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BRIEF REPORT

Cloning of a new fibroblast cell line from an early primary culture from mandarin fish (*Siniperca chuatsi*) fry for efficient proliferation of megalocytiviruses

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Abstract Megalocytiviruses are important emerging pathogens in both freshwater and marine finfish aquaculture. However, a limited number of piscine cell lines are persistently susceptible to these viruses, which greatly limits the study of megalocytiviruses. In this study, a new fibroblast-like cell line was established from an early primary culture from mandarin fish fry by a single cell cloning and was designated as MFF-8C1. The MFF-8C1 cells grow well in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum and had been subcultured more than 60 passages since the initial recovery culture in October 2009. Chromosomal analysis revealed that 91 % of the MFF-8C1 cells maintained a normal diploid chromosome number ($2n = 48$) in the 46th passage. Infection experiments showed that both freshwater-borne and marine-borne megalocytiviruses induce severe cytopathic effects in infected MFF-8C1 cells characterized by the rounding and enlargement of cells, which are highly consistent with the previous description of the infection in other susceptible cells with megalocytivirus. Megalocytivirus infections were further confirmed by a transmission electron microscopy. Furthermore, the MFF-8C1-cultured megalocytiviral suspension was highly virulent to infected mandarin fish. In summary, a new fibroblast cell line from mandarin fish fry that was highly permissive to megalocytiviruses was established. The MFF-8C1 cell line is a promising cellular substrate candidate for cell-cultured vaccine production of megalocytivirus.

Keywords Iridovirus · Megalocytivirus · Infectious spleen and kidney necrosis virus · Cell line · Infectivity

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Introduction

Megalocytivirus is a newly defined piscine iridovirus in the family Iridoviridae (Chinchar et al. 2005) and is considered as one of the most important emerging pathogens in finfish aquaculture (Kurita and Nakajima 2012; Subramaniam et al. 2012). The virus was first documented from a mass mortality of cage-cultured red sea bream *Pagrus major* in Japan in 1990 (Inouye et al. 1992). The causative agent was designated as red sea bream iridovirus (RSIV) (Nakajima and Sorimachi 1994). An early molecular epidemiologic investigation showed that RSIV infects over 30 marine-cultured fish

in Japan (Kawakami and Nakajima 2002; Matsuoka et al. 1996). Simultaneously, RSIV-like viral agents associated with mass mortality of finfish were also described from Australia (Anderson et al. 1993), Singapore (Chua et al. 1994), Chinese Taiwan (Chou et al. 1998), mainland China (He et al. 2000), South Korea (Jung and Oh 2000), and other Southeast Asian countries (Sudthongkong et al. 2002). Most recently, natural outbreaks of megalocytivirus-associated diseases were firstly reported emerging in North America (Marcos-López et al. 2011).

Very few piscine cell lines are susceptible to megalocytivirus (Dong et al. 2008; Imajoh et al. 2007), unlike ranaviruses, which propagate well in a very broad range of cell lines (Pham et al. 2012; Zhang et al. 1999). In most cases, normal piscine cell lines only support the propagation of megalocytivirus in very limited number of viral passages. A dramatic declining infection always occurs after no more than two rounds of viral passages, which indicates that most of these cell lines are actually unsuitable for the isolation and culture of megalocytiviruses (Chou et al. 1998; Imajoh et al. 2007; Nakajima and Sorimachi 1994).

In our previous reports, an epithelial-like cell line called MFF-1 was developed and was shown to be highly susceptible to both freshwater-borne and marine-borne megalocytiviruses (Dong et al. 2008, 2010; Ma et al. 2012; Shuang et al. 2013). However, MFF-1 was suspected to be a naturally transformed cell line due to its high variability of chromosomal distribution (Dong et al. 2008). Development of more susceptible cell lines is necessary to understand the infection mechanism of megalocytiviruses and for a broader screening of suitable cellular substrates for cell-cultured vaccines. In this study, a distinct cell line with normal diploid karyotype (2n = 48) was developed through a single cell cloning assay using the early primary culture material from mandarin fish (*Siniperca chuatsi*) fry. The characteristics of the new cell line were partially analyzed and megalocytiviral susceptibility was confirmed.

