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Effect of CRISPR/Cas9-mediated knockout of either Mx1 or ISG15 gene in EPC cells on resistance against VHSV infection

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ABSTRACT

Although the type I interferon-mediated increase of Mx1 and ISG15 gene expression in Epithelioma papulosum cyprini (EPC) cells has been reported, the antiviral role of Mx1 and ISG15 in EPC cells has not been investigated. In this study, to know the anti-viral hemorrhagic septicemia virus (VHSV) role of Mx1 and ISG15 of EPC cells, either Mx1 or ISG15 gene was knocked-out using a CRISPR/Cas9 system, and the progression of cytopathic effects (CPE) and viral growth were analyzed. Mx1 gene and ISG15 gene knockout EPC cells were successfully produced via CRISPR/Cas9 coupled with a single-cell cloning. Through the sequence analysis, one clone showing two heterozygous indel patterns in Mx1 gene and a clone showing three heterozygous indel patterns in ISG15 gene were selected for further analyses. Mx1 knockout EPC cells did not show any differences in VHSV-mediated CPE progression, even when pre-treated with polyinosinic:polycytidylic acid (poly I:C), compared to control EPC cells. These results suggest that Mx1 in EPC cells may be unfunctional to cytoplasmic RNA viruses. In contrast to Mx1, ISG15 knockout cells showed clearly hampered anti-VHSV activity even when pre-treated with poly I:C, indicating that ISG15 plays an important role in type I interferon-mediated anti-viral activity in EPC cells, which allowed VHSV to replicate more efficiently in ISG15 knockout cells than Mx1 knockout and control cells.

1. Introduction

In vertebrates, type I interferon (IFN) responses are the main innate immune responses against viral infections, and are triggered by the binding of IFN-α or IFN-β on the heterodimeric IFN-α receptor (IFNAR), which phosphorylates receptor-associated Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), and subsequently phosphorylates signal transducer and activator of transcription 1 (STAT1) and STAT2. Finally, STAT1, STAT2 and IFN-regulatory factor 9 (IRF9) form the IFN-stimulated gene factor 3 (ISGF3) complex and move into nucleus to induce the transcription of antiviral genes, such as myxovirus resistance (Mx) and interferon-stimulated gene 15 (ISG15) [1–3].

Viral hemorrhagic septicemia virus (VHSV) causes a serious damage on aquaculture farms in both freshwater and seawater worldwide [4]. VHSV is classified as a member of the genus *Novirhabdovirus* in the family Rhabdoviridae and has a NV gene in the genome [5,6]. Although VHSV is highly susceptible to host's type I IFN responses, the suppression of type I IFN responses in an early infection stage through NV protein has been demonstrated [7,8].

Previously, we showed that CRISPR/Cas9-mediated knockout of IRF9 gene in Epithelioma papulosum cyprini (EPC) cells led to a significant decrease of type I IFN responses, which resulted in a significant increase of VHSV replication [9]. Different from IRF9, Mx and ISG15 proteins are representative interferon-induced antiviral proteins that can act directly to viruses [10,11]. Mx proteins are dynamin-like large GTPases and the antiviral mechanism is closely related to GTP binding and GTPase activity [12–15]. Fish have one to seven Mx genes according to species, and the antiviral role of Mx proteins has also been demonstrated in various fish species [16,17]. ISG15 shows anti-viral activities against diverse kinds of RNA and DNA viruses in mammals. ISG15 can be covalently conjugated to target proteins (ISGylation) via C-terminal LRLRGG motif, or can act as a cytokine to regulate several immune responses when it presents as a free unconjugated form [18]. The ISG15 orthologue has been reported from many fish species, and the enhanced expression of ISG15 in response to viral infection and polyinosinic:polycytidylic acid (poly I:C) stimulation have been demonstrated [19–21].

Although the type I interferon-mediated increase of Mx1 and ISG15 gene expression in EPC cells has been reported [9,22], the antiviral role of Mx1 and ISG15 in EPC cells has not been investigated. In the present study, to know the anti-VHSV role of Mx1 and ISG15 genes of EPC cells, either Mx1 or ISG15 gene was knocked-out using a CRISPR/Cas9

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Table 1
Summary of primers used in this study.

