Studies on molecular pathogenic mechanism and development of RNAi-based antiviral approach for red seabream iridoviral disease

DOCTORAL DISSERTATION

STUDIES ON MOLECULAR PATHOGENIC MECHANISM AND DEVELOPMENT OF RNAi-BASED ANTIVIRAL APPROACH FOR RED SEABREAM IRIDOVIRAL DISEASE

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STUDIES ON MOLECULAR PATHOGENIC MECHANISM AND DEVELOPMENT OF RNAi-BASED ANTIVIRAL APPROACH FOR RED SEABREAM IRIDOVIRAL DISEASE

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DECLARATION

I hereby declare that this thesis has been composed by myself and is a result of my own investigations. It has neither been accepted, nor submitted for any other degrees. All sources of information have been duly acknowledged.

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ABSTRACT

Iridoviruses are considered infectious pathogens that are responsible for causing serious systemic diseases among aquatic animals in many parts of the world. In fish, outbreaks of an iridoviral disease known as red seabream iridoviral disease (RSIVD) have been recorded in at least 31 cultured marine fish species. The causative pathogen was first isolated from diseased red seabream (*Pagrus major*) in Japan in 1992 and hence named red seabream iridovirus (RSIV). Due to the devastating effects of this pathogen to marine aquaculture, the pathogenic mechanism of RSIV infection has been well-studied at the organismal level. Some rapid, sensitive diagnostic methods as well as some kinds of vaccines have also been developed for RSIV. RSIV gene regulation strategies that occur in a common temporal kinetic manner of the family *Iridoviridae* with 3 stages (Immediate-Early, Early and Late) have been well-defined in a cell culture system. However, the molecular pathogenic mechanism or the spread of RSIV in a fish model at the molecular level is still not fully understood. The development of alternative antiviral approaches based on advances in molecular technology may also need to be undertaken. Such studies will be of enormous contribution to the thorough knowledge of RSIV infection and control of iridoviral diseases.

Recent significant advances in molecular genetic technology, such as DNA microarray and RNA interference (RNAi), have been successfully applied in virological studies. The DNA microarray technology has been considered as a powerful tool for the parallel analysis of whole-genome expression levels in a single experiment, allowing a better understanding of viral gene regulation strategies and viral molecular pathogenic mechanisms. While the RNAi technology, a process of sequence-specific gene silencing triggered by small molecules of double-stranded RNAs (siRNAs and miRNAs), has been considered as a promising antiviral tool. The antiviral potency of RNAi can be evoked by the introduction of viral gene-specific siRNAs or plasmid-based virus-encoded hairpin RNA expression system into cells. In this study, therefore, we demonstrated the molecular pathogenic mechanism of RSIV infection in a fish model using viral DNA

microarrays and the potential antiviral activity of RSIV-derived dsRNAs through the introduction of viral gene-specific siRNAs and plasmid expressing virus-encoded pre-miRNAs into a cell culture system.

The transcriptional profile of RSIV was explored over the time-course of the virus infection in infected spleen of red seabream using viral DNA microarrays containing almost all RSIV putative open reading frames (ORFs). cDNAs derived from spleens at different time points upto 14 days post-infection (d.p.i.) were labeled with cy5 and hybridized with cDNAs derived from spleens at 0 d.p.i. that were labeled with cy3 onto the DNA microarrays. In addition, differential gene expression patterns in spleens and kidneys were also investigated throughout the virus infection by hybridization of cy5-labled-cDNAs derived from spleens with cy3-labeled-cDNAs derived from kidneys. Microarray data indicated that the pathogenesis of RSIV infection appears to spread at around day 5 and continued with high levels of viral multiplication until viral clearance by host antiviral defenses starting from around 10 d.p.i. A comparison of RSIV gene expression patterns between spleens and kidneys showed that all viral genes were expressed at higher levels or at similar levels in the spleens when compared with those in the kidneys throughout the infection. The infectious viral concentration in infected spleens, as measured by $TCID_{50}$ assay, was also higher than that in infected kidneys. The microarray data was further confirmed by RT-PCR assay. Our results provided a greater understanding of the pathogenesis of RSIV infection and further confirmed, at the molecular level, that the spleen is a suitable organ for diagnosis of RSIV infection.

Major capsid protein (MCP) gene of RSIV, a gene essential for the formation of viral particles, was selected as the target gene for study of the potential anti-RSIV option based RNAi-related mechanisms. A siRNA specific to the MCP gene (siR-MCP) were synthesized and introduced into HINAE cells in order to test for inhibitory effects on virus replication. The inhibition of virus replication was demonstrated by reduced MCP expression levels and reduced RSIV titers. siR-MCP dose-dependently inhibited the expression of MCP gene in cells that were transiently transfected or stably transfected with a plasmid expressing the target MCP gene (pCMV-MCP). Under RSIV infection, siR-MCP reduced the expression of MCP gene by 55.2% and 97.1% at 84 and 96 hours

post-infection, 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.

With the use of plasmid-based pre-miRNA expression system (Block-iT™ Pol II miR RNAi Expression Vector), we described another potential approach to trigger antiviral RNAi, termed microRNAs (miRNAs). In this miRNA study, another marine fish-pathogenic virus, HIRRV (Hirame rhabdovirus) also was an interesting candidate. By incorporating sequences encoding miRNAs specific to MCP gene (miR-MCPs) and a miRNA targeting to HIRRV (miR-HIRRV) into a murine miR-155 pre-miRNA backbone, we were able to intracellularly express miR-MCPs and miR-HIRRV in HINAE cells. The anti-RSIV activity of miR-MCPs was initially assessed by measuring MCP gene silencing by employing transient transfection of a plasmid expressing the target gene (pCMV-MCP). We then investigated the inhibitory effect of the miR-MCPs on RSIV replication following challenge with RSIV. The inhibitory effect of miR-HIRRV on HIRRV replication was demonstrated by reduced expression of viral glycoprotein (G) gene and reduced HIRRV titers in plasmid-transfected cells over the time-course of the virus infection. Our results suggested that engineered viral-encoded miRNAs were able to trigger the antiviral miRNA-related pathways in fish cells. However, further analyses revealed that the expression of pre-miRNAs also activated IFN-related pathways, correlating with the up-regulation of antiviral IFN-induced Mx protein, resulting in non-specific antiviral effects. The antiviral effects of engineered virus-encoded 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.

Also in this study, some general discussions, suggestions and future perspectives were included.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Overview

Over the past three decades, aquaculture has become the fastest growing food-producing sector all over the world. As the aquaculture industry has expanded, intensified and diversified, numerous aquatic animal diseases have emerged and become widespread as a result of global trade and modern transportation systems. Diseases caused by viruses, bacteria, fungi, parasites and other undiagnosed pathogens have thus become a primary constraint to the culture of many aquatic species, impeding both economic and social development in many countries. Among these pathogens, viruses pose the most serious threat to fish because they affect almost all phases of its developmental stages (Ellis, 2001).

Viral infections that occur as sporadic events in wild fish populations may cause high mortality in intensive aquaculture systems. Hence, for the aquaculture industry to prosper and be sustainable, it is imperative that losses caused by viral diseases and the use of antimicrobials are kept at the minimum. Unlike other pathogens, viruses are very small pathogens that multiply only within the living cells, and must utilize the system of the infected host cells for survival and replication, therefore, viral diseases cannot be controlled with medication. Although innate immune system is the first line of defense against disease, this system only provides adequate protection in conditions of low infection pressures from pathogens, and indeed, dead fish release very large numbers of pathogens into the water, increasing infection pressure, resulting in increased risk for the rest of the population in a farm (Ellis, 2001). Moreover, most pathogens have developed mechanisms for avoiding these defenses. Many RNA and DNA viruses counteract the host antiviral immune defenses through mutation of their genome, by encoding viral suppressors, or both (Zheng et al., 2005). In these cases, antiviral immunity responses need to be enhanced to combat viral infections. Despite significant advances made in management of environment and control of viral diseases, current biochemical agents and vaccines are limited by many factors, such as toxicity, complexity, cost and resistance (Dave and Pomerantz, 2003). For instance, virus-inactivated vaccines show poor induction of cell-mediated immunity, poor immunogenicity and the increased risk of environmental exposure to viral products (Caipang et al., 2006). Moreover, though

vaccination is used routinely to prevent viral diseases in humans and mammals, it is still limited in fish because fish are cold-blooded animals, and their immune response to a vaccine is not as predictable as that of warm-blooded animals. Thus, understanding the molecular pathogenic mechanisms of viral infections in fish will be of enormous help in the proper management and prevention of viral diseases. The development of alternative potential approaches that are experimentally exploited based on relatively antiviral pathways in fish to control viral infections will eventually lead aquaculture towards sustainable production in the aquatic ecosystem. Recent potential advances in molecular genetic technologies, such as DNA microarray and RNA interference (RNAi) could be applied in these directions.

The DNA microarray technology has been considered as a powerful tool for the parallel analysis of whole-genome expression levels in a single experiment and for the comparative analysis of series of samples. The design and construction of a DNA microarray for any given microbial genome are straightforward, facilitating our understanding of various aspects of both sides of the host-pathogen interaction in molecular detail (Cummings and Relman, 2000). While RNAi technology, a process of gene sequence-specific silencing triggered by small molecules of double-stranded RNA (dsRNAs), has been considered as a promising antiviral tool. RNAi-related mechanisms can also be triggered by experimentally introduced synthesis dsRNAs into cells in various ways allowing to target any gene with high specificity and efficiency based on homology in sequences (Schutze, 2004; Sledz and Williams, 2005; van Rij and Andino, 2006; Zheng et al., 2005).

1.2 Major infectious viral diseases in fish aquaculture

Although about 60 different viruses have been detected in fish worldwide, only a few cause severe diseases in aquaculture (Ahne, 1994). The causative agents of major viral diseases in fish worldwide are briefly updated in Table 1. Among the diseases caused by these viruses, epizootic haematopoietic necrosis, infectious haematopoietic necrosis, spring viremia of carp, viral haemorrhagic septicemia, infectious salmon anaemia, red seabream iridoviral disease, and koi herpesvirus disease are recently listed as "notified" viral diseases by Office International des Epizooties (OIE) because they are considered to

Table 1. The causative agents of major viral diseases in fish

Viruses	Distribution of diseases
Iridoviridae	
Epizootic haematopoietic necrosis virus (EHNV)	Australia, Europe, North America
Erythrocytic necrosis virus (ENV)	Worldwide
Lymphocystisvirus (LCV)	Worldwide
Red seabream iridovirus	Japan, East and South-East Asian
White sturgeon iridovirus	Europe, North America
Herpesviridae	
Anguilla herpesvirus	Japan
Cannel catfish virus (CCV)	North America
Coho salmontumorvirus	Japan
Flounder herpesvirus	Japan
Herpesvirus salmonis	USA
Koi herpesvirus (KHV)	Worldwide
Oncorhychus masou virus (OMV)	Japan
Rhabdoviridae	
Anguilla rhabdovirus (EVA, EVX)	Japan, Europe
Hirame rhabdovirus	Japan
Infectious hematopoietic necrosis virus (IHNV)	Asia, USA, Europe
Pike fry rhabdovirus	Europe
Spring viremia of carp virus (SVCV)	Europe
Ulcerative disease rhabdovirus	Burma, Sri lanka, Thailand
Viral hemorrhagic septicemia virus (VHSV)	Japan, USA, Europe
Birnaviridae	
Infectious pancreatic necrosis virus (IPNV)	Worldwide
Nodaviridae	
Viral encephalopathy and retinopathy (VER) or Viral nervous necrosis (VNN)	Worldwide
Orthomyxoviridae	
Infectious salmon anaemia virus (ISAV)	Norway, Canada, UK, USA
Reoviridae	
Grass carp reovirus	China
Reovirus of common carp	China
Reovirus of salmonids	Taiwan
Coronaviridae	
Coronavirus of carp	Japan

be of socio-economic and/or public health importance within countries and are significant in the international trade of animals and animal products (http://www.oie.int/eng/maladies/en_classification2007.htm).

1.3 Viral diseases of marine fish in Japan

The intensification of aquaculture in both the number of species and the amount of production without proper regulations for disease control is considered to be the major factor for the failure of establishing a disease control system in Japan. The viruses infecting marine fish and shellfish were comprehensively discussed by Muroga (2001) (Muroga, 2001). Yellowtail ascites virus (YTAV) isolated from juvenile yellowtail in 1983 was reported as the first marine fish-pathogenic virus in marine aquaculture (Sorimachi, 1985). Since then, several viral diseases have been recorded from marine fish. There have been cases of rhabdoviral diseases that was caused by Hirame rhabdovirus (HRV) (Kimura, 1986) or viral hemorrhagic septicemia (VHSV) (Isshiki, 2001; Takano, 2000), and red seabream iridoviral disease that was caused by red seabream iridovirus (RSIV) (Inouye et al., 1992). Other viruses include flounder herpesvirus (FHV) which causes epidermal hyperplasia in Japanese flounder, tiger puffer virus which causes "white mouth disease" in puffer fish (Wada, 1986), epithelial necrosis virus identified in black seabream (Miyazaki, 1989), and viral nervous necrosis (VNN) that was first identified in Japanese parrotfish (Yoshikoshi, 1990) and has since infected several other marine fish (Nguyen, 1994). These viruses are considered to be important pathogens in marine aquaculture in Japan.

Concerning red seabream iridoviral disease (RSIVD), it is reported as an acute and highly infectious disease that causes a significant mortality in red seabream (Inouye et al., 1992) and 30 other cultured marine fish spanning three taxonomic orders (Table 2) (Kawakami and Nakajima, 2002). The first outbreak of RSIVD was recorded in cultured red seabream in Shikoku Island in 1990 and the disease has since been documented in cultured marine fish populations in 18 prefectures in the country. The disease was severely apparent during the summer time when the water temperature is elevated around 25°C. The pathological signs were lethargy, severe anemia, petechiae of gills, and enlargement of spleen and kidney. The disease is characterized by the appearance of

enlarged cells in the spleen, kidney, liver, heart, and gills that were stained strongly with Giemsa solution. The causative agent was identified and named red seabream iridovirus (RSIV) (Inouye et al., 1992). Iridovirus-like agents of the family *Iridoviridae* are observed in both invertebrates and vertebrates (Hedrick, 1992), and most of them are confirmed to be pathogens that are associated with systemic infection in fish (Wolf, 1988). Because of geographical range and occurrence in fish involved in international trade, the RSIVD is notified to be quarantined by the OIE.

Table 2. Fish species susceptible to RSIV infection in Japan

***Perciformes* (28 species)**	
Sea bass (*Lateolabrax sp*)	Snubnose dart (*Trachinotus blochii*)
Red spotted grouper (*Epinephelus akaara*)	Threeline grunt (*Parapristipoma trilineatum*)
Sevenband grouper (*E. Septemfasciatus*)	Threeband sweetlips (*Plectorhynchus cinctus*)
Malabar grouper (*E. malabaricus*)	Adjutant (*Lethrinus haematopterus*)
Kelp grouper (*E. Moara*)	Spangled emperor (*L. nebulosus*)
Orange-spotted grouper (*E. coioides*)	Red seabream (*Pagrus major*)
Banded grouper (*E. Awoara*)	Crimson seabream (*Evynnis japonica*)
Cobia (*Rachycentron canadum*)	Black seabream (*Acanthopagrus schlegeli*)
Yellowtail (*Seriola quinqueradiata*)	Japanese parrot fish (*Oplegnathus fasciatus*)
Amberjack (*S. dumerili*)	Spotted parrot fish (*O. punctatus*)
Goldstriped amberjack (*S. aureoittata*)	Largescale blackfish (*Girella punctata*)
Striped jack (*Pseudocaranx dentex*)	Albacore (*Thunnus thynnus*)
Buri-hira (*Seriola sp.*)	Japanese mackerel (*Scomberomorus niphonius*)
Horse mackerel (*Trachurus japonicus*)	Chub mackerel (*Scomber japonicus*)
***Pleuronectiformes* (2 species)**	
Japanese flounder (*Paralichthys olivaceus*)	Spotted halibut (*Verasper variegates*)
***Tetraodontiformes* (1 species)**	
Tiger puffer (*Takifugu rubripes*)	

1.4 Red seabream iridovirus: characteristic, diagnosis and control

RSIV is a large, icosahedral, double-stranded (ds)DNA virus, belonging to the family *Iridoviridae*. Each virion measures 200-240 nm in diameter and consisted of a capsid, intermediate lipid membrane and a central core (Inouye et al., 1992). RSIV is believed to have a wide geographical distribution not only in Japan but also in other East and South-East Asian countries (Jeong et al., 2003; Mahardika et al., 2004; Wang et al., 2003), and shares high homology with iridovirus isolated in Taiwan (Wang et al., 2003), Korea (Jeong et al., 2003) and in Indonesia (Mahardika et al., 2004). The high homology of the RSIV ATPase and MCP genes with other iridovirus isolates, SBIV (Sea bass iridovirus), GSDIV (Grouper sleepy disease virus), ALIV (African lampeye virus) and DGIV (Dwarf gourami iridovirus) led to a proposal to name a new genus for this group in the *Iridoviridae* family known as "*Tropivirus*" (Sudthongkong et al., 2002).

Studies on the biological and physicochemical characteristics showed that RSIV produced cytopathic effect (CPE) in several fish cell lines at suitable temperature from 20-25^{o}C; sensitive to low pH (3), chloroform and ether; unstable to heat treatment but stable to ultrasonic treatment. However, the virus titers were reduced by repeated freezing and thawing (Nakajima and Sorimachi, 1994). RSIV induced apoptosis *in vitro* and is dependent on the presence of caspases (Imajoh et al., 2004). The cell apoptosis by RSIV consisted of three stages: cell shrinkage and rounding at the early stage; cell enlargement at the middle stage; formation of apoptotic body-like vesicles at the late stage and phagocytosis by neighboring cells. Caspase-3 and caspase-6 involved in the morphological changes during the mid- and late apoptotic stages and in viral protein synthesis in the later stage of the virus infection.

The complete genome sequences of RSIV is 112, 414 bp containing about 93 putative open reading frames (ORFs) with a main structural component, the major capsid protein (MCP) gene (Kurita et al., 2002). The MCP gene normally occupies upto 45% of total virion protein and is highly conserved among the family *Iridoviridae* (Williams, 1996). The MCP gene of RSIV has been confirmed to be the most suitable gene for detection and measurement of the virus infection (Caipang et al., 2003). Several ORFs have been assigned putative function based on significant matches with the potential proteins of

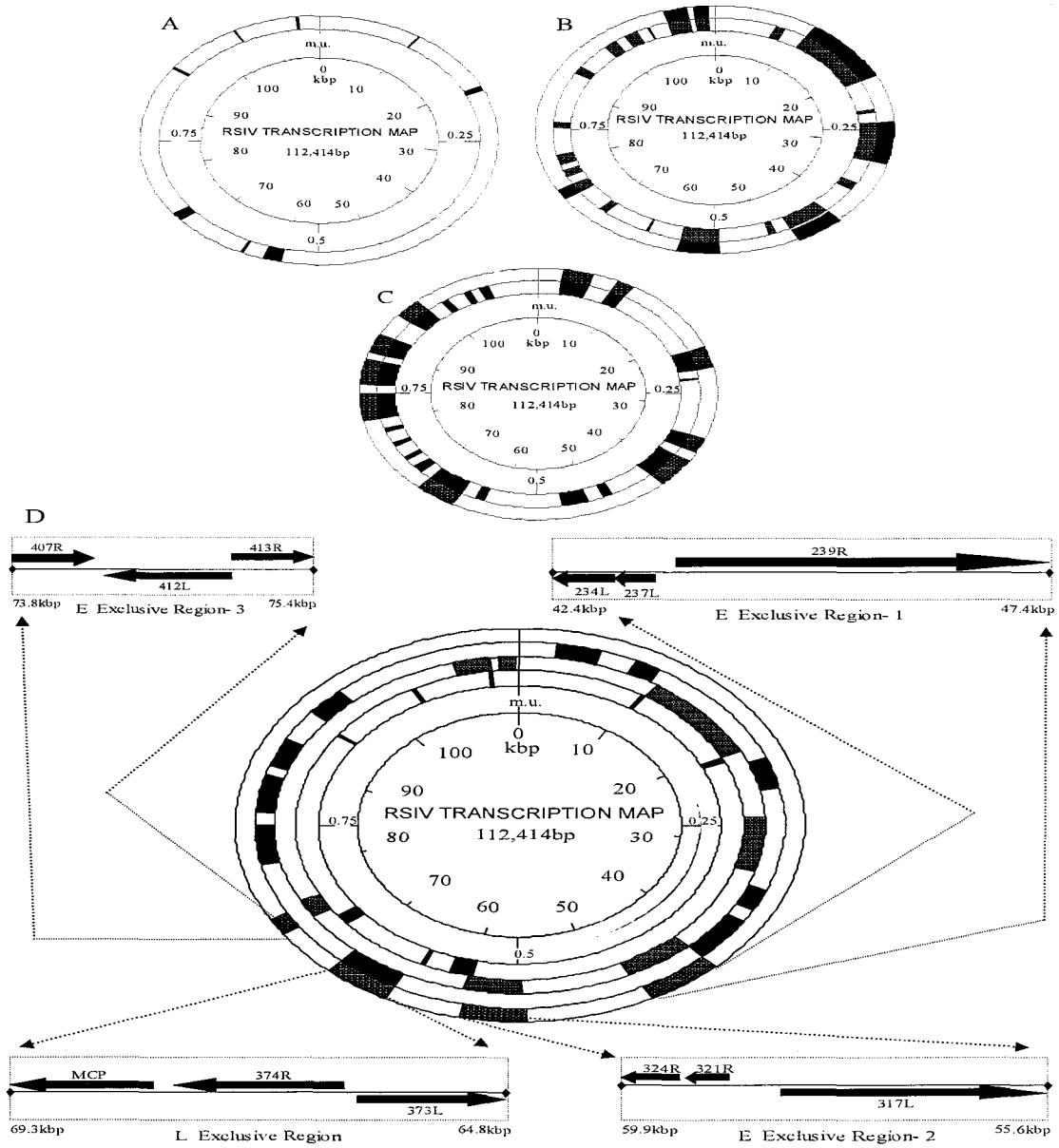

Fig. 1. Distribution of IE, E and L transcripts in the RSIV genome and correlation with genomic sequence data. Transcriptionally active regions are shown in black, while inactive or undetected regions are shown in white. The innermost circle indicates map units (m.u.) and kilobases (kb) from map unit 0. (A) Distribution of IE transcripts in the RSIV genome. (B) Distribution of E transcripts in the RSIV genome. The inner solid circle shows regions of E transcripts. The outer solid circle indicates major clusters of E transcripts. (C) Distribution of L transcripts in the RSIV genome. The inner solid circle shows regions of L transcripts. The outer solid circle indicates major clusters of L transcripts. (D) Distribution of E and L exclusive regions in the RSIV genome. The three inner solid circles show major clusters of IE, E, and L transcription in the innermost, middle, and outermost circles, respectively. The outer solid circle indicates three regions exclusive to E transcripts encoding enzymes associated with viral DNA replication and an L region containing MCP. (Adapted from Lua et al. (Lua et al., 2005)).