Materials and methods

Cell lines and megalocytiviral strains

The MFF-1 and KCF-1 cell lines were developed and characterized from mandarin fish fry and caudal fin of a young koi, respectively, and kept in our laboratory (Dong et al. 2008, 2011). Both MFF-1 and KCF-1 cells were grown in complete DMEM supplemented with 10 % fetal bovine serum (Gibco, Life Technologies, Carlsbad, CA, USA) at 25 °C. ISKNV-NH060831 and SKIV-ZJ07 were isolated and characterized from natural outbreaks of mandarin fish (*S. chuatsi*) and spotted knifejaw (*Oplegnathus punctatus*), respectively (Dong et al. 2008, 2010). Both strains were propagated in MFF-1 cells and stored at −80 °C until use.

Primary cell revival, single cell cloning, and subculture

Cells from mandarin fish fry were primarily cultured on April 25, 2006 (Dong et al. 2008). After several subcultures, the primary cultures in the fourth and fifth passages were stored in liquid nitrogen on May 9 and 12, 2006, respectively. In the present study, cell recovery was conducted on October 20, 2009. One tube of frozen primary cell cultures at the fourth passage was taken out from the liquid nitrogen and thawed in pre-warmed 30 °C water with fast agitation. The thawed cell suspension (about 1.5 ml) was transferred into a 15 ml sterile cell centrifuge tube containing 2 ml of growth DMEM [containing 20 % (v/v) FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B] (Life Technologies Corporation, Carlsbad, CA, USA) and centrifuged at 1,200*g* for 5 min at room temperature. After removing the supernatant, 5 ml of growth DMEM was added to re-suspend the cells, which were seeded into a 25 cm^2 flask, and incubated at 25 °C. After three rounds of subculturing, primary cells in the eighth passage were subjected to single cell cloning using a standard limitation dilution in a 96-well plate (Imajoh et al. 2007; Iwamoto et al. 2000). The colonized cells were kept in a 25 °C incubator with 5 % CO_2 and monitored under an inverted microscope (Nikon, Tokyo, Japan). About half of the old DMEM (containing 20 % FBS) was replaced every 2 days to stimulate rapid cell growth. When the cells grew to approximately 70 % confluence, the cells were trypsinized with a commercial trypsin–EDTA solution (Gibco) and transferred into one well of a 48-well plate. Amplified subcultures were consequently amplified through 12- and 6-well plates and finally to a 25 cm^2 flask. For the first 10 subcultures of MFF-8C1, the FBS concentration was maintained at 20 % and then decreased to 10 %.

Karyotype analysis

Chromosome analysis was conducted for MFF-8C1 at Passage 46, which was similar to our previous operation on MFF-1 cells with a minor modification (Dong et al. 2008). Briefly, MFF-8C1 cells in the 45th passage were seeded into a 25 cm^2 flask until the cells reached about 90 % confluence. Freshly prepared colchicine solution was added to the cells at a final concentration of 0.2 μg/ml. After 2.5 h of incubation at 25 °C, the cells were trypsinized with 0.25 % trypsin–EDTA solution and washed once with sterile PBS. The resulting cells were resuspended in 5 ml of 0.075 M KCl for 25 min at room temperature and fixed for 5 min in 5 ml of Carnoy's fixative solution (methanol:acetic acid = 3:1). After 5 min of centrifugation at 200*g* and 4 °C using a horizontal centrifuge, the cells were resuspended in 2 ml of fresh Carnoy's fixative solution. The fixed cells were dropped onto precooled glass slides and stained with 10 % fresh Giemsa solution (Weijia Technologies Corporation, Guangzhou, China) for 20 min at RT. Finally, the slides were observed and photographed under a phase contrast microscope. A total of 120 chromosome spreads were counted.

Susceptibility to ISKNV and SKIV

ISKNV-NH060831 and SKIV-ZJ07 were propagated in MFF-1 cells and used for susceptibility tests in MFF-8C1. MFF-8C1 cells were seeded into 25 cm^2 flasks and incubated at 25 °C. When the cells reached confluence, ISKNV and SKIV with a MOI of 1.0 were added and maintained at 25 °C. The cytopathic effects (CPEs) were observed daily under an inverted microscope. When advanced CPE was observed, the infected MFF-8C1 cells were stored at −80 °C. After two freezing/thawing cycles, the cell-cultured ISKNV and SKIV were used for another round of infection in MFF-8C1 cells. Both the ISKNV and SKIV were kept passaged through MFF-8C1 cells at least six times each. The virus titers were determined using the 50 % tissue culture infective dose ($TCID_{50}$) method in 96-well culture plates as previously described (Reed and Muench 1938).

