Fishery Technology 54 (2017) : 258 - 264

Development and Characterization of Embryonic Stem-like Cell Culture from *Carassius auratas* (Linnaeus, 1758)

Abhay Kumar[1], Pooja Kanyal[3], Megha K. Bedekar[2], A. Pavan Kumar[2], N. Sushila[2] and Gayatri Tripathi[2]*

[1]*ICAR-Central Institute of Fisheries Technology, P. O. Matsyapuri, Cochin - 682 029, India*
[2]*ICAR-Central Institute of Fisheries Education, Yari Road, Mumbai - 400 061, India*
[3]*NHPC Limited, Siliguri Basin Projects, Kamarangaguri - 734 005, India*

Abstract

Embryonic stem cells are pluripotent cells, capable of differentiating into various cell types. In the present study, feeder cell-free culture of mid-blastula embryos of the Goldfish (*Carassius auratus*) was carried out. Embryonic stem cells were derived from mid-blastula stage embryos using pronase to release the inner cell mass (ICM). The cells were cultured in Leibovitz-15 medium on gelatin-coated cell culture well plates supplemented with 15% fetal bovine serum, 1% goldfish serum, basic fibroblast growth factor and leukaemia inhibitory factors (LIF) and were incubated at 28°C. The ES-like cells were characterized by their unique round and polygonal morphology, elevated activity of alkaline phosphatase, spherical three-dimensional embryoid body formation, chromosomal diploid number (2n=50). The expression of Oct4, a member Oct family of POU transcription factors, which play a key role in regulating stem cell pluripotency and differentiation, was used for characterization of undifferentiated pluripotent ES cells. The expression of Oct4 in embryonic stem-like cell (2nd passage) and gonads of goldfish was studied by RT- PCR. Embryonic stem-like cells culture from the mid blastula embryo of *C. auratus* would be a useful tool for cellular development and differentiation studies.

Keywords: ES-like cells, pluripotency, *Carassius auratus,* immunocytochemistry, RT-PCR

Received 24 April 2017; Revised 04 October 2017; Accepted 06 October 2017

**E-mail: gayatrit@cife.edu.in*

Introduction

Embryonic stem-like cells are undifferentiated cells, derived from early embryos that after cultivation can differentiate into any cell type (Gossler et al., 1986). These cells can be used as an excellent model for analyzing the vertebrate developmental biology *in vitro*, and provide a versatile resource for inducing differentiation into many desired cell types in large quantity for regenerative medicine. These cells are novel experimental systems for the study of molecular events of development and functional genomics, namely, targeting a gene via homologous recombination and their phenotype analysis in a growing organism (Alvarez et al., 2007). The pioneering work and development of stem cells in mice opened new vistas for research in developmental biology and led to guide similar applications in fish using the characterization of embryonic stem cells (Hong et al., 1996). ES-like cells derived from early embryos of animals have full developmental potential in *in vitro* conditions for prolonged periods (Evans & Kaufman, 1981; Martin, 1981). Establishment of embryonic stem cell culture has been utilized in the molecular study of pluripotency including differentiation and developmental biology mechanisms (Nishikawa et al., 2007). Fish embryonic stem-like cells have been used as instruments of genetic engineering in germ-line transmission and have the potential for the production of superior stocks in aquaculture through transgenesis (Gong et al., 2001). Partial characterization of ES-like cells were reported for zebra fish, (*Danio rerio*), medaka (*Oryzias latipes*), sea bream (*Pagrus major*), sea perch (*Lateolabrax japonicas*), turbot (*Psetta maxima*), Barramundi (*Lates calcarife*r), gilt-head sea bream (*Sparus aurata)* and rohu (*Labeo rohita*) (Collodi et al., 1992; Bejar et al., 2002; Chen et al., 2003a,b; Parameswaran et al., 2007; Goswami

et al., 2012). In most of the cases, embryonic stem-like cell were characterized by their morphology, alkaline phosphatase (AP) activity and embryoid body formation. (Hong et al., 1996) developed a feeder cell-free culture condition of Medaka (*Oryzias latipes*) in which mid-blastula embryos (MBE) cells were grown on the gelatin-coated surface. ES-like cell culture systems developed from mid-blastula stage have been characterized for different applications in a few fish species (Chen et al. 2003a, b; Dash et al. 2010). Goldfish (*C. auratus*), a freshwater fish, belongs to the family Cyprinidae of order Cypriniformes. It is an important ornamental fish and one of the earliest fish to be domesticated. Development of embryonic stem-like cell culture systems from mid blastula embryos of *C. auratus* will be instrumental in gene expression and cell differentiation studies in fishes. The present study was aimed to develop embryonic stem-like cell culture of mid-blastula stage embryos of goldfish and to characterize it using morphological description, immunocytochemistry and molecular techniques.

