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Generation of buffalo embryonic stem cell clones on matrigel and fibronectin synthetic matrices and their characterization

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ABSTRACT

The present experiment was carried out to generate embryonic stem cell clones on matrigel and fibronectin synthetic matrices and their characterization in buffalo. *In vitro* derived buffalo embryos were made zona free and were cultured on matrigel and fibronectin synthetic matrices with 4 culture media, viz. control media: media 1: CM + SCF; media 2: M-I + bFGF; and media 3: M-II + IGF -1 in CO_2 incubator. A total of 16 numbers of zona free embryos were cultured in each culture media on both the synthetic matrices and the efficiency of clumped blastomere attachment, stem cell clones formation and their propagation was observed. The primary stem cell clone formation in culture media 1 and 3 both were found to be significantly higher than media–2 and control. The embryos attachment percentages on both the matrices were also better in M-1and M-3 as compare to media-2 and control.The results showed that embryonic stem cell proliferation was better in both SCF supplemented media and combination with IGF for lineage maintenance. It was also observed that the stem cell colony formation was better on matrigel than fibronectin matrices which may be due to the fact that the pluripotent gene expression was better in matrigel based culture than the fibronectin synthetics. The clones formed in all the medium expressed pluripotent markers vide Oct4, Nanog, Klf4 and Foxd3 which implies that the stem cell clones were stem cell like cells. It can be concluded that buffalo ES cell can be established on synthetic matrices.

Key words: ES cells, Matrigel, Fibronectin, Early stage embryos, Buffalo

Traditionally, stem cells are cultured on the embryonic fibroblast cells inactivated by mitomycin-c. The effects of some factors such as calf serum, feeder cell, culture medium and additives on the efficacy of isolation and culture of bovine embryonic stem cells were studied using bovine and murine feeder (Li Long *et al*. 2002). However, many efforts have recently been made to culture stem cells on feeder free condition. Embryonic stem cells can be well maintained without feeder layer on gel or extra cellular matrix substrate in conditioned media or in LIF supplemented media (Wiles 1993). There are also reports of propagating ES cells on extracellular matrix for survival and growth, instead of feeder layer (Zhang *et al.* 2009). Therefore, the present study was aimed at generation of stem cell colony using synthetic matrices and characterizing them with stem cell specific markers.

MATERIALS AND METHODS

In vitro *embryo generation:* Buffalo ovaries were collected from local slaughterhouse and oocytes were aspirated from all the visible non atretic surface follicles in oocytes collection medium (modified phosphate buffered saline supplemented with estrus buffalo serum and BSA (fraction V) 3 mg/ml) and searching of oocytes were carried out under stereo zoom microscope. The cumulus oocyte complexes (COCs) were evaluated and graded by the evenly granulated cytoplasm and expansion of cumulus cells layers. The excellent (>5 layers) and good (>3 layers) quality of cumulus oocyte complexes were taken in 50µl droplets of maturation media (TCM-199 supplemented with 10% fetal bovine serum, BSA (Fraction-V) 3 mg/ml, 5% estrus buffalo follicular fluid,10 ng/ml of epidermal growth factor,50µg/ml FSH and 10 IU/ml hCG) and kept in CO_2 incubator with an atmosphere of 5% CO_2, 21% O_2, 37°C and 95% relative humidity (RH) for 24 h. After maturation, the oocytes were washed several times with sperm TALP and finally with fertilization TALP media and transferred to fertilization TALP media (Totey *et al.* 1996). To this, 20 µl of capacitated spermatozoa (50–100 × 10^3) were added and finally incubated in CO_2 incubator at same atmospheric condition. After 18–20 h of sperm-oocyte co-incubation, the presumptive zygotes were washed in modified synthetic oviductal fluid and cultured in the 100 µl droplet of same media and embryo developments were

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assessed by observing the cleavage rate after 48–72 h of post insemination.

