Establishment and characterization of an astroglial cell line derived from the brain of half-smooth tongue sole (*Cynoglossus semilaevis*)

Tian-Zi WANG[1], Ai SUN[1,2], Na WANG[1,2], Zhong-Kai CUI[1], Song-Lin CHEN[1,*], Zhen-Xia SHA[1,*]

[1] *Yellow Sea Fisheries Research Institute*, *Chinese Academy of Fishery Sciences*, *Qingdao* 266071, *China*

[2] *Function Laboratory for Marine Fisheries Science and Food Production Processes*, *National Lab for Ocean Science and Technology*, 266003, *China*

ABSTRACT

An astroglial cell line was established from the brain of half smooth tongue sole (*Cynoglossus semilaevis*) and was designated as CSAC. CSAC shows the morphological homogeneity of epithelial cells. The cell identity was tested by the presence of glial fibrillary acidic protein (GFAP), which was revealed by RT-PCR and immunofluorescence. The cell line was optimally maintained at 24 °C in minimum essential medium supplemented with HEPES, antibiotics, 20% fetal bovine serum, 2-Mercaptoethanol (2-Me) and basic fibroblast growth factor. Chromosome analysis revealed that the CSAC cells maintained a normal diploid chromosome number (2*n*=42). The fluorescent signals were observed in CSAC after the cells were transfected with green fluorescent protein (GFP) reporter plasmids. The CSAC cell line may serve as a valuable tool for studies on the potential functions of fish astroglial cells.

Keywords: Half-smooth tongue sole; *Cynoglossus semilaevis*; Brain astroglial cell

INTRODUCTION

The brain of fish retains a higher capacity to grow and regenerate compared with the mammalian central nervous system (Schwartz et al, 1985). Astrocyte in fish have long processes and occur as radial glial cells in most of the central nervous systems, which are different from those of mammals (Dahl & Bignami, 1973; Grupp et al, 2010; Kalman, 2002). Astrocytes react to brain diseases and injuries by forming a glial 'scar', which is thought to keep the neuronal environment stable (Yiu & He, 2006). It had been reported that the accumulation of aggregated amyloid-β (Aβ) in amyloid plaques is a neuropathological hallmark of Alzheimer's disease (AD) in humans. Reactive astrocytes can intimately be associated with amyloid plaques (Mack & Tiedemann, 2013). In mice, the astrocytic dopamine D2 receptor (DRD2) modulates innate immunity through αB-crystallin (CRYAB), which suppresses neuroinflammation (Shao et al, 2012). In fish, the role of astroglial cells in immunity and brain injure repair is still unknown. Cell lines devote systems which are similar with *in vivo* environments, thus, they can be widely used in fish developmental biology, immunology (Clem et al, 1996), virology (Lu et al, 2004; Ruiz et al, 2009; Wei et al, 2009), toxicology (Oh et al, 2001), physiology (Fan et al, 2007; Rodea et al, 1997; Wang et al, 2004), carcinogenesis (Salinas et al, 2008), transgenic applications (Fan & Collodi, 2002) and others. An astrogilial lineage cell line was established from the orange-spotted grouper (*Epinephelus coioides*) to study viral pathology and epidemiology (Wen et al, 2008). However, there is no established astrogilial cell line in flatfish.

The half-smooth tongue sole (*Cynoglossus semilaevis*) is an important economic marine fish in China. Several of its established cell lines have been widely used in virology and transgenic applications (Wang et al, 2010; Zhang et al, 2011; Zheng et al, 2012). The establishment of CSAC serves as a useful tool for the studies of fish astroglial cells.

The green fluorescent protein (GFP) gene experiment showed that CSAC can function as foreign gene expression receptors. It was assumed that the morphological homogeneity of CSAC is associated with the adding of 2-Me and bFGF, and the latter was confirmed as a possible neural-inducing morphogen (Kengaku & Okamotq, 1995).

