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Establishment and cryopreservation of a cell line from cavefish *Sinocyclocheilus anshuiensis*

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Abstract: The cyprinid genus *Sinocyclocheilus* is the largest genus in Chinese Cyprinid fishes and more than half of species are cavefish, however, no cell line from any of these has been made available to date. In this study, we established a cell line, SAF (*Sinocyclocheilus anshuiensis* fin) and characterized the caudal fin tissue of *Sinocyclocheilus anshuiensis*, a cave-restricted cavefish. This SAF cell line consists of fibroblast-like cells and has been subcultured more than 30 times over the course of a year. Biological analysis showed that the population doubling time for thawed cells was approximately 73.3 h. PCR amplification confirmed the SAF cells were derived from *Sinocyclocheilus anshuiensis*. When the cells were transfected with pEYFP-N1 plasmid, bright fluorescent signals were observed, which suggests that the cell line is suitable for transfection and gene expression study. In addition, the SAF cell line established in this study can be used for protection of this species and research of adaptive evolution of cave animal. Furthermore, the establishment of cryopreservation of the SAF cell line can provide technical and theoretical support for preserving genetic resources at the cellular level for other cavefish species.

Keywords: *Sinocyclocheilus anshuiensis*; tissue culture; cell line; cryopreservation

Introduction:

The cyprinid genus *Sinocyclocheilus* (Cypriniformes: Cyprinidae) is endemic to the massive southwestern karst area joined to the eastern Qinghai-Tibetan Plateau of China, covering Yunnan-Guizhou Plateau and the surrounding region (including east of Yunnan Province, south central of Guizhou Province, and northwest of Guangxi Province). There are about 50 valid species distributing in the related narrow area (about 270,000 km^2), making it the largest genus in Chinese Cyprinid fish (Zhao & Zhang, 2009). More than half of *Sinocyclocheilus* species are cavefish. As adapting to subterranean environment, cavefish have evolved a series of adaptive characters. The most distinctive phenotypic changes are the degeneration or loss of eyes and pigmentation in adult fish (Jeffery, 2001, 2009; Wilkens & Strecker, 2003; Soares &Niemiller, 2013; McGaugh et al, 2014). Numerous studies about *Sinocyclocheilus* have been reported including description of new species (Gan et al, 2013; Pan et al, 2013), ecology (Pan et al, 2009a; Zhao & Zhang, 2009), artificial propagation (Yang et al, 2007, 2014; Pan et al, 2009b,c), embryonic development (Pan et al, 2014), genetic diversity, and system phylogeny (Zhao & Zhang, 2009). However, there has been no cell culture study in *Sinocyclocheilus* cavefish so far. Although more than 280 cell lines have been established since the first cell line was reported in 1962 (Wolf & Quimby), most of them are derived from freshwater and anadromous fish species (Wolf & Mann, 1980; Fryer & Lannan, 1994; Lakra et al, 2011). In China, more than 50 cell lines derived from 20 species have been established, but *Sinocyclocheilus* species (Chen & Qin, 2011). In the present study, we chose *Sinocyclocheilus anshuiensis* (*S.anshuiensis*) as the representative of cave-restricted cavefish, and successfully constructed a cell line SAF. The establishment of *S. anshuiensis* cell line SAF (SA,*Sinocyclocheilus anshuiensis*; F,fin) provides a good foundation for gene knockout, overexpression and RNA interference study, which is critical for the study of cavefish evolution.

Materials and Methods:

Primary cell culture and subculture

A healthy adult of *S. anshuiensis* (9 cm total length) (Figure 1) was collected from Linyun, Guangxi and cultured in the Endangered Fish Conservation Center (EFCC), Kunming Institute of Zoology, Chinese Academy of Sciences. The fish was disinfected with potassium permanganate at 18-20 ℃ for 1 h, followed by anesthetization in 70% ethanol for 2 min. The caudal fin tissue was clipped aseptically, and transferred to flasks. The tissue was washed five times with Hanks' balanced salt solution (HBSS) supplemented with antibiotics (penicillin, 300 IU/ml; streptomycin, 300 μg /ml; gentamicin, 30 μg/ml). The washed tissue was minced into about 1 mm^3 by using a surgical scissors and transferred into T-25 flasks (Corning) containing 3 mL of growth medium (L-15, Gibco) supplemented with 20% fetal bovine serum (FBS, Gibco), 100 IU/ml of penicillin and 100 ug/ml of streptomycin. Tissues were incubated at 20 ℃ and 5 mL of growth medium was supplemented 24h later.

