Derivation of buffalo embryonic stem-like cells from in vitro-produced blastocysts on homologous and heterologous feeder cells

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Received: 22 February 2011 / Accepted: 20 April 2011 / Published online: 4 May 2011
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Abstract

Purpose The aim of the present study is to compare the ability of homologous and heterologous embryonic fibroblast feeder layers to support isolation and proliferation of buffalo ES-like cells generated from hatched and expanded blastocysts produced by in vitro fertilization and characterization of derived cells through expression of pluripotent markers.

Methods Embryonic stem cells were derived from hatched and expanded blastocysts through intact blastocyst culture and enzymatic method respectively and compared for proliferation rate on homologous (buffalo) and heterologous feeder layers (goat and sheep).

Results A total of 69 hatched and 83 expanded blastocysts were used for isolation of inner cell masses which were seeded on buffalo, goat and sheep embryonic feeder layers. Following seeding, attachment rate, primary colony formation rate and survival to maximum number of passages were observed to be higher on homologous feeder layers.

Conclusions Upon comparison of different feeder layer cells for derivation and maintenance of buffalo ES-like cells from hatched and expanded blastocysts, buffalo embryonic fibroblast cells were able to provide a better environment for maintaining pluripotency in culture conditions.

Keywords Buffalo · Characterization · Fibroblast · NANOG · OCT-4

Introduction

Embryonic stem (ES) cells are undifferentiated, unspecialized cells that can renew themselves for long periods through cell division and give rise to one or more specialized cell types with specific functions in the body. Specialized cells are the cells, which are committed to conduct a specific function, but ES cells remain uncommitted, until they receive a signal for differentiation. To maintain the undifferentiated state in vitro, ES cells require a substrate such as feeder cells or matrix as well as specialized media. The work in this area was initiated by Evans and Kaufmann [1] who discovered that pluripotent cell lines could be derived directly from the inner cell mass (ICM) of blastocysts in mouse. These ES cells were originally derived and maintained on feeder layers although now they can also be maintained in feeder free culture condition in which the medium is supplemented with leukemia inhibitory factor (LIF). Activation of the LIF pathway appears to be essential for self-renewal of mouse ES cells [2, 3]. Human counterparts do not have the same response to LIF [4, 5], suggesting that mouse ES cells and human ES (hES) cells require different signals to maintain

Capsule Influence of the different type of feeder layers and isolation methods on the derivation of buffalo embryonic stem cells from hatched and expanded blastocysts.

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self-renewal and pluripotency. Recently feeder-free system has been adapted especially for culturing hES cells to eliminate the potential issues of infection and cross-species contamination [6, 7]. Some feeder-free systems support the culture of undifferentiated hES cells more efficiently than others [8, 9], however, porcine and bovine epiblast and ICM cells cultured on a feeder free system did not grow continuously and started senescence or differentiation [10–13]. In case of the farm animals, ES cell-like cells are also commonly cultured on feeder cells because the molecular pathways and key molecules required in maintaining pluripotency in these species are still unknown [14]. As stated earlier in various reports, homologous feeder cell layers supported self renewal and maintained pluripotent characteristics of human and monkey ES cells, with characteristics similar to those of ES cells cultured on mouse fetal fibroblast (MEF) [9, 15]. Recently, buffalo embryonic stem cell-like cells have been generated on buffalo fetal fibroblasts [16, 17]; although the buffalo ES (BuES) cell-like cells grew on homologous feeder cell layers but cell colonies did not maintain an undifferentiated state over a long period of time. Whether the absence of specific factors or other unknown attributes of buffalo embryonic fibroblast (BEF) affected the self-renewal of BuES-like cells is not known. So, in the present study, we compared the efficacy of different feeder cells derived from ruminant species of animals for culturing BuES-like cells.

The characterization of ES cell lines is done on the basis of cell morphology, expression of alkaline phosphatase (AP) and expression of transcription markers Oct4, Sox2, Nanog, Foxd3 and Rex1 which exhibit unique expression pattern during early development. Keeping the importance of BuES cells in view, as buffalo is an important animal for developing countries and provides drought power and currency in the form of meat, milk and hide, the present study was carried out with the following objectives 1) to compare the ability of homologous and heterologous embryonic fibroblast feeder layers to support isolation and proliferation of BuES-like cells generated from hatched and expanded blastocysts produced by in vitro fertilization, and 2) to characterize the BuES-like cells grown on homologous and heterologous embryonic fibroblast feeder layers.

