Establishment of Goat Embryonic Stem Cells From In Vivo Produced Blastocyst-Stage Embryos

E. Behboodi,1**, A. Bondareva,1 I. Begin,2 K. Rao,2 N. Neveu,2 J.T. Pierson,2 C. Wylie,2 F.D. Piero,3 Y.J. Huang,2 W. Zeng,3 V. Tanco,1 H. Baldassarre,2 C.N. Karatzas,2 and I. Dobrinski1*

1 Department of Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, Canada
2 PharmAthene Canada, Inc., Montreal, Quebec, Canada
3 Department of Clinical Studies, New Bolton Center, School of Veterinary Medicine, University of Pennsylvania, Kennett Square, Pennsylvania

SUMMARY

Embryonic stem (ES) cells with the capacity for germ line transmission have only been verified in mouse and rat. Methods for derivation, propagation, and differentiation of ES cells from domestic animals have not been fully established. Here, we describe derivation of ES cells from goat embryos. In vivo-derived embryos were cultured on goat fetal fibroblast feeders. Embryos either attached to the feeder layer or remained floating and expanded in culture. Embryos that attached showed a prominent inner cell mass (ICM) and those that remained floating formed structures resembling ICM disks surrounded by trophodermal cells. ICM cells and embryonic disks were isolated mechanically, cultured on feeder cells in the presence of hLIF, and outgrown into ES-like colonies. Two cell lines were cultured for 25 passages and stained positive for alkaline phosphatase, POU5F1, NANOG, SOX2, SSEA-1, and SSEA-4. Embryoid bodies formed in suspension culture without hLIF. One cell line was cultured for 2 years (over 120 passages). This cell line differentiated in vitro into epithelia and neuronal cells, and could be stably transfected and selected for expression of a fluorescent marker. When cells were injected into SCID mice, teratomas were identified 5–6 weeks after transplantation. Expression of known ES cell markers, maintenance in vitro for 2 years in an undifferentiated state, differentiation in vitro, and formation of teratomas in immunodeficient mice provide evidence that the established cell line represents goat ES cells. This also is the first report of teratoma formation from large animal ES cells.


INTRODUCTION

Stem cells are characterized by their ability for self-renewal and their capability to differentiate into a broad spectrum of cell types. Embryonic stem (ES) cell lines have

Abbreviations: ES, embryonic stem [cell]; gES, goat embryonic stem [cells]; ICM, inner cell mass; LIF, leukemia inhibitory factor.
been established in the mouse, nonhuman primates, humans and rat (Evans and Kaufman, 1981; Thomson et al., 1995, 1998; Buehr et al., 2008). However, generation of ES cells from embryos of domestic animals has been more challenging (Keefer et al., 2007). Recently, there have been reports on ES-like cell lines derived from bovine embryos (Stice et al., 1996; Mitalipova et al., 2001; Wang et al., 2005). Bovine ES-like cells have been derived either from 16-cell (Cibelli et al., 1998) or from days 7 to 9 blastocyst-staged embryos (Cibelli et al., 1998; Van Eijk et al., 1999). These cell lines displayed a high nucleus to cytoplasm ratio, competence for extended culture in vitro, and the capacity to differentiate to embryoid bodies similar to mouse ES cells.

A panel of markers indicative of pluripotency of ES cells has been investigated in mouse, human, and bovine cells. Mouse and human ES cells stain positive for alkaline phosphatase activity whereas staining of bovine ES-like cells is more variable (Cibelli et al., 1998; Van Eijk et al., 1999). The transcription factor POUF1 (also known as Oct-4), a key regulator in undifferentiated pluripotent cells, appears to be a primary transcription factor in ES cells. The presence of POUF1 in bovine 16-cell embryos, ICM, and goat blastocysts has been verified previously (Kirchhof et al., 2000; He et al., 2006; Meinecke-Tillmann and Meinecke, 1996). Stage-specific embryonic antigen 1 (SSEA-1) is expressed in mouse ES cells while human ES-like cells exhibit SSEA-3 and SSEA-4 activity, but not SSEA-1. Conflicting results have been reported for these two markers in bovine ES cells.

