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Collagen-IV supported embryoid bodies formation and differentiation from buffalo (*Bubalus bubalis*) embryonic stem cells

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ABSTRACT

Embryoid bodies (EBs) are used as in vitro model to study early extraembryonic tissue formation and differentiation. In this study, a novel method using three dimensional extracellular matrices for in vitro generation of EBs from buffalo embryonic stem (ES) cells and its differentiation potential by teratoma formation was successfully established. In vitro derived inner cell masses (ICMs) of hatched buffalo blastocyst were cultured on buffalo fetal fibroblast feeder layer for primary cell colony formation. For generation of EBs, pluripotent ES cells were seeded onto four different types of extracellular matrices viz; collagen-IV, laminin, fibronectin and matrigel using undifferentiating ES cell culture medium. After 5 days of culture, ESCs gradually grew into aggregates and formed simple EBs having circular structures. Twenty-six days later, they formed cystic EBs over collagen matrix with higher EBs formation and greater proliferation rate as compared to other extracellular matrices. Studies involving histological observations, fluorescence microscopy and RT-PCR analysis of the in vivo developed teratoma revealed that presence of all the three germ layer derivatives viz. ectoderm (NCAM), mesoderm (Flk-1) and endoderm (AFP). In conclusion, the method described here demonstrates a simple and cost-effective way of generating EBs from buffalo ES cells. Collagen-IV matrix was found cytocompatible as it supported buffalo EBs formation, their subsequent differentiation could prove to be useful as promising candidate for ES cells based therapeutic applications.

1. Introduction

The formation of embryoid bodies (EBs) is the principal step in the differentiation of embryonic stem (ES) cells. ES cells are pluripotent cells derived from blastocyst-stage of mammalian embryos, when maintained in the presence of leukemia inhibitory factor (LIF) or co-cultured with mouse embryonic fibroblasts (MEFs), they retain their pluripotency and are capable of prolonged self-renewal. However, when ES cells get dissociated from colonies and cultured in the absence of LIF or MEF feeders, then they spontaneously aggregates to form spherical EBs [1–2]. This structure facilitates multicellular interactions, which consists of ectodermal, mesodermal, and endodermal tissues leading to cell differentiation during early mammalian embryogenesis [3–4]. Therefore, EB formation has been utilized widely as a trigger of in vitro differentiation of both mouse and human ES cells and has proven valuable tool for genetic studies of tissue differentiation [5].

There are several methods to induce EB formation: a common method to form EBs is in vitro culture of ES cells in suspension without anti-differentiation factors. The three basic culture methods namely, liquid suspension culture in bacterial-grade dishes [6], culture in methylcellulose semisolid media [7] and culture in hanging drops [8] are usually used for the formation of EBs from ES cells. However, drawback of these methods is the lack of support from the extra-cellular matrix because most untransformed mammalian cells require an attachment to an appropriate surface for survival and self renewal. In recent years, several researchers have used different polymer scaffolds for the generation of EBs from ES cells and found that ES cells differentiate into tissue-like structures [9–11]. Chen et al., [12] successfully cultured rhesus monkey ES cells on the surface of 3D collagen matrices and induced ES cell differentiation into various cell types. Zhou et al., [13] demonstrated that collagen/Matrigel scaffolds supported mouse EBs formation and their subsequent differentiation in a single three-dimensional environment. Therefore, synthetic matrices are likely to offer reproducibility, time and cost saving advantages over other feeder systems used for in vitro ES cell generation, propagation and differentiation. Considering that formation of EBs remain important for in vitro differentiation of ES cells, there has been no description for derivation, characterization, besides emphasizing differentiation potential of buffalo embryoid bodies. Therefore, the present study was designed to develop a suitable culture system for derivation, characterization and further evaluation of their differentiation potential of buffalo EBs using three-dimensional microenvironment.

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2. Materials and methods

2.1. Materials

All the chemicals used in this study were purchased from Sigma Chemical Company (St Louis, MO, USA), unless otherwise indicated. Buffalo ovaries from random stages of the estrous cycle were collected from local abattoir immediately after slaughter and transported in 0.9% normal saline at 25–30 °C to the laboratory within two hours.

2.2. Experimental design

2.2.1. Experiment I

To find out the best cytocompatible extracellular matrices, pluripotent ES cells ($\sim 1 \times 10^6$ cells/ml in ES culture medium) were cultured separately onto four different extracellular matrices in terms of growth, numbers and cystic embryoid bodies formation.

2.2.2. Experiment II

Developmental competence (growth, numbers and cystic embryoid bodies formation) were compared between two types of culture system viz. hanging drops vs. extracellular matrices. Three independent experiments were conducted for each culture system.

