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Characterization of rock bream (*Oplegnathus fasciatus*) fin cells and its susceptibility to different genotypes of megalocytiviruses

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Genus *Megalocytivirus* cause red sea bream iridoviral disease (RSIVD) and scale drop disease (SDD). Based on the phylogeny of the major capsid protein (MCP) and adenosine triphosphatase (ATPase) genes, megalocytiviruses except for SDD virus (SDDV) could be three different genotypes, red sea bream iridovirus (RSIV), infectious spleen and kidney necrosis (ISKNV), and turbot reddish body iridovirus (TRBIV). In this study, primary cells derived from the caudal fin of rock bream (*Oplegnathus fasciatus*) grew at 25℃ in Leibovitz's medium supplemented with 10% (v/v) fetal bovine serum and primocin (100 μg/mL). Rock bream fin (RBF) cells exhibited susceptibility to infections by different genotypes of megalocytiviruses (RSIV, ISKNV and TRBIV) with the appearance of cytopathic effects with an increase in the viral genome copy number. Furthermore, compared to grunt fin (GF) cells, even though 10 times lower number of RSIV genome copies were inoculated in RBF cells, viral genome copy number produced on RBF cells were 44 times higher than that of GF cells at 7 d post-inoculation. As the isolated RBF cells are sensitive to different genotypes of megalocytiviruses (RSIV, ISKNV and TRBIV), they can be used for future studies regarding *in vitro* viral infection and subsequent diagnosis.

Key words: Rock bream, Primary cell, *Megalocytivirus*, Red sea bream iridoviral disease

Introduction

Members of the genus *Megalocytivirus* (family *Iridoviridae*) comprise double-stranded DNA enclosed within an icosahedral capsid surrounded by an envelope and cause red sea bream iridoviral disease (RSIVD) and scale drop disease (SDD) (Kurita and Nakajima, 2012; de Groof et al., 2015; Chinchar et al., 2017). Phylogenetic analysis of the major capsid protein (MCP) and adenosine triphosphatase (ATPase) genes suggests that megalocytiviruses except for SDD virus (SDDV) could be classified into three major genotypes: red sea bream iridovirus (RSIV), infectious spleen and kidney necrosis (ISKNV), and turbot reddish body iridovirus (TRBIV) (Kurita and Nakajima, 2012). The first RSIV induced disease outbreak was recorded in the Japanese cultured red seabream (*Pagrus major*) in 1990 (Inouye et al., 1992). The acute outbreaks of ISKNV in China, during 1994 to 1997, resulted in significant economic losses in cultured mandarin fish (*Siniperca chuatsi*) (He et al., 2001, 2002). Moreover, the first TRBIV infection in turbot (*Scophthalmus maximus*) in eastern China was

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reported in 2004 (Shi et al., 2004). Notably, RSIV and ISKNV have been causative agents for RSIVD found in a wide range of freshwater and marine fish (OIE, 2021). In Korea, an endemic region for RSIVD, RSIV-type has been a predominant type infecting marine fish since the 1990s (Oh et al., 1999; Kim et al., 2019). Of the species susceptible to RSIVD, rock bream (*Oplegnathus fasciatus*) showed annually mass mortality due to RSIV infection in Korea (Oh et al., 1999; Jung and Oh, 2000; Jeong et al., 2003). Additionally, although rock bream experimentally infected with ISKNV (PGIV-K1 isolate) presented a clinical infection (Jeong et al., 2008), TRBIV (MVSF-12 isolate) infected rock bream showed virus replication with no mortality and a subclinical infection (Jin et al., 2018).

Cell lines have been an ideal tool for *in vitro* studies of cell-virus interactions, virus isolation, diagnosis, and vaccine development. For diagnosing RSIVD in infected fish via cell culture, grunt fin (GF) cells were generally used for viral isolation (OIE, 2021). Although GF cells showed susceptibility for RSIV and ISKNV, the complete cytopathic effect (CPE) was not easily observed in RSIV-infected GF cells (Ito et al., 2013). To overcome the drawback, novel cells derived from a variety of fish that were susceptible to megalocytiviruses (RSIV, ISKNV and TRBIV) have been developed for virus propagation in recent decades (Dong et al., 2008; Imajoh et al., 2004; Ku et al., 2010; Oh and Nishizawa, 2016; Kawato et al., 2017; Yeh et al., 2018; Gardenia et al., 2020; Kwon et al., 2020). Notably, even though several researchers attempted to establish highly permissive cell lines for RSIV from rock bream, cultures of only rock bream embryo-derived cells (RoBE-4 cells), which were susceptible to RSIV, could be established (Oh and Nishizawa, 2016).

