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Development and characterization of cell lines from heart, liver, spleen and head kidney of sea perch *Lateolabrax japonicus*

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Five cell lines (LJHK, LJS, LJL, LJH-1 and LJH-2) were established from the head kidney, spleen, liver and heart of sea perch *Lateolabrax japonicus*. The cell lines LJHK, LJS, LJL, LJH-1 and LJH-2 were subcultured 46, 32, 32, 36 and 34 times in minimum essential medium (MEM) supplemented with foetal bovine serum (FBS), sea perch serum and 10 ng ml^{-1} basic fibroblast growth factor (bFGF). Morphology of primary cultures and subcultures of the five cell lines were observed continuously by microscopy. The suitable temperature for growth was 18 to 30° C for all of these cell lines with the optimum growth at 24° C and a reduced growth rate <18° C. The optimum concentration of FBS was found to be 10% and addition of bFGF to the medium significantly increased the growth rate of the cells. The doubling time of LJS, LJH-1, LJL, LJH-2 and LJHK cells was determined to be 52·7, 54·9, 57, 58·7 and 66 h at a plating density of 1×10^5 cells ml^{-1} at 24° C, respectively. Chromosome analysis revealed that 42, 48, 38, 43 and 45% cells maintained normal diploid chromosome number (48) in the LJH-1, LJH-2, LJHK, LJL and LJS cell lines, respectively. The LJHK cells were successfully transfected with green fluorescent protein (GFP) reporter plasmids and the expression of GFP gene in the cells indicated the possible utility of the cells in gene expression studies. Furthermore, treatment of the LJHK cells with lipopolysaccharide led to increased expression of IL-1β, demonstrating that LJHK cells might be a valuable tool for studying the expression and function of immunomodulatory gene in fishes.

Key words: cell lines; IL-1β; *Lateolabrax japonicus*; sea perch; transfection.

INTRODUCTION

The availability of fish cell lines is crucial for the study of various aspects of cellular physiology, molecular biology, genetics, immunology, endocrinology, nutrition, comparative biology and biotechnology in teleosts (Hightower & Renfro, 1988; Alvarez *et al.*, 1991; Babich & Borenfreund, 1991; Bols, 1991*a*, *b*;

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Bols *et al.*, 1992; Janssens *et al.*, 1994; Fent, 2001). By 1994, >150 cell lines have been established from fishes of which most cell lines are derived from freshwater teleosts (Fryer & Lannan, 1994). In recent years, for purpose of identifying and growing fish viruses in marine fish species, a few cell lines were developed in marine fishes (Bejar *et al.*, 1997; Tong *et al.*, 1998; Chi *et al.*, 1999; Chang *et al.*, 2001; Chen *et al.*, 2003*a*, *b*, 2004*a*).

Sea perch *Lateolabrax japonicus* (Cuvier & Valenciennes) is one of the widely farmed marine fish species in China. In past years, two epithelioid cell lines derived from spleen and heart of the sea perch were already developed for use in virological studies (Tong *et al.*, 1998). In this paper, the development and characterization of five cell lines (LJHK, LJS, LJL, LJH-1 and LJH-2) from various tissues of the sea perch are described, and their possible application in studying exogenous gene manipulation and expression in fish cells explored.

