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ORIGINAL PAPER

Establishment and characterization of a new fish cell line from head kidney of half-smooth tongue sole (*Cynoglossus semilaevis)*

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Abstract A new cell line (TSHKC) derived from half-smooth tongue sole (*Cynoglossus semilaevis*) head kidney was developed. The cell line was subcultured for 40 passages over a period of 360 days. The cell line was optimally maintained in minimum essential medium supplemented with HEPES, antibiotics, fetal bovine serum, 2-Mercaptoethanol (2-Me), sodium pyruvate and basic fibroblast growth factor. The suitable growth temperature for TSHKC cells was 24 °C, and microscopically, TSHKC cells were composed of fibroblast-like cells. Chromosome analysis revealed that the TSHKC cell line had a normal diploid karyotype with $2n = 42$, contained the heterogametic W chromosome. The TSHKC cell line was found to be susceptible to lymphocystis disease virus. The fluorescent signals were observed in TSHKC when the cells were transfected with green fluorescent protein and red fluorescent protein reporter plasmids.

Keywords Cell line · Head kidney · Half-smooth tongue sole · *Cynoglossus semilaevis* · TSHKC · Karyotype

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Introduction

The application and use of established fish cell lines had provided much new information in studies relating to fish developmental biology, fish immunology (Clem et al. 1996), virology (Ruiz et al. 2009), toxicology (Oh et al. 2001), physiology (Rode et al. 1997), carcinogenesis (Salinas et al. 2008), transgenic applications (Fan and Collodi 2002) and others (Hashimoto et al. 2008; Lakra et al. 2011; Zhou et al. 2003; Cosnefroy et al. 2009;Ormonde et al. 2000;Smith et al. 2000; Huang et al. 2005).

Half-smooth tongue sole (*Cynoglossus semilaevis*) was an economically important marine fish species. However, intensive culture of this species was developed and resulted in outbreaks of diseases by bacterium (Wang et al. 2007). Although four cell lines from half-smooth tongue sole (*C. semilaevis)* had been established previously, including the cell lines derived from the embryo (FEC) (Sha et al. 2010), the heart (CSH) (Wang et al. 2010b), the liver (HTLC) (Ren

et al. 2008) and the testis (CGTC) (Zhang et al. 2011), there was not a cell line from half-smooth tongue sole head kidney. The cell line from head kidney would help isolation and identification of viruses in this species. Furthermore, half-smooth tongue sole had the ZW sex-determining system, the growth of the female individuals was 2–4 times faster than the male individuals, the TSHKC cells derived from female individuals was a better in vitro system for this species for identifying growth and immune-related genes and studying their function.

Materials and methods

Primary culture and subculture

Several healthy half-smooth tongue sole (*C. semilaevis)* weighing 250–300 g were bought from a local fish farm and maintained in laboratory aquaria equipped with seawater recirculation for use. The external surface of a fish was wiped with 75 % v/v ethanol solution. The head kidney was removed and immersed in minimum essential medium (MEM) contained antibiotics (400 U ml^{-1} penicillin, 400 ug ml^{-1} streptomycin). The tissues were washed twice with sterile phosphate-buffered salts (PBS), then minced thoroughly with scissors and digested with 1 ml of 0.25 % trypsin solution (0.25 % trypsin and 0.2 % EDTA in PBS) for 20 min. The contents were centrifuged at 2,200 rpm for 2 min, and the pellet was suspended in 1 ml of MEM complete medium (MEM supplemented with 20 % fetal bovine serum (FBS),100 U ml^{-1}penicillin,100 ug ml^{-1}streptomycin,1 mM sodium pyruvate, 10 ng ml^{-1} bFGF) and seeded into 25-cm^2 culture flasks. The cultures were incubated at 24 °C in an incubator set. On the 2nd day, 1 ml of MEM complete medium was added to the flask. The medium was changed every 7 days.

When the cells around the tissue pieces stopped growing, the cell clusters were digested with 1 ml of 0.25 % trypsin solution (0.25 % trypsin and 0.2 % EDTA in PBS) and continued to culture in the original culture flask. A combination of 50 % each of the new and old medium of MEM complete medium was used. When a complete monolayer had formed in primary culture, the confluent cells were digested with 0.5 % trypsin solution (0.5 % trypsin and 0.4 % EDTA in PBS) and transferred into a fresh 25-cm^2 flask. Cell cultures were maintained in the medium with 10 % FBS after 10 passages.

Growth of cells

For growth study, the TSHKC cells at passage 10 were seeded into a 12-well plate at an initial density of 16×10^4 cells ml^{-1} and incubated at 24 °C for 7 days. The number of cells from duplicate wells was trypsinized and counted using a hemocytometer every day.

