Int Aquat Res (2022) 14:275–283
https://doi.org/ 10.22034/IAR.2022.1971400.1344

ORIGINAL RESEARCH

Inhibition of orange-spotted grouper nervous necrosis virus replication by short hairpin RNAs in grouper GF-1 cells

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Received: : 04 October 2022 / Accepted: 28 December 2022 / Published online: 30 December 2022

Abstract Infection with the nervous necrosis virus (NNV) causes viral nervous necrosis, resulting seriouslly economic losses in the aquaculture of marine fish. The viral genome of orange-spotted grouper nervous necrosis virus (OSGNNV) consists of two single-stranded, RNA1 and RNA2, encoding RNA-dependent RNA polymerase (RdRp) and capsid protein (CP), respectively. RNA interference has been shown to have activities against various viruses and is considered a promising antiviral approach. Here, we describe antiviral activities of short hairpin RNAs (shRNAs) that target RdRp and CP gene of OSGNNV in GF-1 cells. We constructed shRNAs for two specific target genes, RdRp and CP genes of OSGNNV. The shRNAs were transfected into GF-1 cells, followed by OSGNNV infection at multiplicity of infection (M.O.I) of 10.0. Then, the efficient inhibition of OSGNNV replication was examined by real-time quantitative RT-PCR analysis of RdRp and CP gene expression and viral titers. The results showed that the expression levels of RdRp and CP gene were reduced by shRNAs-transfected GF-1 cells at 48 and 72-hour post infection (hpi). Besides, the viral titers of shRNAs-transfected GF-1 cells were significantly lower than those of OSGNNV control at period of 48-96 hpi. These results indicated that the plasmid-transcribed shRNAs could inhibit effectively the OSGNNV replication in GF-1 cells, providing a potential approach to control viral disease in aquaculture.

Keywords Orange-spotted grouper nervous necrosis virus (OSGNNV) . shRNAs . RdRp gene expression . CP gene expression . Transfection . Inhibition . GF-1 cells

Introduction

Viral nervous necrosis (VNN) is a serious viral disease affecting more than 40 species of cultured marine fish worldwide, including barramundi, European sea bass, striped jack, Japanese parrotfish and groupers (Muroga 2001; Munday et al. 2002; Shetty et al. 2012). The early satges of development, larvae and juvenile, are more susceptible to VNN disease, causing mortality rates of up to 100% (Yoshikoshi and Inoue 1990; Mori et al. 1991). Infected fish often show clinical signs such as uncoordinated darting, swimming in spirals, abnormal swim sbladder control and vacuolation in the brain and retina (Mori et al. 1992; Grotmol et al. 1997). VNN disease, which is an under the class *Betanodavirus* and family *Nodaviridae*, is caused by the pathogen nervous necrosis virus (NNV) (Schneemann et al. 2005). The genome of OSGNNV consists of two single-stranded, positive-sense RNAs, namely RNA1 and RNA2. The RNA1 (3.1 kb in length) encodes RNA dependent RNA polymerase (RdRp) while RNA2 (1.4 kb in length) encodes capsid protein (CP) (Chien et al. 2017). RdRp catalyzes RNA synthesis directed by RNA template for replication of viral genome in all of positive strand RNA viruses (O'Reilly and Kao 1998) while CPs are involved in the assembly of viral particles (Fischer and James 2004).

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RNA interference (RNAi) plays an important role in silencing target gene expression and thus inhibit viral replication in cell culture. The strategy of RNAi is based on post-transcription gene silencing which is initiated by double strand RNA (dsRNA) > 30 nucleotides, small interference RNA (siRNA) or short hairpin RNA (shRNA) <30 nucleotides (Zhou et al. 2010). Short dsRNAs can be presented into cells from externally or formed internally in the cell nucleus from longer precursors. The prescursors are characteristically RNAs that can fold back on themselves by forming base pairs in the form of hairpin structures. These structures are sliced to produce shorter RNAs (siRNAs) of about 21-23 base pairs and these are exported to the cytoplasm. The short siRNAs amass with a number of proteins into silencing complexes. Targeted mRNA that match one strand of the siRNA is cleaved and destroyed, as a consequence, the expression of its encoding protein cannot be advanced (Carmichael 2002). The regulation of gene expression and protecting the host cells from invading viruses are two of the biological functions of RNAi. It does this by preventing the expression of key viral protein by destroying targeted mRNA (Chen et al. 2004).

