Characterization and establishment of embryonic stem cell-like cell lines from *in vitro* produced buffalo (*Bubalus bubalis*) parthenotes

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ABSTRACT

Embryonic stem cells (ESC) provide potentially unlimited cell numbers for gene targeting, providing great value to agriculture and medicine. This study was conducted to isolate and characterize parthenogenetic ESC (pESC)-like cells from *in vitro* produced buffalo parthenotes. A total of 100 blastocysts were produced through chemical activation. For pESC culture, 20 blastocysts were left intact, while the rest was dissociated mechanically or enzymatically, to isolate inner cell mass (ICM) cells from 44 hatched and 36 non-hatched blastocysts, respectively. ICM cells were seeded and cultured on mitomycin-C treated feeder layers of buffalo fetal fibroblasts. Primary cell colonies formed three to four days after ICM seeding. Colony formation was significantly lower from intact blastocysts (3/20 (15%)) than from ICM cultures (39/80 (49%)) with hatched blastocysts being better starting material than non-hatched ones (30/44 (68%) versus 9/36 (25%), respectively). Up to passage nine, two mechanically isolated pESC-like lines remained viable and expressed specific markers such as alkaline phosphatase activity, as well as Nanog and Oct-4 mRNA. Cells formed embryoid bodies in suspension culture. It has been shown it is possible to isolate buffalo pESC-like cells from parthenogenetically developed embryos and maintain the culture *in vitro* for a prolonged period of time.

Key words: Buffalo; parthenogenetic embryonic stem cell like; inner cell mass; *in vitro* maturation.

INTRODUCTION

Embryonic stem cells (ESC) are immortal pluripotent cells derived by placing preimplantation blastomeres into *in vitro* culture (Evans *et al*., 1981; Martin *et al*., 1981). They are capable of proliferation, self-renewal and the production of differentiating daughter cells. When combined with normal preimplantation embryos as chimeras, they can contribute to any kind of tissue including the germ-line. In addition to mice and human (Thomson *et al*., 1998; Reubinoff *et al*., 2000), attempts have also been made to establish ESC lines from other mammals, like, cattle (Strelchenko, 1996; Mitalipova *et al*., 2001) and rhesus monkey. ESC have been successfully obtained from buffalo (*Bubalus bubalis*) by culturing inner cell mass (ICM) cells isolated from *in vitro* produced embryos (Verma *et al*., 2007).

Parthenogenesis is the process by which an oocyte develops into an embryo without being fertilized by spermatozoa. Parthenogenetic preimplantation embryos have been used as a source of putative ESC lines in mice (Cibelli *et al*., 2002). In buffalo only 8 to 10% of *in vitro* fertilized eggs developed into blastocysts, while parthenogenetic embryo development rates were higher.Therefore, parthenogenetic development could be used to obtain more blastocysts as starting material for parthogenetic embryo stem cells (pESC), in addition to this practical advantage, pESC have properties that make them attractive for *in vitro* studies of imprinting, including a lack of paternal contribution; a potential to divide indefinitely; and a potential to differentiate into all tissues of an adult body. Parthenogenetic embryos can gastrulate and complete early organogenesis, but not develop to term. However, chimeras of parthenogenetic cells with biparentally derived embryonic tissues have generated apparently normal offspring (Boediono *et al*., 1999).

MATERIALS AND METHODS

Oocytes collection and in vitro maturation

Buffalo oocytes were collected and matured *in vitro* as described earlier (Verma *et al*., 2007).

Parthenogenetic activation

After *in vitro* maturation for 24 h, the oocytes were manually denuded by pipeting through a pasteur pipette and vortexing. Oocytes with an extruded first polar body (MII) were assigned to the following treatments: 5 µM calcium ionophore (A23187) for 5 min with subsequent treatment by 2 mM 6-dimethylaminopurine (DMAP) for 4 h under a humidified atmosphere of 5% CO_2 at 38.5°C. Activated oocytes were cultured in mCR2aa supplemented with 10% fetal bovine serum under a humidified atmosphere of 5% CO_2 at 38.5°C. The development of the embryos was monitored for 7 to 8 days.

Preparation of feeder cells

Buffalo feeder layer was prepared as described earlier (Verma *et al*., 2007).