2.2.3. Experiment III

Based on the results of Experiment I and II, in vivo developed EBs from those method and matrices showing significantly higher developmental competence were used to evaluate the differentiation potential by teratoma formation.

2.3. In vitro embryo production

Buffalo embryos were produced in vitro as per the established protocol of our laboratory Sharma et al., [14]. In brief, cumulus oocytes complexes (COCs) were collected by aspiration of antral follicles. Collected COCs were matured in tissue culture medium-199 (TCM-199) supplemented with 10% fetal bovine serum (FBS), 0.25 mM sodium pyruvate, 0.68 mM l-glutamine, 0.5 μg/ml FSH, 5 μg/ml LH, 1 μg/ml estradiol and 20 ng/ml epidermal growth factor for 24 h under sterile embryo culture tested mineral oil at 38.5 °C in a moist atmosphere of 5% CO_2 in air. Frozen-thawed buffalo bull semen was used for in vitro fertilization of matured oocytes. Sperms were washed twice in fertilization (FERT-TALP) medium containing heparin (10 μg/ml), fatty acids free BSA (6 mg/ml) and sodium pyruvate (0.25 mM) by centrifugation at 850 g for 10 min each. In vitro matured oocytes were washed in TALP medium and incubated with 70 μl droplets of sperm suspension (15–20 oocytes/droplet). After 18 h co-incubation of sperms and eggs, presumptive zygotes were removed from fertilization droplets and washed in embryo development medium (EDM) containing BSA (3 mg/ml), 10% FBS, sodium pyruvate (0.25 mM), l-glutamine (0.68 mM) with essential and non-essential amino acids. Presumptive zygotes were washed and cultured (10–15 zygotes/50 μl droplets) in embryo development medium at 38 °C, 5% CO_2 in air with maximum humidity for their development until blastocyst stage.

2.4. Feeder layer preparation

Pregnant uteri were collected from local abattoir to obtain fetus (approx. 40 days old) and head, bones and abdominal viscera were removed, minced into small pieces and transferred into 75 mm^2 conical flasks and cultured in DMEM supplemented with 10% FBS, 2 mM l-glutamine and 50 μg/ml gentamycin in 5% CO_2 in air at 38.5 °C. Primary fetal fibroblast monolayer were passaged by incubating in 0.25% trypsin–EDTA for 10 min at 37 °C and used as feeder layer for embryonic stem cell culture after third passage. Prior to use, feeder layer was inactivated with mitomycin-C (10 μg/ml) for 3 h.

2.5. Embryonic stem cell culture

Inner cell masses (ICMs) of hatched blastocyst were seeded onto feeder cells derived from mitotically inactivated buffalo fetal fibroblast. ES cells maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum, 2% nonessential amino acid, 2 mM l-glutamine, 1% ITS liquid solution, 0.1 mM β-mercaptoethanol, 40 ng/ml basic fibroblast growth factor (bFGF) and 20 ng/ml leukemia inhibitory factor (LIF) at 38.5 °C and 5% CO_2 in humidified air. The formation of dome shaped structure was examined 8–9 days after the ICMs had been cultured. To further propagate, the primary embryonic stem cell colonies were mechanically dispersed into two to three small clumps by using a micropipette and then transferred to a fresh feeder layer. These cells were passaged mechanically up to the fifteenth passages.

2.6. Alkaline phosphatase staining

Primary embryonic stem cell colonies were subjected to alkaline phosphatase staining on day 5 of culture. In detail, ESCs were freed from culture media and fixed in 4% paraformaldehyde for 10 min, fixed cells were washed with DPBS and incubated in AP staining solution containing of 25 mM Tris–HCl, 150 mM NaCl, 8 mM $MgCl_2$, 0.4 mg/ml Naphthol AS-MX Phosphate and 1 mg/ml FastRed TR salt for 30 min at 37 °C.

2.7. Immunocytochemical analysis for markers of ES cells

For characterization, EBs was randomly collected on days 5, 10, 15 and 26 from all four culture groups and fixed in 4% paraformaldehyde for 10 min at room temperature. The cells were permeabilized with 0.1% Triton-X in PBS for 30 min at 37 °C. Non-specific binding was blocked by incubating the ESCs with donkey serum (1:10 dilution in PBS with 2.5% BSA) for 30 min at 37 °C. EBs were then incubated with primary antibodies at 37 °C for 2 h. Primary antibodies included SSEA-1 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), SSEA-4 (1:100), TRA-1–60 (1:100) and TRA-1–81 (1:100). Normal goat serum was used as a negative control. Localization of antigens was done with anti-goat IgG secondary antibodies conjugated with FITC or texas red (1: 500, Santa Cruz). Immunolocalization of SSEA-1, SSEA-4, TRA-1–60 and TRA-1–81 proteins in EBs were viewed under fluorescent microscope (IX 71, Olympus, Japan).

