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Engineered virus-encoded pre-microRNA (pre-miRNA) induces sequence-specific antiviral response in addition to nonspecific immunity in a fish cell line: Convergence of RNAi-related pathways and IFN-related pathways in antiviral response

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ABSTRACT

Transfection with synthesized virus-specific small interfering RNAs (siRNAs) efficiently inhibits viral replication in viral-infected fish cell lines, implying the involvement of RNA interference (RNAi)-related pathways in the antiviral response of fish cells. Here, we demonstrate that plasmid expressing virus-encoded pre-microRNAs (pre-miRNAs) can also inhibit viral replication through these pathways. By incorporating sequences encoding miRNAs specific to major capsid protein (MCP) gene of red sea bream iridovirus (RSIV) and a miRNA specific to hirame rhabdovirus (HIRRV) genome into a murine miR-155 pre-miRNA backbone, we were able to intracellularly express viral pre-miRNAs (miR-MCPs and miR-HIRRV) in a fish cell line. The miR-MCPs and miR-HIRRV, delivered as pre-miRNA precursors in transfected cells, inhibited viral replication when these cells were infected with the target virus. Although this may suggest sequence-specific interference, inhibitory effect on viral replication was also observed in cells transfected with a plasmid expressing pre-miRNA targeting β-galactosidase gene (miR-LacZ) that served as a specificity control. Expression of pre-miRNAs was found to activate interferon (IFN)-related pathways, correlating with upregulation of the antiviral IFN-induced Mx protein. The antiviral effects of viral-miRNAs observed here were partly the result of the antiviral miRNA-related pathways and partly the result of the antiviral IFN-related pathways. We propose that engineered virus-encoded pre-miRNA can engage not only RNAi-related pathways but also IFN-related pathways to induce potent antiviral responses in fish cells.

1. Introduction

Small RNA molecules engage in sequence-specific interactions that inhibit gene expression by RNA silencing. This process fulfils fundamental regulatory roles as well as antiviral functions, through the actions of small interfering RNAs (siRNAs) and microRNAs (miRNAs) involved in RNA interference (RNAi) pathways. The small RNA molecules are incorporated into an RNA-induced silencing complex (RISC) and serve as guides for silencing their corresponding target mRNAs based on complementary base-pairing (Yeung et al., 2005). siRNAs, which are derived by processing of long double-stranded RNAs are often of exogenous origin, degrade mRNAs bearing fully complementary sequences, and are currently being extensively evaluated as potential antiviral tools. In contrast, miRNAs, which are endogenously encoded and derived by processing of long hairpin RNA precursors, can either cleave mRNAs bearing fully complementary sequences or inhibit translation of mRNAs bearing partially complementary sequences (Kusenda et al., 2006; Zeng et al., 2003). A single miRNA can target numerous mRNAs, often in combination with other miRNAs, thus miRNAs operate highly complex regulatory networks (Kim and Nam, 2006; Nair and Zavolan, 2006). It is believed that miRNAs are essential regulators of various processes, such as cellular differentiation, proliferation, development, cell death and pathogen–host interaction (Ambros, 2004; Miska, 2005; Nair and Zavolan, 2006). However, recent reviews on the role of miRNAs concluded that miRNA machinery can also be exploited for defense against viruses (Browne et al., 2005; Kloosterman and Plasterk, 2006).

Although miRNAs can function as siRNAs (Doench et al., 2003; Zeng et al., 2003), virus-encoded siRNAs have been studied for use in antiviral strategies prior to virus-encoded miRNAs. The antiviral potential of viral-gene specific siRNAs has been comprehensively

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discussed in numerous reviews (Dave and Pomerantz, 2003; Pushparaj and Melendez, 2006; Sanchez-Vargas et al., 2004; Stram and Kuzntzova, 2006; Tan and Yin, 2004). While the antiviral potential of viral-gene specific miRNAs has been reported for several viruses, including human immunodeficiency virus type 1 (HIV-1) (Boden et al., 2003; Omoto et al., 2004), simian virus 40 (SV 40) (Sullivan et al., 2005), hepatitis C virus (HCV) (Zhang et al., 2005), and primate foamy virus type 1 (PFV-1) (Lecellier et al., 2005). These studies have involved the introduction of plasmid-based expression systems capable of producing endogenous hairpin miRNA precursors targeting viral-specific genes into cells.

The use of plasmid-based expression systems is an easy and inexpensive way to generate miRNAs. However, one major drawback is that the expression of long RNA in some cases has been shown to trigger sequence-nonspecific interferon responses in the cells, thereby leading to a global inhibition of mRNA translation and limiting how useful they are (Dykxhoorn et al., 2003; Samuel, 2001). Due to its antiviral nature, a cellular interferon response should be given special concern in studies of miRNAs targeting viruses, where reduced viral replication in RNAi-transfected cells is often taken as indicative of successful specific interference (Bhuyan et al., 2004; Kapadia et al., 2003).