Effect of temperature on cell proliferation

To determine how temperature affected the growth of the cells, 2×10^4 cells ml^{-1} at passage 15 were inoculated in a 12-well plate and incubated at 15, 24 and 30 °C for growth tests, respectively. Wells of cells at each temperature were trypsinized, and cell numbers were measured microscopically via a hemocytometer every day during the next 8 days.

Chromosome analysis

For chromosome analysis, the TSHKC cells at passage 22 were used. In brief, the cells were inoculated into 25-cm^2 culture flasks and incubated at 24 °C for 24 h. The cells were dosed with colchicine (0.1 ug ml^{-1}) for 1.5 h in 25-cm^2 culture flasks and harvested by centrifugation (1,000 rpm, 5 min). The single cells were suspended in 5 ml hypotonic solution of 0.075 M KCl for 25 min at 37 °C and then premixed for 5 min in 1 ml of cold Carnoy's fixative (methanol: acetic acid = 3:1) by centrifugation (1,000 rpm, 5 min). The cell pellets were fixed two times in 2 ml cold Carnoy's fixative, 15 min for each time. After the second centrifugation, cells were resuspended in 0.2–0.5 ml Carnoy's fixative according to the size of the cell pellet. Slides were prepared using the conventional drop-splash technique (Freshney 1994) and then air-dried. Chromosomes were stained with 5 % Giemsa for 10 min. One hundred photographed cells at metaphase on the slides were counted under Nikon eclipse 80-I fluorescence microscope, and chromosome karyotype was analyzed according to the method of Levan et al. (1964).

Virus susceptibility

Nodules of the diseased flounder (*Paralichthys olivaceus*) with lymphocystis disease were collected. Lymphocystis disease virus (LCDV) was separated from lymphocystis cells of the nodule by cell disruption and centrifuged according to the method of Cheng et al.(2009). The titration value of LCDV determined based on TCID50 assay was 10^2 $TCID_{50}$ ml^{-1}. The TSHKC was used to detect the susceptibilities to LCDV. In brief, 1×10^6 cells ml^{-1} were seeded into 25-cm^2 flask and incubated at 24 °C. After 24 h culture, the medium was removed, and then the cells were washed twice with PBS, 1 ml of LCDV virus solution was added into 25-cm^2 flasks. After 1 h infection, the virus solution was replaced with MEM complete medium containing 5 % FBS. The cytopathic effect (CPE) was observed daily using an inverted microscope.

Electron microscopy was performed as a routine method. Briefly, after the cells appeared the cytopathic effect, the LCDV-infected cells and uninoculated cells were harvested and fixed with 2.5 % glutaraldehyde in cacodylate buffer (0.1M, pH 7.4) for 4 h at 4 °C, rinsed three times in PBS (0.1M, pH 7.4) for 10 min, and then postfixed with 1 % osmium tetroxide in cacodylate buffer (0.1 M, pH 7.4) for 2 h. Subsequently, the specimens were rinsed three times in PBS buffer for 10 min, dehydrated in graded ethylalcohol (30, 50, 70, 90, 100 %) and embedded in Epon812 epoxy resin. Ultrathin sections were cut with a diamond knife on a Reichert–Jung Ultracut-E microtome, mounted on copper grids, stained with 2 % uranyl acetate-lead citrate. Grids containing ultrathin sections were examined with a JEOL JEM-1200EX transmission electron microscope.

Cell transfection with pEGFP-N_3 and pDsRed$_1$-N_1 reporter gene

The TSHKC cells were seeded at a density of 2×10^5 cells $well^{-1}$ in a 12-well plate at 24 °C. More than 84 % confluent monolayers were transfected with pEGFP-N_3 and pDsRed$_1$-N_1 express vector using lipofectamine™ 2000(Invitrogen). In brief, 2 ul lipofectamine™ 2000 was mixed with 98 ul MEM without FBS and antibiotics in a 0.5-ml centrifuge tube. Meanwhile, 4 ul pEGFP-N_3 (200 ng ul^{-1}) and 8 ul pDsRed$_1$-N_1 (100 ng ul^{-1}) was mixed with 96 and 92 ul MEM without FBS and antibiotics in a 0.5-ml centrifuge tube, respectively. After 5 min, the two solutions were mixed and interacted for 20 min. During this period, the cells were washed twice with PBS, and the medium was replaced with MEM without FBS and antibiotics; subsequently, the aforementioned two 200 ul mixtures were dropped into the wells and cultured at 24 °C for 4.5 h, respectively, and the medium was replaced with normal medium. The green or red fluorescence signals were observed under a fluorescence microscope (Nikon Eclipse TE2000-U) after a 24-h incubation.

