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Inhibition of red seabream iridovirus (RSIV) replication by small interfering RNA (siRNA) in a cell culture system

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Abstract

Small interfering RNAs (siRNAs), mediators of a process of sequence-specific gene silencing called RNA interference, have been shown to have activity against a wide range of viruses and are considered to be potential antiviral tools. Here, we describe an antiviral activity of a siRNA that targets the major capsid protein (MCP) gene of red seabream iridovirus (RSIV), a marine fish-pathogenic virus, in a cell culture system. Inhibition of RSIV replication was demonstrated by reduced MCP expression level and reduced RSIV titer. MCP-targeted siRNA (siR-MCP) dose-dependently inhibited the expression of MCP gene in cells that either transiently expressed or stably expressed the MCP gene. At 84 and 96 h after viral infection, siR-MCP reduced the expression of MCP gene by 55.2% and 97.1%, respectively. Transfection with siR-MCP reduced the production of RSIV particles in supernatants of samples infected with RSIV, while the corresponding mismatched siR-MCP (MsiR-MCP) and nsRNA controls did not exhibit this effect. These results show that MCP-targeted siRNA can effectively and specifically inhibit the expression of the target gene and hinder RSIV replication during an *in vitro* infection, providing a potential approach for the control of viral diseases in aquaculture.

Keywords: RNA interference (RNAi); Small interfering RNA (siRNA); Major capsid protein; MCP; Red seabream iridovirus; RSIV

1. Introduction

RNA interference (RNAi), mediated by double-stranded small interfering RNAs (siRNAs), has been shown to have activity against a wide range of viruses and is promising a new antiviral therapy (Andino, 2003; Coburn and Cullen, 2002; O'Brien, 2007). It is a process of sequence-specific gene silencing in the cytoplasm of eukaryotic cell, in which siRNAs of 21–23 nucleotides (nts) are associated with a multiprotein complex known as the RNA-induced silencing complex (RISC) to target homologous mRNA for degradation based on complementary base pairing. siRNAs can be processed in cells from longer double-stranded RNAs produced by viral infection, by transposons or can also be chemically introduced into cells from the outside (Agami, 2002; Carmichael, 2002). Therefore, introduction of 21–23 nts siRNA duplexes specific for viruses into cells could lead to viral mRNA degradation and inhibition of viral gene expression and viral replication. Recent studies have proven that siRNAs can inhibit replication of many kinds of viruses at several stages of infection in various cells (Ferreira et al., 2007; Haasnoot et al., 2003). siRNAs can be employed to suppress the expression of viral genes in plant cells (Yelina et al., 2002), insect cells (Adelman et al., 2002; Caplen et al., 2002), mammalian cells (Capodici et al., 2002; Novina et al., 2002; Surabhi and Gaynor, 2002), as well as aquatic animal cells (Tirasophon et al., 2005; Xie et al., 2005).

Efficient inhibition has been demonstrated for DNA viruses both in cell culture and animal models. These include hepatitis B virus (HBV) (Giladi et al., 2003; Morrissey et al., 2005; Wu et al., 2007), herpes simplex virus 1 (HSV-1) (Bhuyan et al., 2004), herpes simplex virus 2 (HSV-2) (Palliser et al., 2006), human papillomavirus type 18 (HPV-18) (Hall and Alexander, 2003), human cytomegalovirus (HCMV) (Wiebusch et al., 2004), human herpes virus 6B (HHV-6B) (Yoon et al., 2004), JC virus (JCV) (Orba et al., 2004), murine herpesvirus 68 (MHV-68) (Jia and Sun, 2003), anatid herpes virus 1 (AHV-1) (Mallanna et al., 2006), and tiger frog iridovirus (TFV) (Xie et al., 2005). The results of these studies suggest the possibility of using siRNAs as an antiviral tool against DNA viruses.

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Red seabream iridovirus (RSIV), a fish-pathogenic virus, causes a systemic infectious iridoviral disease known as red seabream iridoviral disease (RSIVD) in cultured marine fish in some parts of the world (Do et al., 2004; Inouye et al., 1992; Kawakami and Nakajima, 2002; Wang et al., 2003). Therefore, it is necessary to develop novel therapeutic approaches that effectively inhibit RSIV replication. RSIV is a double-stranded DNA virus with the genome encoding a major capsid protein (MCP) gene and 92 other putative open reading frames (ORFs) (Kurita et al., 2002). The MCP gene accounts for about 45% of the total protein of the virus and is needed for the cleavage and packaging of viral DNA to form viable virions (Williams, 1996). This gene has been selected to analyze the phylogenetic relationships of iridoviruses (Go et al., 2006; Imajoh et al., 2007; Lu et al., 2005) and has been confirmed to be the most suitable gene for detection and measurement of RSIV infection (Caipang et al., 2003; Dang Thi et al., 2007).