Recently, somes studies reported that siRNA was useful for inhibition of viral replication at different stages of infection in various cells such as plant cells (Yelina et al. 2002), insect cells (Adelman et al. 2002; Caplen et al. 2002) as well as mammalian cells (Capodici et al. 2002; Novina et al. 2002). Furthermore, efficient inhibition of siRNA had been proven for DNA viruses (Xie et al. 2005; Mallanna et al. 2006; Kim et al. 2010; Bronkhorst et al. 2012) and RNA viruses (Kumar et al. 2010; Ma et al. 2011; Huang and Han 2014; Liu et al. 2016; Boylan et al. 2017; Morick and Saragovi 2017; Fouad et al. 2019). Especially, the inhibitory effect of siRNA on DNA viruses, tiger frog iridovirus (TFV) and red seabream iridovirus (RSIV) (Xie et al. 2005; Dang et al. 2008), and RNA viruses, red-spotted grouper nervous necrosis virus (RGNNV), grass carp reovirus (GCRV) and spring viraemia of carp virus (SVCV) (Wu et al. 2010; Ma et al. 2011; Gotesman et al. 2014; Fouad et al. 2019), also have been reported in various fish cell lines. However, the application of siRNAs have been limited by their short half-lives, the high costs of synthetic siRNAs and instability due to degradation by nucleases (Elbashir et al. 2001), whereas shRNA can be used for long-term in silencing expression and can be delivered to specific tissue (Khan 2018). Therefore, the effects of shRNAs targeting RdRp and CP genes on inhibition of OSGNNV replication were investigated in GF-1 cells. The shRNAs were synthesized and transfected into GF-1 cells, followed by OSGNNV infection. The efficient inhibition of OSGNNV replication was examined by gene expression levels and virus titers.

Materials and methods

Cell line and virus

Grouper fin (GF-1) cells were grown in Lebovitz L-15 medium (Gibco) supplemented with 5% fetal bovine serum and 1X antibiotic-antimycotic (Gibco) at 28°C. The nervous necrosis virus (OSGNNV) isolated from orange-spotted grouper (Chien et al. 2018) was inoculated into GF-1 cells with 10.0 multiplicity of infection (MOI). Once complete cytopathic effect was observed, the culture fluid was collected and centrifuged at 2,000×g for 10 min. Then, the supernatant was collected and stored at -80°C until use. The virus titer was determined as described by Reed and Muench (1938).

Construction of plasmids

shRNAs targeting RdRp and CP gene were designed by using the BLOCK-It™ RNAi Designer (Invitrogen). For each target gene, we designed a forward sequence with 5' single stranded overhangs TA and a reverse sequence with 5' single stranded overhangs TCGA. The hairpin sequence contains 21 oligonucleotides specific to RNA targets and a loop sequence separates the two complementary domains (Table 1). Equal amount of two primers (F_RdRp and R_RdRp for RdRp gene; F_CP and F_CP for CP gene) with

Table 1 The sequences of primers for constructing short hairpin RNAs

Name	Sequence (5'-3')	Accession number
F_RdRp	TATGCACC**GCAACAACATTGCCAACTTCA***CCACACC***TGAAGTTGGCAATGTTGTTGC**TTTTG	MF144242
R_RdRp	TCGACAAAA**GCAACAACATTGCCAACTTCA***GGTGTGG***TGAAGTTGGCAATGTTGTTGC**GGTGCA	MF144242
F_CP	TATGCACC**GCAAAGGTGAGAAGAAATTGG***CCACACC***CCAATTTCTTCTCACCTTTGC**TTTTG	MF144241
R_CP	TCGACAAAA**GCAAAGGTGAGAAGAAATTGG***GGTGTGG***CCAATTTCTTCTCACCTTTGC**GGTGCA	MF144241

Bold letters: hairpin sequence
Italic letters: loop sequence
Underline letters: 5' overhang

concentration of 100 µM for each primer were mixed. The mixture was boiled for 3 min, followed by incubation at 80°C for 5 min, and then cooled slowly down to room temperature to anneal. Double-stranded DNA fragments were ligated into pET-23a (+) vector which was digested with restriction enzymes NdeI and SalI, and then were transformed into *E. coli* DH5α. The resulting plasmids were named as pET-RdRp and pET-CP.

