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Establishment, characterization of a new cell line from heart of half smooth tongue sole (*Cynoglossus semilaevis*)

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Abstract A new cell line was established from the heart of a cultured marine fish, half smooth tongue sole (*Cynoglossus semilaevis*), designated as CSH (*Cynoglossus semilaevis* heart cell line). The CSH cells grow over 400 days in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 2 ng/ml basic fibroblast growth factor (bFGF). The suitable temperature for the cell growth was 24–30°C with the optimum growth at 24°C and a reduced growth at 12 and 30°C. FBS and bFGF concentration were the two important components for CSH cells proliferation. Twenty percent FBS in the medium was found to be the optimum concentration and bFGF promoted the growth of CSH cells. The double time of the cells at 24°C was determined to 73.39 h. Chromosome analysis revealed that 44% of the cells maintained a normal diploid chromosome number ($2n = 42$) in the CSH cells at Passage 58. The fluorescent signals were observed in CSH after the cells were transfected with green fluorescent protein (GFP) reporter plasmids. CSH cells showed the cytopathic effect (CPE) after infection with lymphosystis disease virus (LCDV). Moreover, the LCDV particles can be observed in the cytoplasm of virus-infected cells by electron microscopy, and a segment of MCP gene for major capsid protein of LCDV was found by PCR amplification DNA of virus-infected cells.

Keywords Cell line · Half smooth tongue sole (*Cynoglossus semilaevis*) · CSH · Karyotype · GFP · LCDV

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Introduction

In vitro culture of fish cells provides an important tool for studying cellular physiology, molecular biology and toxicology applications (Hightower and Renfro 1988; Bejar et al. 2005; Bahich and Borenfreund 1991; Bols et al. 2005; Lee et al. 2009; Segner 1998; Villena 2003). By 1994, more than 150 cell lines have been established from fishes of which most are derived from freshwater teleosts (Fryer and Lannan 1994). In recent years, for purpose of identifying fish viruses and researching functional genes, more and more cell lines were developed in marine fishes (Chen et al. 1999, 2003a, 2004, 2007; Ye et al. 2006; Parameswaran et al. 2007a, b; Zhou et al. 2007).

Half smooth tongue sole (*Cynoglossus semilaevis*) is a newly exploited and high-valued fish and becoming widely farmed in northern China (Wan

et al. 2004) in recent years. Yet intensive aquaculture of this species has resulted in outbreaks of virus diseases which brought high mortalities of the tongue sole. Little is known about the epidemiology of viral infections and the characterization of the virus. Since the tongue sole is commercially important, cell lines from this species should help the aquaculture industry by allowing diseases to be diagnosed and studied. By now, two other cell lines have been built in our laboratory, the liver cell line (HTLC) (Ren et al. 2008) and embryonic cell line (CSEC) (Sha et al. 2010 in press). In the present study, a heart cell line from the half smooth tongue sole was developed and characterized. The efficiency of transfection and the susceptibility of the cell line to the flounder lymphocystis disease virus (LCDV) were examined as an example of an application for the cell line in gene regulation and virus research.

Materials and methods

Primary cell culture and subculture

A healthy half smooth tongue sole (*C. semilaevis*) was obtained from Haiyang Marine Fish Hatchery (Qingdao, China). The fish was wiped with 70% alcohol and killed by over-anaesthetizing with 20% urethane. The heart was removed aseptically and washed three times with phosphate-buffer saline (PBS). The heart tissue was dipped in 70% alcohol for 5 min and then washed three times with PBS. The tissue was minced thoroughly with scissors and transferred to tissue culture dishes containing 5 ml of 0.25% trypsin solution (0.25% trypsine and 0.2% EDTA in PBS). After the heart tissue was trypsinized for 15 min, the mixture of cells and cell clumps was filtered through 200-mesh nylon gauze. Then, the cells were gathered by centrifuging at 1,000*g* for 5 min. The cells were cultured into 25-cm^2 tissue culture flasks containing 3 ml of MEM. The components of the medium are 20 mM hepes, pH 7.4, antibiotics (penicillin, 100 U ml^{-1}, streptomycin, 100 mg ml^{-1}), 10–20% FBS (Gibco), 2 ng ml^{-1} bFGF (Invitrogen, human, recombinant). The cells were incubated at 24°C in a normal atmosphere incubator.

