Isolation and Characterization of Embryonic Stem Cell-Like Cells From in vitro Produced Goat (Capra hircus) Embryos

Arun Kumar De, Dhruba Malakar, Yogesh S. Akshey, Manoj Kumar Jena & Rahul Dutta

Animal Biotechnology Centre, National Dairy Research Institute, Karnal, Haryana, India

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PLEASE SCROLL DOWN FOR ARTICLE
ISOLATION AND CHARACTERIZATION OF EMBRYONIC STEM CELL-LIKE CELLS FROM IN VITRO PRODUCED GOAT (Capra hircus) EMBRYOS

Arun Kumar De, Dhruba Malakar, Yogesh S. Akshey, Manoj Kumar Jena, and Rahul Dutta

Animal Biotechnology Centre, National Dairy Research Institute, Karnal, Haryana, India

The aim of the present study was to isolate and characterize goat embryonic stem cell-like cells from in vitro produced goat embryos. Inner cell mass (ICM) cells were isolated either mechanically or by enzymatic digestion from 150 blastocysts and 35 hatched blastocysts whereas 100 morulae were used for blastomeres isolation mechanically. The ICM derived cells or blastomeres were cultured on a feeder layer. The primary colony formation was significantly higher ($P < 0.01$) for hatched blastocysts (77.14%) than early/expanded blastocysts (54%) or morula (14%). When ICMs were isolated mechanically the primary colony formation for hatched blastocysts (90%) as well as blastocysts (66%) were significantly more than when ICMs were isolated by enzymatic digestion (60% and 30%, respectively). The colonies were disaggregated either mechanically or by enzymatic digestion for further subculture. When mechanical method was followed, the colonies remained undifferentiated up to 15 passages and three ES cell-like cell lines were produced (gES-1, gES-2, and gES-3). However, enzymatic disaggregation resulted in differentiation. The undifferentiated cells showed stem cell like morphological features, normal karyotype, and expressed stem cell specific surface markers like alkaline phosphatase, TRA-1-61, TRA-1-81, and intracellular markers Oct4, Sox2, and Nanog. Following prolonged culture of the ES cell-like cells were differentiated into several types of cells including neuron like and epithelium-like cells. In conclusion, goat embryonic stem cell-like cells can be isolated from in vitro produced goat embryos and can be maintained for long periods in culture.

Keywords: Blastocysts; Embryonic stem cell-like cells; Feeder layer; Goat; Oct-4

INTRODUCTION

Embryonic stem (ES) cells are cells that have the capacity to self-renew as well as the ability to generate differentiated cells (1, 2). Embryonic stem (ES) cells are most frequently derived from the inner cell mass (ICM) of blastocysts (3, 4). The ICM is used to give rise to an ES cell line that is pluripotent, meaning the cells have the potential to develop into any cell types from all three germ layers-ectoderm, endoderm and mesoderm both in vivo and in vitro. These cell lines can be propagated in vitro.
indefinitely yet maintaining a normal karyotype. ES cells or ES cell-like cells have been produced in mice and other animal models, including chicken (5), bovine (6), equine (7), sheep (8), rabbit (9), hamster (10), pig (11), and buffaloe (12). In primates, ES cell lines have been produced in the common marmoset (13) and the rhesus monkey (14). In 1998, Thomson and coworkers reported the first ES cell lines produced from human blastocyst stage embryos (15). The first bovine ES cell line had remained pluripotent in culture for more than 150 passages (16). The applications of ES cells are wide, including the application of therapeutic medicine in human, study of developmental biology, analysis of the characteristics of totipotent cells, and gene targeting to produce genetically modified livestock.

At present there is a vast need of generating ES cells in domestic animals because domestic animals are immunologically and physiologically more similar to human than rodent models and form better model for studying human pathology (17). Goat is an important livestock species contributing to milk, meat and wool production. ES cells have 10–20-fold higher efficiency as somatic cell nucleus donor in somatic cell cloning than cumulus cells (18). Genetic modification by homologous recombination is higher in ES cells than in somatic cells, hence, there is higher efficiency of transgenic animal production by nuclear transfer (19). The present study was carried out to isolate and characterize embryonic stem cell-like cells from in vitro produced goat embryos.