Material and Methods

Disease-free healthy, two-year-old brooders of goldfish *(C. auratus*) with an average weight of (58±2 g) (mean wt ± SD) were procured from the Kurla Aquarium Shop, Maharashtra, India (Fig.1). Fish were acclimatized in a glass aquarium with the optimum physio-chemical condition for a period of 10 days and fed with commercial goldfish pelleted feed with 30% crude protein. Goldfish was stocked in breeding tanks (150 l), with a sex ratio of 2:1 for male and female. Fish were induced to breed with GONOPRO-FH (Amrit Pharmaceuticals, Mumbai) at a dose of 0.5 mL kg^{-1} body weight (Mahmud et al., 2012) by providing suitable substratum. Post spawning, fertilized eggs of *C. auratus* in good condition were collected from the aquarium. Embryos were observed under a stereomicroscope during their developmental stage within 2-4 h (Fig. 2). Approximately 200 embryos of the mid-blastula stage were taken from cell culture. For the collection of blastomeres, the embryos were disinfected with 70% ethanol, washed seven to eight times with phosphate buffer saline (PBS, pH 7.4). The cell mass was harvested with Proteinase-k (enzyme) at a rate of 10 μg mL^{-1}, with supplementation L-15 medium and incubated for 1 min. Further, the chorion and egg shells were removed.

Fig. 1. Showing mature Carassius *auratus* belly plump

For cell culture, conditioned medium was prepared with commercially available basic media, serum, growth factors and other supplements as reported earlier. Leibovitz-15, with 4·5 gL^{-1} glucose and Ham's F-12 in 2:1:1 ratio and 15% FBS was used. The seeding density (primary culture of *C. auratus* embryonic stem-like cells) was 10^5 cells mL^{-1}. Additional components, such as 15 mM HEPES [4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid], 8 nM sodium selenite, 5 ng mL^{-1} human epithelial growth factor (hEGF), leukemia inhibitory factors (LIF),1 mM β-mercaptoethanol, 1 mM sodium pyruvate, 1mM non-essential amino acid and 100 IU mL^{-1} penicillin and 0·1 mg mL^{-1} streptomycin into the media were added (Sigma-Aldrich, USA). Isolated blastomeres were seeded on a 0·1% gelatin-coated twelve well cell culture plate with the conditioned medium at 28°C in the incubator. The media were changed every third day and cell morphology was examined under an inverted microscope (Zeiss Axio Observer-1). After complete colony formation, passaging was done every sixth day by trypsinization.

The seeded twelve wells plate cells were observed at every 2-3 day's interval for the proliferation of cells and morphological details using an inverted microscope (Zeiss Axio Observer-1), under 100X magnification.

In order to assess the alkaline phosphatase activity, Embryonic Stem-like cells grown in 12-well plates were washed twice with PBS (pH 7.4) and then fixed with 1% glutaraldehyde solution for 10 minutes, after that glutaraldehyde solution was removed and cells were washed twice with PBS (pH 7.4). Then cells were stained for 20 min with bromochloroindolyl phosphate/nitroblue tetrazo-

lium (BCIP/NBT (Sigma-Aldrich, USA) and observed using an inverted microscope (Zeiss Axio Observer-1). The active cells with 60-70% confluence were seeded in duplicate 75 cm^2 tissue culture flask and a fresh L-15 medium with 15% FBS after adding 0.1 μg mL^{-1} colchicine was used. Cells were incubated for 24 h with colchicine to arrest the cell at metaphase stage and observed under a microscope. Further, cells were centrifuged at 500 X *g* for 5 min. and the supernatant was discarded, then Prewarm 500 μl of 0.075 M KCl, was added and incubated for 15-30 min at room temperature. And after addition of 100 μl KCl the cells were allowed to settle down at the bottom. Four drops of fixative (methanol-glacial acetic acid in the ratio of 3:1) were added to avoid clumping of cells. The cells were centrifuged at 500 X g for 5 min and the supernatant was discarded, 200 μL of fixative was again added to the settled cells and the cells were incubated at 4°C overnight, few drops of cell suspension were dropped on clean glass slide from a height of one foot. After drying, cells were stained with Hoechst stain at the rate of 100 μg mL^{-1}. The slide was dried and observed under the microscope with 200 X magnification for chromosome study.