Transmission electron microscopy (TEM)

The protocol for preparing virus-infected cell samples and ultrathin sections were similar to our previous description (Dong et al. 2008). Briefly, infected MFF-8C1 cells and mandarin fish splenic tissues were collected and fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer and post fixed in 0.1 M phosphate buffer containing 2.0 % osmium tetroxide. Ultrathin sections were stained with uranyl acetate–lead citrate and examined using a Philips CM10 electron microscope.

Challenge to mandarin fish

The ISKNV060831- and SKIV-ZJ07-infected MFF-8C1 suspensions in the sixth viral passage were used for an infection experiment. Healthy mandarin fish (average weight = 70 g) were obtained from a local fish farm and kept in 4.5 m × 1.0 m × 1.2 m indoor pools with air-pumped circulating water systems (Dong et al. 2010). The water temperature was kept at 28 °C. Before the infection experiment, the fish were assigned into seven groups with 10 fish in each group and fed a commercial diet once a day. The viral suspensions were diluted 100-fold, 1,000-fold, and 10,000-fold with sterile PBS. For the infection experiments, the fishes in each group were intraperitoneally injected with 200 μl of serially diluted ISKNV and SKIV. Additionally, 10 fish were intraperitoneally injected with 200 μl of sterile PBS and kept in an independent aquarium as a negative control. The fish were observed daily and the diseased fish were collected for virus detection using PCR, tissue imprinting, and TEM analysis, as previously described (Dong et al. 2010).

Results

Establishment of the MFF-8C1 cell line

The recovered primary cultured cells exhibited rapid growth in DMEM containing 20 % FBS during subcultures. The cells were subcultured at a ratio of 1:3–5 and grew to confluence in 3 days. After three passages, cells from the seventh subcultures were seeded into 96-well tissue culture plates using a limiting dilution assay. The wells in which a single cell was observed were marked, monitored, and treated as described above. After culturing for 2 months, one cell mass was observed in a marked well, 8C1, in a 96-well tissue plate. After continuous subcultures, a

fibroblast-like cell line was developed, and designated as MFF-8C1. As shown in Fig. 1b, fibroblast-like cells were predominant in the confluent MFF-8C1 monolayer and few other morphologic cells were observed. Karyotype analysis showed that MFF-8C1 cells in the 46th passage maintained normal diploid numbers with a modal chromosomal number of 48. Moreover, 91 % (109/120) of the MFF-8C1 cells also contained this normal chromosome number (Fig. 2). The MFF-8C1 cells grew well in DMEM supplemented with 10 % FBS and were subcultured for more than 60 passages since the initial cloning assay in October 2009. Thus, a new fibroblast cell line from mandarin fish fry was established.

Susceptibility to ISKNV and SKIV infection

Typical CPEs were observed at 24 hours post infection (hpi) with both ISKNV and SKIV. In this stage, numerous round and enlarged cells appeared, and increased in number with increasing infection time (Fig. 3a, c). Advanced CPEs were observed at 72–96 hpi. Abundant round enlarged cells began to dislodge from the flasks and accumulate at the bottom of the tissue plate (Fig. 3b, d). The infected MFF-8C1 cells were collected and stored at −80 °C. After freezing/thawing, the viral suspensions were used for another round of infection of MFF-8C1 cells. The yielded viral suspensions were still highly infectious to the MFF-8C1 cells. Unique CPEs characterized by cell shrinkage, rounding, enlargement, and breakage were also observed, which are highly consistent with previous descriptions of permissive cell infections with megalocytiviruses (Dong et al. 2008, 2010; Imajoh et al. 2004, 2007). After several continuous viral passages in MFF-8C1, the viral titers stabilized. The resulting $TCID_{50}$ at 7 days post infection were $10^{8.2}$/0.1 ml for SKIV-ZJ07 and $10^{7.9}$/0.1 ml for ISKNV-NH060831 in the sixth viral passage in MFF-8C1 cells. The viral infections were further confirmed by TEM observation. TEM observation showed that a high ratio of infected MFF-8C1 cells was observed with large numbers of viral particles (Fig. 4).