Materials and methods

In vitro oocyte maturation and embryo production

To obtain in vitro matured oocytes, buffalo ovaries were obtained from Delhi slaughterhouse and transported to the laboratory in an insulated container in 0.9% normal saline containing antibiotics 400 IU/ml penicillin and 500 µg/ml streptomycin at 32–37°C within 4–5 h. Oocytes were

collected by aspiration of surface follicles (2–8 mm diameter) with a 19-gauge disposable needle attached to a 10 ml syringe (Henke Saas Wolf GmBH, Tuttlingen, Germany) in the medium consisting of TCM-199 and 0.6% (v/w) bovine serum albumin (BSA). For oocyte maturation, compact cumulus-oocyte-complexes (COCs) with an unexpanded cumulus mass having ≥ 4 layers of cumulus cells and with homogenous evenly granular ooplasm were taken. The oocytes were washed four times with the washing medium (TCM-199+10% FBS+0.81 mM sodium pyruvate+50 µg/ml gentamycin sulphate) and then twice with the IVM medium (TCM-199+10% FBS+5 µg/ml porcine FSH+1 µg/ml estradiol-17 β +0.81 mM sodium pyruvate+5% buffalo follicular fluid+50 µg/ml gentamycin sulphate). The washed COCs were then placed in 80-µl droplets (15–20 oocytes/droplet) of the IVM medium, covered with sterile paraffin oil, in a 35 mm Petri dish (Becton, Dickinson and Co., Lincoln Park, NJ, USA) and cultured for 24 h in a CO₂ incubator (5% CO₂ in air, 90–95% relative humidity) at 38.5°C. In vitro matured oocytes with expanded cumulus cells were subjected to in vitro fertilization (IVF) as described earlier [18]. Briefly, two straws of frozen-thawed ejaculated buffalo semen were washed twice with washing BO medium (BO medium containing 10 µg/ml heparin, 137.0 µg/ml sodium pyruvate and 1.942 mg/ml caffeine sodium benzoate). The pellet was resuspended in 0.5 ml of the washing BO medium. The matured oocytes were rinsed in washing BO medium and transferred to 50 µl droplets (15–20 oocytes/droplet) of the capacitation and fertilization BO medium (washing BO medium containing 10 mg/ml fatty acid-free BSA). The spermatozoa in 50 µl of the capacitation and fertilization BO medium (3 million spermatozoa/ml) were then added to the droplets containing the oocytes, covered with sterile mineral oil and placed in a CO₂ incubator (5% CO₂ in air) for 18 h at 38.5°C. After the end of sperm-oocyte incubation, the cumulus cells were washed off the oocytes by gentle pipetting. The oocytes were then washed several times with the IVC medium i.e. modified Charles Rosenkrans medium with amino acids (mCR2aa), and cultured for 8–10 days as described earlier [18].

Establishment, culture and cryopreservation of embryonic fibroblasts

The three types of feeder cells, which were compared in this study included buffalo, sheep (SEF) and goat embryonic fibroblasts (GEF). BEF were prepared from buffalo fetuses. Briefly, buffalo fetuses (approximately 50–60 days old) obtained from slaughtered animals were separated from uteri and were washed twice with sterile phosphate buffered saline (PBS). Skin biopsies were taken, minced by scissors into small pieces (approximately 1 mm³) and washed 6–8 times with DPBS. The tissue pieces were

transferred to 60-mm cell culture dishes and were cultured in DMEM supplemented with 20% FBS, 2 mM L-glutamine and 50 µg/ml gentamycin sulphate in a CO₂ incubator (5% CO₂ in air) at 37°C. The explants were removed after achieving confluency. Confluent cells were treated with trypsin-EDTA (0.25%), and sub cultured for cell multiplication. At passage-5, the harvested cells were frozen in small aliquots, for that the culture medium was removed and the cells were rinsed with 4–6 ml Ca²⁺ Mg²⁺-free DPBS, after which trypsin-EDTA solution was added to cover the surface of the cells. The flasks carrying confluent cultures of feeder cells were incubated for 3–5 min at 37°C to separate the cells after which an equal volume of FBS containing medium was added to prevent further damaging action of trypsin. The separated single cells were counted by using a hemacytometer and the cells were suspended and mixed in freezing medium (culture medium + 10% DMSO) at 3 × 10⁵ cells/ml concentration. The cells were cryopreserved by first dispensing into cryovials in 1 ml aliquots and keeping them in an insulated container at –80°C overnight followed by their direct transfer to liquid nitrogen. Similar procedures were followed for preparation of feeder layers from goat and sheep fetuses which were approximately 30 days in age. To provide the same feeder layers for all experiments, sufficient stocks of the feeder layers cells were produced and frozen in liquid nitrogen.

