A novel heart-cell line from brown-marbled grouper *Epinephelus fuscoguttatus* and its susceptibility to iridovirus

Y.-B. Wei*, T.-J. Fan†, G.-J. Jiang, X.-H. Xu and A. Sun

Department of Marine Biology, College of Marine Life Sciences, Ocean University of China, Qingdao 266003, China

*(Received 26 February 2009, Accepted 16 December 2009)*

A novel cell line (bmGH) was established from the heart of brown-marbled grouper *Epinephelus fuscoguttatus* and its viral susceptibility was evaluated. The bmGH cells have been subcultured to passage 65 in Dulbecco’s modified eagle medium:Ham’s nutrient mixture F-12 (1:1) medium (DMEM/F12) which was further supplemented with foetal bovine serum (FBS), carboxymethyl-chitosan, basic fibroblast growth factor (bFGF) and insulin-like growth factor-I (IGF-I) at 24°C. The heart cells have a fibroblastic morphology and proliferated to confluence 14 days later. The cells grew at a steady rate during subsequent subculture and had a population doubling time of 40·3 h at passage 60. Karyotype analysis showed that these cells exhibited chromosomal aneuploidy with a modal chromosome number of 48. The results of viral susceptibility characterization revealed that cytopathic effects (CPE) of bmGH cells appeared after infection by two iridoviruses, turbot reddish body iridovirus (TRBIV) and lymphocystis disease virus (LCDV). A large number of TRBIV and LCDV particles were also observed in the infected bmGH cells by electron microscope examination. All of these facts indicate that the bmGH cell line established here may serve as a valuable tool for studies of cell-virus interactions and has potential applications in fish virus isolation, propagation and vaccine development.

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Key words: brown-marbled grouper; cytopathic effects; *Epinephelus fuscoguttatus*; heart-cell line; iridovirus; viral susceptibility.

INTRODUCTION

Brown-marbled grouper *Epinephelus fuscoguttatus* (Forsskål) is one of the most important cultured marine fish species with good flavour and high nutritional qualities. Recently, an outbreak of iridoviral diseases has severely affected cultured groupers with great economic losses (Qin *et al.*, 2002; Villena, 2003; Gibson-Kueh *et al.*, 2004). To control these viral diseases, healthy and sensitive cell lines from *E. fuscoguttatus* are needed to provide efficient approaches for studying the cell–virus interactions.
interactions during viral infection and developing more effective vaccines in vitro (Villena, 2003; Qin et al., 2006).

Many fish cell lines have been established but consist primarily of cells from freshwater and anadromous species (Fryer & Lannan 1994; Villena, 2003). Up to the present, only a few cell lines have been established from commercial marine fishes, such as red sea bream *Chrysophrys major* Temminck & Schlegel (Chen et al., 2003; Imajoh et al., 2007); sea perch *Lateolabrax japonicus* (Cuvier) (Ye et al., 2006; Chen et al., 2007); turbot *Psetta maxima* (Temminck & Schlegel) (Chen et al., 2005; Fan et al., 2007); flounder (*Paralichthys olivaceus*) (Ye et al., 2006; Chen et al., 2007); sea perch *Lateolabrax japonicus* (Cuvier) (Ye et al., 2006; Chen et al., 2007); flounder (*Paralichthys olivaceus*) (Tong et al., 1997; Chen et al., 2004) and groupers (*Epinephelus* spp.) (Qin et al., 2006; Parameswaran et al., 2007; Zhou et al., 2007; Wen et al., 2008). Unfortunately, no heart-cell line has been established from *E. fuscoguttatus*. This study was intended to establish a novel heart-cell line from *E. fuscoguttatus* and to use this model as a means to characterize its susceptibilities to iridovirus, taking advantage of the potential for the studies of virology and vaccine development at the cellular level.

