Development, Characterization, and Pluripotency Analysis of Buffalo (*Bubalus bubalis*) Embryonic Stem Cell Lines Derived from *In Vitro*–Fertilized, Hand-Guided Cloned, and Parthenogenetic Embryos

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Abstract

We present the derivation, characterization, and pluripotency analysis of three buffalo embryonic stem cell (buESC) lines, from *in vitro*–fertilized, somatic cell nuclear–transferred, and parthenogenetic blastocysts. These cell lines were developed for later differentiation into germ lineage cells and elucidation of the signaling pathways involved. The cell lines were established from inner cell masses (ICMs) that were isolated manually from the *in vitro*–produced blastocysts. Most of the ICMs (45–55%) resulted in formation of primary colonies that were subcultured after 8–10 days, leading subsequently to the formation of three buESC lines, one from each blastocyst type. All the cell lines expressed stem cell markers, such as Alkaline Phosphatase, OCT4, NANOG, SSEA1, SSEA4, TRA-1-60, TRA-1-81, SOX2, REX1, CD-90, STAT3, and TELOMERASE. They differentiated into all three germ layers as determined by ectodermal, mesodermal, and endodermal RNA and protein markers. All of the cell lines showed equal expression of pluripotency markers as well as equivalent differentiation potential into all the three germ layers. The static suspension culture–derived embryoid bodies (EBs) showed greater expression of all the three germ layer markers as compared to hanging drop culture–derived EBs. When analyzed for germ layer marker expression, EBs derived from 15% fetal bovine serum (FBS)-based spontaneous differentiation medium showed greater differentiation across all the three germ layers as compared to those derived from Knock-Out Serum Replacement (KoSR)-based differentiation medium.

Introduction

THE VARIOUS *IN VITRO* embryo production (IVEP) techniques, like *in vitro* fertilization, parthenogenesis, and somatic cell nuclear transfer (SCNT), have made it possible to obtain buffalo blastocysts following *in vitro* culture for 7–8 days. It was discovered way back in 1980s that the inner cell mass (ICM) of murine blastocysts could be separated from surrounding trophoctoderm and grown under laboratory conditions to produce embryonic stem cells (ESCs) (Evans and Kaufman, 1981; Martin, 1981), a special category of cells with unique properties of infinite propagation and differentiation to all somatic and germ cell lineages (Bradley et al., 1984; Nagy et al., 1990). A genuine belief that ESC research will usher in a revolution in terms of providing a simple model system to

study the basic processes of early embryonic development, cellular differentiation, and cell-based therapies for human disease and treatment led to initiation of the research for derivation of human ESCs and consequently to development of human ESCs (Shamblott et al., 1998; Thomson et al., 1998). The speculations that development of ESC lines of domestic animals would greatly facilitate the precise genetic manipulation for increased disease resistance, increased milk production, as well as desired composition, increased growth rate, improved carcass composition, and enhanced reproductive performance, demonstrated their usefulness at par with those of human ESC lines. The potential to produce transgenic animals using ESCs and their use as models for human diseases, cell transplantation therapies, and biopharming further added to the zeal (Laible, 2009; Wheeler and Walters, 2001).

Despite considerable differences in mice and farm animal reproductive behavior, generation time, production of sufficient number of elite animal embryos, and development of efficient methodologies to isolate ICMs and grow them under laboratory conditions, ESC research in domestic animals proceeded at a pace next only to human ESC research, and ESC lines or ESC-like lines were developed for bovine (Cao et al., 2009; Evans et al., 1990; Iwasaki et al., 2000; Mitalipova et al., 2001; Saito et al., 2002; Sims and First, 1994; Cibelli et al., 1998; Wang et al., 2005), ovine, caprine, equine (Keefer et al., 1996; Notarianni et al., 1991; Saito et al., 2002), and porcine species (Hall, 2008; Li et al., 2004).

Our laboratory was the first to report buffalo ESC (buESC)-like cells derived from *in vitro*-fertilized (IVF) embryos that survived only up to eight passages (Verma et al., 2007). The poor success rates in developing ESCs in species other than mice may either be due to fundamental biological differences between the species or simply due to technical factors associated with the derivation and culture conditions (Bongso et al., 1994; Piedrahita et al., 1990). Here we describe the successful establishment, characterization, and differentiation analysis of buESC lines derived from ICMs of IVF, parthenogenetic (PAR), and hand-guided cloned (HGC) blastocysts.

Materials and Methods

Chemicals

All chemicals and media used in the present study were obtained from Sigma Aldrich (St. Louis, MO, USA), and the plasticware was from Falcon (Paignton, UK), unless stated otherwise.

Production of IVF, PAR, and SCNT buffalo embryos

In vitro fertilization. Cumulus-oocyte complex (COC) aspiration from buffalo ovarian follicles was performed using an 18-gauge syringe needle. The buffalo ovaries were collected from a local abattoir and transported to the laboratory within 3–4 h of slaughter in streptomycin- (50 µg/mL) fortified normal saline solution (NSS). COCs with homogeneous, evenly granular ooplasm and more than four layers of compact unexpanded cumulus cells were transferred to 100 µL droplets (10–15 COCs per droplet) of *in vitro* maturation (IVM) medium, composed of Tissue Culture Medium-199 (TCM-199) supplemented with 10% fetal bovine serum (FBS), 10% buffalo follicular fluid, porcine follicle-stimulating hormone (FSH; 5 µg mL⁻¹), estradiol-17b (1 µg mL⁻¹), 0.81 mM sodium pyruvate, and 50 µg/mL gentamicin sulfate. The mixture was incubated at 38.5°C for 24 h in a humidified CO₂ incubator (5% CO₂ in air), before subjecting to IVF and *in vitro* embryo culture, following the already established in-house protocol (Zandi et al., 2013).

Parthenogenesis. After 22–24 h of IVM, COCs with an expanded cumulus were transferred to 1.5-mL eppendorf tubes containing 500 µL of hyaluronidase (0.5 mg/mL in T2 medium consisting of TCM-199 + 2% FBS) and vortexed for 2–3 min at high speed. The denuded oocytes were washed twice in mcR2aa medium, and activation was achieved by exposure to 2 µM calcimycin A23187 in T20 (T2 with 20% FBS) for 5 min in a dark CO₂ incubator at 38.5°C. After three washes in T20, the oocytes were incubated individually

for 4 h in 5-µL droplets of T20 medium containing 2 mM 6-dimethylamino purine (6-DMAP). After four washes with Research Vitro Cleave Medium (K-RVCL-50; Cook, Brisbane, Queensland, Australia) supplemented with 1% fatty acid-free bovine serum albumin (BSA), the activated oocytes were cultured in 300 mL of this medium in four-well dishes (10–15 embryos per well), overlaid with mineral oil, at 38.5°C and 5% CO₂ in a humidified incubator for 7–8 days.

Hand-guided cloning. The procedure followed for producing the SCNT buffalo embryos was an protocol developed in-house (Shah et al., 2008). Briefly, the IVM COCs were subjected to cumulus removal and Pronase (2.0 mg mL⁻¹ in T10) treatment for zona digestion. After 15–20 min, the denuded and zona-free oocytes with a prominent protrusion cone were transferred to T20 medium containing 2.5 mg mL⁻¹ Cytochalasin B and bisected with a Microblade. The demi-cytoplasts, so obtained, were treated with phytohemagglutinin (0.5 µg mL⁻¹ in T2) for 3–4 sec, followed by gentle rolling over a single donor cell (buffalo fetal fibroblast) to allow their attachment. The couplets (demicytoplast attached to donor cell) were transferred to fusion medium (0.3 M D-mannitol, 0.1 mM MgCl₂, 0.05 mM CaCl₂, and 1 µg mL⁻¹ polyvinyl alcohol) and laid on to the fusion chamber (BTX Microslide, 0.5-mm gap, model 450; BTX).

To achieve fusion in a single-step fusion protocol, the couplet was aligned with an AC pulse (4 V) using a BTX Electrocell Manipulator 200 (BTX, San Diego, CA, USA) in such a manner that the somatic cell faced the negative electrode. The second demi-cytoplast was then immediately transferred to the fusion chamber as close to the somatic cell as possible, followed by application of a single DC pulse (3.36 kV cm⁻¹ for 4 msec). The resulting triplets were incubated in T20 in a CO₂ incubator at 38.5°C for 6 h to enable them to fuse together, followed by activation and culture for 7–8 days, in a manner similar to parthenogenesis.