MATERIALS AND METHODS

PRIMARY CELL CULTURE

A healthy sea perch weighing *c.* 80 g was obtained from Maidao Marine Fish Hatchery (Qingdao, China) and killed by over-anaesthetizing with 20% urethane. The fish was disinfected with 70% ethanol. The heart, liver, spleen, and head kidney were removed and washed three times with phosphate-buffered saline (PBS), then washed several times in antibiotic minimum essential medium (MEM) containing 400 IU ml^{-1} penicillin and 400 μg ml^{-1} streptomycin (Gibco BRL, Grand Island, NY, U.S.A.). These tissues were minced thoroughly with scissors and transferred to tissue culture dishes containing 5 ml of 0·25% trypsin solution (0·25% trypsin and 0·2% EDTA in PBS) for trypsinization. The contents were gently agitated with a magnetic stirrer at 24° C for 15 min. After settling the larger tissues pieces for 2 min at room temperature, the supernatant containing cells was centrifuged at 800 *g* for 5 min, cells were transferred into complete medium in 25 cm^2 tissue culture flasks and incubated at 24° C in a normal atmosphere incubator. The complete growth medium was MEM supplemented with 20 mM hepes, pH 7·4, antibiotics (penicillin, 100 U ml^{-1}, streptomycin, 100 μg ml^{-1}), 10–20% foetal bovine serum (FBS, Gibco BRL), sea perch serum (1%, pooled homologous serum), basic fibroblast growth factor (bFGF, 10 ng ml^{-1}, Gibco BRL). The primary cells from heart, liver and spleen were cultured in the complete growth medium with 20% FBS, and the primary cells from the head kidney were cultured in complete growth medium with 20% FBS supplemented 100 mg l^{-1} Phytohemagglutinin (PHA, Yi Hua biotech Co., Ltd, Shanghai, China).

MORPHOLOGICAL OBSERVATION

An Olympus inverted microscope (Olympus Optical Co., Ltd) equipped with phase optics was used to observe and photograph living cell cultures every 2–3 days for primary cell cultures and subcultures.

SUBCULTURE AND MAINTENANCE

Confluent primary cell cultures were trypsinized using 0·25% trypsin solution and subcultured at a ratio of 1:3 every 5 or 7 days. The subcultures grew in fresh complete medium (15% FBS). After the initial 10 subcultures, for economy, the concentration of FBS was reduced to 10%.

STORAGE OF CELLS IN LIQUID NITROGEN

For cryopreservation, cell cultures were harvested by centrifugation and suspended in complete medium with 10% dimethyl sulphoxide and 15% FBS. Cryopreservation of the cell cultures was performed as described by Chen *et al.* (2004*a*).

GROWTH OF CELLS

For growth studies, the LJH-1, LJH-2, LJS, LJHK and LJL cells were placed into 25 cm^2 culture flasks at an initial density of 1×10^5 cells ml^{-1} and incubated at 24° C for 5 days. The cells in flasks were trypsinized and counted using a haemocytometer. The experiment was repeated three times.

EFFECT OF TEMPERATURE CELLS PROLIFERATION

To determine the effect of temperature on the proliferation of the cells, LJH-1, LJH-2, LJS, LJHK and LJL cells were cultured in 25 cm^2 tissue culture flasks at 12, 18, 24 or 30° C at an initial density of 1×10^5 cells ml^{-1}. After 5 days, cells were trypsinized and then counted microscopically with a haemocytometer.

EFFECT OF FBS AND BFGF ON CELLS PROLIFERATION

Effect of FBS concentration and bFGF on the proliferation of LJH-1, LJH-2, LJS, LJHK and LJL cells was examined. A total of 1×10^5 cells were inoculated in a 25 cm^2 tissue culture flask and cultured in medium containing various concentrations of FBS (5, 10, 15 and 20%) and bFGF (0 and 10 ng ml^{-1}) for 5 days at 24° C, then the cells were harvested for counting.

CHROMOSOME ANALYSIS

For chromosome analysis, cell cultures (LJH-1, LJH-2, LJS, LJHK and LJL) at passages 17–20 were used. The actively dividing cells were inoculated in colchicine (0·5 μg ml^{-1}) (Sigma) for 4 h in 25 cm^2 culture flask. The cells were harvested by centrifugation (800 *g*, 5 min), single cells were suspended in hypotonic solution of 0.075M KCl for 30 min, and fixed three times in cold Carnoy's fixative, 15 min for each time. Slides were prepared using the conventional drop-splash technique (Freshney, 1994), then air-dried. Chromosomes were stained with 5% Giemsa for 20 min. Finally, chromosomes were observed and counted microscopically.