Although reports of the development of ES-like lines have been published in many species, evidence is lacking for pluripotency as determined by teratoma formation or germline transmission in vivo. There is also limited information on goat ES cells (Meinecke-Tillmann and Meinecke, 1996). The aim of the present study was: (1) to isolate goat ES cell lines from in vivo derived blastocysts; (2) to characterize the growth characteristics and expression of markers indicative of pluripotency in goat ES cells; (3) to demonstrate the capacity of goat ES cells to differentiate into all three germ layers in vivo as a hallmark of pluripotency.

RESULTS

Embryo Culture and ES Cells Derivation

A total of 58 embryos were recovered (avg. 9.7/donor; range 3–16) with only one donor failing to produce any viable embryos. Of those, 30 embryos were degenerated and 28 expanded blastocysts were cultured on goat fetal fibroblast feeder layers. Following 3 days in culture, 25 of 28 embryos hatched. Ten embryos attached to the goat fetal fibroblast feeder layer but the remaining 15 floated in the medium and expanded after 4–5 days in culture. After 3–4 days in culture, the embryos that had attached showed a prominent inner cell mass (ICM) growth (Fig. 1A), and the embryos that remained floating formed structures resembling ICM disks surrounded by trophectodermal cells (Fig. 1B). ICM cells (4/10) and embryonic disks (3/6) were isolated mechanically and cultured on feeder cells (Fig. 1C, D). The ICM and embryonic disks outgrew into ES-like colonies on Days 4–8 post-culture. Colonies that formed from these outgrowths of either ICM or embryonic disks were cultured on feeder cells up to 20 passages (Fig. 1E, F). Overall success of goat embryonic stem (gES) cell derivation is summarized in Table 1. The early stage gES colonies
were mixed, with trophodermal cells but colonies grew uniformly later as shown in Figure 1E. Trophodermal cells appear morphologically different from the ES-like cell colonies. ES cells formed large, uniform size colonies whereas trophodermal cells were flat and large. The morphology of the derived cell lines did not vary in long-term culture, however, some colonies showed differentiation at the edges (Fig. 1F). The gES colonies grown in FCS supplemented medium consisted of relatively large cells with a large nucleus to cytoplasm ratio that were flat and varied in shape. The doubling time for these cells was 19–22 hr based on cell counts at seeding and harvesting. Occasionally, some long-term cultures (over 3 weeks without trypsinization) formed net-like monolayer structures. Two cell lines derived from embryonic epiblast-like structures were maintained in medium supplemented with FCS and growth factors, and proliferated for more than 25 passages; one line proliferated for more than 120 passages, and still maintained ES-like morphology. The other line was frozen at passage 25.

Characterization of Undifferentiated gES Cells

Two goat ES cell lines were selected for long-term culture. These cell lines were similar to mouse ES cells with regards to expression of specific cell markers for pluripotent cells, using mouse ES cells as positive and goat fibroblasts as negative controls. The gES cells were positive for POU5F1 (Oct-4), NANOG, SOX2, SSEA-1, SSEA-4 protein, and AP activity (Fig. 2) regardless of the culture conditions and passage number. Expression of POU5F1, NANOG, SOX2, and LIN-28 was maintained at >120 passages as evident also at the mRNA level (Fig. 3). Mouse-specific primers for NANOG did not amplify a product in gES RNA samples, ruling out any contamination of gES cells with mouse ES cells (Fig. 3E).

Karyotype of gES Cells

The majority (84%; n = 44 spreads) of the cells showed a normal chromosomal count consisting of 60 chromosomes (58 ± 2) at passages 35 and 70, but a high percentage of chromosomal fusion (80–90%) was evident at passage 140.

Generation of a Transgenic gES Line

Stable cell lines expressing eGFP were generated through lipid-mediated gene transfer (Fig. 4A,B). Importantly, after transfection and selection of positive clones, the morphology, and doubling time of the gES cells did not change while fetal fibroblast cells transfected in a control experiment underwent senescence after few additional cell cycles post-transfection (not shown).