Establishment and culture of buESC lines

Preparation of feeder layer. The feeder layer was prepared from buffalo fetal fibroblasts derived from the ear tissue of a buffalo fetus (3–4 months of age). Fetal ear skin explants (~1 mm³) were transferred into 25-cm² tissue culture flasks and incubated at 38°C in a 5% CO₂ incubator for attachment, after which fibroblast culture medium [Dulbecco's Modified Eagle Medium (DMEM) + 10% FBS + 50 µg/mL gentamicin] was slowly added. The medium was replaced with fresh medium every 3 days until 70–80% confluence was achieved. At this time, the monolayer was disaggregated by trypsinization and incubated at 37°C for 2 min to ensure fractional disaggregation of fibroblasts. This was followed by another passage, after 3–4 days, when a portion of the culture was subjected to immunocytochemical characterization for fibroblast-specific proteins like CYTOKERATIN18, VIMENTIN, and β-TUBULIN as well as for epithelial/myoepithelial cell marker α-SMOOTH MUSCLE ACTIN to ensure absence of these cells from the cultures (Fig. S1) (Supplementary Data are available at www.liebertpub.com/cell/). The feeder layer was prepared by treating the cells at 70–80% density with 10 µg/mL mitomycin C for 3 h, followed by trypsinization,

three to four washings with Dulbecco's phosphate-buffered saline (DPBS⁻), and seeding at a final concentration of 2×10^5 viable cells mL⁻¹, in 100- μ L droplets of fibroblast culture medium under sterile mineral oil. The feeder layers were prepared 1 day prior to seeding of ICMs or culture of the ESC colonies.

Isolation and culture of ICM

ICMs of the hatched blastocysts were isolated mechanically by cutting with a MicrobladeTM under a zoom stereomicroscope (Olympus, SZ40, Japan). Only those blastocysts with clear ICMs, definite spherical shape, and normal size were selected. The isolated ICMs were seeded individually and separately onto the feeder layers, overlaid with ESC medium (KoDMEM + 15% KoSR), supplemented with 2 mM L-glutamine, 5 ng/mL basic fibroblast growth factor (bFGF), 1000 U/mL recombinant murine leukemia inhibitory factor (rmLIF), $1 \times$ nonessential amino acids, and 50 μ g/mL gentamicin sulfate and incubated at 38°C in a 5% CO₂ incubator. The culture medium was changed every alternate day, and further colonization of the cells was observed routinely under an inverted microscope. The primary colonies, obtained 8–10 days after seeding, were disaggregated with the aid of a Microblade and individually reseeded onto new feeder layers. The colonies exhibiting typical morphological features of ESC-like cells were subcultured onto the new feeders.

Characterization of buESC lines

Alkaline phosphatase staining. For alkaline phosphatase staining, ESC colonies growing normally on feeder droplets were washed twice with DPBS after removing the culture medium. The staining was performed by an alkaline phosphatase staining kit (Sigma, Catalogue No. 86C) as per the manufacturer's protocol.

Immunocytochemical analysis for stem cell markers. The ESC colonies were examined at regular intervals to determine the expression of various stem cell markers. For immunocytochemistry, colonies were dissociated from their feeders, washed twice with DPBS⁻, and fixed in 4% paraformaldehyde (PFA) for 20 min. After two washes, the colonies were treated with 0.1% Triton X-100 for 15 min, washed twice, and incubated for 1 h in blocking solution

(4% normal goat serum in DPBS) at room temperature. The colonies were exposed to primary antibodies for OCT4, NANOG, SOX2, and FOXD3 (1:50 dilution) and SSEA-1, SSEA-4, TRA-1-60, TRA-1-81, and CD90 (1:100 dilution), and incubated overnight at 4°C. After three thorough washings, the cells were incubated for 1 h at room temperature with the appropriate class-specific secondary antibodies, labeled either with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). After three washings, the colonies were put individually on clean grease-free microscopy glass slides overlaid with Prolong Gold Antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies, USA) for nuclear staining and visualized under a fluorescence microscope (Diaphot; Nikon, Tokyo, Japan). The various primary antibodies, their sources, and class-specific secondary antibodies used against them are listed in Table 1.

Western blot analysis for stem cell markers

A total of 50–60 colonies of the IVF buESC line, at passage 32, were disrupted in liquid nitrogen for total protein extraction using an NE-PER Mammalian Protein Extraction Kit (Thermo-Scientific), as per the manufacturer's protocol. The protein fraction so obtained was resolved in a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the previous protocol (Shah et al. 2015). The bands were transferred to Hybond-LFP [low fluorescent polyvinylidene difluoride (PVDF) membrane; Millipore, Billerica, MA, USA] in a semi-dry transfer apparatus (E5100). After overnight blocking in 5% BSA at 4°C, the blots were treated with the primary antibodies (Table 1) at 1:2000 dilution in blocking solution for OCT4, NANOG, SOX2, FOXD3, SSEA1, SSEA4, TRA-1-60, TRA-1-81, and CD90. After 2 h incubation at room temperature, the blots were rinsed thrice in Tris-buffered saline (TBS), followed by incubation in appropriate class-specific horseradish peroxidase (HRP)-conjugated secondary antibody solution (1 μ g μ L⁻¹) [diluted 1:5000 in Tris-buffered saline (TBS)] for 1 h at room temperature. After thorough washings, the blots were treated with working HRP-substrate, containing equal volumes of Luminol reagent and Peroxide solution (Millipore, USA), and incubated for 5 min. The excess substrate was drained, and the blots were covered in clean plastic

TABLE 1. ANTIBODIES USED FOR IMMUNOSTAINING, THEIR SOURCES, TYPE, AND CLASS-SPECIFIC SECONDARY ANTIBODIES

Antibody	Source	Type	Secondary antibody
SSEA-1	MAB4301	Mouse monoclonal, IgM	Rabbit anti-mouse IgM- FITC labeled
SSEA-4	MAB4304	Mouse monoclonal, IgG	Rabbit anti-mouse IgG-PE labeled
TRA-1-60	MAB4360	Mouse monoclonal, IgM	Rabbit anti-mouse IgM- FITC labeled
TRA-1-81	MAB4381	Mouse monoclonal, IgM	Rabbit anti-mouse IgM- FITC labeled
Oct-4	AB3209	Rabbit polyclonal, IgG	Mouse anti-rabbit IgG-PE labeled
NANOG	AB9220	Rabbit polyclonal, IgG	Mouse anti-rabbit IgG-PE labeled
Sox-2	orb11398	Rabbit polyclonal, IgG	Mouse anti-rabbit IgG-PE labeled
FoxD3	orb69353	Mouse monoclonal, IgG ₁	Rabbit anti-mouse IgG-PE labeled
Thy-1 (CD90)	F15-42-1	Mouse polyclonal, IgG	Rabbit anti-mouse IgG-PE labeled
Cytokeratin 8	Orb10413	Rabbit polyclonal, IgG	Mouse anti-rabbit IgG-PE labeled
GATA4	AB4132	Rabbit polyclonal, IgG	Goat anti-rabbit IgG-FITC labeled
BMP4	MAB1049	Mouse monoclonal, IgG _{2B}	Rabbit anti-mouse IgG-PE labeled

Ig, immunoglobulin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

wrap for exposure to CL-Xposure film (Thermo Scientific, USA) for 5–10 min. The film was developed in the dark-room by dipping in developer and fixer solutions (Sigma-Aldrich, St. Louis, MO, USA), and proteins were observed as dark bands on the X-ray film. The exposed films were scanned using a fluorescent laser imager (Typhoon TRIO+ Variable Mode Imager, GE Healthcare). The protein lysate from buffalo fetal fibroblasts was also subjected to western blotting (negative control), and β -ACTIN was used as the loading control.

RT-PCR detection of stem cell markers

Total RNA was extracted from buESC colonies using an RNeasy RNA Extraction Kit (Qiagen, Germany) following the manufacturer's instructions. The extracted RNA was subsequently treated with DNase (Ambion, USA), quantified, and checked for purity in a NanoDrop Spectrophotometer (TECAN, Austria GmbH). Only those RNA samples with a 260/280 ratio ≥ 1.9 were used for cDNA synthesis, employing a SuperScript III First Strand cDNA Synthesis Kit (Invitrogen, USA). Reverse transcriptase (RT)-negative reactions, which contained all components for cDNA synthesis except the RT enzyme, were also prepared. The primers for various stem cell

markers were synthesized on the basis of the published nucleotide sequences in the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) and ENSEMBL Genome Browser. The primers were designed, using Perl Primer v1.1.21, to include intronic sequences or designed from the exon-exon boundaries.

