A new epithelial cell line, SSK from kidney of striped snakehead *Channa striatus* (Bloch 1793)

D. K. CHAUDHARY, N. SOOD, D. K. VERMA, T. R. SWAMINATHAN*, B. KUSHWAHA,
R. ABIDI AND P. K. PRADHAN

ICAR-National Bureau of Fish Genetic Resources, Canal Ring Road, P. O. Dilkusha, Lucknow - 226 002
Uttar Pradesh, India

*Peninsular and Marine Fish Genetic Resources Centre, ICAR-National Bureau of Fish Genetic Resources
CMFRI Campus, PB No. 1603, Kochi - 682 018, Kerala, India
e-mail: sood_neeraj@rediffmail.com

ABSTRACT

A cell line was established from striped snakehead *Channa striatus* kidney. The cell line, named as SSK, has been passaged for over 62 times. Growth studies at different temperatures and foetal bovine serum (FBS) concentrations revealed that SSK cells show optimum growth at 28°C in L-15 medium containing 20% FBS. The chromosome number of SSK cells was 2n=40. Partial sequencing of mitochondrial cytochrome oxidase 1 gene of the cells confirmed that the cell line originated from *C. striatus*. The SSK cells were primarily epithelial, as determined using immunophenotyping. The cells from SSK cell line were transfected with phrGFP II-N mammalian expression vector. The SSK cell line was stored in liquid nitrogen (-196°C) at different passages and was successfully revived after four months of storage. The cell line could be successfully employed for cytotoxicity studies, as revealed by neutral red assay. The cell line can be a valuable surrogate to the whole fish studies.

Keywords: Cell line, *Channa striatus*, Karyotype, Kidney

Introduction

The striped snakehead *Channa striatus* from the family Channidae, are highly valued food fish in India (Talwar and Jhingran, 1992). The fish inhabits rivers, ponds, canals, swamps, lakes and rice fields (Hossain *et al.*, 2008). It is considered as a potential species for aquaculture (NBFGR, 2011) owing to several desirable traits, namely good price and capability to withstand adverse environmental conditions due to air breathing ability and hardiness (Samantaray and Mohanty, 1997). Moreover, the flesh of *C. striatus* has high nutritive value besides other medicinal value (Courtenay and Williams, 2004). However, overexploitation as well as several man-made factors has resulted in decline in the native stock (Hossain *et al.*, 2008). Aquaculture of this species can serve multiple purposes including conservation and restocking programs as well as species diversification in freshwater aquaculture. However, various diseases are bound to occur during fish culture. Therefore, development of *in vitro* test systems like cell lines and determining their optimal growth requirements are important (Grunow *et al.*, 2011).

Cell lines are important tools for studies on animal physiology, virology and toxicology which can provide highly reproducible results (Buonocore *et al.*, 2011). As per Lakra *et al.* (2011), approximately 283 cell lines have been

developed from finfish. Furthermore, several workers established cell lines from *C. striatus*, namely SSN-1 (Frerichs *et al.*, 1991); CSK and CSG (Abdul Majeed *et al.*, 2013, 2014) and CST (Sood *et al.*, 2015). In the present study, we describe the establishment a cell line from the kidney of *C. striatus*.

Materials and methods

Primary cell culture and maintenance

A healthy snakehead, *C. striatus* weighing 65 g was euthanised with MS222 (Sigma-Aldrich, Missouri, United States of America), swabbed with 70% ethanol and its kidneys were aseptically dissected. The kidney tissue was cleaned four times in phosphate buffered saline (PBS) with antibiotic-antimycotic solution (2X) (Invitrogen, Paisley, UK) and shifted to a fresh petridish containing PBS with antibiotic-antimycotic solution. The tissue was cut into approximately 1 mm³ explants using sterile scissors, which were transferred to a 25 cm² cell culture flask. The PBS was removed with help of micropipette and 250 µl of foetal bovine serum (FBS) was added. The flask was incubated at 28°C overnight to allow attachment of explants. Subsequently, 5 ml of L-15 medium with 20% FBS and 1x conc. of antibiotic-antimycotic solution was added to the flask. The

flask was again incubated at 28°C and about 50% of the medium was replenished every 4th day with fresh medium. The medium was decanted, after the cells reached 90% confluence, followed by two washings with PBS. Thereafter, the cells were harvested using trypsin-EDTA solution (Invitrogen) and passaged at a split ratio of 1:2. The concentration of FBS in medium was gradually reduced from 20 to 10% between 10th and 20th subculture. After every 10th passage, 1x10⁶ cells were resuspended in 1 ml of cryopreservation medium (FBS with 10% dimethyl sulphoxide) and transferred to a cryovial, which was stored overnight at -80°C and then transferred to liquid nitrogen (LN₂). For revival, the cryovial was thawed quickly at 37°C and its contents were transferred drop-wise to a tube containing 10 ml of L-15 medium. The tube was centrifuged at 200 g for 5 min at room temperature, medium was discarded and the pellet was resuspended in fresh medium. The viability of the stored cells after thawing was calculated by trypan blue staining using a haemocytometer. The cells were subsequently seeded in 25 cm² flask