Name of primer	Sequence (5′ to 3′)	
EPC Mx1 SDM	F	TACGTCATTGCTCAGGGTTCTGGAGGCGCTGTCAGGTTTTAGAGCTAGA
	R	TCTAGCTCTAAAACCTGACAGCGCCTCCAGAACCCTGAGCAATGACGTA
EPC ISG15 SDM	F	TACGTCATTGCTCAGGTGAGCACTAATGCCACAGTGTTTTAGAGCTAGA
	R	TCTAGCTCTAAAACACTGTGGCATTAGTGCTCACCTGAGCAATGACGTA
EPC Mx1 sequencing	F	ATGGAGAAAATGAGTTACACATTCAGTCAGC
	R	CTTTAGTTCTTATCATCTTGAGCTCAAGAG
EPC ISG15 sequencing	F	ATGGAACTGACAGTAAAACTGTTGAACGGAG
	R	GTAACTGCTGAGGCTTCTGGAATCATCATC
β-actin-real time	F	AAGGAGAAGCTCTGCTATGTGGCT
	R	AAGGTGGTCTCATGGATACCGCAA
Mx1-real time	F	TGGAGGAACCTGCCTTAAATAC
	R	GTCTTTGCTGTTGTCAGAAGATTAG
ISG15-real time	F	TGATGCAAATGAGACCGTAGAT
	R	CAGTTGTCTGCCGTTGTAAATC

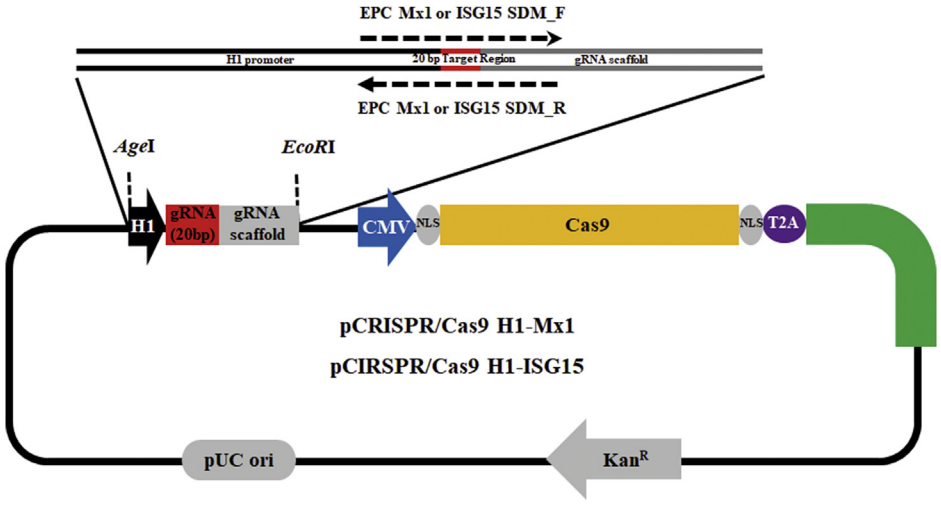

Fig. 1. Construction of a CRISPR/Cas9 vector targeting Epithelioma papulosum cyprini (EPC) cell's Mx1 gene or ISG15 gene by the site-directed mutagenesis of guide RNA sequence from a previously constructed vector pCRISPR/Cas9 H1–HIF-1α [23].

system, and the progression of cytopathic effects (CPE) and viral growth were analyzed.

2. Materials and methods

2.1. Cells and virus

EPC cells (ATCC no. CRL-2872) were cultured in Leibovitz medium (L-15, Sigma) supplemented with penicillin (100 U/ml, Welgene), streptomycin (100 μg/ml, Welgene) and 10% fetal bovine serum (FBS, Welgene). VHSV (VHSV KJ2008) was propagated in a monolayer of EPC cells at 15 °C in the presence of 2% FBS.