Cryopreservation and recovery of cells

For cryopreservation, the cells were trypsinized and suspended in MEM complete medium containing 10 % dimethyl sulphoxide at a density of 2×10^6 cells ml^{-1}. The cell suspensions were dispensed into 2-ml plastic ampoules and kept in NALGEBE™ Cryo 1 °C Freezing Container (Invitrogen) at −70 °C overnight and transferred into liquid nitrogen (−196 °C). The frozen cells were recovered by thawing in a 42 °C water bath. Following the removal of the freezing medium by centrifugation, the cells were suspended in MEM complete medium with 10 % FBS.

Results

Primary culture and subculture

About 2 days after the tissue pieces and cells suspension were inoculated, the cells began to grow from the edges of seeded tissue explants. The cells grew quite fast and formed many colonies or partial cell monolayers gradually, then the first subculture was conducted. After several subcultures, the cells formed a complete monolayer. The TSHKC cell line was composed of fibroblast-like cells (Fig. 1). During the initial 5 passages, the cells were subcultured at 3–7 day intervals. A combination of 50 % each of the new and old medium of MEM containing 20 % FBS was used. After that, the cells could be subcultured every 7 days, and cells were subcultured at a ratio of 1:2. The size of cells at early passages was longer than the later cells. The TSHKC cells had been subcultured for 40 passages.

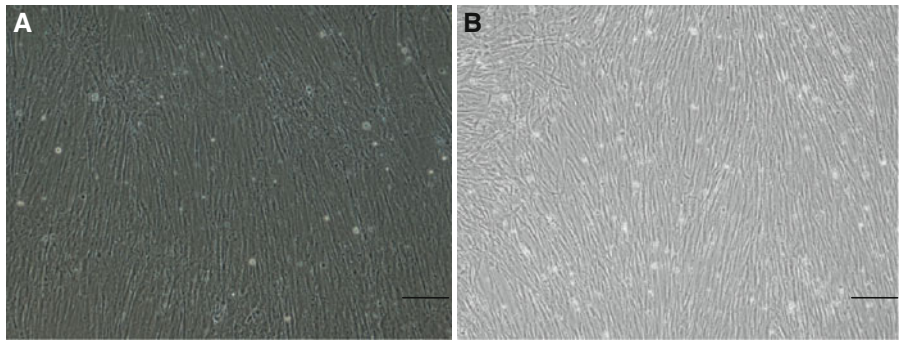

Fig. 1 Monoplayer cells of the TSHKC cell line. **a** At passage 3, **b** at passage 21. Original magnification ×100

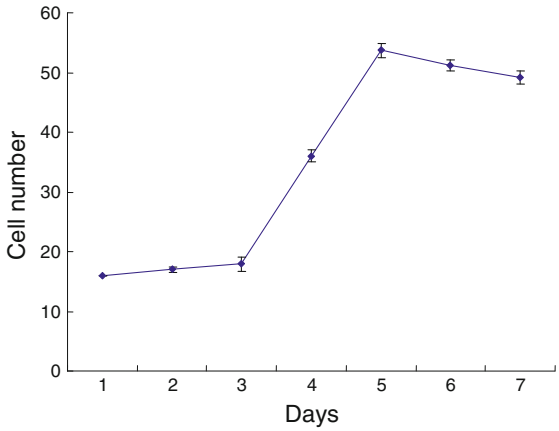

Fig. 2 The TSHKC cells growth curve

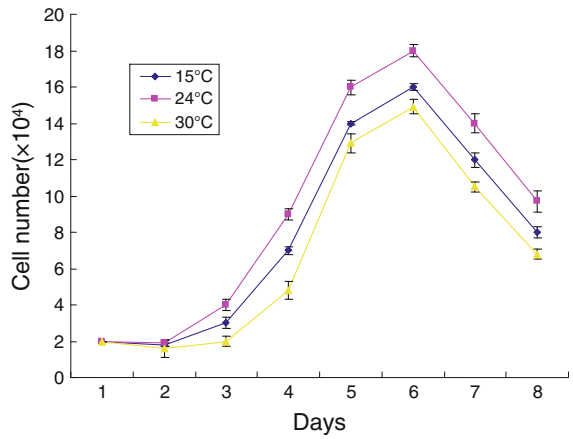

Fig. 3 Effects of temperature on growth of the TSHKC cells

Growth of cells

The growth curve of TSHKC cells was shown in Fig. 2. The cells adhered well to the substrate and achieved confluence in 7 days. Morphologically, the TSHKC cell line was composed of fibroblastic-like cells. After 4 days, the cell number reached 53.7×10^4 cells ml^{-1}.