In vitro RNA transcription

The plasmids, pET-RdRp and pET-CP, were cut into linear DNAs by restriction enzyme SalI. Then, linear DNAs were served as the template for *in vitro* transcription using TranscriptAid T7 High Yield Transcription Kit according to the instruction of the manufacturer (Thermo Scientific). After *in vitro* transcription, the reaction mixture was treated with DNase I, extracted by phenol/chloroform, and precipitated in ethanol. The RNA pellet was washed by 70% cool ethanol and dried at room temperature. Then, it was dissolved in 20 µl of DEPC-treated ddH_2O (DEPC-ddH_2O). RNA transcripts were named as shRdRp and shCP, respectively. The schematic map shows the shRdRp with the added restriction enzyme site and its target position (Fig. 1). The procedure of constructed shCP was the same as shRdRp.

Short hairpin RNA transfection and virus infection

$4x10^5$ of GF-1 cells per well were seeded in a 6-well plates and incubated at 28°C for overnight. The cells were transfected with shRNAs using lipofectamine 3000™ reagent (Invitrogen, USA) according to the manufacturer's protocol. Briefly, single shRdRp (75 pmol) or shCP (75 pmol) or combined stranfection shRdCP (37.5 pmol shRdRp and 37.5 pmol shCP), was diluted in 250 µl of Lebovitz L-15, followed by adding 7.5 µl lipofectamine 3000™ reagent (Invitrogent) per well. The transfection complex was incubated and added into each well. Transfected cells were then infected with OSGNNV at M.O.I of 10.0 and incubated at 28°C. Cells were harvested to extract RNA for gene expression experiment and the viral supernatants were collected for viral titer experiment at 48, 72, 96, and 120 hour post infection (hpi).

RNA extraction

Total RNA was extracted from cells by using TRIzol® Reagent (Ambion, Life technologies, USA) according to the manufacturer's instructions. Then, 5 µg of total RNA was treated with 2U/µl DNase I (Invitrogent). RNA was subsequently extracted by Monarch total RNA miniprep kit (Biolabs), and dissolved in 20 µl of DEPC-ddH_2O.

cDNA synthesis and qPCR

Five hundred ng of RNA was reverse-transcribed by using oligo(dT)18 and reverse transcription primers, RT-RdRp and RT-CP primers (Table 2) with M-MuLV reverse transcriptase according to manufacturer's protocol (Protech). The corresponding cDNA was used for qPCR qualification using iTaq ™Universal SYBR ®Green Supermix.

For detection RdRp and CP gene exprssion, the cDNA was diluted 10 times with ddH_2O and analyzed by CFX connect™ system (Bio Rad). The qPCR mix contained 2 µl of cDNA, 5 µl of 2x iTaq ™Universal SYBR ®Green Supermix, 0.6 µl of 5 µM for each forward and reverse primer (F_RdRp and R_RdRp; F_CP and R_CP; or F_β actin and R_β actin) (Table 2) and 1.8 µl of ddH_2O. The qPCR program was 95°C for 30 s, followed by 40 PCR cycles (95°C for 5 s, 60°C for 20 s) and followed by a standard melting curve analysis. The relative expression levels were normalized to an endogenous reference gene (β-actin) and relative to the positive control group, and calculated by using the $2^{-\Delta\Delta CT}$ method.

Virus titer assay

$1x\ 10^4$ of GF-1 cells per well were seeded into a 96-well plate and incubated at 28°C overnight. The viral supernatants which collected from shRNAs-transfected cells at 48, 72, 96 and 12 hpi were diluted in Lebovitz L-15 medium supplemented with 1% fetal bovine serum and 1X antibiotic antimycotic solution (Gibco) from 10^{-1} to 10^{-10} and used to infect GF-1 cells. Three independent infections with five replications per dilution were performed. Virus titters were calculated following the standard method (Reed and Muench 1938).

