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Expression of pluripotency markers in buffalo (*Bubalus bubalis*) embryonic stem cell like cells

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Abstract
The objective of present study was to culture and characterize the buffalo embryonic stem cell-like cells (BESC) for their pluripotency by analysing Oct-4 and TRA-1-81 markers. Blastomeres from two, four, eight, sixteen, morula and blastocyst of *in-vitro* produced buffalo preimplantation embryos were derived and were seeded on mitomycin inactivated buffalo foetal fibroblasts feeder layer and cultured. The cultured blastomeres were monitored colony formation. The colonies exhibiting typical morphological features of BESC were subcultured. Marker expressions were tested on passages 1, 2, 3 and 4 by RT-PCR, immune-cytochemistry and flowcytometric analysis. RT-PCR study revealed that expression of Oct-4 progressively declined with subsequent passages. The BESC strongly reacted with TRA-1-81 antibodies. In flowcytometry, the cells expressing TRA-1-81 in the first passage and second passage were 98.03±0.03 per cent and 97.13±0.07 per cent respectively. However, in the third and fourth passages the expression levels were reduced to 12.11±0.04 and 4.21±0.09 per cent respectively. Thus the study proved that cultured BESC had pluripotency characters and from third passage they started differentiation process.

Keywords: Buffalo, embryonic stem cell, pluripotency markers, RT-PCR, flow cytometry

Introduction
Embryonic Stem (ES) cells are pluripotent cells that can be cultured indefinitely and either maintained in an undifferentiated state or induced to differentiate into any cell type, including gametes. Stem cell research attracts more public and scientific attention than any other topic in developmental biology. An understanding of the specific properties of stem cells may generate deep insights and crucial impacts in cell biology and also provide great hope for the treatment of a variety of degenerative diseases such as Parkinson's, diabetes mellitus and leukemia. There are some important criteria which need to be satisfied before a stem cell line qualify to be a bonafide ES cell line. These include morphological similarities to ES cells of other species, indefinite undifferentiated proliferation *in vitro*, potential to differentiate into three embryonic germ layers and expression of specific molecular markers and maintenance of normal karyotype. The expression of Oct4 in undifferentiated pluripotent cells has also been shown in various other pecies like canine [2], goat [3], bovine [7] and buffalo [11]. The expression of TRA-1-60 and TRA-1-81 has also been reported in buffalo ES cell like cells [6, 9]. The pluripotent phenotype of buffalo ES like cells were characterized by flowcytometry employing antibodies to SSEA-4, SSEA-3, SSEA-1, TRA-1-81, TRA-1-60 and Oct-4 have been analysed [10]. The objective of present study was to derive the embryonic cells from *in vitro* produced buffalo embryos and to culture and characterize the embryonic cell line for their pluripotency by analysing Oct-4 and TRA-1-81 markers.

Materials and Methods
The study was carried out in the Centralized Embryo Biotechnology Unit, Department of Animal Biotechnology, Madras Veterinary College, Chennai, Tamil Nadu.

In-vitro embryo production
Slaughter house derived buffalo oocytes were used for *in vitro* embryo production. The different stage *in vitro* embryos were produced following *in vitro* maturation (TCM 199 supplemented with 10 per cent fetal calf serum, 0.2 mM pyruvate, 5 µg/ml FSH, 5 µg/ml LH and 1 µg/ml E_2 at 39.3°C, 5% CO_2 and 95 per cent relative humidity (RH) for 27 h)S, *in vitro*

fertilization (TALP medium) and *in vitro* culture (TCM 9 ml, 1ml FCS, 20 µl Gentamicin, 2 µl ITS, 10 µl of EGF and 10 µl of IGF-I). After 24 h, presumptive zygotes were washed in embryo culture medium and transferred to the ECM droplets. Presumptive zygotes were co-cultured with buffalo oviductal epithelial cells (BOEC) obtained from a metestural stage of oviduct in 100 µl IVC droplets of embryo culture medium for 7 - 9 days under a humidified atmosphere of 5 per cent CO_2 at 39.3°C.

Derivation of embryonic cells

Blastomeres from two, four, eight, sixteen, morula and blastocyst of preimplantation embryos were derived by enzymatic digestion of zona pellucida (zonalysis by 0.5 per cent pronase treatment) followed by mechanical method of shearing. The embryos were sheared using a 26G needle (macro dissected) under a zoom stereo microscope.

