J. Aqua., **18** (2010): 9-21

GROWTH AND PROPAGATION OF DIFFERENTIATED CELLS DERIVED FROM EMBRYONIC STEM (ES) LIKE CELLS OF *CIRRHINUS MRIGALA* (HAM.)

S. Tripathy and P. Routray

Central Institute of Freshwater Aquaculture
Kausalyaganga, Bhubaneswar -751002, Odisha, India
**Corresponding author: routray30@yahoo.co.in*

In this study, we describe the growth and propagation of differentiated cells *viz*. keratinocyte-like cells (ectodermal origin), muscle-like cells (mesodermal origin) and hepatocyte-like cells (endodermal origin) generated from embryoid bodies (EBs) in their respective formulated medium. These EBs were developed by culturing mrigal ES-like cells in suspension. When these EBs were attached to a permissive surface and upon treatment with appropriate inducer like retinoic acid (RA) they continued to differentiate into different cell lineages. Here, we formulated three specific growth and propagation media for these derived differentiated cells. The mediums were supplemented with key factors required for their growth and propagation. Keratinocyte growth and propagation medium (KGPM) was supplemented with 5 ng/ml keratinocytes growth factor (KGF), 5 ng/ml epidermal growth factor (EGF), 5 µg/ml insulin and optimum calcium concentration (0.05 mM) for keratinocyte-like cells. Similarly, 10 ng/mL EGF, 0.5 mg/mL BSA and 100 ng/mL insulin-like growth factor -1 (IGF-1) were supplemented in muscle cells growth and propagation medium (MGPM) for muscle-like cells and 10 ng/ml EGF along with 2% dimethyl sulfoxide (DMSO) was supplemented in hepatocyte growth and propagation medium (HGPM) for hepatocyte-like cells.

INTRODUCTION

Embryonic stem (ES) cells are derived from the early stage of developing embryos, and these cells represent an excellent *in vitro* model system to study the molecular mechanism associated with pluripotency and self-renewal activity. The remarkable plasticity of ES cells to transform into various somatic and germinal cells reflects the dynamic events occurring *in vivo* (Wobus *et al*., 1984; Routray *et al.,* 2009). The most reliable method for generating differentiated cells from ES cells is by induction of embryoid bodies (EBs). EBs are three dimensional multicellular aggregates having spherical structure developed by culturing ES cells in suspension (Höpfl *et al*., 2004). Many features of EBs resembles with that of developing embryo (Keller, 1996). These EBs upon attachment to permissive surface like gelatin, collagen and treatment with appropriate inducer continue a programmed differentiation into ectodermal, mesodermal and endodermal lineges.

ES cell technology has been generated from fish, birds and mammals (Familari and Selwood, 2006). Propagation of derived differentiated cell types from ES cells is well studied in mammalian system (Vallier *et al.,* 2009). *In vitro* differentiation potential of ES cells has been investigated in many fish ES cells *viz.* from zebra fish (Sun *et al*., 1995), medaka (Hong *et al.,* 1996), red seabream (Chen *et al*., 2003a), sea perch (Chen *et al*., 2003b), turbot (Holen and Hamre, 2003), Asian seabass (Parameswaran *et al*., 2007), rohu (Dash *et al*., 2008) and catla (Dash *et al*., 2010). But no report is available regarding growth and propagation of these derived differntiated cell types from fish ES cells from Indian major carp, *Cirrhinus mrigala* (Ham.) (commonly known as mrigal). This study infers about the growth and propagation of keratinocyte-like cells (ectodermal origin), muscle-like cells (mesodermal origin) and hepatocyte-like cells (endodermal origin) differentiated from EBs developed from ES cells of this species.

MATERIALS AND METHODS

Broodstock management and induced breeding of mrigal

Broodfish of *C. mrigala* were reared in earthen ponds (0·2 ha) of the farm facility of the Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, Odisha, India. For induced breeding, two to four males and females (1:1) were injected intra-peritoneally with ovaprim (Salmon GnRH + Domperidone, Syndel Laboratories, Canada) at a rate of 0.25 ml and 0·5 ml/kg body mass to males and females respectively. After 5 to 6 h following hormone administration, male and females were stripped for milt and eggs. Fertilization was done by mixing semen and ova over an enamel tray with addition of freshwater to it. Embryos were collected in sterilized petridishes.

