J. Ocean Univ. China (Oceanic and Coastal Sea Research)
DOI 10.1007/s11802-011-1778-5
ISSN 1672-5182, 2011 10 (4): 397-401
http://www.ouc.edu.cn/xbywb/
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Doctor Forum

Establishment of a Spleen Cell Line from Large Yellow Croaker *Pseudosciaena crocea* and its Primitive Application in Foreign Gene Transfection

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(Received June 3, 2010; revised June 25, 2010; accepted March 1, 2011)

Abstract A large yellow croaker, *Pseudosciaena crocea*, spleen (LYCS) cell line was established and the feasibility of using it for foreign gene transfection was evaluaed in this study. Primary culture of LYCS cells was initiated from spleen tissue pieces, which were cultured at 25℃ in Dulbecco's modiced Eagle medium/F12 medium (DMEM/F12, 1:1) (pH7.2), supplemented with 20% fetal bovine serum, carboxymethyl chitosan, chondroitin sulfate, basic fibroblast growth factor (bFGF) and insulin-like growth factor-I (IGF-I). The cultured LYCS cells, in fibroblast shape, proliferated to 100% confluency 20 days later. Chromosome analyses indicated that the LYCS cells exhibited chromosomal aneuploidy with a modal chromosome number of 48 which displayed the normal diploid karyotype of *P. crocea* (6m+6sm+36t, NF=60). A LYCS cell line, with a population doubling time of 48.7 h at passage 60, has been established and subcultured to passage 70. Transgenic feasibility test demonstrated that positive green fluorescence protein (GFP) expression was observed in LYCS cells after pcDNA3.1-GFP plasmid transfection. In conclusion, a continuous foreign gene transfection feasible LYCS cell line has been established successfully. The cell line might serve as a valuable tool for studies of transgenic breeding and has potential applications for different kinds of cytotechnological studies.

Key words large yellow croaker spleen cells; cell line; transgenic feasibility; green fluorescence protein (GFP); *Pseudosciaena crocea*

1 Introduction

Large yellow croaker, *Pseudosciaena crocea*, with good qualities in nutrients and taste, is one of the most important commercial marine fishes in China (Wang *et al*., 2008). Unfortunately, germ degeneration of this species occurs in recent years because of environmental pollution, disease eruption, inbreeding and so on (Wang *et al*., 2006). Therefore, new breeds with high qualities, disease resistance and rapid growth rate need to be developed urgently by transgenic or cytotechnological techniques. Since fish cell lines can be used as ideal tools for *in vitro* studies of transgenic and cytotechnological breeding, it is necessary to develop cell lines of the large yellow croaker (Villena, 2003).

At present, many established fish cell lines come primarily from freshwater and anadromous species (Fryer and Lannan, 1994; Villena, 2003). Only several cell lines have been established from commercial marine fishes, such as sea perch (*Lateolabrax japonicus*) (Ye *et al*., 2006; Chen *et al*., 2007), flounder (*Paralichthys olivaceus*) (Tong *et al*., 1997; Chen *et al*., 2004), turbot (*Scophthalmus maximus*) (Chen *et al*., 2005; Fan *et al*., 2007), groupers (*Epinephelus fuscoguttatus*) (Lai *et al*., 2000; Lai *et al*., 2003; Qin *et al*., 2006; Parameswaran *et al*., 2007; Zhou *et al*., 2007; Wen *et al*., 2008; Wei *et al*., 2009). By now, no cell lines have been established from large yellow croaker *P. crocea*. This study was intended to establish such a cell line and to characterize its feasibility for transgenic studies by using green fluorescence protein (GFP) gene as a reporter.

2 Materials and Methods

2.1 Animals

Healthy large yellow croakers (about 500 g) were obtained from Qingdao Nanshan fish market, Shandong Province, China. And they were maintained in aerated sterile seawater containing 1000 IU mL^{-1} penicillin and 1000 μg mL^{-1} streptomycin at 22-24℃ for 24 h.