Seeding and culturing of embryonic cells

The sheared out blastomeres were seeded on mitomycin inactivated buffalo foetal fibroblasts feeder layer (BFF) and cultured in DMEM (supplemented with 20 per cent FBS, 1,000 IU/ml murine leukemia inhibitory factor, 1 per cent nonessential amino acids, 0.1 mM β-mercaptoethanol, and 2 mM L-glutamine). The culture medium was changed every second day and further colonization of the cells was observed routinely under an inverted microscope. The cultured blastomeres were monitored for time of attachment, aggregation, colony formation, expansion and embryoid body formation. The primary colonies, obtained 5-7 days after seeding blastomeres on the feeder layers were disaggregated mechanically under the microscope. Aggregates of 50–100 cells were individually reseeded onto a new feeder layer in 6-well cell culture plates. The colonies exhibiting typical morphological features of ES cell-like cells were subcultured using mechanical dissociation until the cells remained in an undifferentiated state. Buffalo ES like cell colonies derived from differerent stage embryos and various methods were analysed for characterization. Marker expressions were tested on passages 1, 2, 3 and 4.

RT-PCR amplification of Oct-4

ES like cell colonies were collected and subjected for RNA isolation by Trizol method, followed by one step RT-PCR analysis for Oct-4 gene expression. cDNAs were synthesized with oligo dT primer by Revert Aid ™ H-Minus First strand cDNA synthesis kit (Fermentas) based on company standard protocol (Reaction volume25µl). Amplified RT-PCR products specific for Oct-4, were confirmed by gel electrophoresis as per standard procedure. In negative control samples, there was no addition of reverse transcriptase. For comparison, 100 bp molecular weight marker was used (Sigma) and electrophoresed. The results obtained were recorded in a gel documentation system (GELDOC, USA). Primer sequence of *Bubalus bubalis* OCT 4 (Accession No HM 853818.1), available in Genebank was used in the study.

Oct-4 F 5'- GAG GAG TCC CAG GAC ATC AA – 3'

Oct-4 R 5'- CTC TCC AGG TTG CCT CTC AC - 3'

(Primer3 software, product size: 341, annealing temperature 59˚C).

Amplified RT-PCR products specific for Oct-4 were confirmed by gel electrophoresis. Agarose gel (1.5%) was prepared to which ethidium bromide an intercalating dye of 3 µl was added to stain the products and then visualized on a UV illuminator as per standard procedure. To check the cDNA synthesis amplified GAPDH of 357bp was also loaded together with sample. In negative control samples, there was no addition of reverse transcriptase and template DNA. For comparison, 100 bp molecular weight marker was used (Bangalore Genei, India) and electrophoresed. The results obtained were recorded in a gel documentation system (GELDOC, USA).

Immunohistochemistry of stem cell markers

The primary antibodies to TRA-1-81 were diluted (1:10 dilution) in PBS solution. After washing with PBS, cells were fixed with 4% paraformaldehyde for 10-15 min and washed three times in PBS, followed by incubation with 10% normal goat serum for 30 min. Subsequently, primary antibody TRA-1-81 was added for 40 min; cells were then washed with PBS three times, followed by incubation with secondary antibody 1:50 FITC labelled goat anti-mouse IgM for 40 min, washed and mounted. All incubations were carried out at room temperature. The results were examined by fluorescence microscopy.

Flowcytometric analysis

The cells from the first to fifth passages were analysed using BD-FACS Calibur Flowcytometer for the presence of surface marker TRA-1-81. The ES colonies were dissociated using a 0.1 per cent trypsin and then the cells were centrifuged at 1000 rpm for 10 min. Then the cells were fixed using freshly prepared 2 per cent paraformaldehyde for 30 min. Fixed ES cells (100000 cells) were washed using FACS buffer i.e. 1000 rpm for 10 min and resuspended in the same. Then the ES cells were permeabilized using 0.1 per cent Triton X-100 for 30 min. After washing three times with FACS buffer the cells were incubated with primary antibody (mouse monoclonal antibody TRA-1-81, (Chemicon) for 40 min. After washing with FACS buffer for three times, the cells were incubated with secondary antibody (Anti-mouse FITC antibody) for 40 min. Followed by three washing of FACS buffer the cells were either stored overnight in 0.4 per cent paraformaldehyde or resuspended in 500 µl of FACS buffer. The controls were unstained ES cells, ES cells with secondary antibody only, unstained feeder layer cells (BEFF cells) and feeder layer cells treated with both primary and secondary antibodies. A BD-FACS Calibur flow cytometer with a dual laser source (488 and 635 nm) was used to acquire data and used one parameter staining (FITC only).Data analysis was done using CellQuest software (BD Biosciences).