The sequences of the gene-specific primers were entered into a BLAST search to ensure that they did not show significant homology to other sequences in the genome. The primer sequences, annealing temperatures, amplified product lengths, and GenBank accession numbers of the original sequences are given in Table 2. RT-PCR reactions were set up in a final volume of 25 μ L having 10 $\times$ PCR buffer, 10 mM dNTPs, 1.0 U of DreamTaq DNA polymerase, 10 pmol/ μ L each of forward and reverse primers, and 4 μ L (400 ng) of cDNA. The thermal cycling conditions were 94°C for 3 min, followed by a cycling program of 94°C for 30 sec, X°C (annealing temperature as mentioned for each primer pair in Table 2) for 30 sec and 72°C for 30 sec for 36 cycles, followed by final extension at 72°C for 10 min.

RT-negative PCR reactions were also performed for the respective genes to ensure complete absence of genomic DNA. The PCR products were visualized on 2% agarose gel

TABLE 2. PRIMERS USED FOR RT-PCR AND qPCR

Gene	Sequence(5'-3')	Ta (°C)	Product length	Acc. no
<i>OCT4</i>	F, CGCCCTATGACTTGTGTGGA R, TCGGCTCCAGCTTCTCCTT	58	201	GU997625.1
<i>SOX2</i>	F, ATGGGCTCGGTGGTGAAGT R, GCTCTGGTAGTGTCTGGGACA	59	183	GQ853881.1
<i>NANOG</i>	F, CCGAAGCATCCAACCTCTAGG R, GAGACAGTGTCCGTGTCTGAG	60	100	NM_001025344.1
<i>c-MYC</i>	F, CTCCTCACAGCCCGTTAGTC R, ATTTGCGGTTGTTGCCTATC	53	156	GU296437.1
<i>REX-1</i>	F, CAGCTGCCATAGGCTCTACC R, GTCTGGCCTGACAAAGGTGT	55	239	XM_001255545
<i>STAT3</i>	F, GCGAAGAATCAAGCAGTTCC R, CCAGGGCAGTAAGCATCTGT	60	383	DQ487026
<i>FOXD3</i>	F, CTATTCGTACATCGCGCTCA- R, GGTCCAGGGTCCAGTAGTT	58	228	ENSBTAG00000008501
<i>TELOMERASE</i>	F, GACTTCCAGCCGTACCTGA R, GATCCCCTGACACTGGATGT	58	192	NM_001046242.1
<i>NF-68</i>	F, AGGAAGATGCTGAGGAAGCA R, GTTGACCTGATTTCTGGGAGA	60	183	NM174121
<i>CYTOKERATIN-8</i>	F, GTCTCCAGGCTGAGATCGAGT R, CCAGCAGCTTCTCTGTAGGT	58	241	BC103339.1
<i>BMP-4</i>	F, GGTAACCGAATGCTGATGGT R, CTGGGATGTTCTCCAGATGT	55	385	NM001045877.1
<i>MSX1</i>	F, GGACTCCTCAAACCTGCCAGA R, TACTGCTTCTGGCGGAACCT	58	224	NM_174798
<i>AFP</i>	F, TCTTCCCCATGTTCTTTCAGG R, TTTACAGGCAAATTTCTTCC	55	205	NM_001034262
<i>GATA4</i>	F, CGGAAAGAGGGGATTCAAAC R, ATCTCCTCACTGCTGCTGGT	58	152	DQ487031
<i>GAPDH</i>	F, TCAAGAAGGTGGTGAAGCAG R, CCCAGCATCGAAGGTAGAAG	157	56	GU324291.1
β -ACTIN	F, TCACGAAACTACCTTCAATTC R, TCTTGATCTTCATTGTGCTGG	163	56	NM_001290932.1

The primer sequences, annealing temperatures, PCR product lengths, and GenBank accession numbers of the original sequence from which the primers were synthesized are given.

F, forward; R, reverse.

for the specific products. PCR products were cloned into a TA cloning vector and sequenced using the automated dye-terminator cycle sequencing method in an ABI PRISM 377 DNA Sequencer (Merck Specialties, Bangalore, India). The sequences obtained were subjected to a BLASTn search to ensure that the primer amplification was specific and correct. Some of the sequences (>200 bp) like *OCT4* (acc. no. KJ194832.1), *REX1* (KJ194835.1), *FOXD3* (KJ194833.1), and *STAT3* (KJ194836.1) were submitted to GenBank, and the corresponding accession numbers were obtained.

Analysis of chromosomal integrity

Karyotyping was performed in randomly collected colonies throughout the culture period as per the protocol described by Dyban (1983), with some modifications. buESC colonies were allowed to grow for 3–4 days under standard culture conditions, cut into 1:8 split ratios, washed three times with DPBS[−], and treated with Trypsin-EDTA for 2–3 min to remove any attached feeder cells. This was followed by two washes in DPBS[−], treatment with Accutase, and repetitive pipetting with a finely drawn glass pipette until the cells were completely dissociated into single cells. The cell pellet, obtained by centrifugation at 2000 rpm for 5 min, was treated with Colcemid (0.1 µg/mL) for 3 h at 37°C followed by overnight incubation in 0.56% KCl at 4°C. The cells were pelleted and suspended in 2 mL of chilled fixative solution (glacial acetic:methanol, 1:3) for 30 min, followed twice by the same treatment, each for 10 min.

The supernatant was removed, leaving about 0.5 mL of fluid into which the pellet was mixed by gentle pipetting. Two to three drops of the suspension were dropped from a height of about 25–30 inches on ice cold glass slides and allowed to dry at room temperature for 10–15 min. The slides were immersed in freshly prepared 2% Giemsa stain for 10 min and rinsed with deionized water to remove the excess stain. The slides were dried thoroughly for few hours before screening by microscopy for the well spread-out metaphase plates, which were photographed and evaluated for construction of karyotypes by Cytovision 7.2 software (Leica, USA). A total of 69 (29 for IVF buESCs, 20 for HGC buESCs, and 20 for PAR buESC line) well spread-out metaphase plates were evaluated for karyotyping.

Pluripotency analysis of buESC lines

Quantitative PCR (qPCR) was performed for various pluripotency and self-renewal associated genes such as *OCT4*, *NANOG*, *SOX2*, *c-MYC*, *TELOMERASE*, and *REX1* to examine their relative expression in the three cell lines. cDNA was prepared from total RNA extracted from each cell line at passage 28. The optimized qPCR reactions contained 2 µL (100 ng) of cDNA template, 10 µL of SYBR Green PCR Master Mix Buffer (2×), and 10 pmol each of forward and reverse primers in a total volume of 20 µL, and were performed on a CFX96 instrument (BioRad Hercules, CA, USA). *GAPDH* and β -*ACTIN* were used as the endogenous controls for each sample. The thermal cycling parameters were initial denaturation at 95°C for 10 min, followed by 40 PCR cycles (denaturation at 95°C for 30 sec, annealing at X°C (respective for each gene as given in Table 2) for 30 sec, and extension at 72°C for 30 sec, followed by a dissociation protocol. A no-template control (NTC) of nuclease-free water

was included in each run. All PCR reactions were performed in triplicate, and PCR efficiencies (E) were evaluated by two-fold dilution series of cDNAs (1 to 1:64 dilution) for each pair of primers including the reference genes (*GAPDH* and β -*ACTIN*) using a cDNA pool, obtained from all the buESC lines, as per the previously described protocol (Shah et al., 2015). The relative expressions of the test genes in the cell lines were calculated using $2^{-\Delta\Delta C_t}$ method, taking the corresponding values for IVF buESCs as the calibrator values (Schmittgen and Livak, 2008).

In vitro differentiation of buESC lines

The stem cell colonies were differentiated via formation of embryoid bodies (EBs) as well as on adherent cultures (0.1% gelatin or MaxGel-coated dishes). The EB formation was achieved in static suspension cultures (SSCs) and hanging drop (HD) cultures. The colonies were cut into small clumps (1:8 split ratios) with a margin to the feeder layer to avoid fibroblast contamination, and washed three times in spontaneous differentiation medium (SDM; ESC culture medium without bFGF and rmLIF) to remove any attached feeder cells. Each experiment replicate was performed with 50 colonies from each ESC line. In the HD strategy, the HD cultures were prepared on a Petri dish lid with 25-µL droplets of SDM, with one colony placed into each droplet. The lid was inverted and placed on the Petri dish base containing DPBS and incubated for 5 days at 38°C and 5% CO₂. The presumed EBs were subjected to further growth in SSC for a total period of 14 days. In the SSC strategy, the buESC colonies were placed in low-attachment 35-mm Petri dishes (Nunc, Denmark, cat. no. 150255) in SDM and cultured for the same time period under similar culture conditions.