In the present study, primary cells from the caudal fin of rock bream were cultured and their optimal culture conditions were investigated. As the replicable for different genotypes of megalocytiviruses (RSIV, ISKNV and TRBIV) in rock bream tissues, the susceptibility of rock bream fin cells to different megalocytiviruses was evaluated. Furthermore, viral genome copy number between megalocytivirus-inoculated rock bream fin and GF cells were compared.

Materials and methods

Preparation of primary cells from rock bream fin

Juvenile rock bream (body length 7.4 ± 0.4 cm; body weight 5.4 ± 0.8 g) confirmed to be RSIV-free by PCR (Table 1) was purchased at a fish farm in Geojae. Fish were anesthetized on ice, the surface of the fish was sterilized with 70% ethanol, and the end

Table 1. Primers used in this study

Target gene	Primers	Sequence (5'–3')	Amplicon (bp)	Purpose	Reference
Pst I fragment	1-F 1-R	CTC AAA CAC TCT GGC TCA TC GCA CCA ACA CAT CTC CTA TC	570	Detection	Kurita et al. (1998)
DNA polymerase gene	4-F 4-R	CGG GGG CAA TGA CGA CTA CA CCG CCT GTG CCT TTT CTG GA	568		
Major capsid protein	RSIV 1094F RSIV 1221R RSIV 1177 probe	CCA GCA TGC CTG AGA TGG A GTC CGA CAC CTT ACA TGA CAG G FAM-TAC GGC CGC CTG TCC AAC G-BHQ1	128	Quantification	Kim et al. (2021)
COI gene[a]	RbCOIF RbCOIR	GAC CCT GCA GGA GGA GGA GTT CAA GTG CTG TGG AGG GT	531	Cell origin analysis[b]	In this study

[a]the mitochondrial cytochrome c oxidase subunit 1 (*COI*) gene for rock bream; [b]PCR & Seqeuencing

part of the caudal fin was dissected. Minced small fin pieces (approximately 1 cm^2) were rinsed with phosphate-buffered saline (PBS; Gibco, NY, USA) containing 3 × antibiotic-antimycotic (PBS containing 60 units/mL of penicillin, 60 μg/mL of streptomycin, and 0.15 μg/mL of amphotericin B) (AA; Gibco, NY, USA) for minimization of microbial contamination including mycoplasma. Next, the cells were separated by stirring with 10 mL of 0.25% trypsin-EDTA solution (Gibco, USA) at 20°C for 1 h. The dissociated cells were suspended in 10 mL of Leibovitz's medium (L-15) supplemented with 20% fetal bovine serum (FBS, Performance plus; Gibco) and 2 × AA (L-15 medium containing 40 units/mL of penicillin, 40 μg/mL of streptomycin, and 0.10 μg/mL of amphotericin B) and then filtered twice through a cell strainer (pore size: 70 μm; Falcon, NY, USA). Filtered cells were collected via centrifugation at 500 × *g* for 10 min at 4°C. This process was repeated three times. The cells were resuspended (approximately 10^6 cells/mL) in L-15 medium supplemented with 20% FBS and primocin (100 μg/mL; InvivoGen, CA, USA), an antimicrobial agent, and then seeded in tissue culture (TC)-treated cell culture flasks (25 cm^2; Geriner Bio-one, Germany). Primary cells, designated as rock bream fin (RBF) cells, were incubated at 25°C. For the maintenance of primary cells, the medium was exchanged every 1~2 days with two washes with PBS containing 2 × AA. Subculturing was performed when the primary cells formed a monolayer (confluency above 90%), at a split ratio of 1:2 or 1:3 in subculture every 10~14 days. After continuous subculturing for 20 passages, the culture medium composition was replaced with L-15 medium containing 10% FBS and primocin (100 μg/mL).

Sequence analysis of mitochondrial cytochrome c oxidase subunit 1 (*COI*)

To confirm the cell origin, the phylogeny of the mitochondrial cytochrome c oxidase subunit 1 (*COI*) gene was analyzed. Total genomic DNA was extracted from RBF cells using the Patho Gene-spin™ DNA/RNA Extraction Kit (iNtRON Biotechnology, Gyeonggi, Korea). A primer set (RbCOI F and RbCOI R) targeting the partial *COI* gene of rock bream was designed (Table 1). The cycling conditions for PCR were as follows: initial denaturation at 94°C for 5 min; 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s; and a final extension at 72°C for 7 min. The PCR products were sequenced using an ABI 3730XL DNA Analyzer (Applied Biosystems, MA, USA) and aligned using the Clustal W algorithm in BioEdit software (Ver. 7.2.5.) with fish *COI* genes retrieved from the GenBank of the National Centre for Biotechnology Information (NCBI). A phylogenetic tree was constructed using the maximum likelihood method with 1,000 bootstrap values using the MEGA software (Ver. 11).