When the cells formed a monolayer, the old medium was removed and the cell sheets were washed with PBS twice and dispersed with 0.25% trypsin and distributed into two flasks. The primary cells were cultured in the complete growth medium with 20% FBS, and the subcultured cells were cultured in medium with 10% FBS.

Cryopreservation and thawing of cells

For cryopreservation, CSH cells were harvested by centrifugation and suspended at a density of 10^6 cells ml^{-1} in complete medium with 10% dimethyl sulphoxide and 10% FBS with the progress of 4°C 0.5 h, −70°C, 4 h and then liquid nitrogen forever. When thawed, the cryogenic vials were placed in the water with the temperature of 40°C, agitated gently until the cells were dissolved and then centrifuged at 1,000*g* for 4 min. Then, the cells were suspended in MEM and seeded into 25-cm^2 cell culture flasks.

Growth of cells

For growth studies, CSH cells were seeded into 12-well plate at an initial density of 1.5×10^5 cells well^{-1} and incubated at 24°C for 6 days. The cells were trypsinized and counted using an hemocytometer every day. The experiment was repeated three times. The doubling time was determined using the method described (Hayflick 1973).

Effect of temperature, FBS and bFGF on cells proliferation

To determine the effect of temperature on the proliferation of the cells, the CSH cells were inoculated in 12-well plate at an initial density of 1×10^5 cells ml^{-1} at 12, 18, 24 and 30°C, respectively. After 2, 4, 6 and 8 days, the cells were trypsinized and counted microscopically using an hemocytometer. To determine the effect of different FBS concentration on cell proliferation, 4×10^4 cells were inoculated in 12-well plate in medium containing 25, 20, 15, 10 or 0% FBS, respectively. To determine the effect of different bFGF concentration on cell proliferation, 4×10^4 cells were inoculated in 12-well plate in medium containing 0, 2, 6, 8 ng ml^{-1} bFGF, respectively. Six days later, the cells were trypsinized and counted. The experiment was repeated three times. Data were expressed as mean ± SE.

Chromosome analysis

For chromosome analysis, the CSH cells at passage 57 were used. The preparation of chromosome was done by the method of Ye (Ye et al. 2006). In brief, the cells were dosed with colchicine (0.8 μg ml^{-1}) for 4 h in 25 cm^2 culture flask and harvested by centrifugation (1,000*g*, 5 min), single cells were suspended in hypotonic solution of 0.075 M KCl for 30 min, and fixed two times in cold Carnoy's fixative, 15 min for each time. Slides were prepared using the conventional drop-splash technique (Freshney 1994) and then air dried. Chromosomes were stained with 5% Giemsa for 20 min. Finally, chromosomes were observed and counted microscopically. One hundred photographed cells at metaphase were counted, and chromosome karyotype was analyzed according to the reported method (Levan 1964).

Cell transfection with GFP reporter gene

The ability of the CSH 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. 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 $well^{-1}$ in 12-well plates individually. After monolayer was 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.

Virus susceptibility

Lymphosystis disease virus (LCDV) as an important pathogen can lead to lymphocystis disease in many fish species (Weissenberg 1965; Alonso et al. 2007). The virus was isolated from the infected *Paralichthys olivaceus* as described in previous study (Xu et al. 2000) and used to detect the susceptibility of the CSH cells. The titration value of LCDV determined based on $TCID_{50}$ assay was 10^2 $TCID_{50}$ ml^{-1}. A total of 2×10^5 cells ml^{-1} 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 2 weeks.

The CSH cells DNA was extracted from the infected cells. Cells were homogenized with 1 ml extraction buffer (100 mM NaCl, 10 mM Tris pH8.0, 25 mM EDTA, 1% SDS, 3 μg proteinase K). The mixture were incubated at 55°C for 30 min. Equal volume of phenol/chloroform (v/v 1:1) was added and then the mixture was centrifuged at 13,000 rpm, 4°C for 15 min. Supernatant liquids were collected and extracted twice with equal volume of phenol/chloroform (v/v 1:1). Subsequently, twice volume of anhydrous ethanol was used to precipitate DNA. Finally, DNA was collected and washed with 75% ethanol, and then dissolved in sterile water. For PCR, one pair of primers specific to the major capsid protein segment of LCDV was designed. The sequences were 5′-CCG TTGATTC CAATGGTCA-3′ (forward) and 5′-CACCGTCAAA GATTACAGGAG (reverse). The size of the PCR fragment was 491 bp. Reactions were performed using the following steps: denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 40 s and elongation at 72°C for 1 min, ending with an additional elongation step of 10 min at 72°C.