TRANSFECTION WITH GFP REPORTER GENE

pCMV-EGFP (Clontech) expresses a green fluorescent protein (GFP) under the control of human cytomegalovirus (CMV) promoter. The plasmid DNA was prepared according to the supplier's instruction (Qiagen). Transfection reagent Genejammer (Stratagene) was used for plasmid transfection. In brief, the cell cultures of LJHK were seeded at a density of 1×10^5 cells $well^{-1}$ in 12-well plates individually. Monolayers with 70–80% confluence were transfected with 1 μg pCMV-EGFP in 6 μl Genejammer reagent, and the green fluorescence signals were observed under a fluorescence microscope (CK40, Olympus) equipped with a mercury burner (HBO50W/AC, Osram).

LIPOPOLYSACCHARIDE-INDUCED IL-1β GENE EXPRESSION IN LJHK CELL LINE

LJHK cells were seeded at 2×10^5 cells $well^{-1}$ in six-well plates in serum-free MEM medium supplemented with penicillin and streptomycin. Cells were left undisturbed at

24° C for 24 h to allow for any manipulation-induced gene expression to return to constitutive levels. After this period, 2 ml of fresh medium was added to each well, either without lipopolysaccharide (LPS) (control), or with 500 μg ml^{-1} LPS (Sigma) to achieve a final LPS concentration of 1, 5 and 25 μg ml^{-1}, respectively. The cells were induced for 18 h, then total RNA extraction and cDNA synthesis were performed as described by Chen *et al.* (2004*b*). The polymerase chain reaction (PCR) was conducted by using the following primers designed according to the sequence of sea perch IL-1β cDNA (AY383480): IL-1βN 5′-AGATGCCCAGTGGAATGGACTT-3′ and IL-1βC 5′-GATGTTCAGCCCAGTGGTCTGT-3′ to give a fragment of 569 bp. The PCR reaction (25 μl) consisted of 1 μl of 10 μM of each primer, 4 μl of 2·5 mM of each dNTP, 0·25 U of Taq polymerase (Takara) and 1 μl of cDNA as template. The PCR was run as follows: initial incubation at 94° C for 2 min, followed by 35 cycles of 94° C, 10 s; 55° C, 30 s; 72° C, 2 min, with a final extension of 10 min at 72° C. Expression of 18S rRNA was used as internal control. The primers SP-18SN1 (5′-GGCAGCGTCCGGGAAACCAAATTC-3′) and SP-18SC1 (5′-CCACCC ACA-GAATCGAGAAAGAGC-3′) were used for amplifying 18S rRNA. A fragment of 196 bp was amplified.

STATISTICAL ANALYSIS

Data were expressed as mean ± S.E. The number of cells was analysed with independent-samples test. A value of $P < 0{\cdot}05$ was considered as statistically significant. The statistical analysis was computed using SPSS software.

RESULTS

MORPHOLOGY OF CELLS DERIVED FROM HEART, HEAD KIDNEY, SPLEEN AND LIVER

The cells migrating from the heart covered the bottom of the flask in 4–5 days mainly as epithelioid and fibroblast-like cells. The fibroblast-like cells were more easily brushed off the bottom of flask by trypsinization using 0·25% trypsin solution than epithelioid cells. Consequently, two cell lines, LJH-1 and LJH-2, were developed from the respective cell types after 4–5 passages [Fig. 1(a), (b)].

The cells from the head kidney appeared as either single cells or clumps following incubation with PHA for 2–3 days after plating [Fig. 1(c)]. In initial culture, confluent cultures consisted of two morphologically cells, large rounded cells with clear vesicles around the nuclear area in the low number culture and small rounded cells with clear edges forming the major component of the culture [Fig. 1(d)]. In subculture, within 1 day after attachment, cells were very flat, circular in shape and some with ruffled edges, commonly with vacuolated cytoplasms [Fig. 1(e)]; with continuous incubation, cells became to fibroblast-like morphology and grew into confluent monolayers [Fig. 1(f)].

The cells migrating from spleen and liver completely covered the bottom of flask within a week and 2–3 weeks, respectively, and were mainly fibroblast-like cells. Elongated or fusiform fibroblastic cells, with ovoid nuclei and filiform processes [Fig. 1(g), (h)] form the major component of the culture.