Embryoid bodies were induced to form by hanging-drop method. In brief, the ES-like cells were detached by trypsinization and suspended in L-15 medium supplemented with 20% FBS at a density of $1x10^5$ cells mL^{-1}. Drops of 20 μL cell suspension were placed on the inner side of the lid of 60 mm tissue culture dishes. To avoid loss of nutrients through evaporation, the dishes were filled with PBS and incubated at 28°C for 48 h in the hanging-drop state in the incubator. After 2–3 days, three-dimensional EB were formed. These were transferred into culture flasks (25 cm^2) having L-15 media and observed using an inverted microscope (Zeiss Axio Observer-1) under 200 X magnification.

The expression of the transcription factor Oct4 and undifferentiated ES-like cells were confirmed by immunocytochemistry. For this study, Anti Oct4 (SC-5279) monoclonal antibodies were used as primary antibodies to detect antigen expression and FITC (Fluorescein isothiocyanate) labelled goat anti-mouse IgG (SC-2010) as a secondary antibody for visualization. In brief, cells grown in 12-well plate were washed with PBS (pH 7.4) for 5 min and were fixed in 4% paraformaldehyde (PFA). After fixation, the cells were washed twice with PBS and were blocked with 10% normal goat serum for 30 min at room temperature. Primary antibodies were diluted in a blocking solution (1:50) incubated and were added to the cells for 1 night at 4°C. Next day these were washed three times with 1X Rinse Buffer. Further, secondary antibodies goat anti-mouse IgG (SC-2010) were diluted in 1X PBS and added to cells for 30 min at room temperature and then washed thrice with 1X rinse buffer (PBS). Finally, cells were washed in PBS, covered with a cover slip using 50% glycerol and observed under inverted fluorescence microscope (100X) (Zeiss Axio Observer-A1) using an FITC filter.

Total RNA was isolated using the Trizol reagent (Thermo Scientific, USA) following the instructions of manufacturer, from embryonic stem cell culture (2^{nd} passage), ovary, testis, kidney, liver, heart, brain and muscle. Speed and time for initial homogenization steps were adjusted according to the tissue processed. For ES-like cells homogenization was done for 40 Sec at 8000 X *g*, whereas for other tissues homogenization was done for 1 min at 12000 X g. Further, complementary DNA (cDNA) was synthesized by first strand cDNA synthesis kit (Thermo Scientific, USA). B-actin was used as an internal control for assessing (cDNA) synthesis. *Catla catla* Oct4 mRNA sequence (conserved region) was used to design Oct4 primers (BLAST, NCBI). Primers were designed using gene Runner V. 3.0 software and the set of primers (Table 1) were used for standard RT-PCR. Pluripotency gene fragments were successfully amplified after optimization of

Table 1. Primers used in this study

GENE	SEQUENCE	PRIMER SEQUENCE	ANN. TEMP.	AMP.SIZE	
B-actin	Forward Reverse	GGCCTCCCTGTCTATCTTCC TTGAGAGGTTTGGGTTGGTC	55°C	156 bp	HQ267516.1
Oct4	Forward Reverse	CAGAGGTGGTTGAATGAGGCAG TGTTGGGTTTGAGGCACTTCAC	55°C	134 bp	GU289876.1

annealing temperatures (55ºC) and PCR master mix composition.

Results and Discussion

Embryonic stem–like cells were isolated and proposed from fertilized eggs of *C. auratus.* Blastula stage embryos were observed under a stereomicroscope and embryos reached mid-blastula stage within 3-6 h (Fig. 2). The ES-like cells at initial seeding stage were morphologically round or polygonal (Fig. 3). The cells attained full confluency in the non-gelatin coated flask after 3-4 days. The ES-like cells were sub-cultured after an interval of 3-4 days and were maintained for more than 5 passages (Fig. 4). When the ES-like cells were seeded at low density, the cells were able to form dense colonies in 9–12 days. The cells of these colonies were uniform and healthier in nature. Similar morphology of ES cells was reported by various researchers (Hong et al., 1996; Chen et al., 2003b; Alvarez et al., 2007; Goswami et al., 2012).