Figure 1. Morphology of adult *Sinocyclocheilus anshuiensis*. Scale bars=1cm

Monolayers of primary cells were obtained 30-40 days after tissue culture. Each flask was checked for microbial contamination and the cell morphology was examined. The old medium was replaced by same volume of fresh medium to make a conditioned medium. Cells that grew into a confluent monolayer were subcultured every 5-6 days by trypsinization with 0.25% trypsin-EDTA solution (Invitrogen) at a split ratio of 1:2. After passage 10, the medium was changed from 20%FBS + L-15 to 10% FBS+L-15 without any supplements and the cells were subcultured every 8 days.

Cryopreservation and recovery

For cryopreservation, every three passage of SAF cells at the logarithmic growth phase (80%–90% confluence) were harvested by trypsinization and centrifuged at 200g for 10 min. After removing the supernatant, the harvested cells were resuspended in freezing medium containing 80% FBS + 20% L-15 complete medium supplemented with 10% dimethyl sulfoxide (DMSO) (Sigma, USA). To make a concentration of 5–6×10^7 cells/mL, the cell suspension was counted using a hemocytometer and dispensed into 0.25 mL sterile plastic freeze-tubes (Corning) labelled with animal name, gender and date. The sealed tubes were kept in a Nalgeme Mr Frosty Freezing Containers (Thermo) at –80°C overnight and finally transferred into liquid nitrogen (-196°C).

To recover and reseed cells, frozen tubes were taken from the liquid nitrogen and thawed immediately in 40°C water bath for 5 min to recover to the optimal temperature. After centrifugation at 200 g for 5 min, the cells were suspended in 20% FBS + L-15 medium, transferred into T-25 flasks, and incubated at 20°C. To evaluate cell viability, thawed SAF cells in another freeze-tube were stained with 0.4% trypan blue, and alive and dead cells were counted separately under an inverted microscope (Olympus, CKX41).

Growth properties

SAF cells at passage 30 were trypsinized and resuspended in 10% FBS + L-15 medium as described above. Cells with an initial density of 2.0×10^4 cells/ml were placed in two 24-well plates and incubated at 20℃. Three wells of SAF cells were harvested by trypsinization and resuspended in 1 ml of PBS with 24h intervals. The number of cells in each well was counted, and the average value of three wells at each measurement was used to plot the growth curve. The population doubling time of the cells was calculated.

Identification of SAF cells

Genomic DNA was isolated from fresh *S. anshuiensis* muscle tissue and from SAF cell line using a DNeasy Tissue Kit (BioTeke). A primer set was designed to amply Cytb gene (L14737 5'-CCACCGTTGTTAATTCAACTAC-3' and H15915 5'-CTCCGATCTCCGGATTACAAGAC-3') . The PCR conditions were : 50μl mixture of PCR containing 5μl of 10×buffer, 1μl of 10μM L14737 and H15915 primers, 3μl of 10mM dNTP, 0.25μl Supernew Taq, 1μl DNA and 36.75μl ddH2O (PCR KIT, COMPANY). The reaction condition was 95℃ denaturation for 3 min followed by 35 cycles of 90℃ for 45 s, 52℃ for 45 s and 72 ℃ for 90 s, and a final extension at 72℃ for 10 min. The purified PCR product (KIT, COMPANY) was sequenced by Sangon Biotech company.

