Acta Oceanol. Sin., 2010, Vol. 29, No. 2, P. 81-87
DOI: 10.1007/s13131-010-0025-z
http://www.hyxb.org.cn
E-mail: hyxbe@263.net

Development and characterization of a cell line from the embryos of half smooth tongue sole (*Cynoglossus semilaevis)*

SHA Zhenxia[1§], REN Guocheng[1,2§], WANG Xianli[1,3§], WANG Na[1], CHEN Songlin[1*]

[1] Key Laboratory of Sustainable Development of Marine Fisheries, Ministry of Agriculture, Yellow Sea Fisheries Research Institute, Chinese Academy of Fisheries Sciences, Qingdao 266071, China

[2] College of Biology, Shandong Normal University, Jinan 250014, China

[3] College of Marine Life, Ocean University of China, Qingdao 266003, China

Received 31 December 2008; accepted 15 September 2009

Abstract
A new cell line, CSEC, has been successfully established from embryos at gastrula stage of a cultured marine fish, half smooth tongue sole (*Cynoglossus semilaevis*). CSEC cells grow actively and stably more than 50 passages for over 200 d in DMEM medium supplemented with 15% FBS (fetal bovine serum), 2.5 ng/cm^3 bFGF (basic fibroblast growth factor), 1 ng/cm^3 LIF (leukemia inhibitory factor) and 50 mmol/dm^3 2-ME (2-mecaptoethanol). The cells grew well in the temperature range of 24–30 °C and the optimal growth temperature was 24 °C. FBS and bFGF concentrations are the two key components for CSEC cells proliferation. Chromosome analysis reveals that CSEC cells have a normal diploid karyotype with 2n=42t. The significant fluorescent signals were observed in CSEC after transfection with the GFP reporter gene, suggesting that the CSEC cell line can be used as a useful tool for transgenic and genetic manipulation studies. CSEC cells showed the cytopathic effect (CPE) after infection with lymphosystis disease virus (LCDV) in 2 d. Moreover, the LCDV particles can be observed in the cytoplasm of virus–infected cells by electron microscopy. It suggests that CSEC could be potentially used for the study of aquatic virus.

Key words: embryonic cell line, half smooth tongue sole, karyotype, GFP, LCDV, CSEC

1 Introduction

Cell lines are powerful tool for studying epidemiology, molecular carcinogenesis, toxicology and functional genomics in fish (Wise et al., 2002; Bahich and Borenfreund, 1991; Bols and Lee, 1991; Hightower and Renfro, 1988). Cell line is also essential for isolating and identifying fish viruses (Kang et al., 2003). A large number of cell lines have been developed in fresh water teleost (Fryer and Lannan, 1994), but relatively few cell lines were developed in marine fish (Buonocore et al., 2006; Sahul et al., 2006; Chen et al., 2005, 2004; Chen, Sha et al., 2003; Chen, Ye et al., 2003; Chang et al., 2001; Chi et al., 1999; Bejar et al., 1997).

Half-smooth tongue sole (*Cynoglossus semilaevis*) is a newly exploited and high-valued fish, and has recently emerged as an increasingly farmed species in northern China (Wan et al., 2004). Over the years, there has been a steady demand for tongue sole in China and been doing well in the international market. However, intensive aquaculture of this fish species has resulted in outbreaks of viral diseases with high mortalities. Little is known about the epidemiology of viral infections. Due to the lack of suitable cell lines in this species, the characterization of the virus and function analysis of resistant genes in half smooth tongue sole is still flank. Furthermore, the sex determination system of this species is female heterogametic (ZZ/ZW; Zhuang et al., 2006). The tongue sole shows a typical sexual dimorphism, with females being 1–2 times larger at first maturity and growing to a larger size than males. Genetic studies in this species have been carried out in recent years such as the development of SSR markers (Liu et al., 2008, Liao et al., 2007) and female-specific AFLP markers (Chen et al., 2007). However, most of them have no significant hit to well-characterized genes. So far, identification of sex determination genes and functional analysis in

Foundation item: The State "863" High-Technology Research and Development Project of China under contract Nos 2006AA09Z406 and 2006AA10A401; the National Natural Science Foundation of China under contract No. 40376047; the Taishan Scholar Project of Shandong Province and MOA for returned scientists from abroad.
§Contributed equally.
*Corresponding author, E-mail: chensl@ysfri.ac.cn

this species are still underdeveloped. The present study describes the development and characterization of an embryonic cell line from half smooth tongue sole as the potential basis for gene function analysis and isolation of fish virus.