Infectivity to mandarin fish

MFF-8C1 cells cultured-ISKNV and -SKIV in the sixth viral passage were harvested for infection experiments on mandarin fish. The MFF-8C1 cells cultured-ISKNV and -SKIV were highly pathogenic to mandarin fish. The 100-fold to 10,000-fold diluted viral suspensions caused 100 % mortality among the infected fish within 14 days (Fig. 5). The infectivity also showed dosage-dependent effects. The viral suspensions showed increasing virulence with increasing viral concentrations. No fish died in the PBS injection group. Molecular investigation indicated that all dead fish died due to ISKNV or SKIV infection (data not shown). Large numbers of viral particles were observed in the diseased fish tissues under further TEM analysis (Fig. 6).

Discussion

In an earlier study, an epithelial-like predominant cell line (MFF-1, Fig. 1a) with a variation of chromosomes was established using the same cellular materials from

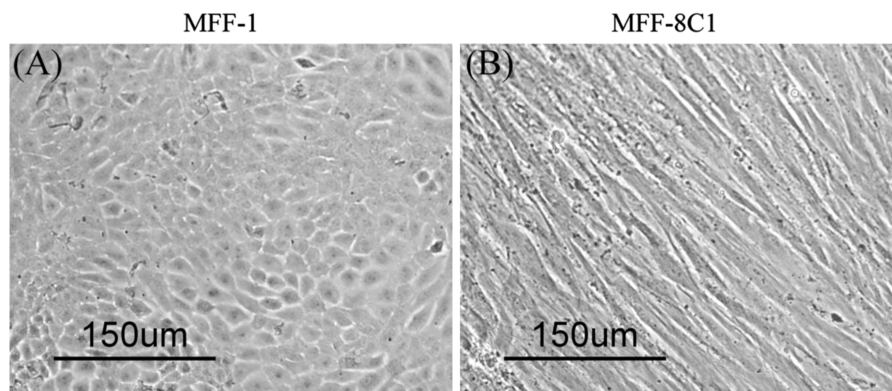

Fig. 1 Comparison of morphological difference between MFF-1 cells and MFF-8C1 cells. **a** Epithelial-like MFF-1 cells at passage 230; **b** fibroblast-like MFF-8C1 cells at passage 78. *Bar* 150 μm

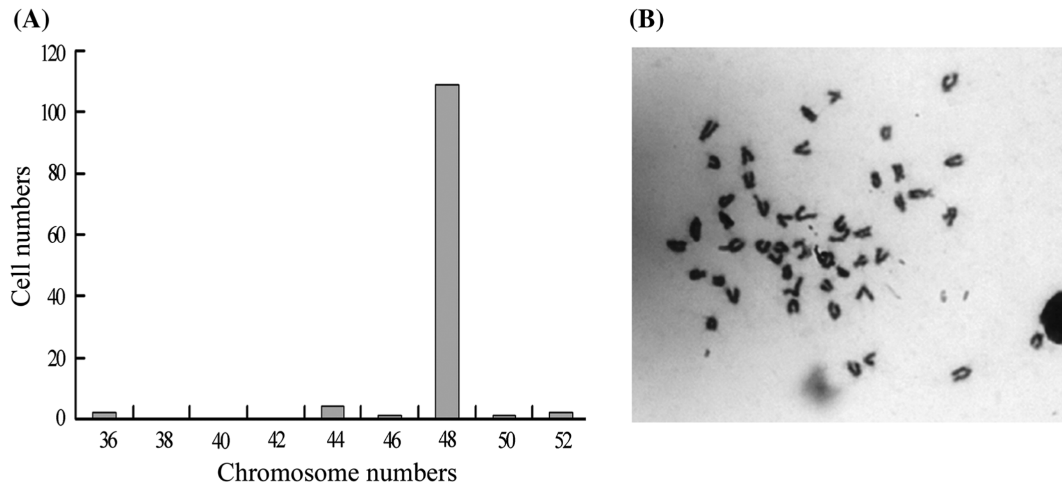

Fig. 2 Distribution of chromosomes in the MFF-8C1 cells (**a**) and the metaphase (**b**) of MFF-8C1 cells in the 46th passage ($n = 120$ cells)