MATERIALS AND METHODS

Chemicals

Except where otherwise stated, all chemicals and cell culture media were purchased from Sigma Chemicals Co. (St. Louis, MO) and were of cell culture/embryo tested. The same batch of fetal calf serum (FCS, 054K8416) was used throughout the study. The plasticware used were purchased from Tarson (Tarsons products Pvt. Ltd. India) and Nunc (Nunc, Denmark) and filters were from Millipore (Millipore India Pvt. Ltd. India).

In Vitro Production of Goat Embryos

In vitro goat embryos were produced according to the methods described in Malakar and Majumdar (20) with slight modifications. Briefly, goat ovaries were collected from local abattoir and transported to laboratory in a thermo flask containing 0.9% normal sterile saline fortified with 50 μL/mL gentamicin at 32–35°C within 3 h. Cumulus oocyte complexes (COCs) were isolated by a puncturing method in medium consisting of TCM-199 (HEPES modified) and bovine serum albumin (BSA) (0.3% v/w). Only COCs with more than three cumulus layers and homogeneous ooplasm were taken for in vitro maturation. COCs were washed five times with maturation medium containing TCM 199 (HEPES modified), 10 μg/mL LH, 5 μg/mL FSH, 1 μg/mL estradiol-17β, 50 μg/mL sodium pyruvate, 3.5 μg/mL L-glutamine, 50 μg/mL gentamicin, 5.5 mg/mL glucose, 3 mg/mL BSA, and 10% EGS (heat inactivated goat serum). COCs (15–20 oocytes) were placed in 100 μL of maturation medium, covered with paraffin oil, and incubated in 5% CO2 in air at 38.5°C with maximum humidity for 27 h. Fresh semen was collected from a
proven buck by artificial vagina (AV) and washed two times by centrifugation at 297 X g for 7 min in sperm TALP medium (21). Then, it was placed in fertilization-TALP (fert-TALP) medium containing sperm TALP supplemented with 50 μL/mL heparin and 3 mg/mL BSA for capacitation in 5% CO₂ in air for 1.5 h. The matured oocytes with expanded cumulus cells were washed in fertilization-TALP medium and co-incubated with capacitated spermatozoa with concentration 2 × 10⁶ sperms/mL in fertilization-TALP medium in 5% CO₂ in air) at 38.5°C with maximum humidity for 10 h. Then the presumptive zygotes were washed 5 times in embryo development medium (EDM) containing TCM 199 (HEPES modification), 30 μg/mL sodium pyruvate, 100 μg/mL L-glutamine, 50 μg/mL gentamicin, 10 μL/mL essential amino acids, 5 μL/mL non-essential amino acids, 10 mg/mL BSA (Fraction-V), 10% FCS, and 50 mM cysteamine to remove the attached spermatozoa and, subsequently, cultured in EDM along with goat oviductal epithelial cells. Cleavage was found after 36–48 h post-insemination. Morulae, blastocyst, and hatched blastocyst were obtained on day 5, day 7, and day 8 post–inseminations, respectively. The cell number of blastocyst was counted by Hoechst 33342 staining (Fig. 1F).

**Preparation of Feeder Layer**

In the present study, goat fetal fibroblast was used as the feeder layer. Goat fetuses of around 60 days of age obtained from a local slaughterhouse were dissected out from uteri and washed 4 to 5 times in sterile Dulbecco’s phosphate buffer saline

![Figure 1](image-url)

**Figure 1** (A–F) Morphology of different stages of goat embryos: (A) Morula stage goat embryos (200X). (B) Group of goat blastocysts (100X). (C) Expanded blastocyst of goat (400X). (D) Hatching of goat blastocysts (400X). (E) Hatched blastocysts of goat (400X). (F) Hoechst 33342 staining of goat Blastocyst (400X). (Figure available in color online.)
Skin explants were taken and washed 8 to 9 times with sterile DPBS. Then, the tissue pieces were cultured in separate 4-well tissue culture dishes in Dulbecco’s modified eagles medium (DMEM) supplemented with 10% FCS and 50 μg/mL gentamicin in 5% CO₂ in air at 37°C with maximum humidity. Monolayer fibroblasts were allowed to grow until confluence. Subsequent cell layers used as feeder layers were obtained by disaggregating the cells with 0.25% Trypsin-EDTA solution. For preparation of feeder layer, the fibroblasts were inactivated by treatment with 10 μg/mL mitomycin-C for 3 h, after which these were washed 5 times in DPBS.