STORAGE AND THAWING OF CELLS

LJH-1, LJH-2, LJS, LJHK and LJL cells in different passages were stored in liquid nitrogen. The cells cryopreserved in liquid nitrogen for 1–2 years

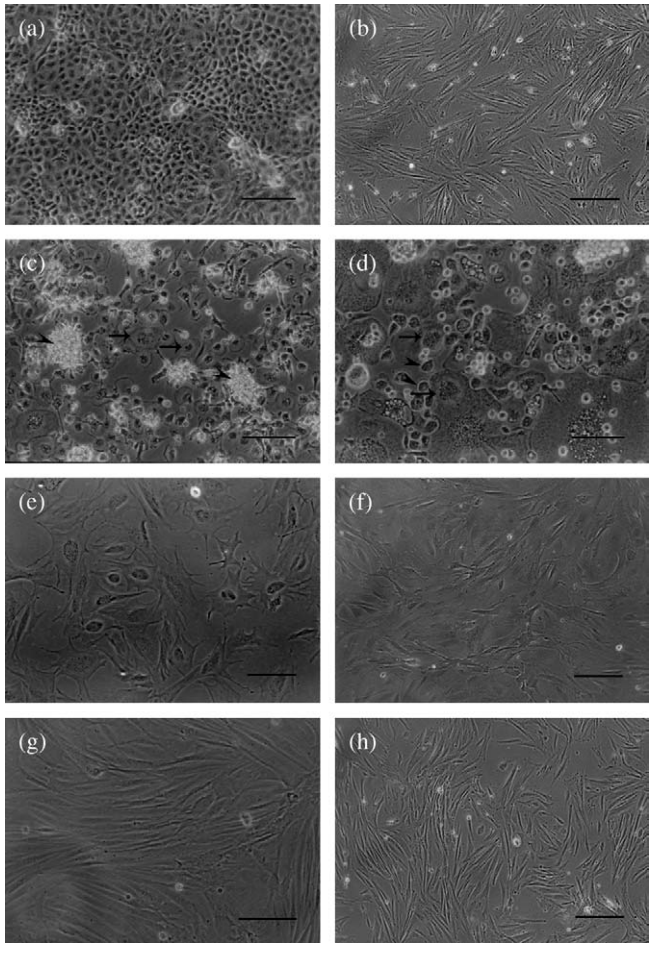

FIG. 1. Morphology of cells derived from sea perch: (a) epithelioid cells (LJH-1) from the heart at passage 12, (b) fibroblast-like cells (LJH-2) from the heart at passage 16, (c) primary LJHK cells from the head kidney activated by PHA in 2–3 days after plating, single cells with small rounded shape and clear edges (⟶) or as clumps of cells (➤), (d) in initial culture (7–8 days), one of a low number of LJHK cells from the head kidney with large rounded cell bodies and clear vesicles around the nuclear area (⟶), another of small rounded cells with clear edges (➤), (e) LJHK cell cultures from the head kidney at passage 15, with very flat, circular shape, large nucleolus and ruffled edges, (f) LJHK cell cultures from the head kidney at passage 15, after confluent monolayers, cells morphology was predominantly fibroblast-like, and fibroblast-like cells (LJS) from (g) the spleen and (h) the liver at passage 18. Bar: 100 μm.

retained viability well after thawing, the viable cells were seeded into flasks and could be grown to confluence within 3–4 days.

GROWTH OF CELLS

The doubling time was determined to be 52·7, 54·9, 57, 58·7 and 66 h for the LJS, LJH-1, LJL, LJH-2 and LJHK cells at a plating density of 1×10^5 cells ml^{-1} at 24° C, respectively, using the formula described elsewhere (Wang & Belosevic,

1994). The growth curves of LJH-1, LJH-2, LJS, LJHK and LJL cells are shown in Fig. 2.

EFFECT OF TEMPERATURE ON CELL PROLIFERATION

The LJH-1, LJH-2, LJS, LJHK and LJL cells exhibited different growth at different temperatures [Fig. 3(a)]. The number of LJH-1, LJH-2, LJS, LJHK and LJL cells increased as culture temperature increased, when the temperature was between 18 and 24° C. Almost no change in cell number was observed at 12° C in any cell line, and the number of cells cultured at 30° C was less than that at 24° C.