2 Materials and methods

2.1 *Primary cell culture*

Gastrula-stage embryos of half smooth tongue sole were harvested approximately 12 h after fertilization and prepared for cell culture. For each culture, a total of 50–70 embryos as one group were disinfected with 70% ethanol and washed with PBS buffer. The chorions were torn with fine forceps for releasing the cell mass. Single cells were obtained by gentle pipetting. After several washes, the cells were transferred into gelatine-coated 24-well tissue plates. The complete growth medium M_0 was used for primary cell culture. The prescription of M_0 was that Dulbecco's Modified Eagles Medium (DMEM) supplemented with 20 mmol/dm^3 Hepes, pH 7.5, antibiotics (penicillin, 100 U/cm^3, Streptomycin, 100 μg/cm^3; Gibco BRL), fetal bovine serum (FBS, 15%), sea perch serum (SPS, 0.5%), basic fibroblast growth factor (bFGF, 2 ng/cm^3; Gibco BRL), human recombinant leukemia inhibitory factor (hLIF, 1 ng/cm^3; Pepro Tech EC, London) and 2-mecaptoethanol (2-ME, 50 mmol/dm^3; Gibco BRL). Cells were cultured at 24 °C in a normal atmosphere incubator. Medium was changed every two or three days.

2.2 *Subculture and maintenance*

When the primary cell cultures were reached 95% confluence, the cells were subcultured at a ratio of 1∶2 according to the standard trypsinization method. Trypsin-EDTA solution (Trypsin 0.05%, EDTA 0.02%, and NaCl 0.08%) was used for trypsinization. Cryopreservation of CSEC cells was performed as described (Chen, Sha et al., 2003). In brief, cell cultures were suspended in complete medium with 10% dimethyl sulfoxide (DMSO) and 20% FBS, and poured into 2-ml cryovials. Then the filled cryovials were placed in a freezing container (Nalgene, USA) and frozen at –80 °C for 4 h, and finally transferred to liquid nitrogen for long-term storage.

2.3 *Cell growth characteristics*

2.3.1 *Effect of temperature on cell growth*

To determine the optional conditions of CSEC, temperature preference on proliferation of CSEC cells was examined. Fifty thousand cells were inoculated in one well of 12-well plate and incubated at 15, 18, 24 or 32 °C for growth test. After 4 d, cell density was measured microscopically by using a hemocytometer (Brand, Germany). The experiments were repeated four times.

2.3.2 *Effect of component of media on cell growth*

The optional medium with different component concentration for cell proliferation was determined. Six media named M_0, M_1, M_2, M_3, M_4 and M_5, respectively. The components of each medium were shown in Table 1. Fifty thousand CSEC cells with complete growth medium were plated to each well of 12-well plate and incubated at 24 °C. After 16–24 h, the old medium was removed and each medium previously described was added. After 3 d intervals, cells were detached and counted by using a hemocytometer (Brand, Germany). The experiments were repeated four times.

Table 1. The component of medium M_0, M_1, M_2, M_3, M_4, M_5

Medium	FBS concentration(%)	2-ME concentration/mmol·dm^{-3}	bFGF concentration/ng·cm^{-3}	LIF concentration/ng·cm^{-3}
M_0	15	50	2.5	1
M_1	15	50		
M_2	15	50	2.5	
M_3	15	50		1
M_4	7.5	50	2.5	1
M_5	15		2.5	1

2.3.3 *The time of CSEC proliferation*

Fifty thousand CSEC cells at Passage 47 with complete growth medium were plated to each well of 12-well plate and incubated at 24 °C for up to 96 h. The cell numbers were counted every 12 h and repeatedly three times.