Isolation of Inner Cell Masses (ICMs) From Blastocysts

The inner cell masses (ICMs) were isolated from expanded and hatched blastocysts using two methods: mechanical isolation and enzymatic isolation.

For mechanical isolation, the zona pellucida of expanded blastocysts was removed by treatment of 1% pronase in DPBS (w/v) until zona pellucida dissolved completely. After the disappearance of zona pellucida, the blastocysts were immediately given 4 to 5 washing in DPBS to inhibit further action of pronase. The ICM cells were dissected out with the help of two fine glass needles under zoom stereomicroscope (Olympus SZ 61, Japan).

For enzymatic isolation, zona free blastocysts were transferred into 0.25% Trypsin-EDTA solution and observed under microscope until the trophectodermal cells became loose and were shed from the ICM by gentle pipetting.

In the case of hatched blastocysts, the ICM cells were easily isolated mechanically or enzymatically as they were clearly visible under zoom stereomicroscope. The isolated ICMs were seeded on mitomycin-C (10 μg/mL) inactivated feeder layers in ES cell medium containing DMEM supplemented with 20% FCS, 1,000 IU/mL murine leukemia inhibitory factor (mLIF), 1% nonessential amino acids, 0.1 mM β-mercaptoethanol, and 2 mM L-glutamine. The medium was changed at every 48 h interval and the formation of colony was observed routinely under inverted microscope (Nikon, Japan).

Subculture of Putative Embryonic Stem Cells

The primary colonies, obtained on 3 to 5 days after seeding of ICMs, intact blastocysts, or hatched blastocysts, were divided randomly into two groups. In the first group, the colonies of putative ES cells were treated with 0.25% Trypsin–EDTA for 5 to 6 min at 38.5°C. The treated putative ES cell colonies were disaggregated with the aid of two fine glass needles under a zoom stereomicroscope. In the second group, the colonies were disaggregated mechanically under the zoom stereomicroscope. Aggregates of 50 to 100 cells were individually reseeded onto a new feeder layer in 4-well culture plates containing 0.5 mL ES cell culture medium containing DMEM, 5 μg/mL L-glutamine, 50 μg/mL gentamycin, 0.007 μL/mL β-Mercaptoethanol, 1 μL/mL NEAA, 20% FCS, and 1000 IU/mL LIF. The colonies exhibiting typical morphological features of ES cell-like cells were taken for sub-culturing.
Characterization of Embryonic Stem Cell-Like Cells

The putative stem cell-like cells was characterized by expression study of surface markers like alkaline phosphatase, TRA-1-61, TRA-1-81, and intracellular markers Oct4, Sox2, and Nanog.

Alkaline Phosphatase Staining

Putative embryonic stem cell-like cell colonies showing typical stem cell like morphology was selected for alkaline phosphatase (AP) staining. The medium was removed and the colonies were fixed in 3.7% paraformaldehyde in DPBS for 15 min. Then, the fixed colonies were washed 4 or 5 times with DPBS and incubated in AP substrate solution containing 25 mM Tris-maleate (pH 9.5), 8 mM MgCl$_2$, 0.4 mg/mL sodium alphanaphthyl phosphate, and 1 mg/mL Fast red for 30 min. The cells were washed 2 or 3 times in DPBS and response of the cells to AP staining was observed under an inverted microscope. The cells that took a red stain, which is an indicator of AP, were considered to be ES cell-like cells.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from putative goat ES cell colonies for expression study of Oct4, Sox2, and Nanog using “Cells-to-cDNA kit-II” (Ambion, Austin, TX, USA) as per manufacturer’s instructions. Briefly, the cells were washed with ice cold PBS after which 20–50 μL of chilled cell lysis buffer was added and the mixture was incubated at 75°C for 10 min in a thermal cycler (BIO-RAD, USA). Genomic DNA was degraded by incubating the cell lysates in DNase-I at 37°C for 30 min and the remaining activity of DNase-I was inactivated by heating at 75°C for 5 min. cDNA was prepared by taking 10 μL of the cell lysates using random primers. The PCR cycle included denaturation (94°C for 2 min) followed by repeated cycles of denaturation (94°C for 30 sec), annealing (for 30 sec at temperature 58°C), and extension (72°C for 45 sec) followed by a final extension (at 72°C for 10 min). A negative RT reaction (i.e., RT reaction but without MMLV enzyme) was set up. A house keeping marker gene β actin was also amplified at each stage of PCR (see Table 1).

