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Lab Resource: Stem Cell Line

Development of buffalo (*Bubalus bubalis*) embryonic stem cell lines from somatic cell nuclear transferred blastocysts

Syed Mohmad Shah, Neha Saini, Syma Ashraf, Manoj K Singh, Radheysham Manik, Suresh K Singla, Prabhat Palta, Manmohan Singh Chauhan *

Embryo Biotechnology Lab, Animal Biotechnology Center, National Dairy Research Institute, Karnal, India , 132001

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ABSTRACT

We developed buffalo embryonic stem cell lines from somatic cell nuclear transfer derived blastocysts, produced by hand-guided cloning technique. The inner cell mass of the blastocyst was cut mechanically using a Microblade and cultured onto feeder cells in buffalo embryonic stem (ES) cell culture medium at 38 °C in a 5% CO_2 incubator. The stem cell colonies were characterized for alkaline phosphatase activity, karyotype, pluripotency and self-renewal markers like OCT4, NANOG, SOX2, c-Myc, FOXD3, SSEA-1, SSEA-4, TRA-1-60, TRA-1-81 and CD90. The cell lines also possessed the capability to differentiate across all the three germ layers under spontaneous differentiation conditions.

Resource table:

Name of Stem Cell construct	Not applicable
Institution	National Dairy Research Institute, Karnal
Person who created resource	Syed Mohmad Shah and Manmohan Singh Chauhan
Contact person and email	Researcher 1—syedalhyderi14@gmail.com Researcher 2—chauhanabtc@gmail.com
Date archived/stock date	Jan, 2014.
Origin	Embryonic stem cells
Type of resource	Embryonic stem cells derived from SCNT blastocysts by using female buffalo fetal fibroblasts as donor cells
Sub-type	Cell line
Key transcription factors	Oct4, Nanog, Sox2, cMyc, Foxd3
Authentication	Characterization confirmed by RT-PCR and immunocytochemistry of stem cell markers (Figs. 2, 3 and 4) and also by differentiation potential to all three germ layers (Figs. 6 and 7).
Link to related literature (direct URL links and full references)	www.ncbi.nlm.nih.gov/pubmed/26168169
Information in public databases	

1. Resource details

Following their mechanical isolation from day 7 blastocysts, the inner cell masses (ICM) were seeded individually onto the feeder layers. After 2 days of incubation, most of the ICMs were found attached to the feeder cells. The cultures were allowed to grow for 8–10 days, with medium changes every alternate day, to develop into primary embryonic stem cell (ESC) colonies. The primary colonies were subcultured onto fresh feeders to produce ESC lines. Out of the initial 20–30 primary colonies, only four to five made it up to 10 passages and beyond. Our main purpose for establishment of these XX-ESC lines was to develop strategies and study mechanisms for their differentiation into oocytes. All 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. (Fig. 1). RT-PCR was positive for various stem cell markers like OCT4, NANOG, SOX2, c-MYC, REX-1, STAT3, FOXD3 and TELOMERASE (Fig. 2). Immunocytochemical analysis for stem cell markers carried out for intact colonies as well as monolayer adherent cells showed positive expression of various pluripotency and self renewal-associated transcription factors like OCT4, NANOG, SOX2 and FOXD3 (Fig. 3) as well as of ESC surface markers like SSEA1, SSEA4, TRA-1-60, TRA-1-81 and CD-90 (Fig. 4). The cell lines karyotyped at various passages (passage 10, 30, and 40) revealed normal chromosomal content throughout the culture interval. In all of the 20 spreads evaluated, karyotyping revealed normal and exact metaphase euploid content of a female murrah buffalo (*Bubalus bubalis*) (48 + XX) genome (Fig. 5). Upon spontaneous differentiation in hanging drop (HD) cultures, the colonies from all three stem cell lines differentiated into EBs. 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. 6). None of these markers

* Corresponding author.