MATERIALS AND METHODS

Primary cell culture and subculture

A one-year old half smooth tongue sole weighing 150 g was obtained from SanXin Fisheries Company in Changyi,

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*Corresponding authors, E-mails: shazx@ysfri.ac.cn; chensl@ysfri.ac.cn

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Shandong Province, China and was disinfected with 75% ethanol for 2 minutes. The brain was taken and transferred to a dish, washed three times with phosphate-buffered saline (PBS) containing antibiotics (penicillin, 1 000 IU/mL; streptomycin, 1 000 μg/mL), and minced with scissors into small pieces (1 mm^3), and digested with 1 mL of 0.2% Collagenase II for 15 min. The contents were centrifuged at 2 200 g for 3 min, seeded into the 25 cm^2 culture flasks. The pellet was suspended in 1 mL of MEM complete medium. The components of the medium were 20 mM HEPES, pH 7.4, antibiotics (penicillin, 100 U/mL; streptomycin, 100 mg/mL), 20% FBS (Gibco), 2 ng/mL bFGF (Invitrogen, human, recombinant), 50 mmol/mL 2-Me (Gibco). The cultures were incubated at 24 °C in an incubator set. The next day, 2 mL of new growth medium was added to the flasks. Monolayers of primary cells formed after 10 days of culture.

Primary cultures were digested with 0.25% trypsin-EDTA solution (Sigma) into single cells and transferred into another fresh 25 cm^2 flask at a split ratio of 1:2 for subculture. Cells were initially maintained in MEM with 20% FBS. After 20 passages the concentration of FBS in MEM was reduced to 10%. Half of the medium was changed every two days. To date, CSAC cell line has been subcultured for more than 56 passages.

Optimal conditions

To analyze the effect of temperature on the proliferation of the cells, the CSAC cells were inoculated in a 12-well plate at an initial density of $2x10^5$ cells/mL at 15 °C, 20 °C, 24 °C and 30 °C, respectively. After 2, 4, 6 and 8 days, the cells were trypsinized and counted microscopically via a hemocytometer. The effect of FBS concentration on cell growth at 24 °C was evaluated in 12-well plates for CSAC. The cells were incubated in MEM containing 5%, 10%, 20% and 25% FBS and incubated at 24 °C. The cells were collected every two days for 10 days and counted for three times in triplicate.

Cryopreservation and recovery of cells

Cells at approximately 90% confluence were trypsinized and centrifuged at 1 200 g for 3 min. The collected cells were suspended at a density of $2x10^6$ cells/mL in pre-cold (4 ^oC) MEM complete medium containing 10% dimethyl sulphoxide, 20% FBS. Cells were dispensed into the 1.8 mL sterile plastic vials, which were put in a styrofoam box, incubated at -80 °C overnight and transferred into liquid nitrogen for cryostorage. The vial containing frozen cells from liquid nitrogen was thawed at 42 °C for 1 min, agitated gently until the cells were dissolved and centrifuged at 2 000 g for 2 min. The cells were suspended in fresh MEM and seeded into a 25 cm^2 cell culture flask.

Chromosome analysis

CSAC cells at passage 28 were inoculated into 25 cm^2 culture flasks and incubated at 24 °C for 20 h. The cells were dosed with colchicine (0.1 μg/mL) for 3 h in 25 cm^2 culture flasks and harvested by centrifugation (1 000 g, 5 min). The collected cells were suspended in 10 mL hypotonic solution of 0.075 M KCl for 25 min at 24 °C and then premixed for 15 min in 3 mL of cold Carnoy's fixative (methanol:acetic acid=3:1) by centrifugation (1 000 g, 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 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 pEGFP-N_3

CSAC cells cultured in 12-well plate at approximately 80% confluence were transfected with pEGFP-N_3 express vector using Trans (TIANGEN). In brief, 3 μL Trans was added into a 0.5 mL centrifuge tube containing 50 μL MEM without FBS and antibiotics. Meanwhile, 4 μL pEGFP-N_3 mixed with 50 μL 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 mixtures were dropped into the wells and cultured at 24 °C for 5 h and the medium was replaced with normal medium. The green fluorescence signals were observed under a fluorescence microscope (Nikon Eclipse TE2000-U). After a 48 h incubation, more than 15% cells were observed green fluorescence signals.

Immunochemical characterization

Cultures were fixed in cold (4 °C) methanol for 10 min. To label astrocyte cells, antibodies were applied directed against glial fibrillary acidic protein (GFAP) isolated from bovine spinal cord (Invitrogen, rabbit polyclonal, diluted 1:100). Following a washing step with PBS after fixation, cells were treated with pre-incubation solution containing 1% bovine serum albumin in PBS at room temperature for 60 min. Afterwards primary antibodies were diluted in PBS and were detected by incubation with donkey anti-rabbit secondary antibodies (Molecular Probes, Invitrogen) for 0.5 h at room temperature. Cells were subsequently washed and the fluorescence signals were observed under a fluorescence microscope (Nikon Eclipse TE2000-U).