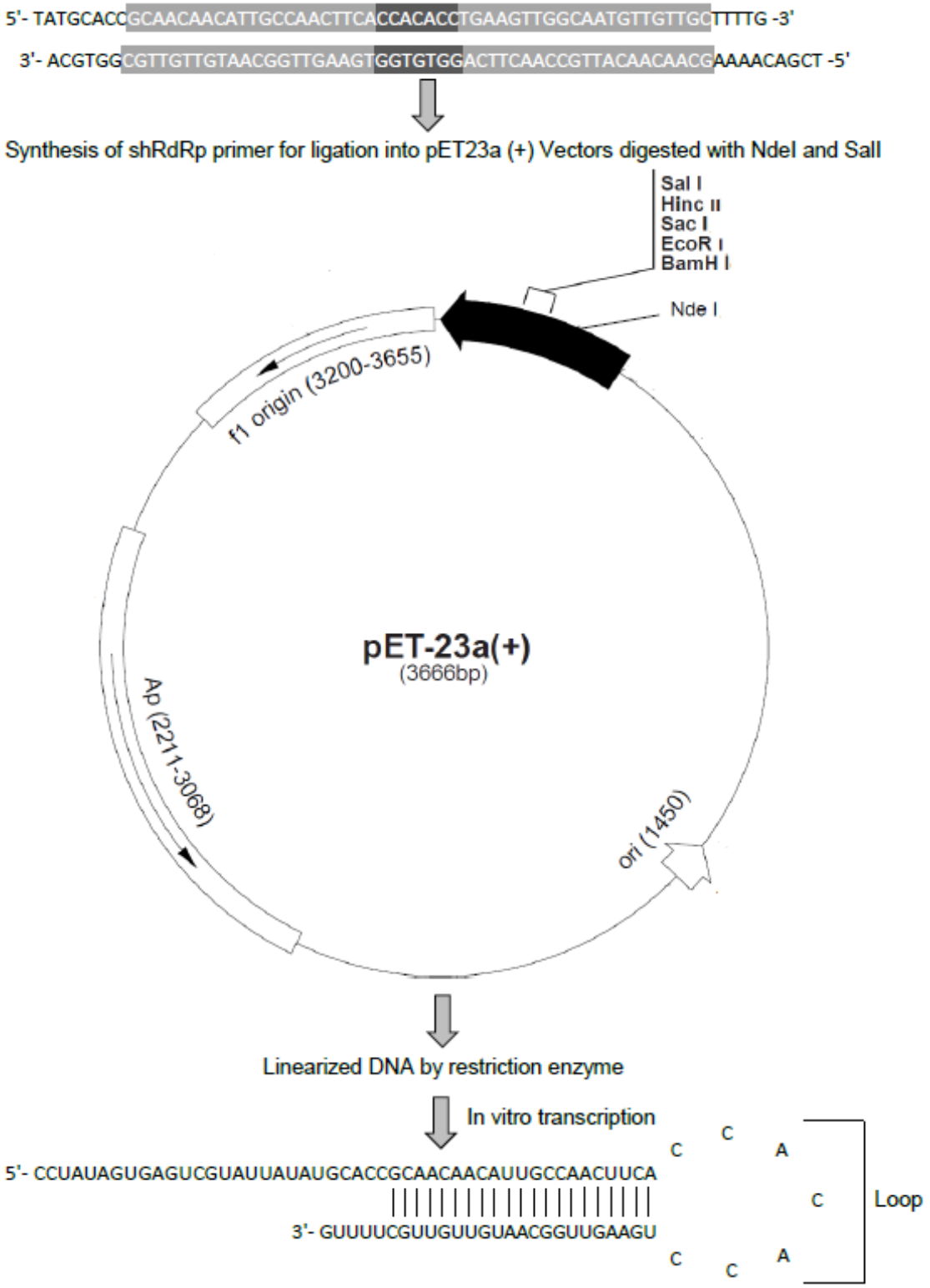

Fig. 1 The schematic map showing the short hairpin RNAs sequences with the added restriction site and their target sequences.