In Vitro Differentiation

We observed frequent, spontaneous differentiation of gES-like cells in the absence of leukemia inhibitory factor (LIF) that resembled epithelial-like or neuron-like cells (Fig. 5A,B). These cells differentiated into neuron and epithelia-like cells (Fig. 5C,D). The presence of LIF inhibited the differentiation of gES cells with support of feeder cells.

Teratoma Formation

Four out of five SCID mice injected with passage-60 gES cells developed tumors under the skin, and two of four developed tumors in the testis. Histological and immunocytochemical analyses demonstrated that the tumors contained differentiated cells representing the ectoderm, endoderm, and mesoderm lineages (Fig. 6). These teratomas were generally well demarcated from the surrounding tissues, and exhibited organized clusters of cells and primitive tissue structures, including cartilage, mesenchyme, mineralized bone, smooth muscle, putative nerve bundles, and various types of epithelia. Immunostaining confirmed the identity of particular lineages.

DISCUSSION

The goat ES cells described here exhibited the hallmarks of pluripotency: Long-term proliferation in culture in an undifferentiated state, expression of known markers of pluripotent cells, and differentiation into derivatives of all three germ layers in vivo. Progress in derivation of ESC from domestic animals has been slow. However, the gES cells reported here were different from those reported previously in farm animals. Mainly, the gES cell lines were derived from later stage embryos (Days 11–14) than were used previously. Here, we derived gES cells from epiblast or embryonic disks of embryos cultured in vitro for 12–14 days. In most farm animals, including cattle, pig, and small ruminants (for recent reviews see Gjørret and Maddox-Hyttel, 2005; Renard et al., 2007), the derivation of ES cell lines has been attempted from the ICM; however, the data are inconsistent.

More recently, generation of ES cells has been reported from the epiblast and the embryonic disk in mice (Tesar et al., 2007; Brons et al., 2007). It has been postulated that human ES cells more closely resemble those pluripotent cells derived from mouse epiblast than ICM-derived mouse ES cells (Brons et al., 2007). It can be hypothesized that the timing of epiblast formation may be a crucial determinant for derivation of pluripotent cells from different species. In mice and humans, where the embryo attaches to the uterus earlier than in domestic animals, the transition from ICM to epiblast cells likely coincides with the successful derivation.

<table>
<thead>
<tr>
<th>TABLE 1. Derivation of Goat ES Cells</th>
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<tr>
<td>Total embryos</td>
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<tr>
<td>Hatched</td>
</tr>
<tr>
<td>Attached</td>
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<tr>
<td>Inner cell mass</td>
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<tr>
<td>Embryonic disk</td>
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<td>ES cell</td>
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tion of pluripotent cell lines. In contrast, in large domestic animal species, where the embryo remains motile in the uterus for prolonged periods of time, this transition, and hence the time window for successful ES cell derivation, may occur somewhat later in embryonic development. This may be a reason why we could successfully derive goat ES cells from in vivo-derived embryonic disks in the current study.

Another factor promoting the successful derivation of gES cells reported here may have been the availability of species-specific feeder cells. Several different cell types have been used as feeder layers for the culture of ESCs. In the bovine, fetal fibroblast (Strelchenko, 1996), bovine uterus epithelial cells, and mouse fetal fibroblasts have been used for ES-like cell culture. However, in farm animals none of these cell types could sustain prolonged ES cell proliferation and prevent spontaneous differentiation. In goats, there was not much information on using different feeder cell lines for ES cell culture. In the present study, the presence of LIF and inactivated goat fetal fibroblast feeder cells had a positive influence on the success of establishing gES cell lines. In other farm animals, studies regarding the utility of LIF and feeder cells have yielded conflicting results (Sims and First, 1993; Galli et al., 1994; Saito et al., 2003;
Our results support the use of human LIF in addition to goat fetal fibroblast, similar to a report on embryoid body formation after culture of sheep ES-like cells in the presence of LIF (Trounson, 2005). Morphologically, the gES cells reported here formed large colonies with distinct boundaries similar to those generated from mouse, monkey, and human (Evans and Kaufman, 1981; Thomson et al., 1995, 1998) but also share some similarity with those reported in previous studies on ES-like cells from large domestic animals that formed mostly monolayers of cells (Cibelli et al., 1998; Mitalipova et al., 2001). gES cells possessed alkaline phosphatase activity and were characterized by expression of POU5F1, SOX-2, NANOG, and LIN-28 as well as SSEA4 and SSEA1. This is similar to expression patterns reported for undifferentiated primate ESC, mouse stem cells and goat ICM cells (Evans and Kaufman, 1981; Thomson et al., 1995; Sasaki et al., 2005; He et al., 2006), but is different from previously reported pluripotent stem cell lines produced from bovine IVF embryos, which were SSEA-1 negative (Wang et al., 2005). The gESC line that was maintained in culture for >120 passages exhibited a cell cycle time of 19–22 hr, which is shorter than that observed for goat fibroblasts (Memili et al., 2004).

