Technical note

Conventional pluripotency markers are unspecific for bovine embryonic-derived cell-lines

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Abstract

Bovine embryonic stem cells are of potentially big value in transgenic research and studies of lineage commitment and development. Nevertheless, key aspects of the establishment of bovine embryonic stem cells such as the identification of specific pluripotency markers need to be clarified to achieve successful results. Bovine blastocysts were produced in vitro and cultured for 8 days up to the expanded or hatched stage. The trophectoderm, the inner cell mass and its embryonic stem cell-derived lines, all showed a common positive immunocytochemical staining for stage-specific embryonic antigen-4, tumour-rejection antigen gp96 and NANOG proteins. The antigenic profile obtained partially agrees with previous data from bovine and other species. Until a validated pluripotent bovine stem cell marker can be identified, it might be advisable to combine the use of epiblast and trophoblast-specific markers to rule out the presence of early committed trophectoderm cells in bovine embryonic stem cell cultures.

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1. Introduction

Embryonic stem cells (ESC) are derived from the epiblast cells of the blastocyst. These cells show ability to self-renew and to result in all three germinal layers of the embryo. These unique features of ESC provide an invaluable tool for studies of development, functional genomics and generation of transgenic animals. Unfortunately, efforts made to establish validated bovine embryonic stem cell (bESC) lines up to now have been fruitless.

Hitherto, bESC culture conditions have mimicked human and mouse ESC cultures (hESC/mESC). Nevertheless, during the past decades it has become clear that

although the general mechanisms that regulate pluripotency and self-renewal are shared by ESC from different species, mESC and hESC differ in morphology, surface marker expression, gene expression patterns, and production of cytokines [1]. Therefore, data extrapolation from one species to another should be made carefully. Recently, Keefer et al. [2] reviewed key aspects for the establishment of domestic ungulate embryonic stem cell lines, suggesting that identification of appropriate ungulate stem cell markers and pluripotency-maintaining factors should be clarified to achieve successful results.

Developmentally regulated stage-specific embryonic antigens, such as SSEA1, SSEA4, TRA-1-60 and TRA-1-81, have been widely used as markers to identify mESC and hESC. Human embryonic carcinoma (EC) cells, hESC and the inner cell mass (ICM) of the human blastocyst share antigen phenotype [3]. To date, variable

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staining patterns have been described for the above markers in bESC-like cultures [4–7]. Previous reports have also described striking differences in “stemness” gene distribution between human, mouse and bovine embryos. *NANOG*, a critical homeobox transcription factor underlying pluripotency, has been found both in the ICM and trophectoderm (TE) of bovine blastocysts [8], but its expression is restricted to the ICM in human and mouse blastocysts [9–11]. Meanwhile, OCT-4 protein, a member of the POU family of transcription factors that plays a crucial role during embryogenesis, localizes to both the ICM and TE in human [12], and the ICM of murine embryos [13]. Controversial data describing OCT-4 expression only in the epiblast [14] or both the ICM and TE of bovine embryos [13] have also been reported. These discrepancies between species and the absence of specific bovine stem cell markers make necessary to validate pluripotency markers before their heterospecific use with bESC.

Several culture systems have been tested for the establishment of ICM-derived bESC, without definitive results for long-term proliferation and avoidance of differentiation [4,15]. Failure to keep bESC cultures in an undifferentiated state might be due to species-specificity problems with some ligand/receptor systems. Human leukaemia inhibitory factor (LIF) has an adverse effect on bovine blastocyst development in vitro [16]. Recently, we have suggested that mouse and human (LIF) should not be used in culture media with bovine embryos or bESC [17]. Therefore, the use of homo-specific feeder layers and the addition of homologous compounds to bESC cultures might be advisable.

In this work, we aimed to improve the characterization of some stem cell and tissue-specific markers currently used to identify bESC lines. We compared the distribution of SSEA1, SSEA4, TRA-1-81, TRA-1-60 and *NANOG* proteins between bovine blastocysts and bESC-like lines derived from the ICM of in vitro produced (IVP) blastocysts.

2. Materials and methods

2.1. Bovine foetal fibroblast feeder layer

A bovine foetus estimated to be 3 months old was released from its embryonic sac and washed in PBS before dissection. After removal of soft tissues, clean carcasses were triturated and tissue was digested with 0.25% trypsin–EDTA. The embryo/trypsin solution was divided between several 175 cm² flasks containing feeder cell growth medium and incubated at 38.5 °C

with 5% CO₂. Subconfluent cultures were treated with trypsin–EDTA and passaged for cell multiplication. Cells harvested from different passages were frozen using a mixture of 90% culture medium and 10% dimethylsulfoxide. Frozen aliquots were thawed for 2 min at 37 °C in a water bath, grown to 80% confluence, and treated with 10 µg/ml mitomycin C for 2 h. Mitomycin-treated fibroblasts were seeded at a density of 10⁵ cells/well on 0.1% gelatin-coated 4-well culture dishes (Nunc). The feeder cell growth medium was DMEM, with 10% FCS, 1% MEM-amino acids and 1% penicillin–streptomycin.

