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Establishment of a pluripotent embryonic cell line from sea perch (*Lateolabrax japonicus*) embryos

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Abstract

Pluripotent embryonic stem (ES) cells provide an efficient approach for genome manipulation with many applications in marine biotechnology and development studies. To develop this technology, we have worked to derive fish ES cells for in vitro studies of embryo cell growth and differentiation and for the generation of transgenic fish. A pluripotent cell line, LJES1, was established from blastula-stage embryos of a cultured marine fish, *Lateolabrax japonicus*. The LJES1 cells were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with fetal bovine serum (FBS), marine fish serum, sea perch embryo extract, selenium, basic fibroblast growth factor (bFGF) and leukemia inhibitory factor. The ES-like cells were small and round or polygonal, and grew actively and stably in culture. The cells exhibited a positive alkaline phosphatase activity upon histochemical staining. When the cells were treated with all-*trans* retinoic acid, differentiation into various types of cells, including neuron-like cells, muscle cells and some unidentified cells were observed, suggesting that the LJES1 cells remained pluripotent in culture. Chromosome analysis revealed that LJES1 cells have a normal diploid karyotype with $2n = 48$. Up to now, LJES1 cells have been continuously cultured for more than 150 days with more than 40 passages. High survival rate has been obtained after cryopreservation of cell cultures. GFP reporter gene were transferred into LJES1 cells and successfully expressed.

Keywords: Differentiation; Embryonic stem cells; *Lateolabrax japonicus*; Pluripotency; Sea perch

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1. Introduction

Embryonic stem (ES) cells are undifferentiated cell lines derived from early developing embryos of animals, retaining their full developmental potential and their capability to differentiate (Evans and Kaufman, 1981; Martin, 1981). When introduced into host embryo, ES cells can participate in normal development and contribute to several tissues of the host including cells of the germ line. These characteristics make embryonic stem cell ideal experimental systems for in vitro studies of embryo cell development and differentiation and a vector for the efficient transfer of foreign DNA into the germ line of an organism (Gossler et al., 1986). Mouse ES cell lines have been established and used for research in a variety of areas including targeted gene inactivation and repair (Thomas and Capecchi, 1987; Thompson et al., 1989), developmentally regulated gene expression and cell differentiation and transformation (Mummery et al., 1990). Since the development of embryonic stem cell technology in mouse in 1980s (Evans and Kaufman, 1981; Martin, 1981) and the production of the first knock out mice (Thomas and Capecchi, 1987), the technique has become feasible to generate mice with desired genetic modifications ranging from simple gene mutation to large genomic deletions. In mice, ES cells were routinely used for gene transfer since they provide an ideal experimental system for efficient genome manipulation and for the investigation of factors controlling vertebrate development.

Transgenesis has shown an enormous potential for genetic improvement of aquatic organisms (Devlin et al., 1994). However, up to now, transgenic fish have been generated by direct introduction of foreign genes into fertilized eggs. The foreign genes integrate randomly into the genome of host, and the fates of transgene are neither controllable nor selectable. In most cases, the transgenics are highly mosaic for the introduced genes. Desired integration of the transgene and its proper expression is rarely achieved (for review, see Iyengar et al., 1996). Transgenic fish research urgently requires development of a novel gene transfer approach for achieving site-directed integration of a transgene into the host genome and regulation of the expression of transgenes (Hong et al., 1998a). ES cell-mediated gene transfer technique will be the most promising approach for producing site-mutated transgenic fishes with enhanced growth rate or disease-resistance and in analyzing functions of fish genes (Melamed et al., 2002; Chen et al., 2002a).

To develop ES cell lines and gene targeting technique in fish, extensive studies have been done in small model fishes, such as zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), because they offer the possibility of combining embryological, genetic and molecular analysis of vertebrate development. ES-like cell lines have been established in medaka (Wakamatsu et al., 1994; Hong et al., 1996) and zebrafish (Collodi et al., 1992; Sun et al., 1995). Recently, one medaka ES-like cell line, MES1, was shown to retain an eudiploid karyotype and the ability to form viable chimeras (Hong et al., 1998b). Homologous recombination vectors for medaka p53 gene were constructed on the basis of genomic sequences of the p53 gene (Chen et al., 2001, 2002b). Also, a positive–negative selection procedure for gene targeting in fish cells has been established (Chen et al., 2002a). Most importantly, the availability of zebrafish germ line chimeras from embryo primary cell cultures (Ma et al., 2001) and development of nuclear transplantation technique for medaka embryonic cells (Wakamatsu et al., 2001) point to the possibility of developing embryonic stem cell technique in fish.

