Tissue and Cell 45 (2013) 219–226

Contents lists available at SciVerse ScienceDirect

Tissue and Cell

journal homepage: www.elsevier.com/locate/tice

A SRCF cell line from snowtrout, *Schizothorax richardsonii*: Development and characterization

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ARTICLE INFO

Article history:
Received 13 March 2012
Received in revised form 15 January 2013
Accepted 18 February 2013
Available online 29 March 2013

Keywords:
Cell line
SRCF
Snowtrout
Schizothorax richardsonii

ABSTRACT

Schizothorax richardsonii, commonly called snowtrout, is an important indigenous coldwater fish of the Himalayas, India with high commercial values as food and game fish. A cell line named as SRCF was developed from the caudal fin tissue of *S. richardsonii*. The cell line has been maintained in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum (FBS) at 24 °C. The cells showed fibroblastic morphology, high plating efficiency and cell doubling time of 48 h. Chromosomal analysis of SRCF cells revealed a diploid count of 98 chromosomes. The origin of the cell line was confirmed by the amplification of 655 and 578 bp of cytochrome oxidase subunit I (COI) and 16S rRNA of mitochondrial DNA (mt-DNA) genes, respectively. Transfection of SRCF cells with pEGFP-C1 plasmid resulted in bright fluorescent signals, suggesting the application of cell line in transgenic and genetic improvement programme. In addition, genotoxicity assessment illustrated the utility of the cell line as an *in vitro* model for aquatic toxicological studies.

1. Introduction

A flurry of interest into the development of cell lines from fish has been observed in the recent years. The cell lines have been potentially used in toxicology (Babich and Borenfreund, 1991; Segner, 1998), virology (Wolf, 1988) and also cytogenetics (Blaxhall, 1983). The fish cell lines have also made significant contributions in biomedical research (Hightower and Renfro, 1988), biotechnology and aquaculture (Bols, 1991), endocrinology (Bols and Lee, 1991), fish immunology (Clem et al., 1996; Bols et al., 2001), ecotoxicology (Fent, 2001; Castano et al., 2003; Schirmer, 2006), disease control (Villena, 2003) and radiation biology (Ryan et al., 2008).

Establishment of cell lines from different organs or tissues of a host species will be valuable for studying species-specific responses to viral infection at the cellular level since some pathogenic viruses are known to be organ or tissue-specific. Since the first cell line reported in 1962 (Wolf and Quimby, 1962), about 283 cell lines have been established from finfish around the world (Lakra et al., 2010a). Most of the cell lines have been previously derived from coldwater fishes of European origin but very few cell lines have been developed from coldwater fishes of Asia and South East Asia including India (Lakra and Bhonde, 1996).

The snowtrout, *Schizothorax richardsonii*, is the principal indigenous food and game fish of the Himalayas available in various upland resources. *S. richardsonii* is a widespread and sometimes abundant species in the Himalayan foothills of Indus, Ganges and Brahmaputra river basins. However, the species is undergoing rapid declines in several locations along the distribution range due to several anthropogenic threats such as dams, overfishing, tourism, alien species introductions, and in some areas fishing for ornamental purposes. *S. richardsonii* is enlisted in IUCN Red List of Threatened Species (Vishwanath, 2010). Hence, the species requires urgent protection and proactive conservation efforts to save it from becoming threatened in most of its range.

Very few cell lines from coldwater fishes have been reported from India except the cell lines from *Tor putitora* (Lakra et al., 2006) and *Tor tor* (Yadav et al., 2011). Hence, the establishment of snowtrout cell line will be instrumental in carrying out *in vitro* studies as well as conservation of germplasm of this important coldwater fish species. The present study was aimed to develop and characterize a cell line named as SRCF from *S. richardsonii*.

2. Materials and methods

2.1. Primary cell culture and subculture

Live fingerlings of *S. richardsonii* weighing 10–20 g were collected from the River Alaknanda, Garhwal, Uttarakhand, India and were maintained in the wet lab of National Bureau of Fish

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0040-8166/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tice.2013.02.002

Genetic Resources (NBFGR), Lucknow, India. Before explant preparation the fish were maintained in aerated, sterile water containing 1000 IU/ml penicillin and 1000 μg/ml streptomycin for 24 h at room temperature. The fish were then anaesthetized in ice-cold water, and wiped with 70% alcohol. The fin tissue was taken out aseptically and chopped in small pieces with scissors in phosphate-buffered saline (PBS, Ca^{2+} and Mg^{2+} free). The tissue fragments from fin were then washed several times in an antibiotic solution (PBS containing 500 IU/ml penicillin and 500 μg/ml streptomycin) and then planted into 25 cm^2 standard cell culture flasks with approximate 50 μl volume of fetal bovine serum (FBS) and incubated at 24 °C. After 24 h of attachment, L-15 medium supplemented with 20% FBS was carefully added to the flasks without unsettling the explants. Every 4–6 days, half of the medium was removed and replaced with fresh medium until passaging.