We recently synthesized a siRNA specific to the major capsid protein (MCP) gene of red sea bream iridovirus (RSIV) and introduced it into HINAE cells (Dang et al., 2008). The results demonstrated that RNAi-related pathways are involved in antiviral defenses and could be evoked by introduction of small RNA molecules into fish cell lines. Herein, we describe another potential approach for delivering small RNAs, using an expression system of pre-microRNAs (pre-miRNAs), and investigate whether engineered viral-encoded miRNAs can exert antiviral activities through antiviral miRNA-related pathways in a cell culture system. Two marine fish-pathogen viruses, including RSIV and HIRRV (hirame rhabdovirus), were used as models in our miRNA studies. Our findings proposed that engineered virus-encoded pre-miRNAs not only trigger the antiviral miRNA-related pathways but also activate the antiviral IFN-related pathways to mount immunity to a viral pathogen in fish cells.

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 the propagation of RSIV stock, while HINAE cells were used for propagation of HIRRV stock, as well as for plasmid transfection and virus infection experiments. The virus titers was determined using the 50% tissue culture infective dose ($TCID_{50}$) method (Reed and Muench, 1938).

2.2. Construction of plasmid expressing virus-encoded pre-miRNA (pcDNA-miR) and plasmid expressing the MCP gene (pCMV-MCP)

Three pairs of oligonucleotides encoding MCP-specific miRNAs of RSIV (referred as miR-MCP-1, miR-MCP-2 and miR-MCP-3), and a pair of oligonucleotides corresponding to HIRRV genome (referred as miR-HIRRV) (Table 1) were designed using the RNAi Designer (www.invitrogen.com/rnai). Each oligonucleotide pair ("top strand" and "bottom strand" oligos) was annealed and ligated into the pcDNA 6.2-GW/EmGFP-miR vector (Block-iT™ Plo II miR RNAi Expression Vector Kits, Invitrogen, U.S.A.) to create plasmids (pcDNA-miR-MCPs and pcDNA-miR-HIRRV) capable of producing virally encoded pre-miRNAs in plasmid-transfected cells. The ligation mixture was then transformed into competent *E. coli*, One Shot TOP10, cells following the manufacturer's protocol. A control expression plasmid (pcDNA-miR-LacZ) that expresses pre-miRNA targeting the β-galactosidase gene (miR-LacZ) was also generated using miR-LacZ-positive ds oligos supplied by the kit.

The MCP-expressing plasmid (pCMV-MCP) constructed in our previous studies (Dang et al., 2008) was used in co-transfection experiments to express the target MCP gene.

Plasmid DNAs were extracted from positive colonies by standard alkaline lysis (Sambrook and Russell, 2001). All constructs were verified by DNA sequencing using ABI Prism® BigDye® Terminator kit on Applied Biosystems 3130 Genetic Analyzer (www.appliedbiosystems.co.jp).

2.3. Transfection of fish cells with plasmid DNA

HINAE cells were seeded into 24-well or 96-well cell culture plates using L-15 medium containing 15% of FBS without antibiotics for about 24 h before transfection at a cell confluence of approximately 85–90%. Cells were transfected with plasmid DNA using Lipofectamine™ 2000 and Opti-MEM I Reduced Serum Medium (Invitrogen, U.S.A.) following the manufacturer's protocol. The transfection mixtures were removed at 6-h post-transfection, and transfected cells were maintained for further processing.

2.4. Anti-RSIV activity of miR-MCPs

miR-MCPs were initially tested for sequence-specific silencing on the target MCP gene by employing transient transfection of a plasmid expressing MCP gene (pCMV-MCP). HINAE cells were co-transfected with pcDNA-miRs and pCMV-MCP (Fig. 1A). Cells transfected with only pCMV-MCP were used as a positive control

Table 1
Oligonucleotide sequences encoding viral pre-miRNAs

Name	Strand	Oligo sequence	Position in genome
miR-MCP-1	Top	TGCTGTAAAGTAGTCTACTCCCATCTGTTTTGGCCACTGACTGACAGATGGGAAGACTACTTTA	68957–68977[a]
	Bottom	CCTGTAAAGTAGTCTTCCCATCTGTCAGTCAGTGGCCAAAAC**AGATGGGAGTAGACTACTTTAC**	
miR-MCP-2	Top	TGCTGAATTAGCATGGCCAGTCTGTTGTTTTGGCCACTGACTGACAACAGACTCCATGCTAATT	68075–68095[a]
	Bottom	CCTGAATTAGCATGGAGTCTGTTGTCAGTCAGTGGCCAAAAC**AACAGACTGGCCATGCTAATTC**	
miR-MCP-3	Top	TGCTGATTACAGTACGGCACACACAAGTTTTGGCCACTGACTGACTTGTGTGTCGTACTGTAAT	69378–69398[a]
	Bottom	CCTGATTACAGTACGACACACAAGTCAGTCAGTGGCCAAAAC**TTGTGTGTGCCGTACTGTAATC**	
miR-HIRRV	Top	TGCTGTCTCTTTGGAGACTTTCTCGTGTTTTGGCCACTGACTGACACGAGAAACTCCAAAGAGA	1821–1841[b]
	Bottom	CCTGTCTCTTTGGAGTTTCTCGTGTCAGTCAGTGGCCAAAAC**ACGAGAAAGTCTCCAAAGAGAC**	

Bold and underlined letters represent sense sequences of engineered miRNAs derived from the target gene.