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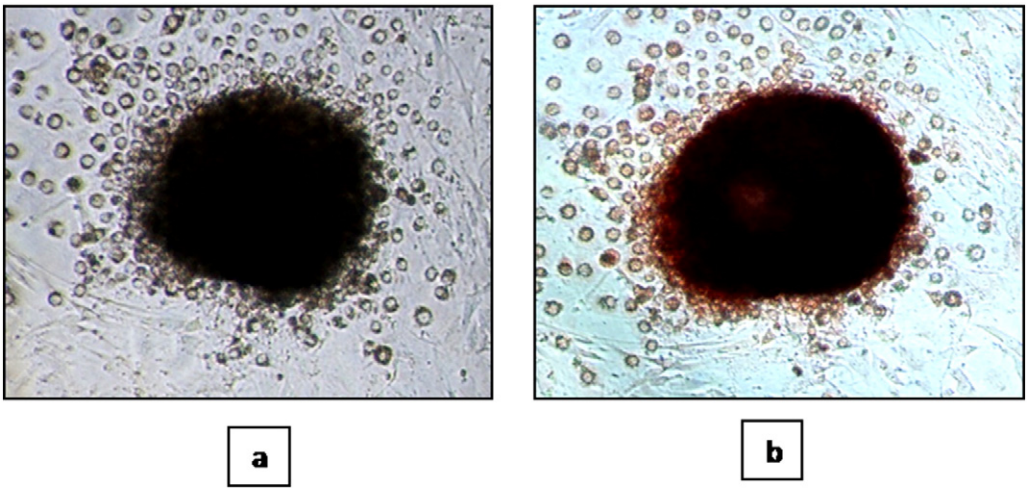

Fig. 1. Morphology and alkaline phosphatase (ALP) staining of putative buESC colonies at passage 15, derived from ICMs of SCNT blastocysts (magnification 200×, scale bar—100 μm). Note the dome shape of buESC colonies (a) and high ALP activity in comparison to the underlying feeder cells (b).

