Journal of Fish Diseases 2011, **34**, 81–85 doi:10.1111/j.1365-2761.2010.01208.x

Short Communication

Establishment, characterization and virus susceptibility of a kidney-derived cell line from southern flounder, *Paralichthys lethostigma* Jordan & Gilbert

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Keywords: karyotype, kidney cell line, *Paralichthys lethostigma,* SFK, southern flounder, virus susceptibility.

Since the establishment of the first teleost fish cell line in the 1960s (Wolf 1962), more than 100 lines have been established and applied in many aspects of virology, immunology and genetics in freshwater and marine fish (Fernández, Yoshimizu, Kimura, Ezura, Inouye & Takami 1993; Fryer & Lannan 1994; Fernández-Puentes & Figueras 1996; Bejar, Borrego & Alvarez 1997; Chen, Sha & Ye 2003; Chen, Ye, Sha & Hong 2003; Chen, Ren, Sha & Shi 2004; Chen, Ren, Sha & Hong 2005; Buonocore, Libertini, Prugnoli, Mazzini & Scapigliati 2006; Parameswaran, Ishaq Ahmed, Shukla, Bhonde & Sahul Hameed 2007; Xing, Lee, Fan, Collodi, Holt & Bols 2008). Only five flounder cell lines have been reported, including the tissue cell lines FG (flounder gill cell line) (Li, Tong & Hetick 1997; Tong, Li & Miao 1997), FFN (flounder fin cell line), FSP (flounder spleen cell line) (Kang, Oh, Kim, Kawai & Jung 2003) and the embryo cell line FEC (flounder embryonic cell line) (Chen *et al.* 2004) derived from Japanese flounder, *Paralichthys olivaceus* (Temminck & Schlegel), and the embryo cell line SFEC (southern flounder embryonic cell line) (Ren, Chen & Sha 2007) from southern flounder, *Paralichthys lethostigma* Jordan & Gilbert. There is no report on the establishment of a southern flounder tissue cell line.

The southern flounder, originally distributed along the southern Atlantic and Gulf of Mexico coasts of the United States, has been introduced into China since 2001 and is now widely cultivated. The intensive aquaculture of this fish species has resulted in outbreaks of viral diseases such as lymphocystis disease (LCD). Little is known about the infection pattern of the LCD pathogen, lymphocystis disease virus (LCDV), in southern flounder due to a lack of suitable cell lines.

In order to establish a kidney cell line, a healthy southern flounder weighing 150 g was obtained from the Haiyang Fisheries Company in Yantai, Shandong Province, China. The fish was anaesthetized with MS-222 and wiped with 70% alcohol. The kidney was removed and placed in Eagle's minimum essential medium (MEM) (GIBCO) containing antibiotics (1000 U mL^{-1} penicillin, 1000 U mL^{-1} streptomycin). The samples were minced thoroughly with scissors and digested with 0.5% trypsin–EDTA solution (0.5 g porcine trypsin and 0.2 g EDTA–4Na per millilitre of 0.9%, sterile-filtered sodium chloride) for 30 min. The residual liquids were filtered with 200 mesh nylon gauze and transferred into 25-cm^2 cell culture flasks containing 3 mL MEM with 20 mM HEPES, 20% foetal bovine serum (FBS) (GIBCO), 0.05% 2-Mercaptoethanol (2-Me), and 2 ng mL^{-1} basic fibroblast growth factor (bFGF) (GIBCO). The primary cells were maintained at 24 °C. One half of

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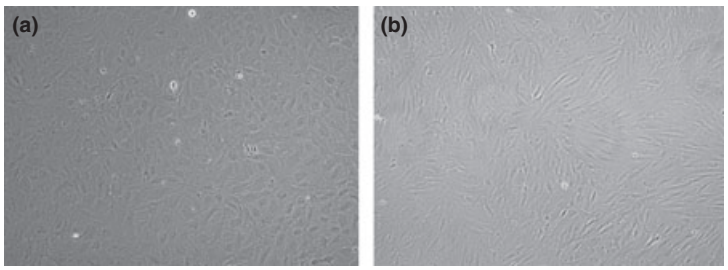

Figure 1 Monolayers of the SFK cell line. (a) Cells of primary passage forming cell monolayer. (b) Cells at passage 16, composed of fibroblastic cells.

the growth medium was changed every 4 days for the first 3 weeks.

Using the protocol described above, the cell monolayer (Fig. 1a) of southern flounder kidney (SFK) was obtained after 30 days incubation. The confluent cells were digested with 0.25% trypsin–EDTA solution and transferred into fresh 25-cm^2 flasks. During the initial eight subcultures, MEM containing 20% FBS was used and the cells were split at a ratio of 1:1 or 1:2 every 10 days. From passage 8 onwards, the cells were subcultured every 6 or 7 days with MEM containing 10% FBS. To date, the SFK cell line has been subcultured through more than 50 passages. The SFK cells are mainly composed of fibroblastic cells (Fig. 1). The SFK cells were cryopreserved from passage 10 onwards following the protocol described in our previous study (Chen *et al.* 2003). After 1–6 months storage, the cells thawed with an average viability of 80%.