Preparation of feeder layers

The frozen aliquots of BEF, SEF and GEF were thawed and plated in 4-well tissue culture dishes (Nunc, Roskilde, Denmark), grown to form a monolayer and were then treated with mitomycin-C (10 µg/ml) for 3 h. These were washed 4–6 times with DPBS and finally with DMEM supplemented with 20% FBS and used within 1–2 days of preparation. Before plating ICM cells, tissue culture medium was replaced with ES medium.

Inner cell mass isolation and culture

ICMs were isolated either by enzymatic treatment or intact blastocyst culture method. For isolation of ICM cells by enzymatic treatment, expanded blastocysts were incubated with 0.1% pronase to dissolve the zona pellucida (~6 min), then treated with 0.25% trypsin-EDTA and observed under a zoom stereomicroscope until the trophoblasts became loose, and dispersed to release the ICM. For isolation of ICMs by intact blastocyst culture, hatched blastocysts were first seeded as such onto mitotically inactivated fibroblast feeder layers, were allowed to attach and proliferate. On the basis of morphological appearance of the colony, ICM was separated from trophectoderm by cutting with the help of fine needle and pasture pipette. The isolated ICMs from

both procedures were washed and then seeded again onto freshly prepared mitomycin-C treated feeder layer from respective species individually. The cells were cultured in 200 µl of ES medium in 4-well plate in 5% CO₂ at 38.5°C and examined at the interval of 24 h with ES medium being replaced every day. The ES medium used was DMEM supplemented with 20% FBS, 2 mM L-glutamine, 1,000 units/ml recombinant murine leukemia inhibitory factor (rmLIF), 1X nonessential amino acids and 50 µg/ml gentamycin sulphate. After 3–4 days of culture, subculture was performed by mechanically cutting out the colonies and dividing it into pieces with the aid of two fine glass needles under a zoom stereomicroscope (Olympus, SZ40, Japan). The primary colony formation rate; primary colony formation time in days, maximum no. of passages and colony morphology were recorded carefully.

Characterization of buffalo ES-like cells colonies

Alkaline phosphatase staining

AP staining was performed as described earlier [17]. Briefly, the ES-like cells were fixed in citrate-acetone-formaldehyde fixative solution for 1 min, washed three times with deionized water, incubated for 15 min at room temperature with the alkaline dye under dark condition, rinsed again with deionized water and counter stained with haematoxylin stain. The cells that stained red, which is an indicator of AP, were considered to be the ES cells.

Karyotyping

Chromosomal integrity of BuES-like cells were analysed by performing karyotyping. The ES-like cells were incubated with 0.1 µg/ml colcemid for 4 h at 37°C. The colonies were washed, trypsinized and resuspended in freshly prepared 75 mM KCl and incubated for 30 min at 37°C. They were washed and then fixed in chilled fixative (3:1, methanol: glacial acetic acid) for 20 min at room temperature and centrifuged at 200 g for 10 min. The pellets were resuspended in 5 ml of chilled fixative for another 10 min and then centrifuged again. The metaphase spreads were prepared by dropping the cells onto ice cold glass slides and stained with 2% giemsa for 5–6 min. The glass slides were rinsed and observed under oil immersion (1000X) using a compound microscope (Nikon, Microphot, FXA, Japan).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from the cells of interest using the “Cells-to-cDNA kit-II” (Ambion, Austin, TX) as per