LCDV-1 (lymphocystis disease virus 1), RBIV and other better-studied viruses. These proteins are those involved in DNA replication, DNA modification and processing, DNA transcription, processing of viral DNA, protein processing and modification and virus-host interaction. There are also other sequences in the genome that are considered RSIV-specific genes, the large submit of DNA-dependent RNA polymerase, DNA polymerase, ATPase and putative ankyrin repeat containing protein genes.

In vitro RSIV replication and gene regulation strategies have been well-studied using DNA microarray technology. Individual RSIV ORFs were characterized at the transcriptional level and were also classified into temporal kinetic classes by their dependence on *de novo* protein synthesis and viral DNA replication. The gene expression of RSIV occurred in a temporal kinetic cascade with 3 stages, which includes Immediate-Early (IE), Early (E) and Late (L) transcripts, following a common feature of the family *Iridoviridae*. The three classes of transcripts were distributed throughout the RSIV genome; however, E transcripts were found to be clustered in at least 6 discrete regions and L transcripts appeared to originate from 7 discrete regions (Fig. 1) (Lua et al., 2005).

Diagnosis of RSIV infection has been achieved through virological, histological and molecular approaches. These diagnostic techniques include cell culture (Nakajima and Sorimachi, 1994), Giemsa staining (Inouye et al., 1992), immuno-assay using a monoclonal antibody (Nakajima, 1995), conventional PCR (Oshima et al., 1998; Oshima et al., 1996), as well as some sensitive, rapid diagnostic techniques, such as quantitative real-time PCR (Caipang et al., 2003) and the loop-mediated isothermal amplification (LAMP) reaction (Caipang et al., 2004).

Control of RSIV infection was made possible through vaccination using virus-inactivated and DNA vaccines. The virus-inactivated vaccine was prepared by addition of formalin to the RSIV-infected cell culture supernatant and cells (Nakajima, 1997), while DNA vaccines were produced by construction of DNA plasmids encoding the MCP and an ORF containing a transmembrane domain of RSIV (Caipang et al., 2006). The RSIV-inactivated vaccine has been found to be highly efficient in protecting the vaccinated-fish both at the laboratory-scale (Nakajima, 1997) and field trials (Nakajima, 1999) and is

now commercially available. The RSIV-DNA vaccines have also been found to be able to induce significant protection in fish against RSIV infection (Caipang et al., 2006).

1.5 DNA microarray technology (gene chip technology)

1.5.1 Background

DNA microarrays, a high-capacity system, was first described and used for quantitative expression measurements of many genes in 1995 by Schena et al. (Schena et al., 1995). The DNA microarrays are basically a miniature form of dot blot, but in a high-throughput format spotting thousands of different target probes on a small solid surface, such as a coated glass slide or nylon membrane. The target probes can be DNA, cDNA or oligonucleotides of selected genes, and are used to determine complementary binding of the unknown sequences. Although several different types of DNA microarrays have been developed based on their target probes, the key unifying principle of all DNA microarray experiments is a labeled nucleic acid hybridization-based technique with high sensitivity and specificity. Each experiment consists of five discrete steps (Sellheyer and Belbin, 2004): (1) fabrication of the DNA microarray, (2) preparation of the biological sample, (3) hybridization of the labeled nucleic acid sample with the array, (4) signal detection and data visualization, and (5) data processing and analysis (Fig. 2).

It is widely believed that thousands of genes and their products in a given living organism function in a complicated and orchestrated way that creates the mystery of life. The same genes are not active or expressed in every cell. Gene expression is a sensitive indicator of cellular metabolism, disease state and toxicant exposure, and thus represents a unique way of characterizing how cells and organisms adapt to changes in the external environment (Lettieri, 2006). The measurement of gene expression levels facilitates the annotation of the molecular mechanisms underlying normal cellular processes as well as the molecular basis for disease. Obtaining such global views at the molecular level was impossible using conventional molecular biological techniques (Choudhuri, 2004; Sellheyer and Belbin, 2004). Thus, DNA microarray technology is revolutionary because it allows for the monitoring of the expressions of thousands of genes in a single experiment, providing a platform to perform genome-wide expression analyses across various biological models. This enormous power of microarrays has already made a

remarkable impact on many fields of biological and biochemical research, including cellular physiology, cancer biology, and pharmacology (Cummings and Relman, 2000). It is an undeniable fact that the number of publications employing microarrays has undergone exponential growth since its inception in 1995 by Schena et al (Schena et al., 1995). Since that time, the publications increased from 239 in 2000 to 4099 in 2005 with an accumulated total of 12,679 microarray papers (Ju et al., 2007).

DNA microarray experiments have been widely used in both basic and applied research ranging from viruses to humans in order to answer different research questions related to gene expression, for instance in gene discovery, drug discovery, disease diagnosis and toxicological research. DNA microarray technology has focused on identification of new genes based on similarities in expression patterns with known genes. Ultimately, these studies may greatly expand the size of existing gene families, reveal new patterns of coordinated gene expression across gene families, uncover entirely new categories of genes, and identify genes involved in the different developmental stages of diseases. The technology has already focused on the field of drug discovery and development by screening for changes in gene expression following exposure of cells to drug treatments. Correlation of gene expression with drug activity may enhance the annotation of molecular details of drug action, while correlation of transcription profiles in untreated cells with drug response may reveal mechanisms for sensitivity and resistance, providing information to synthesize more effective drug targets and reduce their adverse side effects (Choudhuri, 2004). In addition, with the evolution of microarray technology, it can be possible to learn more about different diseases and further classify the types of diseases on the basis of the patterns of gene activity in specific target cells. Microarray technology has also been a valuable tool in toxicology research involving both mammals and aquatic animals by measuring gene expression levels upon exposure to toxicants (Ju et al., 2007; Lettieri, 2006; van Hal et al., 2000).

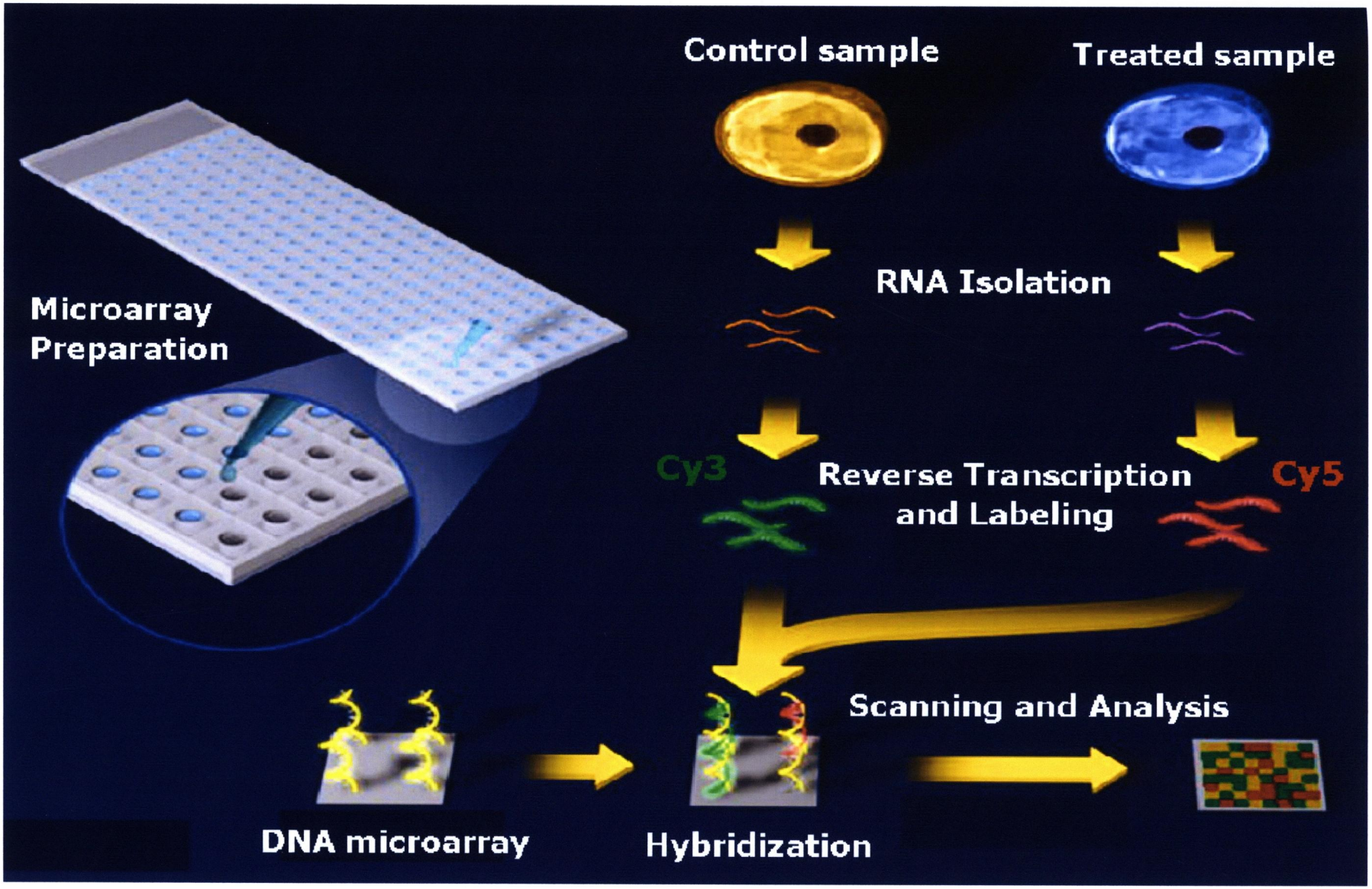

Fig.2. Principle model of DNA microarray experiment

1.5.2 Application of the DNA microarray technology in virological studies

The concurrent development of DNA microarray technology and the complete sequencing of a number of viral genomes are providing the opportunity to speed our understanding of various aspects of both sides of the host-virus interaction at the molecular level. By understanding the molecular details of this interaction, we can identify virulence-associated genes, host-defense strategies and characterize the cues to which they respond and mechanisms by which they are regulated. In fact, DNA microarray technology has been widely applied in virological studies in both cell culture systems and experimental animal models. By monitoring viral gene expression, one can explore viral replication and gene regulation strategies, predict the molecular pathogenic mechanisms, predict the functions of uncharacterized genes, identify genes involved in viral pathogenesis, and test the effects of drugs or inhibitors. Similarly, by using host gene microarrays, one can explore host response at the level of gene expression and provide a molecular description of the events that follow the viral infection (Cummings and Relman, 2000). Firstly, DNA microarrays have been successfully used to study the transcriptional profiles of variety of viruses, including plant (Pasquini et al., 2007), insect (Yamagishi et al., 2003), mammalian (Martinez-Guzman et al., 2003; Paulose-Murphy et al., 2001; Stingley et al., 2000) and aquatic viruses (Lua et al., 2005; Marks et al., 2005). Secondly, they have focused on the host's transcriptional profiles following viral infections (Bigger et al., 2001; Cuadras et al., 2002; Jones and Arvin, 2003; Otsuka et al., 2003). Viruses used as models in DNA microarray technology-based studies are summarized in Table 3.

Basically, the DNA microarray technology has been applied to explore the transcriptional profiles of viruses during an infection or under different conditions, such as different replication stages, drug treatments/inhibitors and toxicity. Transcription of HCMV genome was measured during infection by using a microarray of 75-mer oligonuleotides representing each of the 226 predicted HCMV ORFs (Chambers et al., 1999). By blocking *de novo* viral protein synthesis and viral DNA replication, the authors revealed a detailed classification of HCMV genes into four temporal kinetic classes, and also assigned many ORFs, for which expression data were not previously available, into

Table 3. Viruses used as models in DNA microarray technology-based studies

Virus	References
Autographa californica multiple nuleopolyhedrovirus (AcMNPV)	(Yamagishi et al., 2003)
Coxsackievirus B3	(Taylor et al., 2000)
Echovirus 1 (EV1)	(Pietiainen et al., 2000)
Hepatitis B and C viruses	(Otsuka et al., 2003)
Hepatitis C virus (HCV)	(Bigger et al., 2001)
Herpes simplex virus type 1 (HSV-1)	(Mossman et al., 2001; Stingley et al., 2000)
Herpesvirus of turkeys (HVT)	(Karaca et al., 2004)
Human cytomegalovirus (HCMV)	(Chambers et al., 1999)
Human herpesvirus 8 or Kaposi'sarcoma-associated herpesvirus (HHV-8 or KSHV)	(Jenner et al., 2001; Nakamura et al., 2003; Paulose-Murphy et al., 2001)
HIV-1	(de la Fuente et al., 2002; Geiss et al., 2000; van 't Wout et al., 2003)
Hirame rhadovirus (HRV)	(Kurobe et al., 2005)
Marek's disease virus (MDV)	(Morgan et al., 2001)
Murine grammaherpesvirus-68 (MHV-68)	(Ahn et al., 2002; Ebrahimi et al., 2003; Martinez-Guzman et al., 2003)
Plum pox virus (PPV)	(Pasquini et al., 2007)
Red seabream iridovirus (RSIV)	(Lua et al., 2005)
Rotavirus	(Cuadras et al., 2002)
SARS coronavirus	(Leong et al., 2005)
Varicella-Zoster virus (VZV)	(Cohrs et al., 2003; Jones and Arvin, 2003)
Viral hemorrhagic septicemia virus (VHSV)	(Byon et al., 2005)
White spot syndrome virus (WSSV)	(Khadijah et al., 2003; Liu et al., 2005; Marks et al., 2005; Tsai et al., 2004)
Zaire Ebolavirus (ZEBOV), Reston Ebolavirus (REBOV) and Marburgvirus (MARV)	(Kash et al., 2006)

these groups. Similarly, the temporal kinetic transcriptional profiles of RSIV in cell culture in the presence or absence of *de novo* protein synthesis and viral DNA replication inhibitors have already been described (Lua et al., 2005). In other studies, viral DNA microarrays were used to compare the expression of viral genes during latency and lytic replication (Cohrs et al., 2003; Ebrahimi et al., 2003; Jenner et al., 2001; Martinez-Guzman et al., 2003). These studies provide a better understanding of viral gene regulation strategies, pathogenic mechanisms as well as understanding of virus-host cell interactions. In addition, DNA microarrays have also been applied to investigate the correlation in expression patterns between unknown and known genes under the same conditions. Because virulence-associated genes are often tightly and coordinately regulated, candidate virulence factors are specifically expressed during infection and likely to be co-regulated with known ones. Thus, such studies allow for the determination of virulence-associated genes and for predicting their functions. Moreover, gene expression studies based on the DNA microarray technology may also reveal key regulatory differences that lead to differing virulence between closely related pathogen strains or that account for differences in viral replication between different targeted–cells/organs of viruses (Cummings and Relman, 2000). A comparison of expression profiles of known ORFs in AcMNPV by using a viral DNA microarray (Ac-BmNPV chip) pointed up that most of genes involved in the viral life cycle were regulated differently in two different cell lines, implying the different expression of these viral genes accounts for the differences in viral replication between various targeted-cells (Yamagishi et al., 2003).

On the other hand, the DNA microarray technology is also involved in exploring host cell transcriptional changes in response to virus infections (Bigger et al., 2001; Geiss et al., 2000; Jones and Arvin, 2003). These studies promise to accelerate our understanding of the host side of the host-pathogen interaction, contributing to annotation of pathogenesis as well as host response to viral infections. The first application of DNA microarrays to pathogenesis was to monitor global gene expression in primary human fibroblasts infected by HCMV (Zhu et al., 1998). Among 6,600 spotted human genes, 258 transcripts changed by more than fourfold compared to uninfected cells at either 8 or 24 hours after infection. Some of these changes, such as induction of cytokines, stress-

inducible proteins, and many interferon-inducible genes were consistent with induction of cellular immune responses. By identifying factors expressed in the host under vaccination or stimulation, the DNA microarrays promise to identify vaccine targets, provide a better understanding of host defensive and virus-counter-defensive mechanisms, and contribute guidelines for antiviral therapies (Byon et al., 2005; Kurobe et al., 2005; Yasuike et al., 2007). In addition, a comparison of host responses to related strains of the same virus or family can explain differences in pathogenesis. Comparison of gene expression responses in human liver cells infected with Zaire Ebolavirus (ZEBOV), Reston Ebolavirus (REBOV) and Marburgvirus (MARV) revealed different mechanisms involved in antagonizing interferon (IFN) signaling pathways by the different members of the family *Filoviridae* (Kash et al., 2006).

Recently, to address the limitations of existing viral detection methodologies, DNA microarrays derived from highly conserved sequence regions within viral families have been utilized as diagnostic tools (Clewley, 2004; DeFilippis et al., 2003). Using available sequence data from more than 140 sequenced viral genomes, DeRisi and colleagues designed a long oligonuleotide (70-mer) DNA microarray with the potential to simultaneously detect hundreds of viruses, including essentially all respiratory tract viruses (Wang et al., 2002). Plant viral DNA microarrays were similarly constructed to detect plum pox virus (PPV) and its strains (Pasquini et al., 2007). Historically, standard viral detection techniques relied on isolation and *in vitro* viral culture or immunoassays or even PCR methods can be used to determine the presence or absence of the virus (Storch, 2000). However, in more complex biological situations, such as diseases where many different viruses are present or where no etiologic agent has been identified, the limitations of these methodologies become readily apparent. Some viruses are completely refractory to *in vitro* culture, and immunoassays depend on the quality and availability of the antiserum while PCR methods depend on the specificity of primers (Wang et al., 2002). Thus, the potential use of this technology in routine diagnostics is versatile and greatly expands the spectrum of detectable viruses in a single assay while simultaneously providing the capability to discriminate among viral subtypes. Such a diagnostic tool will undoubtedly have many uses in the study of viral pathogenesis and perhaps equally important has the potential to facilitate novel viral discovery and identification of

diseases of unknown etiology as well as in instances of bioterrorism. Currently, the DNA microarray technology has even known as highly parallel microbial diagnostic microarrays (MDMs) because they have allowed rapid and simultaneous identification of many different microorganisms, not only viruses in a single assay. Recent applications in the MDM field were comprehensively recorded in a review by Loy and Bodrossy (Loy and Bodrossy, 2006).

1.6 RNA Interference (RNAi) technology

1.6.1 Background

RNA interference (RNAi) was initially characterized in the nematode worm *Caenorhabditis elegans* by Fire and colleagues, who found that double-stranded RNAs (dsRNAs) induced a more potent sequence-specific silencing response than single-stranded antisense RNA alone, which was customarily used for this purpose (Fire et al., 1998). It soon turned out that RNAi is not restricted in nematode and can be induced in fruitfly *Drosophila melanogaster* (Kennerdell and Carthew, 1998), parasites *Trypanosoma* (Ngo et al., 1998), fungi *Neurospora crassa* (Romano and Macino, 1992), flowering plant *Arabidopsis thaliana* (Napoli et al., 1990; van der Krol et al., 1990), and animals (Elbashir et al., 2001). These phenomena were termed "post-transcriptional gene silencing" (PTGS) and "co-suppression" in plants, "quelling" in fungi, and "sequence-specific gene silencing" in animals. They are phenotypically different but mechanistically similar forms of RNAi (Agrawal et al., 2003). The core of all these phenomena is the processing of long, double-stranded (ds)RNAs by the RNAse III enzyme Dicer into small 21-25 bp dsRNAs molecules, designated small interfering RNAs (siRNAs) and microRNAs (miRNAs) (Dykxhoorn et al., 2003; Elbashir et al., 2001; Hutvagner and Zamore, 2002; Li and Ding, 2005). These small dsRNAs provide sequence specificity to the related RNA-induced silencing complexes (RISCs), termed siRISC and miRISC, respectively, and serve as guides for cleavage or inhibition of translation of mRNAs that share sequence similarity with the dsRNA trigger.

In the nematode *C. elegans*, the first metazoan in which RNAi was documented, silencing can be experimentally induced by injection, feeding or transgenic expression of dsRNA molecules (Fire et al., 1998; Grishok, 2005; Grishok et al., 2005). The systemic

nature of RNAi in *C. elegans* is a striking example of the ability of cells to detect and internalize extracellular dsRNA to initiate intracellular gene silencing events. A number of cells can naturally take-up exogenous dsRNA and use it to initiate RNAi silencing (Clemens et al., 2000; Worby et al., 2001). Thus, RNAi has been widely employed to manipulate gene expression, elucidate signal pathways and identify gene functions (Agrawal et al., 2003; Haasnoot et al., 2003). Insurmountable evidences also pointed out the efficiency of RNAi in mounting antiviral responses.

1.6.2 Small interfering RNA (siRNAs) and microRNA (miRNAs) molecules: definition, origin and functionality

Small molecules of dsRNAs, mediators of RNA-related gene silencing mechanisms, are classified into siRNAs and miRNAs based on their origin and function (Tang, 2005; Zheng et al., 2005).

siRNAs are small RNAs that are produced in the cytoplasm by Dicer-mediated cleavage of long double-stranded RNA that has been formed by base pairing between two independent transcripts (Sarnow et al., 2006). The long dsRNAs are normally produced from foreign genomes, such as virus infections or transposons. In the cytoplasm of cells, siRNAs are incorporated into RISC containing siRNA (siRISC) for cleavage of the target mRNA with perfect complementary in sequence (Sledz and Williams, 2005) (Fig. 3).