The EBs formed were used for total RNA extraction and immunocytochemical analysis to evaluate their differentiation into three germ layers. RNA extraction and cDNA preparation were performed, according to the already discussed protocols. RT-PCR for various ectoderm (*NF-68*, *CYTOKERATIN8*), mesoderm (*BMP4* and *MSX1*), and endoderm (*AFP* and *GATA4*) markers was performed, according to already mentioned PCR parameters, albeit at different annealing temperatures, as mentioned for each primer pair in Table 2. The same PCR program was followed for control (undifferentiated ESC colonies) as well as for RT-negative and NTC reactions. The EBs as well as undifferentiated ESC control colonies were also characterized by immunocytochemistry for *CYTOKERATIN8*, *BMP4*, and *GATA4*, following the already discussed protocol. In an alternative mode of differentiation that excluded formation of EBs, stem cell colonies were cultured in spontaneous differentiation medium while still attached to MaxGel-coated dishes. Half of the culture medium was changed every alternate day, and the colonies were allowed to differentiate for up to 14 days.

EBs produced in SSCs, from all three buESC lines, were compared for the germ layer marker expression by qPCR. Comparisons were also made between HD- and SSC-produced EBs (HD vs. SSC) for the same germ layer marker expression. In both of the experiments, the EB-inducing medium was the usual ESC medium without bFGF and rmLIF. Finally, the differentiation-inducing potential of KoSR-based medium was compared with that of FBS-based medium (15%

FBS vs. 15% KoSR) by quantification of the same germ layer markers. The qPCR assay was performed as described already. The primer sequence and annealing temperatures are listed in Table 2.

Cryopreservation of buESC Lines

ESC cryopreservation was performed as described earlier (Verma et al., 2007) with slight modifications. buESC colonies were vitrified at different passages in groups of four to five in French mini-straws (IMV, L'Aigle, Cedex, France). Vitrification solutions were based on holding medium (HM; DMEM + 20% FBS). Colonies were incubated in vitrification solution 1 [VS1; HM + 10% dimethyl sulfoxide (DMSO) and 10% ethylene glycol] for 1 min, followed by incubation in VS2 (HM + 20% DMSO + 20% ethylene glycol and 0.5 M sucrose) for 20–30 sec, and finally loaded into the straws. The open ends of the straws were sealed with polyvinyl alcohol and the straws were immediately plunged into liquid nitrogen. For thawing, straws were taken out of liquid nitrogen and plunged into water at 37°C for 30 sec. The sealed end was cut to allow colonies to enter the HM supplemented with 0.2 M sucrose for 1 min of incubation, followed by 5 min in HM with 0.1 M sucrose. The colonies were further incubated twice (5 min each) in HM before seeding onto feeder layers. The reviewed colonies were subcultured and evaluated for the stem cell markers.

Statistical analysis

qPCR data analysis for HD- versus SSC- and KoSR- versus FBS-based differentiation media were compared by Student's *t*-test whereas other qPCR data were analyzed by one-way analysis of variance (ANOVA) using a statistical software program (SPSS 11.5, 2004, IBM, USA).

Results

Establishment of buESC lines

We report three XX buffalo ESC lines, one each from IVF-, HGC-, and PAR-produced bubaline blastocysts. The cell lines were accordingly named as IVF buESCs, HGC buESCs, and PAR buESCs. Following their mechanical isolation from day 7 blastocysts, the ICMs were seeded individually onto the feeder layers. After 2 days of incubation, most of the ICMs were found attached to feeder cells, whereas an approximate 20–25% of the ICMs, irrespective of the maternal blastocyst type, showed no attachment. The cultures were allowed to grow for 8–10 days with medium changes every alternate day to develop into primary ESC colonies (Fig. 1). The primary colony formation rates of the ICMs were not significantly ($p < 0.05$) different from one another, although more ICMs from IVF-derived blastocysts produced primary colonies, followed by ICMs from HGC- and PAR-produced blastocysts (Table 3).

The primary colonies so formed were subcultured onto fresh feeders to produce the ESC lines. Out of the initial 20–30 primary colonies of each type, only three to four made it up to 10 passages and beyond. We cryopreserved all of the cell lines around passages 10–20 for future use except for one to two cell lines of each type that were propagated for the current research work. Our main purpose for generation of buESC lines in general, and an XX buESC line in particular, was to develop the differentiation strategies for differentiation of buESCs into germ cells, especially oocytes, and to elucidate the signaling pathways involved in gametogenesis. The IVF buESC line, at the time of writing this draft, has already survived 100 passages, whereas PAR buESC and HGC buESC lines were cryopreserved at about 40 and 45 passages, respectively, for future studies.

FIG. 1. Establishment of buESC lines from ICMs isolated from IVF (upper panel), HGC (middle panel), and PAR blastocysts (lower panel). Magnification, 100 $\times$; scale bar, 100 μ m.

TABLE 3. PRIMARY COLONY FORMATION RATE OF ICMs DERIVED FROM IVF-, HGC-, AND PAR-PRODUCED BLASTOCYSTS

Type of blastocyst	Total number of blastocysts used for ICM isolation	No. of ICMs seeded	No. of primary colonies formed	Primary colony formation rate (%)
IVF	88	80	44 ± 4.0	55 ± 5 ^a
HMC	48	44	23 ± 3	52 ± 7 ^a
PAR	80	70	32 ± 4	45.5 ± 5.5 ^a

^aThe values do not differ significantly; $p < 0.05$.

The increase in colony area was used as an estimator of proliferation rate. All of the three cell lines exhibited an almost similar growth rate, as determined by the difference in colony areas at day 1 and day 7 after seeding, when measured under an inverted microscope at passages 23–30 (Model Eclipse Ti 5, Nikon, Japan). The difference between day 7 and day 1 colony areas did not vary significantly ($p < 0.05$) between the cell lines, although it was highest for the IVF buESC line ($12,581.90 \pm 628 \mu\text{m}^2$), followed by the HGC buESC line ($11,884 \pm 328 \mu\text{m}^2$) and the PAR buESC line ($11,553 \pm 320 \mu\text{m}^2$).

Characterization of buESC lines

All of the putative cell lines obtained were characterized for various stem cell markers by RT-PCR and immunocytochemistry, according to the already established criteria for human ESC characterization (Heins et al., 2004). All of the putative buESC colonies had a characteristic dome-shaped morphology of buESC colonies and were strongly positive for alkaline phosphatase staining, as shown for IVF buESC (Fig. 2), PAR buESC and HGC buESC (Fig. S2a, b). RT-PCR was positive for various stem cell markers like *OCT4*, *NANOG*, *SOX2*, *c-MYC*, *REX-1*, *STAT3*, *FOXD3*, and *TELOMERASE* in all the three ESC lines (Fig. 3). Immunocytochemical analysis for stem cell markers carried out for intact colonies showed positive expression of various pluripotency and self-renewal-associated transcription factors like *OCT4*, *NANOG*, *SOX2*, and *FOXD3* as well as for stem cell-surface markers like *SSEA1*, *SSEA4*, *TRA-1-60*, *TRA-1-81*, and *CD-90*, as shown for IVF buESC (Fig. 4a, b), HGC buESC (Supplementary Fig. S3a, b), and PAR buESC (Fig. S4a, b) lines. Western blot analysis, using the same primary antibodies, revealed the presence of specific bands for *OCT4*, *NANOG*, *SOX2*, *FOXD3*, *SSEA1*, *SSEA4*, *TRA-1-60*, *TRA-1-81*, and *CD90* proteins in buffalo embryonic stem cell protein lysates (Fig. 4c), whereas no such bands were observed in fetal fibroblast protein lysates except for β -ACTIN.

Karyotyping of buESC lines

The cell lines karyotyped at various passages (passage 10, 30, and 60) revealed the normal chromosomal content throughout the culture. In all of the 69 spreads evaluated, karyotyping revealed normal and exact metaphase euploid content of a female murrah buffalo (*Bubalus bubalis*) ($48 + XX$), as shown for IVF buESC (Fig. 5), PAR buESC, and HGC buESC lines (Fig. S5a, b).

FIG. 2. Morphology and alkaline phosphatase staining of putative buESC colonies at passage 15, derived from ICMs of IVF blastocysts (IVF buESC). Magnification, 200 $\times$; scale bar, 100 μm . Note the dome shape of buESC colonies and high alkaline phosphatase activity in comparison to the underlying feeder cells.