**MATERIALS AND METHODS**

**PRIMARY CELL CULTURE**

A healthy *E. fuscoguttatus* weighing c. 600 g was obtained from Nanshan fish market (Qingdao, China) and killed by over-anaesthetizing with 20% urethane (Fluka; www.fluka.org). The fish was immersed in 75% alcohol for 5 min and its heart tissue was removed aseptically. After being washed twice with phosphate-buffered saline (PBS) and once in Dulbecco’s modified eagle medium:Ham’s nutrient mixture F-12 (1:1) medium (DMEM/F12, Gibco; www.invitrogen.com), the heart tissue was minced into small pieces (c. 1 mm³ in size) in DMEM/F12 (pH 7·2) containing 5% foetal bovine serum (FBS, Hyclone; www.thermo.com). After removal of FBS-DMEM/F12 medium by centrifugation, the heart tissue pieces were digested with 0·125% trypsin (Gibco) for 10 min and the digestion mixture centrifuged at 120 g for 10 min. The collected pellets were resuspended with 5% FBS-DMEM/F12 medium and inoculated into 25 cm² cell culture flasks (Corning; www.corning.com). About 18 h later, 20% FBS-DMEM/F12 medium supplemented with 100 μg ml⁻¹ carboxymethyl-chitosan (provided by Laboratory of Biochemistry I, College of Marine Life Science, Ocean University of China), 10 ng ml⁻¹ basic fibroblast growth factor (bFGF, Peprotech; www.peprotech.com), 40 ng ml⁻¹ insulin-like growth factor-I (IGF-I, Peprotech), 100 IU ml⁻¹ penicillin (Lukang Chenxin Pharmaceutical Co., Ltd; www.lkeisen.com) and 100 μg ml⁻¹ streptomycin (Lukang Chenxin Pharmaceutical Co., Ltd) were added into each flask. These flasks were incubated at 24°C and the media were replaced every 5 days.

**SUBCULTURE AND MAINTENANCE**

Cells were subcultured when confluent monolayers developed. After being washed twice with PBS, heart cells were trypsinized with 0-25% trypsin solution until most of the cells had rounded up (c. 0.5–1 min). Trypsinization was stopped with the complete medium and the cells were subcultured at a split ratio of 1:2. The same method was used during subsequent subcultures.

**GROWTH PROPERTIES**

To determine the growth properties, heart cells at passage 60 were inoculated into each well of two 24 well plates with a density of 1·0 × 10⁶ cells ml⁻¹ at 24°C. From this time onwards, three wells of heart cells were harvested and suspended in 1 ml PBS, respectively,
at 12 h intervals. The number of cells in each well was counted by a cell analysis system (CASY; www.casy-technology.com), and the average value of three wells was used to plot the growth curve of heart cells. The growth curve was determined with the time as the abscissa, and the corresponding cell number as the ordinate. The population doubling time of heart cells was calculated according to the method of Fan et al. (2007).

**CHROMOSOME ANALYSIS**

The passage 60 heart cells at the logarithmic phase were treated with 20 μg ml⁻¹ of colchicine (Fluka) for 10 h at 24°C. After harvest and treatment with 3 ml of 0-3% KCl hypotonic solution for 30 min, the heart cells were fixed with Carnoy’s solution for 20 min. The cells were then resuspended with 1 ml fresh Carnoy’s solution, and the cell suspension was dropped onto chilled glass slides. After being air-dried, the slides were stained with Giemsa for 40–50 min. Chromosomes were observed and counted under a light microscope (Nikon E200; www.nikon.com).

**STORAGE IN LIQUID NITROGEN**

For cryopreservation, the 20th, 40th and 60th passage heart cells at the logarithmic phase were harvested and suspended in 1 ml DMEM/F12 medium supplemented with 20% dimethyl sulphoxide (Amresco; www.amresco-inc.com) and 20% FBS with a density of 7 × 10⁶ cells ml⁻¹, respectively. The freeze-tubes (Gibco) of heart cells were kept at 4°C for 0-5 h, –20°C for 1 h, –80°C overnight and finally transferred into liquid nitrogen (−196°C).

The frozen heart cells were recovered by immediately thawing the cryopreservative medium in 40°C water-bath and then transferred into 24°C water-bath for 1–1.5 min to recover to their optimal temperature. After centrifugation, the cells were washed and suspended in 20% FBS-DMEM/F12 medium and incubated into 25 cm² cell culture flasks at 24°C.

**VIRAL SUSCEPTIBILITY AND REPLICATION EFFICIENCY**

The viral susceptibilities of heart cells to two iridoviruses, turbot reddish-body iridovirus (TRBIV) and lymphocystis disease virus (LCDV), were evaluated at passage 61. These iridoviruses were previously isolated and identified from TRBIV- and LCDV-infected marine fishes (Fan et al., 2006, 2009) and stored at −80°C. After removal of growth medium, 0-3 ml of TRBIV or LCDV solution at a dilution of 10⁻¹–10⁻³ was inoculated onto heart cells by adsorption for 2 h at 24°C. The inoculum was removed and 10% FBS-DMEM/F12 medium was added to the cells. The infected cell monolayers were incubated at 24°C and examined daily for possible cytopathic effects (CPE). Blind passages were performed every 7 days if CPE did not occur in infected heart cells.