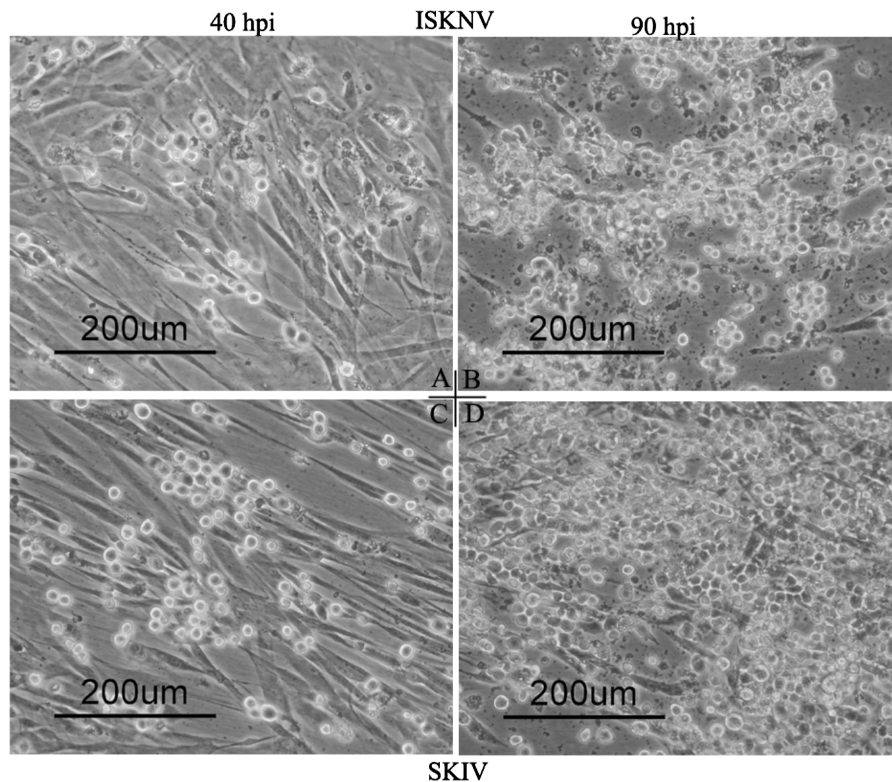

Fig. 3 Cytopathic effects of MFF-8C1 cells infected with ISKNV (**a**, **b**) and SKIV (**c**, **d**) at 40 and 90 h post infection, respectively, using an inverted light microscope. *Bar* 200 μm

mandarin fish fry (Dong et al. 2008). The MFF-1 cells remained highly susceptible to a variety of megalocytiviruses and have been widely used in the study of ISKNV and other megalocytiviruses (Dong et al. 2010, 2013; Ma et al. 2012; Shuang et al. 2013). In the present study, a primary culture was recovered from liquid nitrogen after nearly 3 years of storage and a new fibroblast cell line, designated as MFF-8C1, was