For electron microscopy observation, the CSH cells infected with LCDV were fixed with 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) for 24 h at 4°C and then postfixed with 1% osmium tetroxide in 0.2 M 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.

Results

Primary cell culture and subculture

The cells migrating from heart tissue completely covered the bottom of the flask within 2 weeks. Elongated fibroblastic cells formed the major

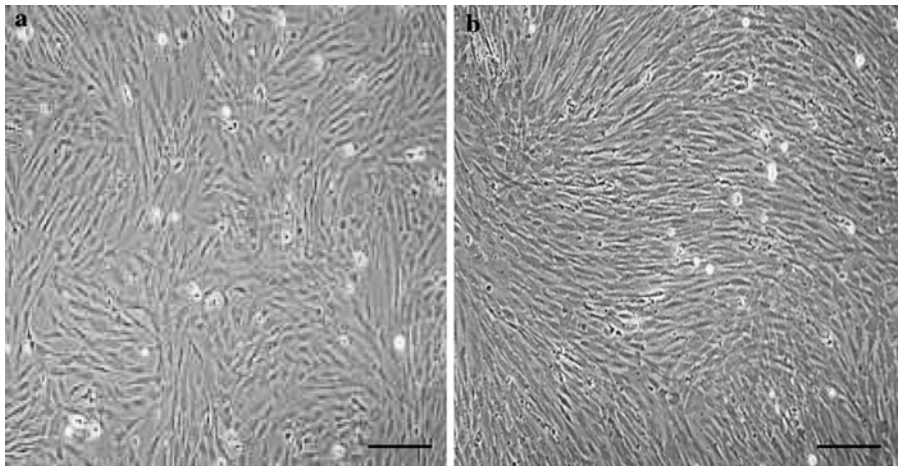

Fig. 1 Morphology characterization of the CSH cells of the half smooth tongue sole: **a** the CSH cells at Passage 8 (×100) and **b** the CSH cells at Passage 53 appear predominantly fibroblast-like. *Bar* 100 μm

component of the culture (Fig. 1). During the initial culture, the cells grew slowly. A combination of 50% each of new and old medium of MEM containing 20% FBS was used. The cells grew stably after a month and subculture could be done once every 10 days. To date, CSH cell line has been subcultured more than 60 times.

Cryopreservation and thawing of cells

The CSH cells were cryopreserved at different passages of 8, 15, 35 and 50. After thawing and being seeded into flasks, the cells recovered with a survival rate of 60–70%. The cells could grow to confluence within 4–5 days.

Growth of cells

The doubling time was determined to be 73.39 h for the CSH cells at a density of 1.5×10^5 cells well^{-1} at 24°C. The growth curves of CSH cells are shown in Fig. 2. After 6 days, the cells number reached 3.6×10^5 cells ml^{-1}.

Effect of temperature, FBS and bFGF on cell proliferation

At 2, 4, 6 and 8 days after inoculation, the CSH cells at each different temperature were trypsinized, and cell numbers were measured. The result showed that the CSH cells were able to grow at different

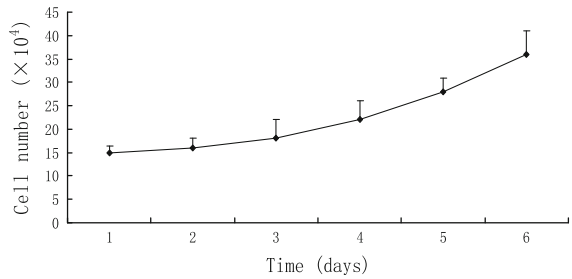

Fig. 2 The growth curve of the CSH cells in MEM supplied with 10% FBS at 24°C. The cell number was counted every day. The starting cell number was 1.5×10^5 cells per well of a 12-well plate. Value are means ± SE ($n = 3$)