manufacturer's instructions. Briefly, the cells were washed with ice cold PBS, 20–50 μ l of cold cell lysis buffer was added and the reaction mixture was incubated in a thermal cycler (MJ Research, Watertown, MA, USA) at 75°C for 10 min. The cell lysate was treated with DNase-I at 37°C for 30 min to degrade genomic DNA and then heated at 75°C for 5 min to inactivate DNase-I. The cell lysate (10 μ l) + 2 μ l random decamer primer + 4 μ l dNTPs were mixed and incubated at 78°C for 1 min after which 2 μ l of 10X RT buffer, 1 μ l RNase inhibitor and 1 μ l MMLV mix were added and the mixture was incubated at 42°C for 60 min and then at 95°C for 10 min. The PCR cycle included heating to 94°C for 2 min, followed by repeated cycles of 94°C for 30 s, annealing temperature 57–60°C for 30 s, and extension at 72°C for 45 s, this cycle was repeated 33–35 times with final extension for 72°C for 10 min. The following primers were used for PCR: primers specific for OCT-4 (accession no. AF022987), 5'-GTTCTCTTTGGAAAGGTGTTTC-3' and 5'-ACACTCG GACCACGTCTTTC-3'; for NANOG (accession no. DQ487022), 5'-GGGAAGGGTAATGAGTCCAA-3' and 5'-AGCCTCCCTATCCAGAAAA-3'; for 18S-rRNA (accession no. AF176811), 5'-GAGAAACGGCTACCACATCC-3' and 5'-GGAACTCAGCTAAGAGCATCG-3'. A –ve RT reaction (parallel RT reaction but without MMLV enzyme) was set with every batch of cDNA preparation to check genomic DNA contamination and a –ve PCR without template was also set. 18S-rRNA was amplified at each stage as a house keeping marker gene. Feeder cells were used as negative control for ES-like cells.

Semi-quantification of RT-PCR products

For resolution of the PCR products, agarose gel electrophoresis (Pharmacia LKB Biotechnology, Piscataway, NJ, USA) was performed with 8 μ l RT-PCR products that were loaded with 2 μ l 6X gel loading dye. A 2% (w/v) agarose gel in 1X TAE buffer with 0.5 μ g/ml ethidium bromide was used. For analyzing the PCR products and capturing the image, Minibis gel documentation system (Labnet, Delhi, India) was used. Densitometry data for band intensities was generated using AlphaDigiDoc™ AD-1201 software under windows™ environment. The relative abundance of different transcripts was estimated by dividing the intensity of the band of the transcript of interest by the intensity of 18S-rRNA from the same sample.

Statistical analysis

Data were analyzed using SYSTAT 7.0 (SPSS Inc. USA) after arcsin transformation. Differences between mean percentages were analysed by one way ANOVA followed by Fisher's LSD test for pair wise comparison of means.

Results

Culture and maintenance of embryonic fibroblasts

In the present study, frozen-thawed embryonic fibroblast cells of buffalo (Fig. 1a), goat (Fig. 1b) and sheep (Fig. 1c) showed normal morphological features. Among the feeder layers from the three species, the proliferation rate was found to be lesser for the SEF compared to that for the BEF and GEF. Also, the goat feeder cells appeared comparatively thinner and longer.

Derivation of buffalo ES-like cells on different feeder layers

A total of 69 hatched blastocysts (Fig. 2a) were used for isolation of ICMs through intact blastocysts culture method out of which 28, 21 and 20 were seeded on the BEF, GEF and SEF feeder layers respectively in individual well of a four-wells plate having mitotically inactivated feeder cells. Following seeding, the percentage of hatched blastocysts that attached to the BEF feeder layer ($85.7 \pm 7.22\%$) was found to be significantly higher ($P < 0.05$) than that on SEF feeder layer (54.0 ± 11.11), whereas attachment time in days was significantly low ($P < 0.05$) upon culture on BEF feeder layer than on SEF feeder layer (Table 1). The time required for the formation of primary colonies (Fig. 2b), on BEF feeder layers was significantly ($P < 0.05$) shorter time than SEF fibroblasts but were similar to GEF. Whereas the primary colony formation rate was not found to be statistically different in the studied feeder layers. The maximum number of passages (i.e. 11) for which the cultured BuES-like cells could survive was highest for the homologous i.e., BEF feeder layer compared to that of passages 6 and 4 for GEF and SEF feeder layers, respectively.