Chromosome analysis

RBF cells (passage 45) were incubated until they reached 70~80% confluency at 25°C in a 25 cm^2 tissue culture flask and were then treated with 0.5 μg/mL of colchicine (Merck, MA, USA) for 3 h at 25°C. After removing the supernatant and washing with PBS, cells were treated with 0.25% trypsin-EDTA and harvested via centrifugation at 500 × *g* for 10 min at 4°C. Cells were then resuspended in 5 mL of 0.075 M KCl (hypotonic solution) for 25 min at 20°C and then pre-fixed with 1 mL of Carnoy's solution (methanol: acetic acid = 3:1) for 2 min. Cells were collected via centrifugation at 500 × *g* for 10 min at 4°C and were then fixed with chilled 7 mL Carnoy's solution for 25 min at 20°C. After centrifugation at 500 × *g* for 10 min at 4°C, the supernatant was removed completely, and the cells were fixed with 1 mL of Carnoy's solution for 18 h at -20°C. Finally, cells were collected via centrifugation at 500 × *g* for 10 min at 4°C and were then resuspended in 500 μL of Carnoy's solution, followed by staining with 10% Giemsa solution for 25 min. The chromosome numbers of RBF cells (n=100) were counted under a light micro-

scope (1,000 × magnification).

Cell growth characteristics

For the optimization of cell growth conditions, RBF cell numbers were analyzed under various conditions, such as different temperatures (20℃, 25℃, and 30℃) and FBS concentrations (L-15 medium containing 2%, 5%, 10%, and 15% FBS). In experiment 1 (effect of temperature on growth), RBF cells were seeded at a density of 5.0×10^4 cells into 12-well plates and grown in L-15 supplemented with 10% FBS at 20℃, 25℃, and 30℃. In experiment 2 (effect of FBS concentration on growth), RBF cells (initially seeded at 5.0×10^4 cells/well) were grown in L-15 medium containing 2%, 5%, 10%, and 15% FBS in 12-well plates at 25℃. After 1 day, all wells were washed with PBS, and the medium was changed to remove non-adherent cells from experiments 1 and 2. The cells were trypsinized using 0.25% trypsin-EDTA (Gibco, USA) and then collected for counting at 1, 3, 5, 7, and 9 days. Cells were counted using a C-Chip disposable hemocytometer (INCYTO, Seoul, Korea). All experiments were performed in triplicate. The statistical significance of differences in cell numbers was determined by two-way ANOVA using GraphPad Prism software (ver. 8.4.3.). Statistical significance was set at $P < 0.05$.

Viral susceptibility analysis

Preparation of virus

To analyze the viral susceptibility of RBF cells, different megalocytiviruses (RSIV, ISKNV and TRBIV) cultured using *P. major* fin (PMF) cells (Kwon et al., 2020) were used as an inoculum. The origin source of RSIV (17RbGs isolate) was diseased rock bream in Goseong in 2017. And ISKNV (PGIV-K1 isolate) was isolated from imported pearl gourami (*Trichogaster leeri*) from an ornamental fish shop in 2004 (Jeong et al., 2008). And TRBIV (FLIV-Ph isolate) was isolated from diseased olive flounder (*Paralichthys olivaceus*) in Pohang in 2010 (Kim et al., 2014).

Virus isolation test

For virus propagation, viral infections were performed in 25 cm^2 flasks (approximately 10^6 cells/mL). The medium was removed, the cells were washed with PBS, and then the viruses were inoculated into RBF cells. Final number of viral genome copies per mL of inoculums were 1.0×10^6 viral genome copies/mL for megalocytiviruses (17RbGs, PGIV-K1, and FLIV-Ph isolates). After 30 min of adsorption at 25 ℃, the L-15 medium was supplemented with 5% FBS (heat-inactivated at 55℃ for 30 min) and primocin (100 μg/mL) were added. Virus-inoculated cells were incubated at temperatures optimum for cell growth, as well as temperatures optimum for viral replication (25℃), and the induction of cytopathic effect (CPE) was observed for 14 days. After the onset of CPE, supernatants were collected via centrifugation at 500 × *g* for 10 min at 4℃. Total DNA was extracted from each supernatant using the Patho Gene-spin™ DNA/RNA Extraction Kit (iNtRON Biotechnology). PCR was performed using virus-specific primers (Table 1), and amplicons were analyzed via agarose gel electrophoresis.