2.8. Extracellular matrices

The matrices of collagen type-IV (extracted from human placenta), laminin (murine sarcoma basement membrane), fibronectin (from bovine plasma) and matrigel (murine sarcoma basement membrane) were used for ES cell culture. In brief, matrix of 1% collagen gel solution (1.0 mg/ml in 0.1 M acetic acid), laminin (2 μg/ml), fibronectin (10 μg/ml) and matrigel (10 μg/ml) were prepared by mixing (v/v) with 2X DMEM (20% FBS, 200 U/mL penicillin, and 200 μg/mL streptomycin). Each of them (200 μl) were pipetted separately into 24 well culture plate (Nunc) and incubated for 20 to 30 min at 37 °C and 5% CO_2 to allow hardening of the mixture. Thereafter, 2.0 ml undifferentiating ES cell culture medium (DMEM, supplemented with 20% FBS, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol, 40 ng/ml bFGF, 20 ng/ml

LIF) was poured into each culture well separately, it was incubated at 38.5 °C, 5% CO_2 with maximum humidity.

2.9. EBs formation under extracellular matrices

To induce EBs formation from ES cells, the undifferentiated primary ES cells were mechanically removed from the feeder layer, these pluripotent ES cells (~1 × 10^6 cells/ml in ES culture medium) were cultured separately onto four different extracellular matrices (Fig. 1A). Finally, 1 ml of ES cell culture medium was added to each well and culture medium was changed every second day.

2.10. EBs formation induced by hanging drop (HD) method

Hanging drops (one droplet, 50 µl contains ~1000 cells) were placed on the lid of a 100 mm bacterial-grade dish filled with phosphate-buffered saline (PBS), and incubated statically for 7 days (Fig. 1B). Standard method of EBs formation was performed as per established protocol [15].

2.11. Morphological evaluation of embryoid bodies

The morphology of the EBs formed using different types of matrices and hanging drops was analyzed using an inverted light microscope (CKX-41, Olympus Inc, Japan). For size analysis, average diameter was calculated in duplicate using Image J 1.33U software (National Institutes of Health, Bethesda, Maryland), based on calibrated ocular micrometer. For number analysis, the average number was calculated by measuring the total EBs per field in 10 fields as described by Koike et al., [16]. Measurement results from three independent experiments are pooled and presented. Cystic embryoid bodies were characterized by appearance of visceral yolk-sac-like structure with clear boundary.

2.12. Teratoma formation

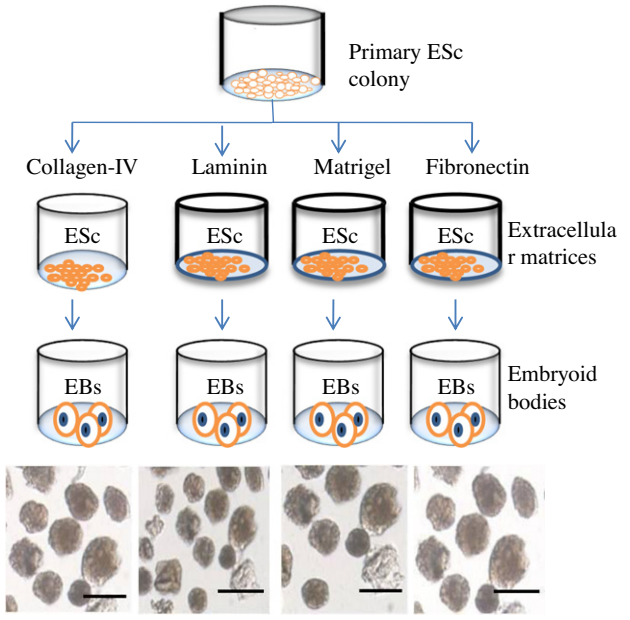
To verify the differentiation potential of EBs in vivo, group of EBs (day 20) were dissociated mechanically and suspended in ES cell culture medium. The EBs suspension (~2 × 10^6 cells) was inoculated subcutaneously in hind leg of immuno-compromised mice for teratoma formation, control group animals were injected with PBS having no cells. Total 6 immuno-compromised mice were selected for 3 trials. After 8 weeks of post inoculation, in vivo developed teratomas were excised surgically, fixed in 4% paraformaldehyde, embedded in paraffin and examined histologically using hematoxylin and eosin staining for germ layer derivatives. Teratoma formation assay was carried out as per our laboratory established protocol [17].