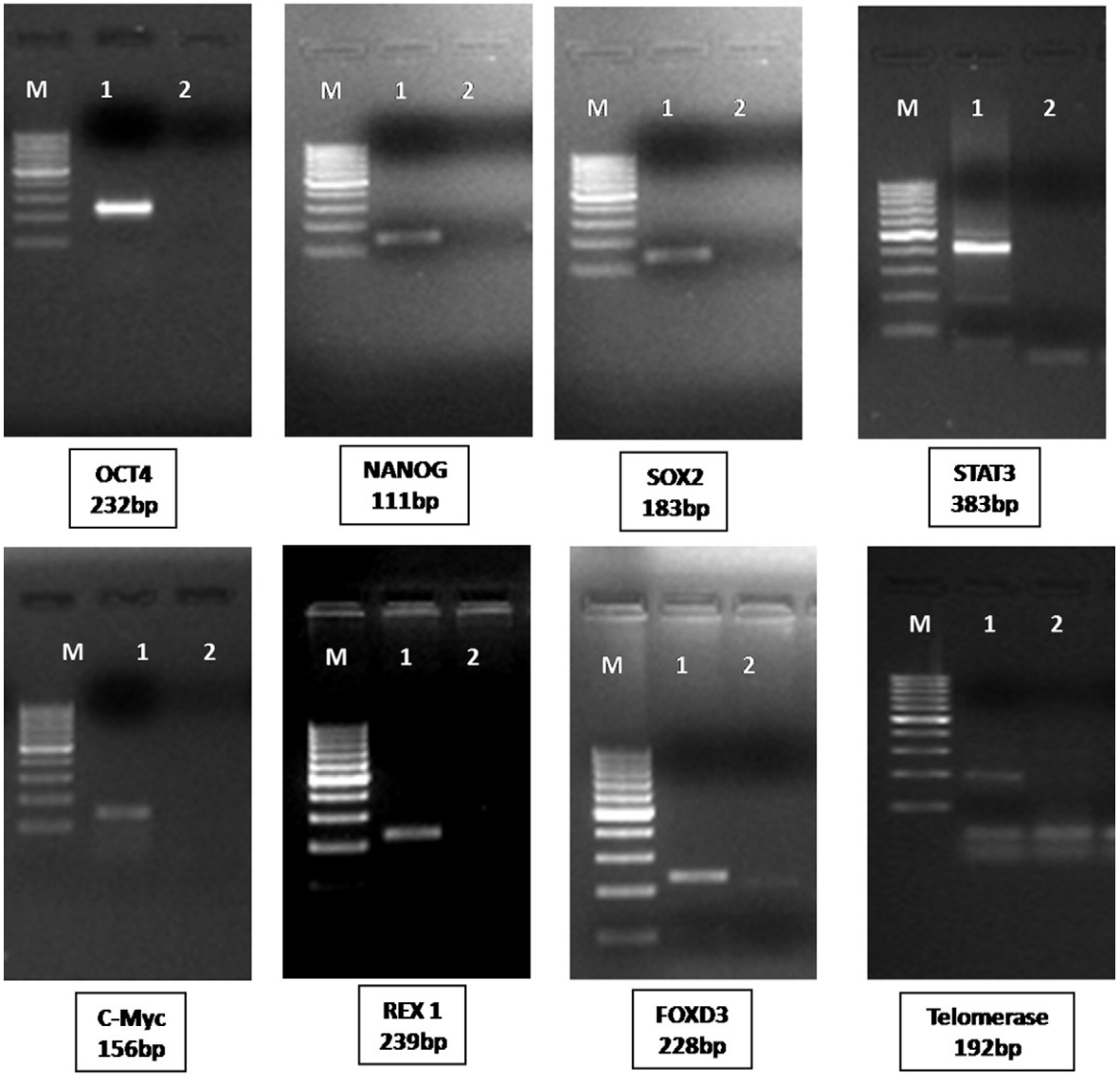

Fig. 2. RT-PCR results for various stem cell markers: 1—OCT4 (232 bp); 2—NANOG (111bp); 3—SOX2 (183 bp); 4—c-MYC (156 bp); 5—REX1 (239 bp); 6—FOXD3 (228 bp); 7—TELOMERASE (192 bp); 8—STAT3 (383 bp); M—100 bp DNA marker; Lane 1—buESC; Lane 2—NTC.