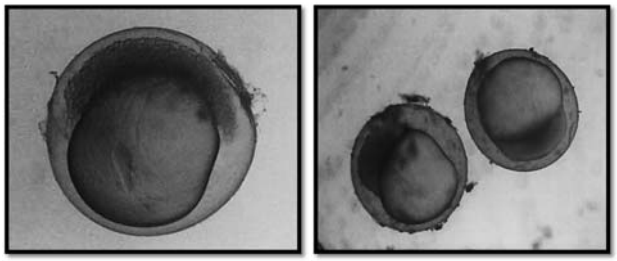

Fig. 2. Embryos of *Carassius auratus* showing mid-blastula stage

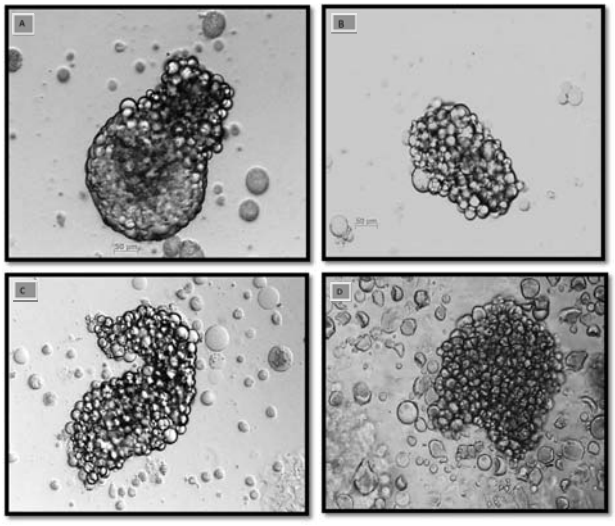

Fig. 3. Seeding of ICM on gelatine coated 12 well plates in A,B,C and D (phase contrast microscopy 100X)

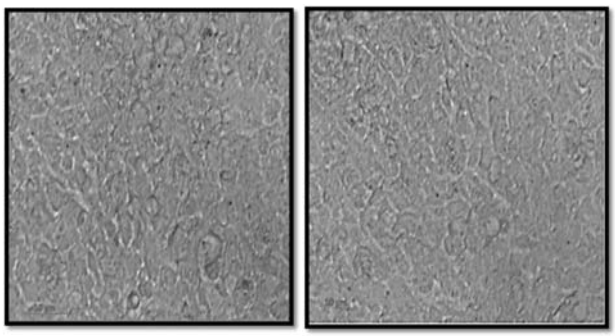

Fig. 4. Embryonic stem-like cells uniform and dense during after 4th passage (Phase contrast microscopy 200X)

Alkaline phosphatase is a hydrolase enzyme that acts on aliphatic and aromatic phosphate esters and hydrolyzes them to release phosphates. The alkaline phosphatase level is reported to be elevated in undifferentiated pluripotent stem cells (Stefkova et al., 2015). In the present study, the ES-like cells exhibited strong morphological alkaline phosphatase (AP) activity (Fig. 5). The activity of alkaline phosphatase has been used as an indicator of undifferentiated pluripotent ES-like stem cells in sea bass *Lates calcarifer* and *L. rohita* earlier (Parameswaran et al., 2007; Goswami et al., 2012). The presence of alkaline phosphatase activity confirmed the pluripotent state of cells in this experiment.