Upon attaining 90% confluency, cells were dislodged with trypsin (0.1% (w/v), in PBS) containing 0.2% EDTA and subcultured at a ratio of 1:2. After the first passage, cells were passaged at an interval of 5–7 days. Initially, subcultured cells were maintained in L-15 medium with 20% FBS and after 10th passage, the concentration of FBS in L-15 medium was reduced to 10%.

2.2. Growth characteristics

Growth characteristics of the SRCF cell line were assessed at selected temperatures, FBS and bFGF concentrations. To determine the growth at optimum temperature, 1×10^5 cells were seeded and incubated at 24 °C for 24 h then incubated at selected temperatures of 16 °C, 20 °C, 24 °C and 28 °C in triplicates. Every alternate day, triplicate flasks at each temperature were trypsinized and cell density was measured with haemocytometer for a total of 7 days. The growth response to various concentrations of FBS (5, 10, 15, and 20%) and bFGF (0, 5 and 10 ng/ml) on cell growth was carried out using the same procedure as for temperature.

2.3. Cell plating efficiency

Plating efficiency of the cell line was determined at seeding concentrations of 200, 500 and 1000 cells per flask (z) (25 cm^2 tissue culture flask) in duplicate at passage 12. Following 14 days of incubation, the medium was removed and cell colonies were fixed with formalin (25%) and stained with crystal violet stain (1%). Colonies were then counted (x) in each flask under the microscope and plating efficiency (y) was calculated using the formula $y = 100xz^{-1}$ (Freshney, 1994).

2.4. Cytotoxicity test

SRCF cells at 15th passage were treated with *Aeromonas* sp. to test the cytotoxicity of bacterial extracellular products (ECP) on the cells. The ECP from *Aeromonas* sp. was prepared according to the protocol described by Balebona (1995). The cells were seeded and grown as a monolayer in 24-well plates using L-15 medium supplemented with 10% FBS at 24 °C. For the toxicity test, the cell line was inoculated with 0.1 ml serial dilutions of ECP. For negative controls, wells were inoculated with sterile saline. Plates were incubated at 24 °C and the effects of ECP on the cells were observed after 24 h and 48 h.

2.5. Chromosome analysis

Chromosome spreads were made from fin cells at different passages. The cells were seeded in a 25 cm^2 culture flask and upon reaching to 70–80% confluency, colchicine (0.05%) was added to the flask and flask was incubated for overnight. Next day, medium was removed and transferred to a conical centrifuge tube. Flask was washed with PBS following trypisinization of cells. Contents were removed and added to the tube containing the medium removed earlier. Tube was centrifuged at 600 × g for 5 min. Supernatant was removed, leaving 0.5 ml solution in the tube and gently resuspended the pellet. Carefully added prewarmed (30 °C) 0.075 M KCl and incubated at 30 °C for 30 min. Centrifugation again carried out at 600 × g for 5 min then cells were fixed with freshly prepared chilled Carnoy's fixative (1:3, acetic acid–methanol). Again centrifuged at 600 × g for 5 min, supernatant was discarded and cells were resuspended in fixative. These fixed cells were then washed two to three times with fresh fixative, and then resuspended in a small amount of fixative. The slides were made by dropping the suspension onto glass slides, air dried and stained with 5% Giemsa (pH 6.8) for 15–20 min. Finally, the slides were observed and chromosome counts were performed in more than 105 metaphase plates.

2.6. Molecular characterization

2.6.1. DNA isolation

DNA was extracted from *S. richardsonii* tissue and SRCF cells at passage 25 according to the method followed by Lo et al. (1996). Briefly, the samples were trypsinized and homogenized in lysis buffer (100 mm NaCl, 10 mm Tris–HCl pH 8.0, 25 mm EDTA pH 8.0, 0.5% sodium dodecyl sulphate and 0.1 mg ml^{-1} proteinase K) then incubated at 65 °C for 1 h, 5 M NaCl was added to a final concentration of 0.7 M followed by a slow addition of 1/10th volume of N-cetyl N,N,N-trimethyl ammonium bromide (CTAB)/NaCl solution (10% CTAB in 0.7 M NaCl). After incubation at 65 °C for 2 h, the digested samples were deproteinized by successive phenol/chloroform/iso-amyl alcohol extraction. Finally the DNA was precipitated with absolute ethanol, washed with 70% ethanol and dissolved in TE buffer. The amount of isolated DNA was quantified by using a UV spectrophotometer at wavelength of 260 nm and DNA was diluted to get a final concentration of 100 ng μl^{-1}.

2.6.2. Amplification and sequencing

Two genes 16S rRNA and cytochrome oxidase subunit I (COI) of mitochondrial DNA were amplified. The 16S rRNA fragment was amplified using the following primers: forward 16SAR (5′-CGCCTGTTTATCAAAAACAT-3′) and reverse 16SBR (5′-CCGGTCTGAACTCAGATCACGT-3′) (Palumbi et al., 1991). The cytochrome oxidase subunit I (COI) fragment was amplified using the following primers: forward FISHF1 (5′-TCAACCAACCACAAAGACATTGGCAC-3′) and reverse FISHR1 (5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′) (Ward et al., 2005).