The morphology of cells did not change during culture in the presence of LIF, but removing LIF from the culture allowed the cells to differentiate into embryoid bodies, as has been reported in mouse and bovine cells (Evans and Kaufman, 1981; Wang et al., 2005), and into epithelial-like cells. Embryoid bodies maintained in culture in plastic dishes differentiated into cells exhibiting neuronal morphology. This spontaneous differentiation into neuronal cells has also been reported in other ruminants (Wang et al., 2005; Verma et al., 2007). Because of the potential limitations of in vitro experiments, it is necessary to examine cell differentiation in vivo after cell transplantation. When pluripotent mammalian stem cells are engrafted into an immunodeficient host, the cells differentiate into complex structures containing derivatives of all three germ layers called teratomas (Evans and Kaufman, 1981; Thomson et al., 1998). Therefore, it is a gold standard of ES cell studies to use the formation of teratomas as an indication of pluripotency (Evans and Kaufman, 1981; Thomson et al., 1995). Similar to teratomas resulting from transplanted human and mouse ESC, transplanted gESC formed teratomas consisting of a range of differentiated tissues representative of all three germ layers, as indicated by tissue morphology and expression of cell type specific markers. While Sritanaudomchai et al. (2007) reported teratoma generation from ES-like cells derived from a buffalo parthenogenic embryo, to our knowledge, the current study is the first report of teratoma formation of ES cells derived from fertilized farm animal embryos.

In conclusion, we established gES cells from in vivo derived epiblast stage goat embryos that expressed known ES cell markers, could be maintained in vitro for 2 years in an undifferentiated state, differentiated into ectodermal cells in vitro and formed teratomas after transplantation to immunodeficient mice. This is the first report of ruminant ES cells, and of teratoma formation from large animal ES cells.

**MATERIALS AND METHODS**

**Embryo Collection**

The embryo donors were hormonally primed according to established methods (Baldassarre and Karatzas, 2004). Briefly, estrus was synchronized in six adult Saanen and
Saanen-cross goats with intravaginal sponges containing 60 mg medroxyprogesterone acetate (Veramix, Upjohn, Orangeville, ON, Canada) for 10 days, with an injection of 125 μg cloprostenol (Estrumate, Schering, Belleville, ON, Canada) at sponge removal. Superovulation was induced with a total equivalent to 160 mg FSH (Folltropin-V, Bio-niche, Pointe Claire, QC, Canada) administered twice daily in decreasing doses over 4 days starting 48 hr prior to sponge removal. A 100 μg dose of GnRH (Factrel, Ayerst, Kirkland, QC, Canada) was given 36 hr after sponge removal, followed by artificial insemination with fresh semen 12 and 24 hr later. Embryos were collected by surgical uterine flushing under general anesthesia 6 days after breeding. A laparoscopic exploration was performed to confirm that the animals had an appropriate response (>3 active corpora lutea), and the reproductive tract was exteriorized through a small, mid-ventral laparotomy. A retrograde uterine flush was conducted by injecting 30 ml EmCare medium (ICP, Christchurch, New Zealand) with a 21G catheter from the oviductal third, and recovering the fluid through a 12Fr. Foley catheter placed at the base of each uterine horn. Care of animals was in accordance with institutional guidelines.