Sea perch (*Lateolabrax japonicus*) is a widely cultivated marine fish species in China. However, diseases of the farmed fish have occurred frequently. Transgenic fish with enhanced resistance to pathogens will be beneficial to aquaculture. With the aim of establishing ES cell-mediated gene transfer technique in this fish, we report here on establishment of an ES-like cell line derived from mid-blastula of this species, its in vitro differentiation and ability to be transfected.

2. Materials and methods

2.1. Sea perch embryo cell cultures

Blastula-stage sea perch embryos were harvested approximately 6 h after fertilization and prepared for cell culture. For each culture, a group of about 50–70 embryos were disinfected with 70% ethanol, washed with PBS and the chorions were torn with fine forceps. The cell mass was released and the chorion was removed. Single cells were obtained by gentle pipetting. After several washes with phosphate-buffered saline (PBS), the cells were transferred into complete medium in gelatin-coated 24-well tissue plates. The complete growth medium was Dulbecco's modified Eagles medium (DMEM) supplemented with 20 mM HEPES, pH 7.5, antibiotics (penicillin, 100 U/ml, streptomycin, 100 μg/ml; Gibco BRL), fetal bovine serum (FBS, 15%), sea perch serum (1%), embryo extract from sea perch (0.1%), sodium selenite (8 nM, Gibco BRL), basic fibroblast growth factor (bFGF, 5 ng/ml, Gibco BRL), human recombinant leukemia inhibitory factor (hLIF, 5 ng/ml; Pepro Tech EC, London), 2-mercaptoethanol (2-ME, 27.5 μM; Gibco BRL), sodium-pyruvate (1 mM) and nonessential amino acids (1 mM). Cells were cultured at 25 °C in a normal atmosphere incubator, the medium was changed every 2 or 3 days. When reaching 95% confluence, the cells were subcultured at a ratio of 1:2 according to the standard trypsinization method. For cryopreservation, cell cultures were suspended in complete medium with 10% dimethyl sulfoxide (DMSO) and 20% FBS, placed in cryovials and stored in liquid nitrogen.

2.2. Preparation of sea perch embryo extract

For preparation of embryo extract, sea perch embryos were obtained 36 h after fertilization (reared at 15 °C) from Maidao Marine Fish Hatchery and rinsed with PBS for several times, and stored at −80 °C until use. To prepare extract, the embryos were thawed at 37 °C and homogenized on ice using a glass homogenizer. The resulting homogenate was centrifuged for 30 min at 13,000 rpm. The supernatant as embryo extract was collected, filter-sterilized, and stored at −20 °C until use.

2.3. Alkaline phosphatase staining

Cultured cells were washed with PBS, fixed in glutaraldehyde solution (1%) for 10 min, washed twice with PBS, and then stained in dark for alkaline phosphatase activity using bromochloroindolyl phosphate/nitroblue tetrazolium (BCIP/NBT, Merck) as sub-

strate. To examine the activity of alkaline phosphatase in blastula-stage embryos, the blastulae were fixed in 1% glutaraldehyde, washed several times in PBS and then stained with BCIP/NBT.

2.4. Chromosome analysis

Cells of LJES1 at passages 7–10 were used for chromosome analysis. The cells were inoculated in culture flask of 25 cm^2 and incubated for 24–48 h. Colchicine was added to the cells to reach final concentration of 10 μg/ml. After 4–6 h, the cells were harvested, treated with hypotonic solution of 0.075 M KCl for 30 min, fixed twice with cold Carnoy's fixative, 15 min for each time, then air-dried. Chromosomes were stained with 5% Giemsa for 20 min.

2.5. Induction of differentiation in vitro

To identify the differentiation potential of LJES1, the cells were inoculated at low density (10^3 cells/well of 12-well plate) or high density (5×10^5 cells/well of 12-well plate) and then cultured for 1–5 weeks with regular medium change. Two days after inoculation, the medium was replaced with the medium supplemented with 2 μM all-*trans* retinoic acid (RA, Sigma). Control cultures were seeded on gelatin-coated wells without retinoic acid. The medium was changed every 2 days. Cell morphology was microscopically examined daily.