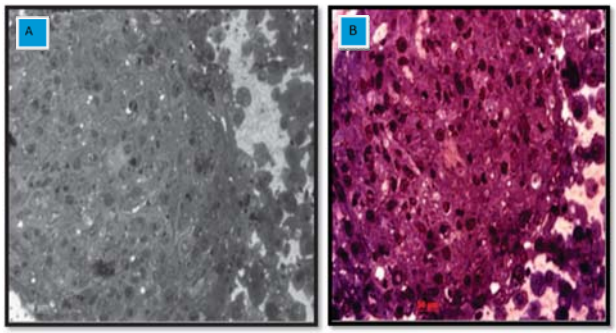

Fig. 5. ES cells expressed a high specific activity of alkaline phosphatase activity; unstained ES- like cells (A), (B) stained of ES-like cells with ALP

Initiation of EB formation was observed on the 2nd day. 4–5 days, cells aggregated to form spherical three-dimensional embryoid bodies. ES formation is a special character of ES-like cells (Fig. 6) and similar embryoid body type morphology of embryonic stem cells was documented in *O. latipes* (Hong et al., 2006) and *L. rohita* (Goswami et al., 2012), earlier. However, in goldfish, this is the first time it is attempted.

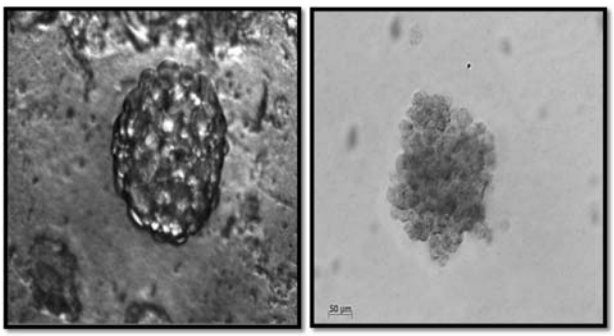

Fig. 6. Showing four to five-day old embryoid body formation of ES-like cell culture 100X

Pluripotency of cultured cells was confirmed by expression of transcription factor Oct4 in immuno-cytochemistry assay, where the expression was found to be high in the nucleus of ES-like cells as compared to the cytoplasmic region (Fig. 7). The elevated Oct4 expression was reported in ES-like cells isolated from *O. latipes* (Yi et al., 2009) and *L. rohita* (Goswami et al., 2012).

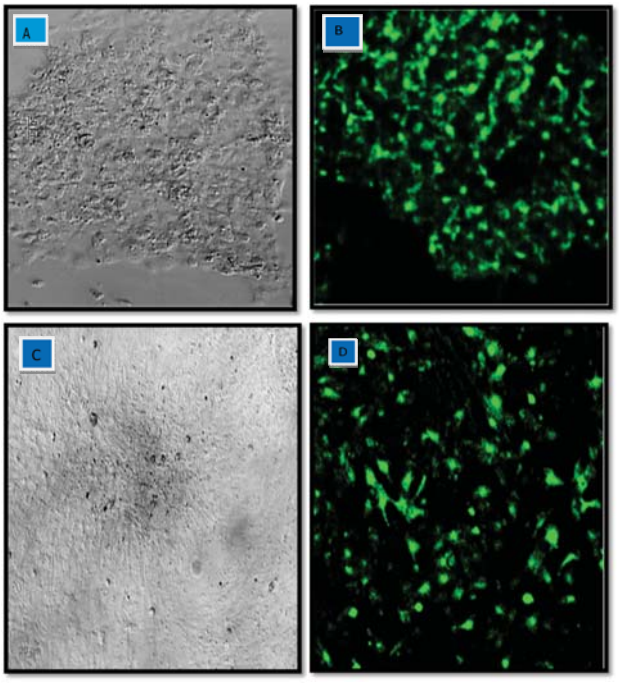

Fig. 7. Immunostaining of Oct4 protein by using anti Oct4 antibodies. Fluorescence density positive suspension of OCT4 protein. (A&C) negative, (B&D) positive of ES-cells Expression of Oct4 at 100X magnification.

Chromosomal analysis of 48 metaphase plates revealed that the number of diploid chromosomes in ES-like cells ranged from 30–52 with a model value of 2n = 50, which is identical with the model chromosome number of *C. auratus* (Fig. 8). Similarly, the diploid state of chromosomes in ES-like cells has been reported in previous studies in *Danio rerio* (Sun et al., 1995), *O. latipes* (Hong et al., 1996) and *Labeo rohita* (Goswami et al., 2012).