2.2. Vector construction for CRISPR/Cas9-mediated knockout of Mx1 or ISG15 gene

Using the CRISPR/Cas9 single-guide RNA (sgRNA) design tools (http://www.rgenome.net/cas-offinder/, http://chopchop.rc.fas.harvard.edu/), the highest-scored guide RNA (gRNA) sequences targeting Mx1 gene (5′-GGTTCTGGAGGCGCTGTCAGGGG-3′) and ISG15 gene (5′-GTGAGCACTAATGCCACAGTCGG-3′) of EPC cells were designed. Site-directed mutagenesis (SDM; Stratagene) was conducted according to the manufacturer's instructions in order to change the gRNA sequence targeting HIF-1α gene of previously constructed vector, pCRISPR/Cas9 H1–HIF-1α [23], with gRNA sequence targeting the Mx1 gene or the ISG15 gene. The primers used for the mutagenesis are described in Table 1, and the constructed vectors were named as pCRISPR/Cas9 H1-Mx1 and pCRISPR/Cas9 H1-ISG15.

2.3. Production of single-cell clones of Mx1 gene or ISG15 gene knockout cells

To produce Mx1 gene or ISG15 gene knockout cells, pCRISPR/Cas9 H1-Mx1 or pCRISPR/Cas9 H1-ISG15 was transfected into EPC cells (3×10^6 cells) using the Neon transfection system (Invitrogen) according to the manufacturer's instructions. At 2 days post transfection, cells were sorted in 96-well plates using Automated High-speed Cytometry Sorter System (BD FACS Aria III) to get single cell clones. After 1 month of culture, genomic DNA was isolated from H1-Mx1/EPC cell clones or H1-ISG15/EPC cell clones using QuickExtract DNA Extraction Solution (Lucigen) according to the manufacturer's instructions, and each fragments containing the 20 bp targeting sequence of Mx1 gene or ISG15 gene were PCR amplified using specific primer pairs listed in Table 1. The PCR products were cloned into pGEM-T easy vector (Promega), and sequenced for the detection of indels.

To analyze the transcript level of Mx1 and ISG15 genes in knockout cells, cell clones of Mx1 knockout, ISG15 knockout, and control cells were cultured in 6 well plates (3.0×10^6 cells/well), and were treated with poly I:C (100 μg/ml, Sigma). After 24 h, total RNA from each cell clone was extracted using Hybrid-R kit (GeneAll, Korea), and treated with DNaseI (GeneAll), then, purified using Riboclear plus kit (GeneAll) according to the manufacturer's instructions. One μg of the RNA was synthesized to first-strand cDNA using a reverse transcription master premix (ELPIS, Korea) with oligo(dT) primer according to the