Effects of temperature on cell growth

Incubation temperature had no effect on cellular morphology. The TSHKC cells were able to grow at temperature between 15 and 30 °C at passage 15. The highest growth rate was obtained at 24 °C, the cell number reaching 18×10^4 cells ml^{-1} after 5 days, and the lower growth rate was obtained at 15 °C, the lowest growth rate was obtained at 30 °C (Fig. 3).

Chromosome analysis

The chromosome assay showed that the number of chromosome in the TSHKC cells ranged from 22 to 81; heteroploidy was observed in the cell line; nevertheless, the modal chromosome number was 42, which came to 43 % in the 100 metaphase cells counted at passage 22(Fig. 4c). The metaphase (Fig. 4a) with a normal diploid number displayed the normal karyotype morphology, all chromosomes were telocentric, contained the heterogametic W chromosome (Fig. 4b).

Virus susceptibility

The susceptibility of TSHKC cells to LCDV was evaluated by the observation of CPE in the cell line. After 4 days infection, the cells inoculated with LCDV exhibited morphological changes including cell shrinkage, most cells rounded and detached from

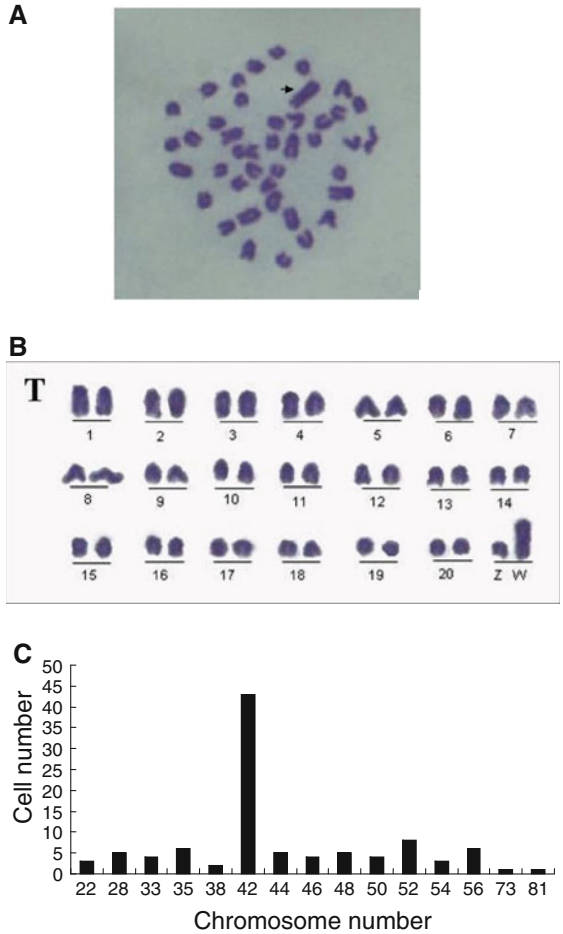

Fig. 4 Metaphase (**a**), diploid karyotype (**b**) and chromosome number distribution (**c**) of TSHKC cells at passage 22. One hundred metaphases were counted

the flask (Fig. 5b). Electron microscopy observation revealed that few virus particles were scattered throughout the cytoplasm of cells infected with LCDV(Fig. 5f). Meanwhile, there were many apoptotic cells, some cells died and fractured (Fig. 5h). These results proved that the TSHKC cells were susceptive to LCDV (Fig. 5).

DNA transfection

When TSHKC cells were transfected with pEGFP-N_3 and pDsRed$_1$-N_1 reporter genes, clear and strong green or red fluorescence signals could be detected after 24 h, respectively (Fig. 6). Furthermore, the number of signals increased gradually during the next few days until the 8th day. The percentage of transfection was 10 and 5 %, respectively. This indicated the suitability of TSHKC cells to transfection.

Cryopreservation and recovery of cells

The TSHKC cells were cryopreserved at different passages. After thawing and being seeded into flasks, the cells recovered with a survival rate of 70–80 %. The cells could grow to confluence after 7 days.

Discussion

In this study, a half-smooth tongue sole (*C. semilaevis*) head kidney cell line designated as TSHKC was developed and characterized. The TSHKC cells could maintain stable growth in MEM and had been subcultured for 40 passages with morphology of fibroblastic-like.