In this paper, we report the inhibition of RSIV replication using a siRNA targeting the MCP gene of the virus in a cell culture system. siRNA is a sequence-specific gene silencing mechanism, and inhibits the target gene in a dose-dependent manner (Colbere-Garapin et al., 2005; Elbashir et al., 2001; Huelsmann et al., 2006; Zheng et al., 2005). Therefore, siRNA was initially assessed for inhibitory effects on the MCP gene silencing in cells either transiently or stably expressing the MCP gene by using a plasmid expressing the target gene, and it was then tested for its inhibitory effect on RSIV replication in terms of MCP gene expression during viral infection and in terms of reduction in viral production. Our data provide evidence that siRNA can be used to selectively block viral gene expression and hence viral replication in fish cell lines.

2. Materials and methods

2.1. Cell culture and virus

Grunt Fin (GF) cells (Clem et al., 1961) and Hirame Natural Embryo (HINAE) cells (Kasai and Yoshimizu, 2001) were maintained following Lua et al. (2005). GF cells were used for propagation of virus stock while HINAE cells were used for transfection experiments.

RSIV was obtained from a spleen homogenate of RSIV-infected red seabream, and was propagated in GF cells following Lua et al. (2005). The virus titer was determined using the 50% tissue culture infective dose ($TCID_{50}$) method (Reed and Muench, 1938), and the virus stock was stored in 1 ml aliquots at −80 °C until use.

2.2. Design and synthesis of siRNAs

Three (3) duplex siRNAs were chemically synthesized for use in this study (Table 1). Among them, a siRNA (referred to as siR-MCP) targeting the MCP gene of RSIV was designed using the siRNA target finder programme of Ambion (http://www.ambion.com/sirnatargetfinder). Two other siRNAs (referred to as MsiR-MCP and nsRNA) were designed for use as controls of siRNA sequence specificity. MsiR-MCP was the corresponding mismatched siRNA of siR-MCP and was designed in accordance with previously published rules (Schyth et al., 2006). nsRNA was identical to the sequence of a siRNA (Si1) specific for MCP of TFV (Xie et al., 2005).

2.3. Construction of MCP-expressing plasmid (pCMV-MCP) and selection of stably MCP-expressing HINAE transformant

The full-length of MCP was amplified from the RSIV genome with primers containing EcoRI and XbaI sites (Table 2) by PCR. The PCR products were purified, and cloned into EcoRI and XbaI sites of pCI-neo mammalian expression vector (Promega, USA) (Fig. 1A). The MCP-expressing plasmid (pCMV-MCP) was extracted and purified using a NucleoSpin plasmid quickpure kit (Macherey-Nagel, USA) according to the manufacturer's protocol.

To generate cells stably expressing the MCP gene, HINAE cells were transfected with pCMV-MCP and cultured with selective medium (Leibovitz's L-15 medium supplemented with geneticin) (Gibco-BRL, USA) following the manufacturer's protocol. The presence of a selectable marker, the neomycin phosphotransferase gene, allowed selecting HINAE transformants that harbored pCMV-MCP under selective conditions. Normal HINAE cells were sensitive while stably MCP-expressing HINAE transformants were stable with geneticin. One month post-transfection, the transformants were checked for the expression of the MCP gene (Fig. 1B) and used to assess the inhibitory effect of siR-MCP on the MCP gene in the case of stable expression.

2.4. Transfection of plasmid DNA and siRNA

HINAE cells were seeded into 24-well or 96-well cell culture plates for about 24 h (90–95% confluent monolayer) using L-15 medium containing 15% of fetal bovine serum (FBS) (JRH Biosciences, USA) without phenol red or antibiotics prior to transfection. Cells were transfected following the manufac-

Table 1
siRNA sequences (sense strand) used in this study

siRNA name	Target sequence	Position in gene sequence	Source
siR-MCP	5′-AACAGACUGGCCAUGCUAAUU-3′	164–182	RSIV
MsiR-MCP[a]	5′-A**G**CAGACUG**A**C**U**A**C**GCUA**G**UU-3′		
nsRNA[b]	5′-CGCCUGGUUGGUACUCAAGUU-3′	237–255	TFV

[a] The corresponding mismatched siRNA of siR-MCP. Mismatched nucleotides are bold and underlined.
[b] The identical sequence of siRNA (Si1) targeting MCP of tiger frog iridovirus (TFV) (Xie et al., 2005).