[a] Sequence position in RSIV genome.

[b] Sequence position in HIRRV genome (NC005093).

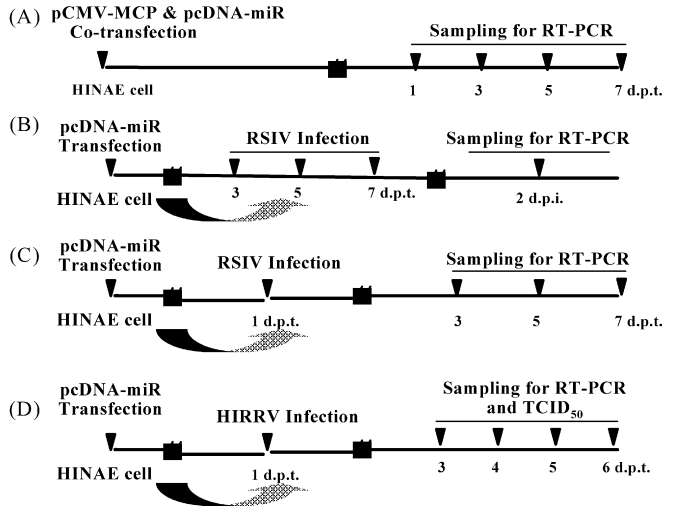

Fig. 1. Experimental scheme of miRNA studies. (A) MCP gene silencing by miR-MCPs; (B) anti-RSIV activity of miR-MCPs in viral-infected cells over the time-course of transfection with pcDNA-miRs; (C) anti-RSIV activity of miR-MCPs in cells over the time-course of plasmid transfection and virus infection; (D) anti-HIRRV activity of miR-HIRRV; d.p.t., days post-transfection; d.p.i., days post-infection.

while Lipofectamine™ 2000-transfected and HINAE cells were used as negative controls. At each indicated time points up to 7-day post-transfection (d.p.t.), total RNA was extracted with TRIzol® (Invitrogen, U.S.A.) from transfected cells and reverse transcribed to cDNA using M-MLV reverse transcriptase (Invitrogen, U.S.A.) according to manufacturer's protocol for reverse-transcription (RT)-PCR analysis.

To elucidate antiviral effects of miR-MCPs on RSIV replication, the expression of MCP gene was monitored in cells transfected with pcDNA-miRs and infected with RSIV in two cases: over the time-course of the plasmid transfection (Fig. 1B) and over the time-course of the plasmid transfection and the virus infection (Fig. 1C). RSIV was inoculated in HINAE cells transfected with pcDNA-miRs or un-transfected at 1, 3, 5 and 7 d.p.t. (Fig. 1B and C). After allowing 2 h 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 indicated time points after plasmid transfection and virus infection, total RNA was isolated from cells with TRIzol® (Invitrogen, U.S.A.), subjected to DNase I treatment (Promega, U.S.A.), and used to synthesize cDNA for RT-PCR analysis.

2.5. Anti-HIRRV activity of miR-HIRRV

HINAE cells were infected with HIRRV at 1 day after transfection with pcDNA-miRs (Fig. 1D). Viral-infected cell debris and cell-free supernatants were collected up to 6 d.p.t. for further processing.

To assess inhibitory effect of miR-HIRRV on HIRRV replication in terms of gene silencing, the expression of Glycoprotein (G) gene, an antigen of HIRRV, was monitored in cell debris and cell-free supernatants of cells transfected with pcDNA-miRs and infected with HIRRV by RT-PCR assay. At indicated time points, total RNA was extracted from plasmid-transfected and virus-infected cells and reverse transcribed to cDNA for RT-PCR analysis.

To assess inhibitory effect of miR-HIRRV on HIRRV replication in terms of production of viral particles, monolayer of HINAE cells seeded in 96-well plates was inoculated with serial 10-fold dilutions of cell-free supernatant samples, and HIRRV titer values were measured according to $TCID_{50}$ method (Reed and Muench, 1938).

Table 2
Primers used for RT-PCR

Primer name	Primer sequence	PCR product size (bp)
RSIV-MCP-F	5′-CCCTATCAAAACAGACTGGC-3′	429
RSIV-MCP-R	5′-TCATTGTACGGCAGAGACAC-3′	
HIRRV-G-F	5′-TGCCTACCCTGCTGTCATCAG-3′	550
HIRRV-G-R	5′-TCCATGGTTTTCCACAGAAGG-3′	
JFMx-F	5′-GCTCTCTGGGTGTGGAGAAG-3′	465
JFMx-R	5′-ACCAGGCTGATGGTTTCTTG-3′	
β-actin-F	5′-ACTACCTCATGAAGATCCTG-3′	510
β-actin-R	5′-TTGCTGATCCACATCTGCTG-3′	

2.6. Expression of Mx in HINAE cells transfected with pcDNA-miRs

cDNAs derived from plasmid-transfected samples and control samples at indicated time points were further used to determine the expression level of Mx gene. We used previously published primers designed for the Japanese flounder Mx (JFMx) protein cDNA (Ooi et al., 2006) to amplify the Mx transcripts by RT-PCR assay.