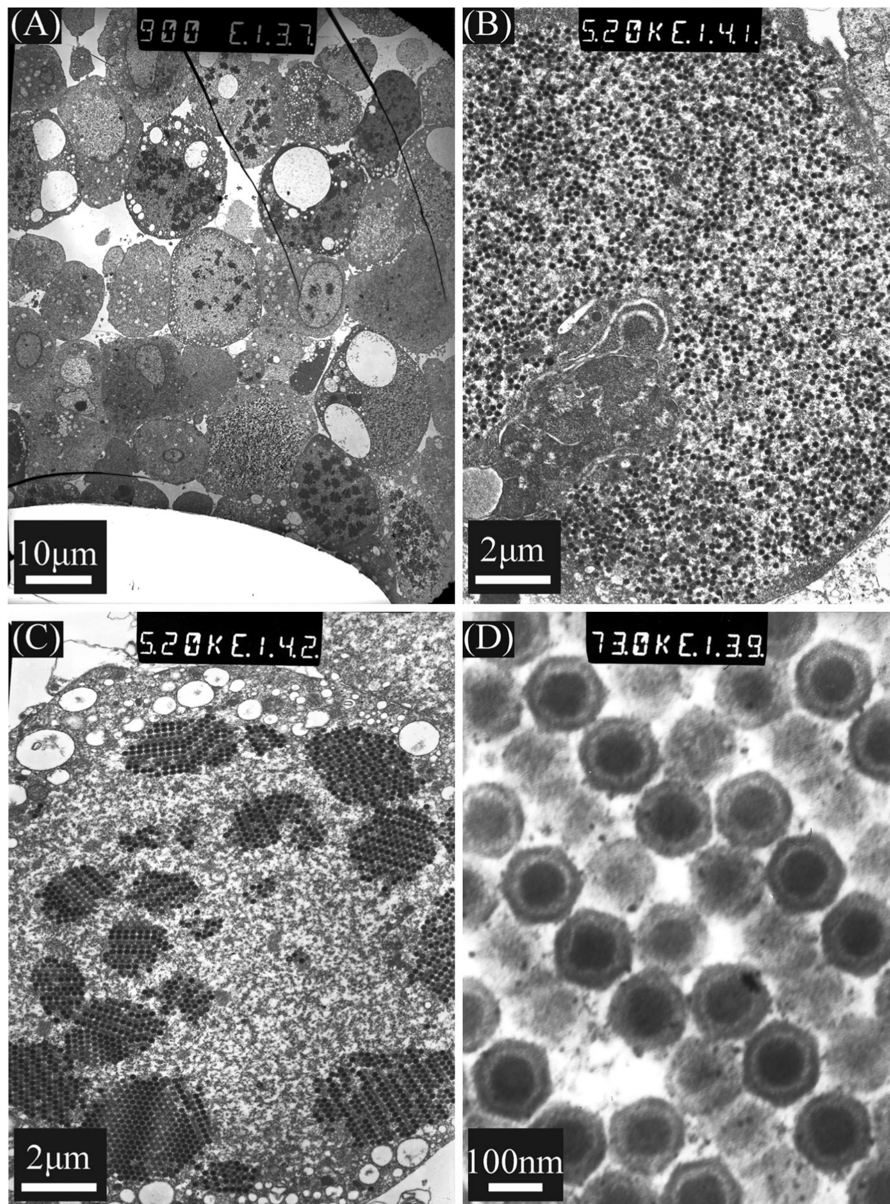

Fig. 4 Transmission electron micrograph of SKIV-infected MFF-8C1 cells under different magnifications. **a** Numerous infected MFF-8C1 cells under low magnification (*bar* 10 μm); **b**, **c** large numbers of random and regular paracrystalline arrays of virus particles in a single infected MFF-8C1 cell (*bar* 2 μm), respectively; **d** micrograph of the regular paracrystalline array of viral particles (*bar* 100 nm)

established by a single cell cloning. In the previous MFF-1 cell line, the cells with 48 chromosomal mode accounted for only a small proportion (about 11 %) of all cells (Dong et al. 2008). However, 48 chromosome–cells were predominant in the newly established MFF-8C1, which strongly suggests that they developed from one common cell clone. Similar phenomena were observed between a mixed primary cell culture and a cloned CRF-1 cell line from the tail fin of the red sea bream (Imajoh et al. 2007). In the study by Imajoh et al. (2007), mixed primary cell cultures exhibited a wide distribution of chromosomes, whereas the cloned CRF-1 cells exhibited a single normal chromosomal type. Aside from the distinct morphologic differences

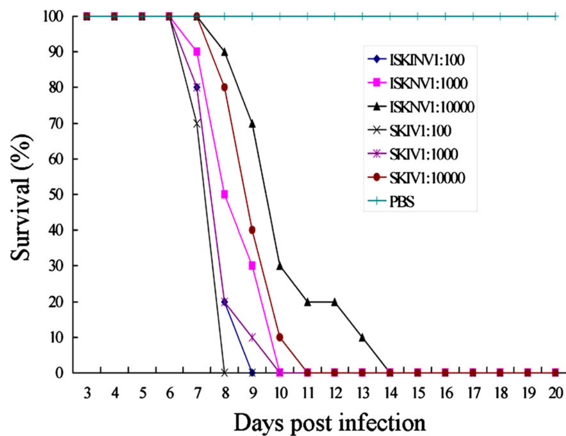

Fig. 5 Accumulated survival rate of mandarin fish after challenge with serially diluted viral suspensions of MFF-8C1-cultured ISKNV and SKIV

between MFF-1 and MFF-8C1, the trypsinized MFF-1 cells were much smaller than the MFF-8C1 cells. The average diameter of the dislodged MFF-1 cells was 8.3 μm, whereas the average diameter of the trypsinized MFF-8C1 cells was 13.1 μm (data not shown).