In contrast to siRNAs, miRNAs are small RNAs that are produced by Drosha- and Dicer-mediated cleavage of RNA-hairpin structures that are encoded in cellular and viral genomes (Sarnow et al., 2006). miRNAs are most often transcribed by RNA polymerase II and the resulting primary miRNAs (pri-miRNAs) are processed in the nucleus by the RNAse type III Drosha to produce precursor miRNAs (pre-miRNAs). Pre-miRNAs are then exported to the cytoplasm by Exportin-5 and processed into mature miRNAs through the action of Dicer. miRNAs are incorporated into RISC containing miRNA (miRISC) and guide for their mRNA targets. Unlike siRNAs, miRNA-armed RISC can enforce either degradation of mRNA (in the case of perfect sequence complementarity) or inhibition of mRNA translation (in the case of imperfect sequence complementarity) (Sullivan and Ganem, 2005; Triboulet et al., 2007) (Fig. 3).

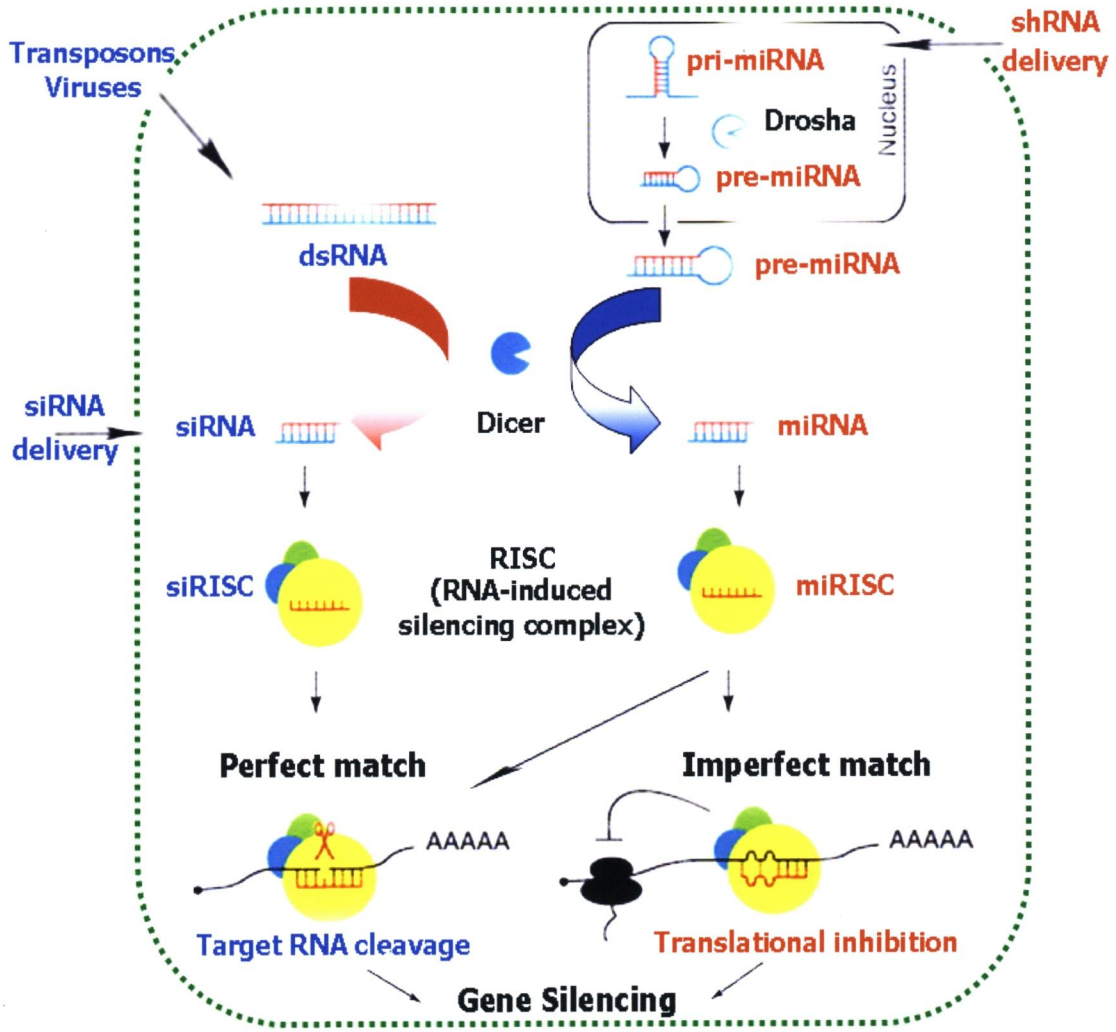

Fig. 3. RNA interference (RNAi) pathway (Adapted from TRENDS in Biotechnology Vol.24 No.4 (2006))

After the milestone discovery of the first miRNA in 1993 (Miska, 2005), hundreds of miRNAs are discovered in nematodes, fruit flies and humans, and it comes as no surprise that viruses, which typically employ many components of the host gene expression machinery, also encode miRNAs (Kim and Nam, 2006). To date, 73 viral miRNAs have been identified in genomes of 7 viral species, including EBV, KSHV, HCMV, MHV68, HSV1, SV40 and rhesus lymphocryptovirus (rLCV) (Pan et al., 2007). It is believed that many miRNAs are ubiquitously expressed, whereas others are expressed in a cell-type-specific manner. A single miRNA can target transcripts from multiple genes and, conversely, several miRNAs can control a single target. Therefore, miRNAs and their targets function as a complex regulatory network (Nair and Zavolan, 2006). miRNAs

influence diverse cellular processes ranging from embryonic development, cellular differentiation, proliferation, apoptosis and metabolism to cancer. A recent study showed that cellular miRNAs have direct antiviral effects in addition to their regulatory functions (Lecellier et al., 2005). In particular, as reviewed by Nair and Zavolan (Nair and Zavolan, 2006), virus-encoded miRNAs are involved both in the regulation of their own genes and in the subversion of cellular defense mechanisms.

1.6.3 Application of RNAi technology in virological studies and RNA-based antiviral approaches

Significant advances in the RNAi technology, together with the completion of several viral genome sequences, paved the way for new approaches in studying molecular virology. It has been applied to study disease-related genes and elucidate their roles in the viral cycle and in virus-host cell interactions as well as to investigate cellular pathways involved in viral pathogenesis (Colbere-Garapin et al., 2005).

RNAi-based option is considered to be of tremendous potential as an antiviral approach against a wide variety of viruses in plants, invertebrate and vertebrate systems. In plants, RNA silencing in the context of viral infections has been extensively studied and is well established that it functions as adaptive antiviral immune response, although viruses have evolved strategies to suppress the RNA-silencing pathways as a counter-defense (van Rij and Andino, 2006). In insects, RNAi pathway was reported to direct innate immunity against viruses. An evidence showed that flock house virus (FHV), a member of the *Nodaviridae* family, is targeted by the RNAi machinery in the insect *Drossophila* (Wang et al., 2006). Furthermore, several studies have established that RNAi-based antiviral approach protects insects from viral infection (Keene et al., 2004; Sanchez-Vargas et al., 2004). Introduction of dengue virus genomic sequences with a Sindbis virus vector into cells of the mosquito *Aedes albopictus* resulted in RNAi-based resistance to subsequent challenges with dengue virus (Sanchez-Vargas et al., 2004). Current reports revealed the existence of both innate (non sequence-specific) and RNAi-related (sequence-specific) antiviral phenomena in other invertebrates, such as crustacean, as reviewed by Robalino et al. (Robalino et al., 2007).

In vertebrate system, it has long been thought that dsRNA, a common virus-associated molecular pattern, is a potent inducer of non-specific immune mechanism involving interferon response, implying RNAi ineffectivity for gene sequence-specific silencing in interferon-producing organisms like mammals (Dykxhoorn et al., 2003; Robalino et al., 2005; Schott et al., 2005). However, the pioneering work by Elbashir et al. (Elbashir et al., 2001) showed that 21-22 nucleotide siRNAs are able to silence genes in a sequence-specific manner, considered as a breakthrough in RNAi studies. This was followed by an outburst of reports that dsRNAs of less than 30 nucleotides bypass the IFN-induced pathways and evoke RNAi-type silencing (Caplen et al., 2001; Dave and Pomerantz, 2003). Thus, RNAi-like mechanisms are also believed to act as an antiviral defense in vertebrates (Browne et al., 2005; Karpala et al., 2005; Li and Ding, 2005) and can be induced through the introduction of naked siRNAs and plasmid expressing short-hairpin (sh)RNAs or pre-miRNAs into cells from the outside (Elbashir et al., 2001; McManus et al., 2002). Plasmid-based expression systems using RNA polymerase II (Pol II) or III (Pol III) promoters have been commonly used (Dykxhoorn et al., 2003). A model of expression vector using Pol II promoter, Block-iT Pol II miR RNAi expression vector with EmGFP, is shown in Fig. 4. This vector is designed to allow cloning ds oligo duplexes encoding a pre-miRNA target sequence and permit high-level expression of the pre-miRNA through co-cistronic expression of EmGFP in most mammalian cells. The expression vector includes flaking and loop sequences from an endogenous murine miR-155 which allow proper processing of the engineered pre-miRNA sequence. Additionally, the vector also contains resistance genes for selection of positive clones in *E. coli* and stable cell lines that stably expressing the miRNA (Invitrogen, Catalog no. K4936-00).

Introduction of naked viral-gene specific siRNAs or plasmid expressing virus-derived miRNAs into cells may lead to inhibit viral gene expression and block viral replication (Zheng et al., 2005). When naked viral-gene specific siRNAs are delivered to cells, they directly guide siRISC without processing by Dicer to their homologous target mRNAs for cleavage. On the other hand, the use of plasmid expressing virus-derived shRNAs or pre-miRNAs is the dependency on Dicer processing. Virus-derived shRNAs or pre-miRNAs are expressed by nucleus enzymes and processed by Dicer into mature

miRNAs prior to being guided to miRISC for cleavage or repression of translation of mRNAs (Dykxhoorn et al., 2003).

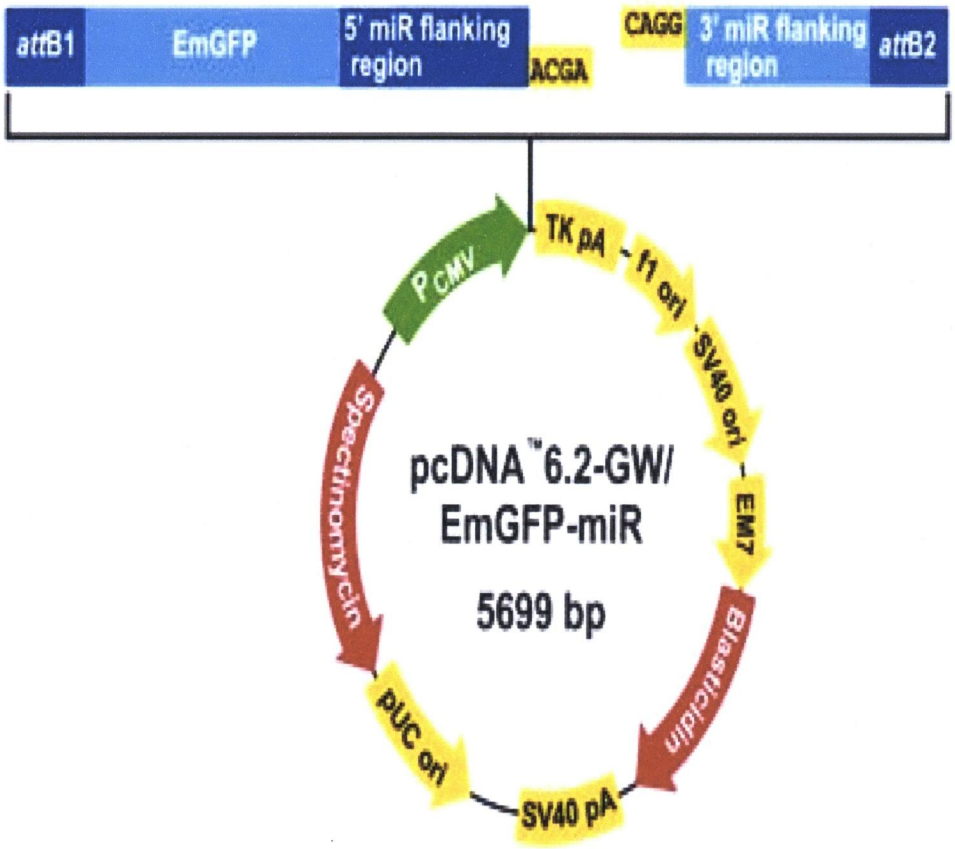

Fig. 4. Model of BLOCK-iT Pol II miR RNAi Expression vector

Roles of viral gene-specific siRNAs and virus-derived pre-miRNAs as antiviral tools are proposed in Fig. 5 and Fig. 6, respectively. Thus, RNAi has been currently considered as a gene-specific therapeutic option for controlling viral infections in many ways, such as, inhibiting the expression of viral antigens and accessory genes, controlling the transcription of viral genome, hindering the assembly of viral particles or blocking viral replication (Tan and Yin, 2004). RNAi mediated by artificial introduction of viral-specific siRNAs or shRNAs has been successfully used to inhibit viral replication of mammalian viruses both in a cell culture system and an experimental animal model, although the outcome of individual RNAi events varies depending on the designed RNAi and their target regions, as well as the escape from RNAi-mediated suppression of viruses through mutations within the targeted regions (Zheng et al., 2005). Poliovirus was one of

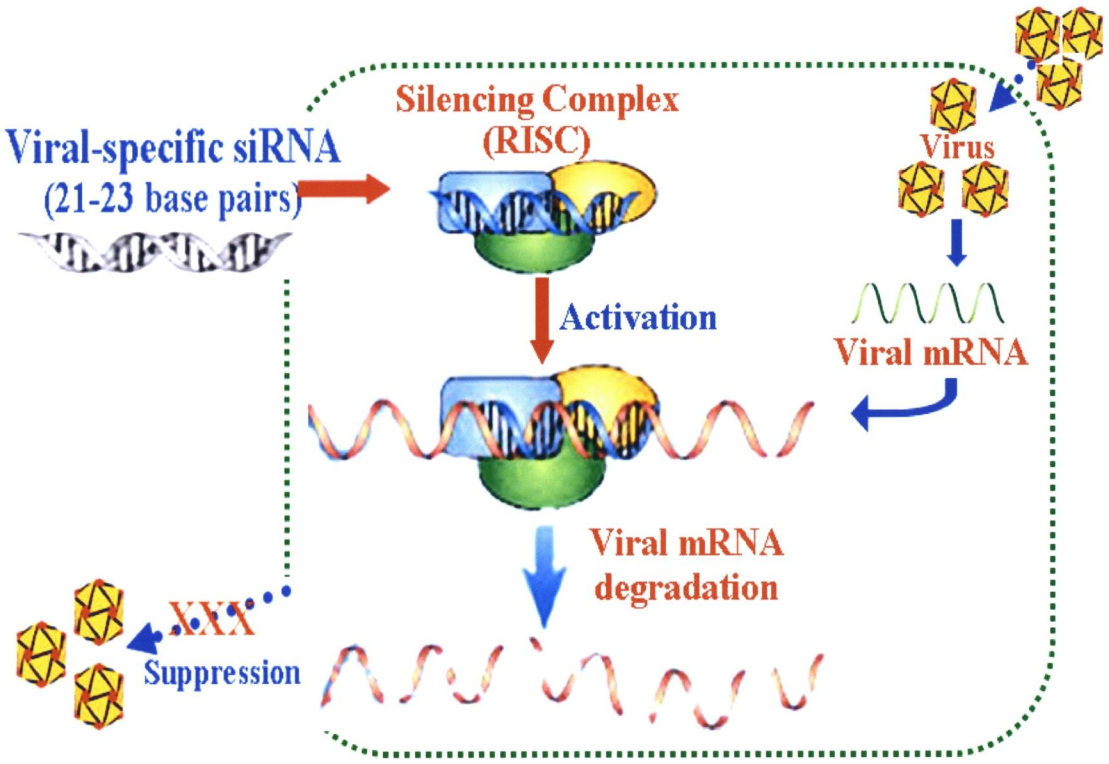

Fig. 5. Proposed role of viral gene-specific siRNA as an antiviral tool

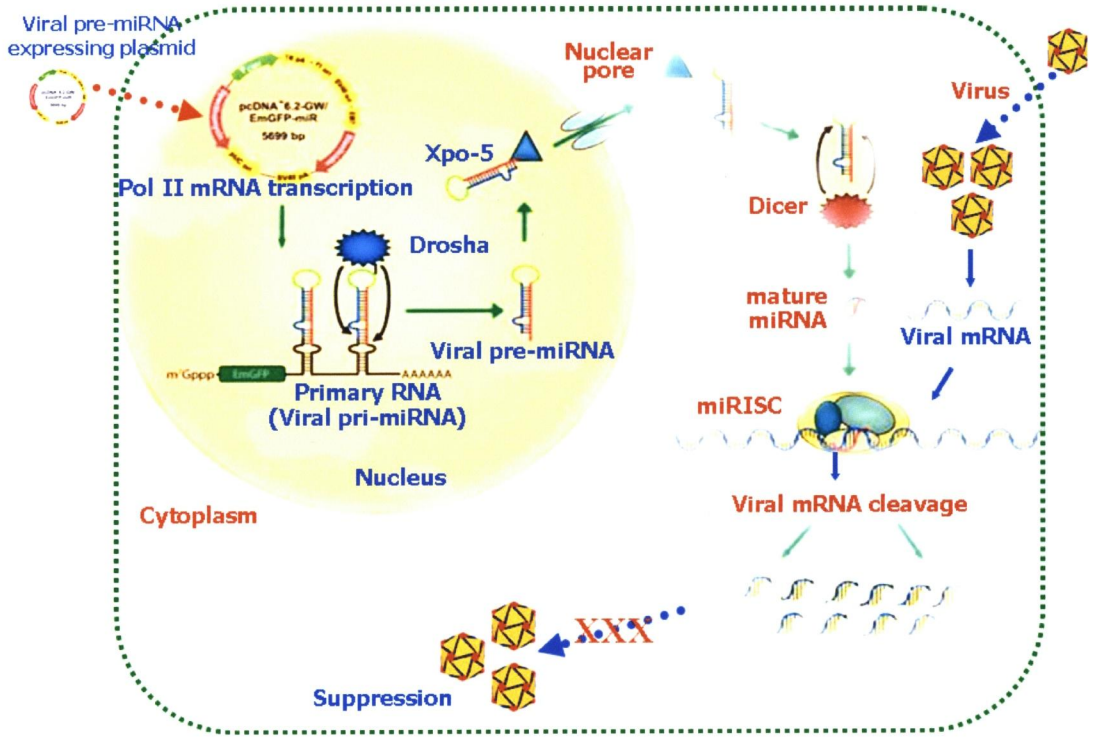

Fig. 6. Proposed role of viral gene-specific miRNA as an antiviral tool using plasmid-based expression system

the first to be tested for siRNA-mediated sequence-specific inhibition. HeLa cells transfected with an siRNA specific for a capsid protein or viral polymerase before infection with Poliovirus markedly reduced the titer of virus progeny (Gitlin et al., 2002). Studies on HIV-1 inhibition by RNAi showed that almost a complete inhibition of HIV-1 production was obtained by the combined expression of siRNAs against Tat and Rev genes (Lee et al., 2002), and HIV-1 production in T-lymphocytes expressing shRNAs targeting Nef gene was reduced to more than 10-fold, and virus replication was inhibited upto 1,000-fold 20 days post-infection (Haasnoot et al., 2003). siRNA molecules targeted to different genome segments of influenza virus, a virus responsible for acute upper respiratory disease causing an estimated 500,000 deaths each year, were also tested and shown that siRNA effectively inhibited virus replication not only *in vitro*, but also in mice *in vivo* (Stram and Kuzntzova, 2006). Various mammalian viruses were targeted for RNAi-based antiviral studies both *in vitro* and in animal models (Table 4 and 5). Almost all these studies have used synthesized viral gene-specific siRNAs or plasmids expressing viral-derived shRNAs, which were introduced into cells either shortly before or after challenge with viruses.

In aquatic animal viruses, applications of RNAi-based antiviral approaches have been ill-studied but this strategy has been reported to be effective against two marine crustacean viruses, including White Spot Syndrome Virus (WSSV) and Yellow Head Virus (YHV), and two marine fish viruses, including Tiger Frog Iridovirus (TFV) and Viral Hemorrhagic Septicemia Virus (VHSV) (Table 6). Using dsRNA specific for WSSV, a model of antiviral immunity in shrimp was proposed, by which viral dsRNA engages not only innate immune pathways but also an RNAi-like mechanism to induce potent antiviral responses *in vivo* (Robalino et al., 2005). Similarly, YHV-specific dsRNAs were effectively inhibited YHV replication in both shrimp primary cell culture and in the shrimp *Penaeus monodon* (Tirasophon et al., 2005; Yodmuang et al., 2006). Unlike aquatic invertebrates such as shrimp, virus-specific siRNAs, instead of using virus-specific long dsRNAs, were used in RNAi-based antiviral approaches in aquatic vertebrates such as frog and fish.