Half-smooth tongue sole could survive under the temperature of 3.5–32 °C, and the suitable temperature was 14–24 °C. The cells could grow under different temperatures ranged from 15 to 30 °C, with optimal growth at 24 °C. It proved the selected temperature at which the primary culture was established within the normal temperature range for fish was the optimum for the cell growth. The optimal temperature of TSHKC cells was similar with other half-smooth tongue sole cell lines, but the effect of temperature on the TSHKC cells growth was not identical with other half-smooth tongue sole cell lines (Wang et al. 2010b; Zhang et al. 2011; Sha et al. 2010; Ren et al. 2008). The advantage of the cells growth over a wide temperature range was their potential suitability to isolating both warm water and cold water fish viruses (Nicholson et al. 1987).

Karyotype analysis revealed that 43 % of the TSHKC cells possessed a diploid chromosome number of $2n = 42$, which was identical with the modal number of HTLC (Ren et al. 2008), CSH (Wang et al. 2010b), CSGC (Zhang et al. 2011) and CSEC (Sha et al. 2010). The diverse chromosome number and the occurrence of aneuploidy were key indicators of immortalized cells (Dong et al. 2008;Zhou et al. 2007). The cause of aneuploidy remained to be elucidated, but might occur as a consequence of chromosome disjoining during cell division (Zhou et al. 2003).