Preparation of matrigel and fibronectin coated culture plate: The feeder free coated plates were made in the laboratory. For Preparation of matrigel and fibronectin coated culture plate, the wells of the tissue culture plates were filled with 200 µl of 0.1 mg/ml geltrex in knockout-Dulbecco's modified Eagle's medium (DMEM) for matrigel and 200 µl of fibronectin (5µg/ml) in PBS and both the plates were kept at 4°C for overnight. The plates were allowed to warm to room temperature in the BOD incubator for approximate 30 min prior to use.

Culture of clumped blastomeres on matrigel and fibronectin coated plates: For derivation of embryonic stem cells, embryos were treated to proteinase-K (0.02%) drops for dissolution of the zona pellucida. After dissolution of zona pellucida, the clumped blastomeres were cultured on matrigel and fibronectin coated plates in each culture media and both the plates were kept at 37°C, 5% CO_2 and 95% relative humidity in CO_2 incubator. For the development of embryonic stem cell clone following media were used; Control media: Knockout DMEM supplemented with 10% FBS, LIF (40ng/ml), ITS (0.3%), NEAA (0.5%); media 1: CM + stem cell factor (SCF) 20 ng/ml; media 2: MI + basic fibroblast growth factor (bFGF) 4ng/ml; and media 3: MII + insulin like growth factor (IGF -1) 100ng/ml.

Comparative gene expression analysis of embryonic stem cell clone: Comparative gene expression of *Oct-4, Nanog, FoxD3* and *Klf4* of the embryonic stem cell clone (ESC) were studied using real time PCR. The cDNA was synthesized from total RNA isolated from ES cell clone using reverse transcriptase kit. The relative expression of gene based on Ct values was calculated as outlined by Ingle *et al.* (2010) with suitable modifications (wherever calibrator was not available, Δct were used to denote the gene expression).

Details of primers used for quantitative reverse transcription–polymerase chain reaction

Primer	Primer sequence (5_–3_) forward and reverse	Annealing temp. (°C)	Product size (bp)
β-actin (reference gene)	F 5'-TCC AAG GCG ACG TAG CAG AG-3' R 5'-CGC ACC ACT GGC ATT GTC AT-3'	60	227
Oct-4	F 5'-GAA AGA CGT CCG AGT GTG GT-3' R 5'-TCC CCC TGT GAA AGG AGA C-3'	60	125
Nanog	F 5'-GTC CCG GTC AAG AAA CAA AA -3' R 5'-TGC ATT TGC TGG AGA CTG AG- 3'	55	107
Klf4	F 5'-GGA AGT TTG CCC GCT CAG AT-3' R 5'-GCC TCT TCA TGT GTA AGG CG-3'	60	129
FoxD3	F 5'-GCT GAC TCT GAG CGG CAT-3' R 5'-TCC TCG GAC TGA GGG TCC A-3'	60	200

Statistical analysis: All the experimental data including relative expression of pluripotent genes were analyzed by Statistical Package for Social Sciences (SPSS-16.0) using one way ANOVA.

RESULTS AND DISCUSSION

The results of the embryonic stem cells culture in feeder free culture condition on matrigel and fibronectin in each culture media have been presented in Tables 1 and 2. The overall average percentage of embryos developed to primary stem cell clones and their attachment on matrigel and fibronectin coated plates were found to be significantly ($P<0.01$) higher in media 1 and media 3 as compared to media 2 and control. There was no significant difference between media 2 and control (Tables 1, 2). The developmental efficacy of embryonic stem cell clone on matrigel and fibronectin matrices did not differ significantly, but matrigel was found better than fibronectin matrices. The overall primary clone formation was similar in both the matrices. Developed stem cell clones on both the synthetic matrices were passaged mechanically up to three passages (Tables 1, 2). The stem cell colony developed in each medium on matrigel and fibronectin expressed pluripotent genes like *Oct4, Nanog, Klf4* and *Foxd3*. The relative expression of these genes was comparatively higher in media 1 and 3 as compared to control and media 2 in matrigel coated plates while in fibronectin coated plates, the expression of these genes was quite low in all the culture medium.