Table 4: RNAi-mediated suppression of mammalian virus replication in cell culture system

Mammalian RNA virus	**RNAi**	**References**
Avian metapneumovirus (AMPV)	siRNA	(Ferreira et al., 2007)
Coxsackievirus B3	siRNA/shRNA	(Kim et al., 2007; Yuan et al., 2005)
Enterovirus 71 (EV71)	siRNA	(Sim et al., 2005)
Foot-and-mouth disease virus	shRNA	(Chen et al., 2004)
Hepatitis A virus	siRNA	(Kanda et al., 2004; Kanda et al., 2005)
Hepatitis C virus	siRNA/shRNA	(Kronke et al., 2004; Yokota et al., 2003)
Hepatitis delta virus	siRNA	(Chang and Taylor, 2003)
HIV-1	siRNA/shRNA	(Capodici et al., 2002; Huelsmann et al., 2006; Novina et al., 2002)
Human parainfluenza virus-3	siRNA	(Barik, 2004)
Human rhinovirus 16	siRNA	(Phipps et al., 2004)
Influenza A virus	siRNA/shRNA	(Ge et al., 2003; McCown et al., 2003)
Poliovirus	siRNA	(Gitlin et al., 2002)
Respiratory syncytial virus	siRNA	(Bitko and Barik, 2001)
Rotavirus	siRNA	(Dector et al., 2002)
SARS-associated CoV	siRNA	(He et al., 2006; Wang et al., 2004; Wu et al., 2005; Zhao et al., 2005)
Vaccinia virus	siRNA	(Dave et al., 2006)
Venezuelan equine encephalitis virus (VEEV)	siRNA	(O'Brien, 2007)
Vesicular stomatitis virus	siRNA	(Barik, 2004)
West Nile virus	shRNA	(McCown et al., 2003)

Table 5: Use of RNAi in *in vivo* animal models of virus infections

Virus	RNAi	Method of delivery	References
SAR-associated CoV	siRNA	Intramuscular injection	(Zhao et al., 2005)
West Nile Virus	siRNA	Hydrodynamic injection	(Bai et al., 2005)
Foot and mouth disease virus	shRNA	Subcutaneous injection	(Chen et al., 2004)
Respiratory syncytial virus	siRNA/shRNA	Intranasal delivery	(Bitko et al., 2005; Zhang et al., 2005)
Parainfluenza virus	siRNA	Intranasal delivery	(Bitko et al., 2005)
Influenza A virus	siRNA/shRNA	Intravenous injection	(Ge et al., 2004)
	siRNA	Hydrodynamic injection, intranasal delivery	(Tompkins et al., 2004)
Hepatitis B virus	siRNA/shRNA	Hydrodynamic injection	(Giladi et al., 2003; McCaffrey et al., 2003)
	siRNA/shRNA	Intravenous injection	(Morrissey et al., 2005; Uprichard et al., 2005)

Table 6: RNAi-mediated suppression of aquatic animal virus replication

Virus	RNAi	Method of delivery	References
Tiger frog iridovirus (TFV)	siRNA	Transfection (cell culture)	(Xie et al., 2005)
Viral hemorrhagic septicemia virus (VHSV)	siRNA	Transfection (cell culture)	(Schyth et al., 2006)
Yellow head virus (YHV)	dsRNA	Transfection (primary cell culture) or injection (shrimp)	(Tirasophon et al., 2005; Yodmuang et al., 2006)
White spot syndrome virus (WSSV)	dsRNA	Injection (shrimp)	(Robalino et al., 2005)

The inhibition of TFV replication by siRNAs targeting the MCP gene was demonstrated by reduced MCP RNA level, postponed emergence of cytopathogenic effect, as well as reduced virus titer and particles in fish cells (Xie et al., 2005). Consistent with the activation of RNA by siRNAs, siRNAs specific to the viral glycoprotein gene of VHSV efficiently inhibited viral replication in infected cell cultures, while the corresponding mismatched siRNAs did not exhibited this antiviral effect (Schyth et al., 2006). Such studies significantly establish RNAi as a potential approach to therapy of viral diseases in aquaculture.

1.7 Objectives and contents of the study

Disease problems particularly of viral origin have continuously plagued the aquaculture industry due to rapid intensification of farming practices. Moreover, the control of viral infection is difficult and complex because of the limited effectiveness of existing antiviral agents and the high speed mutation rate of the viral genome (Lee and Rossi, 2004). Toward this end, understanding the pathogenic mechanisms of viral infections at the molecular level and the development of alternative antiviral approaches based on advances in molecular genetic technology will be of enormous contribution to the control of viral diseases.

This study aimed to better understand of the molecular pathogenic mechanisms of viral infections in fish using RSIV, a marine-pathogenic iridovirus, as a model based on DNA microarray technology, and to develop alternative approaches against iridoviral infections using RSIV-specific siRNAs and miRNAs as antiviral tools based on RNAi technology, instead of vaccination technology. Specific objectives of the study are:

1. to explore the transcriptional profile of RSIV over the time-course of virus infection in a fish model
2. to investigate differential gene expression patterns between RSIV-infected spleen and kidney in order to find out a suitable organ for diagnosis of iridoviral diseases at the molecular level
3. to generate and test the potential antiviral activity of viral gene-specific siRNAs on RSIV replication in a cell culture system

4. to generate and test the potential antiviral activity of virus-encoded miRNAs on RSIV replication in a cell culture system

The contents of this research study are:

1. Chapter 1 (General introduction) provides the background on global views of the research study.
2. Chapter 2 (The pathogenesis of RSIV infection as revealed by viral DNA microarrays) provides understanding of the molecular pathogenic mechanism of RSIV infection in fish and further confirms, at the molecular level, that the spleen is a suitable organ for diagnosis of iridoviral diseases, resulting in the thorough knowledge of iridovirus infection in fish.
3. Chapter 3 (Inhibition of RSIV replication by viral gene-specific siRNAs in a cell culture system) evaluates anti-RSIV activity of viral gene-specific siRNAs on RSIV replication in vitro, providing a promising alternative antiviral approach based on siRNA-related pathways.
4. Chapter 4 (Engineered virus-encoded pre-microRNA (pre-miRNA) induces sequence-specific antiviral response in addition to non-specific immunity in a fish cell line: Convergence of RNAi-related pathways and IFN-related pathways in antiviral response) investigates antiviral activity of viral-encoded microRNAs (miRNAs) on viral replication *in vitro*, providing another option for combating iridoviral infections utilizing the antiviral potency of miRNA-related pathways, as well as better understanding the complex miRNA-related mechanisms.
5. Chapter 5 (General conclusion and perspectives) provides a comprehensive discussion of the proceeding studies, and further discuss perspectives for the use of the antiviral potency of RNAi to control of viral diseases in aquaculture.

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CHAPTER 2

THE PATHOGENESIS OF RED SEABREAM *IRIDOVIRUS* (RSIV) INFECTION AS REVEALED BY VIRAL DNA MICROARRAYS

Abstract

Red seabream iridovirus (RSIV) disease is a serious disease of many marine fish species in Japan and elsewhere. To better understand the molecular pathogenic mechanism, we examined the transcriptional profile of RSIV in infected fish using a DNA microarray. Expression of RSIV open reading frames (ORFs) was first detected at about 5 days post-infection (d.p.i.), and accounted for about 45% of total ORFs. Almost all the ORFs (97%-99%) were expressed at their maximum levels during 7-9 d.p.i. The expression levels and the number of expressed ORFs started to decrease at 10 d.p.i. These results suggest that pathogenesis of RSIV infection began at around day 5, and continued with high levels of viral multiplication until viral clearance by host antiviral defenses starting from around 10 d.p.i. A comparison of viral gene expression patterns in the spleen and kidney over the course of the infection clearly provides a better understanding of the viral pathogenesis in different host cell types, and further suggests that RSIV preferentially targets the spleen. The spleen may thus be a susceptible organ for diagnosis of iridoviral disease.

Keywords: DNA microarrays, RT-PCR, iridoviruses, RSIV, pathogenesis.

2.1 Introduction

Systemic iridoviral infections have caused high mortality in cultured freshwater and marine fish species in many parts of the world (Chao et al., 2004; Iwamoto et al., 2002; Qin et al., 2003). Outbreaks of these diseases have been reported in Australia, France, Germany, Finland, Denmark, United State, Taiwan, Southeast Asia and Japan (Huang et al., 2004). In Japan, an iridoviral disease has been recorded in at least 31 marine fish species (Kawakami and Nakajima, 2002). The iridoviral pathogen was first isolated from red seabream (*Pagrus major*) in 1992 and named red seabream iridovirus (RSIV), and thus the disease was called as red seabream iridoviral disease (RSIVD) (Inouye et al., 1992). RSIV-infected fish showed disease symptoms from 5 days of infection, and mortality commenced at day 6 and increased up to 90% at day 9 (Oshima et al., 1998). The infected fishes displayed enlarged cells in spleen, kidney, liver and gills (Inouye et al., 1992). A similar disease has recently been documented in some other Asian countries (Chao et al., 2002; Chou et al., 1998; Do et al., 2004; Jeong et al., 2003; Mahardika et al., 2004; Wang et al., 2003). Due to the devastating effects of this pathogen to marine aquaculture, various rapid and sensitive diagnostic methods, as well as some control strategies have been developed for RSIV (Caipang et al., 2004; Caipang et al., 2003; Caipang et al., 2006; Jeong et al., 2004; Kurita et al., 1998; Nakajima, 1995; Oshima et al., 1998; Oshima et al., 1996). However, little is known about the pathogenic mechanism and the most susceptible organ to iridoviral infection in molecular detail. Gene expression is a molecular indicator for the annotation of mechanisms underlying normal cellular processes as well as the molecular basis for disease (Clewley, 2004). Therefore, studies of RSIV gene expression patterns are necessary and useful for understanding of the viral molecular pathogenic mechanism, and hence importance of DNA microarray technology, a powerful tool of examining gene expression.

The uses of DNA microarray technology in molecular virology have been reviewed (Clewley, 2004; Cummings and Relman, 2000; Harrington et al., 2000; Ye et al., 2001). Its potential benefits are able to accelerate the annotation of various aspects of both sides of complex virus-host interactions in molecular detail. Viral gene-derived DNA

microarrays provide a better understanding of the viral DNA replication and gene expression strategies; identify virulence-associated genes and their roles involved in viral pathogenesis; and provide possible clues for the pathogenesis of the virus. Moreover, the DNA microarrays derived from highly conserved sequence regions within viral families have been currently utilized as promising diagnostic tools (Pasquini et al., 2007; Wang et al., 2002). On the other hand, host gene-derived DNA microarrays provide a better understanding of host response at the level of gene expression and provide a molecular description of the events that follow the viral infection, as well as explain differences in pathogenesis between related strains of the same virus or family (Cummings and Relman, 2000; Kash et al., 2006).

This technology is well suited for genome-wide transcription studies, and is especially useful for determining the complex transcriptional program of large DNA viruses (Chambers et al., 1999; DeFilippis et al., 2003). It has been successfully used to explore viral gene expression patterns of mammalian and aquatic viruses at different replication stages of viral life cycle or under different conditions both in cell culture systems (Ahn et al., 2002; Chambers et al., 1999; Ebrahimi et al., 2003; Lua et al., 2005; Paulose-Murphy et al., 2001) and experimental animal models (Liu et al., 2005; Marks et al., 2005; Martinez-Guzman et al., 2003; Rochford et al., 2001; Tsai et al., 2004). It has also been used to explore the variability in transcriptional activity for every gene between closely related pathogen strains or between different targeted cells/organs of viruses (Cummings and Relman, 2000). By using a DNA microarray of baculovirus genomes (Ac-BmNPV chip), differential gene expression of autographa californica multiple nucleopolyhedrovirus (AcMNPV) involving in the viral life cycle were revealed between two susceptible insect cell lines, implying the different expression of these viral genes accounts for the differences in viral replication between different targeted-cells (Yamagishi et al., 2003).

We used RSIV as a model to explore the iridoviral transcriptional profiles during the course of an infection both in cell culture and fish model studies by using viral DNA microarrays. Our previous studies in a cell culture system (Lua et al., 2005) provided a better understanding of the RSIV DNA replication and gene regulation strategies.

However, another interest was to focus on the molecular pathogenic mechanisms of the virus. To this end, we examined transcriptional profiles of RSIV in infected spleen and investigated differences in viral gene expression between spleen and kidney over the time-course of an *in vivo* infection in a fish model. We have shown here, for the first time, *in vivo* genome-wide transcription program of an iridovirus by using DNA microarray technology. The present study provided a greater understanding of the molecular pathogenic mechanisms and explored, at the molecular level, a susceptible organ, for RSIV infection, and hence possible clues for early viral screening. Such studies will be of enormous contribution to the thorough of knowledge of RSIV infection and control of iridoviral diseases.

2.2 Materials and methods

2.2.1 Cell culture and virus stock

Grunt fin (GF) cells (Clem et al., 1961) were maintained at 25°C in minimum essential medium (MEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA), 100 IU/ml of penicillin, and 100 μg/ml streptomycin for virus propagation. RSIV stock was prepared in GF cells as previously described (Lua et al., 2005). The virus titer was determined using the 50% tissue culture infective dose ($TCID_{50}$) standard method (Reed and Muench, 1938).

2.2.2 In vivo virus infection and time-course sampling

Red seabream juveniles were experimentally injected with 150 μl of the RSIV inoculum (5.0×10^5 $TCID_{50}$/ml) and held in tanks supplied with running seawater at 25°C. Control fish were injected with the same volume of phosphate buffered saline. Thirty fish were sacrificed immediately after the RSIV infection and were referred to as 0 day post-infection (d.p.i.) fish. Alive fish showed lethargic, severe anemia, petechiae of gills was selected for sampling. Five fish were randomly sampled from the experimental population on each of 2, 3, 5, 7, 9, 10, and 14 d.p.i. The spleens and kidneys were removed from these fish and stored in RNAlater (Ambion, USA) according to the manufacturer's protocol.

2.2.3 Monitoring of RSIV transcriptional profiles during in vivo infection

To examine changing RSIV gene expression levels in infected organs, total RNA was isolated from RSIV-infected spleens at times up to 14 d.p.i. and reverse transcribed to cDNA. cDNA target samples derived from time-course RNA samples were hybridized against cDNA control samples derived from 0 d.p.i. RNA samples on viral DNA microarray chips.

2.2.4 Monitoring of differentially RSIV transcriptional profiles in viral-infected spleen and kidney

To investigate whether RSIV genes were differentially expressed in different targeted-organs and to further explore a susceptible organ for RSIV infection, total RNA was extracted from both RSIV-infected spleens and kidneys at times up to 14 d.p.i. and reverse transcribed to cDNA. At each indicated time point, cDNA target samples derived from the spleens were hybridized against cDNA control samples derived from the kidneys on viral DNA microarray chips.

2.2.5 RNA preparation and labeled cDNA target construction

Total RNA was extracted from the collected spleens and kidneys with TRIzol (Invitrogen, USA) and subjected to DNase I treatment (Promega, USA) according to the manufacturers' protocols. For each time-course target sample and control sample, cDNAs were generated from 50 μg total RNA using an RSIV antisense-strand specific primer mixture. The cDNAs were first labeled with aminoallyl-dUTP using a LabelStarTMArray Kit (Qiagen, USA) and purified with a QIAquick PCR Purification Kit (Qiagen, USA) following the manufacturer's recommendations. The target and control aminoallyl-cDNAs were then coupled with Cy5- and Cy3- monofunctional dyes (Amersham Biosciences, UK), respectively, and purified with MinEluteTM Spin columns (Qiagen, USA) according to the manufacturer's instructions.

2.2.6 Construction of RSIV DNA microarray chip and microarray hybridization

The DNA microarray chips containing almost putative RSIV ORFs (92 ORFs) and control genes used in this study were prepared exactly as described by Lua et al. (2005). Briefly, RSIV ORFs fragments (approximately 300 to 1500 bp in length) were amplified by PCR using viral genome as a template and specific primer sets. All PCR products showing a single band of the appropriate size by gel electrophoresis were purified, and reconstituted in TE buffer at a final concentration of about 500 μg/ml for construction of microarray chip. Piscine β-actin genes from Japanese flounder and red seabream were included as internal controls to normalize the microarray data. In addition, distilled water was also used as a negative control. Each targeted probe was spotted in duplicate at different parts of the chips to assess the consistency of hybridization and facilitate comparison during the analysis. At each indicated time point, the Cy5/Cy3-dUTP labeled cDNAs were combined and hybridized to the microarray chips for 16-18h at 42oC. The chips were rinsed several times and finally dried following the DNA microarray method (Bowtell and Sambrook, 2002.) as modified by Lua et al. (Lua et al., 2005).

2.2.7 Statistical data analysis

The microarray chips were scanned using a GenePix 4000B array scanner and images were analyzed by GenePix Pro 4.0 array analysis software (Axon Instruments, Inc., USA). The Cy5 and Cy3 signal intensities were normalized to equilibrate them with the signal intensity of a spotted housekeeping gene (the β-actin gene) that was set to 1.

The median signal intensity, which was subtracted from the background signal, was used as an appropriate measure of absolute viral gene expression in infected spleens. Only genes exhibiting signal intensities at least two-fold greater than the signal intensities of the reference samples collected at 0 d.p.i. were used for statistical analysis.

The ratio of medians and the cut-off method were used as appropriate differential measure of viral gene expression between spleen and kidney. Genes with median ratios from 2.0 were considered as up-regulated; genes with median ratios less than 0.5 were

considered as down-regulated, and genes with median ratios ranging between 0.5 and 2.0 were considered as unchanged (Byon et al., 2005; Kurobe et al., 2005).

The significance of differences between target samples and reference (control) samples was determined with a paired *t*-test on replicated spots for each gene. *p* values of less than 0.05 were considered significant. The microarray data was also imported into the cluster program 3.0 in conjunction with an average linkage hierarchical clustering algorithm using Euclidian distance as the similarity metric. The cluster analysis allowed grouping of RSIV ORFs with similar expression patterns, and presenting expression levels of each ORF based on color-coded. After clustering, the results were visualized in a tree structure by using a tree view program (Eisen et al., 1998) .

2.2.8 Reverse Transcription (RT) – PCR

A two-step RT-PCR assay, in which cDNA is first synthesized and then used in PCR, was used to determine expression of several RSIV ORFs for confirming the microarray data. Twenty μl of cDNA was synthesized from 5 μg total RNA derived from spleens and kidneys by using M-MLV Reverse Transcriptase (Invitrogen, USA) according to the manufacturer's protocols. RT-PCR was carried out in a 30 μl reaction volume containing 1 μl cDNA using Taq polymerase. The same specific primers for each RSIV ORF used in the amplification of microarray probes were also employed here. The levels of β-actin mRNA, which are assumed to be constant over most experimental conditions and used as an internal control for microarray normalization, were also monitored by using a similar procedure. 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 sec, annealing at 55°C for 30 sec and elongation at 72°C for 1 min, and a final elongation step at 72°C for 5 min. PCR cycles were optimized to determine differences in expression of RSIV transcripts between spleen and kidney at different stages of the infection. A 23 cycle PCR was used to determine differences in expression at the high level spread stage of the infection (7-9 d.p.i.) while a 27 cycle PCR was performed to show differences in expression at the early stage (5 d.p.i.) and late stage (10-14 d.p.i.) of the infection.

2.2.9 Determination of infectious viral concentration in spleen and kidney (TCID50 assay)

RSIV was obtained from spleens and kidneys that were collected from RSIV-infected red seabream during 7 - 9 d.p.i., and was propagated in GF cells as previously described (Lua et al., 2005). $TCID_{50}$ assay was used to determine the infectious viral concentration of RSIV stocks derived from spleens and kidneys. The cells were daily observed for CPE indicative of viral replication and virus titer was calculated following the $TCID_{50}$ standard method (Reed and Muench, 1938).

2.3 Results

2.3.1 In vivo RSIV transcription program

The microarray analysis showed that no viral transcripts were detected in infected spleens at 2 and 3 d.p.i. (data not shown), but viral transcripts showed significant changes in expression from 5 d.p.i. onwards. At 5 d.p.i., 44 viral ORFs were significantly expressed, accounting for about 44.6% ($p < 0.05$) of total RSIV ORFs. Almost all (about 97% to about 99%, $p < 0.001$) of viral ORFs were significantly expressed during the period 7-9 d.p.i. (Table 1). As shown by the cluster analysis (Fig. 1), the expression levels of viral ORFs were at their maximal levels during this period, showing high levels of viral multiplication. However, the numbers and the expression levels of expressed ORFs started to decrease at 10 d.p.i. The expression of only 25% ($p < 0.05$) of the ORFs was detected at 14 d.p.i. (Table 1 & Fig. 1).

2.3.2 Differential RSIV gene expression profiles in viral-infected spleen and kidney

A comparison of RSIV gene expression profiles between viral-infected spleen and kidney at various time points following an in vivo infection, as determined by the RSIV DNA microarray analysis, were detailed in Table 2 and summarized in Table 3. No viral transcripts were detected in either spleens or kidneys at 2 and 3 d.p.i. (data not shown), but viral transcripts were detected starting at 5 d.p.i. (Table 2 & Fig. 2). As shown in detail in Table 2 or summarized in Table 3, all viral genes were expressed at higher levels or no differences in expression in the spleens when compared with those in the kidneys

throughout the infection. In particular, all viral genes were expressed at higher levels in the spleens during the period 7-9 d.p.i. At day 7 after infection, about 60% of expressed genes showed more than tenfold difference in expression between spleens and kidneys (Fig. 2).

2.3.3 Reverse transcription (RT) -PCR analysis

Seven RSIV ORFs were selected for confirming the microarray results by RT-PCR (Fig. 3). Theses ORFs included IE transcript 097R that is associated with *trans*-activation factors, E transcripts 092R, 324R, 407R and 618R that have been predicted to be involved in viral DNA replication mechanisms, and L transcripts MCP (Major capsid protein) and 291L that have been predicted to encode structural proteins of the virus. The β-actin transcript was used as an internal control.

As expected, no viral band was observed at day 2 or day 3 of the infection. Differences in expression of these ORFs between spleens and kidneys during the spreading stage of the virus (7-9 d.p.i.) were detected after both 23 and 27 PCR cycles (Fig. 3A & 3B), while the differences at the early stage (5 d.p.i.) and late stage (10-14 d.p.i.) of the infection were detected after 27 PCR cycles (Fig. 3B). The expression levels of the selected ORFs were all higher in the spleen than in the kidney. In addition, the β-actin transcript levels, as determined by RT-PCR, were similar between samples, confirming that the β-actin gene can be used to normalize the viral gene expression results across the microarrays.