Goat Fetal Fibroblast Feeder Cell Line Preparation

All chemicals and reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Caprine fetal fibroblast cell lines used as feeder lines were derived from 35-day fetuses that were surgically removed and placed in phosphate-buffered saline. Single-cell suspensions were prepared by mincing fetal tissue and cultured in M199 plus 10% goat serum in 25 cm flasks. A confluent monolayer of cells was harvested by trypsinization after 4 days in culture (passage 1). Media were aspirated and 20 ml of fresh DMEM media containing mitomycin (10 μg/ml) were added. Following 1.5–2 hr incubation with mitomycin C at 37°C cells were washed twice with 20 ml PBS. Cell suspensions were produced by the addition of 2–5 ml of 0.1% trypsin/EDTA and gentle pipetting. Cells were seeded at 10^5/well of 4-well IVF culture plates (1.9 cm²/well) (Nalgene, Rochester, NY).

ES Cell Isolation

Twenty-eight in vivo-derived, day 7 embryos were cultured on goat fetal fibroblast feeder layers in goat ES cell media consisting of DMEM with 0.1 mM 2-mercaptoethanol, 0.1 mM MEM nonessential amino-acids, 2 mM l-glutamine, and 10% fetal bovine serum (FBS). Floating embryos formed structures resembling ICM disks surrounded by trophoderm cells. Embryonic disks were isolated mechanically and cultured on goat feeder cells (passage 2) in DMEM medium containing 1,000 U/ml hLIF and 10% FBS. The embryonic disks outgrew into colonies on Days 4–8 post-culture. Compact colonies (70–120 μm) of cells from these outgrowths were isolated mechanically (passage 1) and passed onto fresh goat feeder cells (plated on a 200-mm² gelatinized growth surface) every 4–5 days in the presence of 1,000 U/ml hLIF. The ES cell colonies were collected mechanically (passage 4) under the microscope and were disaggregated by pipetting and seeded on to plastic dishes with seeding density of 5 × 10^5/well in a 4-well culture plate (Nalgene) at early stages. After feeder
removal, seeding was done at $5 \times 10^5$ cells/well in 6-well plates (Fisher Scientific, Ottawa, ON, Canada). In early passages (1–10), mechanical dissection was used to passage the gES cells but 0.25% trypsin was used for the passage of cells beyond passage 15. The colonies attached to plastic plates within hours post-transfer and started growing in 2–3 days. Five cell lines were frozen at passages 8–9. Two cell lines were maintained in medium supplemented with LIF and 10% FBS and proliferated for more than 25 passages. One line was frozen at passage 25 and one line was maintained in culture and proliferated for more than 120 passages.

Characterization of ES Cells for Expression of Pluripotency Markers

Established colonies were tested at passages 4–6 for alkaline phosphates (AP) activity, and expression of POU5F1, NANOG, SSEA-1, and SSEA-4 by immunocytochemistry. For alkaline phosphatase staining, goat ES cells were cultured on feeder layers for 2–3 days prior to staining. Culture medium was removed from the plates, and cells were washed with PBS. Goat ES cells were fixed with 90% methanol and 10% formaldehyde for 2 min. Fixed cells were washed twice with TBST (25 mM Tris–HCl, 0.14 M NaCl, 2.7 mM KCl, 0.1% Tween-20) and stained with Napthol/Fast Red Violet solution (Chemicon International, Inc., Temecula, CA) for 15 min at room temperature, in the dark. Cells were washed with TBST in order to terminate the staining reaction. Stained cells were maintained in PBS.