ICM isolation from blastocysts

The ICM of the hatched blastocysts were isolated mechanically with the aid of two fine glass

needles under a zoom stereomicroscope. ICMs from non-hatched blastocysts were enzymatically isolated. The zona pellucidae were first removed by incubation with 1.0% pronase in phosphate buffered saline (w/v). Then embryos were incubated in 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA) until the trophectodermal cells became loose, and could shed from the ICM using gentle pipetting. Isolated ICMs were individually seeded on mitomycin-C (10 μg/ml) treated feeder layers, and cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 20% foetal bovine serum, 1000 IU/ml murine leukemia inhibitory factor, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol and 2 mM L-glutamine.

Comparison of subculture methods

The primary colonies, obtained three to four days after seeding of ICMs or intact blastocysts, were divided randomly into two groups. In the first group, the colonies of pESC-like cells were treated with 0.25% trypsin–EDTA for five to six min at 37°C. The treated pESC-like cell colonies were disaggregated with the aid of two fine glass needles under a zoom stereomicroscope. In the second group, the colonies were disaggregated mechanically under the microscope. Aggregates of 50 to 100 cells were individually reseeded onto a new feeder layer in 4-well cell culture plates. The colonies exhibiting typical morphological features of pESC-like cells were sub-cultured using enzymatic or mechanical dissociation as long as the cells remained in an undifferentiated state.

Characterization of pESC-like cells

Analysis of marker expression was performed on pESC-1 and pESC-2 at passages 2 to 4 and 6 to 8. Alkaline phosphatase (AP) staining was carried out as described earlier (Verma *et al*., 2007). Nanog was assessed in batches of cells that were found positive for AP activity was assessed on the primary cultures of pESC-like cells by a one step reverse transcription polymerase chain reaction (RT-PCR) performed using the "Cells-to-cDNA Kit-II" (Ambion, Austin, Texas, USA) as per the manufacturer's instructions. The kit had all the reagents for reverse transcription except thermostable Taq DNA polymerase, Nanog primers were purchased from Genetix, Bangalore, India. The specific primers for the amplification of Nanog were – FWD: 5'- ATCCAGCTTGTCCCCAAAG 3'and REV: 5' ATTTCATTCGCTGGTTCTGG 3'(Annealing temperature = 60°C, expected product length = 438 bp). The expression of Oct-4 was detected as described previously (Verma *et al.,* 2007).

TABLE 1: Assessment of culture behavior of embryo–derived cells from different embryonic stages. pESC = Parthogenetic embryo stem cell.

Developmental. stage	Explant	Number of embryos	Number of primary pESC-like colonies (%)
Non-hatched	Inner cell mass	36	9(25)[a]
Non-hatched	Intact blastocyst	20	3(15)[b]
Hatched	Inner cell mass	44	30(68)[c]

Values within the same column with different superscripts differ significantly (P <0.01).

Evaluation of *in vitro* differentiation

Evaluation of the differentiation of pESC-like cells was done as described previously (Verma *et al*., 2007).

Chromosomal integrity of normal pESC-like cells

Karyotyping analysis was performed on pESC1 and pESC2 at passages 2 to 4 and 6 to 8. The cells were subjected to chromosomal analysis as described previously (Verma *et al*., 2007).

Statistical analysis

The differences in the number of embryos giving rise to primary cell colonies were revealed by a chi-square (χ^2) test. A value of P <0.05 was considered to be statistically significant.

RESULTS

***In vitro* production of embryos through parthenogenetic activation**

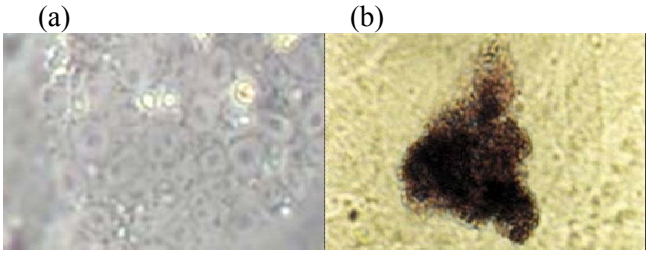
A total of 100 parthenogenetic blastocysts were used for the production of pESCs-like cells.

