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Development and characterization of a continuous embryonic cell line from turbot (*Scophthalmus maximus*)

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Abstract

A continuous cell line, TEC (turbot embryonic cell line), was established from embryos at the gastrula stage of a cultured marine fish, turbot (*Scophthalmus maximus*), and has been cultured for more than 200 days with more than 60 passages. The TEC cells were cultured in DMEM medium supplemented with antibiotics, fetal bovine serum (FBS), sea perch serum (SPS), and basic fibroblast growth factor (bFGF). The cells were small and round, and grew actively and stably in culture. Effect of temperature, fetal bovine serum (FBS) concentration, and bFGF on the growth of TEC cells was examined. The cells grew well in the temperature range of 24–30 °C, but have a reduced growth rate at temperatures below 18 °C. The growth rate of TEC cells in medium containing 15% FBS was higher than that in medium containing 7.5% FBS. Addition of bFGF to the medium significantly increased the growth rate of TEC cells. Chromosome analysis revealed that TEC cells have a normal diploid karyotype with $2n=4m+12st+28t$. High survival rate has been obtained after cryopreservation of cell cultures.
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Keywords: Turbot; *Scophthalmus maximus*; Embryonic cell line; Karyotype

1. Introduction

Cell lines are a powerful tool for studying epidemiology, molecular carcinogenesis, toxicology, and functional genomics in fish (Hightower and Renfro, 1988; Bahich and Borenfreund, 1991; Bols and Lee, 1991; Wise et al., 2002). Cell lines are also essential for isolating and identifying fish viruses. A large number of cell lines have been developed in fresh water teleost (Fryer and Lannon, 1994; Hong et al., 2004), but relatively only a few cell lines were developed in marine fish (Bejar et al., 1997; Chang et al., 2001; Chen et al., 2003a,b, 2004; Chi et al., 1999; Fernandez-Puentes et al., 1993a,b; Tocher et al., 1989).

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Turbot (*Scophthalmus maximus*) is a widely cultivated marine fish species in China. However, intensive aquaculture of this fish species has resulted in outbreaks of viral diseases with high mortalities. Reddish body syndrome (RBS) is one viral disease causing mass mortality of turbot (*S. maximus*). An iridovirus was isolated from diseased turbot with the 'reddish body syndrome' (RBS) and designated as TRBV (turbot reddish body virus) (Shi et al., 2004). Little is known about the epidemiology of viral infections and characterization of the virus in turbot due to lack of suitable cell lines. Thus, cell line is urgently desired in turbot for isolating and identifying viruses that cause viral diseases in this species. So far, few cell lines were developed from turbot tissues (Fernandez-Puentes et al., 1993a,b; Tocher et al., 1989). The present study described the development and characterization of an embryonic cell line from turbot (*S. maximus*).

2. Materials and methods

2.1. Primary cell culture and subculture

Gastrula-stage turbot embryos were harvested approximately 12 h after fertilization and prepared for cell culture. For each culture, a group of about 50–70 embryos was disinfected by immersing the embryos in 70% ethanol for 8–10 s, and then washed with PBS, and the chorions were torn with fine forceps. Then the cell mass was released and the chorion membrane was removed. Single cells were obtained by gentle pipetting. After several washes, the cells were transferred into gelatine-coated 24-well tissue plates. The above operations were performed in a laminar flow cabinet. The complete growth medium (TECM1) was Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 mM Hepes, pH 7.5, antibiotics (penicillin, 100 U/ml, streptomycin, 100 μg/ml; Gibco BRL), fetal bovine serum (FBS; 15%), sea perch serum (SPS; 0.5%), and basic fibroblast growth factor (bFGF, 2 ng/ml; Gibco BRL). Cells were cultured at 24 °C in a normal atmosphere incubator. Medium was changed every 2 or 3 days.

Upon reaching 95% confluence, the cells were subcultured at a ratio of 1:2 according to the standard trypsinization method. Trypsin–EDTA solution (Trypsin 0.05%, EDTA 0.02%, and NaCl 0.08%) was used for trypsinization. Cryopreservation of TEC cells was performed as described (Chen et al., 2003a). In brief, cell cultures were suspended in complete medium with 10% dimethyl sulfoxide (DMSO) and 20% FBS, and poured into 2-ml cryovials. Then the filled cryovials were placed in a freezing container (Nalgene, USA) and frozen at − 80 °C for 4 h, and finally transferred to liquid nitrogen (− 196 °C) for long-term storage.