To detect expression of pluripotency markers, goat ES cells at passages 4–6 were seeded on goat feeder layers in a 4-well slide. Cells were cultured for 1–2 days with goat ES media supplemented with hLIF (1,000 U/ml) and fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were dehydrated by 5 min incubation in increasing concentrations of methanol (25%, 50%, 75%, and 100%). Slides were kept in 100% methanol at –20°C for at least 12 hr. Cells were re-hydrated with incubation for 5 min in decreasing concentrations of methanol. Cells were permeabilized by incubation with 0.1% Triton X-100 for 10 min. Endogenous peroxidase was quenched by 10 min incubation in 3% hydrogen peroxide at room temperature. Cells were incubated overnight at 4°C with monoclonal antibodies against mouse stage-specific embryonic antigen 1 or 4
(SSEA-1 and SSEA-4) or mouse POU5F1 (Abcam, Inc., Cambridge, MA) at concentrations of 1:10, 1:100, and 1:300, respectively. The appropriate secondary antibodies, horse anti-mouse IgG or goat anti-mouse IgM, were used at a dilution of 1:150 and incubated at 37 °C for 1 hr. Detection of specific binding was performed with the Elite ABC peroxidase staining kit and vector® NovaRED™ (Vector Laboratories, Inc., Burlingame, CA) as the substrate. Positive cells stained red. Control experiments were included using either the primary or secondary antibodies alone. Goat fetal fibroblasts were used as negative control cell line and mouse ES cells were included as positive controls. To detect the presence of NANOG by immunocytochemistry, goat ES cells were incubated with a rabbit-anti-human NANOG antibody (1:50; PeproTech, Inc., Rocky Hill, NJ) followed by peroxidase labeled rabbit IgG (1:100; Vector Laboratories, Inc.). Omission of either primary or secondary antibody served as negative control. Mouse ES cells known to express NANOG were processed in an identical fashion to serve as positive control.

RT-PCR Reaction

Total RNA was isolated from cells using the RNeasy Mini kit (Qiagen, Mississauga, ON, Canada) followed by treatment with DNase I (Ambion, Streetsville, ON, Canada) according to the manufacturer’s protocol. Goat fetal fibroblasts, mouse ES (R1) cells, and mouse fetal fibroblasts were used for comparison. The RNA quality and quantity were determined using the NanoVue Plus (GE Healthcare, Chicago, IL) for reverse transcription, 2 μg of total RNA was used in a final volume of 25 μl reaction containing 0.5 μg of Oligo d(T)12–18, RT buffer (1×), 10 mM dithiothreitol, 0.5 mM of dNTP, 5 U of RNase inhibitor, and 10 U of SuperScript II Reverse transcriptase (Invitrogen, Burlington, ON, Canada). Reverse transcription was carried out at 42 °C for 1 hr. RT-PCR amplification was performed using Applied Biosystems ThermoCycler. Primer sequences (5’–3’), length of amplified products, and annealing temperatures were as outlined in the Table 2. PCR reactions were performed by initially denaturing cDNA at 94 °C for 2 min followed by 32 cycles of denaturing at 94 °C for 30 sec, annealing for 30 sec at temperature specified above for each set of primer, and extension at 72 °C for 40 sec, with final 5 min extension. Goat NANOG, SOX-2, and LIN-28 were pre-amplified with additional 15 cycles using external primers. PCR products were resolved in 1.5% agarose gel and imaged using ChemiDoc XRS+ Molecular imager (Bio-Rad, Mississauga, ON, Canada).

Differentiation of Goat ES Cell In Vitro

Undifferentiated goat ES cells were digested by trypsinization and cultured at a concentration of 2 × 10⁶ cells/ml in DMEM + 10% FBS + 10 μg/ml retinoic acid without LIF in 30 mm gelatin coated Falcon dishes. The cells were incubated at 37 °C for 4–5 days and media was changed every 48 hr. When structures resembling neurospheres formed floating in plates, the dishes were rinsed two times and the supernatant was harvested. The neurospheres were allowed to settle by gravity in a 50-ml tube. Neurospheres were then transferred to gelatinized plates, cultured for 3–4 days and examined for differentiation to cells exhibiting neuronal like morphology. To confirm differentiation into the neuronal lineage, a polyclonal antibody raised in rabbits against human nestin (1:100, Abcam, Inc.) was used for immunohistochemistry on cells cultured on chamber slides that had been differentiated to neurons by culturing in the absence of LIF.