Growth studies of cells

The SSK cells were cultured at various temperatures and FBS concentrations to determine the optimum temperature and serum concentration. At 32nd passage, SSK cells (1x10⁵ cells ml⁻¹ of L-15 medium with 20% FBS) were seeded in 25 cm² cell culture flasks, which were subsequently incubated at 28°C overnight. Thereafter, the cells were incubated at various temperatures *viz.* 24, 28, 32 and 37°C. The effect of temperature on SSK cells was studied for 4 days. Daily, three flasks at each temperature were harvested to calculate the cell number. A similar study was conducted with 33rd passage SSK cells at 28°C to know the effect of various FBS concentrations (5, 10, 15 and 20%).

Plating efficiency

To know the plating efficiency of SSK cells, 56th passage cells were seeded at cell densities of 100, 500 and 1,000 cells per flask (25 cm²) and cultured in L-15 medium with 10% FBS at 28°C. About half volume of the medium was replaced with new medium after every 3 days. After 10 days, cells were washed with PBS after decanting the medium. Thereafter, methanol was added to the flasks to fix the cells which were subsequently stained using crystal violet. The flasks were observed microscopically for counting the individual cell colonies. The plating efficiency was calculated following Freshney (2005) as: PE (%) = number of cell colonies/number of cells seeded X 100.

Chromosomal analysis

The karyotype was prepared from 60th passage cells following Freshney (2005). Briefly, the SSK cells at 75-80% confluency were incubated for 2 h with colchicine solution (Invitrogen) at a final concentration of 0.2 µg ml⁻¹. Following

gentle pipetting, the detached cells were collected by centrifugation at 4°C and resuspended in hypotonic solution of 0.56% KCl. The cells were fixed with 1:3 acetic acid : methanol solution. After 10 min, the cells were suspended again in fresh fixative and centrifuged. The cells were finally suspended in 0.5 ml of fixative. Smears were prepared from cell suspension by drop-splash method, air dried and stained with Giemsa solution (5%). Chromosome counting was carried out for 100 metaphase spreads under a light microscope.

PCR for confirmation of origin of cell line

To confirm the origin of SSK cell line, DNA was extracted at 58th passage and muscle tissue of *C. striatus*. A fragment of cytochrome oxidase I (COI) gene was amplified as per Ward *et al.* (2005). The cycling conditions included denaturation at 95°C for 5 min; 30 cycles of 95°C (45 s), 50°C (30 s), 72°C (45 s); and final extension at 72°C for 5 min. The PCR products were sequenced using forward and reverse primers in Applied Biosystems AB 3730 XL. The sequences of PCR fragments were aligned against known sequences from NCBI database using the Basic Local Alignment Search Tool (BLAST).

Gene transfection

The SSK cells were grown in a 6-well plate (10⁵ cells per well). Following attachment, the cells were transfected using 2 µg of phrGFP II-N vector (Stratagene, LaJolla, CA) using SatisFfection reagent. The plate was incubated in dark at 28°C. After 48 h, the cells were observed for fluorescence under fluorescence microscope for expression of GFP (Qin *et al.*, 2006).

Immunophenotyping

SSK cells, grown on cover slips, were fixed with methanol at -20°C. The cover slips were incubated with PBS containing 1% BSA at room temp (RT) for 1 h. The cover slips were incubated at RT with primary antibodies *i.e.* clone AE1/AE3 antibodies (Invitrogen), mouse anti-cytokeratin (pan), or mouse anti-vimentin antibodies (Invitrogen) for 1h. After washing, cells were again incubated for 1h with anti-mouse antibody labeled with FITC (1:50 in PBS) and cover slips were washed and mounted with buffered glycerol. Control cover slips were treated as described above, except that blocking buffer was used in place of primary antibody. Finally, the cover slips were observed for fluorescence under a microscope.