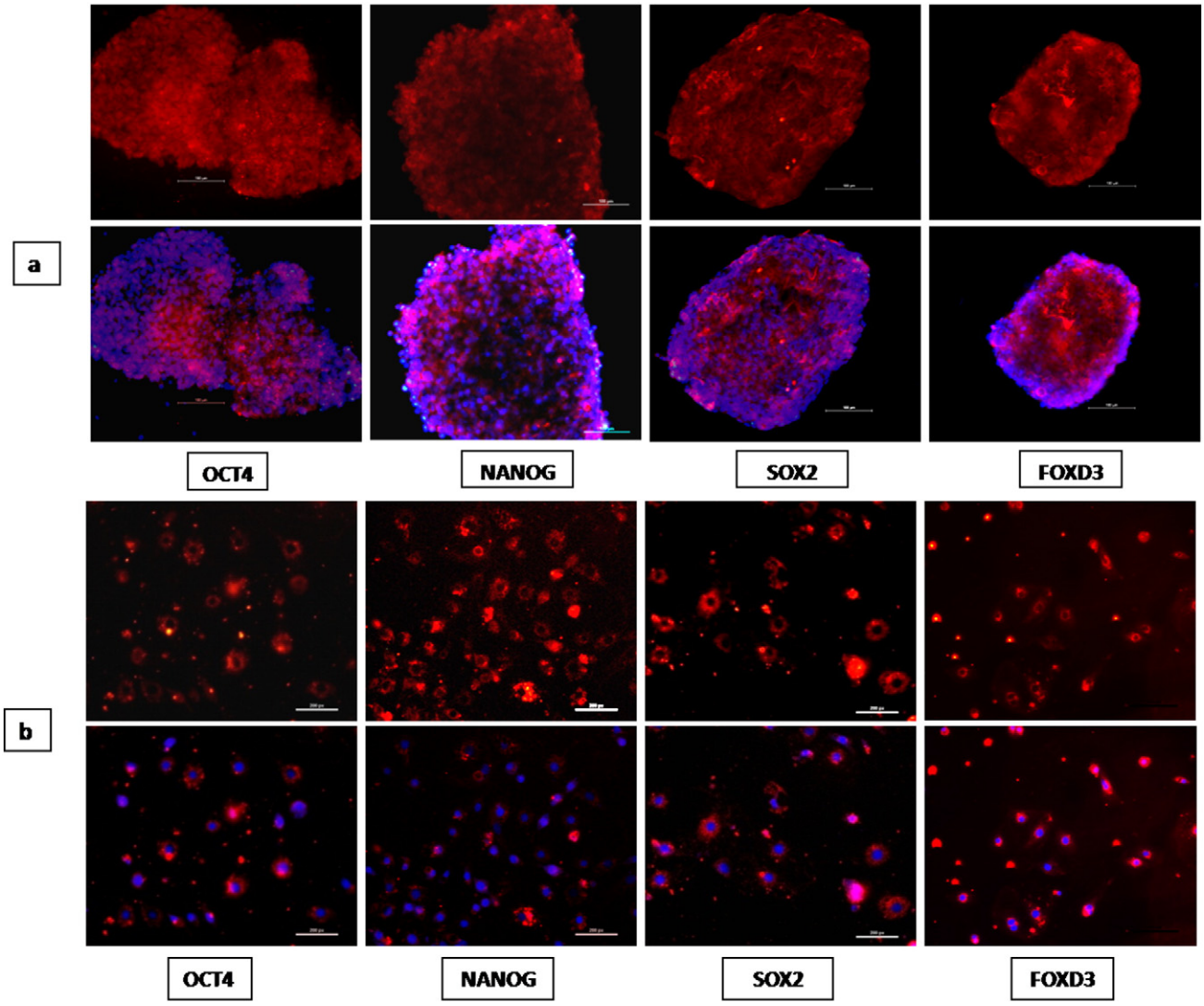

Fig. 3. Immunocytochemical analysis of embryonic stem cells for transcription factors (OCT4, NANOG, SOX2 and FOXD3); a) ESC colonies; b) ESC adherent monolayers; upper panel: secondary antibody stained photographs (PE/FITC conjugated); lower panel: merged photographs of DAPI and secondary antibody stained colonies (magnification—200×, scale bar—100 μm).

was expressed in undifferentiated colonies. Immunocytochemistry also revealed expression of CYTOKERATIN8, BMP4, and GATA4 in the EBs when examined at day 8 of the culture period, whereas no such protein expression was detected in control (undifferentiated) colonies (Fig.7).

2. Materials and methods

2.1. Ethical approval

The study was approved by National Dairy Research Institute Animal Ethics Committee as well as by Department of Biotechnology, New Delhi, for use of animal tissues as well as for development of the embryonic stem cell lines.

2.2. Production of SCNT blastocysts

The cumulus oocyte complexes (COCs) were aspirated from follicles of abattoir obtained buffalo ovaries using an 18-guage syringe needle. A and B grade COCs were matured in vitro in 100 μl droplets of in vitro maturation (IVM) medium at 38.5 °C for 20 h. The IVM COCs were subjected to cumulus removal and pronase (2 mg/ml) treatment for 15–20 min. The denuded and zona-free oocytes, so obtained, were subjected to Cytochalasin B (2.5 mg/ml) treatment and bisected with a Microblade under a zoom stereomicroscope to remove their genetic material. The demi-oocytes, so obtained, were treated with phytohemagglutinin (0.5 μg/ml) for 3–4 s, followed by gentle rolling over a single donor cell (female buffalo fetal fibroblast) to allow their attachment. The couplets so obtained were fused with another demi-oocyte in a fusion chamber by a single-step fusion protocol using a BTX Electrocell Manipulator. The resulting triplets were incubated at 38.5 °C for 6 h, followed by activation with Calcimycin A23187 for 5 min in dark and 6-DMAP for 4 h. This was followed by culture in Research Vitro Cleavage Medium (K-RVCL-50) supplemented with 1% fatty acid free bovine serum albumin for 7–8 days till the blastocysts developed.

2.3. Establishment and culture of buffalo embryonic stem cell lines

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 mm^3) were transferred into 25 cm^2 tissue culture flasks and incubated at 38 °C in a 5% CO_2 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

