficient magnification so that individual cells can be easily seen and evaluated. In some cases, e.g., multinucleated skeletal muscle fibers or hepatocytes, morphological identification is sufficient, but in all cases morphological features and
intermediate filament markers should be consistent. For instance, cells of epithelial morphology would not logically be expected to express muscle-specific markers. Also, cellular filamentous proteins would usually not be expected to produce diffuse or punctate immunocytochemical staining patterns within the cells. Because immunocytochemical proofs are often used, and because they are prone to false positives, it should also be required that proofs include a complete as possible demonstration of negative and positive controls. Form is usually a good reflection of function, and the pluripotent differentiation character of putative ungulate ES cells should be self-evident in the culture dish by microscopic inspection.

Ungulate ES cell lines also afford the opportunity to prove pluripotency by chimera formation through injection of the putative ES cells into host blastocysts followed by embryo transfer into surrogate animals. This can be done in addition to teratoma formation or in vitro differentiation. Perhaps the ultimate test of ES cell competence is proof of contribution by the incorporated ES cells to the germ line through the formation of oocytes or spermatozoa in the chimeric offspring. Proofs of germline transmission are well established in the mouse (Bradley, 1987; Nagy et al., 1993), but have never been achieved with the putative ES cells of ungulates. This is not due to lack of embryonic competence of the starting source material, since it is well established that both in vivo-derived and in vitro-produced ungulate embryos can produce viable pregnancies following embryo transfer. It is more likely that the isolated embryonic cells quickly lose their embryonic competence following propagation under suboptimal in vitro conditions. Even intact blastocysts rapidly lose their competence to establish viable pregnancies during continued in vitro culture. New approaches are needed that allow extension of in vitro embryonic competence.

11. Potential approaches

The establishment of ungulate ES cell lines, either from in vivo or IVP blastocysts, carries many known and unknown challenges. Approaches to the problem may involve innovative genetic manipulation techniques for targeted cell ablation (McWhir et al., 1996), ectopic gene expression (Chambers et al., 2003) or gene expression knock-down. Otherwise, more empirical or observation-based experimental approaches such as traditional cell culture manipulations may still provide a solution to the problem. The key to ES cell line establishment, or at least an appreciation of the problem’s possible complexity, may come from on-going comparative transcriptomic studies being done with mouse and human ES cells and somatic stem cells. The knowledge gained from these studies will hopefully highlight specific genetic factors or signal transduction pathways that will enable the design of genetic interventions or cell culture environments that will yield stable, i.e., self-renewing, continuous cultures of competent ungulate ES cells.

12. Genetic manipulation

Generally, only a few strains of mice have been used in the establishment of germ line competent ES cell lines (McWhir et al., 1996). Very few lines have been established in inbred strains other than the 129 strain, although refinements in conditions have resulted in improved efficiencies (Schoonjans et al., 2003; Cheng et al., 2004). In strains that are considered non-permissive, pluripotent cells within the ICM may be more susceptible to differentiation signals. Constant vigilance and manual removal of differentiating cells can allow propagation of undifferentiated cells in these strains, but this is onerous and time consuming.

An alternate approach is to selectively destroy the differentiating cells. McWhir and co-workers used this approach to obtain ES cell lines from the CBA mouse strain from which no ES cell lines
had previously been established. This selective ablation of differentiating cells involved producing transgenic mice whose genome contained a genetic construct consisting of the \textit{Oct4} promoter driving the neomycin resistance gene (McWhir et al., 1996). Primary cultures of embryonic cells expressing \textit{Oct4} (which is associated with self-renewing, undifferentiated stem cells) also were neomycin resistant and thus could survive and replicate in the presence of a neomycin analog (G418). Differentiating cells were killed and thus apparently prevented from inducing further differentiation. Removal of the selection agent (G418) allowed survival of differentiating cells, which could then contribute to fetal development in chimeras.

Temporal expression of transcription factor genes can also help maintain pluripotency and self-renewal in the mouse. Chambers et al. (2003) demonstrated that over-expression of the \textit{Nanog} gene in mouse ES cells encouraged undifferentiated proliferation even in the absence of LIF. Over-expression of telomerase genes has also aided in the maintenance of ES cells by conferring resistance to apoptosis (Lee et al., 2005b), and, while not preventing differentiation, it could encourage maintenance of pluripotency by inhibiting stress-induced down regulation of \textit{Nanog} (Lin et al., 2005). Inhibition of stress-induced apoptosis, however, may not be the best approach for encouraging stem cell proliferation as it may diminish important checkpoints for genotoxic insults.

These schemes involving ablation of differentiating cells or over-expression of pluripotency-related gene factors should also prove suitable for application to ungulate cells, as the presence of these factors has been demonstrated in ungulate embryos; \textit{OCT4} (van Eijk et al., 1999; He et al., 2004), telomerase (Betts and King, 1999); \textit{NANOG} (He et al., 2004, 2006; Pant and Keefer, 2006). While a similar selective ablation approach could be tried in ungulates, it may be better to use the \textit{NANOG} promoter, because it may be more specific for ungulate ICM cells than the \textit{OCT4} gene, which is also expressed in the trophectoderm of ungulates (van Eijk et al., 1999; He et al., 2004, 2006). While clonal propagation of self-renewing stem cell populations could be encouraged by inducing expression of \textit{NANOG}, telomerase or other factors associated with pluripotency, the use of either an inducible on/off promoter or insertion into a \textit{LoxP} flanked site allowing ready removal of the transgene would be necessary to allow subsequent differentiation either \textit{in vitro} or \textit{in vivo}.