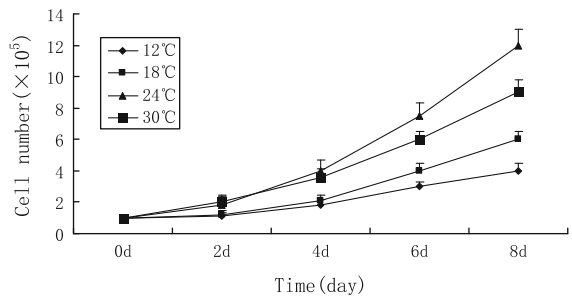

Fig. 3 Effects of temperature on the proliferation of CSH cells. The cells were incubated at different temperature of 12, 18, 24 and 30°C in MEM supplied with 20% FBS, respectively. The cells were trypsinized and counted after growing 2, 4, 6 and 8 days. Value are means ± SE ($n = 3$)

temperatures between 12 and 30°C (Fig. 3). The highest growth rate was obtained at 24°C, the cell number reaching 12×10^5 cells ml^{-1} after 8 days,

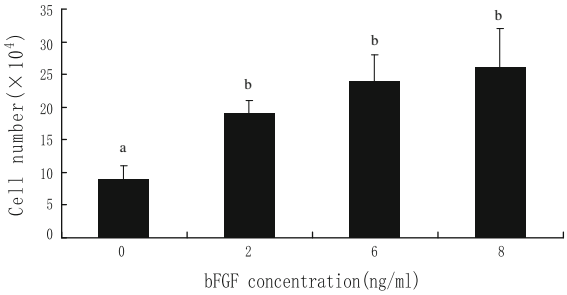

Fig. 4 Effects of FBS on the proliferation of the CSH cells. The cells were cultured in medium containing 25, 20, 15, 10% or without FBS, respectively. Six days later, the cells were trypsinized and counted. Value are means ± SE ($n = 3$). *Different letters* represent significant differences between treatments ($P < 0.05$)

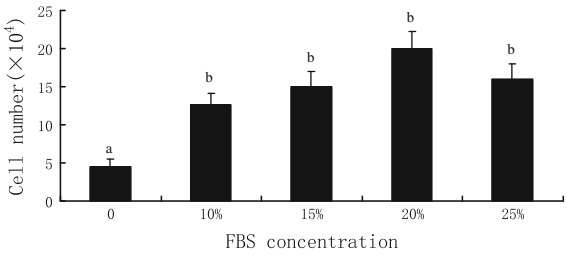

Fig. 5 Effects of bFGF on the proliferation of the CSH cells. The cells were cultured in MEM containing 20% FBS with 0, 2, 6 and 8 ng/ml bFGF, respectively. Six days later, the cells were trypsinized and counted. Value are means ± SE ($n = 3$). *Different letters* represent significant differences between treatments ($P < 0.05$)

and the lowest growth rate was at 12°C, the cell number reaching 4×10^5 cells ml^{-1} after 8 days. The growth of CSH cells was highly dependent on FBS and bFGF concentration in the culture medium. Cells exhibited almost no proliferation without FBS. However, proliferation greatly increased with the increase of FBS concentration and came to the greatest growth with 20% FBS (Fig. 4). bFGF increased significantly proliferation of CSEC cells (Fig. 5). For the reason of economy, 2 ng/ml bFGF was added to the medium.

Chromosome analysis

Chromosome morphology of CSH cells is shown in Fig. 6a. The chromosome morphologies of the cells were similar, with all chromosomes being telocentric (Fig. 6b). The result of karyotype analysis was identical with the modal chromosome number of juvenile tongue sole (Zhou et al. 2005). The result of chromosome counts of 100 metaphase plates revealed that the number of chromosomes in CSH cells ranged from 22 to 80. The modal number of chromosomes for the cell line was 42 (Fig. 7). Heteroploidy was observed in the cell line and 44% cells contained 42 chromosomes.