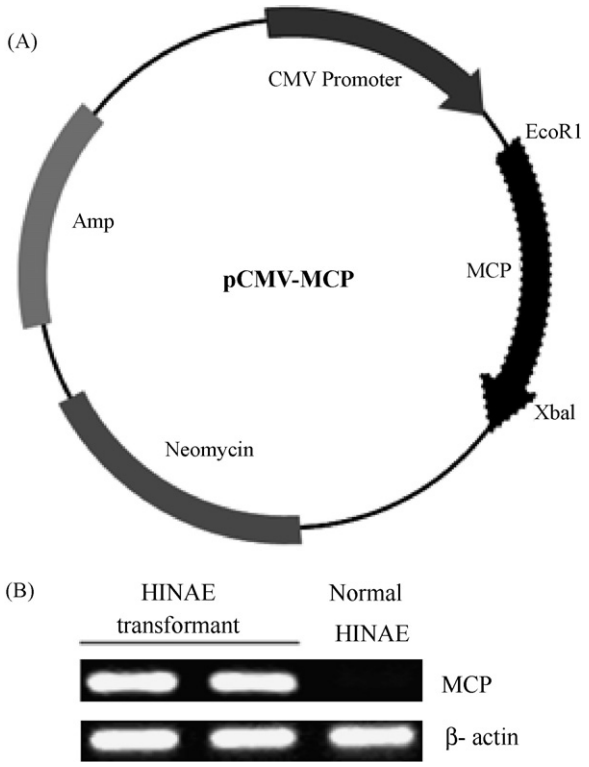

Fig. 1. Expression of MCP gene in HINAE transformants. (A) Construction of pCMV-MCP. The full-length of the MCP gene was amplified from the RSIV genome by using specific primers containing EcoRI and XbaI enzyme sequences and then cloned into pCI-neo expression vector. (B) Expression of MCP gene in HINAE transformants determined by RT-PCR. pCMV-MCP was transfected into HINAE cells and transfected cells were cultured under selective medium to select HINAE transformants. About 1 month post-transfection, total RNA was extracted from the transformants and reverse transcribed to cDNA for RT-PCR.

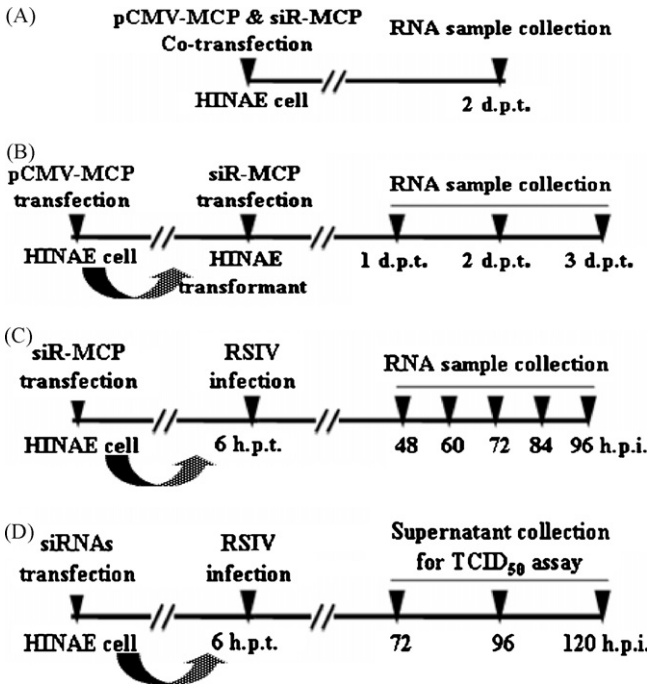

Fig. 2. Experimental scheme of siRNA-mediated RNAi for anti-RSIV. (A) Transient expression of MCP gene by using MCP-expressing plasmid; (B) stable expression of MCP gene by using MCP-expressing plasmid; (C) expression of MCP gene under siRNA transfection and RSIV infection; and (D) sampling for $TCID_{50}$ assay. (d.p.t.) days post-transfection; (h.p.t.) hours post-transfection; and (h.p.i.) hours post-infection.

turer's protocol with lipofectamine 2000 (Invitrogen, USA). Opti-MEM I Reduced Serum Medium (Invitrogen, USA) was used to dilute lipofectamine 2000, plasmid DNA and siRNAs.