2.13. Histological and immunofluorescence analysis

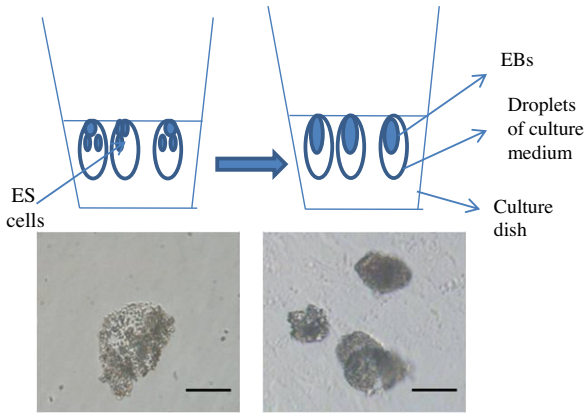
For histological characterization, paraffin embedded sections (5 µm) were stained with hematoxylin and eosin (H & E). For immunofluorescence detection, sections of teratoma were briefly rinsed in PBS, fixed with 3.7% formaldehyde in PBS for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. The fixed sections were pre-incubated with 2.5% bovine serum albumin (BSA) in PBS for 1 h, incubated with primary antibodies {(NCAM) ectoderm, (Flk-1) mesoderm and (AFP) endoderm} for 1 h at 37 °C in moist chamber, rinsed three times with PBS and then further incubated for 1 h with an secondary antibodies (1:500) tagged with Texas red or FITC. After rinsing, coverslips were mounted with neutral gum. All primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted (1:100) in Tris-buffered saline supplemented with 2.5% BSA. Negative controls were processed without the primary antibody. The localization was viewed under fluorescent microscope (IX 71, Olympus, Japan).

2.14. RT-PCR assay

To verify the differentiation potential of EBs in vitro, expression analysis of molecular markers like neural cell adhesion molecule (NCAM), fetal liver kinase-1 (FLK-1) and alpha fetoprotein (AFP) for ectoderm, mesoderm and endoderm, respectively were carried out. Total RNA from EBs was purified with RNeasy Mini Kit (Qiagen, Valencia, CA, USA) as per the manufacturer's instructions.



(A)



(B)

Fig. 1. Schematic representation of EBs formation using four different types of extracellular matrices (A) and hanging drop (B). In vitro derived buffalo ES cells were cultured in four different types of matrices viz. collagen type-IV, laminin, matrigel and fibronectin. ESc = embryonic stem cells, EBs = embryoid bodies. Scale bar = 50 µm.

Approximately 1 μg of total RNA was reverse transcribed using single strand cDNA synthesis kit (Fermentas, USA) as per the manufacturer's instructions. For amplification of NCAM, FLK-1 and AFP gene, StemTAG™ PCR Primer Set for stem cell characterization (Cell Biolabs, Inc., CA, USA) were used. Amplified products were confirmed by the size of respective amplicons and data was normalized to the reference gene (β-actin).

2.15. Statistical analysis

The effects of initial cell concentration on the size and number of EBs formation in different types of matrices were analyzed by one way analysis of variance; a probability level of $P < 0.05$ was considered significant.

3. Results

3.1. Formation of embryoid body in extracellular matrices

As illustrated in Fig. 1, the ES cells were transferred onto two types of culture system, which are suspension culture and attachment culture, to induce the EBs and visceral yolk-sac-like structure in the EBs. ES cells upon seeding over different matrices, gradually grew into aggregates and formed simple EBs showing circular structures at day 5 in all culture groups (Fig. 3A (a)). After 10 days culture, size and the number of EBs increased in both types of culture system however, significant increase in EBs size and number observed under collagen type-IV matrices (Table 1; Fig. (3A) b,c). EBs retained their original spherical structures with almost no outgrowths around even after culturing for 26 days over this matrix (Fig. (3A) d,e). While, cells from EBs were able to migrate outward and develop outgrowths when they are cultured over other scaffolds and in suspension forms. Out of 146 embryos (65.18%) 32 embryos reached to blastocyst stage (21.91%) during in vitro culture (Table 2 and Fig. 2).

3.2. Cavitation of embryoid body in extracellular matrices

Since the cavitation of EBs is a crucial step in the process of ES cell differentiation, the effects of scaffolds composition on EBs cavitation was tested. It was observed that under collagen type-IV and hanging drops, EBs developed a normal central cavity (Fig. (3A) f)

Table 1
Number and size of EBs generated in different extracellular matrices at day 0 and day 26.