2.7. Reverse-transcription (RT)-PCR

One microliter of cDNA that was synthesized from indicated RNA samples was used for RT-PCR in a volume of 30 μl to amplify RSIV-MCP, HIRRV-G and Mx transcripts. The β-actin transcript was used as an internal control. Thermocycler conditions consisted of an initial denaturation at 95 °C for 2 min, followed by 20–30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The primer sequences and the size of PCR products were shown in Table 2.

The PCR products were electrophoresed and visualized in a 1.0% agarose gel stained with ethidium bromide under UV light. MCP expression values were measured using ImageJ software (Abramoff et al., 2004) and normalized to the respective β-actin expression values. Experiments were done in duplicate.

3. Results

3.1. Transfection of pcDNA-miRs in HINAE cells

When pcDNA-miRs were transfected into HINAE cells, they allowed co-cistronic expression of pre-miRNAs with EmGFP gene in cells under the control of the Pol II human CMV promoter. The co-cistronic expression of the pre-miRNAs was monitored microscopically under a fluorescence microscope (data not shown) and the predicted structures of the engineered pre-miRNAs incorporated into the murine miR-155 backbone are shown in Fig. 2.

3.2. Anti-HIRRV activity of miR-HIRRV

Transfection with pcDNA-miR-HIRRV reduced the expression of G gene in both cell debris and cell-free supernatants of viral-infected cells as compared to the positive controls that were only infected with the virus at each indicated time points (Fig. 3A).

As measured by the $TCID_{50}$ method, fewer HIRRV particles were detected in cell-free supernatants of cells transfected with pcDNA-miR-HIRRV expressing miR-HIRRV (Fig. 3B), corroborating the fact that miR-HIRRV efficiently inhibited the replication of HIRRV in transfected cells following challenge with the virus. These results seemed to indicate that the engineered viral-encoded miRNAs worked in a highly sequence-specific manner to trigger only the antiviral potency of miRNA-related pathways.

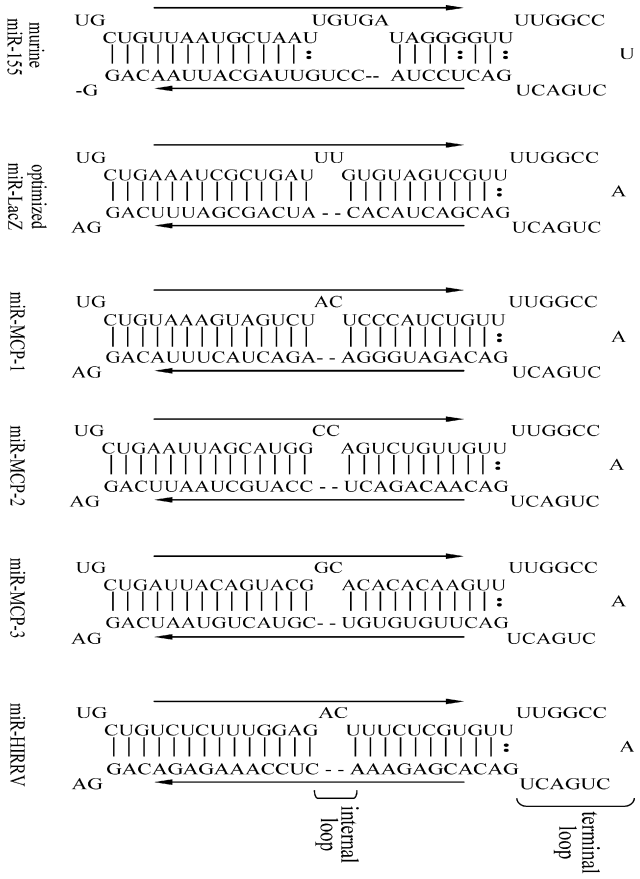

Fig. 2. Schematic presentation of predicted pre-miRNAs sequences. Up-underlined arrows indicate 21 nucleotide antisense target sequence. Down-underlined arrows indicate sense target sequence with 2 nucleotides removed to create an internal loop.

3.3. Anti-RSIV activity of miR-MCPs

When cells were co-transfected with pcDNA-miRs and pCMV-MCP, miR-MCPs were able to significantly silence the expression of the MCP gene when compared to the control samples that were only transfected with pCMV-MCP at 3, 5 and 7 d.p.t. Although all three miR-MCPs exhibited the reduction of the target gene expression levels in co-transfected cells, miR-LacZ seemed able to slightly reduce the expression of MCP gene (Fig. 4A and B).

In the case of transfection with pcRNA-miRs and infection with RSIV, miR-MCPs inhibited RSIV replication, resulting in reduction of the expression level of MCP gene in transfected and infected cells. But a similar pattern of antiviral activity was observed with miR-LacZ in cells transfected with pcDNA-miR-lacZ and infected with RSIV (Fig. 5A and B).

Taken together, these unexpected results led us to hypothesize that the antiviral effect observed here were not solely due to the antiviral potency of RNAi-induced miRNAs. Transfection with plasmids capable of expressing pre-miRNAs could trigger not only the antiviral potency of RNAi, but also activate other cellular mechanisms that interfere in antiviral responses in HINAE cells.