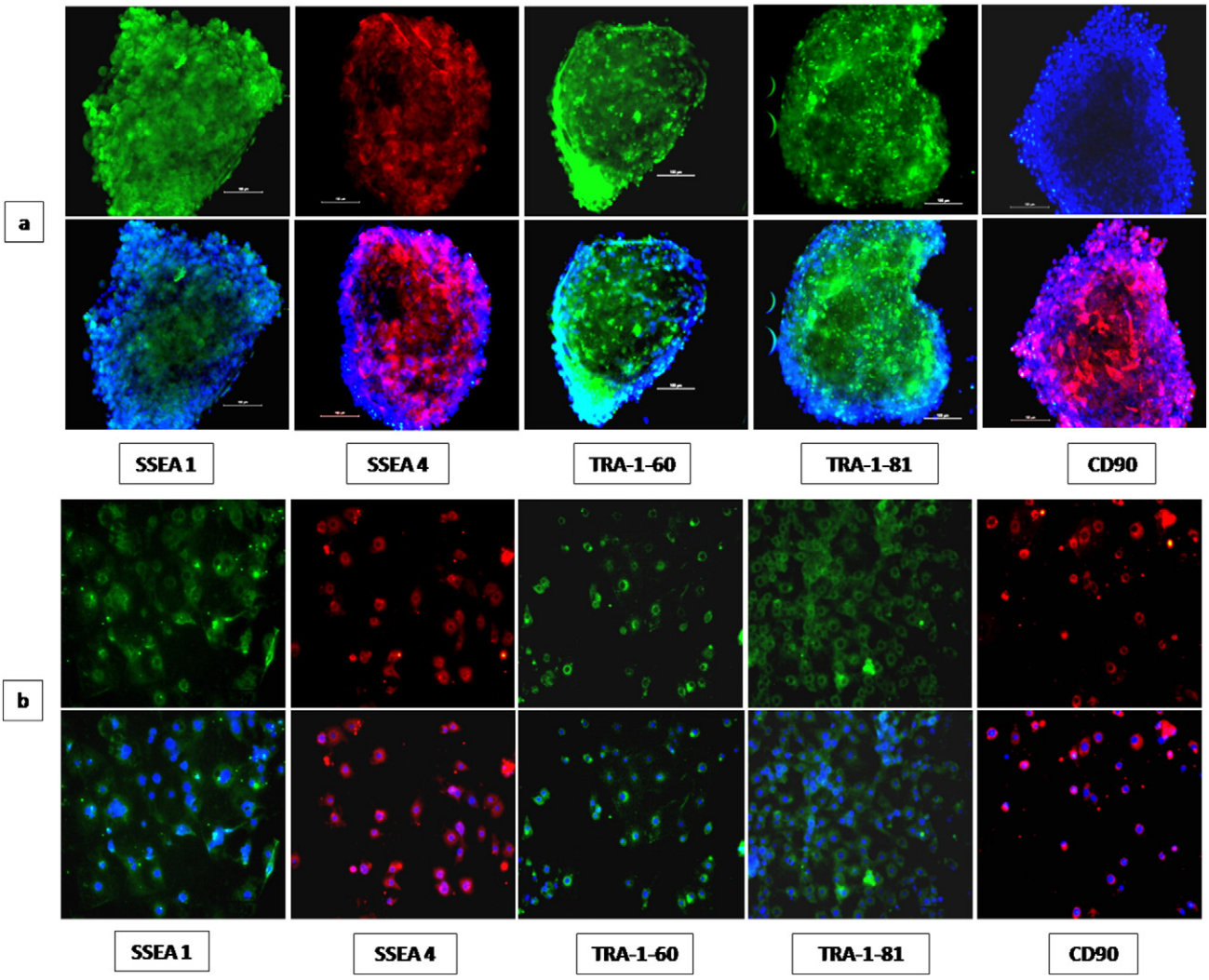

Fig. 4. Immunocytochemical analysis of embryonic stem cells for surface markers (SSEA1, SSEA4, TRA-1-60, TRA-1-81 and CD90). a) ESC colonies; b) ESC adherent monolayers; upper panel: secondary antibody stained photographs (PE/FITC conjugated); lower panel: merged photographs of DAPI and secondary antibody stained colonies (magnification—200×, scale bar—100 μm).

fractional disaggregation of fibroblasts. This was followed by another passage, after 3–4 days. 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 μL 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. 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), 1000IU/mL recombinant murine leukemia inhibitory factor (rmLIF), 1× nonessential amino acids, and 50 μg/mL gentamicin sulfate and incubated at 38 °C in a 5% CO_2 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 sub cultured onto the new feeders.