Neutral red uptake assay

Neutral red uptake assay was carried out following Repetto *et al.* (2008) for cytotoxicity studies using SSK cells that were exposed to various concentrations of mercuric chloride. Briefly, a 96-well plate was seeded with 1x10⁴ SSK

cells per well and incubated overnight at 28°C. Then, the culture medium was replaced with 100 µl of L-15, containing various concentrations of mercuric chloride (SRL, Mumbai, India) (1.96 to 500 µg ml⁻¹). All the above concentrations were used in triplicate. However, in control wells, 100 µl of L-15 medium without mercuric chloride was added. After 24 h, the cells were washed and neutral red solution (40 µg per ml of growth medium) was added and incubated at 28°C for 2 h. Then the neutral red solution was decanted. This was followed by washing of wells with 150 µl of PBS twice and fixation by addition of 5% glutaraldehyde. Subsequently, neutral red was removed by adding 150 µl of neutral red destaining solution to each well by shaking for 10 min. Finally, the optical density (OD) of each well was recorded at 540 nm (Tecan, Austria).

Results

Cell culture

Most of the explants adhered to the flask surface and radiation of the cells was observed by 24 h. These cells grew to form a monolayer by 18 days. After 25 passages, the cells could be passaged at a ratio of 1:3 every 5-6 days. This cell line has been designated as striped snakehead kidney (SSK) cell line. The SSK cells were stored for 4 months in liquid

nitrogen and showed over 80% viability after revival. The morphology of cells did not show any alteration following freezing and thawing. Presently, the cells have been passaged for more than 62 passages during 1 year (Fig. 1).

Growth studies of cells

Variation in growth rate of SSK cells was observed at various incubation temperatures which grew moderately at 24 and 32°C, whereas, maximum cell growth was observed at 28°C (Fig. 2a). At 37°C, the SSK cells showed vacuolation followed by rounding and detachment by 4 days. Similarly, when the FBS concentration in the medium was increased from 5 to 20% at 28°C, the growth rate of SSK cells also increased (Fig. 2b). There was moderate growth of cells at 10 and 15% FBS though maximum growth was noted at 20% FBS.

Plating efficiency

Plating efficiency of SSK cell line was found to be 3.9 ± 1.61 , 16.3 ± 2.24 and 19.2 ± 1.38 at seeding concentrations of 100, 500 and 1000 cells flask⁻¹ respectively.

Chromosomal analysis

The number of chromosomes of SSK cells at passage 60, counted from 100 metaphase spreads varied from 30

Fig. 1. Photomicrographs of SSK cells of *Channa striatus* (10x objectives) (a) Kidney tissue with radiation of cells; (b) Dividing cells observed after 3rd passage; (c) SSK cells at 19th passage and (d) SSK cells at 62nd passage

Fig. 2. (a): Growth response of SSK cells at various temperatures at 32nd passage; (b): various concentrations of foetal bovine serum at 33rd passage

to 51. However, 64% chromosome spreads had a modal chromosome number of 40. Furthermore, karyotype morphology of the spreads with diploid number was found to be normal (Fig. 3).

Confirmation of origin of cell line

Amplification of mitochondrial COI gene from SSK cells and *C. striatus* muscle tissue yielded 700 bp products (Fig. 4). Sequencing of these PCR products revealed 100%

Fig. 3. Chromosomal analysis of SSK cells; A normal metaphase spread from SSK cells at passage 60 revealed 40 chromosomes ($2n = 8m + 2sm + 16st + 14t$)

Fig. 4. PCR amplification COI gene of *Channa striatus*, using universal primers

Lane 1 - Negative control, Lane 2 - SSK cells, Lane 3 - *C. striatus* muscle, Lane M - 100 bp DNA ladder (Fermentas)

(a)

sequence identity between the COI gene from SSK cells and *C. striatus* muscle and 99% similarity with *C. striatus* mitochondrial COI gene sequences in the GenBank database. The sequence of COI gene has been submitted to NCBI GenBank (Accession no. KT347602).

Transfection

The phrGFP II-N vector transfected SSK cells (Fig. 5), exhibited strong green fluorescence signals after 48 h of transfection.

Immunophenotyping

The SSK cells incubated with mouse anti-cytokeratin antibodies exhibited green fluorescence (Fig. 6), whereas, no fluorescence signals was observed in SSK cells incubated with antivimentin antibodies or in SSK cells incubated with 1% BSA.