3.4. IFN activation by the expression of pre-miRNAs

Based on earlier studies, Mx protein is a molecular maker of type I IFN (α/β) production, is induced by dsRNA and have antiviral activity against a wide spectrum of viruses in fish (Nygaard et al., 2000; Ooi et al., 2006; Pakingking et al., 2004). These were confirmed in our applied setup where stimulation of HINAE cells with dsRNA molecules poly (I:C) significantly induced the expression of Mx gene, and poly (I:C)-stimulated cells slightly reduced the expression of MCP gene following challenge with RSIV (Fig. 6). To examine whether transfection of HINAE cells with plasmids expressing pre-miRNAs had any effect on antiviral IFN activity, the expression of Mx gene was investigated in cells co-transfected with pcDNA-miRs and pCMV-MCP (Fig. 7A), and in cells transfected with pcDNA-miRs and infected with either RSIV (Fig. 7B) or HIRRV (Fig. 7C), as well as in cells only transfected with pcDNA-miRs (Fig. 7D).

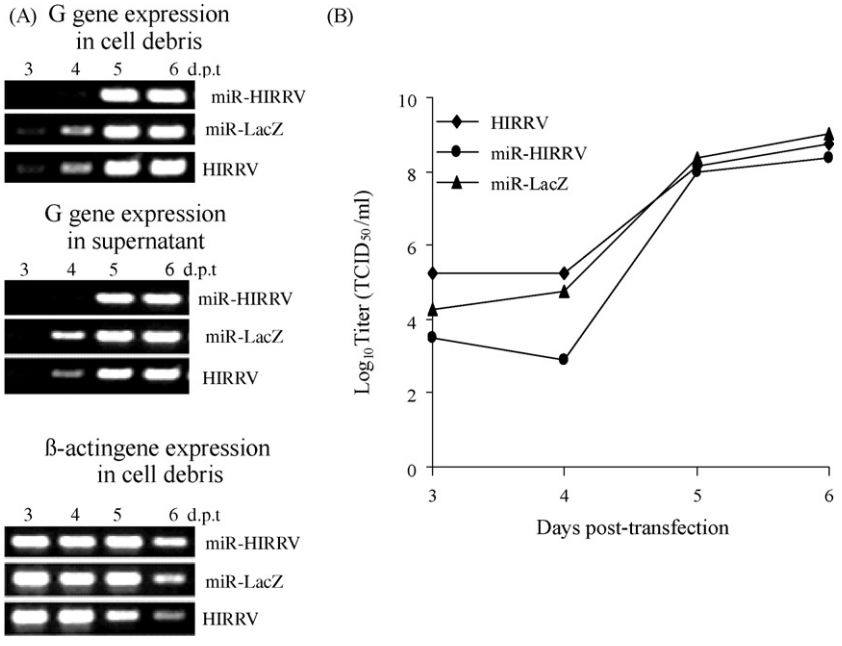

Fig. 3. Anti-HIRRV activity of miR-HIRRV. (A) RT-PCR analysis of G gene expression in cells transfected with pcDNA-miR-HIRRV and infected with HIRRV and (B) reduction of HIRRV titers in cell-free supernatants of cells transfected with pcDNA-miR-HIRRV and infected with HIRRV; (d.p.t.) days post-transfection.

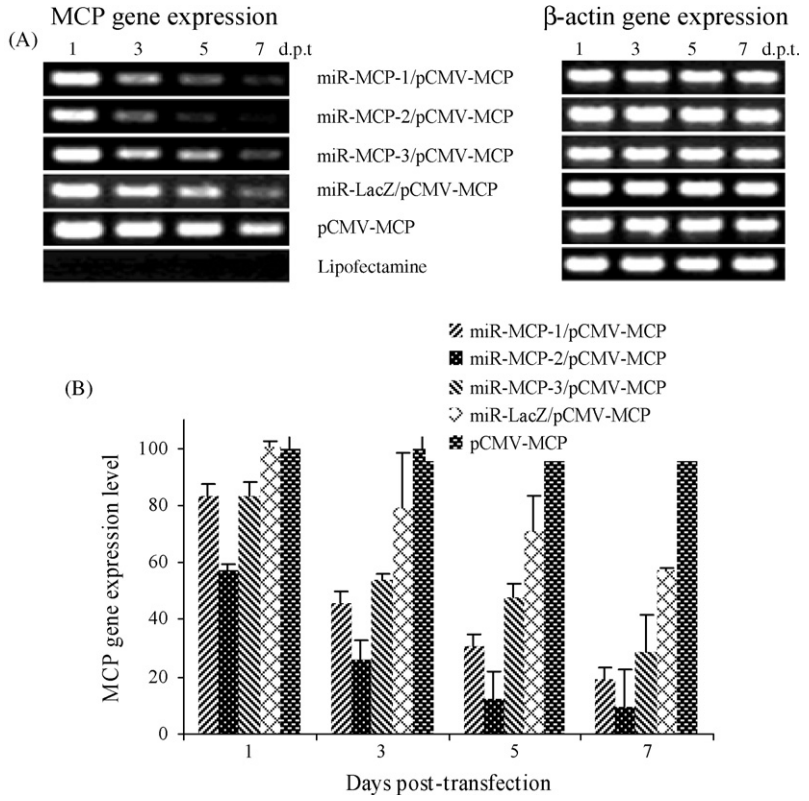

Fig. 4. MCP gene silencing by miR-MCPs in cells co-transfected with pcDNA-miRs and pCMV-MCP. (A) Agarose gel electrophoresis with RT-PCR products and (B) the expression level of MCP is calculated relative to the β-actin expression level. Data represent the mean of two independent experiments ± S.D.