Immunofluorescence Staining of Putative ES Cells

The expression of intracellular marker Oct4 and surface markers TRA-1-60 and TRA-1-81 were examined by immunofluorescence staining of colonies of

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Table 1: PCR primers used for the characterization of putative goat embryonic stem cells

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanog</td>
<td>5'-TGGAGCAATCAGAGCTGGAACAGT-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CAGTGATTGTGCTGGGAACATGA-3'</td>
</tr>
<tr>
<td>Sox2</td>
<td>5'-ACCCCTAAGATGCACAACTGGGA3'</td>
</tr>
<tr>
<td></td>
<td>5'-TCCCGGCAGTGTACTTAATCC-3'</td>
</tr>
<tr>
<td>OCT-4</td>
<td>5'-GTTCTCTTGGGAAGGTGTTC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-AACCTGGACCAGCTTTTC-3'</td>
</tr>
</tbody>
</table>
putative goat ES cells. The putative ES cell colonies were fixed in 4% Para formaldehyde in DPBS for 30 min, washed 3 times with DPBS, and then permeabilized by treatment with 0.1% Triton X-100 in DPBS for 30 min. After thorough washing with DPBS, putative goat ES cell colonies were incubated with the blocking solution (4% normal goat serum) for 30 min and then with the primary antibody (Millipore, USA) at a dilution of 1:10 to 1:20 for 1 h. After washing 3 times with DPBS, putative ES cell colonies were incubated with the appropriate FITC-labeled secondary antibody goat anti-mouse IgG diluted 1:100 to 1:200 for 2 h. The putative goat ES cell colonies were then examined under a fluorescence microscope (Nikon, Tokyo, Japan).

**Chromosomal Integrity of ES Cell-Like Cells**

Karyotyping analysis was performed on ES cell-like cells at 10th passages. The cells were subjected to chromosomal analysis according to the method of Malakar and Majumdar (20) with slight modifications. Briefly, the cell colonies were incubated in DMEM supplemented with 0.1 mg/mL colcemid at 38.5°C for 4 h. The cells were then washed, trypsinized, and resuspended in hypotonic solution (75 mM KCl) for 30 min at 38.5°C. They were washed and then fixed in chilled fixative (3:1 methanol/glacial acetic acid) for 30 min at room temperature and centrifuged at 200 X g for 8 min. The pellets were resuspended in 5 mL of ice-chilled fixative for another 10 min and then centrifuged again. The metaphase spreads were prepared by dropping the cells onto ice cold glass slides. Chromosomes were stained with 2% Giemsa for 6 min and observed under oil immersion (1,000X) using a compound microscope (Nikon, Microphot-FXA, Japan).

**Evaluation of in Vitro Differentiation**

Evaluation of the differentiation of ES cell-like cells was conducted by prolonged culture and suspension culture. Briefly, the passage 10 goat ES cell-like cell colonies were removed from the feeder layers and disaggregated into small clumps using two fine needles. The dissociated single cells or small clumps were transferred to suspension culture dishes containing ES medium without mLIF and in the absence of feeder layer. After 3 days, embryoid bodies were seeded in a 4-well gelatin (0.1%-coated tissue culture dish in differentiation medium for in vitro differentiation.

**Statistical Analysis**

The differences in the number of embryos giving rise to primary cell colony were revealed by Chi-square ($\chi^2$) test. A value of $P < 0.01$ was considered to be statistically significant.

**RESULTS**

**Production of Embryos**

In the present study, in vitro produced 300 morphologically normal blastocysts (expanded + hatched) were used for isolation of ICMs and intact blastocyst culture
and 100 morulae were used to get blastomeres for culturing of embryonic stem cell-like cells.