Karyotype Analysis

Goat ES cells grown in medium supplemented with growth factors at passages 35, 70, and 140 were incubated

### Table 2: Primer Sets for RT-PCR

<table>
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<th>Primers</th>
<th>Sequence (5’–3’)</th>
<th>Annealing T (°C)</th>
<th>Length of the PCR fragment (bp)</th>
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<tr>
<td>gPOU5F1 (goat Oct-4), forward</td>
<td>AGGTGTTTCAGCCAAACGACTATCTGG</td>
<td>60.0</td>
<td>192</td>
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<tr>
<td>gPOU5F1 (goat Oct-4), reverse</td>
<td>TCGTGTCTCAGTACTTGTCGCTT</td>
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<td>mPOU5F1 (mouse Oct 4), forward</td>
<td>AGGTGTTTCAGCCAGCAGACTATCTGG</td>
<td>60.0</td>
<td>192</td>
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<tr>
<td>mPOU5F1 (mouse Oct 4), reverse</td>
<td>TCGTGTCTCAGTACTTGTCGCTT</td>
<td>60.0</td>
<td>201</td>
</tr>
<tr>
<td>gNANOG, forward</td>
<td>AGGACAGCCTGATTTCCTGGACAAG</td>
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<tr>
<td>gNANOG, internal reverse</td>
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<td>60.5</td>
<td>198</td>
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<td>gSox-2 (mouse and goat), internal forward</td>
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<td>gSox-2 (mouse and goat), internal reverse</td>
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<tr>
<td>gLin-28, external forward</td>
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<td>226</td>
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<td>Lin-28 (goat and mouse), internal forward</td>
<td>GATGCTTCTTGTGACCATGAGTGATGGATG</td>
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<tr>
<td>Lin-28 (goat and mouse), internal reverse</td>
<td>GATGCTTCTTGTGACCATGAGTGATGGATG</td>
<td>60.5</td>
<td>226</td>
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<td>GAPDH (goat and mouse), forward</td>
<td>GTGCATCATATTTGGGACGGGTTTCTCC</td>
<td>60.0</td>
<td>253</td>
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<tr>
<td>GAPDH (goat and mouse), reverse</td>
<td>GTGCATCATATTTGGGACGGGTTTCTCC</td>
<td>60.0</td>
<td>253</td>
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overnight in culture medium containing 0.05 g/ml colcemid (KaryoMax, Mississauga, ON, Canada). The cells were harvested by trypsinization, washed twice in PBS, and re-suspended in 0.56% KCl for 20 min at room temperature. Briefly, the cells were fixed in cold Carnoy’s fixative (3:1 methanol/glacial acetic acid), attached to glass slides, and stained with Giemsa stain. The metaphase spreads were analyzed using a CytoVision® cytogenetic analysis system (Applied Imaging Corporation, New Milliton, UK).

Cell Transfection
A fluorescent marker (eGFP) was introduced into undifferentiated goat ES cells and goat fetal fibroblast cells (control) by lipid-mediated gene transfer. The CEE-GFP plasmid used (kindly provided by Dr. T. Takada, National Children’s Medical Research Center, Tokyo, Japan) contained the enhanced, humanized version of the green fluorescent protein reporter gene driven by the human elongation factor-1a promoter and cytomegalovirus enhancer, and the neomycin selection marker under the control of the simian virus-40 promoter. This plasmid was delivered into the cells using Lipofectamine (Gibco, Mississauga, ON, Canada) according to the manufacturer’s instructions. A number of stable clones were generated by selection under G418 for 20 days and assessed for expression of the reporter gene by visualization of the fluorescent signal (Carl Zeiss Canada Ltd., North York, ON, Canada).

Teratoma Formation
Goat ES cells were injected into five immunodeficient mice. Cells were selected manually as aggregates of 10–15 cells and also as single cells by trypsinization and 5–7-suspended in 0.56% KCl for 20 min at room temperature. Briefly, the cells were fixed in cold Carnoy’s fixative (3:1 methanol/glacial acetic acid), attached to glass slides, and stained with Giemsa stain. The metaphase spreads were analyzed using a CytoVision® cytogenetic analysis system (Applied Imaging Corporation, New Milliton, UK).

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We thank Drs. D. Ranourt and L. Tang for critical review of the manuscript, and Terry Jordan for care of laboratory rodents. This work was supported by NIH/NICHD (2 R42 HD044780-02), NIH/NCRR (2 R01 RR17359-06), and PharmAthene Canada, Inc.

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