2.5. Determination of dose-dependent inhibitory effect of siR-MCP on MCP gene expression by using MCP-expressing plasmid (pCMV-MCP)

In the case of transient expression (Fig. 2A), normal HINAE cells were co-transfected with pCMV-MCP and siR-MCP at final concentrations of 30, 60 and 120 nM. Cells transfected with only pCMV-MCP were used as a positive control while un-transfected cells were used as a negative control. Six hours after transfection, the transfection complexes were replaced by fresh L-15 medium containing 15% FBS without phenol red or antibiotics. At 2 days post-transfection (d.p.t.), total RNA was extracted from both transfected and un-transfected cells with TRIzol (Invitrogen, USA) according to the manufacturer's protocol for reverse-transcription (RT)-PCR and real-time PCR analysis.

In the case of stable expression (Fig. 2B), HINAE transformants that stably expressed the MCP gene were transfected with siR-MCP at final concentrations of 30, 60 and 120 nM and total RNA was extracted from both siR-MCP-transfected and

Table 2
Primers used in this study

Primer name	Primer sequence	PCR product size (bp)
pCMV-MCP-F	5′-AT**GAATTC**ATGTCTGCGATCTCAGGTGC-3′	
pCMV-MCP-R	-5′-GC**TCTAGA**TTACAGGATAGGGAAGCCTG-3′	Full-length
MCP (L)—F	5′-CCCTATCAAAACAGACTGGC-3′	429
MCP (L)—R	5′-TCATTGTACGGCAGAGACAC-3′	
MCP (S)—F	5′-CTGCGTGTTAAGATCCCCTCCA-3′	100
MCP (S)—R	5′-GACACCGACACCTCCTCAACTA-3′	
β-actin (L)—F	5′-TTTCCCTCCATTGTTGGTCG-3′	200
β-actin (L)—R	5′-GCGACTCTCAGCTCGTTGTA-3′	
β-actin (S)–F	5′-TGATGAAGCCCAGAGCAAGA-3′	100
β-actin (S)—R	5′-CTCCATGTCATCCCAGTTGGT-3′	

Restriction enzyme sequences are bold and underlined.

un-transfected HINAE transformants with TRIzol (Invitrogen, USA) at 1, 2 and 3 d.p.t. for RT-PCR and real-time PCR analysis.

2.6. Assessment of inhibitory effect of siR-MCP on RSIV replication in terms of MCP gene expression

HINAE cells were transfected with siR-MCP (120 nM) or un-transfected for 6 h prior to infection with RSIV at a multiplicity of infection (m.o.i) of 3 (Fig. 2C). After allowing 90 min for absorption, unattached viruses were removed and infected cells were continuously cultured with L-15 medium supplemented with 15% FBS, 100 IU/ml of penicillin and 100 μg/ml of streptomycin. At intervals up to 96 h post-infection (h.p.i.), total RNA was isolated from both siR-MCP-transfected and un-transfected (control) samples with TRIzol (Invitrogen, USA) and subjected to DNase I treatment (Promega, USA) according to the manufacturer's protocol for RT-PCR and real-time PCR analysis.

2.7. Assessment of reduction in viral production by siR-MCP

HINAE cells transfected with siR-MCP (120 nM) and un-transfected cells were incubated for 6 h prior to infection with RSIV at a m.o.i. of 5 (Fig. 2D). After allowing 90 min for absorption, unattached viruses were removed and infected cells were continuously cultured with L-15 medium supplemented with 15% FBS, 100 IU/ml of penicillin and 100 μg/ml of streptomycin. Viral supernatants were collected from transfected and un-transfected (control) samples at 72, 96, and 120 h.p.i. The supernatants were diluted from 10^{-1} to 10^{-10} and used to infect HINAE cells with eight repetitions per dilution to perform the $TCID_{50}$ assay. Virus titers were calculated following the standard method (Reed and Muench, 1938).

2.8. Assessment of target-specificity of siR-MCP

To examine the specificity of siR-MCP, HINAE cells were transfected with either MsiR-MCP control or nsRNA control for 6 h prior to infection with RSIV. Supernatants were collected at 72, 96 and 120 h.p.i. and assayed for virus titers by using the $TCID_{50}$ assay in the same way as for the target siR-MCP (Section 2.7).

2.9. Reverse-transcription (RT)-PCR

Twenty microlitres (20 μl) of cDNA was synthesized from 5 μg of the purified total RNA using M-MLV reverse transcriptase (Invitrogen, USA) according to the manufacturer's protocol. One microliter (1 μl) of cDNA was used for RT-PCR in a volume of 30 μl. Cycling parameters consisted of an initial denaturation at 95 °C for 2 min, followed by 23–27 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 1 min, and a final elongation step at 72 °C for 5 min. The primers used for RT-PCR are shown in Table 2.