16S rRNA gene was amplified in a 50 μl reaction volume contained 5 μl of 10× Taq polymerase buffer, 0.2 mM of each dNTP, 0.4 μM of each primer, 2.5 U of Taq polymerase and 100 ng genomic DNA using the C-1000 thermal cycler (Bio-Rad). The PCR cycling conditions involved an initial denaturation step at 94 °C for 4 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min with a final extension phase at 72 °C for 10 min and a holding temperature of 4 °C after completion. COI gene was also amplified in a 50 μl reaction volume containing 5 μl of 10× Taq polymerase buffer, 0.25 mM of each dNTP, 0.4 μM of each primer, 2.5 U of Taq polymerase and 100 ng genomic DNA using the C-1000 thermal cycler (Bio-Rad). The PCR cycling conditions involved an initial denaturation step at 95 °C for 2 min followed by 35 cycles of 94 °C for 40 s, 55 °C for 40 s and 72 °C for 1 min 10 s with a final extension phase at 72 °C for 10 min and a holding temperature of 4 °C after completion.

The amplicons were checked on 1.2% agarose gel and the products with high intense bands were selected for sequencing. Products were labeled using the BigDye Terminator V.3.1 Cycle sequencing Kit (Applied Biosystems, Inc.) and sequenced

bidirectionally using an ABI 3730 capillary sequencer following manufacturer's instructions. The obtained sequences of PCR fragments were compared to the known sequences of the species. The obtained sequences of PCR fragments were compared to known sequences in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST).

2.7. Immunocytochemistry

SRCF cells were examined for the expression of monoclonal antibodies directed against Vimentin and Cytokeratin for the morphology confirmation at selected passages. Cells (2 or 4×10^4) were planted on 12-mm diameter uncoated coverslips in 12-well plates. Cells were grown on coverslips at 24 °C for 1–3 days, washed with PBS and fixed in paraformaldehyde (4% in PBS, v/v) for 10 min at room temperature, again washed twice with PBS and blocked with a solution containing 5% sheep serum and 0.1% Triton X in PBS and then incubated at 37 °C for 1 h. Block was removed by washing twice with PBS, dilution of primary antibody either a 1:40 antivimentin clone V9 (V6630-CLONE 9 Sigma) or a 1:400 antipan cytokeratin clone-11 (C2931-Clone C-11 Sigma) in PBS was added and incubated for overnight at 4 °C. Next day cells were washed with PBS and were incubated with 100 μl of a 1:300 dilution of secondary antibody FITC-labeled anti-mouse IgG at 37 °C for 30 min. Nuclei were counterstained with Hoechst 33258 stain (861405 Sigma–Aldrich) for 1 min. Labeled cells were visualized under fluorescence microscope. Negative controls (omission of the primary antibody) were included in each experiment.

2.8. Cell transfection with GFP reporter gene

pEGFP-C1 plasmid was used with transfection reagents lipofectamine LTX and Plus Reagent for transfecting the sub confluent monolayers of SRCF cells at passage 35. In brief, the SRCF cells were seeded at a density of 1×10^5 in a 12 well plate individually and incubated for 18 h at 24 °C in normal atmospheric incubator. Before transfection, cells were washed with PBS and supplemented with 400 μl of fresh L-15 medium devoid of serum and antibiotics. The plasmid DNA (200 ng of pEGFP-C1) was dissolved in 100 μl of optimem and then 0.5 μl of plus reagent was added. The mixture was incubated for 5 min at room temperature. 2 μl of lipofectamine LTX was added to the mixture containing plasmid DNA and incubated for 30 min at room temperature. Finally the mixture was added dropwise on 70–80% confluent SRCF cells in 12 well plate. The medium was changed with fresh medium after an incubation of 6 h at 24 °C. The green fluorescence signals were observed after 18 h under a fluorescent microscope (Olympus).

2.9. Comet assay

SRCF cells at 40th passage were treated with hydrogen peroxide (H_2O_2), a genotoxic model compound, in order to assess the efficiency of the Comet assay for estimating genotoxicity on the cells. For the assessment, cells were first grown in 24 well plate in 1 ml medium for 24–48 h. The medium was then removed and replaced with 1 ml of medium containing serial dilutions of the tested genotoxic compound, H_2O_2 (200, 100 and 50 μM), based on preliminary results obtained for different range of concentration on the cell line. Exposure was carried out for 24 h. After exposure cells were washed with PBS, trypsinized and processed for Comet assay.

The Comet assay (single cell gel electrophoresis assay) was performed following previously described protocol (Singh et al., 1988) with minor modifications. In brief, the cell suspension was mixed with 0.6% low melting agarose and placed on a slide precoated with 1.0% normal melting agarose. Third layer of 0.5% low melting agarose was added and left to solidify. Subsequently, the cells, not the nuclei, were lysed in a lysis buffer (100 mM EDTA, 2.5 M NaCl, 10 mM Tris base, 1% Triton X-100, pH adjusted to 10) at 4 °C for at least 1 h. Single-strand DNA was prepared by unwinding the DNA in electrophoresis buffer (1 mM EDTA, 10% DMSO, 300 mM NaOH, pH 13) for 30 min and then subjected to micro-electrophoresis in the same electrophoresis buffer (25 V, 300 mA) for 15 min. After electrophoresis, the alkalis in the gels were neutralized by rinsing the slides in a neutralization buffer (0.1 M Tris, pH 7.5) for 5 min followed by drying and fixing of slides in methanol. After staining with ethidium bromide (20 μg/ml) for 10 min, the slides were viewed under a fluorescent microscope (Olympus).