2.4 *Chromosome analysis*

CSEC cells at Passages 40 were used for chromosome analysis. The cells were inoculated in culture flask of 25 cm^2 for 24–36 h. Colchicine was added to the cells to reach final concentration of 0.5 μg/cm^3. After about 4 h, the cells were harvested, treated with 5 ml of hypotonic solution of 0.075 mol/dm^3 KCl for 25 min, and then pre-fixed for 2 min by adding 1 ml of Carnoy's fixative to the above cell solution. After centrifugation, the cell pellet was fixed twice with cold Carnoy's fixative for 15 min each time, then air dried. Chromosomes were stained with 5% Giemsa for 25 min. One hundred cells at metaphase were counted, and chromosome karyotype was analyzed according to the reported method (Levan et al., 1964).

2.5 *Cell transfection with GFP reporter gene*

The ability of the CSEC cells to be transfected was determined using pEGFP-N1 plasmid (Clontech,

Germany), which 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 lipofectamine 2000 (Invitrogen) was used for plasmid transfection. In brief, the cells were seeded at a density of 1.5×10^4 cells per well in 12-well plates individually. After monolayer with 80%-90% confluent, the cells were transfected with 1 μg pEGFP-N1 plasmid in 6 μl lipofectamine 2000 reagent and incubated at 24 °C. After 6 h, the medium was changed with normal medium. GPF expression in the cells was detected under a fluorescence microscope (Nikon eclipse, TE2000-U). The green fluorescence images were obtained by using a microscopy camera on the microscope.

2.6 *Virus susceptibility*

Lymphosystis disease virus (LCDV) were used to detect the susceptibility of the CSEC cells. The virus was isolated from Lymphocystis diseased Japanese flounder as described in previous study (Xu et al., 2000). Two hundred thousand cells per millilitres at Passage 25 was seeded into 25-cm^2 cell culture flasks with 80% confluence. After removal of the medium, 1 ml of virus suspension was inoculated into the cell culture. After 1 h, the virus solution was removed and 3 ml of maintenance medium containing 5% FBS was added. The cells were incubated at 24 °C and observed daily for the appearance of CPE up to two weeks.

For electron microscopy observation, the CSEC cells infected with LCDV were fixed with 2.5% glutaraldehyde in 0.2 mol/dm^3 sodium cacodylate buffer (pH 7.4) for 24 h at 4 °C and then postfixed with 1% osmium tetroxide in 0.2 mol/dm^3 sodium cacodylate buffer for 1 h. The infected cells were then embedded in epoxy resin, sectioned, and stained with 2% uranyl acetatelead citrate and examined under a Philips 201C electron microscope.

2.7 *Statistical analysis*

Data were expressed as mean±SD. The cell amount was analyzed with Independent-Samples Tests. A value of P <0.05 was considered as statistically significant. The statistical analysis was computed using SPSS software.

3 Results

3.1 *Establishment of cell line from embryos*

The single cells from 50–70 embryos of *Cynoglossus semilaevis* at the gastrula stage were isolated and inoculated in one well of 24-well plate. These cells with a diameter of about 10–25 μm evenly attached to the surface of the well and then moved to form many dense, aggregates within the first 12–24 h after inoculation. Two or three days later, the cells grew to confluence in the well, and subculture could be performed. The cell division occurred very rapidly and subculture had been done once every three or four days. In early subcultures of up to ten passages, both epithelial-like and fibroblast-like cells were present. After 20 subcultures, epithelial-like cell became the predominant cell type. The CSEC cultures at Passage 17 were shown in Fig.1. So far, the CSEC cells have been cultivated for more than 200 d with more than 50 passages. The cryopreservation tests demonstrated that CSEC cells could undergo cryopreservation in liquid nitrogen with a survival rate of about 70%.