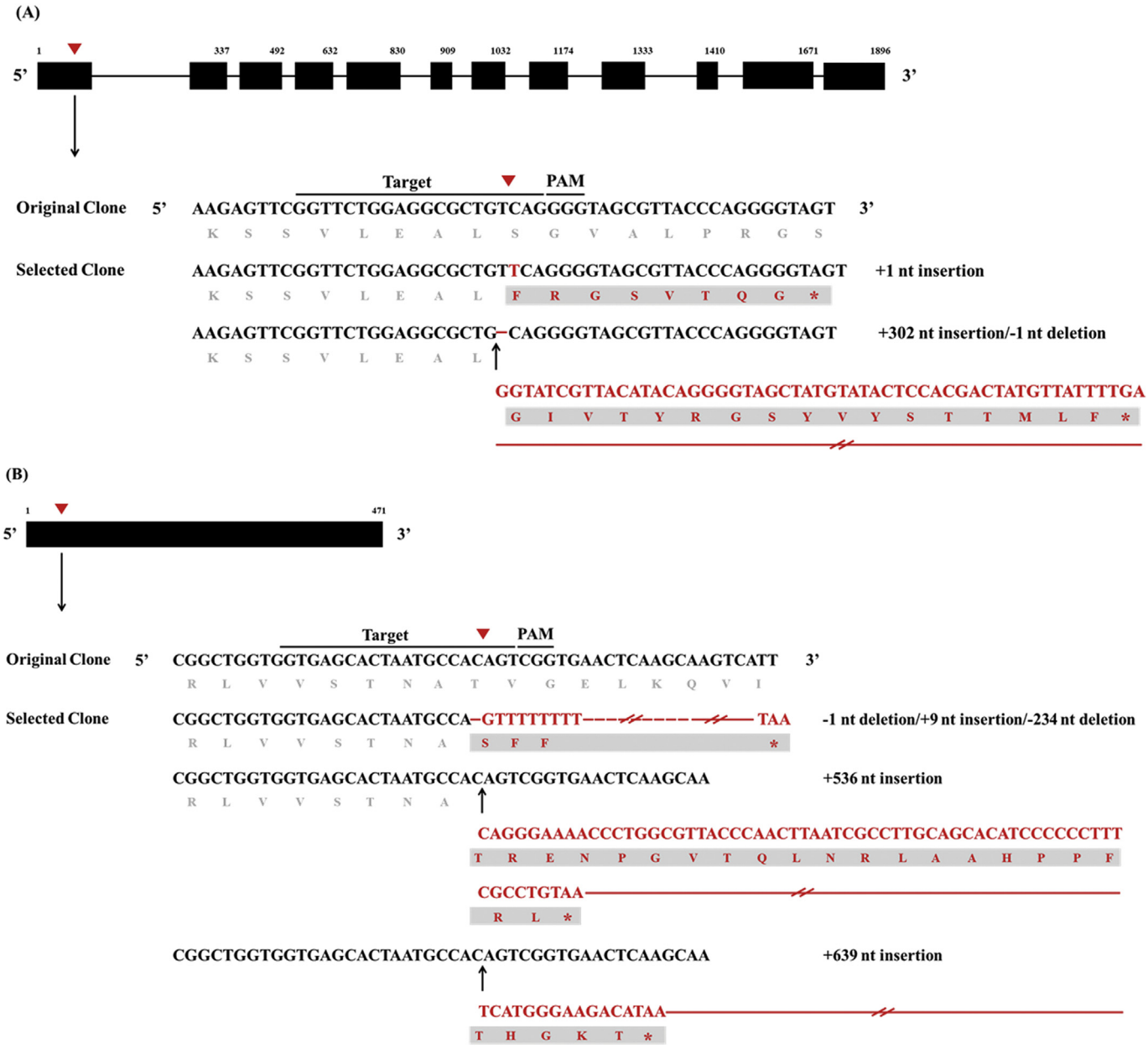

Fig. 2. Sequence alignment of nucleotides and amino acids of Mx1 gene and ISG15 gene. Both a Mx1 knockout cell clone (A) and an ISG15 knockout cell clone (B) showed heterozygous insertion/deletion (indel) mutations. The upper black box(es) represent exon regions of Mx1 gene and ISG15 gene, and upper numbers of the box(es) mean the nucleotides of each gene ORF. The red letters indicate insertion or deletion mutations, and the asterisk represent the generation of a premature stop codon by out-of-frame indels. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was carried out by a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) using the PCR primer sets shown in Table 1. The PCR reactions in a volume of 20 µl were run using 2 × SYBR Green Premix (Enzynomics, Korea) with 5 µl of cDNA and 5 pM of each primer. Thermal cycling condition was 1 cycle of 15 min at 95 °C (preincubation), followed by 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 20 s at 72 °C.

2.4. Effect of Mx1 gene or ISG15 gene knockout on virus replication

Mx1 gene or ISG15 gene knockout EPC cells grown in 6-well plates (3×10^6 cells/well) at 28 °C were pretreated with poly I:C (100 µg/ml). After 24 h, each cell clone was inoculated with VHSV at MOI 0.0001 and observed the cytopathic effect (CPE) progression using a microscope, and also harvested virus supernatant at 1, 3, 5 days post-inoculation. The virus titer was analyzed by the plaque assay.

2.5. Statistical analysis

Statistical analysis was performed using SPSS for Windows (Chicago, IL, USA). Data were analyzed by one-way ANOVA followed by Tukey HSD post-hoc test, and a probability (P) value less than 0.05 was considered statistically significant.

3. Results

3.1. Generation of Mx1 gene and ISG15 gene knockout EPC cells

Single cell clones transfected with pCRISPR/Cas9-H1-Mx1 or pCRISPR/Cas9-H1-ISG15 vector (Fig. 1) were analyzed by the