2.10. Cryopreservation

The viability of SRCF cells after freezing in liquid nitrogen (LN_2) was evaluated. The slow freezing method was used to cryopreserve cells. For cryopreservation, 72 h old cultures of SRCF cells were used at passage 30 and 50. In brief, cells were removed from the flask surface, concentrated by centrifugation, and resuspended in a freezing medium consisting of L-15 medium containing 20% FBS and 10% dimethyl sulphoxide (DMSO) at a density of 10^6 cells per ml. The cell suspensions were poured into 2 ml sterile cryovials and tubes were placed in a −80 °C freezer overnight then transferred to a liquid nitrogen tank (−196 °C) for storage. After 3 months of post-storage the cells were recovered. For recovery the vials were thawed in running water at 24 °C. Following removal of the freezing medium by centrifugation, the cells were suspended in L-15 with 10% FBS and tested for viability by haemocytometer counting after trypan blue staining. The viable cells were seeded into 25 cm^2 cell culture flask and observed.

3. Results

3.1. Primary cell culture and subculture

The radiation of cells from the fin explants started after 72 h of implantation. A confluent monolayer of primary cells was obtained after 7 days (Fig. 1a). Morphologically, initial subcultures of cell line consisted of both epithelial and fibroblast cells (Fig. 1b). After 20 passages, the cell line showed fibroblast morphology (Fig. 1c). The cell line has successfully been subcultured up to 55 passages. The cell line designated as SRCF has been maintained at cell culture laboratory, NBFGR, Lucknow.

3.2. Growth characteristics

The cell line, when examined at different temperatures, exhibited satisfactory growth at 20 °C with optimal growth at 24 °C, while no significant growth was observed at 16 °C (Fig. 2a). The cells were able to grow at 28 °C but as the time progressed, growth declined. Cells exhibited poor growth at 5% concentration of FBS, relatively good growth at 10%, but higher growth occurred with FBS concentrations of 15% and 20% (Fig. 2b). In the presence of bFGF (5 or 10 ng/ml) the cell proliferation was more rapid than the normal one (Fig. 2c). In the absence of bFGF, the proliferation decreased significantly.

3.3. Cell plating efficiency

The plating efficiency of SRCF cells seeded at a density of 200 cells per flask was 39% whereas the plating efficiency increased to 60% and 88% at 500 and 1000 cells per flask respectively with no significant differences between replicates.