2.10. Quantitative real-time PCR

Standard curves for quantification in real-time PCR were prepared following Caipang et al. (2003) using purified PCR products of a 429-bp fragment of the target gene (MCP gene) and a 200-bp fragment of an endogenous reference (β-actin gene) as standard templates. Approximately, 100-bp fragments of the MCP and β-actin genes in unknown samples were amplified by nested primers (Table 2). The calculation of threshold cycle (C_T) and determination of copy number of the target gene were performed using the software of the 7300 real-time PCR system (Applied Biosystems).

For the real-time PCR assay, each cDNA was synthesized in a 20 μl reaction mixture containing 5 μg of total RNA as described in the RT-PCR section, and was then brought up to a final volume of 200 μl with sterilized water. Three microlitres of diluted cDNA was amplified in a 20 μl real-time PCR reaction volume containing 10 μl of SYBR green PCR master mix (Applied Biosystems) and primers at final concentrations of 0.5 μM. The real-time PCR reaction was performed in 7300 real-time PCR system (Applied Biosystems) according to the manufacturer's protocol. Following a denaturation step at 95 °C for 10 min, 40 cycles of amplification were performed at 95 °C for 15 s and 60 °C for 1 min. A dissociation stage was also added to detect non-specific products and to optimize primer concentrations. Each sample was run in quadruplicate.

2.11. Data analysis

Data provided by the 7300 real-time PCR system were normalized according to the comparative threshold cycle ($2^{-\Delta\Delta C_T}$) method (Livak and Schmittgen, 2001). Using the $2^{-\Delta\Delta C_T}$ method, a convenient and accurate method to analyze relative changes in gene expression between a treatment group and an untreated control from real-time, quantitative PCR experiments, the data were presented as the Fold Change ($FC = 2^{-\Delta\Delta C_T}$) in target gene expression (MCP gene) normalized to an endogenous reference gene (β-actin gene) and relative to the positive control. Finally, inhibition percentages were calculated as follows:

$$\text{Inhibition}\,(\%) = \left\{1 - \left(\frac{2^{-\Delta\Delta C_T}\ \text{of tested sample}}{2^{-\Delta\Delta C_T}\ \text{of control sample}}\right)\right\} \times 100$$

All statistical analyses comparing samples un-transfected and transfected with siRNAs were performed by a paired *t*-test.

3. Results and discussion

3.1. Dose-dependent inhibitory effect of siR-MCP on MCP gene expression in HINAE cells transiently expressing the MCP gene

Two days after co-transfection of HINAE cells with pCMV-MCP and different concentrations of siR-MCP, MCP gene expression was effectively reduced by siR-MCP at a final concentration of 120 nM as determined by RT-PCR (Fig. 3A). The real-time PCR data analyzed by the $2^{-\Delta\Delta C_T}$ method indicated that expression of the MCP gene were reduced by 3.85%

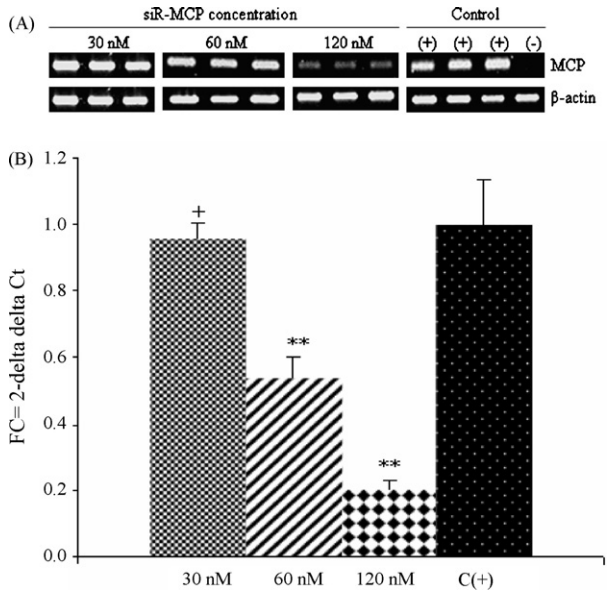

Fig. 3. Dose-dependent inhibitory effect of siR-MCP on the MCP gene in the case of transient expression. (A) RT-PCR analysis of MCP gene expression. HINAE cells were un-transfected or co-transfected with pCMV-MCP and siR-MCP at different concentrations. Total RNA was extracted at 2 d.p.t. for RT-PCR. (+) pCMV-MCP transfection, and (−) un-transfected samples. (B) Real-time PCR and $2^{-\Delta\Delta C_T}$ analysis of relative expression level of the MCP gene. cDNAs were synthesized from 2 d.p.t. RNAs in the same way as for RT-PCR, but were brought up to a final volume of 200 μl with sterile water. Mean values (bars) of three independent experiments plus standard deviation (S.D.) are shown. Fold change (FC = $2^{-\Delta\Delta C_T}$); (30, 60, and 120 nM) siR-MCP concentration; C(+) positive control; (+) $p > 0.05$; (**) $p < 0.01$ when compared to the control.