Transfection with GFP reporter gene

The cells were successfully transfected with pEGFP-N1 by using lipofectamine 2000 according to the

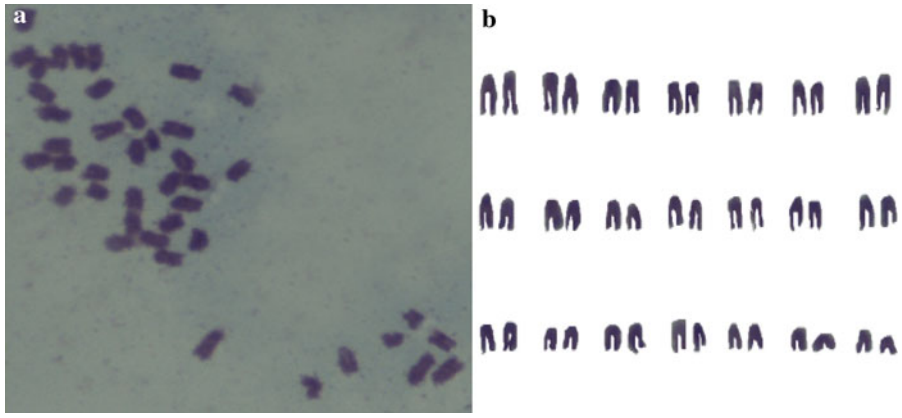

Fig. 6 Photomicrographs of chromosomes (×1,000) (**a**) and karyotype analysis (**b**) of the CSH cells at passage 57. The chromosomes were arrayed according to the chromosome size

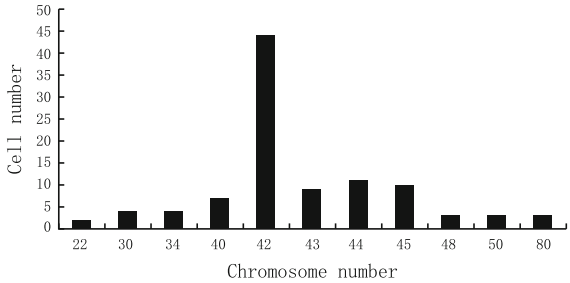

Fig. 7 Chromosome number distribution in 100 metaphases of CSH cells. CSH had a modal chromosomes number of 42. In the counted 100 cells, 2 have 22 chromosomes, 4 have 30, 4 have 34, 7 have 40, 44 have 42, 9 have 43, 11 have 44, 10 have 45, 3 have 48, 3 have 50 and 3 have 80

manufacture's instruction. The expression of EGFP in CSH cells could be detected as early as 16 h after transfection (Fig. 8). The transfection efficiency was found to be about 10% indicating that the suitability of CSH cells for transfection and that CMV promoter can drive the expression of EGFP gene in the cells.

Susceptibility to LCDV

Cytopathic effect (CPE) in the cells was observed to evaluate the susceptibility of the CSH cells to LCDV. CPE appeared in the cells at 72 h after infection of virus. Initially, the specific CPE developed as localized areas of rounded and refractile cells. The CPE with typical multiple vacuolation was observed (Fig. 9b) and then the monolayer completely disintegrated after 10 days of infection (Fig. 9c). Electron microscopy observation showed viral particles in the cytoplasm of CSH cells (Fig. 9d). Intracellular granules were distributed in the cytoplasm of virus-infected cells, which demonstrated viral infection. Virions with enveloped, hexahedral morphology were scattered throughout the cytoplasm of cells. The major capsid protein segment of LCDV was amplified in the DNA of infected cells (Fig. 9e).

Discussion

In the present study, a cell line (CSH) was successfully established from the heart of half smooth tongue sole (*C. semilaevis*). The CSH cells were studied for the growth characterization, transfection with DNA and infection ability by LCDV. The CSH cells could maintain stable growth in MEM medium supplemented with growth factor bFGF and FBS. The cells have been subcultured 60 passages with morphology of all fibroblastic-like with elongated shape.

The tongue sole can survive under the temperature of 3.5–32°C and the suitable temperature is 14–24°C. The vitro cell culture of CSH cells also survived in a wide temperature range. The cells can grow under different temperature ranged from 12 to 30°C, with optimum growth at 24°C, which was identical with that for liver cells and embryonic cells of tongue sole (Ren et al. 2008). One of the advantages of the cells growing over a wide temperature range is their potential suitability of isolating both warm water and cold water fish viruses (Nicholson et al. 1987).