2.2 *In vitro* Culture

After euthanized by etherification, large yellow croakers were immersed in 70% ethanol for 1 min. The spleen tissues were collected aseptically, washed once with 70% alcohol and twice with phosphate-buffered saline (PBS),

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and minced into small pieces (about 1 mm^3 in size) by surgical scissors in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (DMEM/F12, 1:1) medium (Invitrogen) (pH 7.2) containing 5% fetal bovine serum (FBS)(Hyclone). Tissue pieces were inoculated into 25 cm^2 cell culture flasks (Corning) (1 mL in each flask) and cultured at 25℃ for about 18 h. Then each flask was added with 5 mL of 20% FBS-containing DMEM/ F12 medium further supplemented with 100μg mL^{-1} carboxymethyl-chitosan (AK Scientific), 40 μg mL^{-1} Chondroitin sulfate (BBI), 10 ng mL^{-1} basic fibroblast growth factor (bFGF)(Peprotech), 40 ng mL^{-1} insulin-like growth factor-I (IGF-I)(Peprotech), 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Lukang). The flasks were incubated at 25 ℃ and the medium was replaced every five days.

Once large yellow croaker spleen (LYCS) cells grew into a confluent monolayer, the cells were subcultured by trypsinisation (Wei *et al*., 2009). From passage 30, the culture medium was changed from 20% FBS-DMEM/F12 into 20% bovine calf serum (BCS) (Hyclone)- DMEM/ F12 medium (Invitrogen) without any supplements.

2.3 Growth Properties

Spleen cells at passage 60 were trypsinised and resuspended in 20% BCS-DMEM/F12 medium as described above. About 1 mL of LYCS cell suspension with a density of 1.8×10^5 cells mL^{-1} was dispensed into each well of two 24-well plates (Corning) and incubated at 25 ℃ in a 5% CO_2 incubator (Heraeus). Three wells of LYCS cells were harvested by trypsinisation and resuspended in 1 mL PBS at 12 h intervals. The number of cells in each well was counted with a cell analysis system (CASY), and the average value of 3 wells at each time was used to plot the growth curve. The population doubling time of the cells was calculated (Fan *et al*., 2007).

2.4 Chromosome Analysis

The passage 60 LYCS cells at the logarithmic phase were treated with 20 μg mL^{-1} of colchicine (Fluka) for 10 h at 25℃. The cells were harvested by trypsinisation and resuspended in 3 mL 0.3% KCl hypotonic solution for 30 min, fixed with Carnoy's solution for 10 min and stained with Giemsa for 40 min. The chromosome analysis of LYCS cells was performed (Fan *et al*., 2007) and chromosome numbers of 300 metaphase LYCS cells were counted under a Nikon E200 light microscope.

2.5 Storage in Liquid Nitrogen

For cryopreservation, passage 40 LYCS cells at the logarithmic phase were harvested by trypsinisation, centrifuged at 1000 g for 10 min, and resuspended in 20% FBS-DMEM/F12 medium supplemented with 20% dimethyl sulphoxide (DMSO) (Amresco). The cell suspensions with a density of $6\text{-}7\times10^6$ cells mL^{-1} were transferred into sterile plastic freeze-tubes (Corning). Then, the tubes were kept successively at 4℃ for 0.5 h, -20 ℃ for 1 h, and -80℃ overnight and finally transferred into liquid nitrogen (-196℃).

The LYCS cells frozen for 60 d were thawed by immediately incubating the cryopreservative tube into a 40℃ water bath for about 1 min and then transferring it to 25 ℃ water bath for 1.5 min to recover to the optimal temperature of LYCS cells. After being centrifuged at 1000 g for 10 min, the cells were suspended in 20% FBS-DMEM/F12 medium and incubated into 25 cm^2 cell culture flasks at 25℃.

2.6 Transfection with pcDNA3.1-GFP

A pcDNA3.1-GFP plasmid containing a cytomegalovirus (CMV) promoter, a SV40 polyadenylation signal, and a neomycin-resistant gene was constructed (Fig.1). The LYCS cells at passage 65 in 20% FBS-DMEM/F 12 medium without antibiotics were seeded at a density of 4×10^5 cells mL^{-1} into a 24-well plate. After 24 h, the cells were transfected by adding a complex of 0.8 μg pcDNA 3.1-GFP plasmid and 2.0 μL Lipofectamine™ 2000 (Invitrogen) diluted with 100 μL DMEM/F12 medium to each well. After incubated at 25℃ in a 5% CO_2 incubator for 4 h, the old medium were replaced with fresh 20% FBS-DMEM/F 12 medium and cells were cultured at 25 ℃ in CO_2 incubator. The green fluore- scence signals were observed every 24 h under a Nikon Eclipse Ti fluorescence microscope. The transfection efficiency was evaluated by calculating the ratio of cells expressing green fluorescence signals to all cells employed for transfection.