Formation of pESC-like cell colonies from ICM-derived cells

A total of 80 blastocysts (36 non-hatched and 44 hatched blastocysts) were used for studying the culture behavior of embryo-derived ICMs cells (Table1). ICMs were isolated from blastocysts mechanically as well as by enzymatic digestion. Primary colonies were obtained three to four days after seeding them on mitomycin–C treated feeder fibroblast cell layers. The ESC-like cell colonies were formed in 68% (30/44) of ICMs obtained from hatched blastocysts, which was significantly higher (P <0.05), than the 25% (9/36) formed from non-hatched blastocysts. Primary colonies were formed in 15% (3/20) of intact blastocyst cultures, which was significantly lower (P <0.05) than the 49% (39/80) formed from ICM culture.

Morphologically, the cells of ICM-derived primary colonies had a high nucleus: cytoplasm ratio with prominent nucleoli. The colonies were densely packed with a clear border. Furthermore, the primary colonies were observed to be flatter than murine ESC and grew as a monolayer (Figure 1a).When these primary colonies were separated and reseeded onto fresh feeder layers, new colonies were observed within two to three days of subculture. When intact blastocysts were seeded on mitomycin-

FIGURE 1: Morphology of primary parthenogenetic embryonic stem cell (pESC)-like colony and alkaline phosphatase staining at passage-4. (a) pESC-like cell colony after 4 days of culture. (b) Expression of alkaline phosphatase by undifferentiated colonies derived from inner cell mass cells**.** Strong positive staining (Red) was revealed in buffalo pESC-like cells.



C treated feeder layer, the primary cell colonies were observed after 4 to 6 days of culture.

The pESC-like cell colonies were dissected into small sized clumps mechanically or with enzymatic treatment**.** When enzymatic treatment was used for disaggregating the pESC-like colonies, the pESC-like cells differentiated within the first two passages. However, when it was done mechanically, two pESC-like cell lines (pESC1 and pESC2) were obtained, which remained undifferentiated for nine successive passages (Table 2), as indicated by AP activity (Figure 1b), Nanog (Figure 2a) and Oct-4 (Figure 2b) expression measured at passages 2 to 4 and 6 to 8 (Figure 3a).

In addition to this, the chromosomal profile of pESC-like cells analyzed at passages 2 to 4 and 6 to 8 was found to be normal. However, at passage 9, the cells of both the cell lines changed morphologically, did not form colonies and showed negative AP activity. Suspension cultures of pESC-like cells of passage 2 to 4 or 6 to 8 in the absence of murine leukemia inhibitory factor and feeder layer, resulted in the formation of embryoid bodies within 2 to 3 days (Figure 3b). These developed into cystic embryoid bodies after 14 days of culture.

DISCUSSION

To our knowledge this is the first report to document the isolation, culturing and characterization of pESC-like cells from buffalo parthenotes. Our subsequent analysis confirmed that these cells possess key properties of ESC.

In agreement to our previous finding (Verma *et al*., 2007) we found that more primary colonies were formed from hatched blastocysts as compared to those from non-hatched blastocysts perhaps indicating the presence of a higher number of pluripotent cells in embryos at this stage. This study also indicated that the culture of ICMs isolated from blastocysts, either mechanically or enzymatically, was more effective than the culture of intact blastocysts for the establishment of primary colonies. It is possible that trophoblasts affect ICM growth in the intact blastocyst culture and induce ICM cell differentiation in culture at the early stage (Verma *et al*., 2007).

An easy method established by Verma *et al*., 2007 to isolate ICMs mechanically from blastocysts was used, obviating the use of trypsin which caused loss of pluripotency to some extent. The mechanical method, compared to enzymatic treatment was

FIGURE 2: Reverse transcription polymerase chain reaction (RT-PCR) analysis of Nanog and Oct-4 gene expression in buffalo parthenogenetic embryonic stem cell (pESC)-like cells (Passage 4). The pESC-like cells express (a) nanog (438bp) in lanes 1, 2, 3 and 4 (pESC-like cells) and (b) Oct-4 (341bp) in lanes 2, 3 and 4. Lane M: 100 bp DNA ladder. Empty Lanes 1in Figure 2b and lane 5 and 6 in Figures 2a and 2b represent negative control without RTase and without Template cDNA respectively.

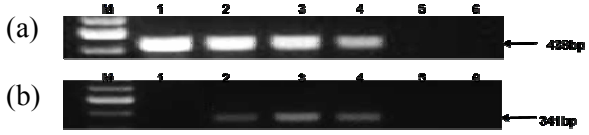


FIGURE 3: Morphology of parthogenetic embryonic stem cell (pESC)-like colony and embryoid body. (a) Morphology of pESC-like colony at 8th passage. (b) A simple embryoid body.