Equivalency of pluripotency marker expression

qPCR quantification for various pluripotency markers like *OCT4*, *NANOG*, *SOX2*, *c-MYC*, *TELOMERASE*, and *REX-1* did not reveal significantly ($p < 0.05$) different expressions in the three cell lines except for *TELOMERASE*, which was significantly ($p < 0.05$) expressed at lower levels in the HGC buESC line (Fig. 6). However, the number of transcripts for *OCT4*, *NANOG*, and *c-MYC*, although nonsignificant, was higher in HGC buESC and PAR buESC as compared to IVF buESC, which expressed *SOX2* and *REX1* at higher levels. *TELOMERASE* showed significantly higher expression in the PAR buESC line when compared to the HGC buESC line (Fig. 6).

In vitro differentiation of buESC lines

Upon spontaneous differentiation in HD and SSCs, the colonies from all three stem cell lines differentiated into EBs. The formation of EBs, both cystic and compact, was detected at day 4 of SSC, whereas in HD culture their presence was detected at day 7 (5 days in HD + 2 days in suspension culture) (Fig. 7a, b). SSCs had a propensity to produce more cystic EBs than the HD method in all the three cell lines. Both SSC and HD culture were maintained

FIG. 3. RT-PCR results for various stem cell markers in buESC lines: 1, *OCT4* (232 bp); 2, *NANOG* (111 bp); 3, *SOX2* (183 bp); 4, *c-MYC* (156 bp); 5, *REX1* (239 bp); 6, *FOXD3* (228 bp); 7, *TELOMERASE* (192 bp); 8, *STAT3* (383 bp). Lane 1, 100 bp DNA marker; lane 2, IVF buESC; lane 3, HGC buESC; lane 4, PAR buESC; lane 5, NTC; lane 6, RT-negative.

for a total of 14 days, after which they were analyzed for germ layer marker expression. RT-PCR analysis of the EBs showed expression of ectodermal (*CYTOKERATIN8* and *NF68*), mesodermal (*BMP4* and *MSX 1*), and endodermal (*AFP* and *GATA4*) markers (Fig. 8). None of these markers was expressed in undifferentiated buESC colonies (Fig. S6). Immunocytochemistry also revealed expression of CYTOKERATIN8, BMP4, and GATA4 in EBs obtained in both SSC and HD culture, when examined at day 8 of the culture period (Fig. 9 and Fig. S7), whereas no such protein expression was detected in control (undifferentiated) buESC

colonies (Fig. S8). The colonies, from all the three cell lines, also differentiated into various cell types (neuron-like cells, muscle-like cells and epithelial-like cells) when allowed to differentiate on MaxGel coated dishes (Fig. 10)

Spontaneous differentiation of the buESC lines in 15% KoSR-based medium in SSCs revealed no significant ($p < 0.05$) differences on qPCR analysis for various germ layer markers like *NF-68* and *CKT8* (ectodermal), *MSX1* and *BMP4* (mesodermal), and *AFP* and *GATA4* (endodermal), except for *AFP*, which showed significantly higher expression in PAR buESCs as compared to the other two cell lines (Fig. 11). The germ layer

FIG. 4. Immunocytochemical analysis for ESC markers in IVF-buESC: (a) Transcription factors (*OCT4*, *NANOG*, *SOX2*, and *FOXD3*). (b) Surface markers (*SSEA1*, *SSEA4*, *TRA-1-60*, *TRA-1-81*, and *CD90*). (Upper panel) Secondary antibody stained photographs [phycoerythrin/fluorescein isothiocyanate (PE/FITC)-conjugated]. (Lower panel) Merged photographs of DAPI and secondary antibody-stained colonies. Magnification, 200 $\times$; scale bar, 100 μ m). (c) Western blot analysis for stem cell markers in IVF buESC line: *OCT4*, *NANOG*, *SOX2*, *FOXD3*, *SSEA1*, *SSEA4*, *TRA-1-60*, *TRA-1-81*, *CD90*, and β -ACTIN.

FIG. 5. Karyogram of IVF buESCs depicting normal diploid 48 + XX karyotype.

markers were expressed to equivalent levels in the three buESC lines. When SSC and HD cultures were compared, mRNA quantification revealed significantly ($p < 0.05$) higher expression of *NF68*, *CK8*, *MSX-1*, *BMP4*, and *GATA4* in SSCs, whereas *AFP* was expressed, although nonsignificantly, at higher level in HD cultures (Fig. 12a). A further comparison of 15% KoSR- and 15% FBS-based media for germ layer differentiation in SSC revealed that FBS had greater capability than KoSR to direct differentiation across all the three germ layers (Fig. 12b). The markers for all three germ layers showed sig-

nificantly ($p < 0.05$) higher expression in FBS-based suspension cultures indicating, perhaps, the usefulness of KoSR in normal ESC culture medium for maintenance of an undifferentiated stem cell state.

Resumption of growth after cryopreservation

All of the cryopreserved buESC lines resumed normal growth after thawing. They showed normal pluripotency marker expression as determined by RT-PCR and immunocytochemistry

FIG. 6. qPCR quantification of various pluripotency associated genes (*OCT4*, *NANOG*, *SOX2*, *c-MYC*, *TELOMERASE*, and *REX1*) in IVF buESCs, HGC buESCs, and PAR buESC colonies. Bars with different superscripts differ significantly ($p < 0.05$). The bars represent $2^{-\Delta\Delta CT}$ values $\pm$ standard error of the of mean (SEM), and are calibrated against the corresponding normalized values of IVF buESC colonies.

FIG. 7. Spontaneous differentiation of buESC colonies into embryoid bodies in SSCs (a) and HD culture (b). (*Upper panel*) IVF buESCs; (*middle panel*) PAR buESCs; (*lower panel*) HGC buESCs. Magnification, 100 $\times$; scale bar, 100 μ m.

that was similar to those of their noncryopreserved counterparts. It was not possible to subject all the reviewed colonies to karyotyping, but a random selection across all the cell lines revealed the normal karyotype, indicating the normal chromosomal stability after cryopreservation.

Discussion

Initial research into the isolation of domestic animal ESCs in our and other laboratories attempted to repeat the work carried out in mice by isolating ICMs directly from

cultured preimplantation embryos. The ICMs could be isolated from the intact blastocysts, either by immunosurgery, enzymatically, or by mechanical isolation. Immunosurgery, involving the removal of outer trophectoderm cells by an antibody–complement reaction, is not a method of choice for isolation of buffalo ICMs, because of potentially harmful effects on ICM due to exposure to antibodies and complement components. Enzymatic removal, achieved by treatment of the blastocysts with either trypsin, collagenase, or dispase (Wang et al., 2007) also has not found good favor with bovine or bubaline ICM isolation, owing to sensitivity

FIG. 8. RT-PCR results depicting expression of various germ layer markers (*CYTOKERATIN8*, *NF68*, *BMP4*, *MSX1*, *AFP*, and *GATA4*) in EBs developed by spontaneous differentiation in SSCs. Lane 1, 100-bp DNA ladder; lane 2, IVF buESC; lane 3, PAR buESC; lane 4, HGC buESC; lane 5, NTC; lane 6, RT-negative.

of ESCs to trypsin and differentiation on subsequent passages (Yu and Thomson, 2008). The mechanical isolation, although cumbersome, is useful for isolation of ICM and propagation of the subsequent colonies. Because no antibody or enzymatic treatment is required, ICMs are not exposed to their harmful effects. Furthermore, the mechanical passaging of ESCs in clumps rather than single cells results in greater stability over time as well as consistent colony

size. Subculturing by enzymatic treatment also leads to individualization of cells, which may accumulate karyotypic abnormalities more readily than manual passaging.

In view of these obvious advantages, we preferred subculture of our ESC lines by manual cutting using a Microblade under zoom stereomicroscope. When seeded onto feeder cells, the attachment and growth of ICM primarily depend on the donor blastocyst health, culture medium

FIG. 9. Immunocytochemical characterization of EBs developed in SSC from IVF, HGC, and PAR buESCs for germ layer marker (CK8, BMP4, and GATA4) expression. Magnification, 200 $\times$; scale bar, 100 μ m.

FIG. 10. *In vitro* differentiation of buESC colonies into various cell types (hepatocyte-like cells, adipocyte-like cells, epithelial-like cells, and neuron-like cells) in adherent cultures on MaxGel-coated dishes. (*Upper panel*) IVF buESC colonies; (*middle panel*) HGC buESC colonies; (*lower panel*) PAR buESC colonies. Magnification, 200 $\times$; scale bar, 100 μ m.