Collection of embryos and *in vitro* culture of mrigal ES like cells

The collected embryos were incubated in water at room temperature (28 °C). After 1.5-2 h of incubation, approximately 200-300 embryos of early blastula stage (64-cell stage) were taken for *in vitro* culture. Embryos were sterilized with 70% ethanol on a cell strainer. The blastomeres were isolated by rupturing chorion with wide mouth pipette. Blastomeres were seeded at a rate of 0.1×10^5 cells/ml on gelatin coated 24-well plates. We followed feeder free procedure (Hong and Schartl, 1996) for culturing mrigal-ES like cells along with complete growth medium. The complete growth medium composed of Leibovitz-15, Dulbecco's modified Eagle's medium with 4·5 g/l glucose and Ham's F12 (LDF) in 50: 35:15 ratio, 10% FBS, additional components supplemented were 15 mM HEPES [4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid], 8 nM sodium selenite, 100 µM ß-mercaptoetanol, 1mM sodium pyruvate, 1 mM non-essential amino acid (NEAA), 100 IU/ml penicillin, 0.1mg/ ml streptomycin, 10 ng/ml basic human fibroblast growth factor (bFGF), 10 ng/ml human leukemia inhibitory factor (hLIF) (All from Sigma, USA), 1% fish serum (FS) and 1% fish embryo extract (FEE). The procedure followed here was adopted from Routray *et al.* (2009).

The cultured cells were incubated at 28 ^{o}C with 5% CO_2 (Contherm, Hult City, New Zealand). They were regularly observed for morphological intactness under inverted microscope (Hund Wetzlar, Germany). The medium was changed at every 3-day interval. When cells attained 80% confluence, sub-culture was done at a splitting ratio (1:2) in 4-5 days interval.

Collection of fish serum (FS) and preparation of fish embryo extract (FEE)

Fish blood was collected from caudal vein of adult mrigal and allowed to clot for 30 min at room temperature. Then kept overnight at 4 ^{o}C, serum separated by centrifugation (200 g for 10 min), sterilized through 0.4 µm syringe filter, heat inactivated at 56 ^{o}C for 30 min and finally stored at -20 ^{o}C in aliquots for further use.

Approximately 300 embryos (2-h post fertilization) of mrigal, rohu and catla were collected for preparation of fish embryo extract (FEE). The main aim of using rohu and catla along with mrigal here for preparation FEE was closely related species is more mitogenic than same species (Hong *et al.,* 2000). Embryos washed thoroughly with 70% ethanol and rinsed several times with phosphate buffered saline, pH 7.4 (PBS) to avoid contamination. The washed embryos were dechorionated manually and homogenized with 2-5 ml of PBS in a tissue homogenizer (Braun, Germany). The resulting homogenate was centrifuged for 10 min at 15,000 g at 4 °C (Heraeus centrifuge, Thermo Scientific, Germany). The supernatant was diluted 1:1 with PBS, passed through the 0.4 µm and 0.22 µm syringe filter. Protein concentration was determined by using method described elsewhere (Bradford, 1976). The solution was made to a concentration of 10 mg ml^{-1} and stored at –20 ^{o}C following a standard protocol (Fan *et al.*, 2004).

Generation of embryoid bodies (EBs)

ES like cells were dissociated using 0.25% trypsin and 0.2% EDTA and sub-cultured in suspension in medium supplemented with Dulbecco's modified Eagle's medium (DMEM) with 15% fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 50 U/ml penicillin, and 50 µg/ml streptomycin (EB medium). $1x10^3$ cells/ml were seeded into 10 cm petridishes (BD Falcon, USA) and incubated at 28 ^{o}C in humidified air with 5% CO_2 to form EBs. After 14 days of culture in EB medium these cells were developed into multicellular aggregate EBs. The supernatant containing EBs was collected from petridishes and were transferred into sterile centrifuge tubes, kept for few minutes to allow the EBs to settle under gravity. These sedimented EBs were then transferred to various types of *in vitro* regimen for differentiation and further analysis.

Alkaline phosphatase activity of embryoid bodies

To assess pluripotency of cells constituting embryoid bodies; EBs were fixed in 1% glutaraldehyde solution for 10 min and then stained in the dark with 0·38 mM BCIP

(5-bromo-4-chloro-3-indolyphosphate p-toluidine salt) and 0.4 mM NBT (nitro-blue tetrazolium) (Roche, USA) in 100 mM Tris–HCl buffer at pH 9·5 containing 100 mM NaCl and 5 mM $MgCl_2$. They were observed under inverted microscope after 90 min.

Immunocytochemical analysis of EBs

To assess expression of stage specific embryonic anigen-1 (SSEA-1) in embryoid bodies, EBs were fixed in 4% paraformaldehyde for 15 min at 4 °C, washed with 1× rinse buffer (20 mM Tris–HCl, pH 7.4, 0.15 g NaCl, 0.05% Tween-20) and permeabilized with 0.1% polyethylene glycol tert-octylphenyl ether (Triton X-100) for 10 min at room temperature. The primary antibodies were diluted at a working concentration of 1:5, incubated for 1 h at room temperature and washed three to four times with rinse buffer. Fluorescein isothyocyanite (FITC)-labeled goat anti-mouse IgG (Sigma, USA) was used as the secondary antibody. It was diluted to a working concentration of 1:50 and those appropriate to the isotype of the primary antibody. Few stained cells were observed under an inverted microscope with fluorescent light at an excitation wavelength of 490 nm (FITC).