Table 1. Microarray analysis of RSIV transcription program *in vivo*

No.	ORF	Putative Function	Accession No.	Signal Intensity Value[a]					Calibrated Expression Ratio[b]					Kinetic class[c]
				5 d.p.i.	7 d.p.i.	9 d.p.i.	10 d.p.i.	14 d.p.i.	5 d.p.i.	7 d.p.i.	9 d.p.i.	10 d.p.i.	14 d.p.i.	
1	ORF016L	Hypothetical protein		430	1287	728	135		0.45	2.38	1.61	0.23		L
2	ORF018R	Hypothetical protein			1177	1398	362			2.18	3.09	0.63		L
3	ORF029R	Hypothetical protein			480	209				0.89	0.46			ND
4	ORF033R	Cytosine DNA methyltransferase	AAT71861		223	157	153			0.41	0.35	0.27		E
5	ORF037R	Hypothetical protein		560	2103	1586	505		0.59	3.89	3.51	0.88		L
6	ORF042R	Hypothetical protein		1370	5346	1650	404	69	1.44	9.90	3.65	0.70	0.14	L
7	ORF049R	RING-finger-containing E3 ubiquitin ligase	AAT71876	192	2212		293	63	0.20	4.10		0.51	0.13	IE
8	ORF054R	Putative RNA guanylyltransferase	AAL98788		242	116	127			0.45	0.26	0.22		E
9	ORF063R	Largest subunit of DNA-dependent RNA polymerase	BAAE2753		505	207				0.94	0.46			E
10	ORF077R	Putative DNA-binding protein	AAT71873		746	492	258			1.38	1.09	0.45		E
11	ORF092R	Putative replication factor	AAS18131	117	3126	938	843	80	0.12	5.79	2.08	1.47	0.15	E
12	ORF097R	Hypothetical protein		471	11601	8886	5031	217	0.50	21.48	19.66	8.75	0.44	IE
13	ORF101R	Hypothetical protein			1115	1318	627	64		2.06	2.92	1.09	0.13	L
14	ORF106R	Hypothetical protein		135	1783	1384	1312	148	0.14	3.30	3.06	2.28	0.30	L
15	ORF111R	Hypothetical protein		804	13835	6500	4051	261	0.85	25.62	14.38	7.05	0.52	L
16	ORF122R	Hypothetical protein			513	608	301			0.95	1.35	0.52		L
17	ORF128R	Hypothetical protein			753	1219	291			1.41	2.70	0.51		E
18	ORF135L	Hypothetical protein		90	847	1415	802		0.09	1.57	3.13	1.39		L
19	ORF140R	Cytosine DNA methyltransferase	AAT71861		530	472	153			0.98	1.04	0.27		ND
20	ORF145R	Hypothetical protein			820	565	259			1.52	1.25	0.45		E
21	ORF151R	Hypothetical protein			387	311	169			0.72	0.69	0.29		E
22	ORF156R	Thiol oxidoreductase	AAP33193		258	232	113			0.50	0.51	0.21		E
23	ORF161L	Hypothetical protein			471	349				0.87	0.77			E
24	ORF162R	Hypothetical protein			358	269	170			0.68	0.60	0.30		E
25	ORF171R	Hypothetical protein			684	289	144			1.27	0.64	0.25		E
26	ORF179L	Hypothetical protein			223	157	180			0.41	0.35	0.31		E
27	ORF180R	Hypothetical protein		138	1171	584	187		0.15	2.17	1.29	0.33		ND
28	ORF186R	Hypothetical protein			300	283	139			0.56	0.63	0.24		L
29	ORF197L	Hypothetical protein		119	2377	1047	460	90	0.13	4.40	2.32	0.80	0.13	L
30	ORF198R	Hypothetical protein			197		103			0.36		0.19		E
31	ORF224L	RNA polymerase beta subunit	AAT71848		584	390	112			1.08	0.86	0.19		L

Table 1. Continued

No.	ORF	Putative Function	Accession No.	Signal Intensity Value[a]					Calibrated Expression Ratio[b]					Kinetic class[c]
				5 d.p.i.	7 d.p.i.	9 d.p.i.	10 d.p.i.	14 d.p.i.	5 d.p.i.	7 d.p.i.	9 d.p.i.	10 d.p.i.	14 d.p.i.	
32	ORF226R	Hypothetical protein		263	501	767	216		0.28	0.93	1.70	0.38		L
33	ORF234L	Deoxyribonucleoside kinase	AAT71846		150	191				0.28	0.42			E
34	ORF237L	Largest subunit of DNA-dependent RNA polymerase	AB018418		896	626				1.66	1.39			E
35	ORF239R	Largest subunit of DNA-dependent RNA polymerase	BAA82753		561	427	118			1.04	0.94	0.21		E
36	ORF256R	DNA repair protein RAD2	BAA82754		1057	1174	507			1.96	2.60	0.88		L
37	ORF261R	Hypothetical protein			208	106				0.39	0.23			L
38	ORF268L	Ribonucleotide reductase small subunit	BAA82755		762	818	333			1.41	1.81	0.58		E
39	ORF291L	Laminin-type epidermal growth factor-like domain	AAT71838	2477	6859	1729	561	157	2.61	12.70	3.83	0.98	0.32	L
40	ORF317L	DNA polymerase	O70736	118	963	405	120		0.12	1.78	0.90	0.21		E
41	ORF321R	DNA polymerase	AB007366		95	146				0.18	0.32			E
42	ORF324R	DNA polymerase	AB007366		870	707	415	143		1.61	1.56	0.72	0.29	E
43	ORF333L	Hypothetical protein		98	1396	870	513		0.10	2.59	1.92	0.89		IE
44	ORF342L	Hypothetical protein		141	1149	698	258		0.15	2.13	1.54	0.45		IE
45	ORF349L	Serine/threonine protein kinase catalytic domain	AAT71828	69	964	1909	766		0.07	1.79	4.22	1.33		L
46	ORF351R	Hypothetical protein			205	265				0.38	0.59			E
47	ORF353R	Hypothetical protein			668	766	465			1.24	1.69	0.81		IE
48	ORF373L	Hypothetical protein		222	1483	1164	367		0.23	2.75	2.58	0.64		L
49	ORF374R	Hypothetical protein		434	3571	6712	1752	54	0.46	15.87	14.85	3.05	0.11	L
50	MCP(380R)	Major capsid protein	BAC56968	309	1645	1464	1317	136	0.33	3.05	3.24	1.77	0.27	L
51	ORF385R	Catalytic domain of std-like phosphatase	AAT71821		411	459	204			0.76	1.02	0.35		E
52	ORF390R	Hypothetical protein			652	365				1.21	0.81			L
53	ORF394R	Hypothetical protein				215					0.48			L
54	ORF396R	Transmembrane amino acid transporter	AAT71816	190	1562	1084	401		0.20	2.89	2.40	0.70		IE
55	ORF401R	Hypothetical protein		140	611	780	251		0.15	1.13	1.73	0.44		L
56	ORF407R	ATPase	AB007367	396	5551	1952	571	138	0.42	10.28	4.32	1.17	0.28	E
57	ORF412L	ATPase	AAO15492	88	532	744	363	53	0.09	0.99	1.65	0.63	0.11	E
58	ORF413R	ATPase	AB007367		303	562	318			0.56	1.24	0.55		E
59	ORF420L	Hypothetical protein			854	362	113			1.58	0.80	0.20		L
60	ORF423L	RING-finger domain-containing protein	AAT71806		123	251				0.23	0.56			L
61	ORF424R	Putative ankyrin repeat protein	AAL98801	115	1673	786	213		0.12	3.10	1.74	0.37		E
62	ORF426R	Hypothetical protein		370	1947	983	335	421	0.39	3.61	2.17	1.45	0.85	L

Table 1. Continued

No.	ORF	Putative Function	Accession No.	Signal Intensity Value[a]					Calibrated Expression Ratio[b]					Kinetic class[c]
				5 d.p.i.	7 d.p.i.	9 d.p.i.	10 d.p.i.	14 d.p.i.	5 d.p.i.	7 d.p.i.	9 d.p.i.	10 d.p.i.	14 d.p.i.	
63	ORF430L	Putative phosphatase	AAT71837	264	5175	1696	610	85	0.28	9.58	3.75	1.06	0.17	E
64	ORF458L	Hypothetical protein		299	5939	3039	952	101	0.31	11.00	6.72	1.66	0.20	L
65	ORF463R	Hypothetical protein			707	507	120			1.31	1.12	0.21		L
66	ORF487L	Proliferating cell nuclear antigen	NP078615		424	389	108			0.79	0.86	0.19		E
67	ORF488R	Putative tumor necrosis factor receptor associated factor	AAL98835		308	277				0.57	0.61			L
68	ORF493R	D5 family NTPase	AAS18067		1244	642	215			2.30	1.42	0.37		L
69	ORF502R	Hypothetical protein			111	212				0.21	0.47			ND
70	ORF506R	Hypothetical protein		190	395	313			0.20	0.73	0.69			L
71	ORF515L	Hypothetical protein			92	106				0.17	0.23			ND
72	ORF522L	Hypothetical protein			171					0.32				L
73	ORF534L	Ankyrin repeat-containing protein	AAT71909		111	230				0.21	0.51			E
74	ORF535R	Hypothetical protein		101	1434	1844	733	122	0.11	2.66	4.08	1.27	0.25	IE
75	ORF543R	RING-finger domain-containing protein	AAT71906	501	5996	2862	1312	222	0.53	11.10	6.33	2.28	0.45	L
76	ORF550R	Hypothetical protein		388	4080	1732	749	202	0.41	7.56	3.83	1.30	0.41	L
77	ORF554R	Hypothetical protein		95	626	548	368	60	0.10	1.16	1.21	0.64	0.12	L
78	ORF562R	Hypothetical protein			1041	631	241			1.93	1.17	0.42		E
79	ORF569R	Hypothetical protein			705	470	191			1.31	1.04	0.33		E
80	ORF575R	Hypothetical protein		103	2386	1222	439		0.11	4.42	2.70	0.76		L
81	ORF586L	Hypothetical protein			463	385	209			0.86	0.85	0.36		E
82	ORF589L	Hypothetical protein			363	273				0.67	0.60			E
83	ORF591R	Hypothetical protein		171	873	674	396		0.18	1.62	1.49	0.69		IE
84	ORF596L	dsRNA-specific ribonuclease	AAT71898	85	1199	830	222		0.09	2.22	1.84	0.39		L
85	ORF600L	Hypothetical protein			283	311	128			0.52	0.69	0.22		E
86	ORF606R	Hypothetical protein		92	396	517	167		0.10	0.73	1.14	0.29		L
87	ORF617L	Hypothetical protein			258	363	125			0.48	0.80	0.22		E
88	ORF618R	Hypothetical protein			137	144				0.25	0.32			E
89	ORF628L	Hypothetical protein		224	1905	782	1027	110	0.24	3.53	1.73	1.79	0.22	E
90	ORF632L	Hypothetical protein		277	4141	1203	362	122	0.29	7.67	2.66	0.63	0.25	E
91	ORF635L	Hypothetical protein		139	2143	1201	708		0.15	3.97	2.66	1.23		IE
92	ORF641L	Putative ankyrin repeat protein	AAL98801	141	1538	576	149		0.15	2.85	1.27	0.26		E
Expressed ORFs (%)				**44.6***	**98.9****	**96.7****	**79.3****	**25***						

[a] Signal intensity value is the background-subtracted median value

[b] The calibrated expression ratio is the ratio of the expression of the RSIV ORF in viral-infected cells compared to the β-actin control gene

[c] Abbreviation: IE, Immediate-Early; E, Early; L, Late; ND, Not Detected (adapted by Lua et al. 2005)

*($p < 0.05$); **($p < 0.001$)

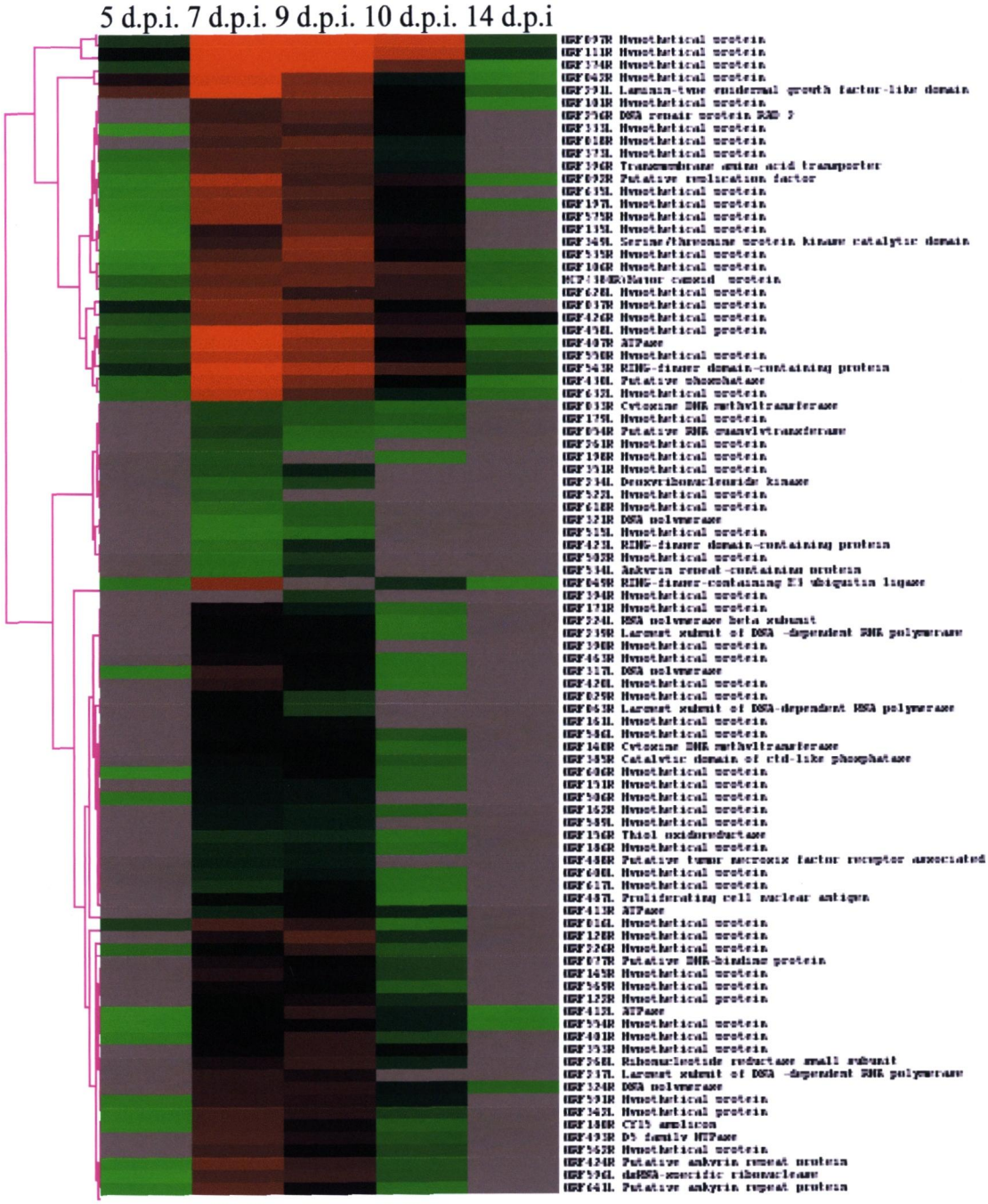

Fig. 1: Hierarchical cluster analysis of RSIV transcription program *in vivo*. Calibrated expression ratios for each ORF were categorized by an average linkage hierarchical clustering program. Each row represents the expression profile of a single ORF, and each column indicates time points after infection. The normalized expression levels across all the time points are color-coded. Green boxes indicate expression ratios lower than the mean. Red boxes indicate expression ratios greater than the mean. Black boxes indicate an intermediate level of expression and gray boxes indicate missing or not detected. The magnitude of up-regulation from the mean is shown by differing intensities of red, with deep red showing lower expression and bright red showing the highest levels of expression.

Table 2. Comparison of RSIV gene expression profiles between spleen and kidney

No.	ORF	Futative Function	Accession No.	Foldchange				
				5 d.p.i.	7 d.p.i.	9 d.p.i.	10 d.p.i.	14 d.p.i.
1	ORF 016L	Hypothetical protein		2.7	2.2	6.5	1.1	
2	ORF 018R	Hypothetical protein			15.2	3.0	1.5	1.8
3	ORF 029R	Hypothetical protein			8.5	3.9	1.4	
4	ORF 033R	Cytosine DNA methyltransferase	AAT71861	1.2	7.8	3.4	1.7	
5	ORF 037R	Hypothetical protein		3.1	3.8	5.1	2.1	1.9
6	ORF 042R	Hypothetical protein		4.3	4.5	6.6	2.4	2.5
7	ORF 049R	RING-finger-containing E3 ubiquitin ligase	AAT71876		11.6	3.5	2.3	3.5
8	ORF 054R	Putative RNA guanylytransferase	AAL98788	1.4	8.5	3.5	2.2	2.2
9	ORF 063R	Largest subunit of DNA-dependent RNA polymerase	BAA82753		4.7	2.5		
10	ORF 077R	Putative DNA-binding protein	AAT71873	4.5	9.1	2.9	2.0	
11	ORF 092R	Putative replication factor	AAS18131	3.7	15.7	3.8	2.0	3.0
12	ORF 097R	Hypothetical protein		5.9	13.1	3.8	2.6	
13	ORF 101R	Hypothetical protein		5.6	25.4	2.6	2.3	4.1
14	ORF 106R	Hypothetical protein		6.2	24.7	3.3	1.8	2.6
15	ORF 111R	Hypothetical protein		4.9	30.8	4.6	2.6	3.7
16	ORF 122R	Hypothetical protein		3.2	14.3	3.3	2.0	2.9
17	ORF 128R	Hypothetical protein		1.6	18.3	4.3	2.3	3.4
18	ORF 135L	Hypothetical protein		3.0	10.6	3.2	2.3	3.3
19	ORF 140R	Cytosine DNA methyltransferase	AAT71861		8.1	2.7	1.3	
20	ORF 145R	Hypothetical protein			13.7	3.3		
21	ORF 151R	Hypothetical protein			10.8	3.2	1.6	
22	ORF 156R	Thiol oxidoreductase	AAP33193		11.2	3.5	1.7	
23	ORF 161L	Hypothetical protein			14.8	3.1	1.6	
24	ORF 162R	Hypothetical protein			10.1	3.2	1.5	
25	ORF 171R	Hypothetical protein			15.2	3.3	1.6	
26	ORF 179L	Hypothetical protein		0.8	5.7	2.5	1.2	
27	ORF 180R	Hypothetical protein			16.8	4.8	1.9	
28	ORF 186R	Hypothetical protein			15.4	3.4	1.7	
29	ORF 197L	Hypothetical protein			19.7	4.0	2.4	3.6
30	ORF 198R	Hypothetical protein			6.7	3.1	1.3	
31	ORF 224L	RNA polymerase beta subunit	AAT71848		7.6	3.5		
32	ORF 226R	Hypothetical protein		2.9	2.9	2.7	1.5	
33	ORF 237L	Largest subunit of DNA-dependent RNA polymerase	AB018418		12.1	2.3	1.5	
34	ORF 239R	Largest subunit of DNA-dependent RNA polymerase	BAA82753		7.4	3.4	2.2	
35	ORF 256R	DNA repair protein RAD2	BAA82754	1.8	9.7	3.6	2.0	
36	ORF 268L	Ribonucleotide reductase small subunit	BAA82755		11.7	3.5	1.7	
37	ORF 291L	Laminin-type epidermal growth factor-like domain	AAT71838	4.5	2.7	4.6	2.4	3.3
38	ORF 317L	DNA polymerase	O70736	1.4	12.2	3.5	1.5	
39	ORF 321R	DNA polymerase	AB007366	2.1	13.9	2.2	1.0	1.5
40	ORF 324R	DNA polymerase	AB007366	15.6	23.7	2.6	1.5	3.5
41	ORF 333L	Hypothetical protein		3.1	17.0	3.3	1.6	
42	ORF 342L	Hypothetical protein			18.2	2.8	2.0	1.8
43	ORF 349L	Serine/threonine protein kinase catalytic domain	AAT71828	4.4	16.4	3.7	1.8	2.1
44	ORF 351R	Hypothetical protein			6.5	2.9		

Table 2. Continued

No.	ORF	Futative Function	Accession No.	Foldchange				
				5 d.p.i.	7 d.p.i.	9 d.p.i.	10 d.p.i.	14 d.p.i.
45	ORF 353R	Hypothetical protein		2.9	8.5	2.8	1.9	2.6
46	ORF 373L	Hypothetical protein		3.3	10.5	3.8	1.9	3.1
47	ORF 374R	Hypothetical protein		2.1	25.0	4.3	1.5	
48	ORF 380R	Major capsid protein	BAC66968	4.0	24.6	3.6	1.8	2.7
49	ORF 385R	Catalytic domain of ctd-like phosphatase	AAT71821		12.2	3.0	1.6	
50	ORF 390R	Hypothetical protein			3.7	3.5		
51	ORF 394R	Hypothetical protein			2.8	3.6		
52	ORF 396R	Transmembrane amino acid transporter	AAT71816		5.3	3.5	1.5	
53	ORF 401R	Hypothetical protein		1.8	11.3	2.8	1.5	
54	ORF 407R	ATPase	AB007367	3.8	16.4	3.1	1.7	3.0
55	ORF 412L	ATPase	AAO16492	1.4	14.8	3.1	1.8	1.8
56	ORF 413R	ATPase	AB007367	1.8	18.1	3.1	1.4	2.1
57	ORF 420L	Hypothetical protein			9.9	4.8	1.8	
58	ORF 423L	RING-finger domain-containing protein	AAT71906		6.2	2.6	1.4	
59	ORF 424R	Putative ankyrin repeat protein	AAL98801		15.2	4.2	1.5	
60	ORF 426R	Hypothetical protein		4.5	41.0	4.5	2.5	4.0
61	ORF 430L	Putative phosphatase	AAT71837	1.7	23.9	3.5	1.7	2.0
62	ORF 458L	Hypothetical protein		2.1	9.3	3.4	2.3	3.3
63	ORF 463R	Hypothetical protein			5.5	4.0		
64	ORF 487L	Proliferating cell nuclear antigen	NP078615	0.8	12.4	2.7	1.8	
65	ORF 488R	Putative tumor necrosis factor receptor associated factor	AAL98835		4.4	3.0		
66	ORF 493R	D5 family NTPase	AAS18067		8.8	3.6	1.5	
67	ORF 502R	Hypothetical protein			5.2	3.2		
68	ORF 506R	Hypothetical protein			3.7	3.1		
69	ORF 515L	Hypothetical protein			5.0	3.0		
70	ORF 534L	Ankyrin repeat-containing protein	AAT71909		3.7	3.9	1.0	
71	ORF 535R	Hypothetical protein		6.5	25.8	2.4	2.0	3.1
72	ORF 543R	RING-finger domain-containing protein	AAT71906	5.0	11.5	3.4	2.2	3.3
73	ORF 550R	Hypothetical protein		4.9	21.5	3.5	2.4	4.3
74	ORF 554R	Hypothetical protein		1.6	19.0	2.6	1.4	1.9
75	ORF 562R	Hypothetical protein		2.8	14.7	2.7	2.1	2.6
76	ORF 569R	Hypothetical protein		1.2	5.5	2.7		
77	ORF 575R	Hypothetical protein		0.8	16.5	4.2	1.6	
78	ORF 586L	Hypothetical protein			10.9	3.1		
79	ORF 591R	Hypothetical protein			3.9	2.1		
80	ORF 596L	dsRNA-specific ribonuclease	AAT71898	0.9	6.3	3.2	1.3	
81	ORF 600L	Hypothetical protein			4.8	2.7	1.1	
82	ORF 606R	Hypothetical protein			6.8	3.8	1.2	
83	ORF 617L	Hypothetical protein			9.7	2.6		
84	ORF 618R	Hypothetical protein			4.6			
85	ORF 628L	Hypothetical protein		1.4	17.3	3.7	2.0	2.2
86	ORF 632L	Hypothetical protein		1.8	18.4	3.6	1.7	2.1
87	ORF 635L	Hypothetical protein		1.3	19.0	3.8	1.7	1.7
88	ORF 641L	Putative ankyrin repeat protein	AAL98801		16.1	4.0	1.7	1.6