The FBS is essential for survival and optimal growth of exogenous cells. In the primary cell culture, 20% FBS at high concentration was favorite

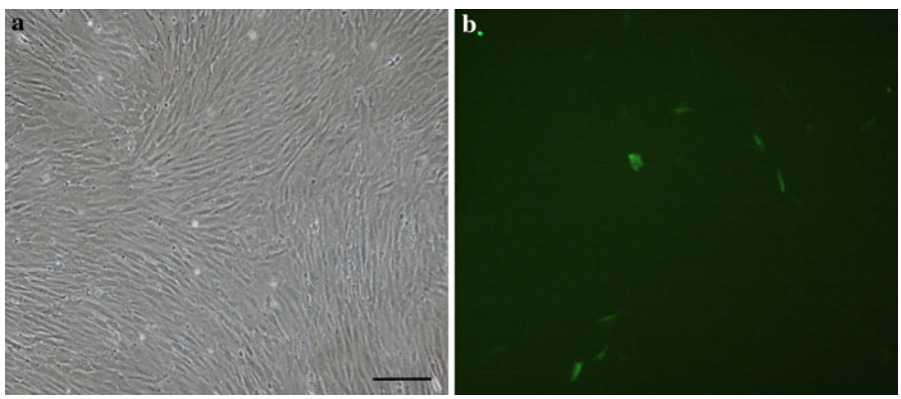

Fig. 8 Green fluorescent protein (GFP) expression in transfected CSH cells at Passage 58 transfected with pEGFP-N1. **a** Control CSH cells, **b** transfected cells. *Bar* 100 μm