***In vitro* differentiation of EBs into cell lineages and their growth and propagation**

The 5 days old EBs were selected for further differentiation purpose. For this EBs (5-20 in number) were plated on various permissive surfaces for attached growth. To get homogenous population of keratinocyte-like cells, EBs were cultured on 0.1% gelatin coated surface along with 10^{-5} mol/l retinoic acid (RA) (Sigma, USA) that was prepared in dimethyl sulfoxide (DMSO) (Sigma, USA). The cultures were observed regularly under inverted microscope. The medium was changed at every third day. After keratinocyte-like cells attain confluency, sub-culture was done following standard procedure after trypsinization. The cell viability was determined by 0.3% trypan blue dye exclusion test and 5×10^5 cell/ml were transferred to keratinocyte growth and propagation medium (KGPM). KGPM composed of DMEM, 10% FBS, 5 ng/ml keratinocytes growth factor (KGF), 5 ng/ml epidermal growth factor (EGF), 5 µg/ml insulin, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 50 U/ml penicillin, and 50 µg/ml streptomycin (all from Sigma, USA) having an optimum calcium concentration (0.05 mM). The cultured cells were incubated at 28 °C and 5% CO_2 for their further growth and propagation.

Muscle-like cells differentiated from EBs. For this, EBs were plated on 0.1% gelatin coated plates in presence of differentiating agent RA at concentration 10^{-4} mol/l that was prepared by dissolving in DMSO. The cultures were observed regularly under inverted microscope. The medium was changed at every third day of culture. After attaining confluency, sub-culture was done and muscle like cells were obtained and seeded at a rate of 2.5×10^5 cells/well to gelatinized 12 well plate in muscle cells growth and propagation medium (MGPM). MGPM composed of DMEM, 10% FBS, 1 mM

sodium pyruvate, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 50 U/ml penicillin, and 50 µg/ml streptomycin in combination with 10 ng/mL EGF and 0.5 mg/mL BSA and 100 ng/mL insulin-like growth factor-1 (IGF-1) (Sigma, USA) for propagation and growth of muscle cells during their culture. Cells were incubated at 28°C and 5% CO_2.

The EBs were transferred to 35 mm petridishes coated with 1 mg/ml collagen. They were cultured in differentiating medium supplemented with 100 ng/ml activin-A (Sigma, USA). The cultures were observed regularly under inverted microscope. The medium was changed at every third day of culture. After attaining confluency, sub-culture was done to obtain hepatocyte-like cells. The dissociated hepatocyte-like cells were seeded at a rate 1×10^6 cells/ml into 60 cm petridishes coated with 1 mg/ml of collagen in hepatocyte growth and propagation medium (HGPM). HGPM composed of L-15 medium supplemented with 20 mM $NaHCO_3$, 5% FBS, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 50 U/ml penicillin, and 50 µg/ml streptomycin and 10 ng/ml epidermal growth factor (EGF) and 2% dimethyl sulfoxide (DMSO). Cells were incubated at 28 °C with 5% CO_2.

RESULTS

The procedures followed here are schematically represented in Fig. 1. *In vitro* differentiation of mrigal-ES like cells was obtained from EBs grown in suspension. Under these conditions, mrigal-ES cells consistently formed multicellular aggregates of elliptical or spherical bodies, EBs in 14 days of culture in suspension as shown in Fig. 2A. To assess the pluripotent status of EBs, we conducted alkaline phosphatase and immunocytochemical assay. Weak expression pattern of alkaline phosphatase activity was observed emitting faint reddish brown color (Fig. 2B). To evaluate pluripotency of these EBs another decisive marker SSEA-1 (positive in mrigal-ES colonies) was checked. Negligible

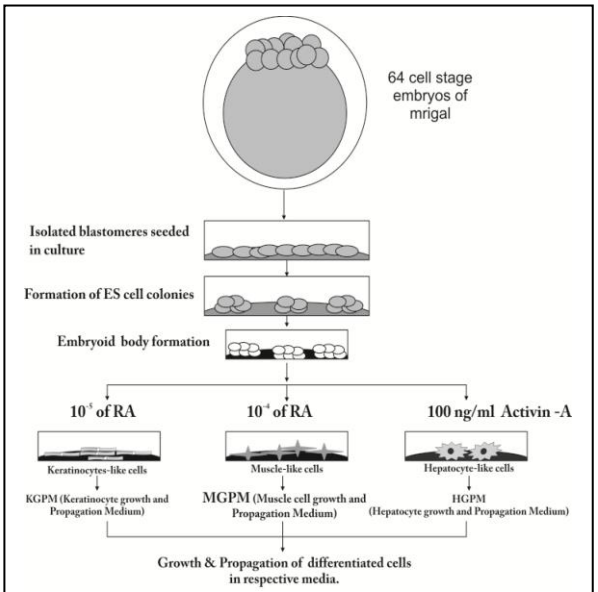

Fig. 1. Schematic representation of the process followed for growth and propagation of differentiated cells derived from ES like cells of mrigal, *Cirrhinus mrigala.*