13. Cytokines and culture conditions

In the past, culture conditions employed for the establishment of ES and ES-like cells in mice, ungulates and other animals have relied on a system of co-culture with mouse embryonic fibroblasts (primary MEF or transformed STO cells) and supplementation of medium with fetal bovine serum (FBS), glutamine and beta-mercaptoethanol. Both FBS and the medium conditioned by feeder cells comprise a poorly defined system of growth factors and cytokines. In the case of mouse ES cells, supplementation with LIF obviates the need for any other additive in feeder- and serum-free conditions. Past attempts to establish ungulate cell lines that fulfill all the criteria of ES cells may not have been successful due, in part, to the inability to optimize culture conditions. The strategy thus far has been to supplement the culture medium with a combination of growth factors and cytokines proven beneficial for mouse ES cells. The most common supplements used to establish and maintain ES-like cells in ungulates include LIF (Strelchenko, 1996; Iwasaki et al., 2000; Saito et al., 2003; Li et al., 2004b; Yadav et al., 2005), bFGF (Strelchenko, 1996; Li et al., 2004b; Yadav et al., 2005), stem cell factor (SCF) and epidermal growth factor (EGF) (Saito et al., 2003). However, no evidence has been provided for the presence in ungulate ES-like cells of receptors for these ligands or the activated components of the signal transduction pathways in
question prior to their use. For example, it has been known for some time that LIF and its related family members (oncostatin M, interleukin-6, ciliary neurotrophic factor, etc.) are key cytokines for the maintenance of mouse ES cell lines (Piquet-Pellorce et al., 1994; Yoshida et al., 1994), whereas they do not appear to function analogously with human ES cell lines (Thomson and Marshall, 1998). Recent studies on human ES cell lines have used a more definitive approach in which the presence and activity of the LIF receptor and signal transduction pathways were studied (Humphrey et al., 2004). While earlier studies indicated that undifferentiated human ES cells could not be maintained with LIF, it was unclear whether this was due to the absence of the LIF receptor and its signal transduction pathway, STAT3 (signal transducer and activator of transcription 3). Recent studies have demonstrated that human ES cells do indeed have LIF receptors, and that these receptors can respond to LIF ligand by phosphorylation of STAT3 and activation of the LIF/STAT3 pathway. Activation of the pathway, however, did not result in proliferation of pluripotent cells but increased differentiation of the cells (Humphrey et al., 2004). Moreover, human ES cell lines can be maintained in an undifferentiated state in the presence of bFGF along with simultaneous inhibition of bone morphogenic protein 4 (BMP4) by the noggin protein, which is in apparent contrast to the response of mouse ES cells (Wang et al., 2005; Xu et al., 2005). Therefore, some of the challenges in establishing ungulate ES cell lines may be reflected in the differences identified in cytokine requirements for the maintenance of undifferentiated mouse and human ES cells.

In preliminary studies, we have found that the bovine ICM and its primary outgrowths do have the LIF receptor and gp130 signal transducer (Pant and Keefer, 2006). However, it may be that, as in human ES cells, stimulation of this pathway will not induce proliferation of ungulate pluripotent cells since LIF has not proven to help in the establishment or maintenance of ES cells in ungulates. It may also be that LIF results in a stronger stimulation of the differentiation-inducing MEK/ERK (mitogen-activated protein kinase kinase/extracellular regulated kinase) pathway than the STAT3 pathway. This increased susceptibility to differentiation in response to LIF has been observed in mouse strains that are normally not amenable to ES cell line derivation (Lodge et al., 2005). The induction of differentiation could be overcome through inhibition of the differentiation-inducing MEK/ERK pathway with simultaneously stimulation of the LIF/STAT3 pathway (Lodge et al., 2005). This combinational approach of simultaneous stimulation and inhibition resulted in 22.6% efficiency in establishment of ES lines from CBA mouse blastocysts versus 0% with either stimulation of STAT3 by LIF or inhibition of the MEK/ERK pathway alone.

It may be that stimulation of specific pathways in ungulate embryonic cells will result in responses differing from those observed for mouse ES cells. Similar to the opposing effects of LIF, stimulation of the transforming growth factor-beta (TGFβ) and bFGF pathways can result in different responses (pluripotency compared with differentiation) depending on whether the pathway is studied in mouse or human ES cells. Furthermore, stimulation of different branches within the TGFβ super-family of receptors can result in either stimulation or inhibition of differentiation depending on the source of cell line tested. A recent report claims that activation of yet another signaling pathway, the Wnt pathway, using a specific inhibitor of glycogen synthase kinase-3 (GSK-3), 6-bromoindirubin-3′-oxime (BIO), could also maintain pluripotency in feeder-free cultures of both mouse and human ES cell lines (Sato et al., 2004). Although our own preliminary testing of compounds related to BIO and the addition to medium of recombinant mouse Wnt protein did not show any inhibition of porcine epiblast cell differentiation, it may be that these types of amendments to the culture environment may eventually prove successful when used in the right combination. The maintenance of pluripotency is probably the most critical problem to be solved in establishing ungulate ES cell lines and should be the major focus of current and future
research. Identification of functional pathways in ruminant embryonic cells will help determine what cytokines are beneficial and which may be inhibiting the establishment of true ES cells.

14. Summary

Although ES cells cannot be strictly considered as embryos, they can contribute to the formation of an embryo and, thus, can display the pluripotency and competence of early preimplantation embryos. While proven ES cell lines have yet to be established in ungulates, the identification of appropriate stem cell markers, functional cytokine pathways, and key pluripotency-maintaining factors along with the release of more comprehensive bovine and porcine genomes (GenBank), provide encouragement for establishment of ungulate ES cell lines in the near future. Moreover, indirectly the successful establishment of ES cell lines provides strong evidence for the competence of embryos from which the cell lines are derived, as well as providing a means to examine differentiation events during early development.

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References