Number of RSIV, ISKNV and TRBIV genome copies in viral-inoculated cells

To analyze the viral genome copy numbers of megalocytiviruses (RSIV, ISKNV and TRBIV), RBF and GF cells (approximately 10^6 cells/mL) in 24-well plates were inoculated with 1.0×10^5 and 1.0×10^6 viral genome copies/well of each virus, respectively. Owing to variable virus susceptibility of GF cells (over 400 passaged cells used in this study), RBF and GF cells were inoculated with the viruses at different inoculum concentrations. The virus-inoculated culture plates were then incubated at 25℃. Experiments were performed in triplicates. For the comparison of viral genome copy number between megalocytiviruses-inoculated RBF cells, the whole supernatants were collected from each well at 1, 3, 5, 7, 9, and 11 days post-inoculation (dpi). Furthermore, for the compar-

ison of viral genome copy number between megalocytivirus-inoculated RBF and GF cells, supernatants from megalocytivirus-inoculated GF cells were collected at 7 dpi in triplicate. Total DNA was extracted from each cell supernatant using the Patho Genespin^{TM} DNA/RNA Extraction Kit (iNtRON Biotechnology). The number of viral genome copies was determined by quantitative real-time polymerase chain reaction (qRT-PCR) targeting partial MCP gene (base position 1,199~1,221 of the consensus sequences which was shared by RSIV, ISKNV and TRBIV) (Kim et al., 2021). Briefly, qRT-PCR was conducted with a 20 μL (total volume) mixture containing 1 μL of DNA extracted from each virus, 0.5 μL of each 200 nM of each primer (RSIV 1094F/1221R) and probe (RSIV 1177 probe) (Table 1), 10 μL of 2× HS Prime qPCR Premix (Genet Bio, Korea), 0.4 μL of 50× ROX dye, and 7.1 μL of DEPC treated water. The amplification was performed on a StepOne Real-time PCR System (Applied Biosystems, USA). The cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 94°C for 10 s and 60°C for 35 s. The standard curve for quantification was generated from a serially diluted plasmid containing the MCP gene (1,362 bp) of RSIV (17RbGs isolate). The statistical significance of the difference in the number of viral genome copies associated with megalocytivirus-inoculated GF and RBF cells was determined by two-way ANOVA using GraphPad Prism software (ver. 8.4.3). Statistical significance was set at $P < 0.05$.

Results and discussion

In this study, the RBF cells from the rock bream caudal fin were cultured successfully over 50 passages under laboratory conditions. Cells in early passages showed heterogeneity, including epithelial-like and fibroblast-like cells (data not shown). After subculturing for at least seven passages, the RBF cell morphology was established to be primarily fibroblast-like cells. Fibroblast-like cells were maintained in L-15 medium supplemented with 10% FBS and primocin (100 μg/mL). Subcultured cells ($10^{5.0}$ -$10^{5.5}$ cells/mL) grew at 25°C within 10~14 days. Furthermore, cells cryo-preserved (38 passages) at both -80 °C and -196°C (in liquid nitrogen) for 30 and 60 days were successfully recovered and formed monolayers comprising fibroblast-like cells (data not shown). The nucleotide sequence of *COI* gene from RBF cells showed 99.4% identity with that of the rock bream gene (GenBank accession no. AP006010) and belonged to the same branch in the phylogenetic tree (Fig. 1), confirming that the RBF cells originated from rock bream. Consistent with the results of a study by Xu et al. (2013), which indicated the diploid chromosome numbers of the rock bream as $2n = 48$, the chromosome numbers of RBF cells at passage number 45 ranged from 39 to 57 with a modal number of 48 (Fig. 2).

To determine the optimal growth temperature, RBF cells were grown at 20°C, 25°C, and 30°C (Fig. 3A) for 9 days. Growth rate was the highest at 25°C followed by 20°C and 30°C, suggesting the optimal temperature for RBF cell growth to be 25°C, which was consistent with the optimal temperature previously determined for RoBE-4 cells (Oh and Nishizawa, 2016). In addition, for determining the optimal FBS concentration, RBF cells were grown in L-15 medium containing 2%, 5%, 10%, and 15%, FBS (Fig. 3B). In a medium supplemented with 2% FBS, although the cells grew for 1-day post subculture, cells continuously detached from the well plate; moreover, cell divisions were not observed at 3 days post subculture, indicating that a FBS concentration of more than 2% (v/v) in the medium might be an essential aspect of RBF cell growth conditions. RBF cells proliferated significantly in media containing 10% and 15% FBS compared to media supplemented with 2% and 5% FBS at 9 days post subculture. Serum contains various ingredients, including proteins, hormones, minerals, and other growth factors, and is a key component for