expression of SSEA-1 in EBs was observed (Fig. 2C). Further 5 days old EBs were subjected to lineage specific differentiation upon treatment with appropriate inducers. RA treaded EBs showed ectodermal and mesodermal lineages. When these EBs were treated in presence of 10^{-5} mol/L of RA they differentiated into keratinocyte-like cells after 10 days of treatment (Fig. 2D).

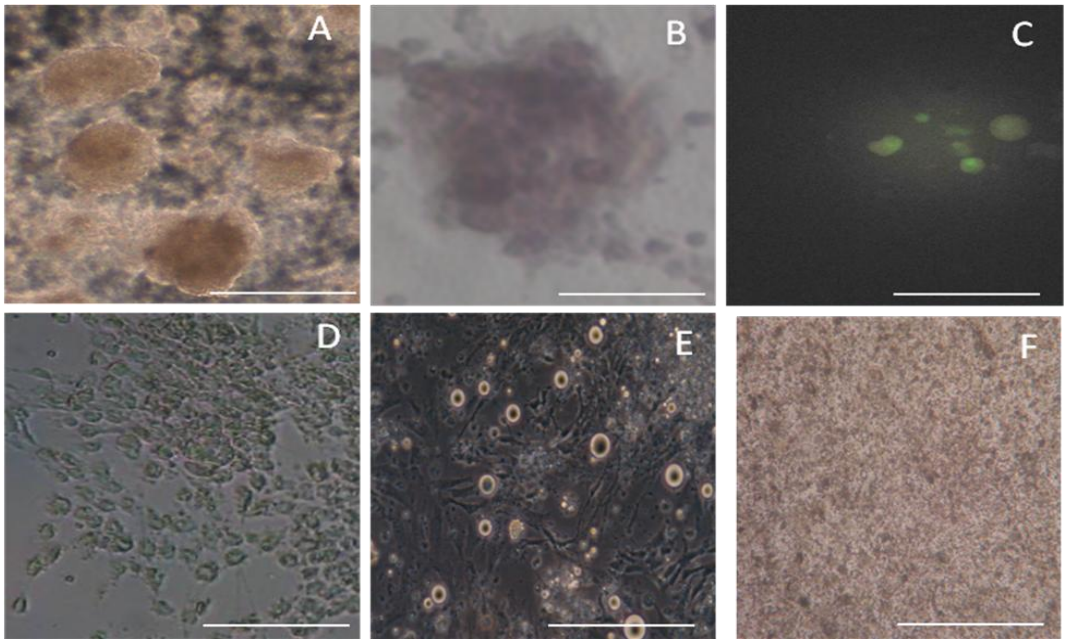

Fig. 2. Differentiated cells from EBs of mrigal, *Cirrhinus mrigala*: (A) embryoid body formed in suspension (bar = 100 µm) (B) weak alkaline phosphatase activity shown by embryoid body (C) weak expression of SSEA-1 shown by embryoid body (D) keratinocyte-like cells (E) muscle-like cells (F) hepatocyte like cells.

These Keratinocytes were predominent cell type of epidermis characterized by network like morphology and dendrite like processes spread in a continuous fashion. But on treatment with 10^{-4} mol/L of RA, EBs differentiated into muscle-like cells after 14 days (Fig. 2E). These muscles like cells were identified by their unique morphology: giant length up to 1 mm, multiple nuclei and striated structures.

When EBs were transferred to collagen coated plates and cultured along with activin-A, they differentiated into flattened, polygonal or round shaped hepatocytes-like cells (Fig. 2F). Hepatocyte-like cells were characterized by their round, polygonal morphology. After the culture attained confluency, cells were sub-cultured and seeded in their respective growth and propagation medium. Subsequent rounds of sub-cloning

showed reduction in their growth and propagation of these differentiated cells. The keratinocyte like cells were seeded at a rate of 5×10^5 cells/ml in formulated medium and maintained up to 12th passages without losing viability significantly (50%) in mrigal. The differentiated muscle-like cells generated from EBs upon sub-culture and seeded at a rate 2.5×10^5 cells/well muscle cells growth and propagation medium (MGPM) support their proliferation and growth up to 12th passage without losing their muscle-cell like morphology. Differentiated hepatocyte-like cells from EBs upon sub-culture and seeded at a rate 1×10^6 cells/ml in formulated HGPM retained their growth and proliferation capacity and maintained their growth till 12th passages. However, viability percentage of these differentiated cells decreased as the passages progressed. It seems as if these cocktail may not be optimal for further growth and propagation of keratinocyte, muscle cell and hepatocytes-like cells (Fig. 3).