Data analysis

Data provided by CFX Conect™ real-time PCR system (BIO-RAD) were analyzed according to the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen 2001). Using the $2^{-\Delta\Delta CT}$ method, the relative changes in gene expression between a transfected-shRNAs group and OSGNNV control from real-time quantitative PCR experiments, was presented as the fold change (FC = $2^{-\Delta\Delta CT}$) of target genes expression

Table 2 The sequences of primers were used in this study

Name	Sequence (5'-3')		Accession number
RT_RdRp	TCAGCTGGATCAGTGTCG	Reverse transcription	MF144242
RT_CP	GTCGTTGTCAGTTGGATC	Reverse transcription	MF144241
F_RdRp	TGAGTACGGTGCTGAGTTGG	RdRp gene	MF144242
R_RdRp	GATGATAGCTGCCTCCTTGC		
F_CP	CCATCACCGCTTTGCAATCAC	CP gene	MF144241
R_CP	ACGACGTATCCGTCAGTTCC		
F_β actin	CTCTTCCAGCCTTCCTTCCT	β actin gene	AY510710
R_β actin	ACAGGTCCTTACGGATGTCG		

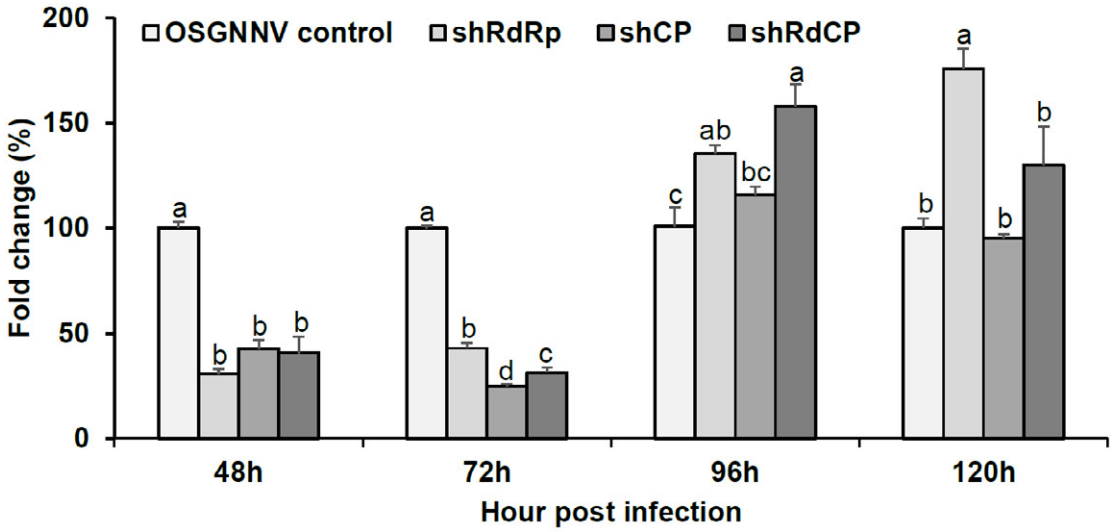

Fig. 2 The expression of RNA-dependent RNA polymerase gene in shRNAs transfected GF-1 cells infected with OSGNNV. GF-1 cells were transfected with shRdRp, shCP, or shRdCP, and then infected with OSGNNV. Total RNA was extracted at 48, 72, 96, and 120 hour post infection and reverse transcribed to cDNA. The expression level of RNA-dependent RNA polymerase gene was determined by qPCR. Non-transfected cells were infected with OSGNNV as a control. The data are presented as means ± SD (n = 3). Bars with different letters significantly differ (P <0.05) among treatments.

(RdRp or CP gene) normalized to an endogenous reference gene (β-actin) and relative to OSGNNV control. The data about viral titers are presented as the means ± standard deviation, and analyzed by SAS computer software version 9.4 (SAS institute Cary, NC, USA).

Results

Inhibition of RNA-dependent RNA polymerase gene expression by shRNAs

The RdRp gene expression levels were reduced by 69.39% ± 2.34, 57.57% ± 4.43 and 59.33% ± 7.83 at 48 hpi in shRdRp-, shCP- and shRdCP-transfected cells, respectively. No significant difference in RdRp gene expression levels was found among shRNAs-tranfection at 48 hpi (Fig. 2). At 72 hpi, the highest reduction of RdRp gene expression was observed in shCP-transfected cells (75.28% ± 1.29), followed by shRdCP-transfected cells (68.37% ± 2.56), whereas the lowest one was found in shRdRp-transfected cells (57.11% ± 2.6). However, RdRp expression levels was not reduced by shRNAs-transfected cells at 96 and 120 hpi (Fig. 2).