**Formation of Embryonic Stem Cell-Like Cell Colonies From Morulae, ICM-Derived Cells and Intact Blastocysts**

In the present study, a total of 100 morulae (Fig. 1A), 185 blastocysts (80 early blastocysts, Fig. 1B; 70 expanded blastocysts, Fig. 1C; and 35 hatched blastocysts, Fig. 1E) were used to study the culture behavior of embryo derived ICM cells for production of embryonic stem cell-like cells (Table 2). ICMs (Fig. 2A) were isolated from blastocysts either mechanically or by enzymatic digestion. Primary cell colonies were obtained (Fig. 2C and 2D) after 4 to 5 days of seeding them on mitomycin-C inactivated feeder layer. ES cell-like colonies were formed in 77.14% of ICMs derived from hatched blastocysts which was significantly higher than \( P < 0.01 \) that for 54% early and expanded blastocysts and 14% morulae (Table 2). On the other hand, when ICMs were isolated mechanically the primary colony formation for hatched blastocysts (90%) as well as early + expanded blastocysts (66%) were significantly more \( P < 0.01 \) than when ICMs were isolated by enzymatic digestion (60% and 30%, respectively, Table 3). The primary colonies were formed in 27% of intact blastocyst (early + expanded) culture and 40% of intact hatched blastocyst culture (Fig. 3B, C), which were significantly less \( P < 0.01 \) than ICM culture (58.37%, Table 4).

Morphologically, the ES cell-like colonies were densely packed and the cells had a higher nucleus:cytoplasm ratio with a prominent nucleus and a clear boarder. The cells were dome shaped and achieved confluency after 10 to 15 days (Fig. 2E, F). Then, the primary colonies were disaggregated and reseeded on fresh feeder layers (Fig. 4A). New primary colonies were formed on day 2 to 3 post-seeding (Fig. 4B, C). When intact hatched blastocysts or intact blastocysts were seeded on feeder layers (Fig. 3A) the trophectodermal cells along with ICM cells were attached (Fig. 3B), later ICM derived cells took over other cells. Primary colonies were formed after 3 to 4 days post-seeding. When zona free morulae were seeded on mitomycin-C treated feeder layer, the cells attached and formed primary colony after 4 to 5 days.

The ES cell-like cells were disaggregated into small clumps either by mechanical disaggregation or by enzymatic digestion. When enzymatic digestion method was used for passages, the ES cell-like cells differentiated after 4th passages but when mechanical disaggregation method was followed three ES cell-like cell lines (gES-1, gES-2, and gES-3) were established. The cell morphology was dome shaped with a clear boarder and a prominent nucleus. The colonies were confluent after 10 to 15 days (Fig. 2E, F). The cell lines were passaged up to 20 times with no significant difference in cell morphology or growth rate. The cell lines were characterized by their ability to differentiate into multiple cell lineages when exposed to specific differentiation factors. The cell lines were used for further studies on the biology and potential therapeutic applications of embryonic stem cells.

**Table 2** Comparison of culture behavior of embryo derived putative stem cells from different stages of embryos

<table>
<thead>
<tr>
<th>Embryonic stage</th>
<th>Cell type</th>
<th>Number of embryos</th>
<th>Number of primary ES cell-like colonies n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morulae</td>
<td>blastomeres</td>
<td>100</td>
<td>14 (14%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blastocysts (early + expanded)</td>
<td>ICMs</td>
<td>150</td>
<td>81 (54%)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hatched blastocysts</td>
<td>ICMs</td>
<td>35</td>
<td>27 (77.14%)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Values within the same column with different superscripts differ significantly \( P < 0.01 \).
gES-2, gES-3) were established which maintained their undifferentiated state up to 15th successive passages (Table 5). The undifferentiated state were indicated by alkaline phosphatase activity (Fig. 5A, B) and Oct4 expression (Fig. 6) measured at passages 5 (lane 1), passage 10 (lane 2), and passage 15 (lane 4). Immunofluorescence staining in ES cell-like cells was also revealed that Oct4, TRA-1-61, and TRA-1-81 marker expressed positively in passage 10 cells (Fig. 5C). In the present culture protocol in our lab, 15 passages ES cell-like cells were growing continuously in the stem cell medium with mLIF. Moreover, the ES cell-like cells showed normal karyotyping measured at passages 10 (Fig. 7A, B). Suspension culture of ES cell-like cells of passage 10 without mLIF and in the absence of feeder layer resulted in embryoid body (EBs) formation within 3 days (Fig. 8A) and further cultured