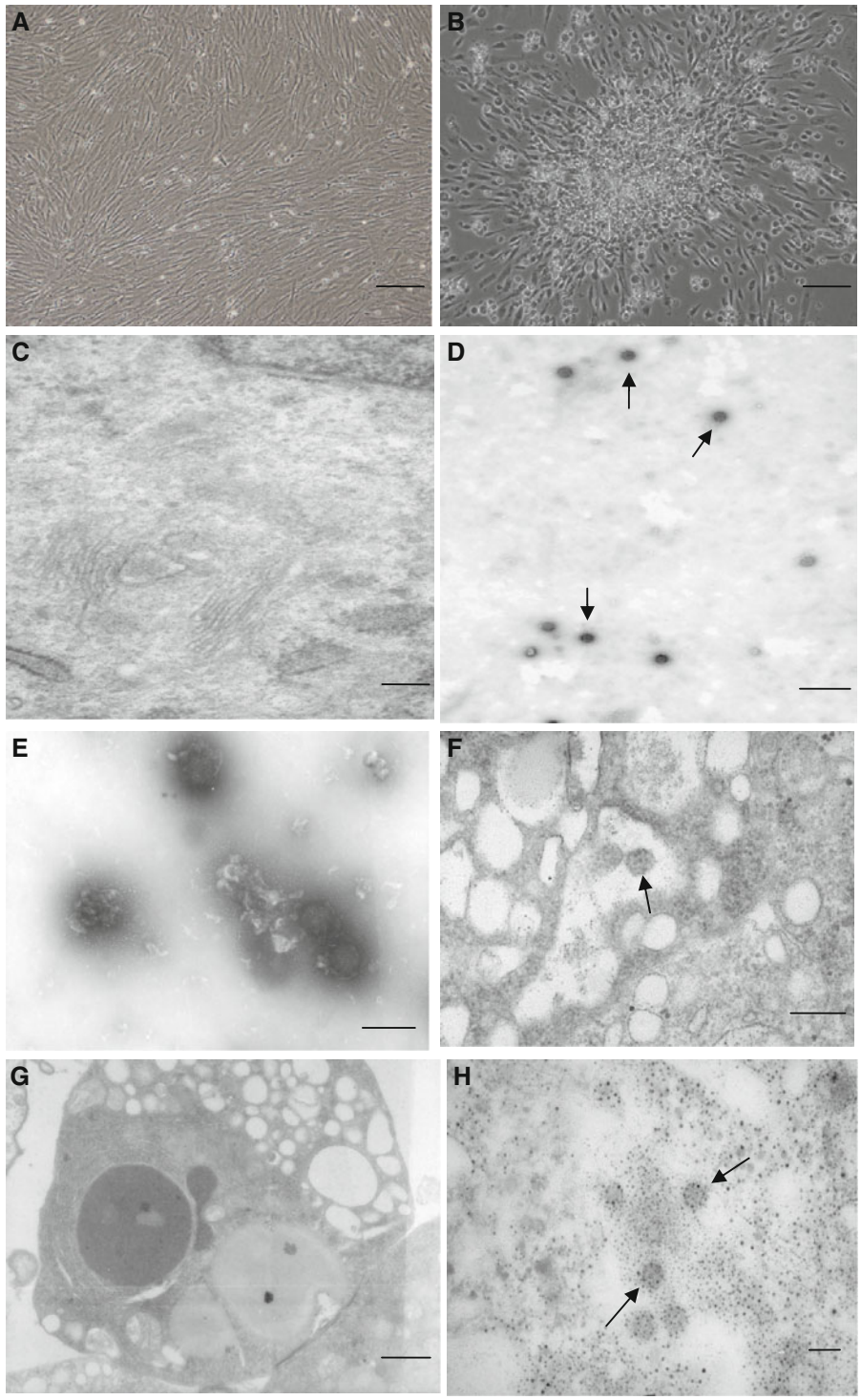

Fig. 5 The results of virus susceptibility assay. **a** Uninfected cells, bar 100um; **b** LCDV-infected cells, bar 100um; **c** electron micrograph of uninfected cells, bar 200 nm; **d**, **e** electron micrograph of LCDV particles that separated from lymphocystis cells, **d** bar 1 um, **e** Bar 200 nm; **f**, **h** electron micrograph of LCDV-infected cells, f Bar 200 nm, **h** Bar 100 nm; **g** electron micrograph of LCDV-infected apoptosis cells, bar 500 nm. *Arrows indicated* the virus particles in the virus extract or the cytoplasm

The establishment of healthy and sensitive fish cell lines was essential for isolation, identification and characterization of infectious viruses from fish (Lee and Loh 1975; Williams et al. 2003; Kang et al. 2003; Rougée et al. 2007;Wen et al. 2008; Sahul Hameed et al. 2006; Zhao and Lu 2006;Lu et al.

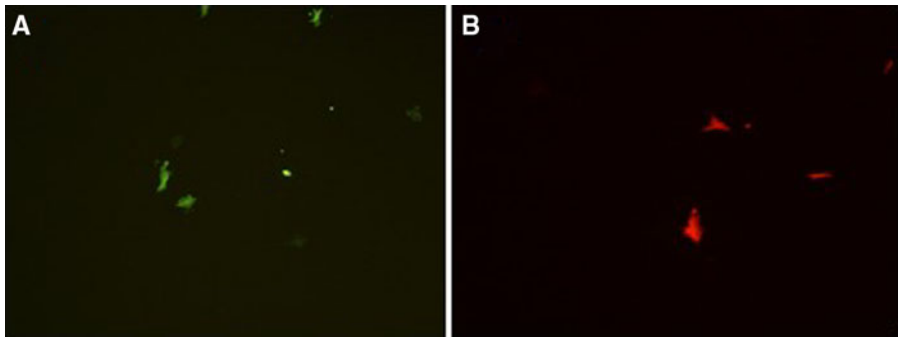

Fig. 6 The expression of GFP and RFP genes in TSHKC cells. **a** *Green signals* photograph in transfected TSHKC cells using pEGFP-N_3 vector DNA, which indicated the expression of pEGFP-N_3 reporter genes. **b** *Red signals* photograph in transfected TSHKC cells using pDsRed$_1$-N_1 vector DNA, which indicated the expression of pDsRed$_1$-N_1 reporter genes. (Color figure online)

1990;Parameswaran et al. 2006; Qin et al. 2006; Chen et al. 2004) .

LCDV was firstly isolated from Japanese flounder (Sun et al. 2000) and could infect also other cell lines of marine fish species (Wang et al. 2010b; Zhang et al. 2011; Sha et al. 2010;Wharton et al. 1977; Sun et al. 2002). Application of TSHKC cell line for virus detection was assessed by its ability to support the infection of LCDV. The results showed that TSHKC cell line was susceptible to LCDV. Electron microscopy detected few replicated virus particles in the cytoplasm. But the TSHKC cell line was not as high sensitive to LCDV as other two half-smooth tongue sole cell lines, CSH and CSGC (Wang et al. 2010b; Zhang et al. 2011). According to relevant report, some pathogenic viruses were known to be organ and tissue specific (Swaminathan et al. 2010), we concluded that it may be due to the three fish cell lines derived from different organs of the same species. The experiment proved that fish LCDV had cell-specific susceptibility to different fish cell lines (Wang et al. 2010a; Ellender et al. 1979). The present study suggested that TSHKC cell line could be used to isolate virus and study cell-pathogen interaction.

In the transfection experiments with pEGFP and pDsRed$_1$ plasmid DNA, the TSHKC cell line expressed the reporter gene, and we could observe signals at 24 h after transfection. Although transfection efficiency needed to improve in our subsequent work, we could observe the signals at the 8th day after transfection. It suggested that the TSHKC cell line could be served as an in vitro system for exogenous research.

In conclusion, the development of half-smooth tongue sole aquaculture was dependent on strategies focused on growth and disease control. A new cell line designated TSHKC derived from the head kidney of half-smooth tongue sole could provide a sensitive diagnostic method for the identification of viral infections in half-smooth tongue sole hatcheries. Furthermore, as the accomplishment of whole genome sequencing of half-smooth tongue sole, the TSHKC cells line would be a useful research tool for studying the function of some immune-related gene.