2.2. Effect of temperature on cell growth

The effect of temperature on proliferation of TEC cells was examined. 5×10^4 cells were inoculated in one well of 12-well plate and incubated at 12, 18, 24, or 30 °C for growth test. After 2, 4, and 6 days, cell density was measured microscopically using a hemocytometer (Brand, Germany). The experiments were repeated four times.

2.3. Effect of FBS and bFGF on cell growth

The effect of FBS concentration and addition of bFGF on proliferation of TEC cells was examined. 1×10^5 cells were inoculated in one well of 12-well plate and incubated at 24 °C. After 16 h, the old medium was removed and various media containing different concentrations of FBS or bFGF were added. After 3 days, cell density was measured microscopically by using a hemocytometer. The experiments were repeated four times.

2.4. Chromosome analysis

Chromosome preparation from cell cultures was performed as described with some modifications (Earley, 1975). In brief, TEC cells at passages 15–20 were used for chromosome analysis. The cells were inoculated in culture flasks of 25 cm^2 and incubated for 24–36 h. Colchicine was added to the cells to reach a final concentration of 0.5 μg/ml. After about 4 h, the cells were harvested, treated with 5 ml of hypotonic solution of 0.075M KCl for 25 min, and then pre-fixed for 2 min by adding 1 ml of Carnoy's fixative to the above cell solution. After centrifugation, the cell pellet was fixed twice with cold Carnoy's

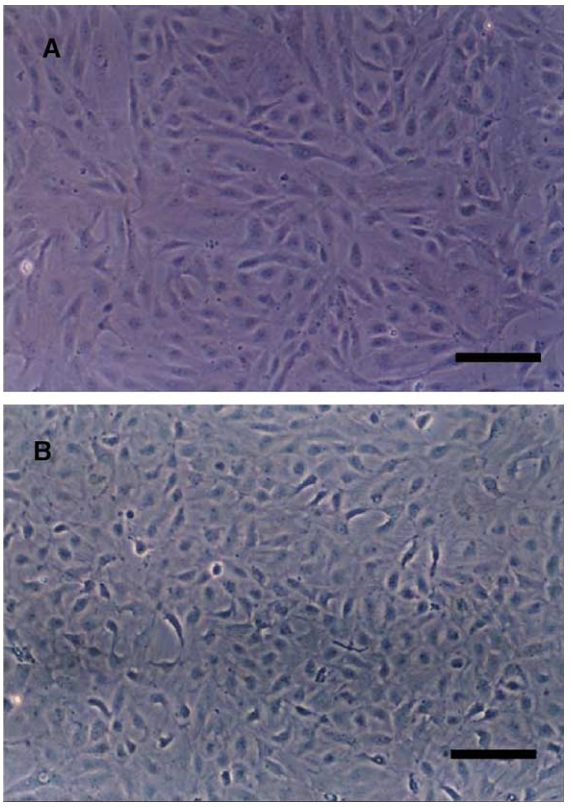

Fig. 1. Morphology and culture of TEC cells at passage 16 (A) and passage 35 (B).

fixative, 15 min for each time, then air-dried. Chromosomes were stained with 5% Giemsa for 25 min. 100 cells at metaphase were counted, and chromosome karyotype was analyzed according to the reported method (Levan, 1964).

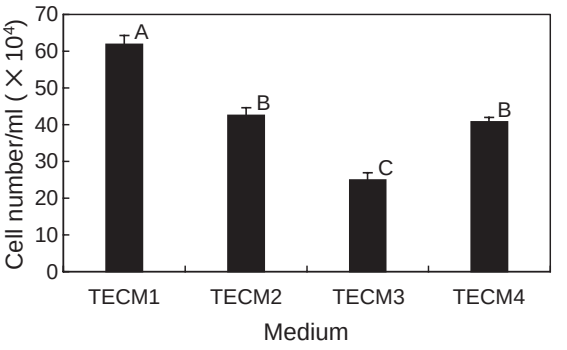

Fig. 3. Effect of fetal bovine serum (FBS) concentration and addition of bFGF on the growth and proliferation of TEC cells. The values having different letters above the column were significantly different ($p<0.05$). Values were expressed as mean ± S.E.M. ($n=4$). TECM1: 15% FBS, 2 ng/ml bFGF, 0.5% SPS; TECM2: 7.5% FBS, 2 ng/ml bFGF, 0.5% SPS; TECM3: 15% FBS, 0.5% SPS; TECM4: 7.5% FBS, 2 ng/ml bFGF.