2.2. Inner cell mass isolation and culture.

Blastocysts were produced after in vitro maturation, in vitro fertilization and in vitro culture in simple medium as previously reported [17]. Inner cell masses were isolated from Day 7 and Day 8, expanded and hatched blastocysts by immunosurgery (IS) [16]. After removal of zona pellucida with pronase (4 mg/ml), embryos were incubated in 50 µl bovine antiserum (SIGMA B1520) during 40 min at 38.5 °C and 5% CO₂. After two washes in PBS-PVA, embryos were placed in complement rabbit serum (SIGMA S7764) for 40 min at 38.5 °C, 5% CO₂ and washed once in PBS-PVA. The TE was eliminated by moving embryos gently through a narrow gauge pipette. Isolated ICMs were seeded on fibroblast feeder layers, allowed to attach and monitored daily. Every 7–10 days, putative bESC colonies were mechanically dissociated into 4–6 clumps and replated. Culture medium was DMEM with 20% FCS (Hyclone), 0.1% MEM-amino acids and 0.1% penicillin–streptomycin.

2.3. Immunocytochemical analysis

Developmentally regulated stage-specific embryonic antigens and pluripotency markers were analyzed by immunocytochemistry. Day-8 blastocysts (*n* = 8 per antibody) and bESC-like colonies (from every passage) were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 in PBS. Subsequently, to block non-specific binding of the primary antibodies, the samples were incubated in Image-iT FX Signal Enhancer (Molecular Probes) and in PBS + 5% BSA. Primary antibodies to stage-specific embryonic antigens 1 and 4 (SSEA1, SSEA4), tumour-rejection antigen gp96 (TRA-1-60 or TRA-1-81) (ES Cell Characterization Kit, Chemicon) and *NANOG* (ab21603; Abcam) were diluted in blocking solution containing 5% BSA and applied to samples overnight at 4 °C. Samples were

Fig. 1. Stage-specific embryonic antigens and NANOG immunostaining of bovine embryos (E–H) and bovine embryonic stem cell-like colonies (A–D). Small squares show DAPI and cytoplasmic staining for SSEA4, TRA-1-81 and TRA-1-60 and nuclear staining for Nanog. Bar = 100 μ m.

incubated with the appropriate secondary antibody, mounted on glass slides and examined by fluorescence microscopy. Staining controls using secondary antibody alone were included.

3. Results

A total of 36 expanded and 10 hatched blastocysts were used for ICM isolation. Within the initial 48-h culture period, 20 out of 29 seeded ICM's attached to the feeder layer. Seven to ten days later, five putative bESC colonies were mechanically dissociated into 4–6 clumps and replated. Cultures were allowed to develop over 7–10 days before being passaged. Six passages were performed over a period of 2 months. From passages 1–4, all the seeded fragments developed outgrowths and formed new colonies. From passages 5–6 half of the fragments were able to form a colony.

SSEA4, TRA-1-81, TRA-1-60 and NANOG proteins were detected both in the ICM and the TE of bovine blastocysts (Fig. 1E–H). SSEA4 staining was cytoplasmic and distributed homogeneously all over the embryo (Fig. 1E). Immunoreaction for TRA-1-81 and TRA-1-60 was also cytoplasmic and present in the ICM and TE cells; however some cells stained stronger than others (Fig. 1F and G). NANOG was detected in the nuclei of both ICM and TE cells (Fig. 1H). Bovine blastocysts did not show positive immunoreaction for SSEA1 antigen (data not shown).

The use of bovine foetal fibroblasts as feeder layers (Fig. 2) has allowed us to establish five bovine ES-like cell lines (out of 29 seeded inner cell masses) during 2

months. Morphological analysis of these bESC-like lines, derived from outgrowth of the ICM of Day-8 IVP blastocysts, showed cells with large nucleus surrounded by a narrow band of cytoplasm that could be clearly distinguished from the feeder layer (Fig. 3). Although the morphology of the cells in the ICM-derived colonies was quite homogeneous, cells with different morphology (i.e. neuron-like or epithelial-like) did appear some times in the edge of the colonies. These cells were mechanically removed when splitting the colonies. Our bESC-like colonies showed an antigenic profile similar to that observed in bovine embryos (Fig. 1A–D). SSEA4 and NANOG were detected in all cells of the colonies in a homogeneous pattern (Fig. 1A and D), whereas two distinct populations of cells within the colonies were positive for TRA-1-81 and TRA-1-60 (Fig. 1B and C). The expression of all the pluripotency markers employed in this study was homogeneous

Fig. 2. Bovine embryonic fibroblast feeder layer.