RT-PCR analysis of the expression of GFAP

Total RNA in CSAC cells at passages 40-45 was isolated for RT-PCR using Trizol (ambion). The concentration and purity of RNA were determined by measuring the absorbance at 260 nm and 280 nm, respectively. RNA (3 μg or 3 μL per sample) was used to generate cDNA using the PrimeScript™ RT reagent kit with gDNA eraser (Perfect Real Time, TaKaRa). Primers were designed (F: 5′-TCCAAGTTCGCTGACCTGACNGAYGCNGC-3′; R: 5′-CTACAGGTGTCGAGCCATNYNYTC-3′) using Consensus-Degenerate Hybrid Oligonucleotide Primer (CODEHOP), from crusian carp (*Carassius auratus*, L23876.1), white rhinoceros (*Ceratotherium simum simum*, XM_004432578.1), painted turtle

(*Chrysemys picta bellii*, XM_005282866.1), wild common carp (*Cyprinus carpio*, S66473.1), zebra fish (*Danio rerio*, AY151284.1), saker (*Falco cherrug*, XM_005447001.1), jungle fowl (*Gallus gallus*, XM_418091.4), gecko (*Gekko japonicas*, GU045301.1), the house mouse (*Mus musculus*, NM_00113-1020.1), nile tilapia (*Oreochromis niloticus*, AB109167.1), medaka (*Oryzias latipes*, XM_004071186.1), cichlid (*Pundamilia nyererei*, XM_005726223.1) and fugu (*Takifugu rubripes*, XM_003964955.1).

PCR amplifications were performed using DNA Taq^{TM} (TaKaRa). cDNA (1 μL), 1 μmol each of the forward and reverse primers, and 13 μL of Master Mix were added to a 0.2 mL thin-wall PCR tube. The reactions were performed as the following steps: 32 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 40 s, ending with a final extension at 72 °C for 5 min. PCR products were electrophoresed on a 1% agarose gel. The observed sizes corresponded to the predicted values were cut from the agarose and then purified with Gel DNA Recovery kit (ZYMO) according to the manufacturer's protocols. Sequencing of the DNA fragments was performed commercially (BGI, Beijing, China). Sequences were identified using BLASTN (http: //www.ncbi.nlm.nih.gov/blast/Blast.cgi).

RESULTS

Primary cell culture and subculture

A monolayer of primary cultures was obtained from the brain of half- smooth tongue sole at 10 day after tissue adherence. From passage 20 onwards, the cells showed with uniform configuration with large body and long protuberances (Figure 1). The cells were subcultured at 3-4 day intervals during the initial 20 passages and were then subcultured every 7 days.

Optimal conditions

The passage 33 was cultured under four different temperatures. CSAC showed tolerance to temperatures from 15 °C to 30 °C and exhibited optimal growth temperature at 24 °C (Figure 2A).

During the first 48 hour, CSAC cells had a similar proliferation in MEM with 5% FBS and 10% FBS. The highest growth rate was obtained in the medium with 20% FBS. The cell number reached 12×10^5 cells/mL after 8 days. Cells in MEM with 25% FBS decreased dramatically in limine probably because the high concentration of FBS was harmful to the cells and caused the death of cells (Figure 2B). Similar effects were not found in other lower concentrations of FBS.

Chromosome analysis

At passage 22, the chromosome number of the CSAC cells was ranged from 32 to 56. Heteroploidy was observed in the cell line (Figure 3A). The modal chromosome number was 42 (60% of the counted metaphase cells (n=100)) (Figure 3B).

Cell transfection with pEGFP-N_3

Clear and strong green fluorescent signals were detected after the CSAC cells were transfected with pEGFP-N3 reporter genes at 48 h (Figure 4). With the percentage of transfection reached 15%, the CSAC was proved to be suitable for transfection. Green signals were observed in transfected CSAC cells using pEGFP-N_3 vector DNA.