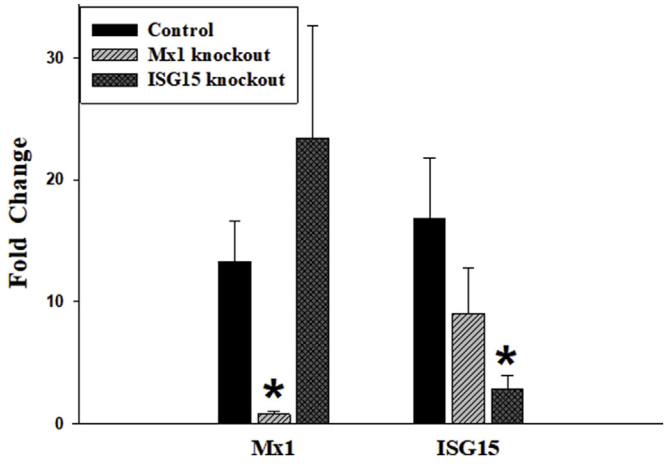

Fig. 3. Comparison of Mx1 and ISG15 mRNA level among control EPC cells, Mx1 knockout and ISG15 knockout EPC cells. Cells were treated with poly I:C (100 μg/ml) or mock-treated. After 24 h, total RNA was extracted from cells, and the transcript level of Mx1 gene and ISG15 gene was analyzed by qRT-PCR. All the values of fold change were calculated relative to the mock of control EPC cells. The asterisk on the bar represents statistically significant at $P < 0.05$.

sequencing of target region to find indels. Among 96 replicates of cell clones having pCRISPR/Cas9-H1-Mx1, eight clones were grown and only one clone showed two heterozygous indel patterns in Mx1 gene (Fig. 2A). In cells containing pCRISPR/Cas9-H1-ISG15, six clones were grown and one clone showed three heterozygous indel patterns in ISG15 gene (Fig. 2B). All the heterozygous indels led to the generation of premature stop codons.

In the analysis of Mx1 and ISG15 mRNA expression level by qRT-PCR, both Mx1 and ISG15 mRNA level in control EPC cells were significantly increased by the treatment with poly I:C, while Mx1 mRNA level in Mx1 knockout EPC cells and ISG15 mRNA level in ISG15 knockout EPC cells were significantly decreased compared to control EPC cells irrespective of exposure to poly I:C (Fig. 3).

3.2. Effect of Mx1 gene or ISG15 gene knockout on CPE by VHSV

In the experiment to analyze the effect of Mx1 gene or ISG15 gene knockout on VHSV infection-mediated CPE progression, Mx1 knockout cells did not show any differences with control EPC cells in CPE progression by VHSV infection, however, ISG15 knockout cells showed clearly more severe CPE at 3 days post-infection than control and Mx1 knockout cells (Fig. 4A).

In the experiment to analyze the effect of poly I:C pre-treatment on the CPE formation by VHSV infection in Mx1 gene or ISG15 gene knockout cells, pre-treatment of EPC cells with poly I:C completely inhibited the CPE formation by VHSV infection in control and Mx1 knockout cells. In contrast, ISG15 gene knockout cells showed clear CPE at 3 days post-infection and complete CPE at 5 days post-infection (Fig. 4B).

3.3. Effect of Mx1 gene or ISG15 gene knockout on VHSV replication

The titers of VHSV in ISG15 knockout EPC cells were significantly higher than in both control EPC cells and Mx1 knockout EPC cells (Fig. 5A). When cells were pretreated with poly I:C, the replication of VHSV was greatly inhibited in control cells and Mx1 knockout cells throughout the experimental days (Fig. 5B). However, the growth of VHSV in ISG15 knockout cells was not affected by the poly I:C pre-treatment (Fig. 5B) and showed a similar growth pattern with ISG15 knockout cells that were not pre-treated with poly I:C in Fig. 5A.