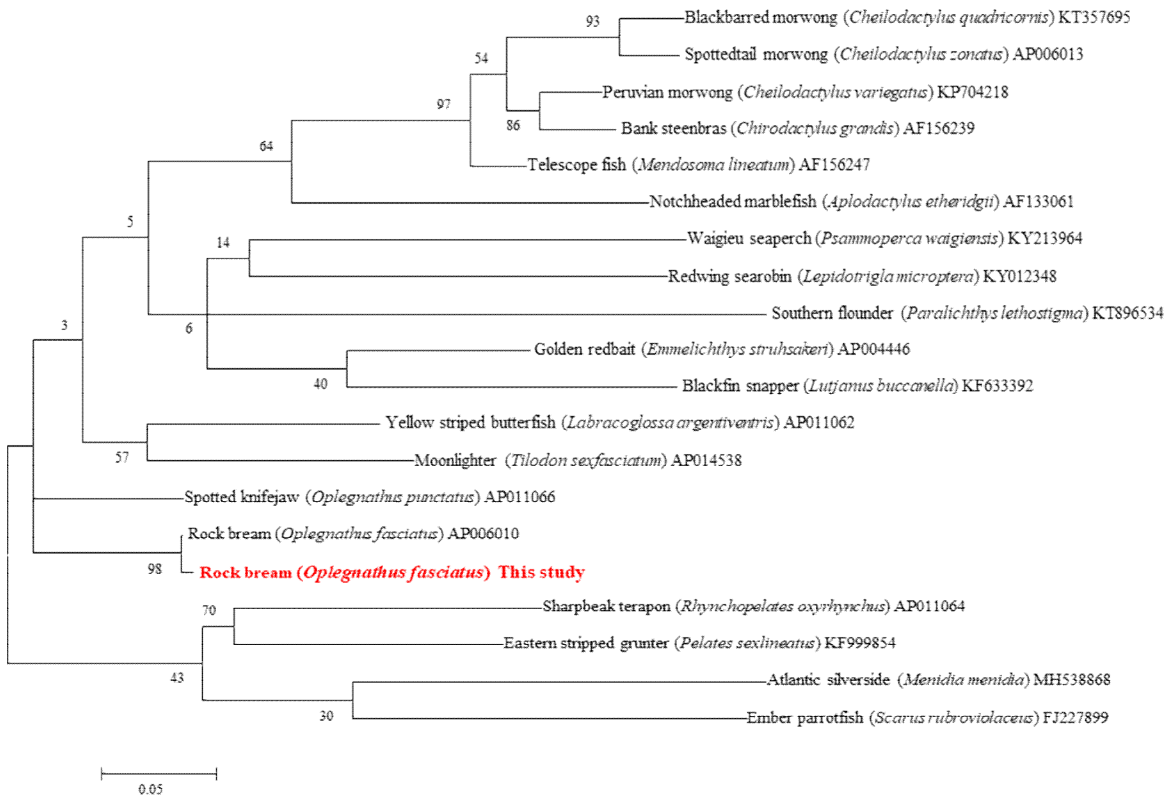

Fig. 1. Identification of mitochondrial cytochrome c oxidase subunit 1 (*COI*) gene from rock bream fin (RBF) cells. Phylogenetic analysis based on *COI* genes identified from various fish species.

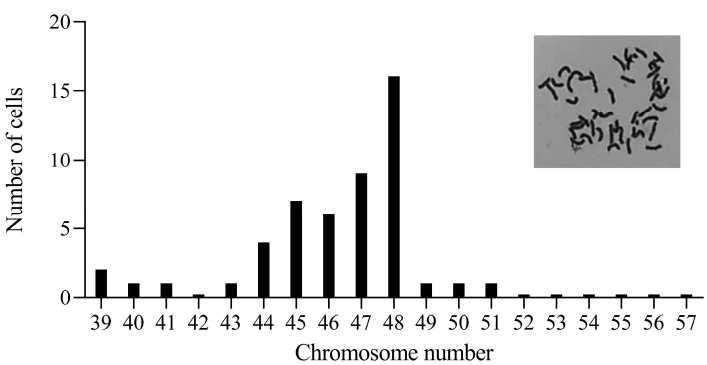

Fig. 2. Chromosome analysis of the rock bream fin (RBF) cells. Chromosome number and distribution of metaphase RBF cells at passage 45, and a representative picture for chromosomes of the RBF cell.

growing and maintaining cells in culture conditions (Butler, 2004). For these reasons, it was expected that the cell would be grown with a rapid cell division time at a higher serum concentration in the culture medium. However, the highest growth rate of RBF cells was observed in a medium supplemented with 10% on day 9. This result is similar to that reported in GP cells derived from giant gourami (*Osphronemus goramy*) (Gardenia et al., 2020), and it was determined that serum concentrations above a certain level in complex cellular growth mechanisms may have served as an inhibitory factor in cell growth. Thus, the opti-