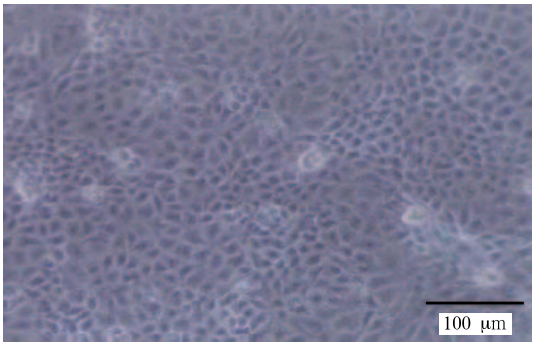

Fig.1. Morphology and culture of CSEC cells at Passage 17. The bar is 100 μm.

3.2 *Effect of temperature on growth of CSEC cells*

CSEC cells were able to grow at incubation temperatures between 15 and 32 °C(Fig. 2). The growth of CSEC cells increased when temperature was at 20 and 24 °C, the optimal growth temperature was 24 °C. The growth rate of the cells cultured at 28 °C began to decrease although cells grew well. The growth rate

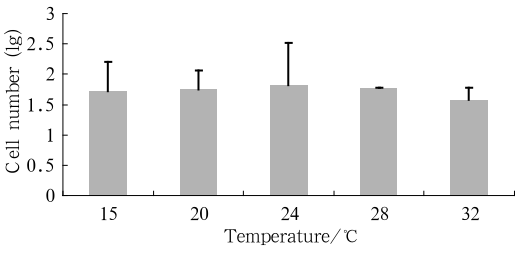

Fig.2. Effect of temperature on the growth and proliferation of CSEC cells. Values were expressed as mean±SE (n=4).

declined markedly at 32 °C and no obvious cell growth was observed at 15 °C (P <0.05).

3.3 *Effect of FBS concentration, bFGF and the other components of medium on growth of CSEC cells*

The growth of CSEC cells was influenced by different components of medium. To determine the significant factor for the growth of this cell line, six kinds of media with different components were designed and shown in Table 1. The effect of those components on proliferation of CSEC cells were shown in Fig.3. The growth rate of CSEC cells in M_4 containing 7.5% FBS was the lowest in the other media containing 15% FBS (P <0.05). While the absence of bFGF decreased significantly proliferation of CSEC cells (M_1 and M_3) (P <0.05), the absence of 2-ME and LIF almost didn't influence the growth of CSEC cells (M_2 and M_5). Taken together, FBS and bFGF concentrations are the two key components for CSEC cell proliferation.

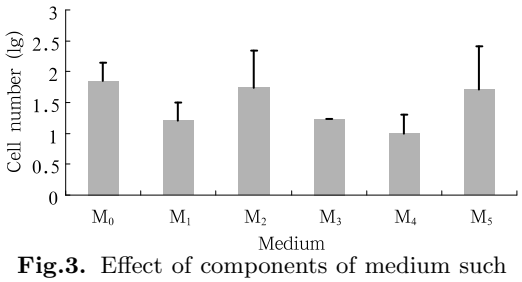

Fig.3. Effect of components of medium such as FBS, bFGF, 2-ME and LIF on growth of CSEC cells. The cells had been cultured for 3 d. Values were expressed as mean±SE (n=4).

3.4 *The time of CESC proliferation*

CESC cells were evenly attached to the surface of the well within the 5 h after inoculation. The cells grew fast and underwent exponential growth from 24 h to less than 60 h. Then the growth rate slowed as a result of nutrient of media depletion and accumulation of toxic products. The cell growth reached stationary phase at 96 h. So the time of CESC proliferation was about 24 h (Fig. 4).

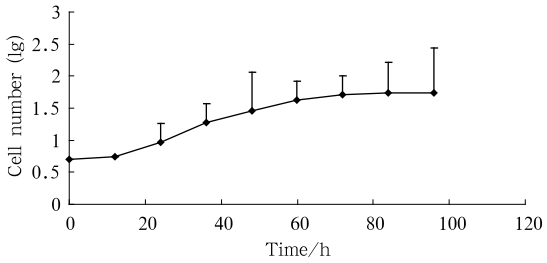

Fig.4. Growth curve of CSEC at Passage 47. Value are means±SE (n=3).