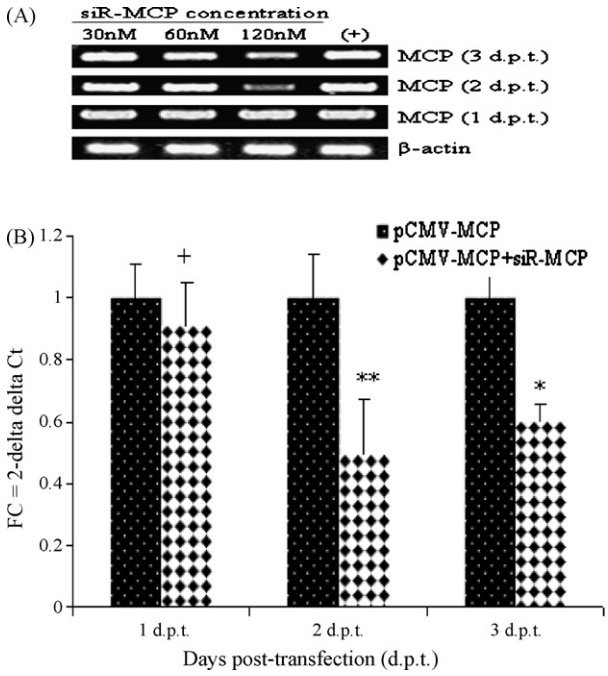

Fig. 4. Dose-dependent inhibitory effect of siR-MCP on the MCP gene in the case of stable expression. (A) RT-PCR analysis of MCP gene expression. HINAE transformants that stably expressed MCP gene were not transfected or transfected with siR-MCP at final concentrations of 30, 60 and 120 nM. Total RNA was extracted at 1, 2 and 3 d.p.t. and reverse transcribed to cDNA for RT-PCR. (+) un-transfected transformants. (B) Real-time PCR and $2^{-\Delta\Delta C_T}$ analysis of relative expression level of MCP gene. cDNAs were synthesized from total RNAs in the same way as for RT-PCR, but were brought up to a final volume of 200 μl with sterilized water. Mean values (bars) of three independent experiments plus S.D. are shown. Fold change (FC = $2^{-\Delta\Delta C_T}$); (+) $p > 0.05$; (*) $p \leq 0.05$; (**) $p < 0.01$ when compared to the control.

(FC = 0.9615 ± 0.044, $p > 0.05$), 45.95% (FC = 0.5415 ± 0.06, $p < 0.01$) and 79.55% (FC = 0.2045 ± 0.028, $p < 0.01$) in cells transfected with siR-MCP at 30, 60 and 120 nM, respectively (Fig. 3B). Thus, siR-MCP is dose-dependently inhibits MCP gene expression and is highly effective at a final concentration of 120 nM.

3.2. Dose-dependent inhibitory effect of siR-MCP on MCP gene expression in HINAE cells stably expressing the MCP gene

siRNAs were also documented silencing of stably expressed target genes (Cao et al., 2004; Takada et al., 2005). Accordingly, we tested the inhibition ability of siR-MCP in HINAE transformants that stably express MCP gene. As shown by RT-PCR, MCP gene expression was not different between un-transfected HINAE transformants and siR-MCP-transfected HINAE transformants at day 1 after transfection at any of the siR-MCP concentrations tested. However, a decline in the silencing of MCP gene was observed at days 2 and 3 after transfection with 120 nM siR-MCP (Fig. 4A). At 120 nM siR-MCP, as determined by real-time PCR and the $2^{-\Delta\Delta C_T}$ analysis, the decrease after 1 day was only 8.84% (FC = 0.9116 ± 0.14, $p > 0.05$), but after 2 and 3 days, the decreases were significant (50.74% (FC = 0.4926 ± 0.182, $p < 0.01$) and 39.69% (FC = 0.6031 ± 0.057, $p < 0.05$), respectively) (Fig. 4B). These results confirm that siR-MCP dose-dependently suppresses the expression of MCP gene and effectively suppresses expression at a final concentration of 120 nM. As a result, siR-MCP was used at a concentration of 120 nM in the following experiments.