To analyse the growth conditions of the SFK cells, cells at passage 14 were inoculated in five wells in a 12-well plate at 1.5×10^4 cells per well and three replicate plates were incubated at 15, 24 and 30 °C, respectively. At 2, 3, 4, 5 and 6 days after inoculation, cells from three wells for each different temperature were trypsinized, and cell numbers were measured using a haemocytometer. SFK cells gave the best growth curve at 24 °C with 5.2×10^4 cells mL^{-1} at 6 days after inoculation and a good growth curve at 30 °C with 5.0×10^4 cells mL^{-1} at 6 days after inoculation. No obvious cell growth

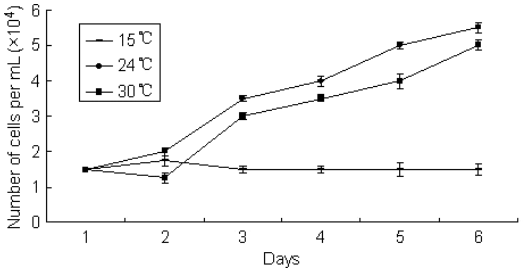

Figure 2 Growth of SFK cells at different temperatures. There was no obvious growth of passage 14 cells at 15 °C, and cell growth was faster at 24 °C than at 30 °C with 5.2×10^4 cells mL^{-1} at 6 days after inoculation.

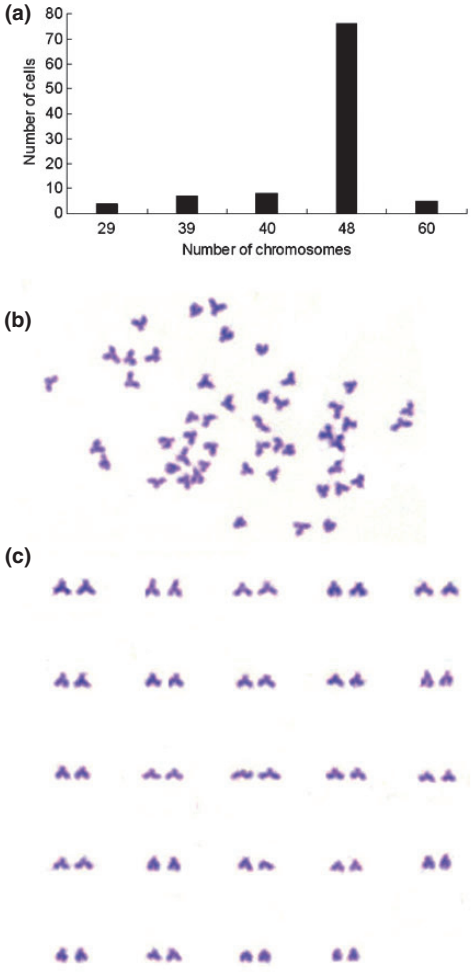

Figure 3 Chromosome number distribution (a), metaphase (b) and diploid karyotype (c) of SFK cells at passage 15. The main chromosome number was 48. The normal diploid karyotype consisted of subtelocentrics ($2n$ = 48st).

was observed at 15 °C (Fig. 2). The optimal growth temperature of SFK cells was similar to that of the southern flounder embryonic cell line (SFEC) (Ren *et al.* 2007) and the Japanese flounder gill cell line (Li *et al.* 1997).

Southern flounder kidney cells at passage 15 were used for chromosome analysis. The protocol was carried out as described previously (Chen *et al.* 2005). One hundred cells at metaphase were counted, and chromosome karyotype was analysed according to Levan (1964). The chromosome number of SFK cells ranged from 29 to 60. The main chromosome number was 48, comprising 76% of the 100 metaphase cells (Fig. 3a). Comparing the metaphase (Fig. 3b) with a normal diploid chromosome number, the normal karyotype morphology (Fig. 3c) consisted of nearly all subtelocentrics with a chromosome number of $2n = 48$, which was identical with the modal number of SFEC (Ren *et al.* 2007). Unlike the karyotype $2n$ = 6st+42t of SFEC, the karyotype of SFK was composed of subtelocentrics ($2n$ = 48st).