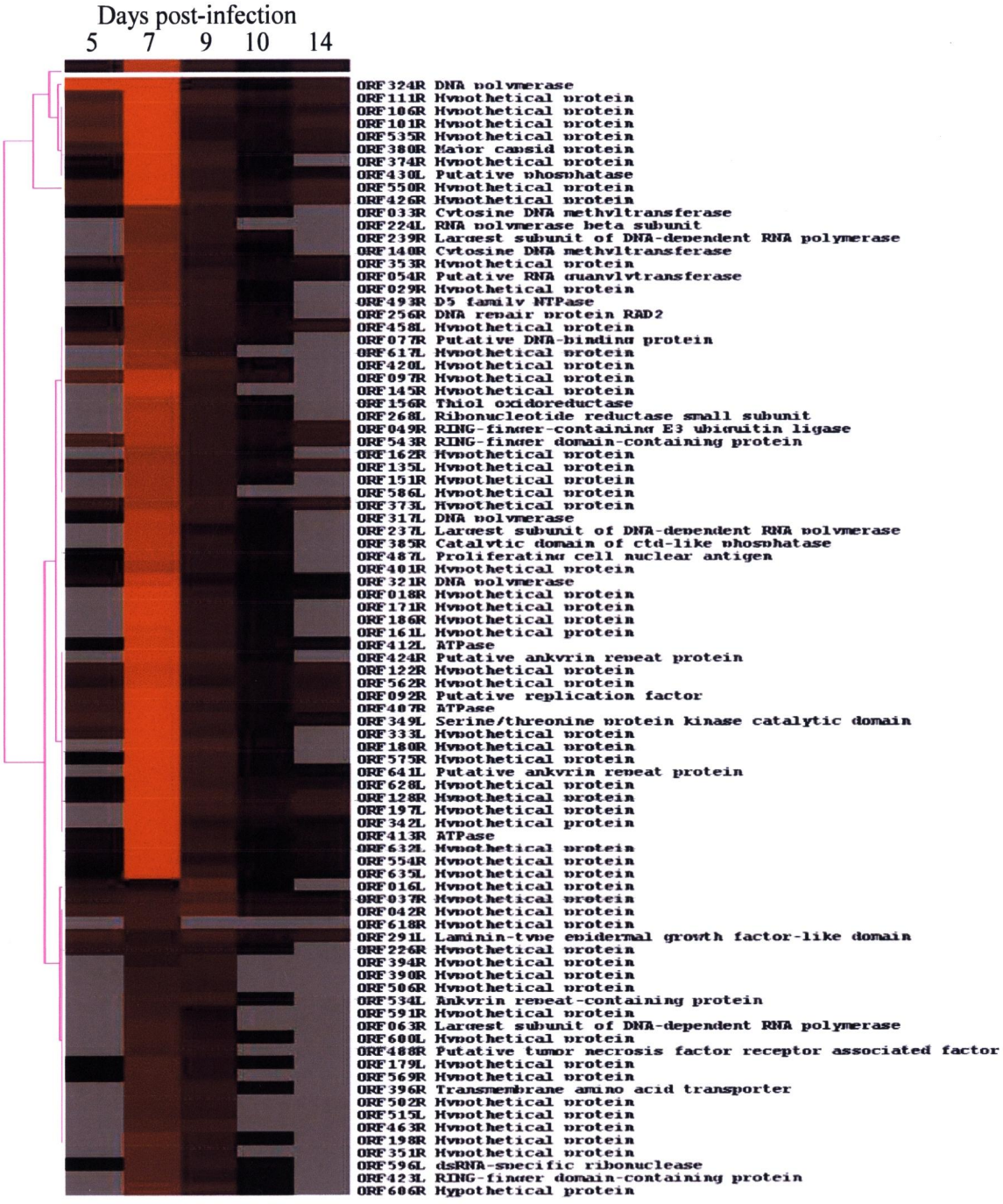

Fig. 2: Hierarchical cluster analysis of differentially RSIV gene expression between spleen and kidney. Expression ratios for each ORF were categorized by an average linkage hierarchical clustering program. Each row represents the expression level of a single ORF in the spleen when compared to that in the kidney, and each column indicates time points after infection. The normalized expression levels across all the time points are color-coded. Red boxes indicate up-regulation in expression (median ratios from 2.0). Black boxes indicate no change in expression (median ratios during 0.5 – 2.0), and gray boxes indicate missing or not detected. The magnitude of up-regulation is shown by differing intensities of red, with deep red showing lower expression and bright red showing the highest levels of expression.

Table 3. Summary of microarray analysis of differential RSIV gene expression between spleen and kidney over the time-course of an *in vivo* infection

Spotted genes					92
Days post infection	5 d.p.i.	7 d.p.i.	9 d.p.i	10 d.p.i.	14 d.p.i.
Expressed genes	46	88	87	72	44
No. of genes expressed at higher levels in spleen	28	88	87	27	36
% of genes expressed at higher levels in spleen	60.9	100	100	37.5	81.8
No. of genes expressed at similar levels	18	0	0	45	8
% of genes expressed at similar levels	39.1	0	0	62.5	18.2

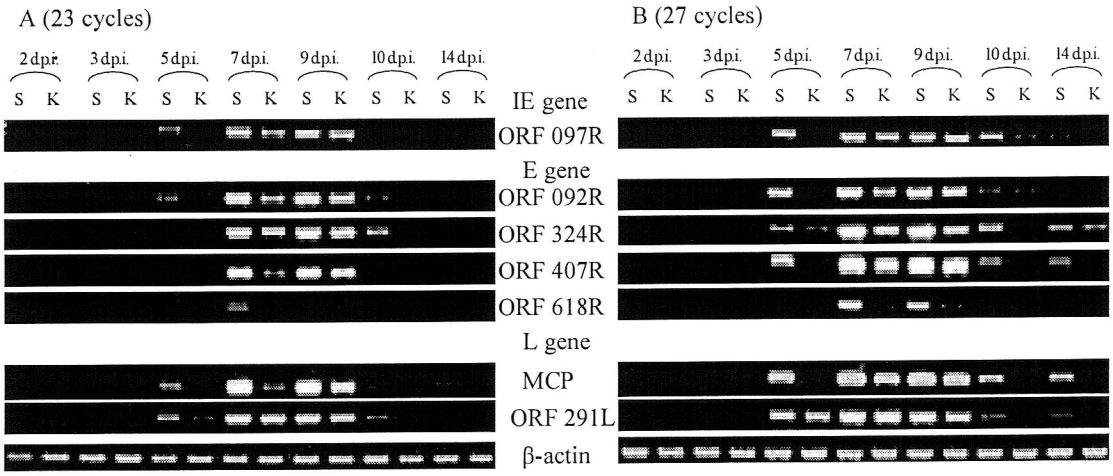

Fig. 3: RT-PCR analysis of RSIV transcripts in spleen (S) and kidney (K) of RSIV-infected red seabream. cDNAs were synthesized from 5 μg total RNA taken from the same samples used for the microarray experiments. One μl of cDNA was used for 30 μl RT-PCR reaction with cycling conditions as follows: an initial denaturation at 95°C for 2 min, followed by 23 and 27 cycles of denaturation of 95°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 1 min, and a final elongation step at 72°C for 5 min. A 23 cycle PCR (Fig. 3A) was used to determine differences in expression of RSIV transcripts between spleen and kidney at the high level spread stage of the infection while a 27 cycle PCR (Fig. 3B) was performed to show expression differences at the early and late stages of the infection. β-actin transcript was used as an internal control.

2.3.4 Determination of the infectious viral concentration in the spleen and kidney

RSIV titers were measured to determine the infectious viral concentration in infected spleens and kidneys. The virus titer of the spleen-derived RSIV stock ($RSIV_S$), as determined by $TCID_{50}$ assay, was higher than that of the kidney-derived RSIV stock ($RSIV_K$) (Table 4).

Table 4. Determination of the infectious viral concentration in the spleen and kidney

Value of virus titer		
Stock	Source of stock	Virus titer ($TCID_{50}$/ml)
$RSIV_K$	Kidney	10^5
$RSIV_S$	Spleen	$2.1 \pm 0.34 \times 10^6$

2.4 Discussion

DNA microarray technology has been successfully used to explore viral gene expression patterns of mammalian and piscine viruses under different conditions in both cell culture systems and animal models (Lua et al., 2005; Ye et al., 2001). In our previous study (Lua et al., 2005), RSIV DNA microarrays were used for rapid analysis of the RSIV gene transcriptional profile over the time-course of an *in vitro* infection in a cell culture system and for grouping genes into temporal kinetic classes, providing a global picture of transcription and kinetics of RSIV genes during the replication cycle. In the present study, the same RSIV DNA microarray was used to characterize the viral gene expression profiles over the time-course of an *in vivo* infection in a fish model, providing a better understanding of the pathogenic mechanisms of RSIV infection at the transcription level. In addition, a comparison of the RSIV gene expression patterns was performed between viral-infected spleens and kidneys to detect the differential viral gene expression between different targeted organs of RSIV infection, resulting in the better understanding of the viral pathogenesis, and to further confirm a susceptible organ to RSIV infection at the molecular level. This study describes the first use of DNA microarrays to explore gene expression patterns of a fish marine-pathogenic virus in aquaculture.

Spleen and kidney were used in this study because they are potential target sites of RSIV during infection. High viral loads were observed in both the spleen and kidney by using real-time PCR (Caipang et al., 2003). Enlarged cells, which are reported as indicators of piscine iridovirus infections, were also found in the spleen and kidney in fish infected with RSIV, TGIV, SGIV and grouper sleepy disease iridovirus (GSDIV) (Chao et al., 2004; Gibson-Kueh et al., 2003; Inouye et al., 1992; Mahardika et al., 2004;

Qin et al., 2002). In addition, the spleen is suggested to be the target organ for RSIV diagnosis (Manual of Diagnosis Tests for Aquatic Animals - 2003; http://www.oie.int/esp/normes/fmanual/A_00032.htm).

The time-course experiments have allowed us to monitor the expression of each RSIV ORF through an *in vivo* infection. The timing of viral transcripts that we observed (beginning at 5 d.p.i. and peaking at 7-9 d.p.i.) is similar to what has been observed in previous studies (Nakajima, 1995; Oshima et al., 1998). In an immunoassay of RSIV-infected red seabream (Nakajima, 1995), the virus was not detected in the spleen at 1 or 3 d.p.i., was moderately detected at 5 d.p.i. and was strongly detected at 7 d.p.i. Using a PCR assay, PCR products corresponding to a portion of the ribonucleotide reductase small subunit gene were not amplified from RSIV-infected red seabream at 1 and 2 d.p.i. but were amplified starting at 5 d.p.i. (Oshima et al., 1998). Similar results were observed in Taiwan grouper iridovirus (TGIV) infection, which is caused by a piscine iridovirus classified into the same group with RSIV (Chao et al., 2002). Particles of this virus were detected in some internal organs of groupers at 4-5 days after intramuscular infection (Chao et al., 2002). In Singapore grouper iridovirus (SGIV) infection, viral antigens were detected in virus-infected fish blood at 3 d.p.i. by using a Western blot analysis (Qin et al., 2002). Taken together, our results suggest that the pathogenic mechanism of RSIV is similar to that found in other piscine iridoviruses, such as TGIV and SGIV. Although viral particles were detected at slightly different times in the above studies by using either traditional viral techniques, such as immunoassay, or molecular techniques, such as PCR, western blot assays or even the DNA microarray technology, piscine iridoviruses seem to begin to spread at around 4-5 d.p.i. in infected fish.

Different viral genes reached peak expression levels at different time points. Almost all Immediately-Early (IE) genes, including ORFs 049R, 097R, 333L, 342L, 396R, 535R, 591R, and 635L, peaked early at 5 d.p.i. and continued to increase throughout the course of the infection (Table 1 & Fig. 1). Similarly, most Early (E) genes, which are thought to be involved in DNA replication, including ORFs 033R (Cytosine DNA methyltransferase); 092R (Putative replication factor); 237L and 239R (Largest submit of DNA-dependent RNA polymerase); 268L (Ribonucleotide reductase small submit); 317L,

321R and 324R (DNA polymerase); 407R, 412L and 413R (ATPase); 424R and 641L (Putative ankyrin repeat protein), as well as 433L (Putative phosphatase), peaked in expression at 5 to 10 d.p.i., indicating preparation for viral DNA replication. Other E genes (Table 1) peaked at later time points (7 to 10 d.p.i.). In general, Late (L) genes, which are thought to encode structural proteins and DNA packaging proteins peaked at later times post-infection (7 to 14 d.p.i.). However, some other L genes, such as ORFs 037R, 042R, 106R, 111R, 197L, 291L, 374R, MCP, 426R, 458L, 543R, 550R, and 554R (Table 1), although expressed early, also peaked later or continued to express throughout the infection. Our results have shown that IE genes and genes involved in viral DNA replication reached peak levels of expression first, followed by genes encoding structural and packaging proteins. The *in vivo* temporal kinetic expression of RSIV genes found here are consistent with the *in vitro* findings reported in our previous studies (Lua et al., 2005). Taken together, as revealed by viral DNA microarrays in both in vitro and in vivo studies, the transcriptional profiles of RSIV occur into a temporal kinetic manner, following a common feature of the family *Iridoviridae* in particular, and a classical virus gene expression cascade of DNA viruses in general.

Our finding that the *in vivo* expression profiles of RSIV gradually declined in both the numbers and the expression levels after 10 d.p.i. (Table 1 & Fig. 1) indicates that the virus was being gradually cleared by host antiviral immune defenses. Similarly, Caipang et al. (2003) showed with real-time PCR that RSIV was cleared from both the vaccinated and unvaccinated red seabream after viral challenge, and Chao et al. (2004) showed with histological, ultrastructural and *in situ* hybridization that the number of basophilic enlarged cells (virus-containing cells) gradually decreased from 7 d.p.i. in TGIV-infected groupers. Chao et al. attributed the viral clearance to either an improved host defense or to depletion of susceptible cell types (Chao et al., 2004).

Differences in viral gene expression between different targeted-organs or cell types enhance a better understanding of pathogenesis of the virus (Cummings and Relman, 2000; Yamagishi et al., 2003). A comparison of expression profiles of known ORFs in AcMNPV between two viral-infected cell types clearly provided information on the replication and pathogenesis of the virus in a variety of host cell types (Yamagishi et al.,

2003). Similarly, differential viral gene expression patterns in different targeted organs of RSIV infection in fish further confirmed the viral pathogenesis. The finding that all RSIV ORFs were expressed at either higher levels or similar levels in spleens when compared with those in kidneys throughout the infection indicates that RSIV appears to preferentially target the spleen. The higher expression of all viral ORFs in the spleens from 7 to 9 d.p.i., together with over tenfold higher of about 60% ORFs at day 7 after infection (Table 2 and Fig. 2)) strongly suggest that the spleen is a susceptible organ for detection of RSIV disease. Interestingly, IE genes (ORFs 049R, 097R, 333L, 353R, 535R) all expressed early and continued with higher expression levels in the spleens when compared to those in the kidneys over the time-course of the infection. In addition, the MCP and some genes that function in nucleotide metabolism or DNA replication (ORFs 092R, 128R, 324R, 407R, 412L, 413R, 562R) also peaked early and expression levels remained higher in the spleens than those in the kidneys (Table 2). The higher virus titer of $RSIV_S$ (Table 4) indicated that the infectious viral concentration in the RSIV-infected spleens was higher than that in the RSIV-infected kidneys. These results are in line with previous studies demonstrating that the spleen is a most susceptible organ for RSIV infection (Caipang et al., 2003; Inouye et al., 1992; Wang et al., 2003) as well as other iridoviral infections in fish (Chao et al., 2004; Huang et al., 2004). The spleen also appears to be where TGIV begins replicating (Chao et al., 2004), and thus has been suggested to be used for early screening of TGIV. Our results support this conclusion.

The RT-PCR data (Fig. 3) confirmed the microarray results, showing the higher expression of selected ORFs in infected spleens when compared to those in infected kidneys. Differences in expression of viral ORFs between spleens and kidneys at the high spread stage of the infection could be observed with only 23 PCR cycles (Fig. 3A). On the other hand, differences in expression at the early and late stages of the infection were not detectable after 23 cycles but they were detectable after 27 cycles (Fig. 3B). Among the selected ORFs, the MCP gene was found to be expressed at higher levels in the spleen than in the kidney over the time-course of infection. MCP gene contains highly conserved domains and codes for the major structural component of viral particles (Schnitzler and Darai, 1993; Tidona et al., 1998; Williams, 1994; Williams, 1996). The MCP gene has been used to detect and measure RSIV as well as other iridoviruses (Caipang et al., 2003;

Kurita et al., 2002; Tidona et al., 1998). Thus, our results further confirm the hypothesis that the spleen is the target organ for RSIV infection. Also seen in RT-PCR analysis, IE genes were also observed early and were expressed throughout the virus infection, and followed by the expression of E and L genes. Therefore, RT-PCR results confirmed the microarray data and further confirmed that the transcriptional profiles of RSIV follow a temporal kinetic cascade.

In conclusion, the present study provides the first global analysis of RSIV gene expression patterns in a fish model. Our results show that the pathogenesis of RSIV spread from 5 d.p.i. and continued with high levels of viral multiplication until viral clearance by host antiviral defenses starting from around 10 d.p.i. A comparison of viral gene expression patterns between infected spleens and kidneys provides a better understanding of the viral pathogenesis, and further confirms, at the molecular level, that the spleen is a susceptible organ of RSIV infection. This study is the continued analysis of RSIV gene expression patterns *in vivo* to complete transcriptional profiles of RSIV both in cell culture and fish model systems. Such studies would enhance our understanding of the molecular pathogenic mechanisms of RSIV infection and further provide a possible clue for selection of the most susceptible organ for detection of iridoviral diseases in fish.

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CHAPTER 3

INHIBITION OF RED SEABREAM *IRIDOVIRUS* (RSIV) REPLICATION BY VIRAL GENE-SPECIFIC siRNAs IN A CELL CULTURE SYSTEM

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 hours 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

3.1 Introduction

RNA interference (RNAi), mediated by small molecules of double-stranded (ds)RNAs, is a natural biological mechanism for silencing genes that is widely conserved in diverse organisms ranging from plants to man, and is shown to have activity against a wide range of viruses and thus 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 small interfering RNAs (siRNAs) of 21-23 nucleotides (nt) are associated with a multiprotein complex known as the RNA-induced silencing complex containing siRNA (siRISC) 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 nt 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 systems and experimental 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), vaccinia virus (Dave 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.

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 in previous studies (Caipang et al., 2003; Dang Thi et al., 2007), and in this study (chapter 2).

In the present study, we have utilized the MCP gene of RSIV as a target for siRNAs in order to determine whether viral gene-specific siRNAs can be used as an antiviral tool against aquatic iridoviral infections. MCP-specific siRNA was tested for the inhibition of RSIV replication 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.

3.2 Materials and methods

3.2.1 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. (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.

3.2.2 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).

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

siRNA name	Target sequence (5' – 3')	Position in gene	Source
siR-MCP	AACAGACUGGCCAUGCUAAUU	164 -182	RSIV
MsiR-MCP	A**G**CAGACUG**A**C**U**A**C**GCUA**G**UU		
nsRNA	CGCCUGGUUGGUACUCAAGUU	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)

3.2.3 *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 *Eco*RI and *Xba*I sites (Table 2) by PCR. The PCR products were purified, and cloned into *Eco*RI and *Xba*I 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.

Table 2. Primers used in this study

Primer name	Primer sequence	PCR product size (bp)
pCMV-MCP-F	5'-AT**GAATTC**ATGTCTGCGATCTCAGGTGC-3'	Full length
pCMV-MCP-R	5'-GC**TCTAGA**TTACAGGATAGGGAAGCCTG-3'	
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

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.