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References

Chen SL, Ren GC, Sha ZX, Shi CY (2004) Establishment of a continuous embryonic cell line from Japanese flounder *Paralichthys olivaceus* for virus isolation. Dis Aqua Org 60(3):241–246

Cheng SF, Sheng XZ, Zhan WB (2009) Purification and western blotting of three strains of lymphocytic disease virus. Prog in Fish Sci 30(3):49–54

Clem LW, Ely IE, Wilson M, Chinchar VG, Stuge T, Barker K, Luft C, Rycyzyn M, Hogan RJ, Lopik TV, Miller NW (1996) Fish immunology: the utility of immortalized lymphoid cells—a mini review. Vet Immunol Immunop 54(1–4):137–144

Cosnefroy A, Brion F, Guillet B, Laville N, Porcher JM, Balaguer P, AÏt-AÏssa S (2009) A stable fish reporter cell line to study estrogen receptor transactivation by environmental (xeno) estrogens. Toxicol In Vitro 23(8): 1450–1454

Dong CF, Weng SP, Shi XJ, Xu XP, Shi N, He JG (2008) Development of a mandarin fish *Siniperca chuatsi* fry cell line suitable for the study of infectious spleen and kidney necrosis virus (ISKNV). Virus Res 135(2):273–281

Ellender RD, Wharton JH, Middlebrooks BL (1979) An established spleen cell line from *Bairdiella chrysura*. In Vitro Cel Dev Biol Pl 15(2):112–113

Fan LC, Collodi P (2002) Progress towards cell-mediated gene transfer in zebrafish. Brief Funct Genomics 1(2):131–138

Freshney RI (1994) Culture of animal cells. In: Freshney RI (ed) A manual of basic techniques. Wiley, New York, pp 387–389

Hashimoto E, Miyadai T, Ohtani M, Kitamura SI, Oh MJ (2008) Proliferation of kuchijirosho causative agent in a fugu-derived cell line. J Fish Dis 31(6):443–449

Huang CJ, Jou TS, Ho YL, Lee WH, Jeng YT, Hsieh FJ, Tsai HJ (2005) Conditional expression of a myocardium-specific transgene in zebrafish transgenic lines. Dev Dynam 233(4): 1294–1303

Kang MS, Oh MJ, Kim YJ, Kawai K, Jung SJ (2003) Establishment and characterization of two new cell lines derived from flounder, *Paralichthys olivaceus* (Temminck & Schlegel). J Fish Dis 26(11–12):657–665

Lakra WS, Swaminathan T, Joy KP (2011) Development, characterization, conversation and storage of fish cell lines: a review. Fish Physiol Biochem 37(1):1–20

Lee MH, Loh PC (1975) Some properties of an established fish cell line from the marine fish, *Caranx mate* (Omaka). Proc Soc Exp Biol Med 150(1):40–48

Levan A, Predga K, Sandberg AA (1964) Nomenclature for centromeric position on chromosomes. Hereditas 52(2): 201–220

Lu YA, Lannan CN, Rohovec JS, Fryer JL (1990) Fish cell lines: establishment and characterization of three new cell lines from grass carp (*Ctenopharyngodon Idelia*). In vitro cell Dev Biol 26:275–279

Nicholson BL, Danner DJ, Wu JL (1987) Three new continuous cell lines from marine fishes of Asia. In Vitro Cell Dev Biol 23(3):199–204

Oh YB, Lee JS, Park EH (2001) Fish cell line as an in vitro test system for analyzing chromosome aberrations. Bull Environ Contam Toxicol 67(1):6–11

Ormonde P, HÖrstedt P, O'Toole R, Milton DL (2000) Role of motility in adherence to and invasion of a fish cell line by *Vibrio anguillarum*. J Bacteriol 182(8):2326–2328

Parameswaran V, Shukra R, Bhonde RR, Sahul Hameed AS (2006) Splenic cell line from sea bass, Lates calcarifer: establishment and characterization. Aquaculture 261(1): 43–53

Qin QW, Wu TH, Jia TL, Hegde A, Zhang RQ (2006) Development and characterization of a new tropical marine fish cell line from grouper, *Epinephelus coioides* susceptible to iridovirus and nodavirus. J Virol Methods 131(1):58–64

Ren GC, Chen SL, Sha ZX (2008) Development and characterization of a liver cell line from half-smooth tongue-sole (*Cynoglossus semilaevis*). Chin High Technol Lett 18(6):657–660