Cell transfection

Cells were seeded in 24-well plates and transfected with a plasmid construct that expresses fluorescent proteins (pEYFP-N1) by using Lipofectamine2000 (Invitrogen). The plasmid DNA (μg) to Lipofectamine 2000 (μl) ratio was 1:2. The medium was renewed 6 hours after transfection, and cells were observed after 24h transfection under a Leica inverted fluorescence microscope. The transfection efficiency was determined by counting the number of fluorescent-positive cells as well as the total cell number in 20 independent optical fields at 400x magnification after 60 hours post-transfection.

Results:

Primary cell culture and subculture

The cells migrated out from the edge of the caudal fin tissues at 5 or 6th day after the first tissue culture. The cells grew well and formed a monolayer within 30 days at 20℃. They showed fibroblast-like morphology (Figure 2a) and we named the cell line as SAF (SA, *Sinocyclocheilus anshuiensis*; F, Fin). The cells were subcultured every 5-6 days with 20%FBS + L-15 medium without any supplement. The cells have been subcultured more than 30 passages fo far, and are still in a good proliferating state (Figure 2b), which indicates successful establishment of *S.anshuiensis* fin cell line (SAF).

Cryopreservation and recovery

The average cell viability of SAF cells at passage 30 before and after thawing was 95.7% and 86.2%, respectively. This indicates that culture and cryopreservation conditions were appropriate for cells to survive after one freeze-thaw cycle.

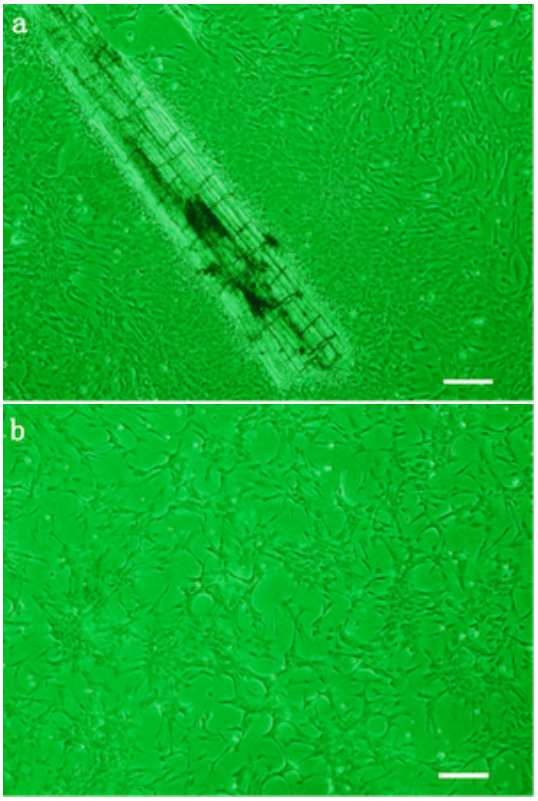

Figure 2. Photographs of *Sinocyclocheilus anshuiensis* caudal fin at the fifteenth day (a) and morphology of SAF cells at passage 30 (b). Scale bars indicate1mm.

Growth of cells

The growth curve of SAF cells at passage 30 (Figure 3) shows that the SAF cells progress through a characteristic growth pattern of lag phase (day 1-2), exponential phase (day 2-7) and stationary phase (day 7-9)

(Figure 2). the SAF cells grew and proliferated with a steady rate and their doubling time was calculated to be 73.3 hours at a seeding density of 2.0 $\times 10^4$ cells/ml with 10% FBS + L-15 at 20℃.

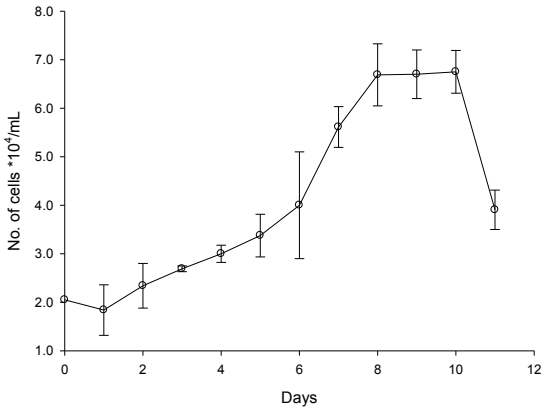

Figure 3. Growth curve of SAF from *Sinocyclocheilus anshuiensis* at passage 30 with 10%FBS+ L-15 at 20℃. (Data are represented as mean ± SEM)

Identification of SAF cells

The amplified PCR product targeting Cytb gene was shown as a single band near 1000 bp on the agarose gel. The sequencing analysis revealed that the amplified DNA sequences of SAF were consistent with ones of Cytb of fresh *S. anshuiensis* muscle tissue. This data confirmed that the origin of the established SAF cells was from *S. anshuiensis*.