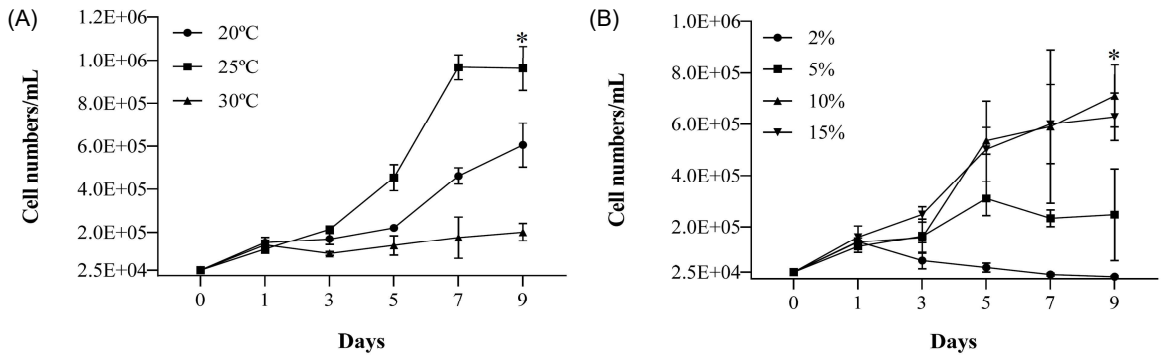

Fig. 3. Growth curves of rock bream fin (RBF) cells. (A) Number of RBF cells (passage 37) in Leibovitz's L-15 medium (L-15) containing 10% fetal bovine serum (FBS) at different temperatures (20°C, 25°C, and 30°C), and (B) number of RBF cells (passage 38) in L-15 medium containing different FBS concentrations (2%, 5%, 10%, and 15%) at 25°C.

mal culture conditions for RBF cells were determined to be L-15 medium supplemented with 10% FBS at 25°C.

From the viral susceptibility test of RBF cells, different genotypes of megalocytiviruses (RSIV, ISKNV and TRBIV) were successfully isolated upon the appearance of CPE within 7 dpi (Fig. 4). After CPE induction, megalocytivirus-infected cells became thinner and rounding and then, began to detach from the cultured well, thereby destroying the monolayer. The rounded appearance of infected cells was considered to be the manifestation of CPE, which was similar to the rounded appearance of RSIV-infected RoBE-4 cells (Oh and Nishizawa, 2016) and ISKNV-infected GP cells (Gardenia et al., 2020). Notably, even at the same inoculum concentration (1.0×10^6 viral genome copies/mL), the onset of CPE was observed under a light microscope at 4 dpi for RSIV (17RbGs isolate)-inoculated RBF cells and at 7 dpi for ISKNV (PGIV-K1 isolate)- and TRBIV (FLIV-Ph isolate)-inoculated cells, suggesting that RBF cells have different susceptibility for each type.

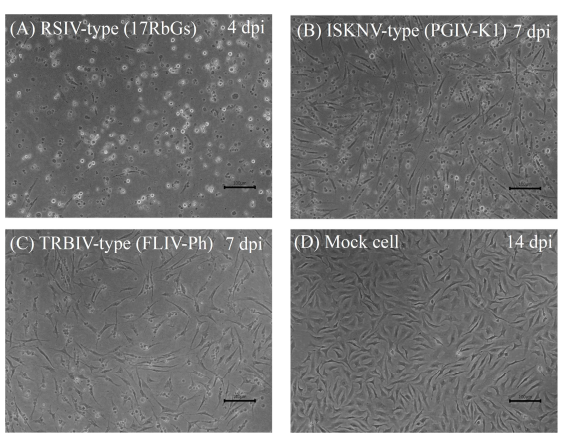

Fig. 4. Virus susceptibility of rock bream fin (RBF) cells to megalocytiviruses. (A) red sea bream iridovirus (RSIV, 17RbGs isolate) (B) infectious spleen and kidney necrosis virus (ISKNV, PGIV-K1 isolate); (C) turbot reddish body iridovirus (TRBIV, FLIV-Ph isolate); (D) negative control (mock). Scale bar = 100 μm.