When the CPE in the LCDV- or TRBIV-infected heart cells affected 70% of the cell monolayer, the cells were collected and frozen-thawed. Then, the cell fragments were centrifuged at 7700 g for 40 min to obtain the viral supernatant. The supernatant was collected for the test of viral replication efficiency and reinfection study in the same cell line. The titres of TRBIV and LCDV in heart cells were expressed as the 50% tissue culture infective dose (TCID₅₀) assay according to the method of Reed & Muench (1938).

**ELECTRON MICROSCOPY**

Once CPE was observed, the infected heart cells were collected and centrifuged at 120 g for 10 min. The heart-cell suspension was fixed with 2.5% glutaraldehyde (Fluka) in 0.2 M sodium cacodylate buffer (pH 7.4) for 8 h at 4°C. After being washed with sodium cacodylate buffer and post-fixed with 1% osmium tetroxide for 1.5 h, the pelleted cells were dehydrated in a graded series of alcohol, embedded in epoxy resin, sectioned and stained with 2% uranyl acetate-lead citrate. Ultra-thin sections were observed and photographed under a transmission electron microscope (JEOL JEM1200EX; www.jeol.com).
RESULTS

PRIMARY CULTURE AND SUBCULTURE

The primary cultured heart cells from *E. fuscoguttatus* had a fibroblastic morphology and proliferated to confluence 14 days later [Fig. 1(a)]. The cells grew at a steady rate in 20% FBS-DMEM/F12 medium supplemented with 100 μg ml\(^{-1}\) carboxymethyl-chito-oligosaccharide, 10 ng ml\(^{-1}\) bFGF and 40 ng ml\(^{-1}\) IGF-I. Subculture was carried out every 6–7 days at a ratio of 1:2. After 10 passages, the proliferation of heart cells became more rapid and the addition of carboxymethyl-chito-oligosaccharide and growth factors was stopped in later subcultures. To date, heart cells have been subcultured up to passage 65 and still retained a good proliferating status [Fig. 1(b)]. A novel *E. fuscoguttatus* heart-cell line named bmGH has therefore been successfully established. The density of heart cells reached a maximum at the fourth day on one passage and the population doubling time was 40.3 h (Fig. 2).

KARYOTYPIC FORMULAE

The results of chromosome counts of 300 metaphase plates from bmGH cells at passage 60 revealed that the chromosome numbers varied from 32 to 52, with a mode of 48 [Fig. 3(a)]. The distribution was asymmetrical and both aneuploidy and heteroploidy appeared in the bmGH cell line, which were in a small proportion of cells. The metaphase chromosomes [Fig. 3(b)] with a normal diploid number of bmGH cells consisted of a pair of subtelocentrics (st) and 23 pairs of telocentrics (t): 2\(n\) = 48, 2st + 46t, NF = 50 [Fig. 3(c)].

CRYOPRESERVATION AND RECOVERY

The cryopreserved bmGH cells in different passages retained viability after thawing. The bmGH cells could be grown to confluence within 1–2 days. The morphology and proliferation ability of bmGH cells were the same before and after cryopreservation.

![Fig. 1. Cell monolayer of the brown-marbled grouper *Epinephelus fuscoguttatus* heart-cell line (bmGH).](image)

(a) Photomicrograph of the bmGH cell monolayer in primary culture; (b) phase-contrast photomicrograph of the bmGH cell monolayer at passage 65. Bar = 100 μm.
The suitability of the bmGH cell line for viral diseases investigations was evaluated by its susceptibility to two iridoviruses, TRBIV and LCDV. Following inoculation with TRBIV, CPE was observed at second to third day [Fig. 4(b)]. About 30–40% of infected cells became round in morphology and began to shrink with cell aggregation. As viral CPE became apparent, additional cells became granular and vacuolated after 3–4 days [Fig. 4(c)]. LCDV-infected bmGH cells also showed CPE at the third day [Fig. 4(e)]. Many cells became round, crimped and granular with cell aggregation, but no obviously vacuolated cells were observed. No CPE was observed in normal bmGH cells [Fig. 4(a)]. As CPE in TRBIV and LCDV-infected bmGH cells affected 70% of the cell monolayer in 5 days, most cells became damaged and detached [Fig. 4(d), (f)]. The in vitro propagated TRBIV and LCDV had infective abilities to reinfect other bmGH cells. The viral titres of TRBIV and LCDV in bmGH cells reached $10^{4.5}$ TCID$_{50}$ ml$^{-1}$ and $10^{4.0}$ TCID$_{50}$ ml$^{-1}$, respectively, within 7 days.
Fig. 3. Chromosome analysis of the *Epinephelus fuscoguttatus* heart (bmGH) cells at passage 60. (a) Chromosomal aneuploidy of bmGH cells with chromosome numbers ranging from 32 to 52; (b) the metaphase chromosomes of one bmGH cell with a diploid number of 48, bar = 10 μm; (c) diploid karyotype morphology of one bmGH cell, 2n = 48, 2sm + 46t, NF = 50.
Fig. 4. Viral susceptibilities of turbot reddish-body iridovirus (TRBIV) and lymphocytis disease virus (LCDV) infected *Epinephelus fuscoguttatus* heart (bmGH) cells. (a) Normal bmGH cells; (b) typical cytopathic effects (CPE) of TRBIV-infected bmGH cells at third day; (c) vacuolations of TRBIV-infected bmGH cells after 3–4 days; (d) CPE in TRBIV-infected bmGH cells became more than 70% after 5 days; (e) typical CPE of LCDV-infected heart cells at third day; (f) CPE in LCDV-infected bmGH cells became more than 70% after 5 days; bar = 100 μm.