Inhibition of capsid protein gene expression by shRNAs

The expression levels of CP gene were reduced by 29.59% ± 1.45, 51.03% ± 1.0, and 57.75% ± 2.12 at 48 hpi in shRdRp-, shCP- and shRdCP-transfected cells, respectively. A significant decrease in CP gene expression was found in shRNAs-transfected cells compared to OSGNNV control at 72 hpi. The highest reduction of CP gene expression was found in shRdCP-transfected cells (74.88% ± 1.0), followed by shCP-transfected cells (63.05% ± 1.84), whereas the lowest one was found in shRdRp-transfected cells

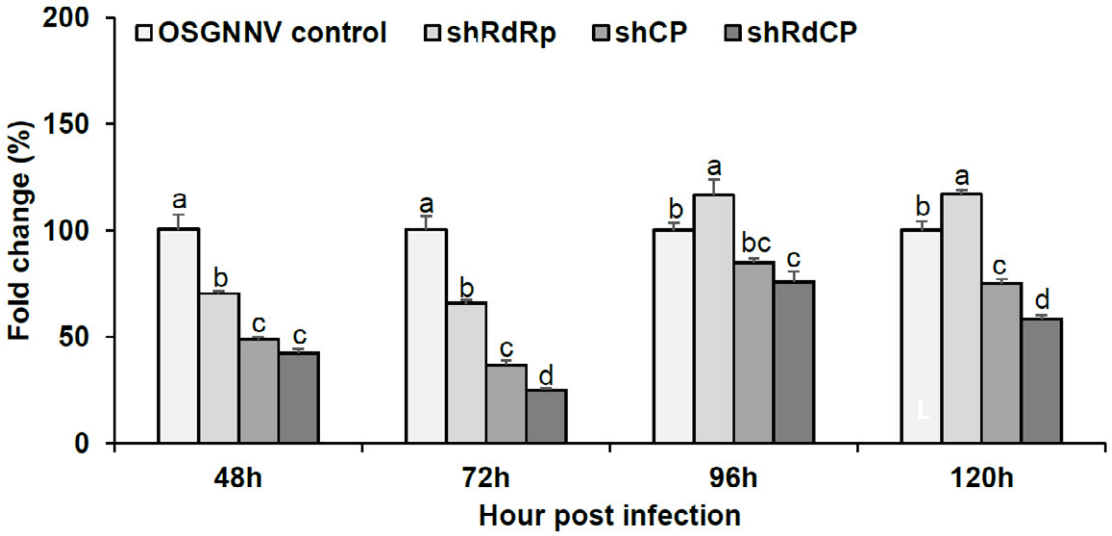

Fig. 3 The expression of capsid protein gene in shRNA-transfected GF-1 cells infected with OSGNNV. GF-1 cells were transfected with shRdRp, shCP, or shRdCP, and then infected with OSGNNV. Total RNA was extracted at 48, 72, 96, and 120 hour post infection and reverse transcribed to cDNA. The expression level of capsid protein gene was determined by qPCR. Non-transfected cells were infected with OSGNNV as a control. The data are presented as means ± SD (n = 3). Bars with different letters significantly differ (P <0.05) among treatments.

(34.14% ± 1.49) at 72 hpi. The CP gene expression levels were slightly increased by shRNAs-tranfected cells at 96 and 120 hpi (Fig. 3).

Inhibition of OSGNNV replication by shRNAs

Viral titer was not found in GF-1 cells uninfected with OSGNNV, whereas the viral titer appeared in OSGNNV-infected cells with or without shRNAs-transfection from 48 to 120 hpi. The average titers of shRdRp-transfected cells were 0.15 ± 0.03 $\log_{10}$TCID50 ml^{-1}, 0.38 ± 0.10 $\log_{10}$TCID50 ml^{-1} and 2.8 ± 0.61 $\log_{10}$TCID50 ml^{-1} at 48, 72, 96 hpi, respectively. These titers were significantly lower than those of OSGNNV control. The average titers of shCP-transfected cells were 0.07 ± 0.01 $\log_{10}$TCID50 ml^{-1}, 0.67 ± 0.33 $\log_{10}$TCID50 ml^{-1} and 2.24 ± 0.14 $\log_{10}$TCID50 ml^{-1} while those of shRdCP-transfected cells were 0.08 ± 0.02 $\log_{10}$TCID50 ml^{-1}, 0.71 ± 0.03 $\log_{10}$TCID50 ml^{-1} and 2.24 ± 0.14 $\log_{10}$TCID50 ml^{-1} at 48, 72, 96 hpi, respectively. These titers were also significantly lower than those of OSGNNV control at 48, 72, 96 hpi. At 120 hpi, the average titer of shRdCP-transfected cells (3.81 ± 0.41 $\log_{10}$TCID50 ml^{-1}) was significantly lower than that of shRdRp-, shCP-transfected cells and OSGNNV control (Fig. 4). These results show that OSGNNV replication was inhibited by shRNA-transfected cells at 48, 72 and 96 hpi.