Cytotoxicity assay

Following incubation of SSK cells with mercuric chloride at increasing concentration, there was a decrease in O.D. which indicated a decrease in neutral red uptake (Fig. 7).

Discussion

In this study, the explant method was used to establish a cell line from kidney of striped snakehead *Channa striatus*,

Fig. 6. Photomicrograph of SSK cells with fluorescence following incubation with anti-cytokeratin antibodies

(b)

Fig. 5. The SSK cells transfected with phrGFP II-N vector expressing GFP gene at 48 h with passage 44 cells

Fig. 7. Cytotoxicity assay; SSK cells incubated with mercuric chloride at increasing concentration levels (1.96 to 500 µg ml⁻¹) exhibited a decrease in neutral red uptake

which has been named as SSK cell line. Till date, the SSK cells have been successfully passaged for over 62 passages. The optimum growth temperature of SSK cells was determined to be 28°C. This finding is in conformity with cell lines developed from freshwater fishes in several tropical countries (Ku *et al.*, 2010; Chaudhary *et al.*, 2013, 2014; Sood *et al.*, 2015; Swaminathan *et al.*, 2016;). Similarly, L-15 medium containing 20% FBS was found as optimum for the growth of SSK cells. Importantly, medium containing 10% FBS was also found to support moderate growth in the cells and this can be used under routine conditions for maintenance of this cell line. These findings are in accordance with previous reports of several fish cell lines (Sood *et al.*, 2015; Swaminathan *et al.*, 2016;). Moreover, after 4 months storage in LN₂, the SSK cells retained good viability (>80%). Monolayer was formed within 6 days after revival and importantly, without any morphological alterations, in accordance with previous studies (Ku *et al.*, 2010; Abdul Majeed *et al.*, 2013, 2014; Swaminathan *et al.*, 2016).

Plating efficiency is an important test to measure colonies developing from single cells. The test is generally employed to know the nutritional requirements of cells, test different lots of sera and measure the effects of exogenous growth factors. In case of SSK cell line, though the plating efficiency was low but it was found to improve when more cells was seeded. The low plating efficiency, as reported with SSK cell line, has been reported earlier by various workers (Ku *et al.*, 2010; Sood *et al.*, 2015) but other researchers reported high plating efficiency for *C. striatus* cell lines (Abdul Majeed *et al.*, 2013, 2014).

The chromosomal counting of metaphase spreads reported a diploid chromosome number of 40 in SSK cells and in accordance with that reported in literature for this species (Kumar *et al.*, 2013; Abdul Majeed *et al.*, 2013, 2014; Sood *et al.*, 2015). The variation in chromosome

number of SSK cells could possibly be due to additions or loss of chromosomes from neighbouring cells during preparation of chromosome (Swaminathan *et al.*, 2010). It has been suggested by various workers that mitochondrial genes viz. 18S rRNA, 16S rRNA, 12S rRNA and COI can be used for identifying the species of origin of cell lines (Cooper *et al.*, 2007; Cheng *et al.*, 2010). COI gene sequence has been used to identify species including fish (Hebert *et al.*, 2003; Ward *et al.*, 2005). The alignment of sequencing data of COI gene from SSK cells, *C. striatus* muscle and available sequences in NCBI GenBank confirmed the origin of SSK cell is *C. striatus*.

The transfection efficiency of SSK cells was high in this study. This suggests that heterologous promoter can be used for foreign gene expression in this cell line and useful in conducting studies on functional genomics, as reported previously with many fish cell lines (Qin *et al.*, 2006; Wang *et al.*, 2010; Sood *et al.*, 2015). The immunological markers of cytoskeleton have been used to differentiate the two cell types *i.e.* fibroblastic and epithelial in fish cell lines (Chaudhary *et al.*, 2014). The lineage of SSK cells, as determined by reactivity with antibodies to fibroblastic and epithelial markers, revealed that the cells of the SSK cell line are of epithelial type.

NR uptake assay is commonly used for cytotoxicity assays (Repetto and Sanz, 1993) and in the present study, the SSK cell's lysosomal integrity was affected following incubation with mercuric chloride and it was confirmed by decrease in NR uptake when the concentration of mercuric chloride increased. The results suggest that SSK cell line can be employed for preliminary screening of different compounds for their cytotoxic effects.

In conclusion, SSK cell line developed from kidney of *C. striatus* appears to be suitable for short-term cytotoxicity studies as well as for expression of recombinant proteins. The cell line will be useful for investigation of disease outbreaks in snakeheads, suspected to be of viral etiology.

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