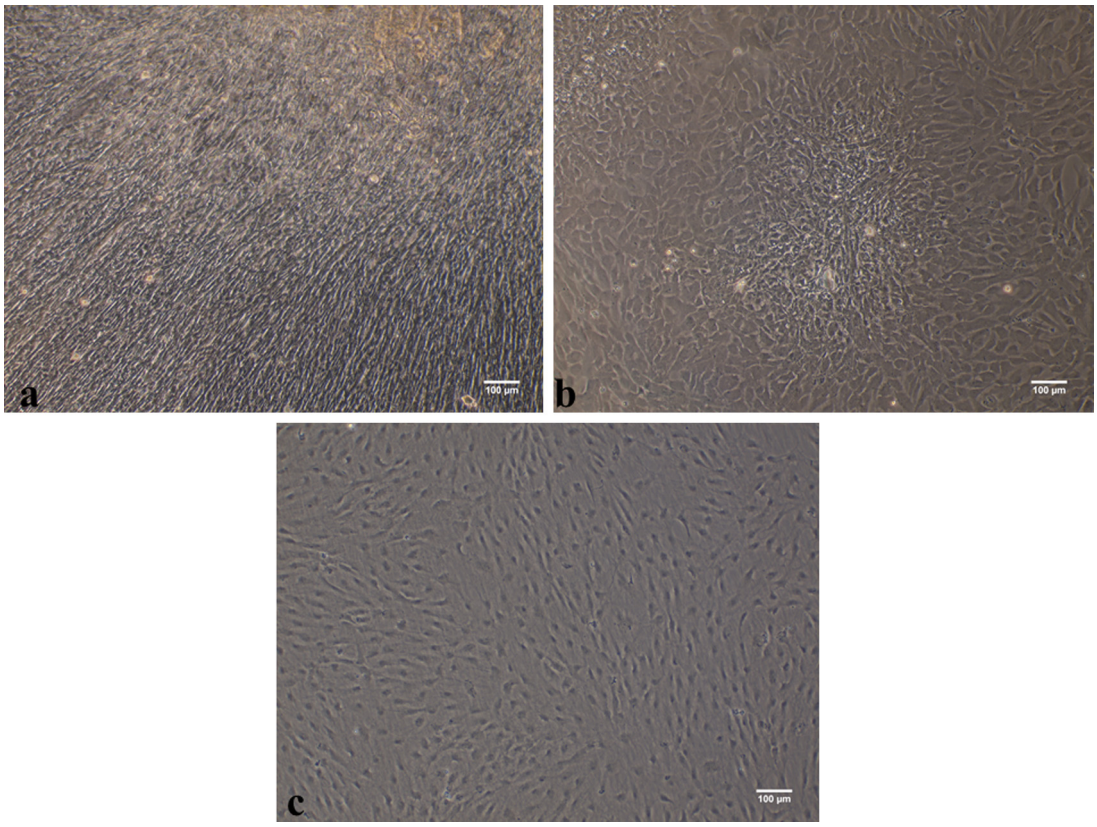

Fig. 1. Derivation of SRCF cell line. (a) SRCF cells primary culture at day 7. (b) Cells at passage 5. (c) Fibroblastic cells at passage 25.

3.4. Cytotoxicity test

The ECP from *Aeromonas* sp. was cytotoxic for SRCF cells. Cytotoxic effects could be observed within 12 h after inoculation of ECP. Cells showed rounding, shrinking, detachment and finally obliteration of the monolayer (Fig. 3).

3.5. Chromosome analysis

The chromosome counts of 105 metaphase plates from SRCF cells revealed that the chromosome number varied in a range from 49 to 119 (Fig. 4a) with a modal value of 98 (Fig. 4b). Distribution of chromosomes was asymmetric and aneuploidy was observed in small proportion of cells.

3.6. Molecular characterization

Amplification of 16S rRNA and COI genes of SRCF cells revealed the expected gene sequences of 578 bp and 655 bp, respectively (Fig. 5). The 16S rRNA and COI gene sequences derived from SRCF cell line showed 100% similarity with the gene sequences amplified from tissue sample of *S. richardsonii*. The gene sequences were submitted to GenBank and accession numbers obtained were JQ435845–JQ435846 and JQ435847–JQ435848 for 16S rRNA and COI genes, respectively.

3.7. Immunocytochemistry

Immunocytochemistry showed that SRCF cells were strongly positive for Vimentin and were negative for Cytokeratin (Fig. 6).

3.8. Cell transfection with GFP reporter gene

The SRCF cell line transfected with pEGFP-C1 plasmid using lipofectamine LTX and Plus Reagent showed clear and strong green fluorescent signals. The expression of EGFP in SRCF cells could be detected as early as after 6 h post transfection (Fig. 7a and b).

3.9. Comet assay

The derived Comet upon exposure of the SRCF cells with H_2O_2 is shown in Fig. 8a. There was a significant difference between the comet tail-length of SRCF cells exposed to H_2O_2 in comparison to the control (Fig. 8b). All the exposed concentrations of H_2O_2 were found to be toxic on cell line.

3.10. Cryopreservation

The cryopreserved SRCF cells following dimethyl sulphoxide (DMSO) slow freezing procedure showed significant viability of cells after thawing. The revived cells recovered well and grew to confluency within 7 days. Ability of cryopreserved cells to adhere after thawing was good and most of the cells could be recovered after freezing. Average estimated recovery percentage was 80–85% of the initial cell population.

4. Discussion

The developed cell line SRCF from *S. richardsonii* in the present study consisted of fibroblastic cells and the cell line has been maintained in L-15 medium supplemented with 10% FBS at 24 °C for up to 55 passages. The cell line exhibited characteristics of continuous cell line (Bejar et al., 1997; Lakra and Goswami, 2011). Cells did not show any adverse effect by trypsin used for subculture, low