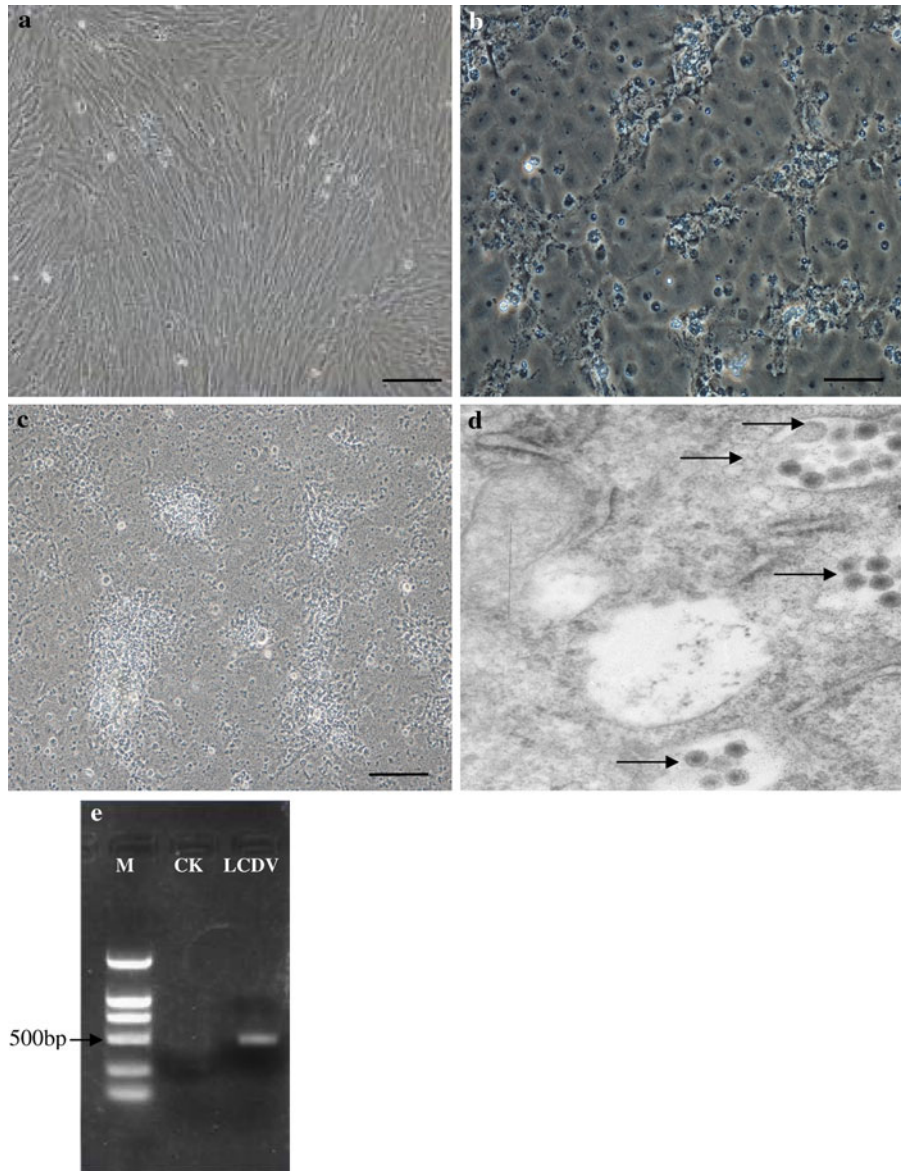

Fig. 9 Susceptibility of infected CSH cells to LCDV at passage 25. **a** Confluent uninfected CSH cells, *Bar* 100 μm, **b** the CPE of the infected cells with typical multiple vacuolation, **c** the monolayer was completely disintegrated after 10 days in LCDV-infected cells, **d** electron micrograph of infected CSH cells. Enveloped hexagonal viral particles (*arrow*) were found in the cytoplasm of LCDV-infected CSH cells (×1,50,000), **e** confirmation of viral infection in CSH cells by PCR [*M* marker, *CK* LCDV uninfected cells, *LCDV* LCDV-infected cells (the segment was 491 bp)]

for the CSH cell survival and proliferation. After subculture, the proliferation of the cells increased as the FBS concentration increased from 10 to 20% and the cells grew slowly in FBS-without medium. Although 20% FBS increased the cell growth, 10% FBS to medium reduced the cost of the CSH cell culture and was selected for subculturing the cells.

bFGF is a potent mitogen for embryonic stem cells derived from medaka *Oryzias latipes* (Hong and Schartl 1996) and sea perch (Chen et al. 2003b), lymphoid cells from grass shrimp *Penaeus monodon* (Hsu et al. 1995), embryonic cells from Japanese flounder *Paralichthys olivaceus* (Chen et al. 2004) and various cell types in culture from mammals

(Matsui et al. 1992; Halaban et al. 1988). In zebrafish, bFGF was mitogenic and suppressed melanogenesis (Bradford et al. 1994). In the present study, bFGF was found to stimulate proliferation of CSEC cells, which was the same as others research before. Now bFGF has been become the indispensable stimulus to induce cell proliferation of different cell lines in our laboratory.

Application of CSH cells for exogenous gene manipulation was assessed by the cells' ability to express the GFP gene. The transfection experiment using pEGFP plasmid DNA showed that the CSH cells expressed the EGFP and produced green fluorescent signals, which suggested that the CSH cells could serve as an in vitro system for exogenous gene manipulation study. The ability of the cells expressing GFP makes it possible of transplantation experiments using the CSH cells. It also showed that the CMV promoter could drive expression of GFP gene in the CSH cells. But in this experiment, the transfection efficiency is low, only about 10%. More attempts such as changing the lipsome kind and the proportion of DNA and lipsome will be needed to improve the efficiency in the future.

Susceptibility to viral infection is important for a cell line in isolating and characterizing fish viruses. In the present study, the CSH cells were found to be susceptible to lymphosystis disease virus (LCDV). The result was further confirmed by electron microscopy observation and PCR. The results indicate that LCDV existed in the CSH cells and then confirm the potential of the cells as a powerful tool for isolating and identifying LCDV from half smooth tongue sole. Only LCDV was determined to validate the infection ability of the cell line, more viruses will be attempted for the wide application of the cell line in separating and characterization of fish viruses in the future.

In conclusion, a new cell line was established from the heart of half smooth tongue sole (*C. semilaevis*). It provides an important tool for studying exogenous gene manipulation and studying the infectious viruses of half smooth tongue sole. It develops a cell model for tongue sole studies to replace the whole fish.

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