In order to determine the susceptibility of SFK cells to fish viruses, the viruses LCDV, Singapore grouper iridovirus (SGIV), and spring viraemia of carp virus (SVCV) were used to infect SFK cells at passage 14. LCDV was isolated from lymphocystis-diseased Japanese flounder as described by Xu, Piao, Jiang & Wang (2000) and based on a TCID50 assay titre was 10^2 $TCID_{50}$ mL^{-1}. One day before virus infection, cells at passage 14 were inoculated into 25-cm^2 flasks. The infection was carried out by adding 1 mL of virus suspension into the cells, followed by new medium after 1 h. After infection, the cytopathic effect (CPE) was observed using a Nikon ECLIPSE TE2000-U fluorescence microscope every day. Infection assays for SGIV (Qin, Lam, Sin, Shen, Chang, Ngoh & Chen 2001) and SVCV (Bjorklund, Higman & Kurath 1996) were carried out in the laboratory of Prof. Q. Qin with a titre value of 1 multiplicity of infection (MOI). A specific CPE was observed only in LCDV infected cells, with cell shrinkage and aggregation at 2 days after infection (Fig. 4a) which became more marked at 4 days (Fig. 4b). In contrast, no significant CPE was observed in SGIV and SVCV infected cells.

Southern flounder kidney cells infected with LCDV were prepared for electron microscopy as

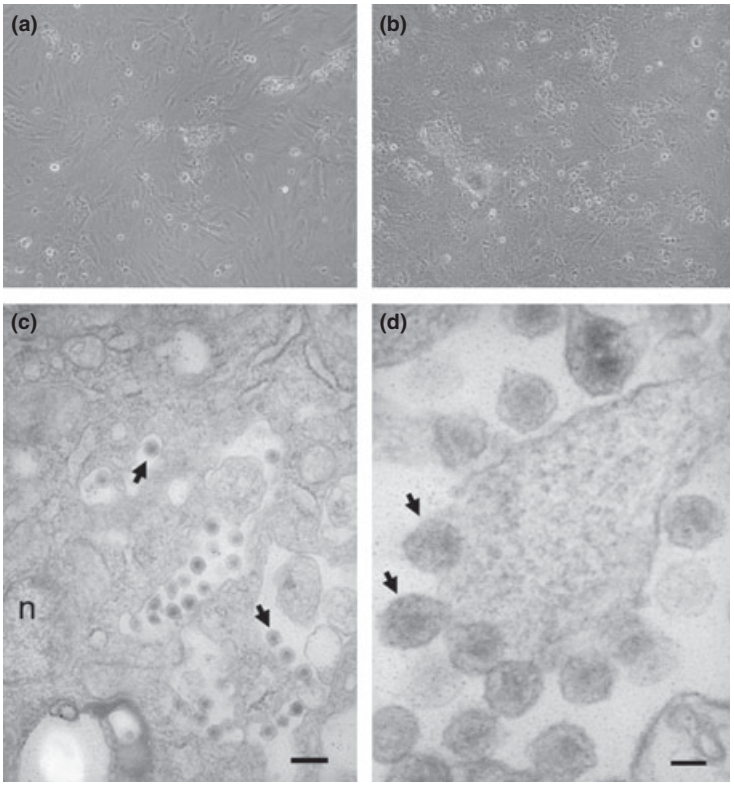

Figure 4 LCDV infection of SFK cells at passage 14. Cells exhibited a greater CPE 4 days after infection (b) than after 2 days (a). (c) Virus particles (arrows) scattered throughout the cytoplasm of cells infected with LCDV, *n* = cell nucleus (bar = 200 m). (d) Virus particles (arrows) invading cells (bar = 50 nm).

described by Chen *et al.* (2004). Virus particles were found scattered throughout the cytoplasm, including near the nucleus (Fig. 4c,d).

The SFK cell line thus exhibited a high susceptibility to LCDV isolated from Japanese flounder (Xu *et al.* 2000; Sun, Qu & Zhang 2000; Zhang, Ruan, Li, Yuan & Gui 2003) but showed no susceptibility to SGIV isolated from diseased brown spotted grouper and SVCV isolated from diseased cyprinids. This indicates that the SFK cell line is highly susceptibility to virus from related species to southern flounder.

Acknowledgements

We gratefully acknowledge Y.Y. Hou and J.S. Tan from the College of Medical Science, Qingdao University for their kind assistance in the electron microscopic preparation. In addition, we acknowledge Q.W. Qin and Y.H. Huang of the South China Sea Institute of Oceanology, Chinese Academy of Sciences for their technical assistance with the SGIV and SCVC virus infection. This study was supported by grants from the National 863 High Technology Research Foundation of China (2006AA09Z406, 2006AA10A401), the E-Institute of Shanghai Municipal Education Commission (Project No E030090), the Taishan Scholar Project Fund and the Basic Scientific Research Fund of YSFRI (2008-ts-02).

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Received: 20 February 2010
Revision received: 6 June 2010
Accepted: 24 June 2010