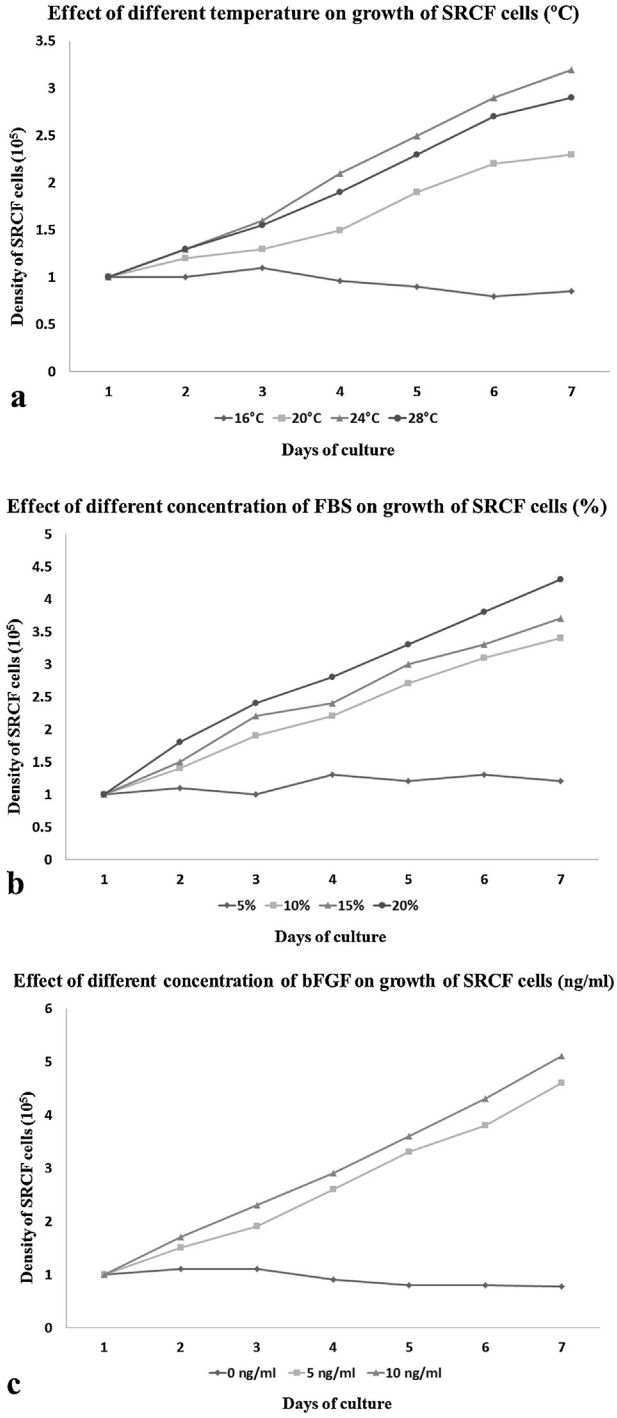

Fig. 2. Growth requirement of SRCF cells. (a) Different temperature. (b) Different FBS concentration. (c) Different bFGF concentration.

concentration of trypsin was used for dislodging the cells. However, increasing trypsin concentration can speed up the digestion of SRCF cells. The major advantage of maintaining the SRCF cell line was that no additional antibiotic used except during explant preparation, whereas the use of antibiotics have been previously reported in maintenance of many fish cell lines (Cheng et al., 2010; Ou-Yang et al., 2010). This indicates that antibiotics will not become a variable factor for bacterial pathogenesis studies using this cell line.

Basic fibroblast growth factor (bFGF) is a growth factor that shows important regulatory abilities in cell proliferation,

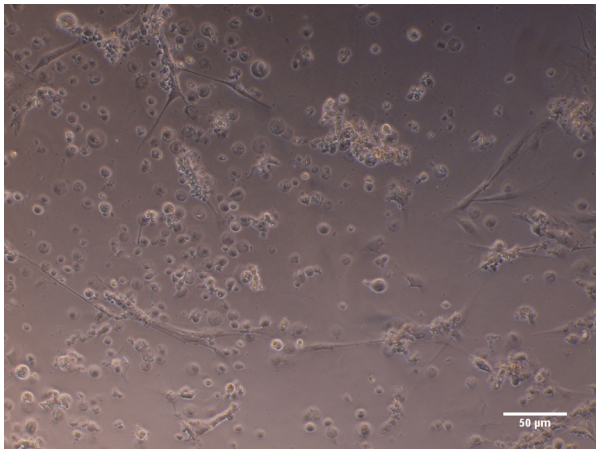

Fig. 3. Cytotoxic effect of extracellular products of *Aeromonas* sp. on the SRCF cell line.

migration and differentiation. It probably activates tyrosine kinase by binding to tyrosine kinase receptor and speed cell proliferation *via* various pathways (Hrzenjak and Shain, 1995). SRCF cells were found to grow optimally at 24 °C. Cells lines established from 'cold water' fish appear to have lower optimum growth temperatures as nine cell lines from salmonids showed best growth between 21 and 24 °C (Lannan et al., 1984). FBS is an essential component for cell growth and proliferation, the higher the concentration (up to 20%) the higher the cell growth. Similar results were also found in

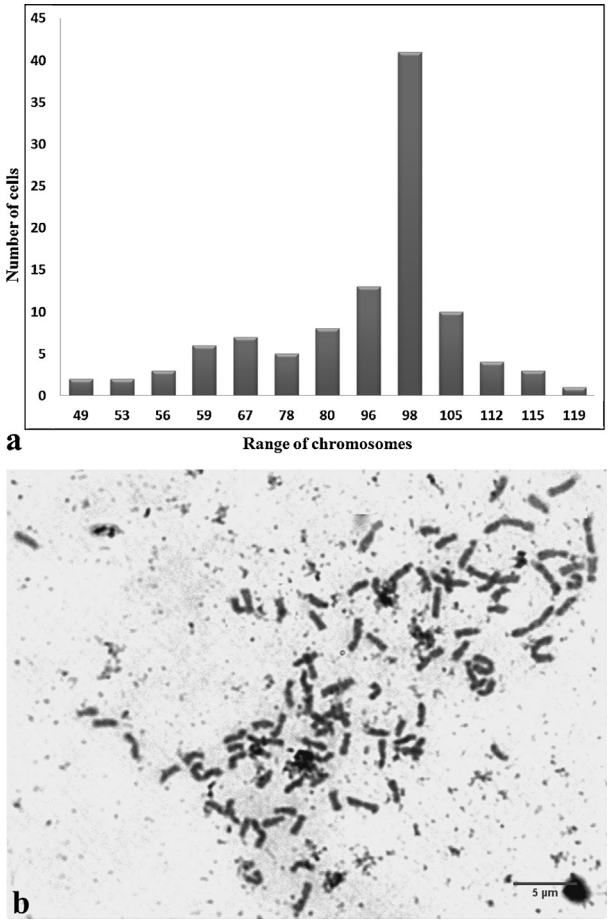

Fig. 4. Chromosome analysis. (a) Chromosome number distribution. (b) Metaphase spread.