[Fig. 5(c)]. The ultra-thin sections of these virions with inner electron-dense central core formed paracrystalline arrays in infected bmGH cells, which also had typical characteristics of LCDV infection (Flügel, 1985).

**DISCUSSION**

In this study, a healthy and sensitive heart-cell line (bmGH) from *E. fuscoguttatus* was successfully established. In primary culture, heart tissues were digested with 0-125% trypsin to lyse the extracellular matrix for cell migration. Similar trypsinization methods have been reported in the establishment of some marine fish cell lines (Lai *et al*., 2003; Qin *et al*., 2006). The bmGH cells can be readily grown and maintained in 20% FBS-DMEM/F12 medium at 24°C, when supplemented with bFGF, IGF-I and carboxymethyl-chito-oligosaccharide. bFGF and IGF-I show important regulatory roles in cell proliferation, migration and differentiation. They probably
activate tyrosine kinase by binding to the tyrosine kinase receptor and speed cell proliferation via the ras, MAPK (mitogen-activated protein kinase) and protein kinase C pathway (Hrzenjak & Shain, 1995; Sivaprasad et al., 2004). Carboxymethyl-chitosan-oligosaccharide possesses various biological activities. It can be used for promoting cell attachment and proliferation in vitro (Fan & Wang, 2002, 2003; Jin et al., 2008). Similar effects of the supplements on acceleration of cell attachment and growth have also been reported in some established marine fish cell lines (Fan et al., 2006; Chen et al., 2007). To date, the bmGH cell line has been successfully established and subcultured for over 65 passages. The fibroblastic cells still have good proliferation status with a population doubling time of 40.3 h.

The euploid karyotype is an important parameter for characterizing a cell line. Karyotype analysis revealed that over 56% of the bmGH cells possessed a diploid
chromosome number of 48, which was identical to the modal diploid karyotype study of *E. fuscoguttatus* (Liao et al., 2006).

The main purpose of this study was to develop a susceptible cell line for the isolation and propagation of iridovirus, the causative agents of severe infectious diseases of *E. fuscoguttatus* in aquaculture (Qin et al., 2002; Gibson-Kueh et al., 2004). Iridoviruses are large double-stranded DNA viruses, icosahedral, 120–300 nm in diameter and contain a spherical deoxyribonucleoprotein core surrounded by a lipid membrane containing protein subunits (Qin et al., 2006). The lack of *E. fuscoguttatus* cell lines has limited both the study of these viruses and vaccine development. The bmGH cell line exhibited high susceptibilities to TRBIV and LCDV, yielding a significant titre of $10^{4.5} \text{ TCID}_{50} \text{ ml}^{-1}$ and $10^{4.0} \text{ TCID}_{50} \text{ ml}^{-1}$, respectively, within 7 days. CPE could be observed after 3 days of infection. Electron microscopy detection confirmed a large number of the replicated TRBIV or LCDV particles scattered throughout the cytoplasm of bmGH cells. TRBIV and LCDV can be readily propagated in bmGH cells. To date, only a few marine fish cell lines could be used for the isolation and propagation of TRBIV and LCDV (Chen et al., 2004; Fan et al., 2006). The present study indicates the potential use of the bmGH cell line as a powerful tool in fish virus isolation, propagation and vaccine development.

In conclusion, a novel heart-cell line (bmGH) from *E. fuscoguttatus* was successfully established, and its viral susceptibility to different types of iridoviruses was evaluated. The bmGH cell line will serve as a useful tool in studies of fish viral diseases and has potential applications in genetics and environmental toxicology as an alternative to fish.

This study was supported by grants from the National 863 High Technology Research Foundation of China (2006AA10A401; 2006AA09Z406).

References