EFFECT OF FBS AND BFGF ON CELL PROLIFERATION

The growth of LJH-1, LJH-2, LJS, LJHK and LJL cells was highly dependent on FBS concentration in the culture medium. Cells exhibited almost no proliferation at 5% concentration of FBS, however, proliferation greatly increased with the increase of FBS concentrations to 10% [Fig. 3(b)]. The addition of bFGF stimulated the proliferation of LJH-1, LJH-2, LJS, LJHK and LJL cells, but the absence of bFGF significantly decreased the proliferation of these cells [Fig. 3(c)].

CHROMOSOME ANALYSIS

Chromosome morphology of LJH-2 cell is shown in Fig. 4. The LJH-1, LJS, LJHK and LJL cell lines had similar chromosome morphology to that of LJH-2. The results of chromosome counts of 100 metaphase plates revealed that the number of chromosomes in LJH-1, LJH-2, LJS, LJHK and LJL cells ranged from 27 to 96, 32 to 112, 24 to 89, 29 to 76 and 25 to 103, respectively (Fig. 5). Both heteroploidy and aneuploidy were observed in the five cell lines though they were small in proportion. Nevertheless, the modal number of chromosomes for all of the cell lines was 48 (Fig. 5), and 42% LJH-1, 48% LJH-2, 45% LJS, 38% LJHK and 43% LJL cells contained 48 chromosomes, respectively.

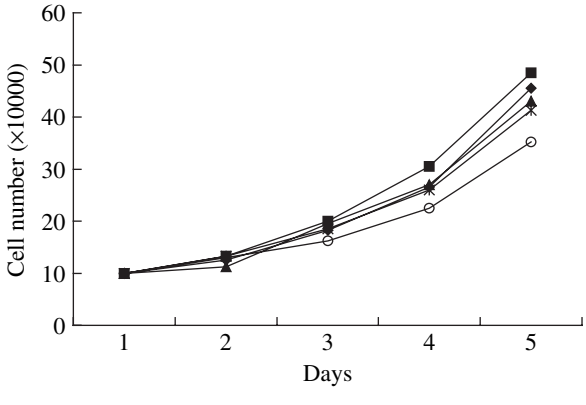

FIG. 2. The growth curves of LJH-1 (–✳–), LJH-2 (–◆–), LJS (–■–), LJHK (–○–) and LJL (–▲–) cells.