FIG. 11. qPCR analysis for germ layer marker expression in EBs, formed from the three buESC lines in SSCs upon spontaneous differentiation in KoSR-based media. Bars represent $2^{-\Delta\Delta CT}$ values $\pm$ SEM and are calibrated against the corresponding normalized values of IVF buESC colonies. Bars with different superscripts differ significantly ($p < 0.05$).

FIG. 12. qPCR analysis of day 14 EBs for: (a) SSC and HD cultures for differentiation into three germ layers in KoSR-based spontaneous differentiation media. (b) Comparison of KoSR- and FBS-based spontaneous differentiation media for differentiation into three germ layers in SSCs. Bars represent $2^{-\Delta\Delta CT}$ values $\pm$ SEM and are calibrated against the corresponding normalized values of SSC differentiated EBs. Bars with different superscripts differ significantly ($p < 0.05$).

composition, and feeder cell type (Van Stekelenburg-Hamer et al., 1995). In our experiment, ICMs were isolated from 7- to 8-day hatched blastocysts with a definite spherical morphology. The health and developmental potential of some sister blastocysts (blastocysts obtained in the same trial but selected randomly for differential staining) was also inferred from total as well as ICM cell number, as revealed by differential staining of the blastocysts. The total cell number and ICM to trophectoderm ratio (ICM/TE) of all the blastocysts was in the range of 160–220 and 0.20–0.24, respectively (data not shown), which are accepted as characteristic features of healthy and developmentally competent embryos (Vajta et al., 2001, 2003). The viability of feeder cells, as examined by Trypan Blue dye exclusion, revealed 97% live cells. These two factors (embryonic health and feeder viability) together with same culture media and growth conditions ensured almost similar primary colony formation rates from ICMs derived from the three different types of blastocysts. These might be the reasons for statistically nonsignificant ($p < 0.05$) differences in primary colony formation rates.

Before the cell line qualifies to be called a bona fide ESC line, it is required to fulfill certain criteria: (1) Derivation from a pluripotent cell population; (2) stable diploidy and normal karyotype; (3) capability for indefinite propagation; (4) ability of spontaneous differentiation into multiple cell types representative of all the three embryonic germ layers,

both in teratomas after grafting or *in vitro* under appropriate conditions; and (5) potential to give rise to any cell type in the body, including germ cells, when allowed to colonize a host blastocysts (Pera et al., 1989). To further ensure that the line so established is a true stem cell line, various other attributes, like species-specific morphological resemblance, pluripotency-related marker expression, growth requirements, and self-renewal capability, are also taken into account. The ESC colonies of the three stem cell lines reported in this article were dome-shaped, a morphological feature characteristic of buESCs (Verma et al., 2007). The ESCs of mouse, human, bovine, canine, sheep, and porcine species have been shown to express high alkaline phosphatase activity (Gjorret and Maddox-Hyttel, 2005; Hamano et al., 1998; Polejaeva et al., 1995; Robertson, 1987; Resnick et al., 1992). Thus, the expression of alkaline phosphatase activity in our ESC lines qualifies them to be putative ESC lines. The presence of transcripts for *OCT4*, *NANOG*, *SOX2*, *c-MYC*, *REX1*, *STAT3*, and *TELOMERASE*, as observed in RT-PCR, further confirms their pluripotency and self-renewal capability.

Transcription factors like *OCT4*, *SOX2*, *NANOG*, *FOXD3*, and *REX1* play a critical role in stem cell self-renewal and are thought to be central to transcriptional regulatory hierarchy specifying ESC identity (Avilion et al., 2003; Chambers et al., 2003; Hart et al., 2004; Lee et al., 2004; Mitsui et al., 2003; Nicholas et al., 1998; Scholer

et al., 1990). The expression of these transcription factors has been used for characterization of ESC lines across species like mouse (D'Amour and Gage, 2003; Okamoto et al., 1990; Sperger et al., 2003), human (Abeyta et al., 2004; Avilion et al., 2003; Mitsui et al., 2003; Rodda et al., 2005), bovine (Yadav et al., 2005; Van Ejik et al., 1999), canine (Hatoya et al., 2006), caprine (He et al., 2006), and porcine (Carlin et al., 2006). *TELOMERASE* expression is also regarded as a characteristic of human ESCs (Zeng et al., 2014). The expression of all these genes in our established ESC lines confirms their bona fide ESC identity. Furthermore, the expression of these pluripotency markers showed no significant ($p < 0.05$) differences among the three cell lines, except for *TELOMERASE*, which was expressed at a slightly but significantly lower level in HGC buESCs. This could be the effect of diminished *TELOMERASE* activity during *in vitro* culture of fetal fibroblasts, which were used as donor cells in our study. The shortening of telomere length, due to diminished telomerase activity, has been reported during *in vitro* culture of bovine fetal fibroblasts (Betts et al., 2001), and SCNT has been reported not to enhance the *TELOMERASE* activity of a donor cell (Jeon et al., 2005).

The immunocytochemical and western blot examination for stem cell-associated transcription factors and surface markers like OCT4, NANOG, SSEA1, SSEA4, TRA-1-60, TRA-1-81, FOXD3, SOX2, and CD-90 revealed expression of these proteins in our buESC lines, irrespective of their origin, whether from IVF-, HGC-, or parthenogenote-embryos. SSEA1, SSEA4, and tumor rejection antigens (TRA-1-60 and TRA-1-81) are regarded as markers of human (Carpenter et al., 2003; Henderson et al., 2002), primate (Liu et al., 2004; Thomson et al., 1995), and bovine ESCs (Dattena et al., 2006; Mitalipova et al., 2001; Saito et al., 2002). The FOXD3 forkhead transcription factor is required for maintaining pluripotent cells in early mouse embryo and for establishment of murine ESC lines (Liu and Labosky, 2008), whereas CD90 has been used as human ESC marker (Fang et al., 2005) as well as a marker for spermatogonial and mesenchymal stem cells (Kobayashi et al., 2009). A decrease in SOX2 expression in human ESCs by RNA interference has been shown to result in loss of the undifferentiated stem cell state, as observed from changes in cell morphology, altered stem cell marker expression, and increased expression of trophectoderm markers (Fong et al., 2008).

The expression of these proteins in our ESC lines thus serves as an indicator of their pluripotency and self-renewal capability. Our ESC lines also maintained the stable diploid karyotype throughout the culture period. The expression of various markers like *NF68*, *CYTOKERATIN8* (ectoderm), *BMP4*, *MSX-1* (mesoderm), *AFP*, and *GATA4* (endoderm), as detected by RT-PCR and immunocytochemistry (for *CYTOKERATIN8*, *BMP4*, and *GATA4*), by all of our buESC lines proves their potential for *in vitro* differentiation to all the three germ layers. qPCR analysis showed almost equal differentiation potential of all the three ESC lines when subjected to SSC differentiation in 15% KoSR-based ESC differentiation medium.

A comparison of HD and SSC differentiation strategies revealed better efficiency of the latter for inducing germ layer differentiation. This could probably be due to ample space and nutritional availability (2–3 mL of medium) in

suspension cultures as compared to HD cultures (20–30 μ L of medium). However, the HD method is advantageous to SSCs in terms of providing uniform sizes of EBs and preventing physical aggregation of EBs into spheroids and premature attachment to the plate (Dang et al., 2002). A further comparison between FBS- and KoSR-supplemented ESC differentiation media revealed significantly higher potential of FBS-based medium to induce differentiation into all three germ layers, as revealed by mRNA quantification in our study. FBS is chemically undefined and contains a larger number of growth factors than KoSR, which is a commercial serum replacement designed primarily for maintaining stem cells in an undifferentiated state under normal culture conditions. The active ingredient in serum replacement has been found to be lipid-rich albumin, which, unlike the albumin of FBS, has a strong positive effect on the expansion of undifferentiated hESCs in culture (Garcia-Gonzalo and Izpisua, 2008). These arguments support a higher expression level of various germ layer markers in FBS-based SSCs. The differentiation of our ESC lines into various cell types (hepatocyte-like cells, adipocyte-like cells, neuronal-like cells, and epithelial-like cells) on gelatin- and MaxGel-coated dishes further proves their *in vitro* differentiation potential.