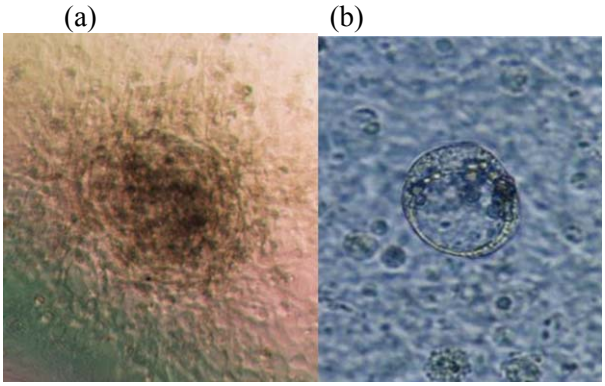


TABLE 2: Loss of viability of parthenogenetic embryonic stem cell-like cell lines following prolonged *in vitro* culture using mechanical dissociation or enzymatic treatment with 0.25% Trypsin–EDTA.

Cell dissociation methods	Number of primary cultures used	Number of cell lines surviving passage								
		1	2	3	4	4	6	7	8	9
Mechanical	26	21	16	11	10	10	7	4	4	2
Enzymatic	10	4	2	0	0	0	0	0	0	0

found to be very effective for the isolation of ICM derived from buffalo blastocysts produced through chemically activated oocytes. Traditionally, ESC-like cells have been obtained from ICMs of blastocysts (Evans and Kaufman, 1981; Martin, 1981; Thomson *et al*., 1998; Reubinoff *et al*., 2000).

Culture conditions used for the buffalo pESC-like cells derived from parthenotes were the same as that used for ESC-like cells derived from *in vitro* produced embryos (Verma *et al*., 2007). It has been reported in earlier studies that mouse (Evans and Kaufman, 1981; Martin, 1981) and pig ESC formed dome shaped colonies, but human and monkey ESC formed flat colonies (Thomson *et al*., 1998). In agreement to our previous studies (Verma *et al*., 2007) we found that most of the pESC-like colonies were flatter than murine ESC colonies and grew as a monolayer. The mechanical method for dissociation of these colonies was found to be more effective than enzymatic treatment as indicated by the capability of the pESCs to survive for a longer period while maintaining their morphological features. Trypsin has been used to dissociate murine ESC (Evans & Kaufman, 1981; Martin, 1981). However, buffalo, cattle and rabbit ESC were found to loose pluripotency when trypsin was used for passaging (Verma *et al*., 2007; Strelchenko, 1996). The results of our study also support the findings that cell dissociation by various enzymes, such as pronase, or collagenase, was not suitable for culturing pESC-like cells, compared to mechanical dissociation.

We used morphological features, AP activity, Oct-4 and Nanog expression to characterize buffalo pESC-like cells of pESC1 and pESC2 at passages 2 to 4 and 6 to 8. AP expression is a preliminary marker for evaluating pluripotent cells and has been used to identify ESC in many species (Thomson *et al*., 1998;). In the present study, AP expression was clearly detected in buffalo pESC-like cells. The expression of Oct-4 is widely detected in early embryos, and becomes restricted to the ICM at the blastocyst stage. The expression of Oct-4 has also been detected *in vitro* in undifferentiated ESC by various other workers (Reubinoff *et al*., 2000; Verma *et al*., 2007) and has been widely accepted as a pluripotent cell marker. Nanog transcription is controlled by the synergistic actions of OCT-4 and SOX2.We have shown that buffalo pESC-like cells also express Oct-4 and Nanog using RT–PCR. The RT-PCR product was of 341bp for Oct-4 and 438bp for Nanog. When karyotypic analysis was performed on pESC-1 and on pESC-2 at passage levels 2 to 4 and 6 to 8, chromosome morphology was normal.

The pluripotency of buffalo pESC-like cells *in vitro* differentiation was tested through spontaneous differentiation. In this study, buffalo pESC-like cells were capable of forming embryoid bodies *in vitro*. The formation of these bodies *in vitro* is considered to be an important characteristic of ESC. In brief, buffalo pESC-like cells could be isolated from parthenogenetically developed embryos and maintained *in vitro* for prolonged periods of time.

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