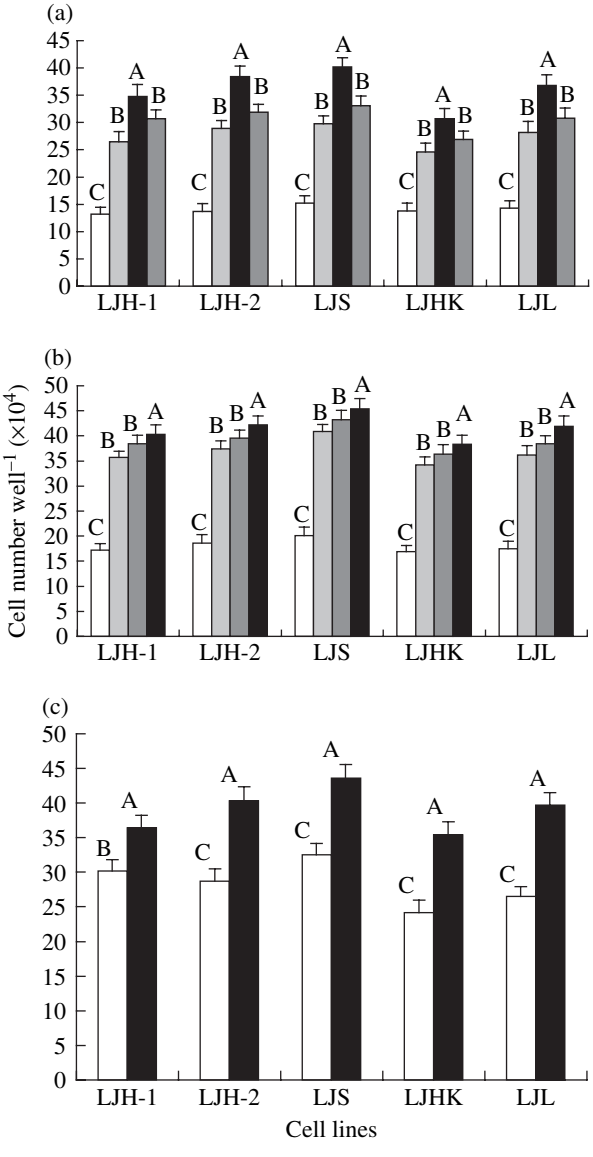

FIG. 3. Effect of (a) temperature (□, 12° C; ▒, 18° C; ■, 24° C; ▓, 30° C), (b) foetal bovine serum (FBS) (□, 5%; ▒, 10%; ▓, 15%; ■, 20%) and (c) basic fibroblast growth factor (bFGF) (□, 0 ng ml^{-1}; ■, 10 ng ml^{-1}) on the growth of cell lines LJH-1, LJH-2, LJS, LJHK and LJL. Values having different capital letters above the column were significantly different ($P < 0{\cdot}05$). Values are means ± S.E. ($n = 3$).

TRANSFECTION WITH GFP REPORTER GENE

LJHK cells were successfully transfected with pCMV-EGFP by using Genejammer reagent according to the manufacturer's instructions. The expression of EGFP in LJHK cells could be detected as early as 24 h after transfection [Fig. 6(a)], indicating that CMV promoter can drive the expression of EGFP gene in LJHK cells.

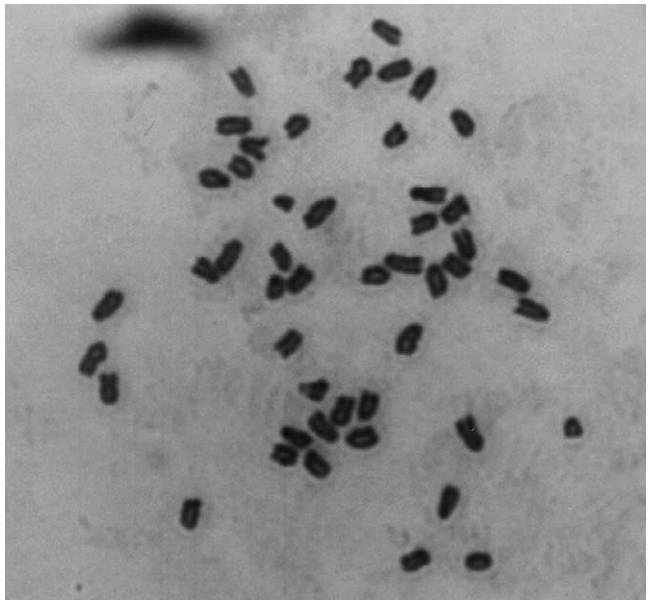

FIG. 4. Giemsa-stained metaphase chromosomes from LJH-2 cells.

EFFECT OF LPS TREATMENT ON IL-1β GENE EXPRESSION IN LJHK CELLS

Reverse transcriptase-PCR demonstrated the presence of IL-1β transcripts in normal LJHK cells (Fig. 7). The treatment of LJHK cells with LPS (1, 5 and 25 μg ml^{-1}) significantly increased the expression of IL-1β in LJHK cells. The stimulating effect of the highest concentration LPS (25 μg ml^{-1}), however, was not as strong as lower concentration LPS (1 and 5 μg ml^{-1}).

DISCUSSION

In recent years, many problems have been arisen within the aquaculture industry in China. Viruses and pollution, for example, have caused disease and death in marine fishes and resulted in a huge amount of economic loss. Fish cell lines are increasingly playing an important role in toxicological and diagnostic studies of viruses, however, at present only a few cell lines have been established from marine fishes (Bejar *et al.*, 1997; Tong *et al.*, 1997, 1998; Chang *et al.*, 2001; Kang *et al.*, 2003; Chen *et al.*, 2003*a*, *b*, 2004*a*). Usually, a single cell line is just susceptible to one or a few kinds of viruses, so the development of more cell lines is necessary. Development of multiplicate cell lines from various tissues of marine fishes will be desirable for developing cell models for study of cellular physiology, molecular biology, genetics, immunology, endocrinology, nutrition, comparative biology and biotechnology in teleosts.