3.5 *Karyotypic analysis*

The results of chromosome counts of 100 metaphase plates from CSEC cells at Passage 45 revealed that the chromosome numbers varied from 18 to 92; nevertheless, the modal number of chromosome

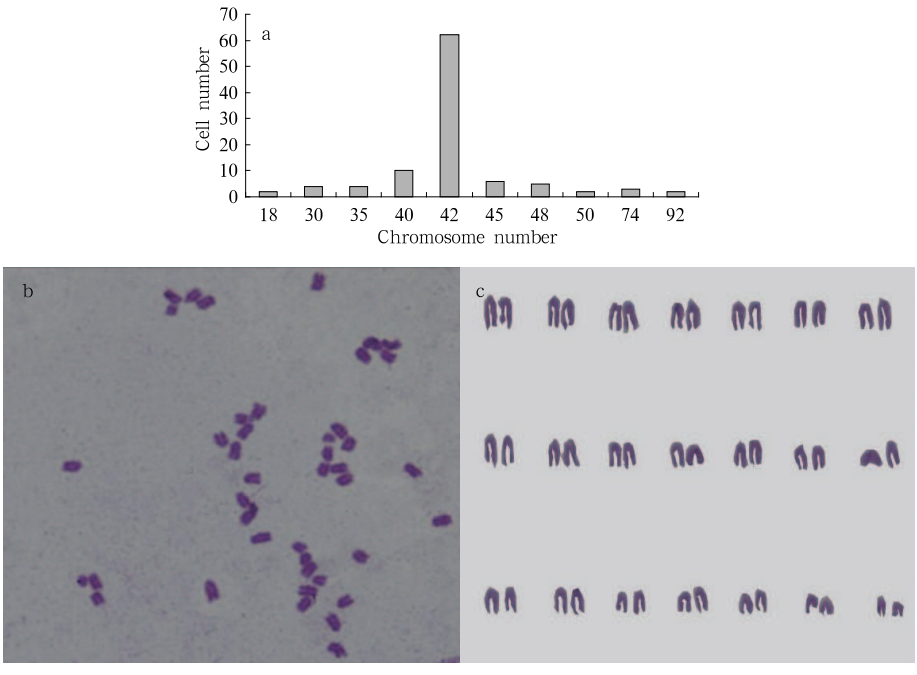

Fig.5. Chromosome number distribution (a), metaphase (b) and diploid karyotype (c) of CSEC cells at Passage 45. One hundred metaphases were counted.

was 42% and 62% cells contained 42 chromosomes (Fig. 5a). The distribution was asymmetrical, most 2n values appearing clustered below the modal value. Both aneuploidy and heteroploidy appeared in CSEC cell line in small proportion. The metaphase (Fig. 5b) with normal diploid number displayed the normal karyotype morphology (Fig. 5c) consisting of 21 pairs of telocentrics (2n=42t).

3.6 *Transfection with GFP reporter gene*

The CESC cells were successfully transfected with pEGFP-N1 by using lipofectamine 2000 according to the manufacture's instruction. The expression of EGFP in CSEC cells could be detected as early as 16 h after transfection (Fig. 6). The transfection efficiency was found to be 10% indicating that the suitability of CESC cells for transfection and CMV promoter can drive the expression of EGFP gene in the cells.

3.7 *Susceptibility to LCDV*

Lymphosystis disease virus (LCDV) was used to determine the susceptibility of the CSEC cells. Cytopathic effect (CPE) in the cells was observed to evaluate the susceptibility of the CSEC cells to LCDV. CPE appeared in the cells at 96 h after infection of