Megalocytivirus infection always results in unique CPE changes in infected cells, which are characterized by cell rounding and enlargement (Dong et al. 2008, 2010; Imajoh et al. 2004, 2007; Nakajima and Sorimachi 1994). However, persistent high infectivity only occurred in a small number of cell lines, which are mainly represented by GF, CRF-1, and MFF-1 (Dong et al. 2008, 2010; Imajoh et al. 2004, 2007). In non-permissive and low permissive cell lines, viral infectivity always declined dramatically after no more than 3 rounds of viral passages. These typical cell lines mainly included BF-2 and KRE (Chou et al. 1998; Imajoh et al. 2007; Nakajima and Sorimachi 1994). Similar phenomena were also observed in the newly established KCF-1 cell line from the caudal fin of a young koi (*Cyprinus carpio*) (Dong et al. 2011). Round enlarged cells were observed in the KCF-1 cells infected with MFF1-cultured SKIV-ZJ07 suspension. However, the number of round cells decreased dramatically with continuous blind passages of the infected KCF-1 cells. After three blind passages, the infected KCF-1 cells recovered and almost no rounded cells were observed in the KCF-1 monolayer (Fig. S1). Thus, the decreasing megalocytivirus infection was classified as a pseudo-infection. Consequently, verifying continuous viral activity is essential when a newly established cell line is considered a susceptible cellular substrate for megalocytivirus. In the present study, MFF-8C1 cells remained highly susceptible to ISKNV and SKIV after continuous viral passages, which were further confirmed by TEM observation (Fig. 4). Importantly, the viruses remained highly virulent to mandarin fish (Figs. 5, 6). Future studies will be conducted to assess the antigenicity of the MFF-8C1-cultured megalocytiviruses for the development of effective vaccines against megalocytiviruses.

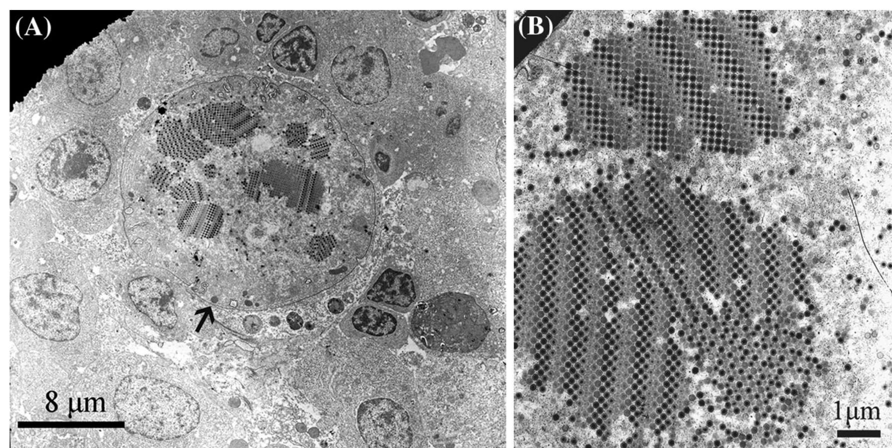

Fig. 6 Transmission electron micrograph of diseased mandarin spleen tissues infected with MFF-8C1-cultured SKIV under low (**a**) and high (**b**) magnification (*bar* 8 and 1 μm, respectively). The *arrow* indicates an abnormally enlarged cell with a large number of paracrystalline array viral particles

In conclusion, a new fibroblast cell line with normal diploid karyotype from mandarin fish fry was developed and was shown to be highly permissive to megalocytiviruses. The cells are a promising cellular substrate candidate for cell-cultured megalocytiviral vaccine production.

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