3.3. Inhibition of RSIV replication by siR-MCP

The inhibitory effect of siR-MCP (120 nM) was assessed on RSIV replication in terms of MCP gene expression in HINAE cells over the time-course of viral infection. siR-MCP effectively reduced RSIV replication with time, as shown by RT-PCR (Fig. 5A). The real-time PCR data analyzed by the $2^{-\Delta\Delta C_T}$ method indicated that the suppression was reduced by 13.04% (FC = 0.8696 ± 0.161, $p > 0.05$), 55.16% (FC = 0.4484 ± 0.113, $p < 0.01$) and 97.14% (FC = 0.0286 ± 0.142, $p < 0.001$) at 72, 84 and 96 h.p.i. in siR-MCP-transfected samples, respectively (Fig. 5B). These results show that siR-MCP inhibits RSIV replication by silencing the expression of the MCP gene.

3.4. Reduction in production of RSIV particles by siR-MCP

Major capsid proteins are involved in the assembly of viral particles (Tan and Yin, 2004; Williams, 1996), and a reduction in the MCP expression levels correlates with a reduction in production of infectious new particles (Radhakrishnan et al., 2004; Xie et al., 2005). Therefore, siR-MCP-transfected cells were

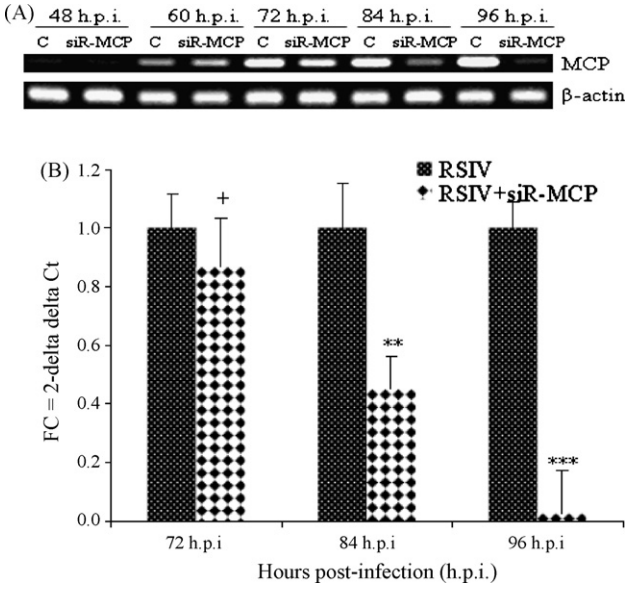

Fig. 5. Suppression of RSIV replication in terms of MCP gene expression by siR-MCP (120 nM). (A) RT-PCR analysis of MCP gene expression. HINAE cells were not transfected or transfected with 120 nM siR-MCP for 6 h prior to infection with RSIV (m.o.i. of 3). Total RNA was extracted at 48, 60, 72, 84 and 96 h.p.i and reverse transcribed to cDNA for RT-PCR. (C) RSIV infection; (siR-MCP) siR-MCP transfection and RSIV infection. (B) Real-time PCR and $2^{-\Delta\Delta C_T}$ analysis of relative expression level of MCP gene. cDNAs were synthesized from time-course RNA samples in the same as for RT-PCR, but were brought up to a final volume of 200 μl with sterilized water. Mean values (bars) of three independent experiments plus S.D. are shown. Fold change ($FC = 2^{-\Delta\Delta C_T}$); (+) $p > 0.05$; (**) $p < 0.01$; (***) $p < 0.001$ when compared to the control.

infected with RSIV and cell supernatants were collected at 72, 96 and 120 h.p.i. to determine the production of viral particles.

In $TCID_{50}$ assays, the average titers of culture medium of siR-MCP-transfected samples were about 23.7-, 86.6- and 48.7-fold lower than those of the un-transfected controls at 72, 96 and 120 h.p.i., respectively (Fig. 6). siR-MCP treatment clearly

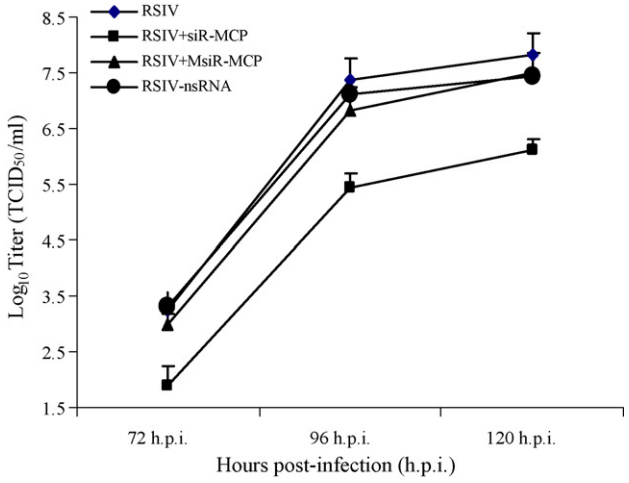

Fig. 6. Reduction in viral production by siRNAs (120 nM) at different time-intervals after RSIV infection (m.o.i. of 5). siRNA-transfected cells were infected with RSIV at 6 h.p.t. Supernatants were collected at 72, 96 and 120 h.p.i. and assayed for virus titration. The data show the average titers of two independent experiments at three time intervals in $\log_{10}TCID_{50}$ plus S.D.