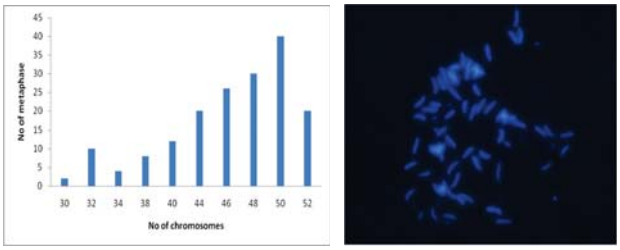

Fig. 8. Chromosome number distribution and range of chromosomes obtained in metaphase

The expression profile of Oct4 gene was restricted to embryonic stem-like cell (2nd passage) and gonads (Fig. 9). In the present study, the presence of high number of Oct4 mRNA transcripts in embryonic stem- like cell and a considerable amount of transcripts in the adult gonads *i.e.* testis and ovary are in accordance with many studies reported previously including humans (Anderson et al., 2007), mouse (Pesce et al., 1998), medaka (Wang et al., 2011; Sanchez Sanchez et al., 2010) and zebrafish (Lachnit et al., 2008). The expression of Oct4 transcripts was found to be highly gonadal-specific. In mouse, Oct4 gene is considered as an ideal pluripotency marker in mammals, because it is maternally transferred hence expresses specifically throughout the totipotent cycle, including embryo, spermatogonia and oocytes in the adulthood (Pesce et al., 1998). In the present study, low level of Oct 4 transcript in somatic adult tissues like kidney, liver, brain, muscle and heart was found. Similarly, insignificant expression of Oct4 transcript in somatic adult tissues was reported in medaka (Wang et al., 2011).

ES cells are an important tool which paves the way to study the molecular events of developmental and functional genomics. In the present study, ES cells were developed from mid-blastula embryos and were characterized by their unique morphology, alkaline phosphatase activity, embryoid body formation tendency, karyotype and expression of Oct 4 through immunocytochemistry. The number of diploid chromosomes in ES-like cells ranged from 30–52 with a model value of 2n = 50, which is identical to the model chromosome number of

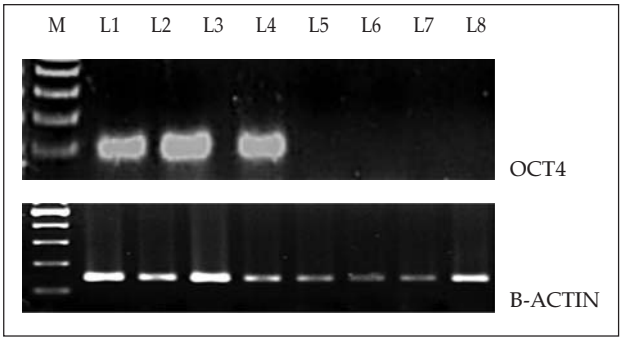

Fig. 9. Expression patterns obtained in semi-quantitative RT-PCR of different genes in all the tissues. Lane M: GeneRuler 100bp Ladder (Thermo scientific USA); lane 1: Embryonic stem like cells; lane 2: Ovary; lane 3: Testis; lane 4: Liver; lane 5: Heart; lane6 : Kidney; lane7:Brain;lane 8: Muscle

C. auratus. The expression of Oct4 in embryonic stem-like cell (2nd passage) and gonads of *C. auratus* were studied by RT-PCR. This is the first report of the development and characterization of ES-like cells from *C. auratus* and can be pivotal to progress further in stem cell biology. However, more molecular markers related to pluripotency need to be characterized and studied.

Acknowledgment

The authors are thankful to Dr. Gopal Krishna, the Director and Vice Chancellor Central Institute of Fisheries Education, Mumbai, India, for providing all the facilities required to carry out the present study.

References

Alvarez, M., Bejar, J., Chen, S. and Hong, Y. (2007) Fishes cells and applications to biotechnology. Mar. Biotechnol. 9: 117-127

Anderson, R. A., Fulton, N., Cowan, G., Coutts, S. and Saunders, P.T. (2007) Conserved and divergent patterns of expression of DAZL, VASA and OCT4 in the germ cells of the human fetal ovary and testis. BMC. Dev. Biol. **7**: 136

Bejar, J., Hong, Y. and Alvarez, M. (2002) An ES-like cell line from the marine fish Sparus aurata characterization and chimaera production. Transgenic Res. 11: 279-289

Bradley, A., Evans, M., Kaufman, M.H. and Robertson, E. (1984) Formation of germ-line chimeras from embryo-derived teratocarcinoma cell lines. Nature. 309:255–256