2.6. Cell transfection with GFP reporter gene

pCMV-EGFP, a reporter construct expressing the cDNA for red-shifted, humanized green fluorescent protein (EGFP) under the control of the human cytomegalovirus (hCMV) promoter, was purchased from Clontech. The plasmid DNA was prepared according to the supplier's instruction (Qiagen). For transfection experiment, plasmids were linearized with restriction enzyme *Bgl*II. The LJES1 cells at passages 20–30 were transfected with the linearized plasmids by the calcium phosphate precipitation method (Chen et al., 2002a). LJES1 cells were plated at 3×10^5 cells/well of 12-well plates. Linearized plasmid DNA (2 μg) was used for one well. The cells were shocked with 15% glycerol 12–18 h after transfection. After another 48 h, GFP expression in the cells was detected by fluorescence microscope (CK40, Olympus) equipped with mercury burner (HBO50W/AC, Osram). Fluorescent images were obtained by using a microscopy camera (PM10, Olympus) mounted on the microscope.

3. Results

3.1. Establishment of sea perch ES-like cell line

To develop an ES-like cell line in cultured marine fish, embryos of *L. japonicus* at mid-blastula stage were used. The single blastomeres from 50 to 70 embryos were isolated and

inoculated in one well of 24-well plate. These blastomeres with a diameter of about 40–50 μm were first evenly attached to the surface of the well and grew to form many dense, homogeneous aggregates within the first 6–12 h after inoculation. The blastomeres in the aggregates began to adhere to the well surface after one or two days of culture. Two or three days later, the cells grew to confluence in the well, and subculture could be done. The cell division occurred very rapidly and subculture should be done every 3 or 4 days. Eight primary cultures were obtained from isolated blastomeres of sea perch mid-blastula embryos. Six of the eight cultures differentiated and gradually died, the remaining two cultures developed into cell lines that exhibited stable growth and morphology similar to that of mouse ES cells (Evans and Kaufman, 1981), one of which was designated as LJES1 (*L. japonicus* embryonic stem cell). The LJES1 cultures at passage 15 were shown in Fig. 1A. The cells were small (20–40 μm) and round or polygonal with a big nuclei and sparse cytoplasm when their densities was not high. As their densities increased, the cells became smaller (10–20 μm) (data not shown). After culture of more than 150 days with more than 40 passages, LJES1 cells displayed stable growth and ES-like morphology. Thus, LJES1 cell culture was considered as a stable cell line. The cryopreservation tests demonstrated that LJES1 cells could undergo cryopreservation in liquid nitrogen (– 196 °C) with a survival rate of about 60%.

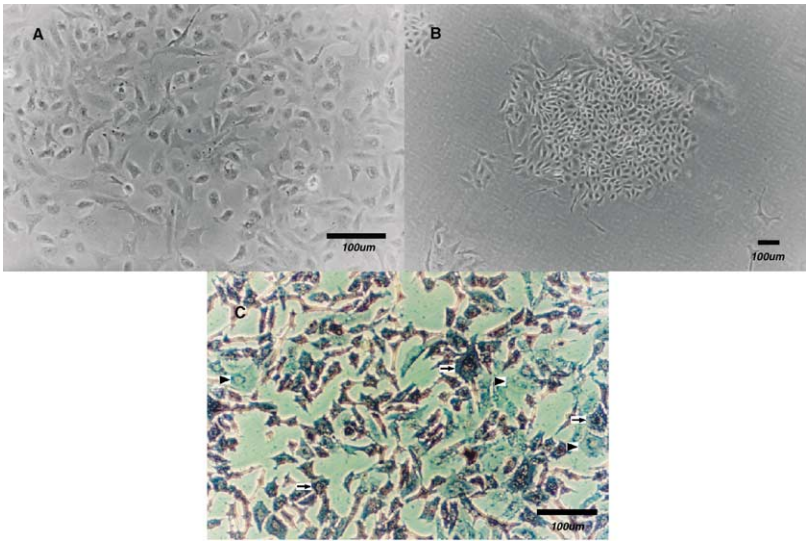

Fig. 1. Culture and alkaline phosphatase (AP) staining of LJES1 cells. (A) LJES1 cells at passage 15, showing small and round or polygonal cells with big nuclei and sparse cytoplasm. (B) ES-like cell colony of LJES1 cells at passage 10. (C) Alkaline phosphatase (AP) staining of LJES1 cells at passage 10, showing many small ES-like cells with strong AP staining (arrows) and a few differentiated cells (arrowheads). Bar, 100 μm.