2.4. Characterization for stem cell marker expression

2.4.1. 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, Catalog No. 86C) as per the manufacturer's protocol.

2.5. Immunocytochemical analysis for stem cell markers

The ESC colonies were examined at regular intervals to determine the expression of various stem cell markers. The staining was performed as per the previously described protocol (Shah et al., 2015a and 2015b). Briefly, the 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

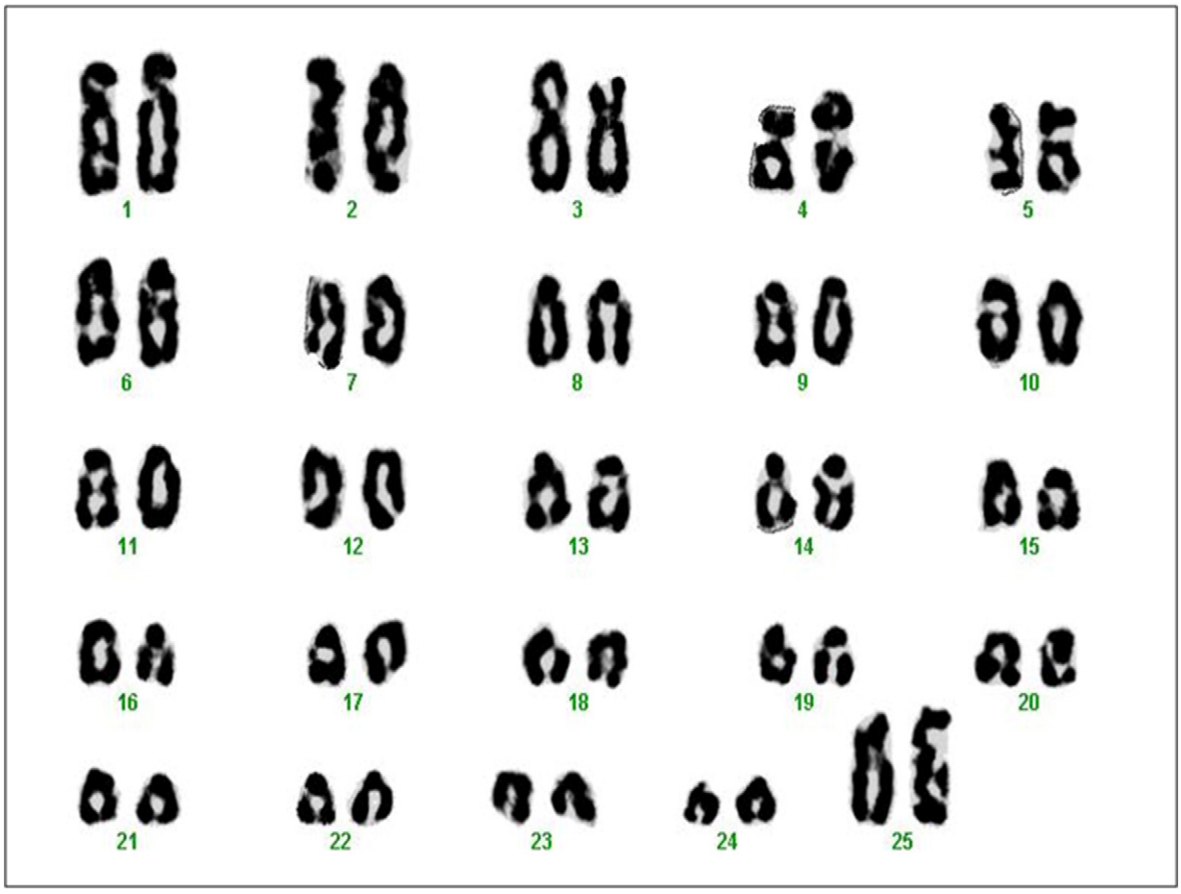

Fig. 5. Karyogram of buESCs depicting normal diploid 48+ XX karyotype.

with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). After three washings, the colonies were put individually onto clean grease-free microscopic 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 have been previously discussed (Shah et al., 2015a).