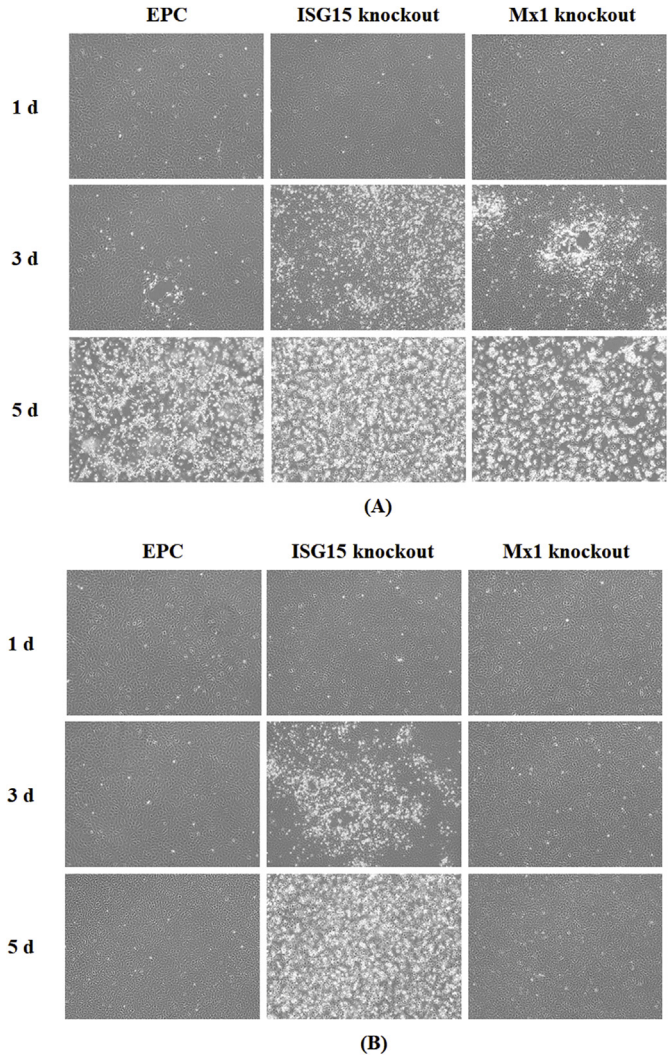

Fig. 4. Effect of poly I:C pre-treatment on CPE progression by VHSV infection in control EPC cells, ISG15 knockout EPC cells, and Mx1 knockout EPC cells. Cells were mock-treated (A) or treated with 100 μg/ml of poly I:C (B) for 24 h, then, infected with VHSV at MOI 0.0001. At 1, 3, and 5 d post-infection, the CPE was observed using a microscope.

4. Discussion

Since transfection efficiencies in fish cells are very low, a transient transfection cannot provide a sensitive detection of the effect of a specific gene knockout by a CRISPR/Cas9 system. Furthermore, as the expression level of transfected gene(s) in a cell population selected using antibiotics is widely variable, a single-cell cloning would be an efficient way to get a homogeneous fish cell population. In the present study, we could successfully produce Mx1 gene and ISG15 gene knockout EPC cells via CRISPR/Cas9 coupled with a single-cell cloning.

During evolution, a third round whole-genome duplication (WGD) had occurred in teleosts (teleost-specific WGD) [24,25], which can be a reason why three heterozygous indel patterns were produced in the ISG15 knockout cell clone by a CRISPR/Cas9-mediated gene editing in this study.

The significant decrease of Mx1 and ISG15 transcripts in Mx1 knockout cells and ISG15 knockout cells, respectively, suggests that the presence of premature termination codons in the mRNAs of Mx1 and ISG15 in each knockout cells induced the mRNAs degradation by nonsense-mediated mRNA decay (NMD) that is a post-transcriptional regulatory mechanism in eukaryotic cells [26–28].