Matrices/Hanging drops	The size of EBs (μm)		The number of EBs	
	Day 0	Day 26	Day 0	Day 26
Collagen type-IV	56.12 ± 2.34	342.45 ± 3.45[b]	68.24 ± 1.23	96.56 ± 1.56[b]
Laminin	52.31 ± 2.12	229.34 ± 3.32[a]	64.12 ± 1.28	79.45 ± 1.74[a]
Matrigel	55.56 ± 1.74	296.75 ± 4.65[a]	62.45 ± 1.18	86.28 ± 1.86[a]
Fibronectin	52.78 ± 1.26	211.24 ± 2.78[a]	64.26 ± 1.12	77.22 ± 1.72[a]
Hanging drops	53.48 ± 2.34	239.84 ± 3.21[a]	66.28 ± 1.74	81.45 ± 1.68[a]

Mean values having different superscripts in column differed significantly ($P < 0.05$).

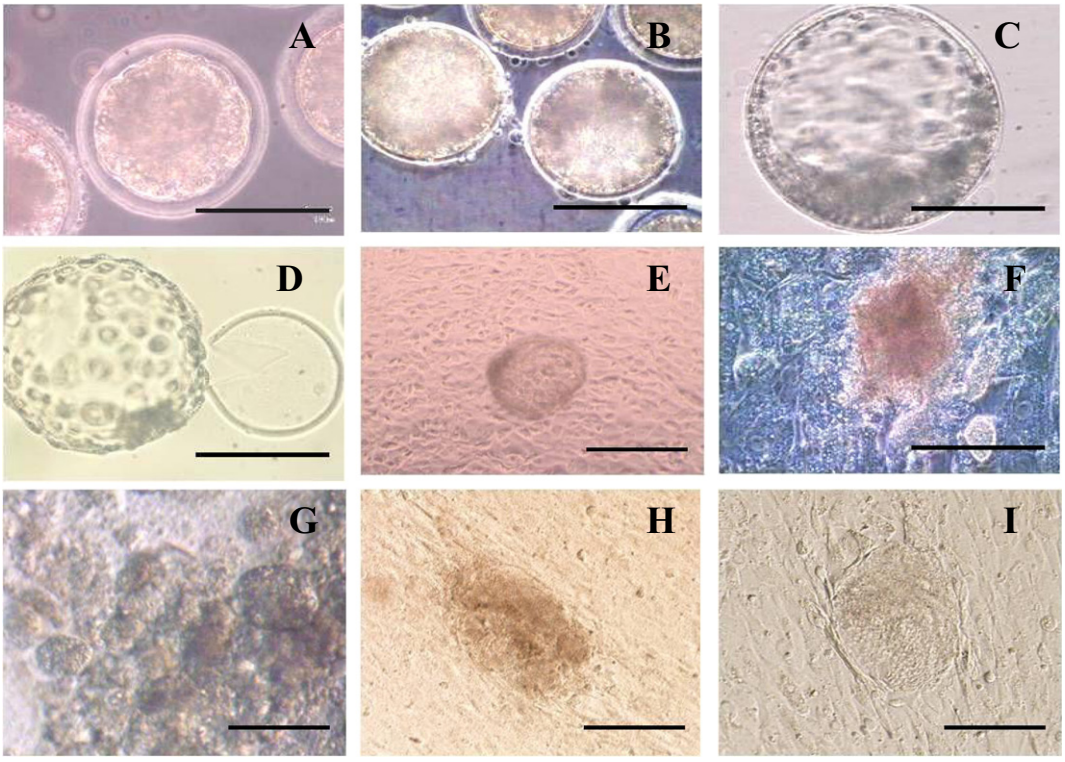


Fig. 2. Derivation of embryonic stem cell colonies from in vitro produced buffalo blastocyst. Different developmental stages of buffalo embryos produced in vitro (A) compact morula, (B) blastocyst, (C) expanded blastocyst, (D) hatching blastocyst showing clear inner cell masses (ICMs) and trphoectodermal cells (TE), (E) ICMs over fetal fibroblast monolayer (day 0), (F) ES cells primary colonies (day 7), (G) 5th passages, (H) 10th passages and (I) 15th passages ES cells colonies over fibroblast monolayer. Scale bar = 100 μm (A–D) and = 80 μm (E–I).

Table 2
Cleavage and blastocyst percentage in buffalo embryos produced in vitro.