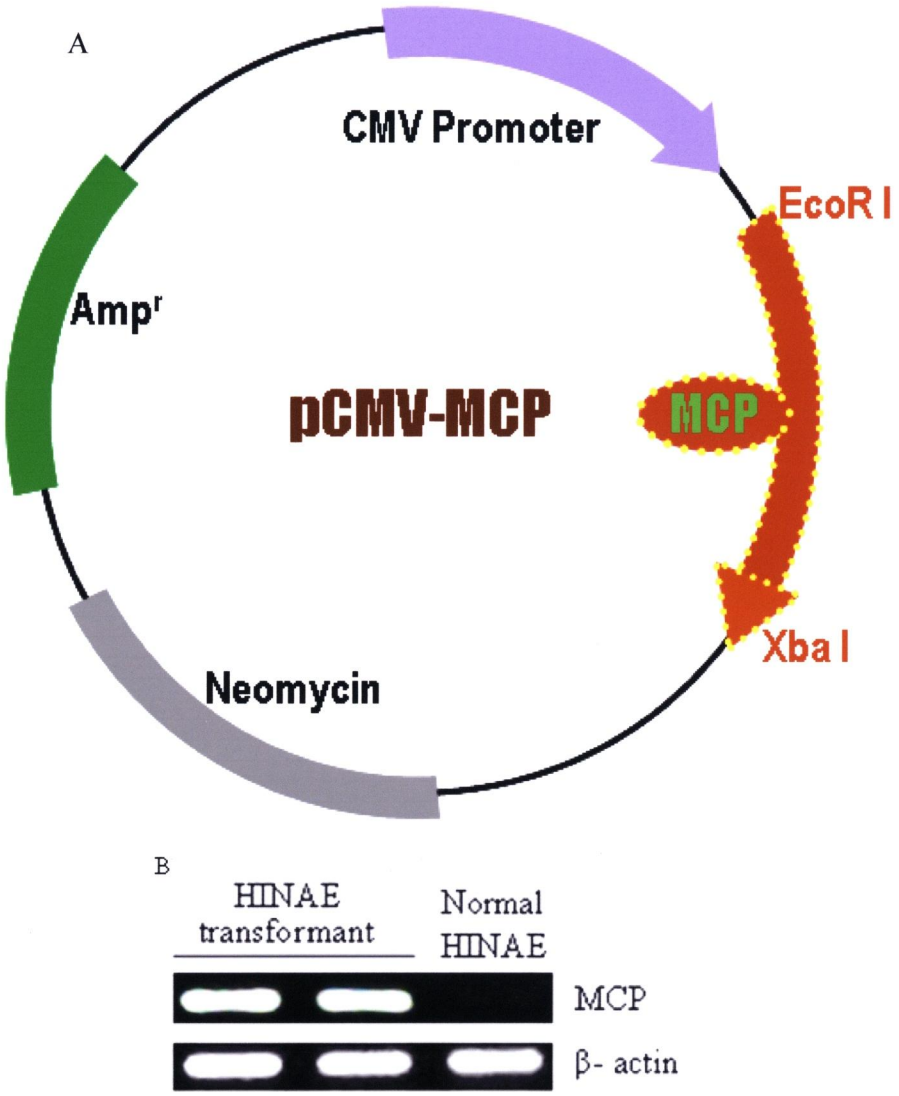

Fig. 1. Expression of MCP gene in HINAE transformants. (A) Construction of pCMV-MCP. The full-length of the MCP gene was amplified from the RSIV genome by using specific primers containing *Eco*RI and *Xba*I enzyme sequences and then cloned into pCI-neo expression vector. (B) Expression of MCP gene in 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 one month post-transfection, total RNA was extracted from the transformants and reverse transcribed to cDNA for RT-PCR.

3.2.4 Transfection of plasmid DNA and siRNA

HINAE cells were seeded into 24-well or 96-well cell culture plates for about 24 hours (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 manufacturer'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.

3.2.5 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 nM, 60 nM 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 nM, 60 nM and 120 nM and total RNA was extracted from both siR-MCP-transfected and un-transfected HINAE transformants with TRIzol (Invitrogen, USA) at 1, 2 and 3 d.p.t. for RT-PCR and real-time PCR analysis.

3.2.6 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 hours 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 hours 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.

3.2.7 Assessment of reduction in viral production by siR-MCP

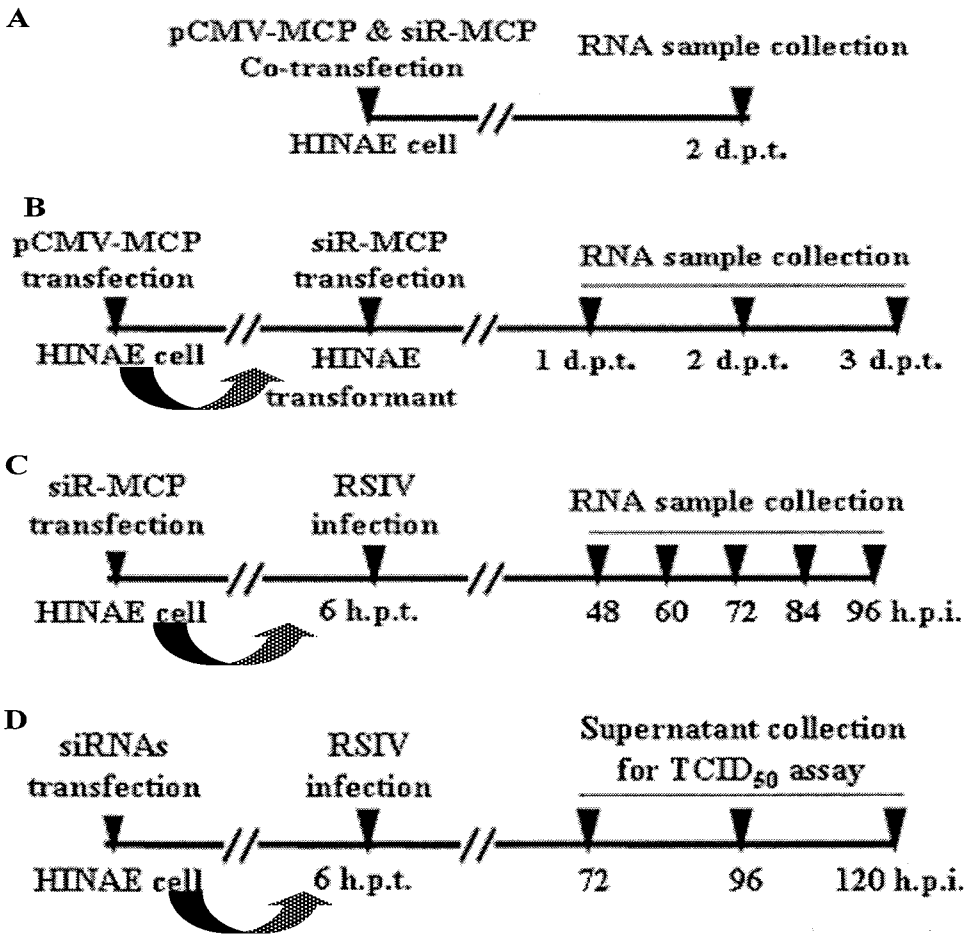

Fig. 2. Experimental scheme of siRNA-mediated RNAi for anti-RSIV. (A) Transient expression of MCP gene by using MCP-expressing plasmid; (B) Stable expression of MCP gene by using MCP-expressing plasmid; (C) Expression of MCP gene under siRNA transfection and RSIV infection; and (D) Sampling for $TCID_{50}$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

HINAE cells transfected with siR-MCP (120 nM) and un-transfected cells were incubated for 6 hours 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 8 repetitions per dilution to perform the $TCID_{50}$ assay. Virus titers were calculated following the standard method (Reed and Muench, 1938).

3.2.8 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 hours 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).

3.2.9 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 95oC for 2 min, followed by 23-27 cycles of denaturation at 95oC for 30 sec, annealing at 55oC for 30 sec and elongation at 72oC for 1 min, and a final elongation step at 72oC for 5 min. The primers used for RT-PCR are shown in Table 2.

3.2.10 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 sec 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.

3.2.11 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:

$$\text{Inhibition (\%)} = \{1 - (2^{-\Delta\Delta C_T} \text{ of tested sample} / 2^{-\Delta\Delta C_T} \text{ of control sample})\} * 100$$

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

3.3 Results and discussion

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

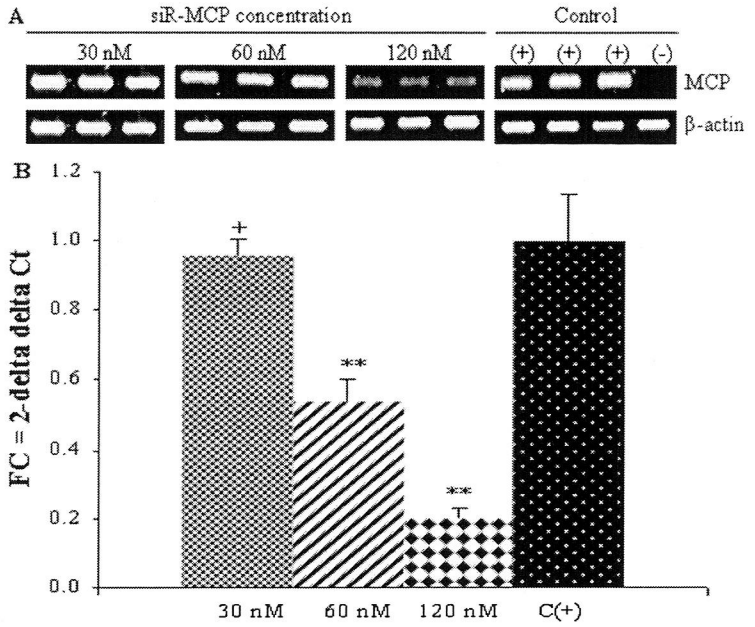

Fig. 3. Dose-dependent inhibitory effect of siR-MCP on the MCP gene in the case of transient expression. (A) RT-PCR analysis of MCP gene expression. HINAE cells were un-transfected or co-transfected with pCMV-MCP and siR-MCP at different concentrations. Total RNA was extracted at 2 d.p.t. for RT-PCR. (+) 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 stand deviation (S.D.) are shown. Fold change (FC = $2^{-\Delta\Delta C_T}$); (30 nM; 60 nM; and 120 nM) siR-MCP concentration; C(+) positive control; (+) $p > 0.05$; (**) $p < 0.01$ when compared to the control.

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% (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 nM, 60 nM and 120 nM, respectively (Fig. 3B). Thus, siR-MCP dose-dependently inhibits MCP gene expression and is highly effective at a final concentration of 120 nM.

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

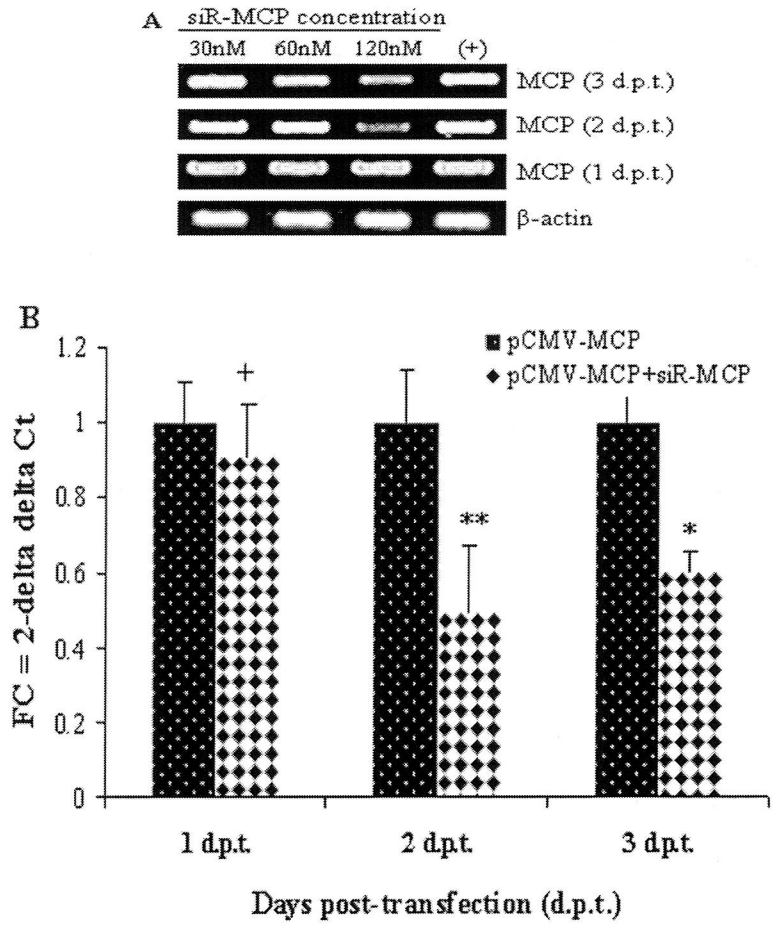

Fig. 4. Dose-dependent inhibitory effect of siR-MCP on the MCP gene in the case of stable expression. (A) RT-PCR analysis of MCP gene expression. HINAE transformants that stably expressed MCP gene were not transfected or transfected with siR-MCP at final concentrations of 30 nM, 60 nM and 120 nM. Total 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

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 day 2 and day 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.

3.3.3 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 analyzied 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.

3.3.4 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 infected with RSIV and cell

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

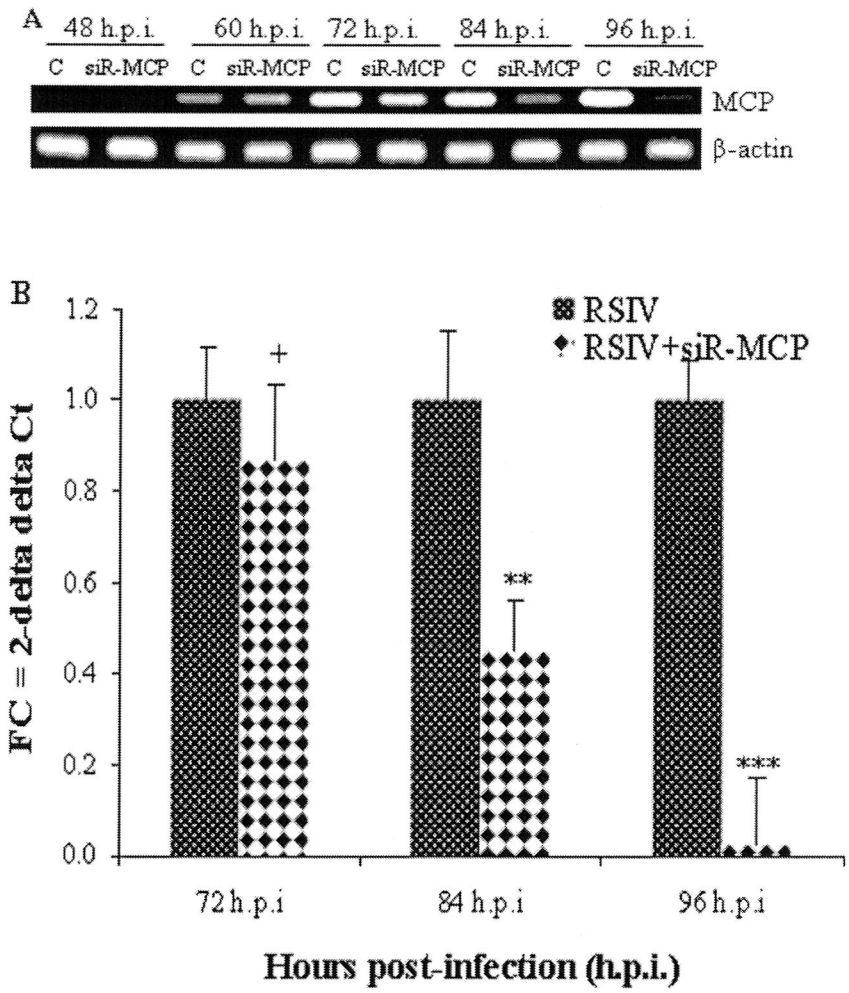

Fig. 5. Suppression of RSIV replication in terms of MCP gene expression by siR-MCP (120 nM). (A) RT-PCR analysis of MCP gene expression. HINAE cells were not transfected or transfected with 120 nM siR-MCP for 6 hours prior to infection with RSIV (m.o.i. of 3). Total RNA was extracted at 48, 60, 72, 84 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.

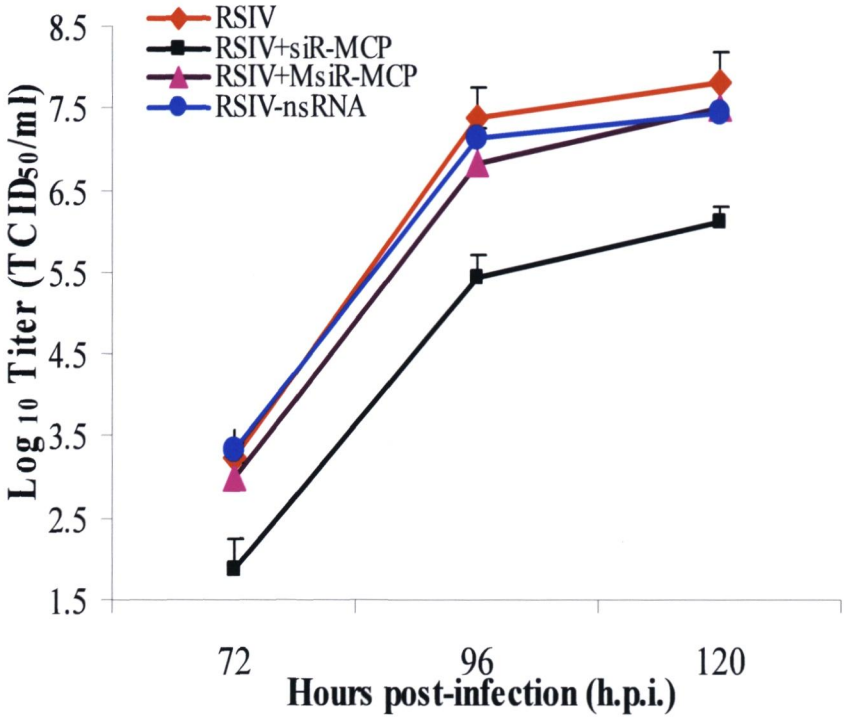

Fig. 6. Reduction in viral production by siRNAs (120 nM) at different time-intervals after RSIV infection (m.o.i. of 5). siRNA-transfected cells were infected with RSIV at 6 h.p.t. Supernatants were collected at 72, 96 and 120 h.p.i. and assayed for virus titration. The data show the average titers of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

In $TCID_{50}$ assays, the average titers of culture medium of siR-MCP-transfected samples were about 23.7-fold, 86.6-fold 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 showed a dramatic effect on MCP mRNA (Fig. 5A and 5B) 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-fold, 3.7-fold 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-fold and 2.4-fold, respectively. These results indicate that siR-MCP specifically targets RSIV.

3.4 General discussion and conclusion

Viral infection is a severe public health problem with significant personal, social, and economical consequences. More effective approaches are needed to prevent and treat viral infection. The exploitations of the RNAi pathway that RNAi is a sequence-specific gene silencing process through the action of siRNAs and can be induced in cultured cells by introducing synthetic siRNAs pave a new way for the use of RNAi as antiviral approaches. This potential use has been comprehensively discussed in numerous reviews (Andino, 2003; Tan and Yin, 2004; van Rij and Andino, 2006; Wang et al., 2006). Highly effective inhibition of virus replication by RNAi has been achieved both *in vitro* and *in vivo*. In comparison with other conventional drugs, the inhibition of viral replication by siRNA molecules has some advantages. The most advantage is that siRNAs are much easier and more flexible to select target sites because target mRNA and siRNA are sequence-specific and complementary (Tan and Yin, 2004). siRNAs can suppress viral replication at several stages of infection, including the very early stages, when viruses are most vulnerable. More importantly, virus infection not only be prevented by re-treatment or co-treatment with siRNAs (reviewed by Andino, 2003), but also be prevented in cells already infected with viruses (Orba et al., 2004).

Although assembly of viral particles varies with different virus families, many capsid proteins are involved in the formation of virus particles. Genes code for proteins needed

for the cleavage and packaging of virus particles are commonly selected as targets for silencing in siRNA studies of DNA viruses. For instance, siRNAs targeting UL-6 gene, a structural gene, were applied to AHV-1 (Mallanna et al., 2006), while siRNAs targeting MCP gene were applied to TFV (Xie et al., 2005). Taken this fact, the MCP gene of RSIV was targeted for siRNAs in this study, and our data demonstrated that siRNA specific for MCP gene efficiently inhibited RSIV replication in terms of MCP gene expression, and hindered viral production in a cell culture system, and further indicated that the MCP gene is essential for life cycles of 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.

3.5 Acknowledgments

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CHAPTER 4

ENGINEERED VIRUS-ENCODED pre-microRNA (pre-miRNA) INDUCES SEQUENCE-SPECIFIC ANTIVIRAL RESPONSE IN ADDITION TO NON-SPECIFIC IMMUNITY IN A FISH CELL LINE: CONVERGENCE OF RNAi-RELATED PATHWAYS AND IFN-RELATED PATHWAYS IN ANTIVIRAL RESPONSE

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 responses 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 seabream 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 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 anti-viral 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.

Keywords: RNAi; Interferon; IFN; siRNA; miRNA; Major capsid protein; MCP; RSIV; HIRRV

4.1 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 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 seabream 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. 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 miR-MCPs and miR-HIRRV in cells transfected with plasmids capable of expressing pre-miRNAs (pcDNA-miRs). The anti-RSIV activity of miR-MCPs was initially assessed by measuring MCP gene silencing by employing transient transfection of a plasmid expressing the target gene (pCMV-MCP). We then investigated the inhibitory effect of the miR-MCPs on RSIV replication following challenge with RSIV. The inhibitory effect of miR-HIRRV on HIRRV replication was demonstrated by reduced expression level of the viral glycoprotein (G) gene and reduced HIRRV titers in plasmid-transfected cells

over the time-course of HIRRV infection. Our results demonstrate the utility of expressing viral-encoded miRNAs through a miR-155 precursor stem-loop structure, and suggest that viral-encoded miRNAs are able to trigger antiviral miRNA-related pathways in fish cells. However, further analyses revealed that the expression of pre-miRNAs also activated IFN-related pathways correlating with upregulation of the antiviral IFN-induced Mx protein, resulting in non-specific antiviral effects. 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.

4.2 Materials and methods

4.2.1 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. (Lua et al., 2005). GF cells were used for 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).

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

Three (3) 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, USA) 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).

4.2.3 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 hrs 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, USA) following the manufacturer's protocol. The transfection mixtures were removed at 6 hours post-transfection, and transfected cells were maintained for further processing.

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

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**AGATGGGAGTAGACTACTTTA**C	
miR-MCP-2	Top	TGCTGAATTAGCATGGCCAGTCTGTTGTTTTGGCCACTGACTGACAACAGACTCCATGCTAATT	68075-68095[a]
	Bottom	CCTGAATTAGCATGGAGTCTGTTGTCAGTCAGTGGCCAAAAC**AACAGACTGGCCATGCTAATT**C	
miR-MCP-3	Top	TGCTGATTACAGTACGGCACACACAAGTTTTGGCCACTGACTGACTTGTGTGTCGTACTGTAAT	69378-69398[a]
	Bottom	CCTGATTACAGTACGACACACAAGTCAGTCAGTGGCCAAAAC**TTGTGTGTGCCGTACTGTAAT**C	
miR-HIRRV	Top	TGCTGTCTCTTTGGAGACTTTCTCGTGTTTTGGCCACTGACTGACACGAGAAACTCCAAAGAGA	1821-1841[b]
	Bottom	CCTGTCTCTTTGGAGTTTCTCGTGTCAGTCAGTGGCCAAAAC**ACGAGAAAGTCTCCAAAGAGA**C	

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)

To elucidate antiviral effects of miR-MCPs on RSIV replication, the expression of MCP gene was monitored in cells transfected with pcDNA-miRs over the time-course of either the plasmid transfection or the virus infection. RSIV was inoculated in HINAE cells transfected with pcDNA-miRs or un-transfected at 1, 3, 5 and 7 d.p.t. (Figs. 1B and 1C). After allowing 2 hrs 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, USA), subjected to DNase I treatment (Promega, USA), and used to synthesize cDNA for RT-PCR analysis.