In case of BuES-like cells from expanded blastocysts, a total of 83 expanded blastocysts (Fig. 2c) were taken and their ICMs were isolated through enzymatic treatment method and they were cultured on different feeder layer cells. The percentage of ICMs attachment rates was observed to be $57.24 \pm 6.40\%$ on BEF, $38.89 \pm 5.57\%$ on GEF and SEF in 3.31 ± 0.28 , 3.46 ± 0.14 and 3.58 ± 0.29 days respectively. However, when the proliferated ICM cells were cultured on fresh feeder layers, the primary colony (Fig. 2d) formation rate was found to be significantly higher ($P < 0.05$) for BEF compared to that for GEF or SEF feeder layers (Table 2). The time required to form the primary colonies was, however, similar for BEF, GEF and SEF feeder layers. When the colonies were cultured further, they maintained their ES cell characteristics till six passages on BEF feeder layer cells; however on GEF they survived till five passages and on SEF they were maintained till three passages only.

Fig. 1 Morphology of different feeder cells at passage 5. (A) BEF, (B) GEF and (C) SEF. Scale bars: 100 μ m

Buffalo ES-like cells developed into colonies with a significant difference in the morphological appearance, where they showed tight or compact morphology with dome shape and had a clear margin on BEF feeder layers (Fig. 3a), however, the colony morphology was thinner and flatter but with clear margins on GEF feeder layers (Fig. 3b). On the other hand the cells of the ES cell colonies appeared to carry loose cells with increased intercellular spacing on SEF feeder layers (Fig. 3c) and lacked clear cut and prominent margins.

Characterization of buffalo ES-like cells

The expression of AP was positive in all BuES-like cells colonies grown on all three feeder layers. But, the expression was strong in the colonies cultured on BEF feeder layers (Fig. 4a), and the signal appeared to be lower in colonies cultured and maintained on goat (Fig. 4b) and SEF feeder layers (Fig. 4c). In addition to that, chromosomal integrity was analyzed at different passages of BuES-like cells and was found normal (Fig. 4d).

Fig. 2 Isolation of ICMs from in vitro produced hatched (A) and expanded blastocysts (C), subsequently cultured on feeder layers to form primary colony (B and D) from respective group. Scale bars: 50 μ m

Table 1 Effect of feeder layer cells from different species on derivation of BuES cell-like cells cultured from inner cell masses isolated from hatched blastocysts

Source of feeder cells	Hatched blastocysts seeded (n)	Hatched blastocysts attached n (%)	Attachment time (days)	Primary colonies formed n (%)	Primary colony formation time (days)	Passage number up to which cells survived
Buffalo	28	24 (85.7±7.22) ^a	2.1±0.14 ^a	21 (76.4±6.05)	3.2±0.22 ^a	11
Goat	21	14 (66.7±9.32) ^{ab}	2.4±0.14 ^{ab}	13 (61.9±4.76)	4.9±0.92 ^{ab}	6
Sheep	20	11 (54.0±11.11) ^b	2.6±0.20 ^b	9 (43.7±15.81)	5.2±0.42 ^b	4

Values are mean ± SEM

a,b values with different superscripts within a column differ significantly ($P<0.05$)

We also preformed the RT-PCR for assessing the expression of OCT-4 and NANOG in AP positive colonies of BuES-like cells and its level of expression, grown on different feeder layer cells. It was found that the expression of OCT-4 (Fig. 5a) and NANOG (Fig. 5b) were positive in BuES-like cells on all the three feeder layers. The relative abundance of OCT-4 and NANOG is shown in Fig. 5c, the level of expression of OCT-4 was almost similar on all feeder layers where as expression of NANOG was significantly higher ($P<0.01$) in BuES-like cells grown on BEF as compared to GEF and SEF cells.

Discussion

Continuous proliferation of ES cells requires optimal in vitro conditions to maintain self-renewal and feeder layer secretes important growth factor for preventing differentiation of ES cells. The present study show that homologous feeder layers allow efficient derivation of BuES-like cells from hatched and expanded blastocysts than heterologous feeder layers. Successful use of homologous embryonic fibroblast feeder layers to produce ES cells has been previously done in mink [19], marsupials [20], human [21–23], pig [24, 25] and buffalo [16, 17, 26]. In contrast, in an early study, Piedrahita et al. [27] determined that porcine embryonic fibroblasts (PEF) did not promote attachment of the porcine ICM to the feeder layer. Also, in some other species like sheep [24] and chicken [28, 29] homologous feeder layer have proved unsuccessful sug-