No. of cleaved embryos	No. of blastocysts formed	Cleavage% (Mean ± SEM)	Blastocyst% (Mean ± SEM)
146	32	65.18 ± 1.42	21.91 ± 1.53

whereas, in other scaffolds no cavity could be observed even after 26 days of culture.

3.3. Alkaline phosphatase and immunocytochemical analysis for markers of ES cells

Expression of pluripotency related marker genes like SSEA-1 (Fig. 3B (c and d)), SSEA-4 (Fig. 3B (e and f)), TRA-1–60 (Fig. 3B (g and h)) and TRA-1–81 (Fig. 3B (i and j)) was positively localized in EBs of 5 and 10 days of culture. The day 5 EBs was also found positive for alkaline phosphatase activity (Fig. 3B (a and b)). Localization of these markers was cytoplasmic and distributed homogeneously all over the ES cell like-cells. The staining pattern of these markers genes did not differ in different culture groups.

3.4. Identification of three germ lineage during the differentiation of embryoid bodies

The histochemical and immunofluroscence staining pattern of in vivo developed teratoma demonstrated the presence of all germinal layers (ectoderm, mesoderm and endoderm) in teratoma (Fig. 4) respectively. RT-PCR analysis showed amplicons of 140 bp for NCAM (ectoderm marker), 175 bp for Flk-1 (mesoderm marker) and 136 bp AFP (endoderm marker) in developed teratoma (Fig. 4). The reference gene β-Actin (327 bp) as an endogenous control was expressed in all samples.

4. Discussion

In this study, a novel method for generating EBs from buffalo ES cells using three dimensional collagen gel culture system was successfully established. The research on ES cells differentiation is important as it provides an efficient in vitro system to screen for effects of small molecules and bioagents, it is also an alternative to embryo and live animal studies. The differentiation of ES cells into various lineages is an area of intense study because of the direct applicability of ES cells to the field of regenerative medicine [18–20]. Several recent reports on the ES cells differentiation and development highlighted that EBs play an important role in the differentiation of ES cells to get desired cell lineages however, the induction efficiency and quality of EBs formed from ES cells affected by composition of culture medium and culture conditions. The traditional and simple method of EBs formation from ES cells is by removal of LIF and the feeder cell layer followed by culture in hanging drop and in bacterial grade petri dishes [2,6]. In this study, we compared four different types of extracellular matrices