Cell transfection

Yellow fluorescent proteins were observed in cell line 24 hours after transfection (Fig.4). The efficiency of pEYFP-N1 transfection was 25%, indicating the suitability of this cell line for transfection and gene expression studies.

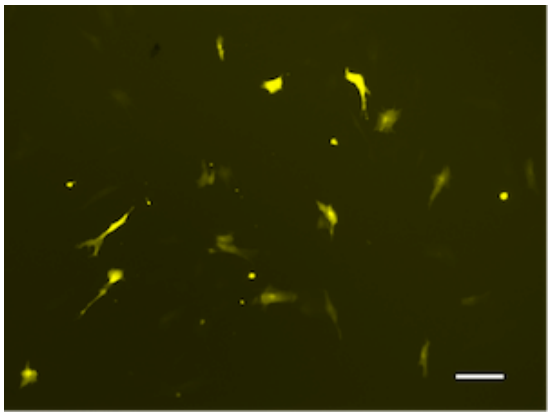

Figure 4. Fluorescent micrograph of yellow fluorescent protein (YFP) expressing cells in transfected SAF cells. Scale bars=1mm

Discussion:

The SAF is the first cell line of .*S.anshuiensis* as well as the first cell line of Chinese cavefish. The morphology of initial cells of SAF is same as other fin cell lines (Zhou et al, 2008; Ku et al, 2010).

Culture condition and identification

Contrary to wild fish, primary culture of cultured fish showed lower contamination rate, faster velocity of fin tissue migration and higher success. Long-term pond culture may contribute to the differences. Moreover, duration of primary culture and population doubling time of SAF cells were found longer than other Cyprinid fish, such as Zebrafish (Collodi et al, 1992), *Carassius auratus* (Shima et al, 1980) and *Anabarilius grahami* (Wang et al, 2012), which may be due to lower temperature for SAF culture. The SAF cells were incubated at 20°C and gained normal cell morphology. Thus 20°C is likely a suitable incubation temperature although it is higher than optimal growth temperature (16-18℃) for adult fish, such as *S. anshuiensis*. Indeed, *Anabarilius grahami* was also found that higher temperature was more suitable (24 - 28℃ vs 23-26℃). Long-term aquarium environment may relate to this difference as the temperature for *A. grahami* could adapt to 28-29℃ in artificial breeding (Wang et al, 2012). The origin of cell was identified once the cell line was stabilized by conducting PCR and sequencing analysis, which is the most commonly used method (Freshney, 2010; Lakra et al, 2011). Since Cytb has been used for phylogenetic analysis of *Sinocyclocheilus* (Zhao & Zhang, 2009), we chose the gene to verify whether the SAF was actually derived from *S. anshuiensis*.

Cell application

As many cell lines are capable to propagate and express exogenous genes (Zhou et al, 2008; Ou et al, 2010; Cheng et al, 2010), *S. anshuiensis* cell line was examined for exogenous gene manipulation by transfection.. Different cell lines show different transfection efficiencies (Zhou et al, 2008; Ku et al, 2010), and the ability of a cell to express different genes is also various (Hameed et al, 2006). These could explain why transfection efficiency of *S. anshuiensis* was lower than that of *A.grahami* (Wang et al, 2012).

In summary, a cavefish cell line, SAF, was established from the caudal fin of *S. anshuiensis*, and subcultured more than 30 passages. This cell line could provide an important tool for the study of adaptive evolution of cave at the cellular level. We also expect that more researchers will be attracted through the present study to be interested in cell culture of cavefishes.

Competing interests

The authors declare no conflict of interest.

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