Results and Discussion

In the present study, ES like cell colonies were observed as multicellular with distinct boundaries (Fig.1). Primary colonies had a high nucleus to cytoplasm ratio and were densely packed with a clear border. Individual cells were large and round with indistinguishable cell to cell boundaries with a smooth surface. The results were in agreement with previous reports in buffalo ES-cells [4, 11] and bovine putative ES cells [12].

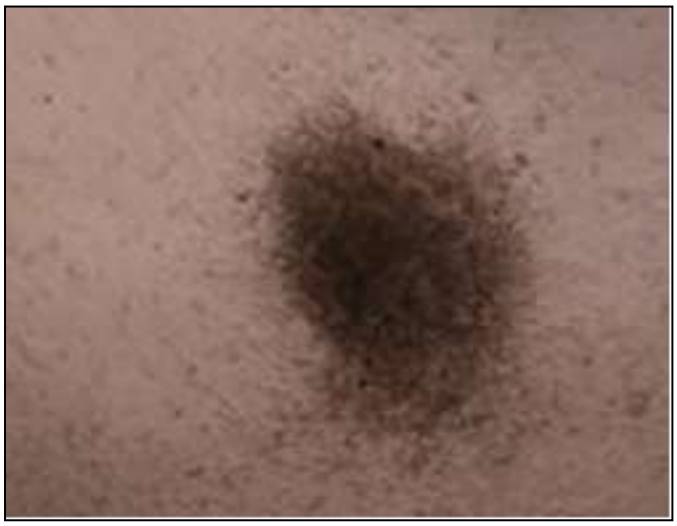


Fig 1: Buffalo ES like cell primary colony

Expression of transcriptional marker

In the present study, the expression of Oct-4 progressively declined with the increase in passage number as indicated by the intensity of the RT-PCR band (Fig.2).

Oct-4 expression declined when stemness of the cells was lost due to differentiation. The findings were in concurrence with the previous reports [5, 9, 11].

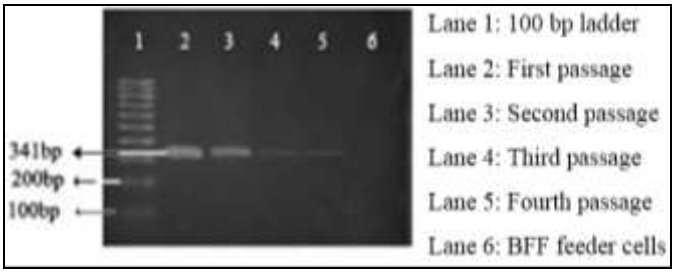
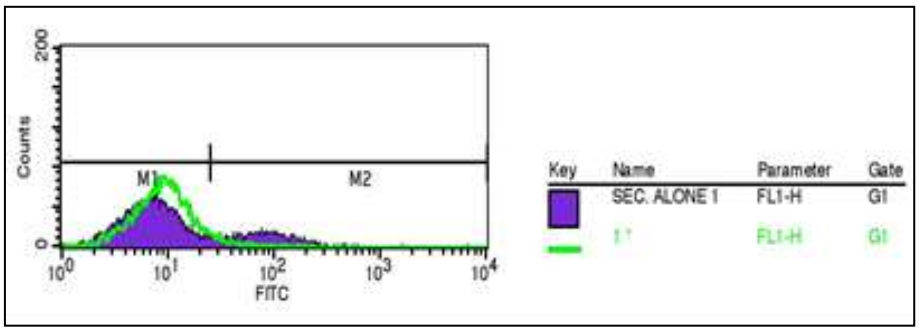