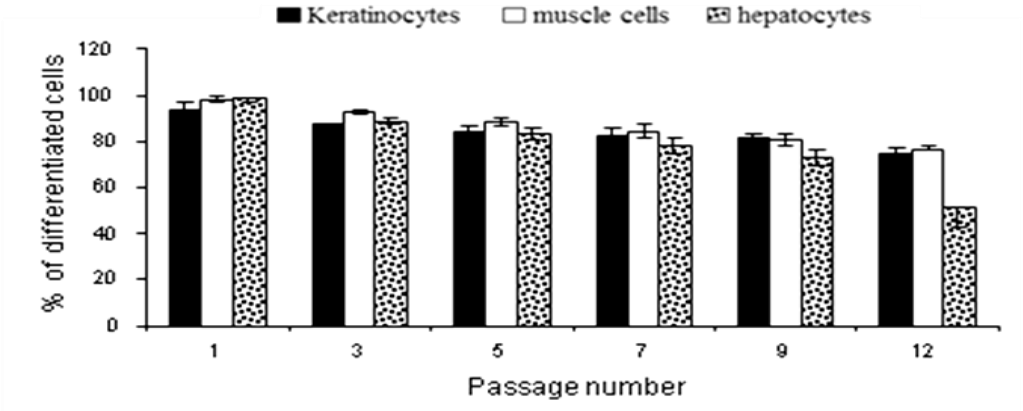

Fig. 3. Percentage (%) of viable differentiated cells of mrigal, *Cirrhinus mrigala* at different passages

DISSCUSION

Embryonic stem (ES) cells derived from early embryonic cells of developing embryos are pluripotent in nature and therefore, can serve as a putative source of numerous types of differentiated cell derivatives of the three germinal layers (Wobus *et al.*, 1984). The integrity and applications of ES cells largely depend upon the perpetuation of their descendent progenitor cells used in cell based therapies, drug discovery, regenerative medicine, tissue engineering and transplantation research (Groebner *et al.*, 2006). ES cells spontaneously differentiate into heterogeneous population of many cell types upon change in any parameter of *in vitro* system. So an optimum *in vitro* condition is prime requirement that facilitate differentiation to homogenous population of desired cell types (Klimanskaya *et al.,* 2008).

Morphologically, generated EBs are spherical structure of ephemeral nature. Here, the EBs developed from mrigal-ES like cells resembled with those derived from mouse ES cells and other fish ES-cells (Ginis *et al*., 2004; Alvarez *et al*., 2007). ES cells in mouse and other animals including fish express high alkaline phosphatase activity, a decisive marker of pluripotency (Alvarez *et al*., 2007). Alkaline phosphatase [orthophosphoric-monoester phosphohydrolase active in alkaline condition], (EC 3.1.3.1) belongs to this class of enzymes (Hass *et al*., 1979). Here, we checked pluripotency in EBs developed from mrigal-ES cells and showed low expression of this enzyme activity. This suggests that EBs are in the transitory phase between pluripotency and multipotency. So, an intermediary expression was observed in EBs developed here. Similar results were also reported by Bernstine *et al.* (1973).

Stage specific embryonic antigen-1 (SSEA-1) is a carbohydrate epitope expressed in *O. latipes* embryos and known to be a potential marker of ES cells in several species (Sasado *et al*., 1999; Familari and Selwood, 2006; Dash *et al*., 2010). Here EBs developed from mrigal-ES cells showed low expression of this marker. This may be due to the conglomeration of both undifferentiated and differentiated cells present within EBs. This further confirmed that EBs are of ephemeral nature.

In this study, keratinocyte differentiation (ectodermal lineage) was observed by supplementing culture medium with 10^{-5} mol/l RA. This is in consistent with other published report (Hong *et al*., 1996). EBs are suitable candidates to induce direct differentiation towards a specific lineage upon introduction of appropriate inducer (Itskovitz-Eldor *et al*., 2000). Retinoids are natural and synthetic derivatives of vitamin-A, which has pleiotropic biological effects through receptor-mediated changes in gene expression (Miano *et al*., 1998). Retinoic acid effectively promotes differentiation of ectodermal and mesodermal lineages (Schuldiner *et al*., 2000). But RA induced differentiation precisely depend on its concentration (Holen and Hamre, 2003). For keratinocyte-like cell differentiation (ectodermal lineage) we used 10^{-5} mol/l and muscle like cell differentiation (mesodermal lineage) was observed upon addition of RA at concentration 10^{-4} mol/l to the culture medium. Our results are in similar lines with other published reports (Hong *et al*., 1996; Parameswaran *et al*., 2007; Chen *et al*., 2003b).