gesting that embryonic fibroblasts from these species do not secrete the necessary factors to support pluripotent self-renewal. A clear species-specific relationship between feeder layers and derivation of cat ES-like cell lines was observed, where higher numbers of cat ES-like cell lines were generated on homologous cat feeder layers than from those derived on heterologous mouse feeder layers [30]. For the derivation of bovine ES cells several different cell types have been used as feeder layers, including bovine fetal fibroblasts, bovine uterus epithelial cells, mouse fetal fibroblasts, STO cells and human lung fibroblasts but none of these cell types has proven feasible to sustain prolonged proliferation of bovine ES cell-like cells [31], whereas mixed fibroblast feeder cells were more efficient for long-term culture of bovine ES cell lines than using only mouse or bovine fibroblasts [32]. On the other hand, the use of homologous or heterologous feeder cells (mouse) equally supported the generation of canine ES cell lines, but the use of heterologous mouse feeder cells was required to continue canine ES cell propagation [33]. This further complicates the use of either homologous or heterologous feeder layer suggesting the species specific differences in the use of feeder layers for ES cell derivation and culture. Here we have compared the three feeder layers i.e. BEF, GEF and SEF for their ability to support growth of BuES-like cells and examined the characteristics of these cells on each feeder layer. When hatched blastocysts were seeded on BEF feeders, it allowed better attachment rates in significantly lesser time period as compared to SEF. Also the primary colony formation time was significantly reduced on

Table 2 Effect of feeder layer cells from different species on derivation of BuES cell-like cells cultured from inner cell masses isolated from expanded blastocysts

Source of feeder cells	ICMs seeded (n)	ICMs attached n (%)	Attachment time (days)	Primary colonies formed n (%)	Primary colony formation time (days)	Passage number up to which cells survived
Buffalo	31	18 (57.2±6.40)	3.3±0.28	16 (51.2±3.37) ^a	4.0±0.49	6
Goat	26	10 (38.9±5.57)	3.5±0.14	7 (25.6±7.60) ^b	5.2±1.01	5
Sheep	26	10 (38.9±5.57)	3.6±0.29	8 (31.0±4.56) ^{ab}	6.0±0.58	3

Values are mean ± SEM

a,b values with different superscripts within a column differ significantly ($P<0.05$)

Fig. 3 Morphology of BuES-like cell colony grown on BEF appears compact and dome shaped (A), on GEF appears compact and flat (B), whereas on SEF appears scattered (C). Scale bars: 50 μ m (A, B) and 100 μ m (C)

BEF, and similar results were also observed in porcine [25], rat and mice [34]. In the process of ES cell derivation, the GEF appeared to be intermediate between BEF and SEF in all the above mentioned criteria. Furthermore, GEF and SEF could maintain BuES-like cells in undifferentiated state up to 6 and 4 passages respectively, whereas BEF allowed them to survive till maximum no. of 11 passages. Thus, BuES-like cells could be cultured on GEF and SEF and were successfully grown on BEF. The establishment of BuES cells on homologous feeder layer prevents the risk of cross species contamination and allows their use as a source material for production of cloned and transgenic animals.

Our observation suggests that the source of ICM is an important criteria for ES cell derivation in buffalo where hatched blastocysts had significantly higher ($P < 0.01$)

attachment rate and primary colony formation rate, obtained in relatively shorter time period over the expanded blastocysts. This observation prompts towards better ICM compaction, presence of more viable ICM or higher ICM cell no. leading to an increased inclination for BuES-like cell derivation from hatched blastocysts compared to expanded blastocysts. In earlier studies too, the isolation of ICMs either from morula, early stage of blastocyst or late stage of blastocyst gave an unsatisfactory result as compared to hatched blastocyst in various species [16, 17, 35, 36]. In our earlier study from our laboratory [17], the comparison of ICM isolation method on buffalo ES cell generation was evaluated. The authors reported that the ICM isolation method affects the generation of BuES-like cells. Likewise, in the present study, we observed that

Fig. 4 The BuES-like cell colony showing positive expression of alkaline phosphatase cultured on (A) BEF, (B) GEF and (C) SEF (D) BuES-like cells exhibited a normal karyotype. Scale bars: 100 μ m

Fig. 5 RT-PCR analysis of gene expression in BuES-like cells cultured on BEF, GEF and SEF feeder layers. Expression of OCT-4 (A) and NANOG (B) shown in lane 2, whereas lane 1. Marker (100 bp), 3. Feeder cells, 4. 18-S rRNA (337 bp), 5. –ve RT and 6. –ve PCR, (C) Expression levels of OCT-4 and NANOG in BuES-like cells grown on BEF, GEF and SEF feeder layers

BuES-like cells which were isolated by intact blastocyst culture of hatched blastocysts were maintained in an undifferentiated state for more numbers of passages than that generated following the enzymatic isolation method of expanded blastocysts. The similar kind of method was used in ES cell derivation from parthenogenetic buffalo embryos [37] and porcine IVF embryos [38]. Thus upon comparison of intact blastocysts culture method with the widely used enzyme treatment method we observed a better efficiency of former in terms of BuES-like cells derivation.