Rode M, Berg T, GjØen T (1997) Effect of temperature on endocytosis and intracellular transport in the cell line SHK-1 derived from salmon head kidney. Comp Biochem Physiol A 117(4):531–537

Rougée L, Ostrander GK, Richmond RH, Lu YA (2007) Establishment, characterization, and viral susceptibility of two cell lines derived from goldfish *Carassius auratus* muscle and swim bladder. Dis Aquat Org 77:127–135

Ruiz S, Schyth BD, Encinas P, Tafalla C, Estepa A, Lorenzen N, Coll JM (2009) New tools to study RNA interference to fish virus: fish cell lines permanently expressing siRNAs targeting the viral polymerase of viral hemorrhagic septicemia virus. Antiviral Res 82(3):148–156

Sahul Hameed AS, Parameswaran V, Shukla R, Bright IS (2006) Establishment and characterization of India's first marine fish cell line (SISK) from the kidney of sea bass (*Lates calcarifer*). Aquaculture 257(1–4):92–103

Salinas I, Meseguer J, Esteban MÁ (2008) Antiproliferative effects and apoptosis induction by probiotic cytoplasmic extracts in fish cell lines. Vet Microbiol 126(1–3):287–294

Sha ZX, Ren GC, Wang XL, Wang N, Chen SL (2010) Development and characterization of a cell line from the embryos of half-smooth tongue sole (*Cynoglossus semilaevis*). Acta Oceanol Sin 29(2):81–87

Smith RW, Palmer RM, Houlihan DF (2000) RNA turnover and protein synthesis in fish cells. J Comp Physiol B 170(2): 135–144

Sun XQ, Qu LY, Zhang JX (2000) Pathogenicity and immunogenicity of lymphocystis virus of Japanese flounder (*Paralichthys olivaceus*). Chin High Technol Lett 9:19–21

Sun XQ, Hong XG, Zhang JX, Zhang SC, Wang M (2002) Propagation in continuous fish cell line FG29307 from gill of flounder *paralichthys olivaceus*. Chin High technol Lett 12:94–96

Swaminathan TR, Wazir WS, Gopalakrishnan A, Basheer VS, Khushwaha B, Sajeela KA (2010) Development and characterization of a new epithelial cell line PSF from caudal fin of Green chromide, *Etroplus suratensis* (Bloch 1790). In Vitro Cell Dev Biol An 46(8):647–656

Wang Y, Han Y, Li Y, Chen JX, Zhang XH (2007) Isolation of photobacterium damselae subsp. piscicida from diseased tongue (*Cynoglossus semilaevis Gunther*) in China. Acta Microbiol Sinica 47(5):763–768

Wang N, Wang XL, Sha ZX, Tian YS, Chen SL (2010a) Development and characterization of a new marine fish cell line from turbot (*Scophthalmus maximus*). Fish Physiol Biochem 36(4):1127–1234

Wang XL, Wang N, Sha ZX, Chen SL (2010b) Establishment, characterization of a new cell line from heart of half-smooth tongue sole (*Cynoglossus semilaevis*). Fish Physiol Biochem 36(4):1181–1189

Wen CM, Lee CW, Wang CS, Cheng YH, Huang HY (2008) Development of two cell lines from *Epinephelus coioides* brain tissue for characterization of betanodavirus and megalocytivirus infectivity and propagation. Aquaculture 278(1–4):14–21

Wharton JH, Ellender RD, Middlebrooks BL, Stocks PK, Lawler AR, Howse D (1977) Fish cell culture: characteristics of a cell line from the silver perch *Bairdiella chrysura*. In Vitro Cell Dev Biol Pl 13(6):387–397

Williams LM, Crane MJ, Gudkovs N (2003) Development and characterization of pilchard (*Sardinops sagax neopilchardus*) cell lines derived from liver and heart tissues. Methods Cell Sci 25(3–4):105–113

Zhang B, Wang XL, Yang CG, Liu SS, Wang N, Chen SL (2011) Establishment and characterization of a testicular cell line from the half-smooth tongue sole, *Cynoglossus semilaevis*. Int J Biol Sci 7(4):452–459

Zhao Z, Lu Y (2006) Establishment and characterization of two cell lines from Bluefin trevally *Caranx melampygus*. Dis Aqua Org 68(2):91–100

Zhou LR, Deane EE, Woo NYS (2003) Development of a black sea bream fibroblast cell line and its potential use as an in vitro model for stress protein studies. Fish Physiol Biochem 29(4):255–262

Zhou GZ, Li ZQ, Yuan XP, Zhang QY (2007) Establishment, characterization, and virus susceptibility of a new marine cell line from red spotted grouper (*Epinephelus akaara*). Mar Biotechnol 9(3):370–376