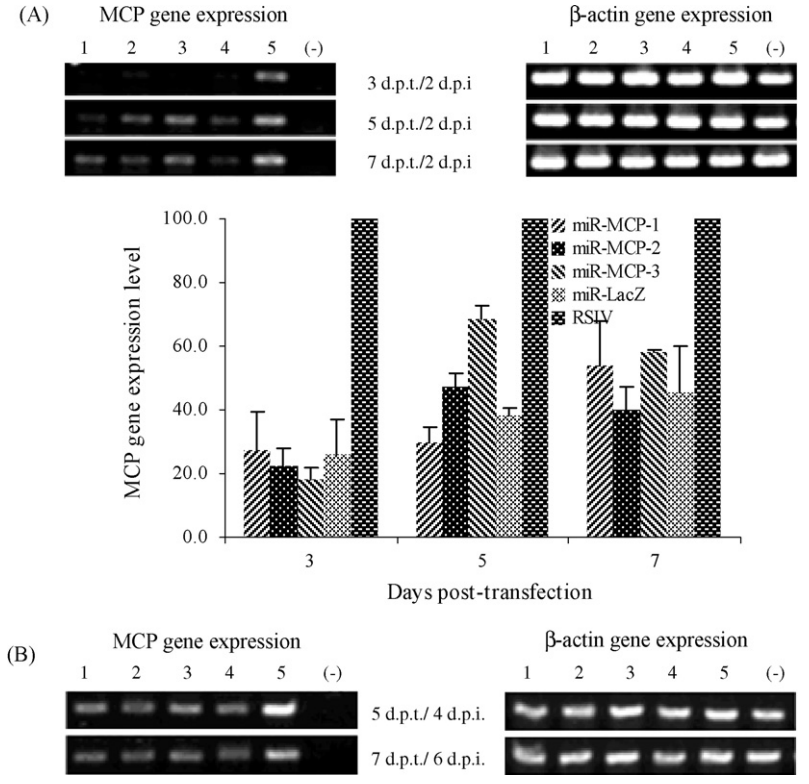

Fig. 5. Anti-RSIV activity of miR-MCPs: (1) miR-MCP-1 transfection/RSIV infection; (2) miR-MCP-2 transfection/RSIV infection; (3) miR-MCP-3 transfection/RSIV infection; (4) miR-LacZ transfection/RSIV infection; (5) RSIV infection. (−) HINAE cells without any treatment; d.p.t., days post-transfection; d.p.i., days post-infection. (A) Over the time-course of plasmid transfection. Top panel: agarose gel electrophoresis with RT-PCR products. Bottom panel: the expression level of MCP is calculated relative to the β-actin expression level. Data represent the mean of two independent experiments ± S.D. and (B) over the time-course of plasmid transfection and RSIV infection.

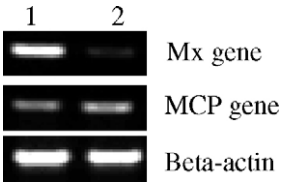

Fig. 6. Effect of IFN stimulation on RSIV replication. HINAE cells seeded onto 24-well plates were stimulated with 50 μg/ml poly (I:C) to induce IFN production. Induced cells and un-induced cells were infected with RSIV at 6 h after stimulation. After allowing 2 h for absorption, unattached viruses were removed and infected cells were continuously cultured with fresh growth medium containing 50 μg/ml poly (I:C) and further maintained for 72 h. The expression of Mx and MCP genes was monitored by RT-PCR. (1) HINAE cells with stimulation of poly (I:C) and infection of RSIV and (2) HINAE cells without stimulation but infection of RSIV.

As determined by RT-PCR in Fig. 7A, HINAE cells co-transfected with pcDNA-miRs and pCMV-MCP showed higher expression levels of Mx than the background expression level observed in cells transfected with pCMV-MCP or Lipofectamine™ 2000 at each of the indicated time points of the transfection. The up-regulation of Mx gene was also observed in plasmid-transfected and viral-infected cells when compared to the controls that were only infected with viruses (Fig. 7B and C). More importantly, transfection with various pcDNA-miRs differentially induced the expression of Mx gene (Fig. 7D). These results indicated that the expression of pre-miRNAs was responsible for the upregulation of Mx gene, and further suggest that each pre-miRNA exhibited its own level of expression in the cells, resulting in different expression levels of Mx gene.