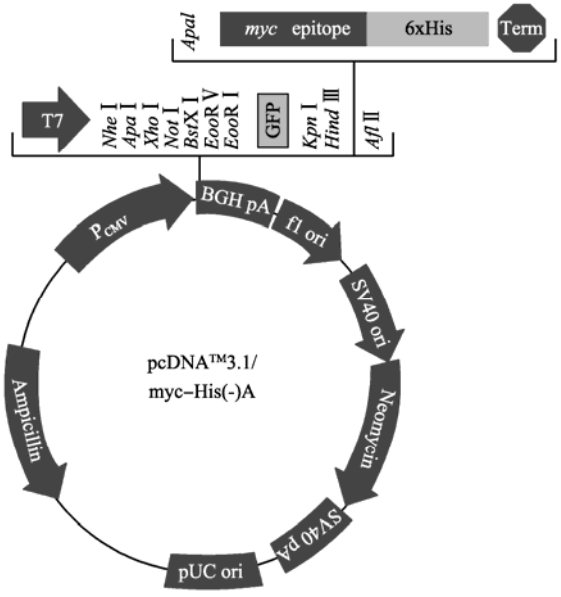

Fig.1 Sketch map of the constructed pcDNA3.1-GFP plasmid.

3 Results

3.1 *In vitro* Culture of LYCS cells

The LYCS cells from *P. crocea* began to migrate from minced spleen tissues 72 h later in primary culture

(Fig.2A). The cells were fibroblastic morphology and proliferated to 100% confluency 20 d later (Fig.2B). The cells grew in 20% FBS-DMEM/F12 medium supplemented with carboxymethyl-chitosan, chondroitin sulfate, bFGF and IGF-I could be subcultured at 4-5 d intervals. To date, the LYCS cells have been subcultured to passage 70 and are still in a good proliferating state (Fig.2C). A continuous large yellow croaker spleen cell line (LYCS-1) has been established.

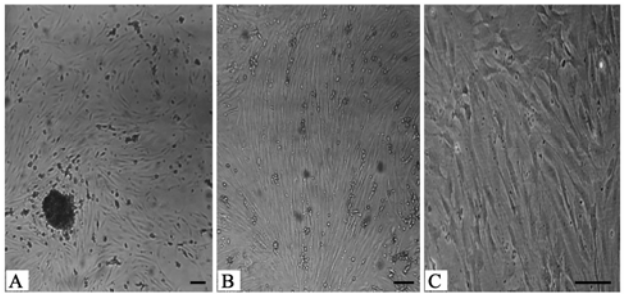

Fig.2 *In vitro* cultured large yellow croaker spleen (LYCS) cells. A, LYCS cells migrated from spleen tissues 72 h after primary culture initiation. B, the confluent monolayer formed by the LYCS cells with a fibroblastic morphology after they were cultured for 20 days. C, subcultured LYCS cells at passage 70. The bar=100 μm.

3.2 Growth Properties of LYCS cells

The growth curve of passage 60 LYCS cells in Fig.3 shows that the LYCS cells were at latent stage in the first day and went into logarithmic stage from day 1 to day 3.5. The cell number remained steady between day 3.5 and day 5 began to decline after day 5. The LYCS cells grew and proliferated at a steady rate and their doubling time was calculated to be 48.7 h at passage 60.

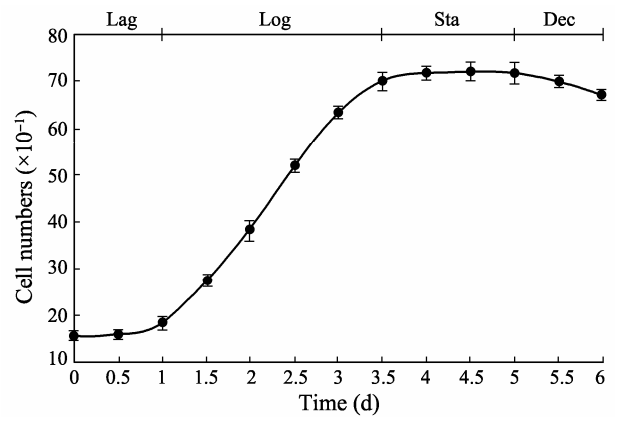

Fig.3 The growth curve of large yellow croaker spleen (LYCS) cells at passage 60. The lag phase (Lag), logarithmic phase (Log), stationary phase (Sta), and decline phase (Dec) are shown.