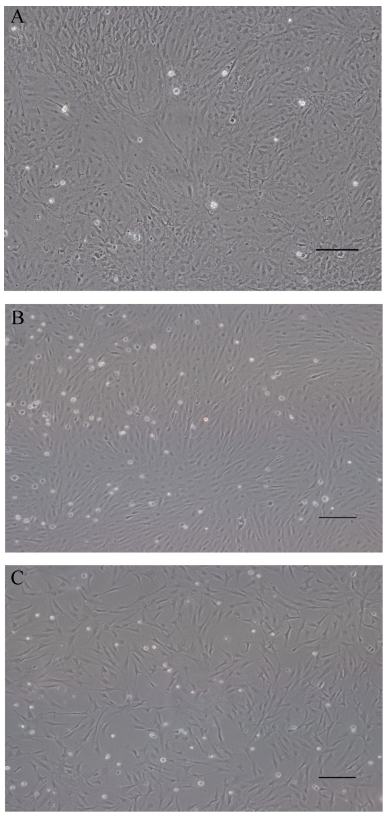

Figure 1 CSAC Cell line derivation

A: Brain cells primary culture at day 3; B: Cells at passage 35; C: Cells at passage 40; Cells in serial culture exhibited an epithelio-like phenotype; Scale bars=100 μm.

Immunochemical characterization

At passage 35, the cultures were tested by immunohistochemical stains, which revealed that the cells were positive for GFAP albeit with high intensities, and were identified as astrocyte cells (Figure 5).

Specific gene expression of GFAP

The size of purified DNA fragments from the CSAC cell line used for sequencing was corresponded with the predicted values. The nucleotide sequences were deposited in GenBank (Access Number: KF912949) (http: //www.ncbi.nlm.nih.gov/Genbank/index.html).

The BLAST program revealed that the nucleotide sequences of GFAP had 83%, 82%, 82%, 81% and 81% identities with that of the medaka (XM_004071186), (XM_003964955), nile tilapia (XM_003441987), cichlid (XM_005726223) and zebra mbuna (*Maylandia zebra*) (XM_004552438), respectively.

DISCUSSION

To isolate astroglial cells from the brain of fish is difficult due to

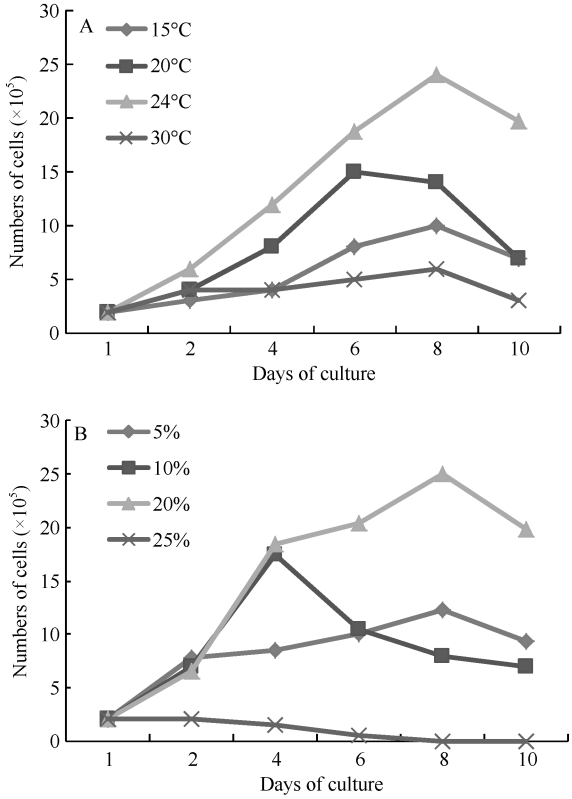

Figure 2 Growth curve of CSAC cell line in different temperature (A) and different concentrations of FBS (B)