2.6. RT-PCR detection for stem cell markers

Total RNA was extracted from the 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 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 provided elsewhere (Shah et al., 2015a). RT-PCR reactions were set up in a final volume of 25 μL having 10X PCR buffer, 10 mM dNTPs, 1.0 U of DreamTaq DNA polymerase, 10 pmol/μL each of forward and reverse primers, and 4 μL (400 ng) of cDNA. The thermal cycling conditions were 94 °C for 3 min, followed by a cycling program of 94 °C for 30 s, X°C (annealing temperature as mentioned (Shah et al., 2015a) for each primer pair) for 30 s and 72 °C for 30 s 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 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.

2.7. Karyotype analysis

Karyotyping was performed on 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 complete dissociation. 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

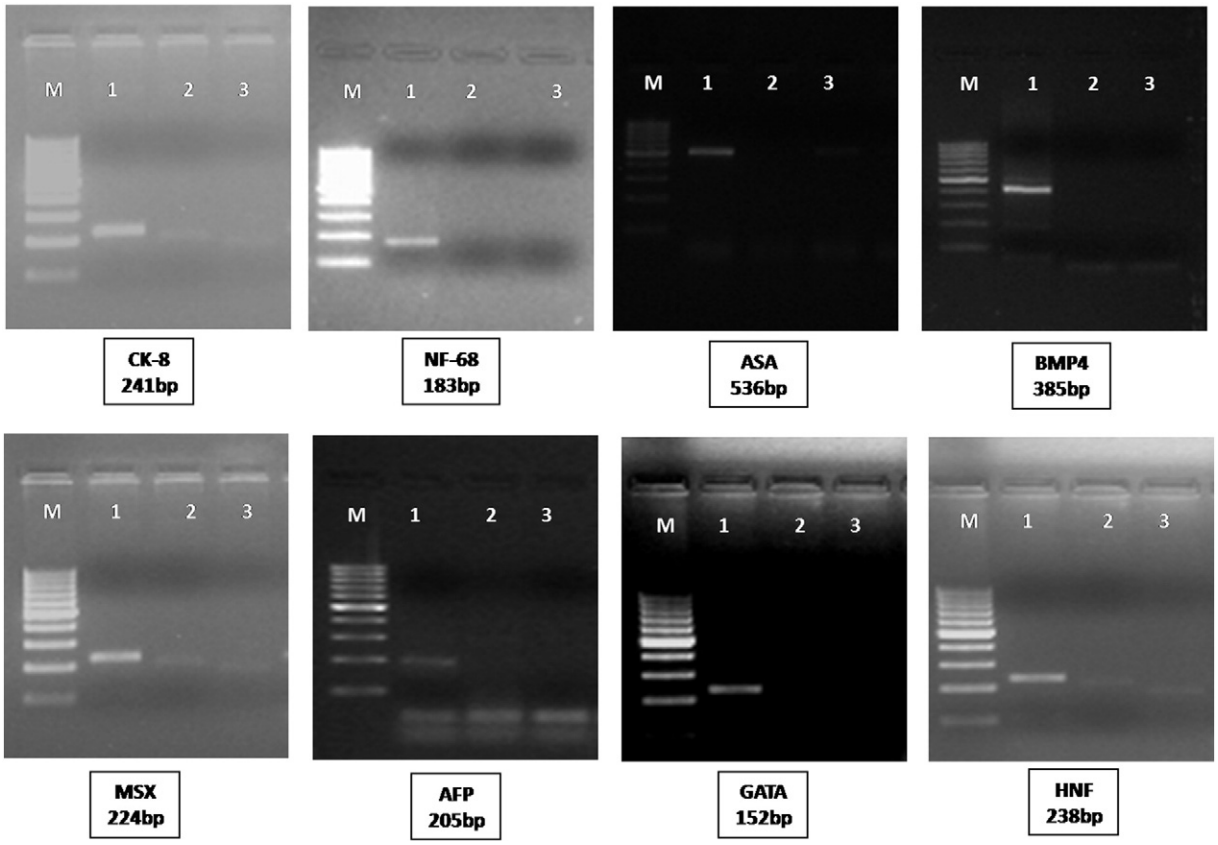

Fig. 6. RT-PCR results depicting expression of various germ layer markers (CYTOKERATIN8, NF68, ASA, BMP4, MSX1, AFP, GATA4 and HNF) in EBs developed by spontaneous differentiation in static suspension cultures. M-100 bp DNA ladder; Lane 1—buESC; Lane 2—NTC; Lane 3—RT-Negative.