A variety of susceptible cells for megalocytiviruses including RSIV and ISKNV were reported (Table 2) and these previous studies suggested that cell-origin and/or each isolate might affect proliferation in cell culture. During culturing for RSIV, similar to SKF-9 cells (Kawato et al., 2017) and PMF cells (Kwon et al., 2020), the genome copy number of RSIV (RSIV subtype II, 17RbGS isolate) in the RBF cell supernatant were significantly increased at 3 dpi (approximately 780 times higher than that of the initial inoculum) and then reach a plateau at 1.0×10^9 viral genome copies/mL at 7 dpi (Fig. 5A and Table 2). Furthermore, the number of RSIV genome copies was significantly higher than that of ISKNV (PGIV-K1

Table 2. Infectivity titer of megalocytiviruses in various cells

Cell	Biological source	Genotype[a]	Virus culture condition		Virus titer[c]	Reference
			Temperature	DPI[b]		
GF	Bluestriped grunt (*Haemulon sciurus*)	RSIV (Infected rock bream)	25°C	8 dpi	about 10^7 $TCID_{50}$/mL	Imajoh et al. (2004)
		RSIV (RbHad09)	25°C	14 dpi	$10^{5.7}$ $TCID_{50}$/mL	Oh and Nishizawa. (2016)
		RSIV (KagYT-96)	25°C	3 dpi	$10^{4.0}$ $TCID_{50}$/mL ($10^{6.8}$ genome copies/mL)	Kawato et al. (2017)
		RSIV (IVS-1)	25°C	7 dpi	$10^{7.21}$ genome copies/mL	Kwon et al. (2020)
		RSIV (17RbGS)	25°C	7 dpi	2.3×10^7 genome copies/mL	This study
MFF-1	Mandarin fish (*Siniperaca chuatsi*)	ISKNV (NH060831)	27°C	5-7 dpi	$10^{7.6\text{-}8.4}$ $TCID_{50}$/ml	Dong et al. (2008)
GBC4	Orange-spotted grouper (*Epinephelus coioides*)	RSIV (GSIV-K1)	25, 30°C	5 dpi	10^9 $TCID_{50}$/mL	Wen et al. (2008)
RSBF-2	Red seabream (*Pagrus major*)	RSIV (Infected fish)[d]	28°C	-	$10^{7\text{-}9}$ $TCID_{50}$/ml	Ku et al. (2010)
RoBE-4	Rock bream (*Oplegnathys fasciatus*)	RSIV (RbHad09)	25°C	14 dpi	$10^{7.7}$ $TCID_{50}$/mL	Oh and Nishizawa. (2016)
SKF-9	Spotted knifejqw (*Opegnathus punctatus*)	RSIV (KagYT-96)	25°C	8 dpi	$10^{6.5}$ $TCID_{50}$/mL ($10^{8.5}$ genome copies/mL)	Kawato et al. (2017)
		ISKNV (ISKNV-seabass)	25°C	15 dpi	$10^{5.1}$ $TCID_{50}$/mL	
ARB8	Green terror (*Aequidens rivulatus*)	RSIV (GSIV-K1)	25, 30°C	7 dpi	$10^{7.6}$ $TCID_{50}$/mL	Yeh et al. (2018)
		ISKNV (RSIV-Ku)			$10^{7.2}$ $TCID_{50}$/mL	
GP	Giant gourami (*Osphronemus goramy*)	ISKNV (GGIV)	25°C	5 dpi	about 5×10^8 genome copies/mL	Gardenia et al. (2020)
PMF	Red seabream (*Pagrus major*)	RSIV (IVS-1)	25°C	7 dpi	$10^{8.36}$ genome copies/mL	Kwon et al. (2020)
RBF	Rock bream (*Oplegnathys fasciatus*)	RSIV (17RbGS)	25°C	7 dpi	1.0×10^9 genome copies/mL	This study
		ISKNV (PGIV-K1)		11 dpi	1.3×10^7 genome copies/mL	
		TRBIV (FLIV-Ph)			2.1×10^7 genome copies/mL	

[a]Isolate or Biological source; [b]Days post inoculation; [c]Viral genome copy numbers/mL or $TCID_{50}$/mL; [d]Diseased red seabream, giant grouper and asian sea bass (barramundi)