Conclusions

We have described the development, characterization, and spontaneous differentiation of clonally derived buESC lines from IVF, HGC, and PAR blastocysts. To our knowledge, this is perhaps the first study that reports the maintenance and propagation of buESC lines for more than 100 passages. Our results also suggest that the spontaneous differentiation potential of ESCs is a function of the differentiation strategy followed, as indicated by greater germ layer marker expression in SSCs as compared to the HD cultures, despite equivalent pluripotent marker expression by the parent stem cells.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

References

- Abeyta, M.J., Clark, A.T., Rodriguez, R.T., Bodnar, M.S., Pera, R.A., and Firpo, M.T. (2004). Unique gene expression signatures of independently-derived human embryonic stem cell lines. *Hum. Mol. Genet.* 13, 601–608.
- Avilion, A.A., Nicolis, S. K., Pevny, L.H., Perez, L., Vivian, N., and Lovell-Badge, R. (2003). Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev.* 17, 126–140.
- Betts, D., Vilceu, B., Jonathan, R., Winger, Q., Westhusin, M.E., Smith, L.C., and King W.A. (2001). Reprogramming of

- telomerase activity and rebuilding of telomere length in cloned cattle. *Proc. Natl. Acad. Sci. USA* 98, 1077–1082.
- Bongso, A., Fong, C.Y., Ng, S.C., and Ratnam, S. (1994). Isolation and culture of inner cell mass cells from human blastocysts. *Hum. Reprod.* 9, 2110–2117.
- Bradley, A., Evans, M., Kaufman, M.H., and Robertson, E. (1984). Formation of germ-line chimeras from embryo-derived teratocarcinoma cell lines. *Nature* 309, 255–256.
- Cao, S., Wang, F., Chen, Z., Liu, Z., Mei, C., Wu, H., Huang, J., Li, C., Zhou, L., and Liu, L. (2009). Isolation and culture of primary bovine embryonic stem cell colonies by a novel method. *J. Exp. Zool. A Ecol. Genet. Physiol.* 311, 368–376.
- Carlin, R., Davis, D., Weiss, M., Schultz, B., and Troyer, D. (2006). Expression of early transcription factors Oct-4, Sox-2 and Nanog by porcine umbilical cord (PUC) matrix cells. *Reprod. Biol. Endocrinol.* 4, 8.
- Carpenter, M.K., Rosler, E., and Rao, M.S. (2003). Characterization and differentiation of human embryonic stem cells. *Cloning Stem Cells* 5, 79–88.
- Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* 113, 643–655.
- Cibelli, J.B., Stice, S. L., Golueke, Kane, J.J., Jerry, J., Blackwell, C., Ponce de León, F.A., and Robl, J.M. (1998). Transgenic bovine chimeric offspring produced from somatic cell derived stem like cells. *Nat. Biotech.* 16, 642–646.
- D'Amour, K.A., and Gage, F.H. (2003). Genetic and functional differences between multipotent neural and pluripotent embryonic stem cells. *Proc. Natl. Acad. Sci., USA* 100, 11866–11872.
- Dang, S.M., Kyba, M., Perlingeiro, R., Daley, G.Q., and Zandstra, P.W. (2002). Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. *Biotechnol. Bioeng.* 78, 442–453.
- Dattena, M., Chessa, B., Lacerenza, Accardo, C., Pilichi, S., Mara, L., Chessa, F., Vincenti, L., and Cappai, P. (2006). Isolation, culture and characterization of embryonic cell lines from vitrified sheep blastocysts. *Mol. Reprod. Dev.* 31, 31–39.
- Dyban, A.P. (1983). An improved method for chromosome preparations from preimplantation mammalian embryos, oocytes or isolated blastomeres. *Biotech. Histochem.* 58, 69–72.
- Evans, M.J., and Kaufman, M. (1981). Establishment in culture of pluripotent cells from mouse embryos. *Nature* 292, 154.
- Evans, M.J., Notarianni, E., Laurie, S., and Moor, R.M. (1990). Derivation and preliminary characterization of pluripotent cell lines from porcine and bovine blastocysts. *Theriogenology* 33, 125–128.
- Fang, Z.F., Jin, F., Gai, H., Chen, Y., Chen, Y., Wu, L., Liu, A.L., Chen, B., and Sheng, H.Z. (2005). Human embryonic stem cell lines derived from the Chinese population. *Cel. Res.* 15, 394–400.
- Fong, E., Hohenstein, K., and Donovan, P. (2008). Regulation of self-renewal and pluripotency by Sox2 in human embryonic stem cells. *Stem Cell* 26, 1931–1938.
- Garcia-Gonzalo, F.R., and Izpisua, J.C. (2008). Albumin-associated lipids regulate human embryonic stem cell self-renewal. *PLoS One* 3:e1384. doi:10.1371/journal.pone.0001384.
- Gjorret, J.O., and Maddox-Hyttel, P. (2005). Attempts towards derivation and establishment of bovine embryonic stem cell-like cultures. *Reprod. Fertil. Dev.* 17, 113–124.
- Hamano, S., Watanabe, Y., Azumi, S., and Toyoda, Y. (1998). Establishment of embryonic stem (ES) cell-like cell lines derived from bovine blastocysts obtained by in vitro culture of oocytes matured and fertilized in vitro. *J. Reprod. Dev.* 44, 297–303.
- Hall, V. 2008. Porcine embryonic stem cells: A possible source for cell replacement therapy. *Stem Cell Rev.* 4, 275–282.
- Hart, A. H., Hartley, L., Ibrahim, M., and Robb, L. (2004). Identification, cloning and expression analysis of the pluripotency promoting Nanog genes in mouse and human. *Dev. Dyn.* 230, 187–198.
- Hatoya, S., Torii, R., Kondo, Y., Okuno, T., Kobayashi, K., Wijewardana, V., Kawate, N., Tamada, H., Sawada, T., Kumagai, D., Sugiura, K., and Inaba, T. (2006). Isolation and characterization of embryonic stem-like cells from canine blastocysts. *Mol. Reprod. Dev.* 73, 298–305.
- He, S., Pant, D., Schiffmacher, A., Bischoff, S., Melican, D., Gavin, W., and Keefer, C. (2006). Developmental expression of pluripotency determining factors in caprine embryos: Novel pattern of Nanog protein localization in the nucleolus. *Mol. Reprod. Dev.* 73, 1512–1522.
- Heins, N., Englund, M.C., Sjoblom, C., Dahl, U., Tonning, A., Bergh, C., Lindahl, A., Hanson, C., and Semb, H. (2004). Derivation, characterization and differentiation of human embryonic stem cells. *Stem Cells* 22, 367–376.
- Henderson, J.K., Draper, J.S., Baillie, H.S., Fishel, S., Thomson, J.A., Moore, H., and Andrews, P.W. (2002). Pre-implantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. *Stem Cells* 20, 329–337.
- Iwasaki, S., Campbell, K.H., Galli, C., and Akiyama, K. (2000). Production of live calves derived from embryonic stem cell like cells aggregated with tetraploid embryos. *Biol. Reprod.* 62, 470–475.
- Jeon, H., Hyun, S., Lee, G., Kim, H.S., Kim, S., Jeong, Y.W., Kang, S.K., Lee, B.C., Han, J.Y., Ahn, C., and Hwang, W.S. (2005). The analysis of telomere length and telomerase activity in cloned pigs and cows. *Mol. Rep. Dev.* 71, 315–320.
- Keefer, C.L., Karatzas, C.N., Lazaris-Karatzas, A., Gagnon, I., Poulin, S., and Downey, B.R. (1996). Isolation and maintenance of putative embryonic stem cells derived from Nigerian dwarf goat embryos. *Biol. Reprod.* 54, 462.
- Kobayashi, H., Nagao, K., Nakajima, Miura, K. and Ishii, N. (2009). Thy-1+ cells isolated from adult human testicular tissues express human embryonic stem cell genes OCT3/4 and NANOG and may include spermatogonial stem cells. *Repr. Med. Bio.* 8, 71.
- Laible, G. (2009). Enhancing livestock through genetic engineering—recent advances and future prospects. *Comp. Immunol. Microbiol. Infect. Dis.* 32, 123–137.
- Lee, J. B., Song, J. M., Lee, J. E., Park, J.H., Kim, S.J., Kang, S.M., Kwon, J.N., Kim, M.K., Roh, S.I., and Yoon, H.S. (2004). Available human feeder cells for the maintenance of embryonic stem cells. *Reproduction* 128, 727–735.
- Li, M., Li, Y.H., Hou, Y., Sun, X.F., Sun, Q., and Wang, W.H. (2004). Isolation and culture of pluripotent cells from in vitro produced porcine embryos. *Zygote* 12, 43–48.
- Liu, S., Liu, H., Tang, S., Pan, Y., Ji, K., Ning, H., Wang, S., Qi, Z., and Li, L. (2004). Characterization of stage-specific