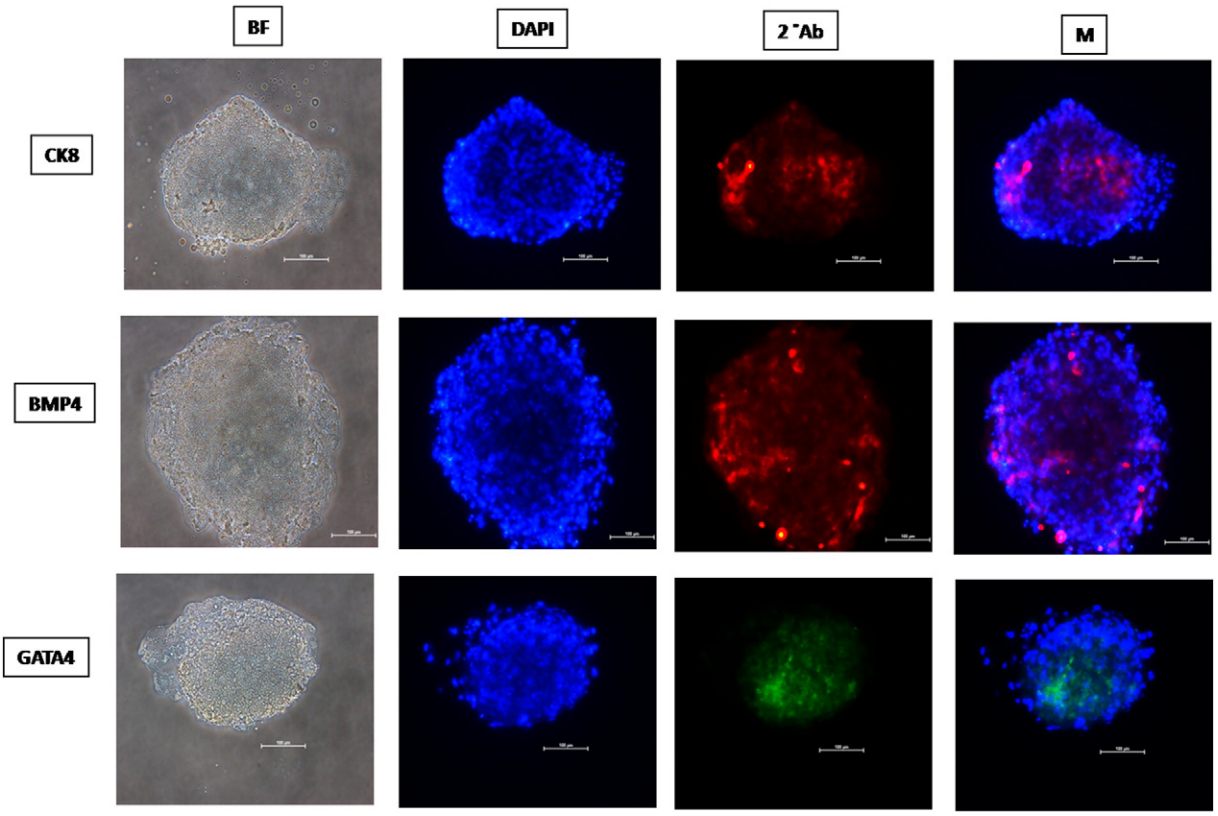

Fig. 7. Immunocytochemical characterization of EBs developed in Static Suspension Culture for germ layer marker (CK8, BMP4 and GATA4) expression (magnification—200×, scale bar—100 μm).

the pellet was mixed by gentle pipetting. Two to three drops of the suspension were dropped from a height of about 25–30 in 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).

2.8. Differentiation into three germ layers

The stem cell colonies were differentiated via formation of embryoid bodies (EBs) which was achieved by hanging drop (HD) culture method. 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. 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% CO_2. The presumed EBs were subjected to further growth in static suspension culture for a total period of 14 days, where they were placed in low-attachment 35-mm Petri dishes (Nunc, Denmark, cat. no. 150255) in SDM and cultured up to 14 days. 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 already mentioned for each primer pair (Shah et al., 2015a). 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, under spontaneous differentiation conditions.

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