<table>
<thead>
<tr>
<th>Material</th>
<th>Procedure of ICM isolation</th>
<th>Number of embryos</th>
<th>Number of primary ES-like colonies n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early + Expanded blastocysts</td>
<td>Mechanical</td>
<td>100</td>
<td>66 (66%)(^a)</td>
</tr>
<tr>
<td>Early + Expanded blastocysts</td>
<td>Enzymatic</td>
<td>50</td>
<td>15 (30%)(^b)</td>
</tr>
<tr>
<td>Hatched blastocysts</td>
<td>Mechanical</td>
<td>20</td>
<td>18 (90%)(^c)</td>
</tr>
<tr>
<td>Hatched blastocysts</td>
<td>Enzymatic</td>
<td>15</td>
<td>9 (60.0%)(^d)</td>
</tr>
</tbody>
</table>

\(^a,b,c,d\) Values within the same column with different superscripts differ significantly (\(P < 0.01\)).
These EBs when cultured in 0.1% gelatin coated tissue culture dishes without feeder layer and mLIF differentiated into neuron and epithelium-like cells (Fig. 9A, B).

**DISCUSSION**

More primary colonies were formed from hatched blastocysts than that of early or expanded blastocysts. This is due to the increased number of pluripotent cells in hatched blastocysts. In mechanical isolation of ICMs, the primary colony formation rate was more than isolation by enzymatic digestion because Trypsin has some differentiating effect on ES cell-like cells. In some previous studies, other workers indicated ICM isolation by immunosurgery (15) but its effectiveness was

<table>
<thead>
<tr>
<th>Material</th>
<th>Number of embryos</th>
<th>Number of primary ES cell like colonies n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICMs</td>
<td>185</td>
<td>108 (58.37%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Intact blastocysts (early + expanded)</td>
<td>100</td>
<td>27 (27%)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Intact hatched blastocysts</td>
<td>15</td>
<td>6 (40%)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup>Values within the same column with different superscripts differ significantly ($P < 0.01$).
found to be low and it becomes laborious in terms of making antibodies. Generally, ES cell-like cells are established from ICMs of blastocysts (3, 4, 22, 23). In the present study, an easy method of isolation of ICMs from blastocysts was described using two fine needles and a sharp surgical blade under zoom stereomicroscope. The efficacy of morulae in formation of ES cell-like cells was found to be low probably due to the mechanical injury to the blastomeres during mechanical isolation of them from morulae. Successful attempts were made to produce pluripotent ES cells from morulae in different species like mouse, bovine, and human (8, 24, 25). Feeder layer cells secrete leukemia Inhibitory Factor which prevents differentiation of ES cells and also provides nutrients to ICM derived cells.

In reference to morphology of ES cell-like cell colonies, dome shaped colonies found were reported in mouse (3, 4), porcine (15, 26), and buffalo (12). In the present study, it was found that most of the colonies were dome-shaped with clear boundaries. The morphology of the colonies was almost similar to buffalo reported (12). Trypsin

Table 5 Progressive loss of viability of goat embryonic stem cell-like cells in subsequent passages

<table>
<thead>
<tr>
<th>Methods of cell dissociation</th>
<th>Number of primary colonies used</th>
<th>Number of cell lines surviving to passage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st 2nd 3rd 4th 5th 6th 7th 8th 9th 10th</td>
<td></td>
</tr>
<tr>
<td>Mechanical</td>
<td>12 11 9 8 7 5 5 4 4 4</td>
<td></td>
</tr>
<tr>
<td>Enzymatic</td>
<td>14 8 5 2 1 0 0 0 0 0</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4 (A–F) Subculturing of ES cell like cells: (A) Group of cells seeded on feeder layer (400X). (B) Proliferation of cells after 2 days of culture (400X). (C) Primary colony formation after 4 days of culture (400X). (D) ES cell like cells after 7 days of culture (400X). (E) ES cell like cells after 10 days of culture (400X). (F) ES cell like cells after 15 days of culture (400X). (Figure available in color online.)
had been used to dissociate murine ES cells (4). But, bovine and rabbit ES cells were found to lose its pluripotency when Trypsin was used for passaging (8, 27). In the present study, a similar type of result was obtained. When mechanical disaggregation was used, three ES cell-like cell lines continued their undifferentiated state up to 15th passage whereas subculture by Trypsin digestion leaded to differentiation after 4th passage. Cell dissociation was also attempted with other enzymes such as pronase or collagenase in rat ES cell-like cells, but the efficacy was less compared to mechanical dissociation (28).