Activin A, a member of the transforming growth factor beta superfamily is a known potent inducer of definitive endoderm cells, largely dependent on concentration, culture conditions and time of application (Sulzbacher *et al*., 2009). Here, activin-A at 100 ng/ml applied to 5 days old EBs on feeder free condition, hepatocyte-like cell differentiation occurred.

Further, these differentiated cells were grown and propagated up to 12^{th} passages without losing their viability in their respective growth and propagation medium. The

KGPM was formulated for growth and propagation of keratinocytes and supplemented with key factors *viz*; keratinocytes growth factor (KGF), epidermal growth factor (EGF), insulin, and $CaCl_2$. KGF is produced by dermal fibroblasts which possess receptors in epithelial cells. The main characteristic of KGF is to stimulate keratinocytes' proliferation (Gragnani *et al.*, 2010). Insulin and EGF are essential for optimal multiplication of keratinocytes (Tsao *et al*., 1982). There is high need for Ca^{++} supplement in the growth and maintenance medium of keratinocytes. Calcium acts as a trigger for ectodermal epithelial cell growth and differentiation. Addition of calcium to formulated culture medium induces a terminal differentiation program including specific structural changes, cell cycle withdrawal, and induction of differentiation-related genes. So supplement of an optimum concentration of external source of Ca^{++} is prime requisite for keratinocytes' growth and propagation. Here, very large colonies of flattened epithelial cells are obtained in the formulated medium when supplemented with low calcium concentration (0.03 mM) and did not favor keratinocyte propagation. Less growth and more differentiation are obtained with higher calcium concentration (>0.07mM). So calcium concentration at 0.05 mM was found to be optimal for keratinocytes' growth, proliferation and propagation. A comparable result has also been documented by Tsao *et al.* (1982).

The key supplements of MGPM medium were EGF, BSA and IGF-1. The EGF plays a significant role in differentiation and propagation of ectodermal and mesodermal derivatives (Schuldiner *et al.,* 2000). The combination of EGF and BSA further enhances survivability of muscle-like cells during culture period even in serum-free condition (St Clair *et al*., 1992).

The IGFs in fact, have biphasic effects on differentiation. They stimulate both proliferation and differentiation at relatively low concentrations but inhibit differentiation at higher, non-physiological concentrations (Florini and Magri 1989). Here, addition of IGF-1 to the formulated muscle cells growth and propagation medium (MGPM) further stimulates growth, proliferation and maintenance of muscle-like cells as reported by Allen *et al*. (1985) and, Florini and Magri (1989).

Here, differentiated hepatocyte-like cells were maintained in 2% DMSO up to 12^{th} passage without losing their viability. DMSO has the ability to enter into biological membranes. This drug may function as a carrier for specific nutrients and hormones. DMSO also seems to alter the structure of proteins and nucleic acids and, as such, may directly alter gene expression. It facilitates to maintain differentiation of normal adult hepatocytes (Cable and Isom, 1997). Here DMSO might have acted in similar manner either directly controlling expression of differentiated liver-specific gene products or indirectly altering hepatocyte responsiveness to other components, such as hormones or nutrients. However, the percentage viability of these differentiated cells declined as

passage number proceeded similar to reported by Isom *et al.* (1985), which might be due to the cocktail used here did not support further growth and propagation.

Cell seeding has been shown to play a crucial role in the initial cell spatial distribution and subsequent cellular activities essential for development of new tissue (Zhou *et al*., 2008). Tissue organization and maintenance within multicellular organisms is in part dependent on the number of cells involved to generate that respective organ, ability of cells to undergo programmed cell death or apoptosis. So the initial cell concentration significantly affects propagation of specific cell type *in vitro* (Swift et al., 2010). Here, 5×10^5 cells/ml of differentiated keratinocytes were able to maintain keratinocyte phenotype and their colony forming ability during culture period (Kalyanaraman and Boyce, 2007). Similarly, seeding at a density of 2.5×10^5 cells/well of differentiated muscle-like cells was able to maintain the phenotype and their colony forming ability during *in vitro* culture (Westerman *et al*., 2010). Cell concentration of 1×10^6 cells/ml supported growth and propagation of hepatocyte-like cells (Novik, 2007).

Here, differentiation into specific cell types from ES like cells and their growth and propagation *in vitro* paves the way for further understanding of ES cell biology in general and differentiation patterns in particular in fish. However, for characterization of terminally differentiated cells and better understanding of the microenvironment guided differentiation more studies are needed.

ACKNOWLEDGEMENTS

The authors are thankful to the Director, CIFA for providing the facilities to carry out the research work. The financial assistance provided by the Department of Biotechnology, Government of India is gratefully acknowledged.

REFERENCES

Allen, R. E., L. S. Luiten and M. V. Dodson, 1985. Effect of insulin and linoleic acid on satellite cell differentiation. *J. Anim. Sci.*, **60**: 1571-1579.