Discussion

The present study indicated that efficient replication of OSGNNV can be controlled by targeting the RdRp and CP gene using shRNAs at 48 and 72 hpi. The findings desmonstrated that RNAi techonology works efficiently in GF-1 cells. The RNAi can inhibit the expression of key protein by targeting mRNA thus controlling gene expression and acting as a defense system (Chen et al. 2004). Hence, RNAi can be an ideal method in the management of viral fish diseases (Denli et al. 2004; Sui et al. 2009). The RdRp and CP genes in this study as target for shRNAs interference to inhibit gene expression levels and virl replication (Fig. 2-4). It was said that interference with RdRp gene expression correlated with a reduction in viral replication in the host cells (Foual et al. 2019). A previous study demonstrated that targeting of SVCV-N and SVCV-P gene expression by siRNA reduced SVCV replication in EPC cell line (Gotesman et al. 2014). Similarly, siRNA targeting RdRp inhibit viral protein expression in GF-1 cells (Wu et al. 2010). Recently, RdRp gene expression was significantly reduced by siRNA targeting RdRp at 72 hpi (Fouad et al. 2019). Results of this study showed that RdRp gene expression was significantly reduced by shRNAs-transfected cells at 48 and 72 hpi (Fig. 2). Besides, another study reported that major capsid protein (MCP) gene expression was reduced by 13.04%, 55.16% and 97.14% in MCP-targeted siRNA (siR-MCP)-transfected