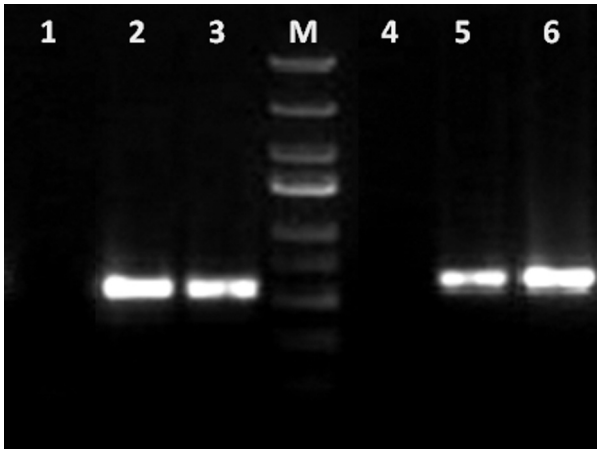

Fig. 5. PCR amplification of 578 bp 16S rRNA and 655 bp COI mt-DNA genes of *S. richardsonii*. Lane 1, 16S rRNA negative control; lane 2, SRCF 16S rRNA; lane 3, postive control 16S rRNA; M, 100 bp Generuler express DNA ladder (Fermentas); lane 4, COI negative control; lane 5, SRCF COI; lane 6, postive control COI (negative control: without template; positive control: muscle tissue of *S. richardsonii*).

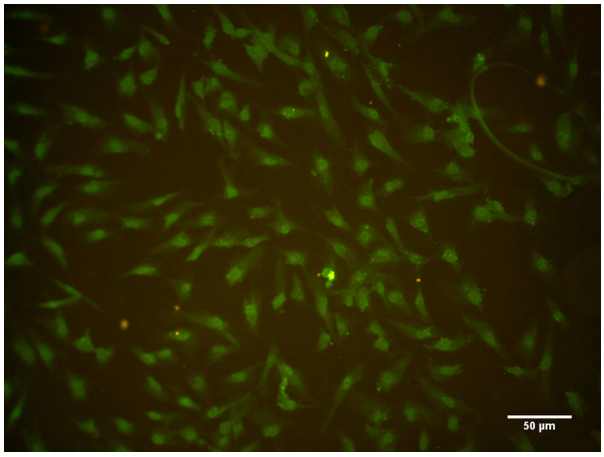

Fig. 6. Expression of fibroblastic protein in SRCF cells labeled with Vimentin and FITC-conjugated secondary antibody.

other fish cell lines (Tong et al., 1997; Chen et al., 2005; Lai et al., 2000; Parameswaran et al., 2007a,b). However, 5% FBS can also support growth of the SRCF cell line but relatively good growth was observed at 10% FBS. Hence, the cell line can be maintained at low cost using 10% FBS. Several fish cell lines were maintained at lower serum concentration (10%) to make the practice cost effective (Lakra et al., 2010b; Lakra and Goswami, 2011).

The SRCF cells showed moderately higher plating efficiency and decreased contact inhibition as the passage number increased which suggests spontaneous transformation might take place during the later passages. Many properties associated with transformation *in vitro* were reported to be a consequence of cell surface modifications (Freshney, 1994). The SRCF cell line was very sensitive to the ECP of *Aeromonas* sp. and morphological changes were seen as described by others (Bejar et al., 1997; Hameed et al., 2006). This indicates the suitability of this cell line to test the cytotoxic effects of bacterial extracellular products. Occurrence of changes in chromosome number as a result of chromosome instability shows the adaptation of cells to *in vitro* conditions. This instability can be generated by culture conditions that damage chromosomes. In this way the diploidy status is a good indicator of chromosome stability that may be relevant to the proximity of a cellular system to the original physiological condition. Chromosome analysis revealed that SRCF cells exhibited a modal chromosome number of $2n = 98$, which is identical with the reported modal chromosome number of *S. richardsonii* (Barat et al., 1997).

Species-specific DNA markers have been used to identify species origin of established cell lines. Hence, cell line authentication using molecular marker is essential especially when contamination with other cell lines is suspected. COI gene sequence is a useful DNA marker and its analysis has demonstrated it as the core of global bio-identification systems for animals (Hebert et al., 2003). It has been used to identify species and to study relationships among organisms (Song and Toh, 1994; Ward et al., 2005). Recently, COI region has been used for identification of cell lines (Lakra et al., 2010b; Lakra and Goswami, 2011; Cooper et al., 2007). The present study revealed the efficiency of COI sequence as a diagnostic marker for species identification. Other alternative marker such as 16S ribosomal RNA gene sequence was used to confirm the origin of muscle and fin cell lines of bluefin trevally (Zhao and Lu, 2006). The 16S rRNA gene sequence of SRCF cell line also confirmed the originality of the cell line.