Alvarez, M. C., J. Béjar, S. Chen and Y. Hong, 2007. Fish ES cells and applications to biotechnology. *Mar. Biotech.*, **6**: 117–127.

Bernstine, E. G., M. L. Hooper, S. Grandchamp and B. Ephrussi, 1973. Alkaline phosphatase activity in mouse teratoma. *Proc. Natl. Acad. Sci. USA*, **70** (12): Part II, 3899-3903.

Bradford, M. M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.*, **72**: 248–254.

Cable, E. E. and H. C. Isom, 1997. Exposure of primary rat hepatocytes in long-term DMSO culture to selected transition metals induces hepatocyte proliferation and formation of duct-like structures. *Hepatology*, **26**(6): 1444-1457.

Chen, S. L., H. Q. Ye, Z. X. Sha and Y. Hong, 2003a. Derivation of a pluripotent embryonic cell line from red sea bream blastulas. *J. Fish Biol*, **63**: 795–805.

Chen, S. L., Z. X. Sha and H. Q. Ye, 2003b. Establishment of a pluripotent embryonic cell line from sea perch (*Lateolabrax japonicus*) embryos. *Aquaculture*, **218**: 141–151.

Dash, C., P. Routray, S. Tripathy, D. K. Verma, B. C. Guru, P. K. Meher, S. Nandi, and A. E. Eknath, 2010. Derivation and characterization of embryonic stem-like cells of Indian major carp *Catla catla*. *J. Fish Biol*., **77**: 1096–1113.

Dash, S. N., P. Routray, C. Dash, B. C. Guru, P. Swain and N. Sarangi, 2008. Use of the non toxic cryoprotectant trehalose enhances recovery and function of embryonic stem cells following cryogenic storage. *Curr. Stem Cell Res. Ther.*, **3**: 277–287.

Familari, M. and L. Selwood, 2006. The potential for derivation of embryonic stem cells in vertebrates. *Mol. Reprod. Dev*., **73**: 123–131.

Fan, L., J. Crodian and P. Collodi, 2004. Culture of embryonic stem cell line from zebra fish. *Meth. Cell Biol.*, **76**: 149–158.

Florini, J. R. and K. A. Magri, 1989. Effects of growth factors on myogenic differentiation. *Am. J. Physiol.*, **256**(4): C701-711.

Ginis, I., Y. Luo, T. Miura, S. Thies, R. Brandenberger, S. Gerecht-Nir, M. Amit, A. Hoke, M. K. Carpenter, J. Itskovitz-Eldor and M. S. Rao, 2004. Difference between human and mouse embryonic stem cells. *Dev. Biol.*, **269**: 360–380.

Gragnani, A., M. B. Rossi, J. C. Albuquerque, G. S. Brito, A. C. Aloise and L. M. Ferreira, 2010. Keratinocyte growth factor protected cultured human keratinocytes exposed to oxidative stress. *Acta. Cir. Bra.,* **25**: 93-97.

Groebner, M., R. David and W. M. Franz, 2006. Embryonic stem cells. Future perspectives. *Internist (Berl),* **47**: 502-508.

Hass, P. E., H. G. Wada, M. M. Herman and H. H. Sussman, 1979. Alkaline phosphatase of mouse teratoma stem cells: immunochemical and structural evidence for its identity as a somatic gene product. *Proc. Natl. Acad. Sci., USA,* **76**: 1164-1168.

Holen, E. and K. Hamre, 2003. Towards obtaining long term embryonic stem cell like cultures from a marine flatfish, *Scopthalmus maximus*. *Fish Physiol. Biochem.*, **29**: 245–252.

Hong Y. and M. Schartl, 1996. Establishment and growth responses of early medakafish *(*Oryzias latipes*)* embryonic cells in feeder layer-free cultures. *Mol. Mar. Biol. Biotechnol.*, **5**: 93–104.

Hong, Y., C. Winkler and M. Schartl, 1996. Pluripotency and differentiation of embryonic stem cell lines from the medaka fish (*Oryzias latipes*). *Mech. Dev.*, **60**: 33–44.

Hong, Y., S. Chen and M. Schartl, 2000. Embryonic stem cells in fish: current status and perspectives. *Fish Physiol. Biochem.*, **22**: 165-170.

Höpfl G., M. Gassmann and I. Desbaillets, 2004. Differentiating embryonic stem cells into embryoid bodies. *Meth. Mol. Biol.*, **254**, 79-98.

Isom, H. C., T. Secott, I. Georgoff, C. Woodworth and J. Mummaw, 1985. Maintenance of differentiated rat hepatocytes in primary culture. *Proc. Natl. Acad. Sci., USA*, **82**(10): 3252–3256.

Itskovitz-Eldor, J., M. Schuldiner, D. Karsenti, A. Eden, O. Yanuka, M. Amit, H. Soreq, and N. Benvenisty, 2000. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol. Med.*, **6**(2): 88-95.