The BuES-like cells were successfully grown and maintained on different feeder layers as they exhibited a typical morphology of high nucleus-to-cytoplasm ratio with prominent nucleoli, had tight or compact and dome shaped colonies with clear margins on BEF feeder layers. In a present study, the morphology of BuES-like cells on BEF was observed to be similar to our previous study [16], and closely resembled porcine [39] and cat [30] ES cells. However, the colony morphology was found to vary on heterologous feeder layers where they appeared compact, thinner and flatter but with clear margins on GEF feeder layers and appeared to carry loose cells with increased intercellular spacing and showed lack of clear margins on SEF feeder layers. The variation in morphology among species may occur due to species-specific characteristics or because of influence due to culture conditions. In fact, cat ES-like colonies that were cultured in stem cell medium supplemented with knockout serum (20%) had a dome shape, while colonies cultured in stem cell medium

supplemented with FBS (20%) were flattened in morphology [40].

Although the proliferation activity was confirmed by the expression of AP which was observed to be present in all BuES-like cell colonies grown on the three different feeder layers but the intensity of expression was found to vary; the expression was more intense on homologous feeder layer (BEF). In addition to this, the BuES-like cell colonies showed expression of transcription based markers OCT-4 and NANOG. The BuES-like cells maintained almost similar expression levels of OCT-4 gene transcripts but differed significantly ($P < 0.01$) in the expression of NANOG transcripts on the different feeder layers indicating that the morphological changes do not affect the expression pattern of pluripotent markers. Corresponding results were reported by [30], in which cat ES-like cells expressed the “stemness” transcription factors and the levels of expression of OCT-4, NANOG and C-MYC were reduced in cat ES-like colonies as compared to that seen in blastocysts. In other study, expression of transcription factors like Oct 4 and Nanog were used as molecular markers for ES cells in various species [16, 17, 41–43]. Moreover, when undifferentiated primary colonies were analyzed for the expression of pluripotent transcription factors, Gómez et al. [30] found that OCT-4 was down regulated regardless of the expression of NANOG. Maintenance of Oct-4 expression at a critical concentration is necessary to sustain ES cell self-renewal, increased expression of Oct-4 triggers differentiation into endoderm or mesoderm, while its suppression

causes ES cells to become trophectoderm [44, 45]. In contrast, over-expression of Nanog, did not induce ES cell differentiation but actually augmented the self-renewal capacity of ES cells in the absence of LIF [46, 47]. Various workers used LIF as a supplement for maintaining pluripotency of ES cells along with feeder layer cells. We have also used 1,000 IU/ml rmLIF with all three feeder layer cells for culture and maintenance of BuES-like cells. However, we found that SEF and GEF only support up to 4 and 6 passage of BuES-like cells respectively, which indicate either the lesser secretion of LIF from feeder cells or a higher requirement of LIF for maintaining pluripotency at greater passage no. of BuES-like cells.

Conclusions

In conclusion the results of the present study show that although all the three used feeder layers support the derivation of ES-like cells from in vitro produced blastocysts but homologous feeder cells are capable of maintaining their proliferation to comparatively longer periods. Also, the hatched blastocysts provide a better source of BuES-like cell isolation compared to expanded blastocysts. The characteristic feature of BuES-like cells was demonstrated by assessing the morphology of colony, karyotype analysis and analysis of pluripotent markers expression like AP, OCT-4 and NANOG. From the above mentioned comparison of different feeder layer cells for derivation and maintenance of BuES-like cells from hatched and expanded blastocysts, we found that BEF were able to provide better environment for maintaining the pluripotency in culture conditions.

Acknowledgements This work was supported by the Department of Biotechnology, Ministry of Science & Technology, Government of India, New Delhi.

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