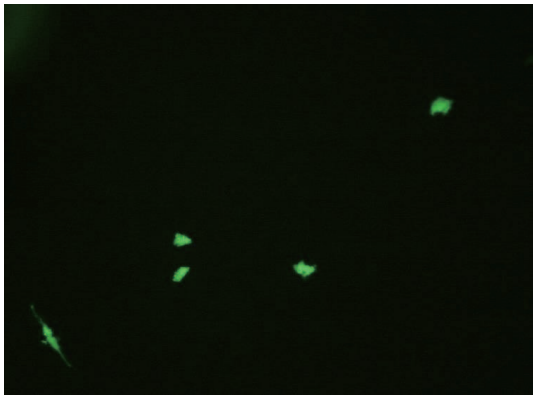

Fig.6. Green fluorescent protein (GFP) expression in transfected CSEC cells transfected with pEGFP-N1.

virus. The monolayer completely disintegrated after 10 d in LCDV-infected cells (Fig. 7b). Electron microscopy observation showed viral particles in the cytoplasm of virus-infected CSEC cells (Fig. 7c). Intracellular granules were distributed in the cytoplasm of virus-infected cells, which demonstrated viral

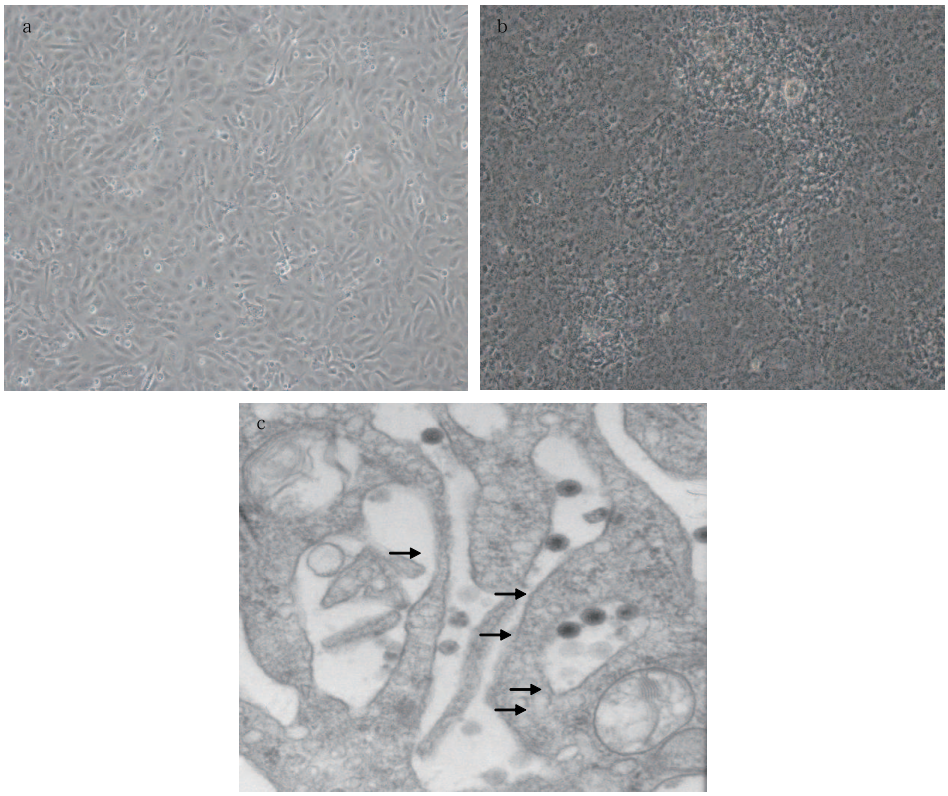

Fig.7. Susceptibility of infected CSEC cells to LCDV at Passage 25. a. Confluent uninfected CSEC cells, b. the monolayer completely disintegrated after 10 d in LCDV-infected cells and c. electron micrograph of infected CSEC cells. Enveloped hexagonal viral particles (thin arrow) were found in the cytoplasm of LCDV infected CSEC cells (×15 000).

infection. Virions with enveloped, hexahedral morphology were scattered throughout the cytoplasm of cells.