The relative pluripotent gene expression of ES cells was better in matrigel based culture than the fibronectin culture may lead to better propagation of embryonic stem cells in matrigel rather than fibronectin culture. Growth factors support the growth and proliferation of embryonic stem cells over long term cultures and multiple passages, without compromising their stemness. For growth and proliferation of undifferentiated embryonic stem cells for a long term culture, several growth factors, viz. bFGF, IGF-1, epidermal growth factor (EGF) and SCF are needed (Shim and Anderson 1998). Supplementation of SCF growth factor in culture media may serve as guidance cues that direct to their stem cell niche (the microenvironment in which a stem cell resides) and it plays an important role in stem cell maintenance and further propagation of cells (Broudy 1990). Buffalo embryonic stem cells expresses c-kit as it is well known that the role of SCF for lineage maintenance and cell renewal depends on the cells that express c-kit (Arman *et al.* 1998), so that, stem cell factor might have more influences on stem cell formation on synthetic matrices as compare to other growth factors. However, bFGF and IGF-1 were also found to promote cell mitogenesis and proliferation in early embryonic developmental stages of embryo and maintained undifferentiated state of bovine ES-like cells. Among the different media tested on feeder free conditions, media 1 and media-3 supported and development of maximum number of stem cell clones and their further propagation followed by media-2 and control media. In media 1, the development of stem cell clones was slightly higher than media-3, but the

Table 1. Culture of embryonic stem cell in feeder free culture condition on matrigel in different culture medium

Media used	No. of embryo cultured	No. of embryo attached (% attachment)	Time taken for attachment (h)	No. of primary clone formed (% of P0 clone formed)	P-1	P-2	P-3
Control media	16	4 (25)	72–96	08 (50.0)	√	√	x
Media 1	16	6 (37.5)	48–72	13* (81.25)	√	√	√
Media 2	16	5 (31.25)	72 –96	10 (62.50)	√	√	x
Media 3	16	6 (37.5)	48–72	11* (68.75)	√	√	√

Control media, CM; media 1: CM +SCF; media 2: MI + βFGF; and media 3: MII + IGF-1; P0: primary ES clone, P1, first passage, P_2; second passage; P_3, third passage; * level of significance (P<0.01).

Table 2.Culture of embryonic stem cell in feeder free culture condition on fibronectin in different culture medium

Media used	No. of embryo cultured	No. of embryo attached (% attachment)	Time taken for attachment	No. of primary clone formed (% of P0 clone formed)	P-1	P-2	P-3
Control Media	16	3 (18.75)	72–96	6 (37.50)	√	√	x
Media 1	16	5 (31.25)	72	12* (75.0)	√	√	√
Media 2	16	4 (25)	72–96	9 (56.25)	√	√	√
Media 3	16	5 (31.25)	72–96	11* (68.75)	√	√	√

Control media, CM; Media 1: CM +SCF; media 2: MI + βFGF; and media 3: MII + IGF-1; P0: primary; P1, first passage; P_2, second passage; P_3, third passage; * level of significance (P<0.01).

difference was non-significant. The mean percentage of embryos developed to stem cell clones did not differ significantly between the coated plates, but matrigel coated plates was found to be slightly better than fibronectin coated plate. When embryonic stem cells are grown in the absence of feeder cells, extracellular matrix components are necessary to maintain the pluripotent, undifferentiated state. Xu *et al.* (2001) first describe feeder cell free hESC culture medium conditioned with matrigel. Hakala *et al.* (2009) reported that the undefined, xenogeneic matrigel to be a superior matrix for hESC culture compared with the purified human ECM proteins, serum matrices, and the biomaterials tested. The embryonic stem cells remained undifferentiated after culture on the fibronectin-coated surfaces, similar to standard culture techniques (Loh *et al.* 2009). Although, extracellular matrix and biomaterials are used successfully for development of stem cells, but their success is not comparable to the fibroblast monolayer.

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