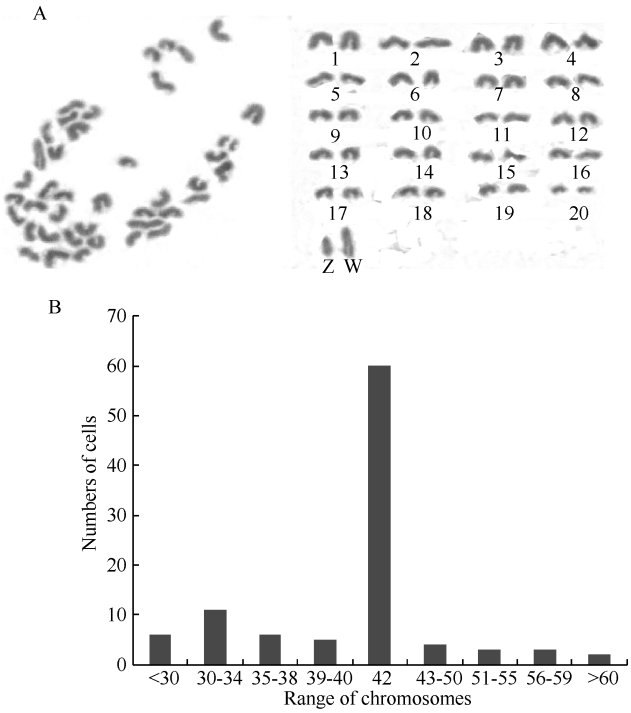

Figure 3 Chromosome analysis of the CSAC cell line

A: Diploid karyotype of CSAC cells at passage 22 (the female specific W chromosome is shown); B: Chromosome number distribution (the main chromosome number was 42).

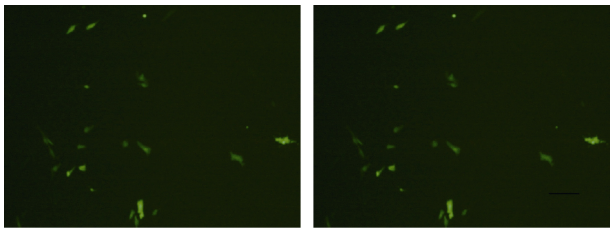

Figure 4 GFP expression of the CSAC cell line

Scale bars=100 μm.

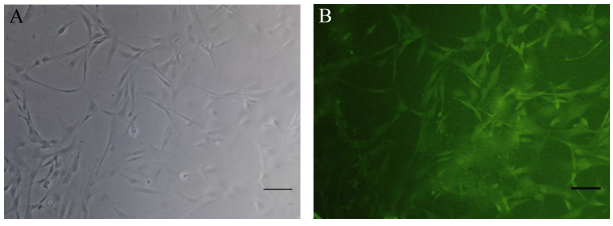

Figure 5 CSAC cells in culture (A) with glial markers revealed expression of glial fibrillary acidic protein (GFAP, green) (B)

Scale bars=100 μm.

the relatively low numbers of glial cells and the small size of the brain. Only a few studies attempted to take fish astrocytes in culture (Frjd et al, 2002). Mack & Tiedemann (2013) set up primary cultures from brain tissue of mature cichlid fish (*Astatotilapia burtoni*) to study fish astroglial cells in hypoosmotic conditions and their growth during axonal elongation but the cells were only passaged for several generations. In this study, we established a cell line designated as CSAC from the brain of half-smooth tongue sole. The results indicate that CSAC is an astroglial lineage cell line. CSAC has brain origin; shows the morphological homogenization of epithelial cells; and expresses a unique intermediate filament molecular marker of astroglial cells, GFAP (Kalman, 1998).

The half-smooth tongue sole has high economic values and survive under the temperature ranging from 3.5 °C to 32 °C. CSAC grows in a wide temperature range with an optimum growth at 24 °C and has the potentiality of isolating both warm and cold water fish virus. CSAC keeps proliferating with the FBS concentration reduced from 20% to 5% and showed optimal growth at the concentration of 20%. The result is in accordant with that of the other half smooth tongue sole cell lines (Wang et al, 2010; Zhang et al, 2011; Zheng et al, 2012).

The transfection efficiency of CSAC suggests that the CSAC could be used as an *in vitro* system to study the exogenous fish astrocyte cell functions. Comparing with the mixed cell morphology of the previously studied other fish brain cell lines, the homogenized cell morphology of CSAC may be affected by 2-Me and bFGF (Wen et al, 2009; Wen et al, 2010).

In conclusion, a new cell line designated as CSAC was derived from the brain of half smooth tongue sole. As the first

neurogenic cell line of flatfish, CSAC would be useful in studying fish nervous-related gene function, especially with the accomplishment of whole genome sequencing of half-smooth tongue sole.

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