2.5. Statistical analysis

Data were expressed as mean ± S.D. The cell amount was analyzed with independent-samples tests. A value of $p<0.05$ was considered as statistically significant. The statistical analysis was computed using SPSS software.

3. Results

3.1. Establishment of embryonic cell line

To develop an embryonic cell line in marine cultured fish, embryos of *S. maximus* at the gastrula

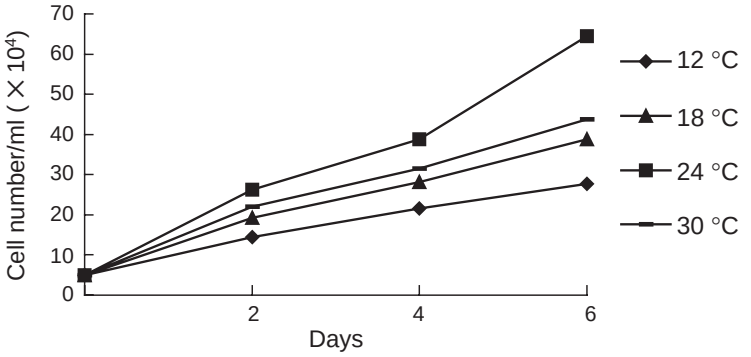

Fig. 2. Effect of temperature on the growth and proliferation of TEC cells. The values having different letters above the column were significantly different ($p<0.05$). Values were expressed as mean ± S.E.M. ($n=4$).

stage were used. The single cells from 50–70 embryos were isolated and inoculated in one well of 24-well plate. These cells with a diameter of about 10–25 μm were first evenly attached to the surface of the well and then moved to form many dense, homogeneous aggregates within the first 12–24 h after inoculation. Two or 3 days later, the cells grew to confluence in the well, and subculture could be done. Cell division occurred very rapidly and subculture should be done once every 3 or 4 days. The TEC cultures at passages 16 and 35 were shown in Fig. 1. In early subcultures of up to 20 passages, both epithelial-like and fibroblast-like cells were present (Fig. 1A). After 20 subcultures, epithelial-like cells became the predominant cell type (Fig. 1B). So far, the TEC cells have been cultivated for more than 200 days with more than 60 passages. The cryopreservation tests demonstrated that TEC cells could undergo cryopreservation