showed a dramatic effect on MCP mRNA (Fig. 5A and B) but not very clearly on the production of virus particles (Fig. 6). This result may be influenced by differences in amounts of virus inoculums. The siR-MCP-transfected cells were infected with RSIV at m.o.i of 3 in case of assessment of viral replication, whereas the transfected cells were infected with the virus at m.o.i of 5 in case of assessment of production of virus particles. Because viruses replicate naturally in infected cells, therefore, high doses of virus may enhance fast production of virus particles, interfere in the inhibitory effect of the siR-MCP, resulting in difficulty in the detection of changes under the siRNA treatment. However, this hypothesis needs to be confirmed further. Taken together, although a dramatic effect on the production of virus particles under the siRNA treatment could not be seen, a reduction in virus particles was determined in siR-MCP-transfected samples when compared to un-transfected samples (Fig. 6), clearly indicating that siR-MCP has anti-RSIV activity.

As also shown in Fig. 6, titers in control samples transfected with MsiR-MCP were decreased only about 1.8-, 3.7- and 2.1-fold at 72, 96 and 120 h.p.i., respectively and titers in control samples transfected with nsRNA were similar, and decreased 1.8- and 2.4-fold, respectively. These results indicate that siR-MCP specifically targets RSIV.

Several kinds of controls, such as non-specific siRNAs and mismatched or scrambled siRNAs, have been used to measure the target specificity in antiviral siRNAs. Non-specific siRNA controls have been used against housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Kapadia et al., 2003) and reporter genes such as green fluorescent protein (GFP). However, these controls do not fully demonstrate the specificity of the siRNAs used in these studies because their sequences are so different from those of the siRNAs (Schyth et al., 2006). Therefore, mismatched or scrambled siRNA controls that differ from the active siRNA by about 1–4 nucleotides have frequently been used (Kapadia et al., 2003; Schyth et al., 2006; Xie et al., 2005). The controls used in the present study were MsiR-MCP, a mismatched siRNA of siR-MCP and nsRNA, an identical sequence of Si1 specific for MCP of TFV (Xie et al., 2005), an iridovirus belonging to different genus from that of RSIV. Neither control showed any complementarity to any regions of the whole RSIV genome sequence. Our finding of no differences in virus titers between siRNA control-transfected and un-transfected samples indicates that the siRNA controls have no antiviral activity. These results show that siR-MCP participates in sequence-specific gene silencing of siR-MCP and confirm that it has antiviral activity.

Taken together, our data demonstrate that siR-MCP efficiently and specifically inhibited RSIV replication in terms of MCP gene expression and hindered viral production in a cell culture system. These results suggest that siRNA methodology can be used to induce gene silencing in fish cell lines, such as HINAE cells. The success of siRNA *in vitro* has led to growing interest in *in vivo* applications of siRNA, leading to a revolution in the control of aquatic viruses. Delivering siRNA *in vivo* to fish/animal tissues is complicated and challenging and involves using physical, chemical or biological approaches (Xie et al., 2006). Because the main goal of *in*

vivo delivery is to have active and stable siRNAs in the target cells, efficient delivery system of siRNA molecules is the most challenging issue. Although delivery strategies for siRNAs toward treatment of aquatic viruses have not been reported yet, siRNAs have been successfully delivered to animal models in organ systems such as liver, spleen, kidney, lung and pancreas, and even in central nervous system (Kumar et al., 2007; Luo et al., 2005). Various delivery methods have been used, including hydrodynamic delivery (Behlke, 2006), viral vector-mediated delivery (Barton and Medzhitov, 2002; Morris and Rossi, 2006; Tiscornia et al., 2003), and lipid, antibodies, ligands and peptides-based delivery (Behlke, 2006; Leung and Whittaker, 2005; Takeshita et al., 2005). Thus, although further studies for *in vivo* siRNA delivery are needed, we have shown here, for the first time, the potential use of siRNAs as an antiviral tool against marine fish-pathogenic iridoviruses in *in vitro*. On the other hand, siRNAs will enable new experimental approaches to analyzing both viral and cellular gene functions in iridovirus-infected cells. Such studies could provide basic information for control of infectious viral diseases in aquatic systems.

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