3.3 Chromosome Analysis of LYCS Cells

The results of chromosome count of 300 metaphase LYCS cells at passage 60 revealed that the chromosome numbers varied from 30 to 62 with a modal chromosome number of 48, which accounted for 55% of the metaphase cells (Fig.4A). The distribution was asymmetrical and both aneuploidy and heteroploidy appeared in the LYCS cell line. The metaphase chromosomes (Fig.4B) with a normal diploid number of 48 displayed the normal karyotype morphology, consisting of 3 pairs of metacentrics (m), 3 pairs of submetacentrics (sm), and 18 pairs of telocentrics (t): 2n=6m+6sm+36t, NF=60 (Fig.4C).

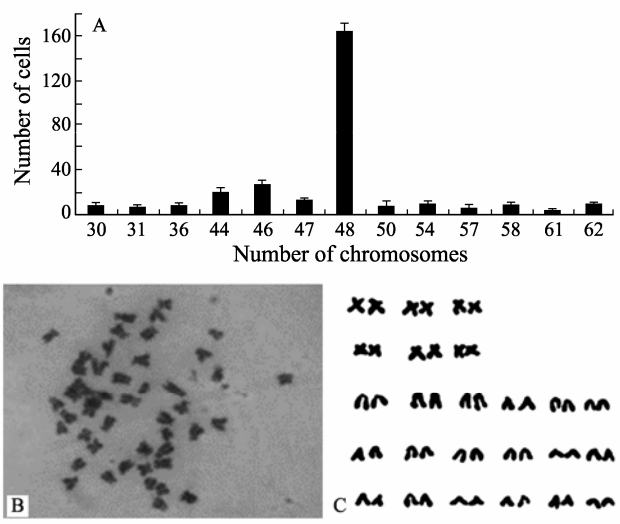

Fig.4 Chromosome analysis of large yellow croaker spleen (LYCS) cells at passage 60. A, chromosomal aneuploidy of LYCS cells with chromosome numbers ranging from 30 to 62. About 55 % of LYCS-1 cells has a chromosome number of 48. B, chromosomes from a LYCS-1 cell with a diploid number of 48; C, the diploid karyotype of LYCS-1 cells, 2n=48, 6m+6sm+36t, NF=60.

3.4 Cryopreservation and Recovery of LYCS Cells

The thawed cells from cryopreserved passage 40 LYCS cells proliferated to confluency in 4-5 d. The morphology and proliferation ability of LYCS cells were the same before and after cryopreservation. The cell morphology and the formed monolayer of thawed passage 40 LYCS cells are shown in Fig.5.

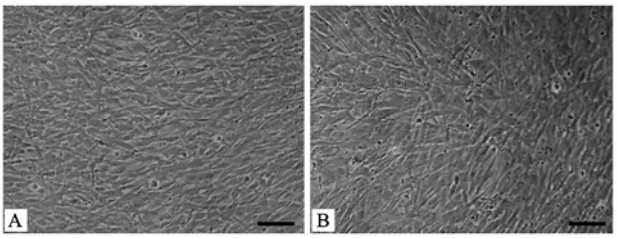

Fig.5 Large yellow croaker spleen (LYCS) cells at passage 40 before and after cryopreservation. A, The monolayer of passage 40 LYCS cells before cryopreservation. B, The monolayer of passage 40 LYCS cells thawed 4 d later. The bar =100 μm.

3.5 LYCS Cells Transfected with pcDNA3.1-GFP

After the LYCS cells were transfected with pcDNA3.1-GFP plasmid by Lipofectamine™ 2000, green fluorescence signals could be detected 48 h later (Fig.6).

This indicates the LYCS cell line has the feasibility for transfection by using Lipofectamine™ 2000 and CMV promoter, and can derive positive expression of the GFP gene in transfected LYCS cells. The transfection efficiency was calculated to be about 8%.

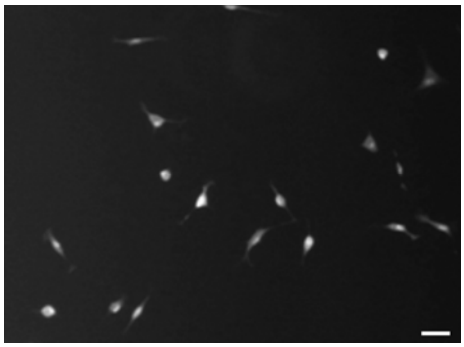

Fig.6 Fluorescent image of LYCS cells transfected by pcDNA3.1-GFP plasmid 48 h later. The positive green signals derived from expression of GFP are shown. The bar =100 μm.