Fig. 3. Day 7 Bovine ES-like colony (10×).

through all passages. Such as observed in bovine blastocysts, bovine ES-like cell colonies were negative for SSEA1 (data not shown).

4. Discussion

In the present work, the ICM of bovine embryos and cultured ES-like cells share a common antigen phenotype, which is similar to that described for human embryos and hES cells. However, the bovine antigen phenotype differs from their murine counterparts (summarized in Table 1, as modified from [3,19]).

We have shown that the ICM and TE of Day 8 IVP bovine embryos express SSEA4, TRA-1-60 and TRA-1-81 proteins. In contrast, in human embryos the expression of the above markers is restricted to the ICM [3], while both hESC- and bESC-like colonies are characterized by the same cell surface antigenic profile. Unlike the profile described in human and bovine, SSEA4, expressed by unfertilized oocytes and early cleavage mouse embryos, does not localize to either murine blastocysts or ES cell colonies [20,21].

However, SSEA1 appears during late cleavage stages in mice and is strongly expressed by murine ICM, TE and ESC [18,20]. SSEA-1 has not been detected either in human and bovine embryos or ESC colonies. Furthermore, human EC and ES cell differentiation is characterized by up-regulation of SSEA1 [3,19,22,23]. Controversial data have been reported for stage-specific embryonic antigen expression in bESC-like cell lines [4–7]. Our results, partially in agreement with those described by other authors [5,7], show that these antigens might be unsuitable to characterize bESC lines. Nanog is known to characterize human and mouse pluripotent cells. *Nanog* null mouse embryos consist entirely of disorganized extraembryonic tissues with no discernible epiblast [10]. Moreover, Nanog down regulation can induce both mouse and human ES cells differentiation to extra-embryonic lineages [24,25].

During mouse development, *Nanog* mRNA is first detected in the innermost cells of the compacted morula, being it onwards expression confined to the ICM [9,10]; similarly, *NANOG* is also detected in the ICM of human blastocysts [11]. *NANOG* transcripts have been localized both in the ICM and TE of the bovine and caprine blastocyst [8,26], which is consistent with our results. Human and mouse ESC strongly express Nanog [19], in accordance with the positive immunostaining found for NANOG in our bESC-like colonies.

Several reports have shown that pluripotency competence is achieved through a delicate network of positive and negative regulation of gene expression [27]. This finely balanced regulation is consistent with a period in bovine development during which TE cells are still transcribing epiblast genes while already transcribing trophoblast-specific genes [8]. At this stage of early TE lineage commitment, the co-expression of epiblast-specifying and extra-embryonic differentiation effector genes probably contributes to a slow differentiation phenotype typical of late implanting bovine embryos

Table 1

Comparative cell surface expression of stage-specific embryonic antigens 1 and 4 (SSEA1 and SSEA4), tumour rejection antigens 1-81 and 1-60 (TRA-1-81 and TRA-1-60), and NANOG expression by human, mouse and bovine embryos and inner cell mass (ICM)-derived cell lines

Marker	ICM			Trophectoderm			ICM-derived cell lines		
	Human	Mouse	Bovine	Human	Mouse	Bovine	Human	Mouse	Bovine
SSEA1	–	++	+	+	+	+	–	+	–
SSEA4	++	–	++	–	–	++	+	–	+
TRA-1-81	++	–	++	–	–	++	+	–	+
TRA-1-60	+++	–	++	–	–	++	+	–	+
NANOG		+	+		–	+	+	+	+

Data from human and mouse ICM and trophoctoderm are from Henderson et al. [3]; data from human and mouse embryonic stem cells are from Boiani and Schöler [19]. Data from bovine summarizes this work.

[8]. The presence of “intermediate” transitory cell types that co-express both features of undifferentiated stem cells and their differentiated derivatives could explain the results described in our work. Thus, NANOG, an epiblast specifying gene, and SSEA4, TRA-1-60 and TRA-1-81, cell surface antigens used to identify human pluripotent cells, colocalized to the ICM and TE in our blastocysts. Different culture conditions have been used for the establishment of ICM-derived bovine ES cell lines, however, none of them has resulted in long-term proliferation and avoidance of differentiation (for review see [2]). The use of bovine embryonic fibroblasts allowed establishment of five bovine ES-like cell lines for 2 months whose pluripotency needs to be confirmed. Bovine ES cell lines have been previously derived on mouse embryonic fibroblast feeder layers, with similar results to those described in this work. However, the use bovine fibroblasts will help to remove some species-specificity problems actually encountered when using mouse fibroblasts.

In conclusion although SSEA4, TRA-1-60, TRA-1-81 and NANOG are widely used to identify mouse and human stem cells, their use as single markers might be inappropriate to identify bovine pluripotent cells. Further research is necessary to identify reliable pluripotency markers for bESC.

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