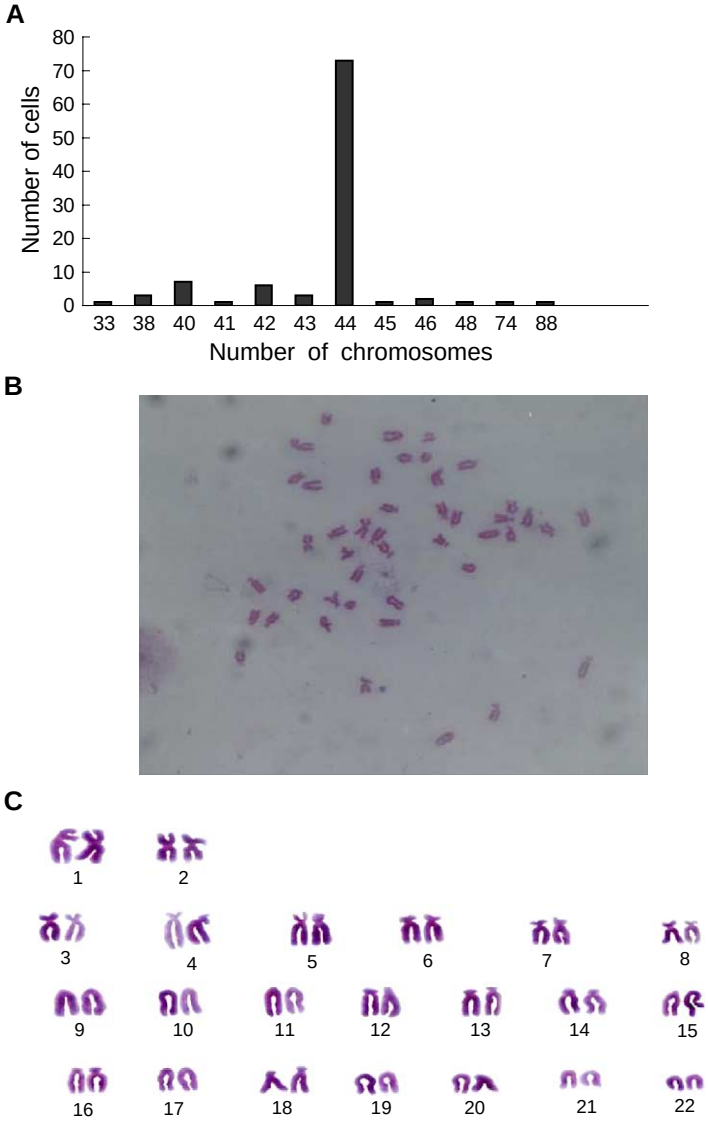

Fig. 4. Chromosome number distribution (A), metaphase (B), and diploid karyotype (C) of TEC cells at passage 17. 100 metaphases were counted.

in liquid nitrogen (− 196 °C) with a survival rate of about 60%.

3.2. Effect of temperature on growth of TEC cells

TEC cells exhibited different growths at different temperatures (Fig. 2). The growth of TEC cells increased with an increase of culture temperature when temperature was between 12 and 24 °C. Although cells grew well at 24 and 30 °C, the growth rate of the cells cultured at 30 °C began to decrease. The growth rate declined markedly at 18 and 12 °C ($p<0.05$).

3.3. Effect of FBS concentration and addition of bFGF on growth of TEC cells

The effect of FBS concentration and addition of bFGF on proliferation of TEC cells was shown in Fig. 3. The growth rate of TEC cells in medium containing 15% FBS (TECM1) was higher than that of TEC cells in medium containing 7.5% FBS (TECM2) ($p<0.05$). The absence of bFGF decreased significantly the proliferation of TEC cells (TECM3) ($p<0.05$). The absence of 0.5% SPS did not influence the growth of TEC cells (TECM4).

3.4. Karyotypic analysis

The results of chromosome counts of 100 metaphase plates from TEC cells at passage 17 revealed that the chromosome numbers varied from 33 to 88; nevertheless, the modal number of chromosome was 44, and 73% cells contained 44 chromosomes (Fig. 4A). The distribution was asymmetrical, with most $2n$ values appearing clustered below the modal value. Both aneuploidy and heteroploidy appeared in the TEC cell line in small proportions. The metaphase (Fig. 4B) with a normal diploid number displayed the normal karyotype morphology (Fig. 4C) consisting of 2 pairs of mediocentrics, 6 pairs of subtelocentrics, and 14 pairs of telocentrics ($2n=4m+12st+28t$).

4. Discussion

Cell lines have been recognized as powerful experimental tools in vertebrate immunology and biology. The importance of animal cell cultures, especially for cultured marine fish, for the study of viruses has gained increasing attention (Wise et al., 2002). So far, only a few cell lines have been developed in cultured marine fish, especially in turbot (Tocher et al., 1989; Fernandez-Puentes et al., 1993b). The present study reports the establishment of an embryonic cell line, TEC, derived from the gastrula-stage embryos of the turbot. The TEC cell line maintained stable growth and normal diploid karyotype over 200 days of culture in vitro with more than 60 passages.

Basic fibroblast growth factor (bFGF) is a potent mitogenic agent for human melanocytes (Halaban et al., 1988). In zebrafish, bFGF was mitogenic and suppressed melanogenesis (Bradford et al., 1994). In turbot, the present study demonstrated that bFGF stimulates the proliferation of TEC cells and can be used as a potent mitogenic factor in TEC and other cell cultures.

The euploid karyotype is an important parameter for characterizing a cell line. Karyotype analysis revealed that over 73% of the cells possessed a diploid chromosome number of $2n=44$, which was identical with the modal chromosome number of turbot (Bouza et al., 1994). This diploid rate is similar to, or higher than, other reports in other fish cell lines (Chen et al., 2003a; Sun et al., 1995; Hong et al., 1996).

Regarding turbot cell culture, Fernandez-Puentes et al. (1993a) developed a cell line from the posterior end of a turbot and subcultured the cell line for 44 times, but the authors found that the cell line is heteroploid and had a bimodal distribution of the chromosome numbers with values of 60 and 100, with tighter grouping around a mode of 85. In addition, Tocher et al. (1989) examined polyunsaturated fatty acid metabolism in turbot fin cell cultures.

In summary, an embryonic cell line, TEC, was established from gastrula-stage embryos of turbot, and could be potentially used for the study of infectious viruses in turbot and for developing cell models for screening immune-related functional genes in this species.

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