- embryonic antigen-1 expression during early stages of human embryogenesis. *Oncol. Rep.* 12, 1251–1256.
- Liu, Y., and Labosky, P. (2008). Regulation of ES cell self renewal and pluripotency by Foxd3. *Stem Cell* 28, 2475–2484.
- Martin, G. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma cells. *Proc. Natl. Acad. Sci. USA* 78, 7634.
- Mitalipova, M., Beyhan, Z., and First N. L. (2001). Pluripotency of bovine embryonic cell line derived from precompacting embryos. *Cloning* 3, 59–67.
- Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., and Yamanaka, S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* 113, 631–642.
- Nagy, A., Gocza, E., Diaz, E.M., Prideaux, V.R., Iványi, E., Markkula, M., and Rossant, J. (1990). Embryonic stem cells alone are able to support fetal development in the mouse. *Development* 110, 815–821.
- Nicholas, J., Zevnik, B., Anastasiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Schöler, H., and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* 95, 379–391.
- Notarianni, E., Galli, C., Laurie, S., Moor, R.M., and Evans, M.J. (1991). Derivation of pluripotent, embryonic cell lines from the pig and sheep. *J. Reprod. Fertil.* 43, 255–260.
- Okamoto, K., Okazawa, H., Okuda, A., Sakai, M., Muramatsu, M., and Hamada, H. (1990). A novel octamer binding transcription factor is differentially expressed in mouse embryonic stem cells. *Cell* 60, 461–472.
- Pera, M.F., Cooper, S., Mills, J., and Parrington, J.M. (1989). Isolation and characterization of a multipotent clone of human embryonal carcinoma cells. *Differentiation* 42, 10–23.
- Piedrahita, J., Anderson, G., and BonDurant, R. (1990). On the isolation of embryonic stem cells: Comparative behaviour of murine, porcine and ovine embryos. *Theriogenology* 34, 879–901.
- Polejaeva, I.A., While, K., Ellis, L.C., and Reed, W.A. (1995). Isolation and long-term culture of mink and bovine embryonic stem-like cells. *Theriogenology* 43, 300.
- Resnick, J.L., Bixler, L.S., Cheng, L., and Donovan, P.J. (1992). Long-term proliferation of mouse primordial germ cells in culture. *Nature* 359, 550–551.
- Robertson, E.J. (1987). Embryo-derived stem cell lines. In: Robertson EJ, ed. *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*. Oxford: IRL Press. pp. 71–112.
- Rodda, D., Chew, J.L., Lim, L.H., Loh, Y.-H., Wang, B., Ng, H.-H., and Robson, P. (2005). Transcriptional regulation of Nanog by Oct4 and Sox2. *J. Biol. Chem.* 280, 24731–24737.
- Saito, S., Ugai, H., Sawai, K., Yamamoto, Y., Minamihashi, A., Kurosaka, K., Kobayashi, Y., Murata, T., Obata, Y., and Yokoyama, K. (2002). Isolation of embryonic stem-like cells from equine blastocysts and their differentiation in vitro. *FEBS Lett.* 531, 389–396.
- Schmittgen, T.D., and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* 3, 1101–1108.
- Scholer, H.R., Dressler, G.R., Balling, R., Rohdewohld, H., and Gruss, P. (1990). Oct-4: A germline-specific transcription factor mapping to the mouse t-complex. *EMBO J.* 9, 2185–2195.
- Shah, R.A., George, A., Singh, M.K., Kumar, D., Chauhan, M.S., Manik, R., Palta, P., and Singla, S.K. (2008). Hand-made cloned buffalo (*Bubalus bubalis*) embryos: Comparison of different media and culture systems. *Clon. Stem Cell.* 10, 435–442.
- Shah, S.M., Saini, N., Ashraf, S., Zandi, M., Singh, M.K., Manik, R.S., Singla, S.K., Palta, P., and Chauhan, M.S. (2015). Comparative expression analysis of gametogenesis-associated genes in foetal and adult bubaline (*Bubalus bubalis*) ovaries and testes. *Reprod. Domest. Anim.* 50, 365–377.
- Shamblott, M.J., Axelman, J., Wang, S.P., Bugg, E.M., Littlefield, J.W., Donovan, P.J., Blumenthal, P.D., Huggins, G.R., and Gearhart, J.D. (1998). Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc. Natl. Acad. Sci. USA* 95, 13726–13731.
- Sims, M., and First, N.L. (1994). Production of calves by transfer of nuclei from cultured inner cell mass cells. *Proc. Natl. Acad. Sci. USA* 91, 6143–6147.
- Sperger, J.M., Chen, X., Draper, J.S., Antosiewicz, J.E., Chon, C.H., Jones, S.B., Brooks, J.D., Andrews, P.W., Brown, P.O. and Thomson, J.A. (2003). Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumours. *Proc. Natl. Acad. Sci. USA* 100, 13350–13355.
- Thomson, J.A., Kalishman, J., Golos, Durning, M., Harris, C.P., Becker, R.A., and Hearn, J.P. (1995). Isolation of a primate embryonic stem-cell line. *Proc. Natl. Acad. Sci. USA* 92, 7844–7848.
- Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. (1998). Embryonic stem cells derived from human blastocysts. *Science* 282, 1145–1147.
- Vajta, G., Lewis, I. M., Hyttel, P., Thouas, G.A., and Trounson, A.O. (2001). Somatic cell cloning without micromanipulators. *Cloning* 3, 89–95.
- Vajta, G., Lewis, I.M., Trounson, A.O., Purup, S., Maddox-Hyttel, P., Schmidt, M., Pedersen, H.G., Greve, T., and Callesen, H. (2003). Handmade somatic cell cloning in cattle: Analysis of factors contributing to high efficiency in vitro. *Biol. Reprod.* 68, 571–578.
- Van Eijk, M.J., van Rooijen, M.A., Modina, S., Scesi, L., Folkers, G., van Tol, H.T., Bevers, M.M., Fisher, S.R., Lewin, H.A., Rakacolli, D., Galli, C., de Vaureix, C., Trounson, A.O., Mummery, C.L., and Gandolfi, F. (1999). Molecular cloning, genetic mapping, and developmental expression of bovine POU5F1. *Biol. Reprod.* 60, 1093–1103.
- Van Stekelenburg-Hamers, A.E., Van Achterberg, T.A., Rebel, H.G., Fléchon, J.E., Campbell, K.H., Weima, S.M., and Mummery, C.L. (1995). Isolation and characterization of permanent cell lines from inner cell mass of bovine blastocysts. *Mol. Rep. Dev.* 40, 444–454.
- Verma, V., Guatam, S. K., Singh, B., Manik, R.S., Palta, P., Singla, S.K., Goswami, S.L., and Chauhan, M.S. (2007). Isolation and characterization of embryonic stem cell-like cells in vitro produced buffalo (*Bubalus bubalis*) embryos. *Mol. Reprod. Dev.* 74, 520–529.
- Wang, L., Duan, E., Sung, L.Y., Jeong, B.S., Yang, X., and Tian, X.C. (2005). Generation and characterization of pluripotent stem cells from cloned bovine embryos. *Biol. Reprod.* 73, 149–155.
- Wang, S., Tang, X., Niu, Y., Chen, H., Li, B., Li, T., Zhang, X., Hu, Z., Zhou, Q., and Ji, W. (2007). Generation and

- characterization of rabbit embryonic stem cells. *Stem Cells* 25, 481–489.
- Wheeler, M.B., and Walters, E.M. (2001). Transgenic technology and applications in swine. *Theriogenology* 56, 1345–1369.
- Yadav, P.S., Wilfried, A.K., Herrmann, D., Carnwath, J.W., and Niemann, H. (2005). Bovine ICM derived cells express Oct4 ortholog. *Mol. Reprod. Dev.* 72, 182–190.
- Yu, J., and Thomson, J.A. (2008). Pluripotent stem cell lines. *Genes Dev.* 22, 1987–1997.
- Zandi, M., Muzaffar, M., Shah, S.M., Kaushik, R., Singh, M.K., Palta, P., Singla, S.K., Manik, R.S., and Chauhan, M.S. (2013). WNT3A signaling pathway in buffalo (*Bubalus bubalis*) embryonic stem cells. *Reprod. Fertil. Dev.* 26, 551–561.
- Zeng, S., Liu, L., Sun, Y., Xie, P., Hu, L., Yuan, D., Chen, D., Ouyang, Q., Lin, G., and Lu, G. (2014). Telomerase-mediated telomere elongation from human blastocysts to embryonic stem cells. *J. Cell. Sci.* 15:752–762.

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