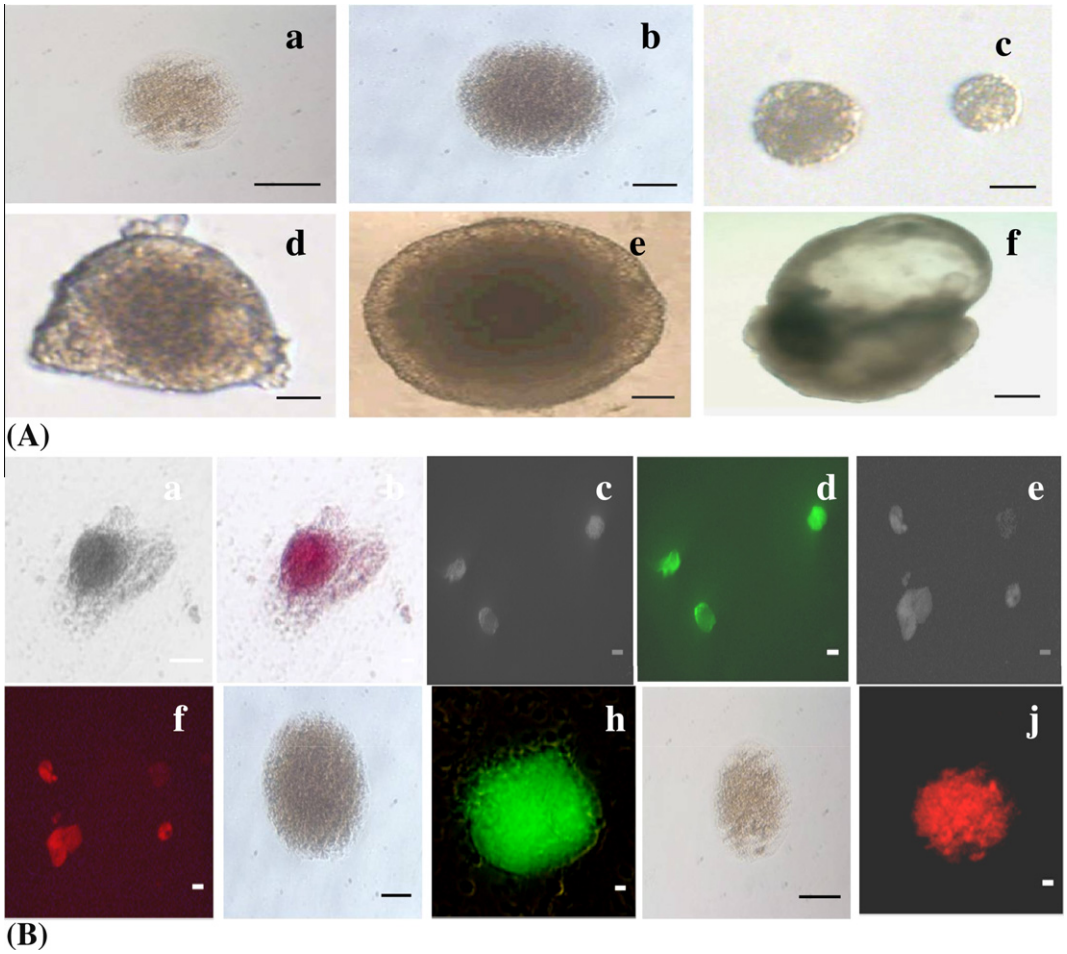


Fig. 3. Derivation, cystic embryoid bodies formation (A) and immunolocalization of pluripotency surface marker genes (B). EBs formation in collagen type-IV matrix during different day of culture (a–e) and cystic embryoid bodies formation in collagen matrix at day 26 (f). Expression of SSEA-1 (d, c), SSEA-4 (f, e), TRA1-61 (h, g), TRA1-81 (j, i) and alkaline phosphatase staining (b, a) in In vitro derived buffalo embryoid bodies. For localization, embryoid bodies were fixed and subjected to antigen retrieval prior to incubation with non-immune normal goat serum and were further incubated in embryonic stem cell related specific primary antibodies and stained by FITC or Texas red conjugated secondary antibodies.