3.2. ES-like colonies

Colony-forming ability is an important feature of ES-like cells. When plated at a very low cell density (20–40 cells/cm^2), LJES1 cells were able to grow into dense ES-like cell colonies 2 weeks after plating. Most of the cells in the colonies were ES-like cells that exhibited a strong alkaline phosphatase staining (data not shown). These colonies were tightly compacted and uniform in morphology. The cells in the colonies were small, round or polygonal in shape (Fig. 1B).

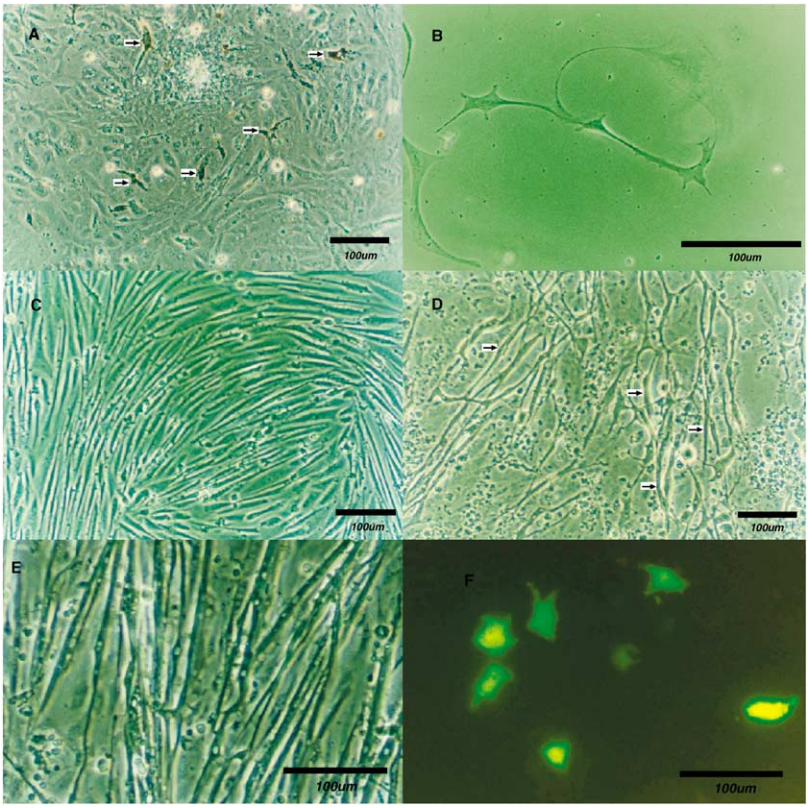

Fig. 2. In vitro differentiation and expression of GFP gene in LJES1 cells. (A) Melanocytes (arrows) appeared at passage 2 after 4–6 days culture. (B) Neuron-like cells appeared in the culture 10–15 days after treatment with retinoic acid at passage 11. (C) Unidentified fiber-like cells being arranged parallel to each other that appeared 2 weeks after treatment with retinoic acid at passage 30. (D) Muscle cells (arrows) appeared in the culture 3 weeks after treatment with retinoic acid at passage 15. (E) Differentiated muscle cells, showing their huge length and multiple nuclei structure. (F) Expression of GFP gene in LJES1 cells at passage 30 transfected with pCMV-EGFP. Bar, 100 μm.

3.3. Alkaline phosphatase activity

In mice, alkaline phosphatase staining was commonly used to evaluate pluripotency of ES cell. In the present study, similar staining was done for identifying LJES1 cell cultures. LJES1 cells at passage 10 were stained by BCIP-NBT, most of the cells exhibited strong alkaline phosphatase (AP) activity, and these cells are round or polygonal, with a larger nucleus and sparse cytoplasm, whereas those with a larger size and differentiated morphology showed weak activity (Fig. 1C). Blastoderms of sea perch embryos at mid-blastula were strongly stained (data not shown).

3.4. Differentiation potential of LJES1 in vitro

A few of LJES1 cells in either primary or early passage cultures spontaneously differentiated into melanocyte cells under normal culture conditions (Fig. 2A). These melanocyte cells began to appear after 4–6 days of culture. These differentiated melanocyte cells did not, however, exert big influence on subsequent cell culture, because they would die of frequent subculture during subsequent cultivations.