4 Discussion

For studies of transgenic and cytotechnological breeding, a continuous LYCS cell line from large yellow croaker was established. This cell line was subcultured to passage 70 and transgenic feasibility test demonstrated that the LYCS cells could be a valuable tool for transgenic and cytotechnological breeding investigations.

To initiate the primary culture of LYCS cells, *P. crocea* spleen tissues were minced into pieces and cultured in flasks without any digestion. This is because the spleen tissues are loose enough for cells to migrate out and enzyme digestion will damage the cells (Ian Freshney, 2004). Similar methods have been reported in the establishment of sea bass, *Lates calcarifer,* kidney and spleen cell lines (Sahul Hameed *et al*., 2006; Parameswaran *et al*., 2007).

To induce *in vitro* cell proliferation, attempts were made to replenish the culture medium with different supplements (Fan and Wang, 2002; Fan *et al*., 2003; Yu *et al*., 2005; Jin *et al*., 2008; Wei *et al*., 2009). Among them, carboxymethyl chitosan (a kind of chitosan derivative) and chondroitin sulfate (a kind of glycosaminoglycan of extra cellular matrix) were both found to have a positive effect on cell attachment and growth (Fan and Wang, 2002; Fan *et al*., 2007a). Growth factors such as bFGF and IGF-I have important regulatory abilities in cell proliferation, migration and differentiation, and similar effects of the supplements on acceleration of cell attachment and growth were reported in the establishment of fin cell line from Brown-marbled grouper, *Epinephelus fuscoguttatus,* embryonic stem cell line from the sea perch, primary culture of embryonic cells from shrimp, *Penaeus chinensis,* and cartilage cells from shark, *Heterodortus japonicus* (Iida *et al*., 1998; Fan *et al*., 2002; Yu *et al*., 2005; Chen *et al*., 2007; Wei *et al*., 2009). Addition of all these supplements in the culture medium was probably the key premise of inducing cell proliferation in primary culture and successful subculture of LYCS cells in this study.

The fibroblastic cells of the established LYCS cell line proliferated actively during subculture and had a population doubling time of 48.7 h at passage 60. The population doubling time was similar to that of grouper fin cell line (Wei *et al*., 2009), higher than that of seabream fin cell line (Bejar *et al*., 1997), and lower than that of turbot fin cell line (Fan *et al*., 2007b). This implies that the LYCS cell line still maintains active proliferating ability and a continuous LYCS cell line has been successfully established.

Karyotype analysis showed that the LYCS cells at passage 60, exhibiting chromosomal aneuploidy, still had a modal chromosome number of 48. The diploid karyotype of 2n=6m+6sm+36t (NF=60) of the LYCS cells was identical to that of *P. crocea* reported (Wang *et al*., 2006). This indicates that the continuous LYCS-1 cell line is a firstly established large yellow croaker cell line.

To examine the feasibility of using the LYCS cell line as an *in vitro* tool for transgenic and cytotechnological breeding investigation, transfection with pcDNA3.1-GFP plasmids was performed. The positive expression of transfected GFP implies that the LYCS cell line can be utilized for transgenic study, and transfection by the pcDNA3.1-GFP plasmids containing a CMV promoter, a SV40 polyadenylation signal, and neomycin resistant gene via Lipofectamine 2000 is feasible. Successful GFP transfection was also reported in other marine fish cell lines, but the transfection efficiency was not mentioned in these cell lines (Chen *et al*., 2003; Qin *et al*., 2006; Parameswaran *et al*., 2007). The feasibility for GFP transfection means LYCS cell line can be applied for transgenic and cytotechnological breeding of large yellow croakers.

In conclusion, a continuous large yellow croaker spleen cell line (LYCS-1) was established and its transgenic feasibility was evaluated. The LYCS cell line could be used as a useful tool for studies of transgenic and cytotechnological breeding techniques and has potential applications in high quality large yellow croaker breeds development.

Acknowledgments

This study was supported by grants from the National High Technology Research and Development Program (‘863’ Program) of China (Grant Nos. 2006AA10A401 and 2006AA09Z406). We thank Mr. Wei Yunbo and Mr. Xu Xiaohui for their technical assistance.

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(Edited by Ji Dechun)