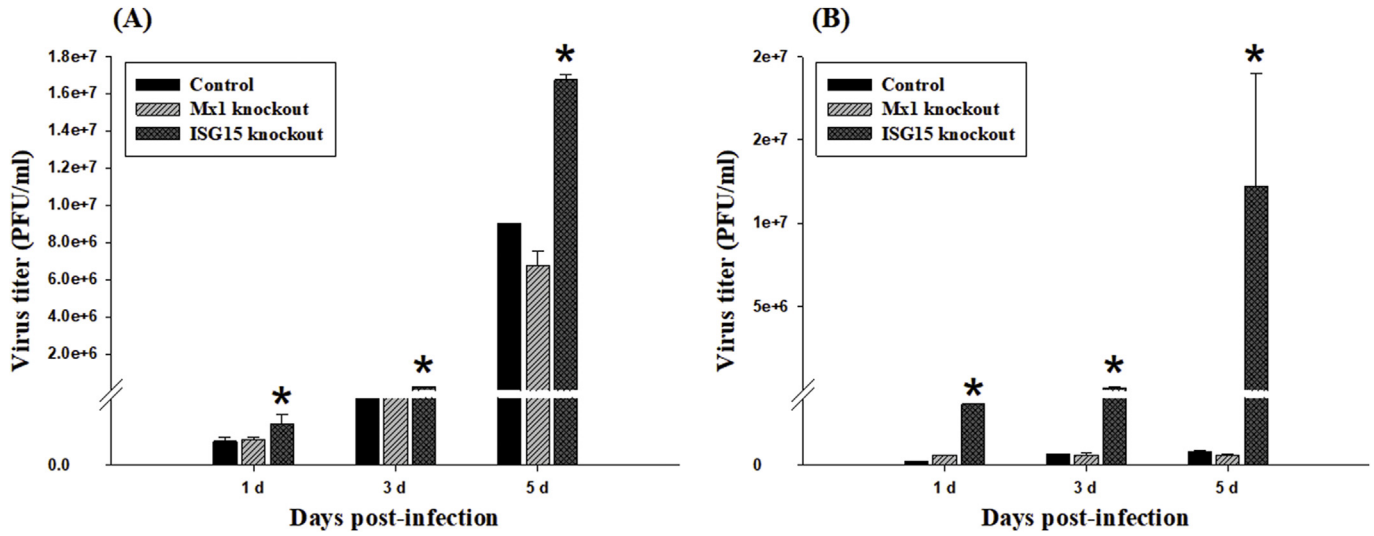

Fig. 5. Effect of poly I:C pre-treatment on VHSV replication in control EPC cells, Mx1 knockout EPC cells, and ISG15 knockout EPC cells. Cells were mock-treated (A) or treated with 100 μg/ml of poly I:C (B) for 24 h, then, infected with VHSV at MOI 0.0001. At 1, 3, and 5 d post-infection, the virus titer was measured by plaque assay. The asterisk on the bar represents statistically significant at $P < 0.05$.

Most mammals possess at least two Mx genes that can be distinguished by subcellular localization of the proteins [29]. Human MxA and mouse Mx1 proteins are nuclear Mx proteins and are involved in the protection against viruses in the nucleus [15,30]. While human MxB and mouse Mx2 proteins are cytoplasmic Mx proteins and inhibit the replication of viruses in the cytoplasm [31–33]. The differential antiviral activity of Mx proteins has also been reported in various fish species [34–36], in which Mx proteins inhibited the replication of cytoplasmic RNA viruses but failed to inhibit the replication of nucleic DNA viruses. Fernández-Trujillo et al. [37] reported that three Mx proteins (SauMx1, SauMx2 and SauMx3) from gilthead seabream (*Sparus aurata*) showed differential antiviral activities according to viral species; SauMx1 against VHSV and lymphocystis disease virus (LCDV), SauMx2 against European sheatfish virus (ESV) and LCDV, SauMx3 against VHSV. Our previous study had showed that the pretreatment of EPC cells with poly I:C, a representative inducer of type I interferon responses, induced no CPE formation by VHSV infection [9] Therefore, if Mx1 of EPC cells plays an important role in the inhibition of VHSV replication, a pre-treatment of Mx1 knockout cells with poly I:C would not efficiently protect cells from CPE formation by VHSV infection. However, in this study, Mx1 knockout EPC cells did not show any differences in VHSV-mediated CPE progression, even when pre-treated with poly I:C, compared to control EPC cells. These results suggest that Mx1 in EPC cells may be unfunctional to cytoplasmic RNA viruses. Further researches on the subcellular localization of EPC Mx1 and its antiviral activity against other viruses including DNA viruses are needed to know the exact function of EPC Mx1 protein.

In mammals, ISG15 protein has shown wide antiviral activities against not only RNA viruses but also DNA viruses [18,38]. In fish, the ISG15 was firstly identified from rainbow trout leukocytes that were infected with VHSV [39], since then, ISG15 gene has been reported from various fish species. In the present study, in contrast to Mx1, ISG15 knockout cells showed clearly hampered anti-VHSV activity even when pretreated with poly I:C, indicating that poly I:C pre-treatment did not induce any inhibitory effect on CPE formation in ISG15 knockout cells. These results suggest that ISG15 plays an important role in type I interferon-mediated anti-viral activity in EPC cells, which allowed VHSV to replicate more efficiently in ISG15 knockout cells than Mx1 knockout and control cells. The viral titers in ISG15 knockout cells were significantly higher than those in Mx1 knockout cells and control cells, indicating the advantage of the present ISG15 knockout cells in the efficient production of VHSV.

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