Differentiation of LJES1 cells into a variety of cell types could be induced by all-*trans* retinoic acid. When LJES1 cells at passages 10–30 were treated with retinoic acid, most of cells lost their stem cell-like morphology and differentiated into various cell types, including neuron-like cells, muscle cells and unidentified cells (Fig. 2B–D). Neuron-like cells began to appear 10–15 days after induction when cells were inoculated at very low densities (Fig. 2B). In some cases, LJES1 cells could be induced into fiber-like, bipolar cells which were arranged parallel to each other 12–20 days after induction (Fig. 2C). These cells remain to be identified for their identity. Muscle cells occurred between 15 and 20 days after induction when the cells were grown at high density. These differentiated muscle cells featured a huge length (up to 1 mm) and multiple nuclei (Fig. 2D,E).

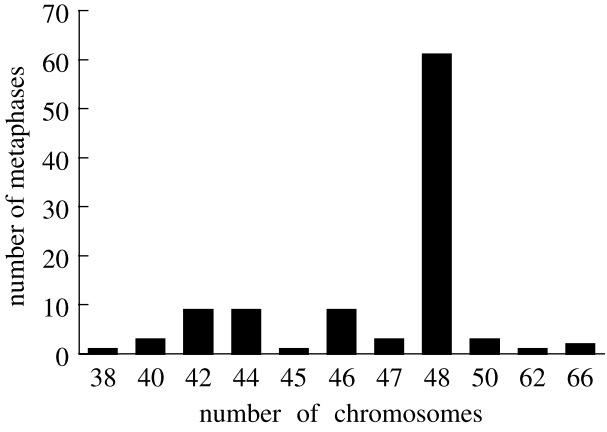

Fig. 3. Chromosome number distribution of LJES1 cells at passage 9. One hundred metaphase plates were counted.

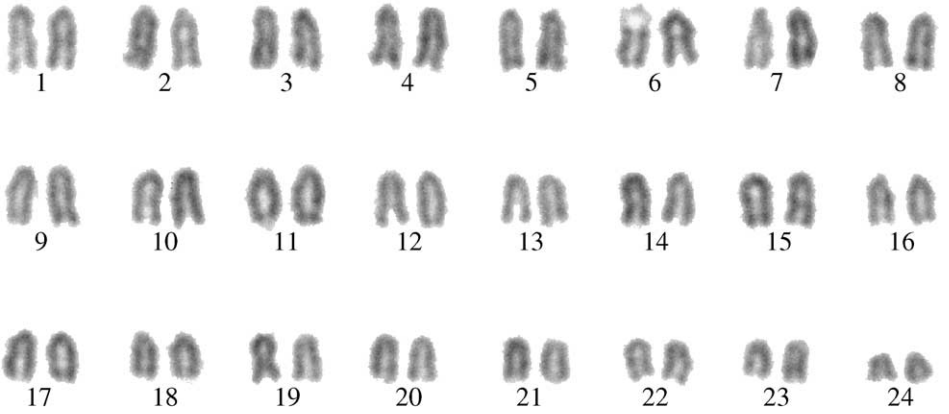

Fig. 4. Diploid karyotype corresponding to a euploid cell from LJES1 cells at passage 9. Homologous chromosomes were paired according to their size.

3.5. GFP expression in LJES1

LJES1 cells were successfully transfected with linearized pCMV-EGFP by the means of calcium phosphate precipitation method. The expression of EGFP in LJES1 could be detected as early as 48 h after transfection. About 2% of the cells were positive for the EGFP expression (Fig. 2F), indicating that calcium phosphate precipitation method was feasible for transfecting LJES1 cell cultures, and cytomegalovirus (CMV) promoter can drive the expression of EGFP gene in LJES1 cell.

3.6. Karyotypic analysis

The results of chromosome counts of 100 metaphase plates from LJES1 cells at passage 9 revealed that the chromosome numbers varied from 38 to 66. Nevertheless, the modal number of chromosome was 48 (Fig. 3). The distribution was asymmetrical, most $2n$ values appearing clustered below the modal value. Both aneuploidy and heteroploidy appeared in LJES1 cell line in small proportion. The diploid karyotype of LJES1 cell at passage 9 is shown in Fig. 4. The metaphase with normal diploid number displayed the normal karyotype morphology, consisting of 24 pairs of telocentrics ($2n = 48$t).

4. Discussion

Since the establishment of ES cell lines in mice by Evans and Kaufman (1981) and by Martin (1981), these totipotent cells have been recognized as powerful experimental system in vertebrate developmental biology. The genetic manipulation of these ES cells, via homologous recombination, allows the site-directed integration of foreign gene into the genome of host and knock-out of specific gene from host genome. Thus, ES cells provide a

promising approach to the generation of transgenic animals with site-directed mutation and to the study of gene function during embryogenesis (Jasin et al., 1996; Muller, 1999).

