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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