AP expression is a preliminary marker for evaluating pluripotent cells and has been used to identify ES cells in many species (9, 13, 22, 28). In our present study, goat ES cell-like cells expressed AP expression clearly (Fig. 5A, B). The present study confirmed that putative goat ES cells express TRA-1-60 and TRA-1-81. The TRA-1-60 and TRA-1-81 are keratin sulfate-associated antigens that are characteristic of human ES cells (29).

It was found that the putative goat ES cells expressed Oct4 marker strongly. The findings were similar to those of earlier studies in which its expression was reported in ES cells in many species like mouse (30), human (31), cattle (32), and buffalo (12).
Among the genes that have been identified to be required for the maintenance of the epiblast cell population and establishment of ICM-derived ES cells in vitro, Oct4 occupies a prominent place. The results of RT-PCR reaffirmed the expression of Oct4 and confirmed the expression of two other important pluripotency markers namely Nanog and Sox2. Nanog mRNA in trophectoderm was significantly down

Figure 6 (A) Expression of Oct-4. Lane M- 100 bp ladder marker, Lane 1-ES cells from passage 2, Lane 2-ES cells from passage 4, Lane 3-Minus template control, Lane 4-ES cells from passage 8, Lane 5-Minus RT control, Lane 6-feeder layer negative control. (B) Expression of pluripotency markers Oct-4, Sox-2, and Nanog in putative goat ES cell at 10 passage. (Figure available in color online.)

Figure 7 (A & B) Chromosomal profile of goat ES cell like cells: (A) Normal No. of metaphage chromosome in passage 4 ES cell like cells (1000X). (B) Normal karyotyping.
regulated as compared with that in ICM (33). Taken together, this novel expression pattern of Nanog in goat pre-implantation embryos suggests that Nanog could serve as marker of pluripotency in goats and may be useful in derivation and characterization of caprine ES cells. Sox2, the expression of this transcription factor, was detected in human (34), rhesus monkey (35), and porcine ES cells (36, 37).

Formation of embryoid bodies by the ES cells is taken as a measure of their pluripotency as embryoid bodies contain all the three germ layers- ectoderm, mesoderm, and endoderm. The ability of the putative ES embryonic stem cells to form embryoid bodies was examined in the present study. When the embryoid bodies were harvested from the hanging drops on day 3 of the suspension culture, they were observed to be in the form of a compact mass. The “hanging drop” method and “static suspension culture” are the two most common methods used for the formation of embryoid bodies from ES cells. The microenvironment, which the ES cells are subjected to culture, can be controlled more precisely in the hanging drop method. This method has, therefore, been used for the formation of embryoid bodies across many species (38, 39). The results of the present study indicate that goat ES cell-like cells have a unique pattern of expression of markers like alkaline phosphatase, TRA-1-60, TRA-1-81, Oct4, Sox2, and Nanog.

![Figure 8](image1.png)  
**Figure 8** (A & B) Embryoid body formation: (A) Embryoid body formation from 10th passage of goat ES cells on 2nd day of culture (400X). (B) Proliferation of embryoid body on 7th day of culture in differentiation medium (200X). (Figure available in color online.)

[![Figure 9](image2.png)](image2.png)  
**Figure 9** (A & B) in vitro differentiation of ES cell like cells derived from ICM: (A) Epithelium like cells (400X). (B) Neuron like cells (400X). (Figure available in color online.)
In vitro differentiation of ES cells to a variety of cell types have been reported in different species like Murine (3), human (22, 23), cattle (6), pig (11), horse (7), and buffalo (12). In the present study, in suspension culture in absence of mLIF and feeder layer embryoid bodies were formed, which is considered to be an important characteristic of ES cells. In prolonged culture, goat ES cell-like cells spontaneously differentiated into neuron and epithelium-like cells.

CONCLUSIONS

In conclusion, goat ES cell-like cells could be isolated from in vitro produced embryos and mechanical isolation of ICMs from hatched blastocysts could be the best method to establish goat ES cell-like cells. Several properties associated with the undifferentiated state and pluripotency of ES cells like AP, TRA-1-61, TRA-1-81, Oct4, Sox2, and Nanog expression, EBs formation and in vitro differentiation were demonstrated.

REFERENCES