It was reported that two cell lines, SPS and SPH, from spleen and heart of sea perch were established and were epithelial cells (Tong *et al.*, 1998). Here, the establishment of five cell lines, LJH-1, LJH-2, LJS, LJHK and LJL, derived

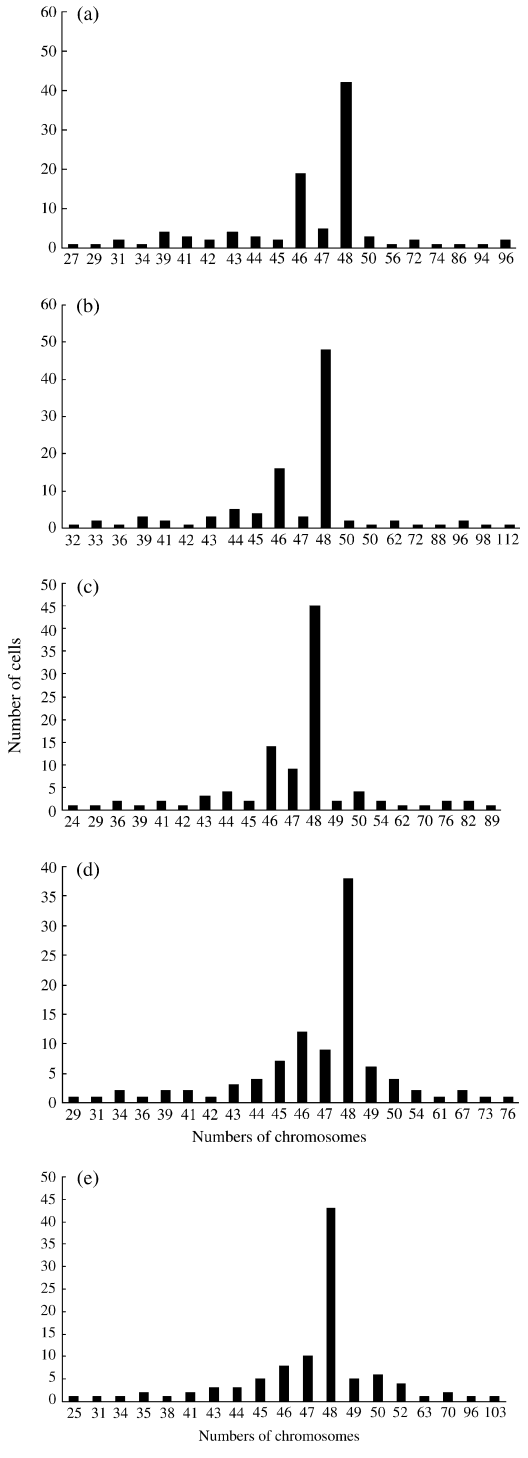

FIG. 5. Frequency distribution of chromosomes in five cell lines from sea perch: (a) LJH-1, (b) LJH-2, (c) LJS, (d) LJHK and (e) LJL.

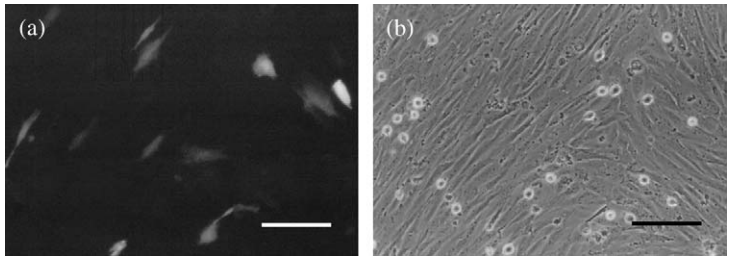

FIG. 6. (a) Green fluorescent protein (GFP) expression in transfected LJHK cells at passage 16 and (b) non-fluorescent control.

from the heart, spleen, head kidney and liver of sea perch, respectively, is reported. All cell lines maintained stable growth in a single medium supplemented with growth factor, bFGF. Initially, cells derived from the heart were mixtures of fibroblastic-like and epithelial cells, however, after several subcultures, fibroblastic-like and epithelial cells transformed into two cell lines, LJH-1 (an epithelial cell line) and LJH-2 (a fibroblastic-like cell line). Cells derived from spleen and liver were all fibroblastic-like with elongated or fusiform shape.