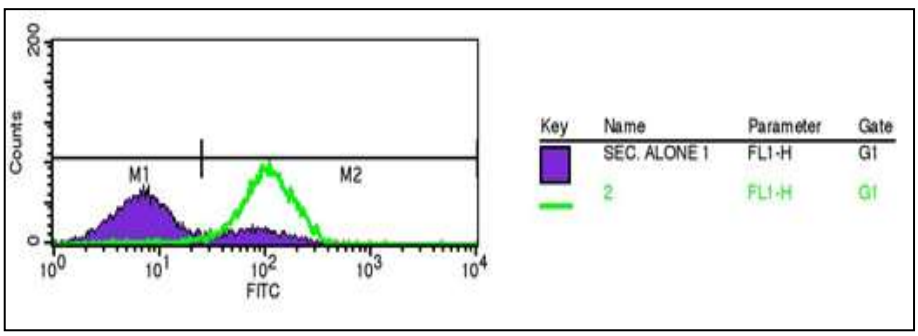
Fig 2: Expression of Oct-4 gene during various passages of ES like cells

Expression of surface marker TRA-1-81

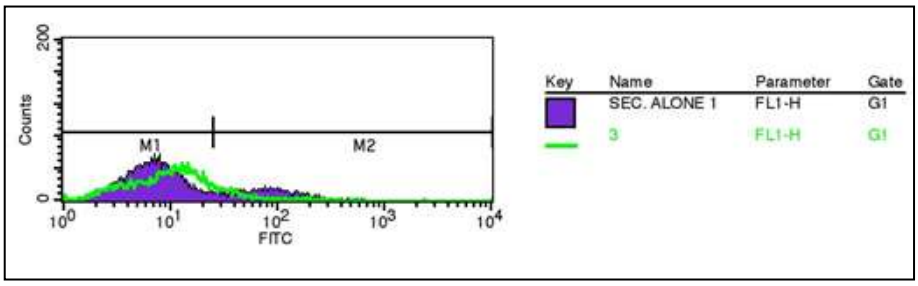
In immunostaining, buffalo ES-like cells strongly reacted with TRA-1-81 antibodies. BFF cells were not positive for TRA-1-81 expression. In flowcytometry, the cells expressing TRA-1-81 in the first passage and second passage were 98.03±0.03 per cent and 97.13±0.07 per cent respectively (Fig.3 a and b). However, in the third and fourth passages the expression levels were reduced to 12.11±0.04 and 4.21±0.09 per cent respectively (Fig.3c and d). Per cent of cell expressing TRA-1-81 declined progressively with each passage clearly indicating that stem cells march towards differentiation. The results were in agreement with previous report [9].The expression of TRA-1-60 and TRA-1-81 has been reported in buffalo ES cell like cells earlier [6, 9]. Flowcytometry has the added advantage of accurately quantifying the percentage of marker expressing cells while the conventional fluorescent staining methods would only qualitatively measure its presence. The results of flowcytometry in the present study were similar to previous report [10].



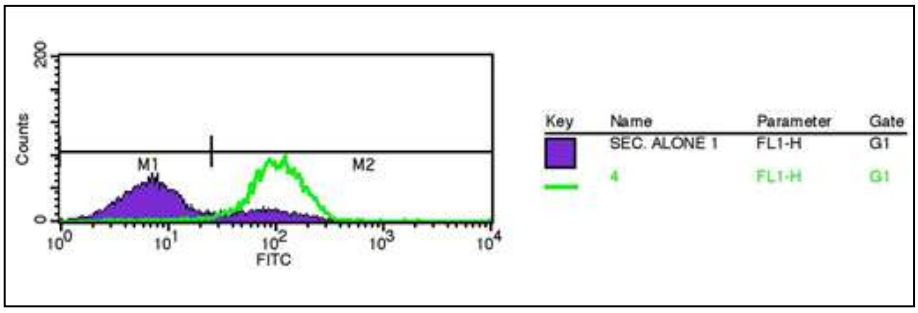
3a: First passage



3b: Second passage



3c: Third passage



3d: Fourth passage

Fig 3: Flowcytometry study: Expression TRA-1-81 in cells of different passages

Conclusion

Buffalo embryonic stem like cells obtained in this study not only showed morphological similarity to authenticated ES cells but also were positive for Oct-4 and TRA-1-81 markers. In addition to the above said properties flow cytometric analysis showed that first passage of the cell colonies were 98.03±0.03 per cent positive for TRA-1-81 surface marker. Thus the study proved that cultured buffalo ES like cells had pluripotency characters and from third passage they started differentiation process.

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