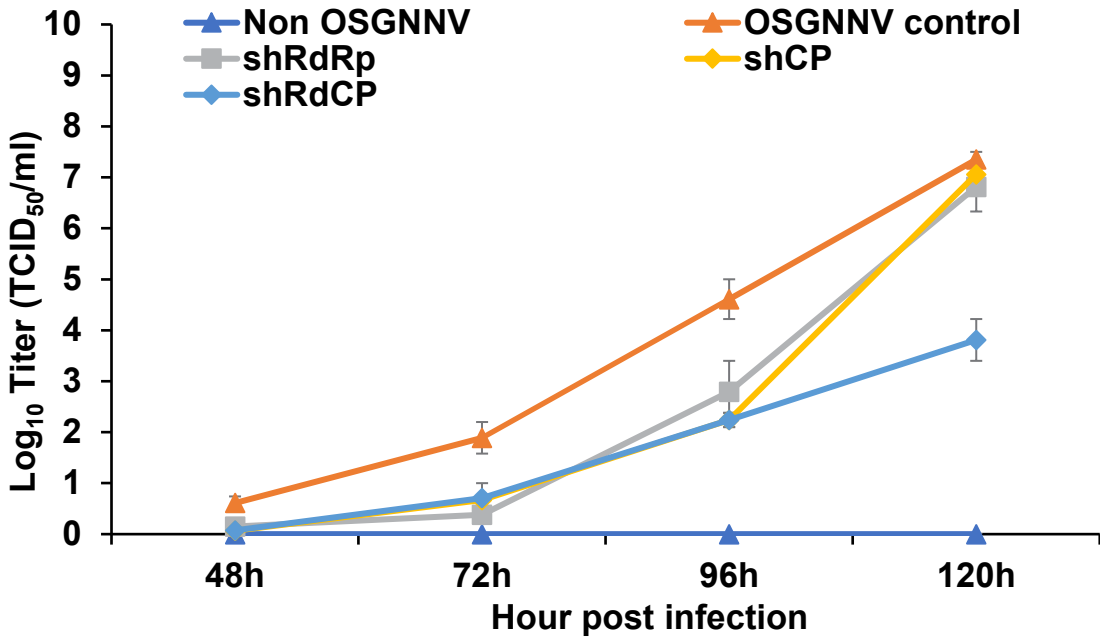

Fig. 4 The virus titer of the culture medium in shRNA-transfected GF-1 cells infected with OSGNNV. GF-1 cells were transfected with shRdRp, shCP, or shRdCP, and then infected with OSGNNV. The culture medium was collected at 48, 72, 96, and 120 hour post infection and assayed for virus titter (Log_{10} $TCID_{50}$ ml^{-1}). Non transfected cells were infected with OSGNNV as positive control and without OSGNNV as negative control. The data are presented as means ± SD (n = 3).

cells at 72, 84 and 96 hpi, respectively (Dang et al. 2008). It was proven that replaced are involved in the assembly of viral particles (Fischer and James 2004) and a reduction in the MCP expression levels correlates with a reduction in production of infectious new particles (Xie et al. 2005). In addition, another study indicated that the expression levels of RdRp and outer capsid protein (OCP) gene were reduced by 89%. and 73% at 120 hpi in pSi-RdRp and pSi-OCP-transfected cells, respectively (Ma et al. 2011). Like RdRp expression, CP gene expression was significantly reduced by shRNAs-tranfected cells at 48 and 72 hpi (Fig. 3). The results of this study showed that the combination of two different target genes also inhibited efficiently expression of RdRp and CP gene (Fig. 2-3). The previous studies have indicated that inhibition of two or more specific siRNAs are more efficiently than only one siRNA (Ji et al. 2003; Ruiz et al. 2009; Fouad et al. 2019). However, the reduction of RdRp and CP gene expression by shRNAs-tranfectd cells at 96 and 120 hpi was less efficient than at 48- 72 hpi (Fig. 2-3). This results may be related to the efficiency of transfection in GF-1 cells. It was reported that microinjection silenced 100% of siRNA-transfected cells while transfection using cationic liposomes silenced 30% of siRNA-transfected cells, indicating that low transfection efficiency can be a major concern for gene inhibition utilizing siRNAs in zebrafish cell lines (Gruber et al. 2005). In this study, lipofectamine 3000™ reagent was used for shRNAs delivery. The efficient transfection of shRNA-transfected GF-1 cells was (10-20%). Therefore a few of GF-1 cells with shRNAs can inhibit OSGNNV while a large number of GF-1 cells without shRNA would infected by OSGNNV and replicated after 96 or 120 hpi, the resulting low efficient inhibition in at 96-120 hpi (Fig. 2-3). Futhermore, the OSGNNV titer in shRNAs-transfected GF-1 cells was significantly lower than that of OSGNNV group at period of 48-96 hpi (Fig. 4). The explaination for this could be that the amount of virus inoculums may also have related to efficient inhibition OSGNNV. Since the virus replicates naturally in infected cells, high dose of virus of this study (M.O.I of 10) may enhance fast production of virus particles which have been accumulated at 120 hpi more than that of 48-96 hpi, resulting less inhibitory effect of OSGNNV replication at 120 hpi (Fig. 4). Besides, RNAi experiment can be affected by serveral parameters, selected target gene, length of fragment, nucleotide sequnce, delivery rout, tranfection reagent, administration frequency, target pathogent which were reviewed by Lima et al. (2013). Therefore, the efficent inhibition and OSVNNV replication of shRNAs-tranfected cells for each target gene RdRp and CP should be different efficency in GF-1 cells (Fig. 2-4).

In conclusion, the present study demonstrated that the shRNA targeting the RdRp or CP effectively suppressed the expression of RdRp or CP gene and OSGNNV replication in GF-1 cells. Indicating that RNAi can be applied as a potential approach to manage aquatic viral disease. However, the antivial machanism of RNAi inhition OSGNNV has not been proven in this study so it is nessary to determine antiviral machanism

of RNAi in GF-1 cells or other fish cells in further study.

Acknowledgements We would like to acknowledge financial support of the Fisheries Agency, Council of Agriculture, Taiwan, ROC (109AS-9.3.4-FA-F1).

Competing interests The authors have no competing interests to declare.

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