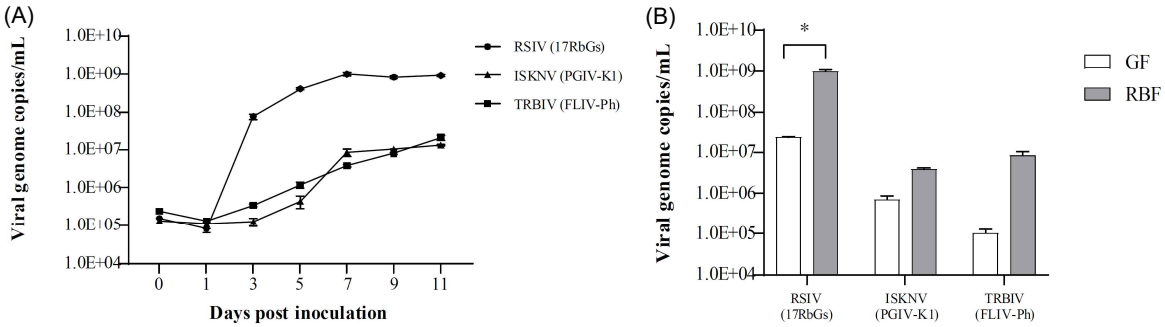

Fig. 5. Viral genome copy numbers of megalocytiviruses (17RbGs isolate for red sea bream iridovirus, RSIV; PGIV-K1 isolate for infectious spleen and kidney necrosis virus, ISKNV; FLIV-Ph isolate for turbot reddish body iridovirus, TRBIV) in inoculated cells at 25 ℃. (A) Viral genome copy numbers from megalocytivirus-inoculated rock bream fin (RBF) cells at 1, 3, 5, 7, 9 and 11 days post-inoculation (dpi) (B) Comparison of the number of viral genome copies of viruses from megalocytivirus-inoculated RBF and grunt fin (GF) cells at 7 dpi. RBF and GF cells in 24-well plates were inoculated with 1.0×10^5 and 1.0×10^6 viral genome copies/well of each virus, respectively. $^*P < 0.05$.

isolate, 1.3×10^7 viral genome copies/mL) and TRBIV (FLIV-Ph isolate, 2.1×10^7 viral genome copies/mL) at 11 dpi (Fig. 5A). For the RoBE-4 cells derived from embryos originating from the same fish species with RBF cells, RSIV (RbHad09 isolate)-infected RoBE-4 cells showed a maximum infectivity titer at 14 dpi (Oh and Nishizawa, 2016). Of note, even though the difference of initial concentration for viral inoculation compared to a previous study (Oh and Nishizawa, 2016), the cells derived from rock bream could be suitable to propagate RSIV.

For ISKNV culturing, MFF-1 cells could be efficiently used to produce a high titer within 7 days (NH060831 isolate, $10^{7.6\text{-}8.4}$ $TCID_{50}$/mL; Table 2) (Dong et al. 2008). Although RBF cells did not produce a higher titer than MFF-1 cells, ISKNV (PGIV-K1 isolate) could be replicated in RBF cells (Fig. 5A). Interestingly, the genome copy number of TRBIV (FLIV-Ph isolate) in the RBF cell increased, suggesting that the RBF cells could be susceptible to TRBIV. This result is concordant with that of a previous study (Jin et al., 2018), TRBIV (MVSF-12 isolate)-inoculated primary cells derived from rock bream embryos formed a general CPE. Similarly, in this study, TRBIV was cultured on RBF cells.

Generally, subculture passages and/or virus strain susceptibility might play a role in virus culture. In GF cells, infection with a high titer of RSIV with the induction of CPE was established using a specific method (Ito et al., 2013). In addition, the RSIV could not consistently be propagated in GF cells (over 400 passaged GF cells used in this study) after inoculation of RSIV (17RbGs isolate) at 1.0×10^5 viral genome copies/mL (data not shown). For these drawbacks of RSIV propagation in the GF cells, we inoculated each virus at different viral copy numbers in RBF (1.0×10^5 viral genome copies/mL) and GF (1.0×10^6 viral genome copies/mL) cells. Even though 10 times lower numbers of RSIV genome copies were inoculated in RBF cells, the number of RSIV genome copies in the culture supernatant of RBF reached 1.0×10^9 viral genome copies/mL, which was 44 times higher than those in RSIV-inoculated GF cells (2.3×10^7 viral genome copies/mL) at 7 dpi (Fig. 5B). Although the increment of viral genome copy number was identified from the culture supernatant of ISKNV-inoculated RBF and GF cells, there was no significant difference (PGIV-K1 isolate, 8.43×10^6 viral genome copy number/mL in RBF cells, and 7.12×10^5 viral genome copy number/mL in GF cells at 7 days).

Moreover, TRBIV was propagated in RBF cells (FLIV-Ph isolate, 3.8×10^6 genome copy numbers/mL at 7 days) (Fig. 5B). But, the TRBIV could not be propagated in the GF cells. These results suggest that RBF cells have varying susceptibilities to different genotypes of megalocytiviruses (RSIV, ISKNV and TRBIV), with respect to the appearance of CPE and viral production.

In conclusion, the culture conditions, including growth temperature and FBS concentration, for rock bream fin cells were optimized and cells were successfully maintained. RBF cells exhibited a variation in susceptibility to different genotypes of megalocytiviruses (RSIV, ISKNV and TRBIV) and induced CPE, which presented as a rounded appearance of infected cells. Furthermore, the number of cultured RSIV genome copies in RBF cells was significantly higher (44 times) than that in GF cells. Thus, RBF cells could be a useful tool for further studies involving diagnosis and mechanistic evaluation of virus infection *in vitro*.

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Conflicts of interest

The authors declare no conflict of interest.

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