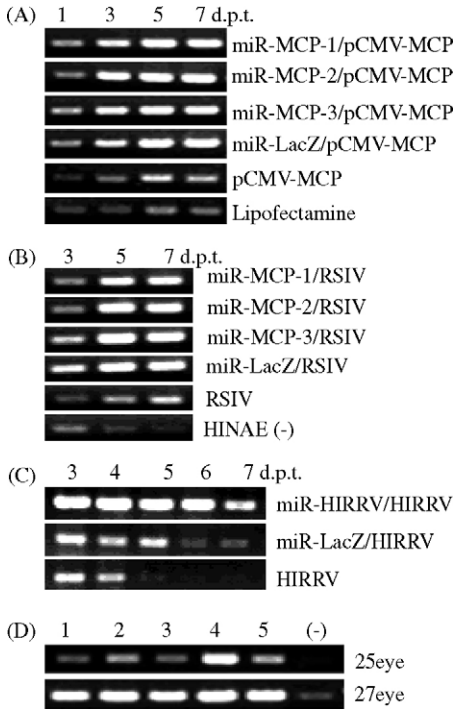

Fig. 7. Induction of the IFN-related pathway in cells transfected with pcDNA-miRs: d.p.t., days post-transfection; cyc., cycles of RT-PCR; (A) expression of Mx gene in cells co-transfected with pcDNA-miRs and pCMV-MCP; (B) expression of Mx gene in cells transfected with pcDNA-miRs and infected with RSIV; (C) expression of Mx gene in cells transfected with pcDNA-miRs and infected with HIRRV; (D) expression of Mx gene in cells transfected with pcDNA-miRs. (1) miR-MCP-1; (2) miR-MCP-2; (3) miR-MCP-3; (4) miR-HIRRV; (5) miR-LacZ; (−) HINAE cells.

4. Discussion

RNAi triggered by small RNA molecules, including siRNAs and miRNAs, offers a new approach for controlling viral infections. Thus far, viral-specific RNAi has been generated in a fish cell line by the introduction of synthetic viral gene-specific siRNA in our previous study (Dang et al., 2008), and by using plasmids capable of intracellular expression of virus-encoded pre-miRNAs (pcDNA-miRs) in the present study. In the previous study, MCP-targeted siRNA (siR-MCP) effectively and specifically inhibited the expression of the target gene and hindered RSIV replication during an *in vitro* virus infection, providing a potential approach for the control of viral diseases in aquaculture. In the present study, we describe another approach to trigger antiviral RNAi-related pathways through the action of miRNAs by using RSIV and HIRRV as models. By incorporating sequences encoding miRNAs specific to the MCP gene of RSIV (miR-MCPs) and a miRNA specific to HIRRV genome (miR-HIRRV) into a murine miR-155 pre-miRNA backbone under control of Pol II promoter, we were able to intracellularly express miRs (miR-MCPs and miR-HIRRV) in cells transfected with plasmids capable of expressing pre-miRNAs (pcDNA-miRs). Antiviral activity of miRs was assessed in transfected cells following viral infections.

In case of miR-HIRRV study, transfection with miR-HIRRV silenced the expression of G gene, and reduced HIRRV particles in cells transfected with miR-HIRRV and infected with HIRRV when compared to the controls that were only infected with HIRRV over the time-course of the transfection (Fig. 3A and B). The G gene is an antigen of HIRRV that is commonly used to detect and produce vaccines against the virus (Yasuike et al., 2007), therefore, this gene was selected to evaluate the inhibitory effect of miR-HIRRV on the virus replication. The reduction in Glycoprotein (G) mRNA could be clearly seen in both supernatants and cell-debris samples at 3 and 4 d.p.t., but not be clearly seen in samples after 5 d.p.t. (Fig. 3A). A similar observation in production of HIRRV particles was detected by $TCID_{50}$ assay. The miR-HIRRV curve was lower than the miR-LacZ curve and the HIRRV alone curve at 3 and 4 d.p.t. The differences between the three curves were also not apparent at 5 and 6 d.p.t. (Fig. 3B). These results may be influenced by the amount of virus inoculum. Infection with a high dose of virus may enhance fast multiplication of virus in infected cells, interfere in the inhibitory effect of the miR-HIRRV, resulting in difficulty in the detection of changes under the miRNA treatment.

In case of miR-MCP study, anti-RSIV activity of miR-MCPs was assessed by measuring MCP gene silencing by employing transient transfection of a plasmid expressing the target gene alone (pCMV-MCP) and following challenge with RSIV. Under RSIV infection, inhibitory effects of miR-MCPs on the expression level of MCP gene were assessed in both cases: over the time-course of miR-MCP transfection and the same time of RSIV infection (Fig. 5A), and over the time-course of miR-MCP transfection and RSIV infection (Fig. 5B). miR-MCPs inhibited the expression of MCP gene in cells co-transfected with pcDNA-miRs and pCMV-MCP (Fig. 4), as well as in cells transfected with pcDNA-miRs and infected with RSIV (Fig. 5). The MCP gene is commonly selected to detect and measure RSIV infection (Caipang et al., 2003; Dang et al., 2008; Lua et al., 2005). Taken together, these results suggest that engineered viral-encoded miRNAs had antiviral activity, showing inhibitory effects on replication of the target virus. The engineered viral-encoded pre-miRNAs seemed to work in a highly sequence-specific manner to evoke the antiviral potential of miRNA-related pathways in transfected cells.