Kalyanaraman, B. and S. Boyce, 2007. Assessment of an automated bioreactor to propagate and harvest keratinocytes for fabrication of engineered skin substitutes. *Tissue Eng.*, **13**(5): 983-993.

Keller, G. M., 1996. *In vitro* differentiation of embryonic stem cells. *Curr. Opin. Biol.*, **7**(6): 862-869.

Klimanskaya, I., N. Rosenthal and R. Lanza, 2008. Derive and conquer: sourcing and differentiating stem cells for therapeutic applications. *Nat. Rev. Drug Dis.*, **7**(2): 131-142.

Miano, J. M., L. A. Kelly, C. A. Artacho, T. A. Nuckolls, R. Piantedosi and W. S. Blaner, 1998. All-*trans*- retinoic acid reduces neointimal formation and promotes favorable geometric remodeling of the rat carotid artery after balloon withdrawal injury. *Circulation*, **98**: 1219 – 1227.

Novik, E. I., 2007. Differentiation of mouse embryonic stem cells along a hepatocyte lineage. Ph.D. Dissertation, The State University of New Jersey and University of Medicine and Dentistry of New Jersey.

Parameswaran, V., R. Shukla, R. Bhonde and A. S. S. Hameed, 2007. Development of pluripotent ES-like cell line from Asian Sea Bass (*Lates calcarifer*) – an oviparous cell line mimicking viviparous ES cell. *Mar. Biotechnol.*, **9**: 766–775.

Routray, P., C. Dash, S. N. Dash, S. Tripathy, D. K. Verma, S. K. Swain, P. Swain and B. C. Guru, 2009. Cryopreservation of isolated blastomeres and embryonic stem like cells of Leopard danio, *Brachydanio frankei*. *Aquaculture Research*, **41**(4): 579-589.

Sasado, T., S. Kani, K. Washimi, K. Ozato and Y. Wakamatsu, 1999. Expression of murine early embryonic antigens, SSEA-1 and antigenic determinant of EMA-1, in embryos and ovarian follicles of a teleost medaka (*Oryzias latipes*). *Dev. Growth Differ.*, **41**: 293–302.

Schuldiner, M., O. Yanuka, J. Itskovitz-Eldor, D. A. Melton and N. Benvenisty, 2000. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc. Natl. Acad. Sci., USA*, **97**(21): 11307-11312.

St Clair, J. A., S. D. Meyer-Demarest and R. G. Ham, 1992. Improved medium with EGF and BSA for differentiated human skeletal muscle cells. *Muscle Nerve*, **15**(7): 774 -779.

Sulzbacher, S., I. S. Schroeder, T. T. Truong, and A. M. Wobus, 2009. Activin A-induced differentiation of embryonic stem cells into endoderm and pancreatic progenitors-the influence of differentiation factors and culture conditions. *Stem Cell Rev.*, **5**(2): 159-173.

Sun, L., C. S. Bradford, C. Ghosh, P. Collodi, and D. W. Barnes, 1995. ES-like cell cultures derived from early zebrafish embryos. *Mol. Mar. Bio. Biotech.*, **4**: 193–199.

Swift, B., W. Yue, and K. L. Brouwer, 2010. Evaluation of ^{99m}technetium-mebrofenin and ^{99m}technetium-sestamibi as specific probes for hepatic transport protein function in rat and human hepatocytes. *Pharm. Res.*, **27**(9): 1987-1998.

Tsao, M. C., B. J. Walthal and R. G. Ham, 1982. Clonal growth of normal human epidermal keratinocytes in a defined medium. *J. Cell Physiol.*, **110**(2): 219-229.

Vallier, L., T. Touboul, Z. Chng, M. Brimpari, N. Hannan, E. Millan, L. E. Smithers, M. Trotter, P. Rugg-Gunn, A. Weber and R. A. Pedersen, 2009. Early cell fate decisions of human embryonic stem cells and mouse epiblast stem cells are controlled by the same signalling pathways. *PLoS ONE,* **4**(6): e6082.

Westerman, K. A., A. Penvose, Z. Yang, P. D. Allen and C. A. Vacanti, 2010. Adult muscle 'stem' cells can be sustained in culture as free-floating myospheres. *Exp. Cell Res.*, **316**: 1966–1976.

Wobus, A. M., H. Holzhausen, P. Jakel, and J. Schoneich, 1984. Characterization of a pluripotent stem cell line derived from a mouse embryo. *Exp. Cell Res.*, **152**: 212–219.

Zhou, J-M., F-Y. Xing, J-J. Shi, Z-F. Fang, X-J. Chen and F. Chen, 2008. Quality of embryonic bodies and seeding density effects on neural differentiation of mouse embryonic stem cells. *Cell. Bio. Int.*, **32**(9): 1169-1175.