The FBS is essential for survival and optimal growth of cells from sea perch. In primary cell cultures, FBS at high concentrations (20%) is favourable for cells growth and attachment. After subculture, the replication rate of LJH-1, LJH-2, LJS, LJHK and LJL cells increased as the FBS concentration increased from 5 to 20%. These observations are similar to previous reports on the establishment of cell lines from other marine fishes (Kang *et al.*, 2003; Lai *et al.*, 2003), however, the concentration of FBS was reduced to 10% because it reduced the cost of cell maintenance.

bFGF is a potent mitogen for embryonic stem cells derived from medaka *Oryzias latipes* (Temminck & Schlegel) (Hong & Schartl, 1996) and sea perch (Chen *et al.*, 2003*a*), lymphoid cells from grass shrimp *Penaeus monodon* (Hsu *et al.*, 1995), embryonic cells from Japanese flounder *Paralichthys olivaceus* Temminck & Schlegel (Chen *et al.*, 2004*a*) and various cell types in culture from mammals (Matsui *et al.*, 1992). In the present study, bFGF was found to stimulate proliferation of various cells from sea perch.

Karyotype analysis revealed all of the cell lines possessed a modal chromosome number of $2n = 48$, which were identical with the modal chromosome number of embryonic stem cell from sea perch (48) (Chen *et al.*, 2003*a*).

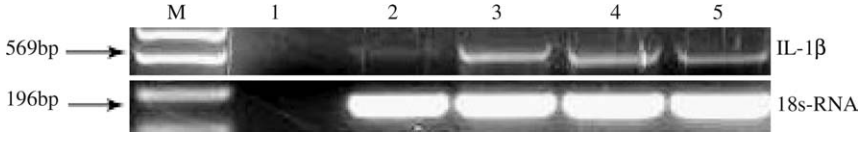

FIG. 7. Constitutive and LPS-induced expression analysis of IL-1β in LJHK cells. RT-PCR was conducted for 35 cycles using RNA isolated from LJHK cells; 18S rRNA was used as RT-PCR control. M, DL2000 markers; 1 no-cDNA control; 2, 3, 4 and 5, products amplified from LJHK cells with stimulation for 18 h with 0, 1, 5 and 25 μg ml^{-1} LPS.

Application of these sea perch cell lines for exogenous gene manipulation was assessed by LJHK cells' ability to express the GFP gene. The present paper demonstrated that LJHK cell lines could be transfected with pCMV-EGFP plasmids, which implied that LJHK cell lines could serve as an *in vitro* system for exogenous gene manipulation. It showed that the CMV promoter could drive expression of GFP gene in LJHK cells.

IL-1β is an immunomodulatory cytokine. Fish IL-1β is homologous to mammalian forms (Secombes *et al.*, 1998). In macrophage-like cell line (RTS11) from rainbow trout *Oncorhynchus mykiss* (Walbaum), study shows that expression of IL-1β gene in RTS11 cells is constitutive, and which is far greater in LPS-induced cells than in non-induced RTS11 cells (Brubacher *et al.*, 2000). The IL-1β gene expressed in LJHK cells, and LPS treatment significantly up-regulated the expression of IL-1β gene in LJHK cells in the present study, which implied the LJHK cells might be used as a cell model for screening immune-regulated functional genes in this fish species.

In conclusion, five cell lines were established from various tissues of sea perch and subcultured more than 30 passages. These cells provide an important tool for the study of exogenous gene manipulation from marine fishes, and lay the foundation for developing cell models for screening immuno-relevant genes.

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