Vimentin, which is a typical intermediate filament protein in the fibroblasts, was found to be expressed in SRCF cells. The result revealed fibroblast morphology of SRCF cells. Strong Vimentin labeling in the cytoplasm of the cell line indicates the immaturity of cells (Wen et al., 2008). The successful transfection of SRCF cells with pEGFP-C1 plasmid illustrates the possibility for this cell line to be genetically manipulated for gene targeting and expression studies. After exposure to H_2O_2 significant increase of DNA damage was observed in the SRCF cells as compared to cells that were not exposed to genotoxic compound in the Comet assay. This suggests that Comet assay can be applied successfully in fish cell lines, for genotoxic monitoring of toxic compounds. The Comet assay detects primary DNA lesions such as DNA strand breaks, but may also detect genomic instability, repair of double-strand breaks, DNA-adduct

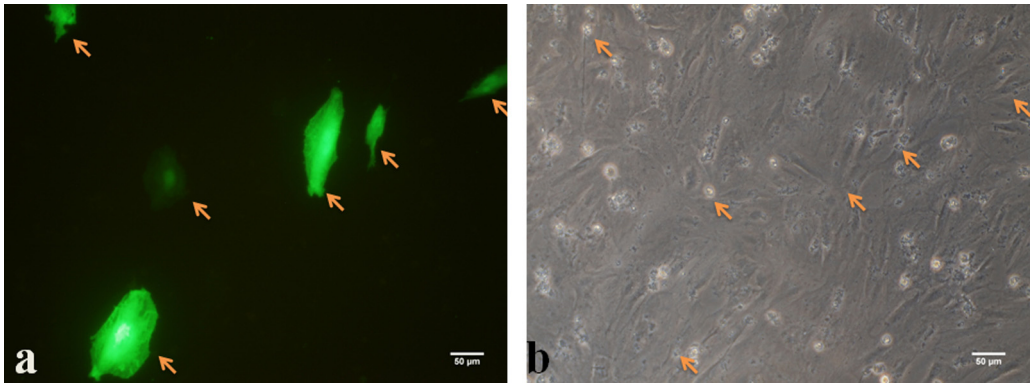

Fig. 7. Expression of green fluorescent protein in SRCF cells transfected with pEGFP-C1 vector: (a) fluorescent view and (b) phase contrast view.

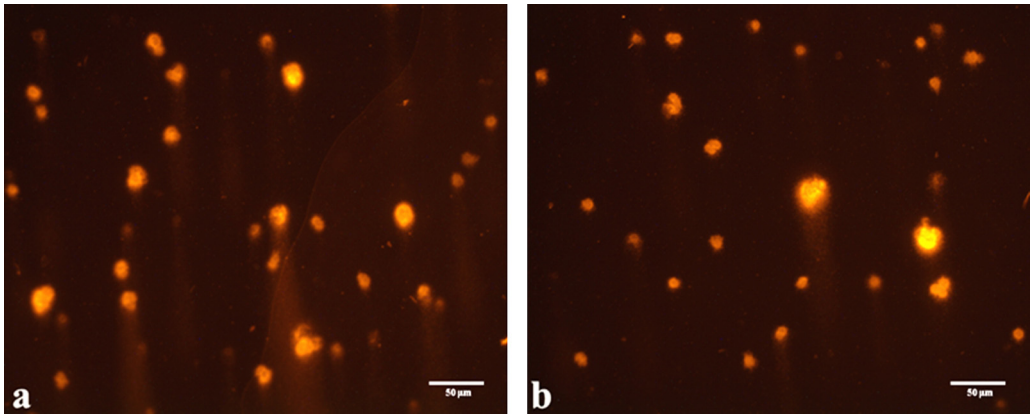

Fig. 8. Comets after single-cell gel electrophoresis of SRCF cells: (a) treated SRCF cells with H_2O_2 and (b) untreated SRCF cells (control).

formation and DNA cross-links (Hartmann and Speit, 1995). The present study explored the possibility of using SRCF cell line as an *in vitro* model in genotoxicology studies.

Cryopreservation of cell lines is necessary for long-term storage. DMSO is the most commonly used cryoprotectant for cultured cells, including fish cells (Wolf and Quimby, 1969) due to its low molecular weight and penetration capacity. Because of the slow penetration of the cryoprotectant into the fin cells, slow freezing method of cryopreservation proved to be highly efficient. However, decreased percentage recovery of cells demonstrates the mild toxic effect of DMSO (Mauger et al., 2006). For cryopreservation of SRCF cells, the optimal concentration of DMSO used was 10%. However, the concentrations of DMSO used for frozen cell suspensions are 10–20% (Donahoe et al., 1977; Jezek et al., 2002).

In conclusion, the cell line developed from caudal fin tissue of *S. richardsonii* could potentially be used as a powerful tool for conservation genetics and biotechnological applications.

Acknowledgments

The authors thank Director, NBFGR, Lucknow for his support and encouragement for the research program. Department of Biotechnology, Govt. of India, New Delhi is highly acknowledged for financial support.

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