Although virus-encoded miRNAs inhibited replication of the target virus, miR-LacZ also had inhibitory effects on viral replication (Figs. 3 and 5). This observation led us to hypothesize that the antiviral effect observed here was not only due to antiviral RNAi.

Long dsRNA induces a sequence-nonspecific IFN response in many mammalian cells, leading to a global inhibition of mRNA translation (Dykxhoorn et al., 2003). In vertebrates, dsRNA induce not only gene silencing but also a complex antiviral program mediated in part by type I IFN, which plays a prominent role during the response to viruses (Robalino et al., 2007; Smith et al., 2005). *In vitro* transcribed siRNAs and hairpin RNAs on DNA vectors appear to induce the antiviral IFN-mediated Jak–Stat pathway and global upregulation of IFN-stimulated genes (Karpala et al., 2005; Kim et al., 2004; Schyth et al., 2006; Sledz et al., 2003). For instance, in EPC (epithelioma papulosum cyprinid) cells, a fish cell line, *in vitro* transcribed-siRNAs induced an antiviral type I IFN response, correlating with the expression of Mx protein (Schyth et al., 2006). Mx protein is a well-characterized IFN-induced protein with antiviral activity, and is considered an indicator of antiviral type I IFN expression (Ooi et al., 2006; Samuel, 2001). Thus, the present results regarding Mx gene expression show that the same problem was observed in another fish cell line, HINAE cells. The upregulation of Mx gene in pcDNA-miRs-transfected cells (Fig. 7) indicates that the intracellular expression of our engineered pre-miRNAs evoked an antiviral IFN-related response in transfected cells. Therefore, we propose that the engineered virus-encoded pre-miRNAs not only trigger the antiviral potency of RNAi, but also evoke an antiviral IFN-related response in a fish cell line. This study provides, for the first time, evidence that the expression of pre-miRNAs, long hairpin RNAs, induced the antiviral type I IFN-related response, correlating with the upregulation of the Mx gene, in fish cells.

Taken together, our data suggest that both miRNA-related pathways and antiviral IFN-related pathways contributed to the observed antiviral effects of virus-encoded miRNAs. A convergence of RNAi and innate immunity in antiviral response was also described in shrimp injected with viral sequence-specific dsRNA by Robalino et al. (2005). The authors reviewed and proposed a model of antiviral immunity in shrimp by which viral dsRNA engages both innate immune pathways and an RNAi-like mechanism to induce potent antiviral responses (Robalino et al., 2005, 2007). Therefore, the possibility of activation of both RNAi and IFN-related pathways by pre-miRNAs must be of great interest to the development of antiviral therapeutics for the control of diseases because these two pathways interact functionally to mount immunity to a viral pathogen.

Our results also revealed that miR-LacZ had strong inhibitory effect on RSIV replication (Fig. 5), but less on HIRRV replication (Fig. 3). Given that miRNA-silencing pathways do not require absolute complement of base-paring with target mRNA, we sought to identify targets of miR-LacZ and found that ORF 424R (putative ankyrin repeat protein) of RSIV genome contains sequences with partial homology to miR-LacZ sequence. While the sequence of miR-LacZ is partial homology to the non-coding sequence region between the non-virion protein gene and the RNA polymerase gene of HIRRV genome (data not shown). ORF 424R is classified as an Early (E) gene that is involved in viral DNA replication and has positive feedback on the regulation of Late (L) genes, such as MCP gene (Lua et al., 2005; Williams, 1996). Our findings led us to hypothesize that miR-LacZ silenced the expression of ORF 424R through homology with the target gene, resulting in significant inhibition of RSIV replication. This hypothesis is supported further by evidences shown in Figs. 4 and 5. The effect of miR-LacZ on the MCP gene was as great as the effects of the three miR-MCPs in transfected cells following RSIV infection (Fig. 5), but was less than those of the miR-MCPs in cells co-transfected with pCMV-MCP (Fig. 4). Transfection with pCMV-MCP expressed the MCP gene alone, instead of the whole RSIV genome. Thus, miR-LacZ may recognize a region (ORF 424R) of the RSIV genome, initiating an apparent block in RSIV function (Fig. 5). Overall, miRNAs seem to have broad-spectrum effects beyond the selective silencing of homologous target genes when experimentally introduced into cells.

In conclusion, our results indicate that antiviral RNAi-related pathways can be triggered through the action of both siRNAs and miRNAs into a fish cell culture system, providing a new door for the future development of strategies to control viral diseases in aquaculture. Antiviral activities of miRNAs observed here are the results of at least part of the effect via direct miRNA-mediated pathways, and the part of the effect via the induction and action of IFN, correlating with upregulation of Mx protein. Although pre-miRNAs activated an antiviral IFN-related response as shown by upregulation of IFN-induced Mx protein, it is possible that other cellular signaling pathways are also activated. Therefore, the results of experiments using miRNA-related pathways should be interpreted with caution. The side effects elicited by miRNAs should be given special concern because cellular interferon responses in some cases cause an unintended stimulation and/or a global inhibition of mRNA translation.

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