Although much effort has been made to develop ES cell lines in several species, cultures that are able to contribute to the germ-cell lineage of a host embryo have been successfully reported only for mice (Evans and Kaufman, 1981; Labosky et al., 1994). In fish, ES-like cells have been cultured in small model fish, zebrafish (Collodi et al., 1992; Sun et al., 1995) and medaka (Wakamatsu et al., 1994; Hong et al., 1996). Also, the germ line chimeras were produced from embryo primary cell cultures of zebrafish (Ma et al., 2001). The ES cell technique has been attracting the attention of fish breeders and molecular biologists due to its possible importance in producing transgenic fish with site-directed integration of foreign gene and in studying gene function in fish (Wakamatsu et al., 2001; Ma et al., 2001; Chen et al., 2002a). Establishment of ES cells and gene targeting technique in cultured marine fish will make it possible to produce genetically modified fish with site-specific mutation, which provides a novel approach for genetic improvements, developmental biology and analysis of gene function in fish, and thus ES cell lines are urgently desired for use in basic and applied research. The present study reports the establishment of an ES-like cell line, LJES1, derived from mid-blastula-stage embryos of sea perch. The LJES1 cells exhibited typical ES-like cell morphology (Evans and Kaufman, 1981). The cell line maintained stable and rapid growth over 150 days of culture with more than 40 passages in the absence of feeder layer cells. The ES cells in various species were successfully cultured using feeder layers technique (Evans and Kaufman, 1981; Wakamatsu et al., 1994; Sun et al., 1995) or using feeder-free system (Nichols et al., 1990; Wurst and Joyner, 1993; Hong et al., 1996). The present study demonstrated that feeder-free technique was also feasible for the derivation of ES-like cells in sea perch. This feeder-free system may facilitate in vitro manipulation of ES cells and will be applicable to the cultivation of ES cells from other fish species.

LJES1 cells possessed strong alkaline phosphatase (AP) activity which is an indication of pluripotency in mouse ES cells (Wobus et al., 1984; Pease and Williams, 1990) as well as in early stages of mouse embryos (Mulnard and Huygens, 1978). In LJES1 cultures, most of the cells exhibited intensive alkaline phosphatase staining which is similar to other reports (Wakamatsu et al., 1994; Hong et al., 1996), indicating their undifferentiated state. The heterogeneity in AP staining of LJES1 cells showed that a few LJES1 cells underwent spontaneous differentiation and lost pluripotency during culture.

The in vitro differentiation ability is one of important criteria for evaluating the pluripotency of ES cells in other animal species including fish (Martin, 1981; Thomson et al., 1998). The LJES1 cells exhibited the ability to undergo differentiation in vitro into melanocytes, muscle cells, neuron-like cells and unidentified cell type in response to all-*trans* retinoic acid induction. However, the direction of differentiation was only partially controllable by regulating initial plating densities of LJES1 cells. The potential of LJES1 cells to differentiate into the above mentioned cell types under specific conditions suggested that the sea perch ES-like cell line described in the present paper remained pluripotent and in undifferentiated state. Similar observations were made in mouse and medaka (Nakano et al., 1994; Wakamatsu et al., 1994; Hong et al., 1996). However, ultimate proof will have to come from transplantation of LJES1 cells into sea perch blastulae to test their germ line chimera-forming ability.

The euploid karyotype is the most important for ES cells to maintain the ability to form functional germ line chimeras (Bradley et al., 1984). Karyotype analysis revealed that over 60% of the cells possessed a diploid chromosome number of $2n=48$, which was identical with the modal chromosome number of sea perch. This diploid rate is similar to or higher than other reports in other ES cells (Sun et al., 1995; Hong et al., 1996). The successful transfection of LJES1 cells with pCMV-EGFP plasmids points to the possibility for this cell line to be genetically manipulated for gene targeting. The availability of LJES1 cells expressing GFP lay foundation for studying in vivo differentiation of the cells into various tissues in host embryos. Transplantation experiments of GFP-positive LJES1 cells into sea perch embryos are under way.

In summary, a pluripotent ES-like cell line, LJES1, was established from sea perch blastulae, and exhibited in vitro differentiation potential. The availability of sea perch ES-like cells lays the foundation for developing gene targeting technique, and also provides a powerful cellular vehicle for the production of genetically modified stock in this fish.

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