Chen, S, L., Ye, H., Sha, Q. and Shi, C, Y. (2003a) Derivation of a pluripotent embryonic cell line from red sea bream blastulas. J. Fish. Biol. 63: 10

Chen, S, L., Sha, X. and Ye, H. (2003b) Establishment of a pluripotent of embryonic cell line from sea perch (Lateolabrax japonics) embryo. Aquaculture. 218:141-151

Collodi, P., Kamei, Y., Sharps, A., Weber, D. and Barnes, D. (1992) Fish embryo cell cultures for derivation of stem cells and transgenic chimera. Mol. Mar. Biol. Biotechnol. 11: 642-646

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

Evans, M. J. and Kaufman, M.H. (1981) Establishment in culture of pluripotent cells from mouse embryos. Nature. 292: 154-156

Familari, M. and Selwood, L. (2006) The potential for derivation of embryonic stem cell in vertebrates. Mol. Reprod. Dev. 73: 123-131

Gong, Z., Ju, B. and Wan, H. (2001) Green fluorescent protein (GFP) transgenic fish and their applications. Genetica. 111: 213-225

Gossler, A., Doetschman, T., Korn, R., Serfling, E., and Kemler, R. (1986) Transgenesis by means of blastocyst-derived embryonic stem cell lines. Proc. Natl. Acad. Sci. 83: 9065-9069

Goswami, M., Lakra, W.S., Yadav, K. and Jena, J.K. (2012) Development of an ES- like cell culture system (RESC) from rohu, Labeo rohita (Ham.). Fish Physiol. Biochem. DOI 10.1007/s10695-012-9674-5

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

Hong, Y. and Schartl, M. (2006) Isolation and differentiation of medaka embryonic stem cells. Mol. Biol. 329: 3-16

Lachnit, M., Kur, E. and Driever, W. (2008) Alterations of the cytoskeleton in all three embryonic lineages contribute to the epiboly defect of Pou5f1 / Oct4 deficient MZ spg zebrafish embryos. Dev. Biol. 315: 1-17

Mahmud, Z., Faysal, A., Ghosh, A.K., Azad. A.K., Bir, J. and Rahaman, S.M.B.(2012) Induced breeding, embryonic and larval development of comet gold fish (Carassius auratus) in Khulna, Bangladesh. Int. J. Biosci. 10: 28-38

Martin, G. R. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci., U.S.A. 78: 7634-763

Muller, U. (1999) Ten years of gene targeting: targeted mouse mutant, from vector design to phenotype analysis. Mech. Dev. 82: 3-21

Nishikawa, S. I., Jakt, L. M. and Era, T. (2007) Embryonic stem-cell culture as a tool for developmental cell biology. Nature. 8: 502-507

Parmeswaran, V., Shukala, R.., Bhonde, R. and Hameed, A. S. (2007) Development of a pluripotent ES-like cell Line from Asian sea bass (Lates calcarifer) an oviparous cell stem cell line mimicking viviparous ES cells. Mar. Biotechnol. 9: 766-775.

Pesce, M., Wang, X., Wolgemuth, D.J. and Scholer, H. (1998) Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation. Mech. Dev. 71: 89-98

Sanchez-Sanchez, A. V. (2010) Medaka Oct4 is expressed during early embryo development, and in primordial germ cells and adult gonads. Dev. Dynam. 239: 672-679

Stefkova, K., Prochazkova, J. and Pachernik, J. (2015) Alkaline phosphatise in stem cells. Stem Cells Int.DOI: 10.1155/2015/628368

Sun, L., Bradford, C.S., Ghosh, C., Collodi, P. and Barnes, D.W. (1995) ES-like cell cultures derived from early zebra fish embryos. Mol. Mar. Biol. Biotechnol. 4: 193-199

Wang, D., Manali, D., Wang, T., Bhat, N., Hong, N., Li, Z., Wang, L., Yan, Y., Liu, R. and Hong, Y. (2011) Identification of pluripotency genes in the fish medaka. Int. J. Biol. Sci. **7**: 440-51

Yi, M., Hong, N. and Hong, Y. (2009) Generation of medaka fish haploid embryonic stem cells. Science. 326: 430-433