4 Discussion

Cell lines have been recognized as powerful experimental tool in vertebrate immunology and biology. The importance of animal cell cultures, especially for cultured marine fish, and for the study of virus, gained increasing attention (Wise et al., 2002). So far, few cell lines were developed in cultured marine fish. The present study reports the establishment of an embryonic cell line, CSEC, derived from gastrula-stage embryos of the half smooth tongue sole. The cell line maintained stable growth and normal diploid karyotype over 200 d of culture with more than 50 passages.

The CSEC cells grow in the temperature range of 15–32 °C with best growth at 24 °C. The growth rate of CSEC cells is higher in medium containing 15% FBS than in medium containing 7.5% FBS. Basic fibroblast growth factor (bFGF) is a potent mitogenic agent for human melanocytes (Halaban et al., 1988). In zebrafish, bFGF was mitogenic and suppressed melanogenesis (Bradford et al., 1994). In half smooth tongue sole, the present study showed that the addition of bFGF stimulated proliferation of CSEC cells. 2-ME and LIF also had effect on the growth of CSEC cells but not significantly as bFGF. Cryopreservation of cell lines is necessary for long-term storage. The cryopreservation tests demonstrated that the CSEC cells could undergo cryopreseration in liquid nitrogen with a survival rate of more than 70%.

A euploid karyotype is an important parameter for characterizing a cell line. A karyotype analysis revealed that over 61% of the cells possessed a diploid chromosome number of 2n=42. This diploid rate is similar to those reported in other fish cell lines (Chen, Sha et al., 2003; Chen, Ye et al., 2003; Hong et al., 1996; Sun et al., 1995). During the process of culturing, the chromosome fragment unceasingly lost. According to the number of chromosome ranging from 18 to 92, we infer that Robertsonian translocation and chromosome reorganization may occur.

The application of CSEC cells for exogenous gene manipulation was assessed by the cells' ability to express the EGFP gene. The transfection experiment using pEGFP plasmid DNA showed that the CSEC cells expressed the EGFP and produced green fluorescent signals, which suggested that the CSEC cells could serve as an in vitro system for exogenous gene manipulation study. It also showed that the CMV promoter could drive expression of GFP gene in the CSEC cells.

Susceptibility to viral infection is necessary for a cell line in isolating and characterizing fish viruses. In the present study, the CSEC cells were found to be susceptible to the LCDV virus. The observation has been further confirmed by Electron microscopy observation. The results indicate that LCDV can be propagated in the CSEC cells and then confirm the potential of the cells as a powerful tool for isolating and identifying LCDV from half smooth tongue sole.

In summary, an embryonic cell line, CSEC, was established from gastrula-stage embryos of half smooth tongue sole, and could be potentially used for the study of infectious viruses in half smooth tonguesole and for developing cell models for screening immune-related functional genes in this species.

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作者： SHA Zhenxia， REN Guocheng， WANG Xianli， WANG Na， CHEN Songlin

作者单位： SHA Zhenxia,WANG Na,CHEN Songlin(Key Laboratory of Sustainable Development of Marine Fisheries,Ministry of Agriculture,Yellow Sea Fisheries Research Institute,Chinese Academy of Fisheries Sciences,Qingdao 266071,China)， REN Guocheng(Key Laboratory of Sustainable Development of Marine Fisheries,Ministry of Agriculture,Yellow Sea Fisheries Research Institute,Chinese Academy of Fisheries Sciences,Qingdao 266071,China;College of Biology,Shandong Normal University,Jinan 250014,China)， WANG Xianli(Key Laboratory of Sustainable Development of Marine Fisheries,Ministry of Agriculture,Yellow Sea Fisheries Research Institute,Chinese Academy of Fisheries Sciences,Qingdao 266071,China;College of Marine Life,Ocean University of China,Qingdao 266003,China)

刊名： 海洋学报（英文版） SCI

英文刊名： ACTA OCEANOLOGICA SINICA

年，卷(期)： 2010,29(2)

被引用次数： 1次

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引用本文格式：SHA Zhenxia.REN Guocheng.WANG Xianli.WANG Na.CHEN Songlin Development and characterization of a cell line from the embryos of half smooth tongue sole(Cynoglossus semilaevis)[期刊论文]-海洋学报（英文版） 2010(2)