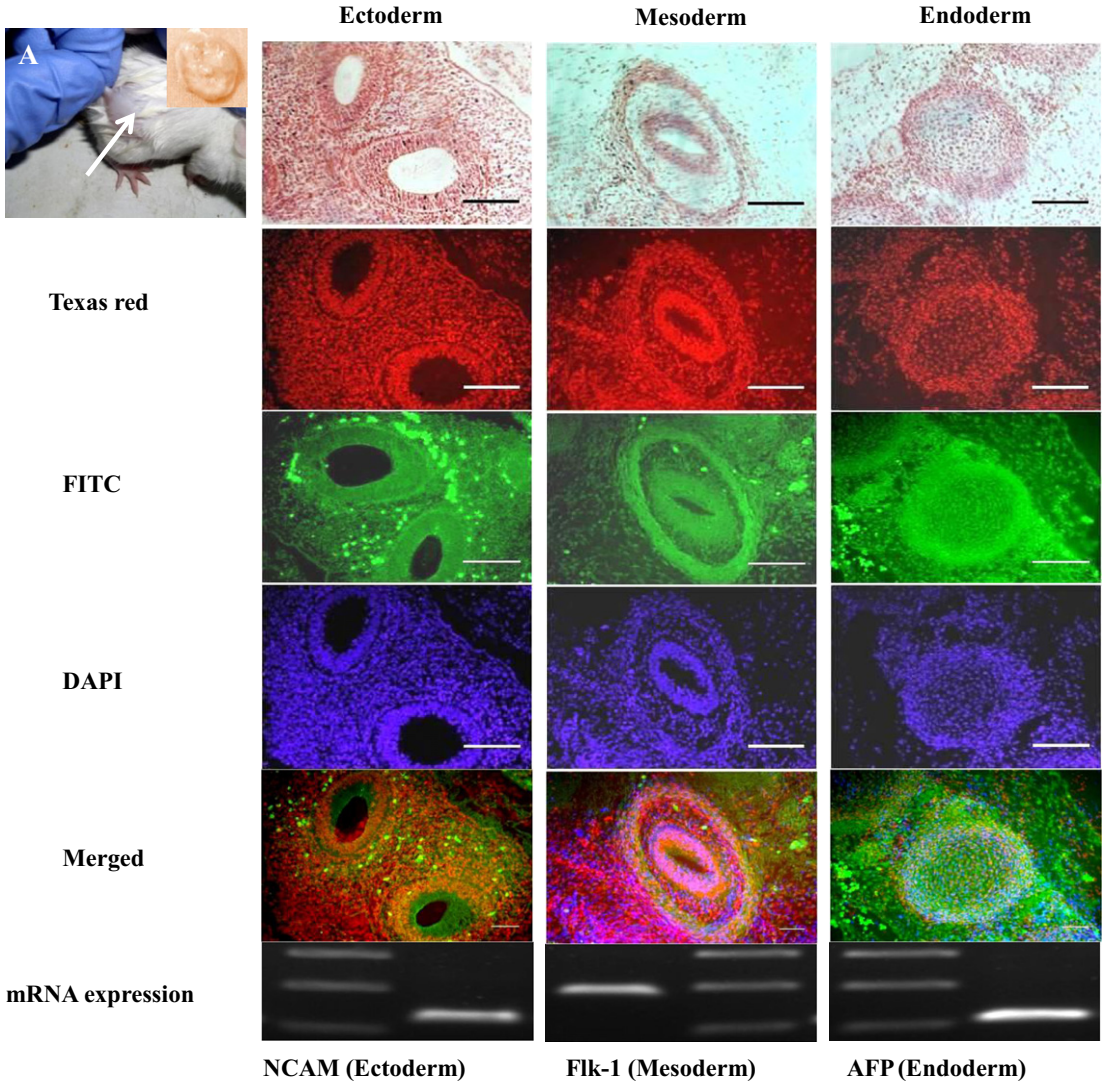


Fig. 4. Derivation, characterization and expression of germinal layers marker genes of in vivo developed teratoma. Histological sections (H & E stain) and immunofluroscence of teratoma showing three different germinal layers derived from buffalo ES cells transplanted into the immune deficient mice for 8 weeks (A). Retinal like pigmented and ectodermal epithelia like structures (ectoderm derived tissues), bone and connective tissue like structures (mesoderm derived tissue), tubular and gland like structures (endodermal derived tissues). mRNA expression studies showing the presence of AFP (endoderm marker, 175 bp); NCAM (ectoderm marker, 140 bp) and FLK (mesoderm marker, 136 bp) transcript. 100 bp DNA ladder. Scale bar = 50 μm.

and hanging drop method for derivation of buffalo EBs from ES cells and their further differentiation. Our results revealed that collagen type-IV matrices supported the EBs formation and their subsequent differentiation as compared to other matrices and hanging drop. Over collagen type-IV and collagen type IV/matrigel matrices, EBs developed a normal central cavity whereas, over other matrices no cavity could be found even after 26 days of culture, indicating that collagen could provide a better physical support for EBs formation, while the presence of matrigel facilitates the differentiation of ES cells. This pattern of differentiation is accomplished in a three dimensional extra-cellular matrix composed of collagen and matrigel mimic in vivo environment.

Formation of differentiated cells representing the three embryonic germ layers could be initiated from ES cells by the formation of progenitor-like cell populations in the EBs. Moreover, prolonged culture of EBs may produce more differentiated cell types, including neural cells [21], cardiomyocytes [22], hematopoietic cells [23] and smooth muscle cells [24]. To the best of our knowledge this study has demonstrated for the first time the formation of simple and cystic buffalo EBs comprising three germ layers in concert with expression of their lineage markers. Thus, buffalo EBs could successfully generate three germ layers and possessed the potential for differentiating into a variety of cell types in vitro. In addition, expression of pluripotent stem cell markers such as SSEA-1 and 4, TRA1-61 and 1–81 explains that early EBs group do consist some undifferentiated ES cells. The mRNA expression of tissue-specific genes in EBs during in vitro differentiation indicated that the early processes of in vivo development into ectoderm, mesoderm and endoderm lineages were recapitulated in vitro.

In the present study, paraffin sectioning of excised teratoma was performed and subjected to hematoxylin and eosin (H & E) staining to observe histological structure of teratoma. Immunofluroscence results showed retinal-like pigmented epithelium and ganglia like structures (ectoderm-derived tissues), muscle and connective tissue like structures (mesoderm-derived tissues) tubular and gland like structures (Endoderm-derived tissues) in developed teratoma were further strengthen our results. Brivanlou et al., [25] reported that paraffin sections of human ES cell-derived teratomas

were highly informative although variable degrees of differentiation are often present, many tissue types as well as three-dimensional organ architecture may be noted.

In summary, this study has successfully established an optimized in vitro culture system for the generation of buffalo EBs and further differentiation into three germ layers. The collagen/Matrigel matrices provide a better 3-D micro-environment for ES cells development and differentiation compared to the other matrices used. Temporal expression pattern of germ lineage markers during in vitro differentiation of ES cells into EBs, provide novel insights for the buffalo EBs which possess germ cell state. Further therapeutic applications of ES cells, it is necessary to produce large number of EBs for the specific purpose to enhance the understanding and ability to differentiate desired cell types from ES cells.

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