4.2.5 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 7 d.p.t. for further processing.

To assess inhibitory effect of miR-HIRRV on HIRRV replication in terms of gene silencing, the expression of 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).

4.2.6 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.

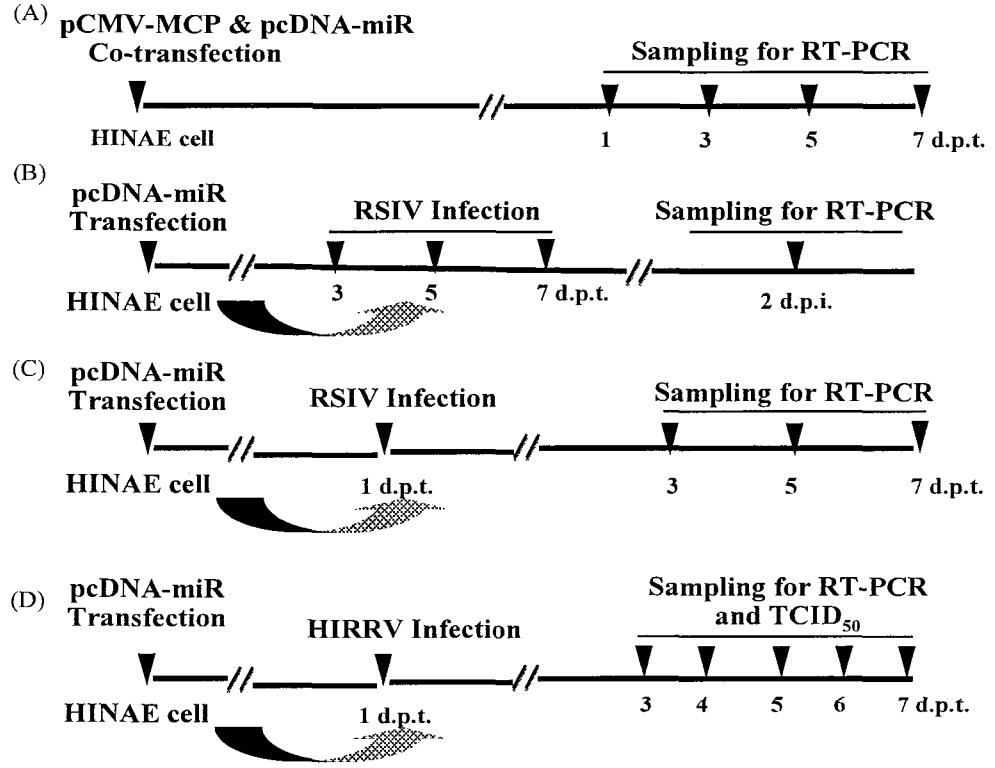

Fig.1. Experimental scheme of miRNA studies; (A) MCP gene silencing by miR-MCPs; (B) Anti-RSIV activity of miR-MCPs in viral-infected cells over the time-course of transfection with pcDNA-miRs; (C) Anti-RSIV activity of miR-MCPs in cells over the time-course of plasmid transfection and virus infection;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

4.2.7 Reverse-transcription (RT) - PCR

One microliter (1 μl) 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 sec, 55°C for 30 sec 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.

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'	

4.3 Results

4.3.1 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.

4.3.2 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 control that were only infected with the virus at each indicated time points (Fig. 3A).

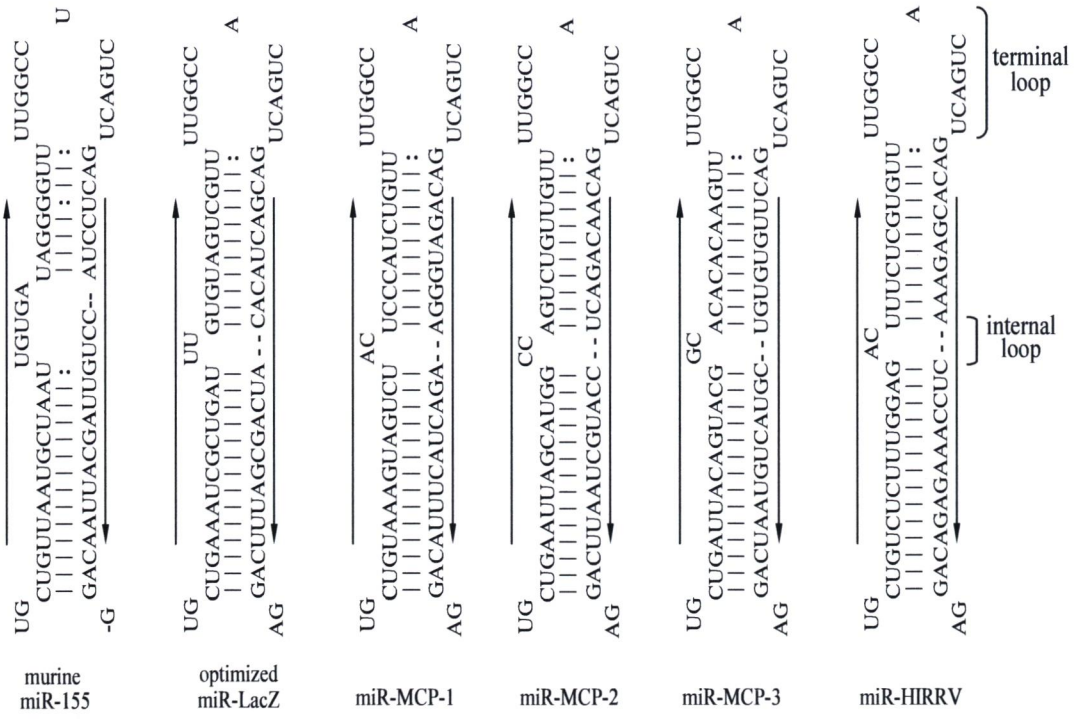

Fig.2. Schematic presentation of predicted pre-miRNAs sequences. Up-underlined arrows indicate 21 nucleotide antisense target sequence. Down-underlined arrows indicate sense target sequence with 2 nucleotides removed to create an internal loop.

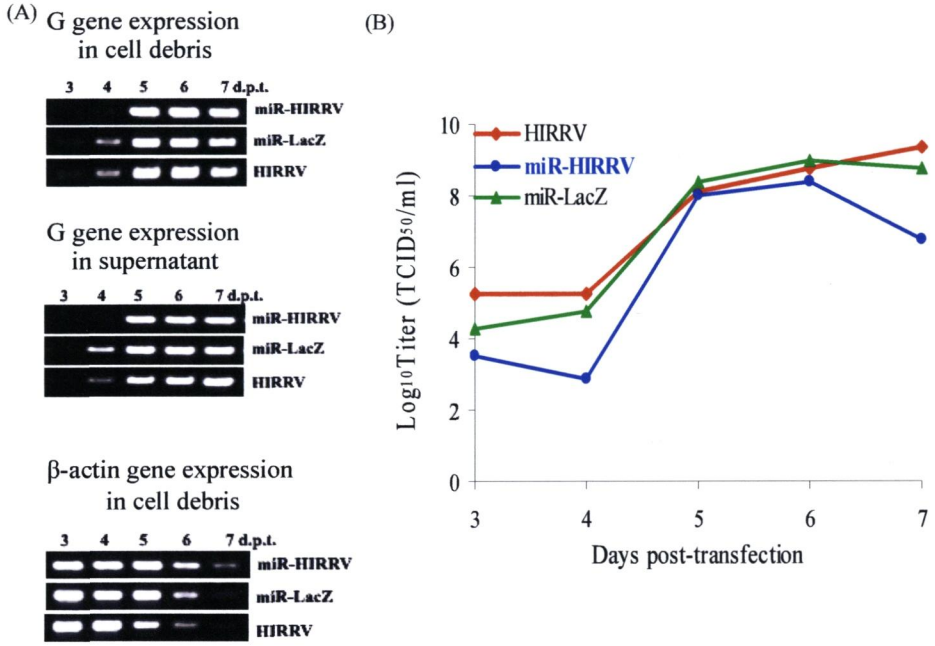

Fig. 3. Anti-HIRRV activity of miR-HIRRV (A) RT-PCR analysis of G gene expression in cells transfected with pcDNA-miR-HIRRV and infected with HIRRV; (B) Reduction of HIRRV titers in cell-free supernatants of cells transfected with pcDNA-miR-HIRRV and infected with HIRRV; (d.p.t.) days post-transfection.

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 specific manner to trigger only the antiviral potency of miRNA-related pathways.

4.3.3 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, miR-LacZ seemed able to slightly reduce the expression of MCP gene (Figs. 4A and 4B).

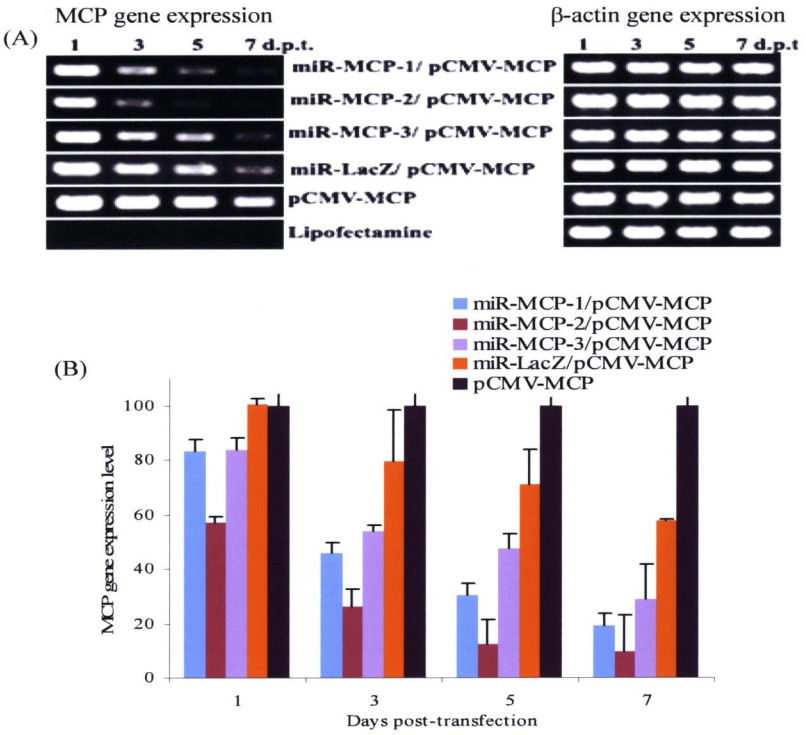

Fig. 4. MCP gene silencing by miR-MCPs in cells co-transfected with pcDNA-miRs and pCMV-MCP; (A) agarose gel electrophoresis with RT-PCR products; (B) the expression level of MCP is calculated relative to the β-actin expression level. Data represent the mean of two independent experiments ± SD.

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 infected cells. But a similar pattern of antiviral activity was observed with miR-LacZ in cells transfected with pcDNA-miR-lacZ (Fig. 5A and 5B).

Taken together, these unexpected results let 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.

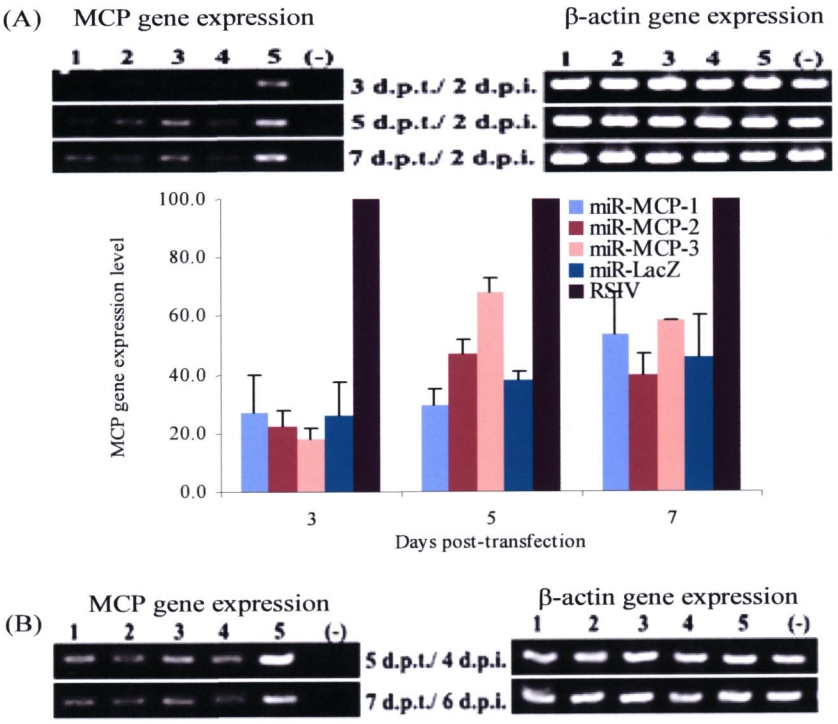

Fig. 5. Anti-RSIV activity of miR-MCPs; (A) over the time-course of plasmid transfection. Top panel: agarose gel electrophoresis with RT-PCR products. Bottom panel: the expression level of MCP is calculated relative to the β-actin expression level. Data represent the mean of two independent experiments ± SD. (B) over the time-course of plasmid transfection and RSIV infection; (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.

4.3.4 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 in fish (Nygaard et al., 2000; Pakingking et al., 2004) and have antiviral activity against a wide spectrum of viruses (Ooi *et al.*, 2006). 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).

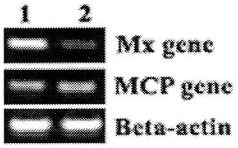

Fig. 6. Effect of IFN stimulation on RSIV replication. HINAE cells seeded onto 24-well plates were stimulated with 50 μg/ml poly (I:C) to induce IFN production. Induced cells and un-induced cells were infected with RSIV at 6 hrs after stimulation. After allowing 2 hrs for absorption, unattached viruses were removed and infected cells were continuously cultured with fresh growth medium containing 50 μg/ml poly (I:C) and further maintained for 72 hrs. 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; (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 unregulation of Mx gene was also observed in plasmid-transfected and viral-infected cells when compared to the controls that were only infected with viruses (Figs. 7B and 7C). 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.

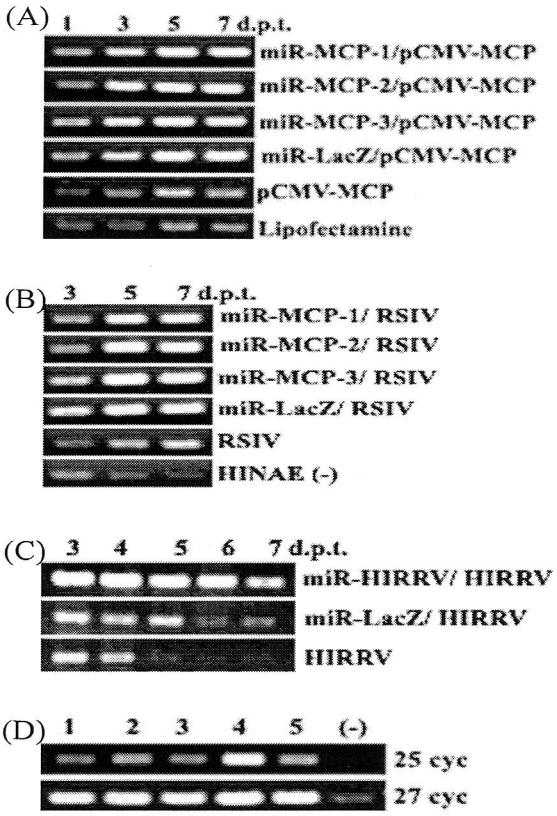

Fig. 7. Induction of the IFN pathway in cells transfected with pcDNA-miRs; (d.p.t.) days post-transfection; (cyc) cycles of RT-PCR; (A) expression of Mx gene in cells co-transfected with pcDNA-miRs and pCMV-MCP; (B) expression of Mx gene in cells transfected with pcDNA-miRs and infected with RSIV; (C) 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.

4.4 Discussion

RNAi triggered by small RNA molecules, including siRNAs and miRNAs, offers a new approach to 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. The present results showed that transfection with miR-HIRRV silenced the expression level of G gene and reduced HIRRV particles in infected cells (Fig. 3), and that miR-MCPs inhibited the expression of MCP gene in cells co-transfected with pCMV-MCP (Fig. 4) as well as in cells infected with RSIV (Fig. 5). 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.

Our finding that virus-encoded miRNAs inhibited replication of the target virus, but miR-LacZ also had inhibitory effects on viral replication (Figs. 3 and 5) strongly supports our hypothesis 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; Robalino et al., 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 stronger inhibitory effect on RSIV replication (Fig. 5) than on HIRRV replication (Fig. 3). In addition, miR-LacZ showed stronger inhibitory effect on the expression of MCP gene in transfected cells following RSIV infection than in cells co-transfected with pCMV-MCP that only expressed the MCP gene (Fig. 4). 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). Taken together, our results suggest that miR-LacZ silenced the expression of ORF 424R through its partial complementarity with the target, resulting in significant inhibition of RSIV replication. 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 by the introduction 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 quaculture. However, the introduction of plasmid-based expression systems capable of intracellularly producing pre-miRNA precursors activates both antiviral miRNA-related pathways and antiviral IFN-related pathways, and possibly other cellular signaling pathways in the vertebrates. 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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CHAPTER 5

GENERAL CONCLUSION AND PERSPECTIVE

Viruses are minute infectious agents which are characterized by a lack of independent metabolism and the inability to replicate outside living host cells. Viruses must utilize the system of the infected host cells for survival and replication; therefore, they have evolved many strategies to prevent infected cells from being apoptosis and to evade the innate and adaptive immune responses of their hosts. They would exploit the biosynthesis machinery of host cells to synthesize various components meanwhile inactivate the innate defense mechanisms of the host, and even deflect the host RNA silencing machinery to their advantages. Many viruses encode proteins that specifically against host-cell defenses, or make tiny miRNAs a particularly efficient tool to turn off the expression of specific genes (Qi et al., 2006). In deed, several viruses produce a persistent carrier state in the host, rendering their control quite difficult. Thus, understanding the molecular pathogenic mechanisms of viral infections is necessary and will be of enormous help towards the development of antiviral approaches.

Gene expression represents a unique way of characterizing how cells and organisms adapt to changes in the external environment (Lettieri, 2006). Therefore, analyses of gene expression profiles upon viral infections can both facilitates the annotation of the molecular pathogenic mechanisms of the virus and further provide clues for control of diseases. The development of high-quality, commercially available DNA microarrays has allowed this technology to become a standard tool in molecular virology. With the advantage of the analysis of thousands of genes at the same time, DNA microarrays have been used to develop a much deeper insight into the mechanism of pathogenesis at the molecular level.

The involvement of biotechnology in the control of viral diseases in aquaculture includes the development of disease-resistant stock, dietary improvements, nonspecific immunostimulants, vaccines and currently antiviral RNAi-based approaches. Recently, the availability of the genome sequences of a range of pathogenic viruses provides the basis for the development of new antiviral therapies (DeFilippis et al., 2003). Antiviral approaches based on RNAi take advantage of this sequence-specific gene silencing

mechanism triggered by small dsRNA molecules, including siRNAs and miRNAs. Due to its high specificity, the simplicity of its design, antiviral RNAi technology has advanced rapidly, opening the possibility for new novel therapeutic procedures. Attractive targets using RNAi are either viral genes that are essential for virus replication, or host genes that are essential for virus entry or that play an essential role in the virus life cycle (Leung and Whittaker, 2005). Small dsRNA molecules can now be obtained in various ways allowing for numerous *in vitro* and *in vivo* applications. All these are worthwhile and much research efforts have been done to test their efficiency for disease control. The antiviral potency of viral-gene specific siRNAs has been comprehensively discussed in numerous reviews (Dave and Pomerantz, 2003; Pushparaj and Melendez, 2006; Qi et al., 2006; Sanchez-Vargas et al., 2004; Stram and Kuzntzova, 2006; Tan and Yin, 2004). siRNAs can inhibit viral replication at several stages of infection, including the very early stages, when viruses are most vulnerable (Carmichael, 2002). Due to its sequence-specific gene silencing, viral-gene specific siRNAs are currently evaluated as promising antiviral tools. It is also reviewed that, although miRNAs are involved in complex regulatory networks, miRNAs can use as antiviral tools against virus infection. However, the antiviral potency of viral-specific miRNAs has just been reported for several viruses.

Both DNA microarray and RNAi technologies have been widely applied in various mammalian viruses, it is rather new in aquaculture and has just been tested in few aquatic viruses *in vitro*. This study, therefore, was carried out to better understand the pathogenic mechanisms of viral infections in fish utilizing DNA microarray technology, and to develop alternative antiviral approaches based on RNAi technology using RSIV as a model. The transcriptional profile of RSIV was explored over the time-course of the virus infection in infected spleen of red seabream using viral DNA microarrays containing almost all RSIV putative ORFs. The microarray data analysis indicates that the pathogenesis of RSIV infection appears to spread at around day 5 and continued with high levels of viral multiplication until viral clearance by host antiviral defenses starting from around 10 d.p.i. In addition, the findings that all viral genes were expressed at higher or similar levels in the spleens when compared with those in the kidneys

throughout the virus infection further confirm, at the molecular level, that the spleen is a suitable organ for diagnosis of iridoviral infections in fish.

The antiviral potency of RNAi, triggered by siRNAs and miRNAs, was applied to RSIV in *in vitro* studies prior to their further application for efficient *in vivo* studies. The anti-RSIV activity of virally small RNA molecules was investigated in a cell culture system by the introduction of a naked synthesized viral-specific siRNA and by using plasmid-based viral-encoded pre-miRNA expression system.

A siRNA specific to MCP gene of RSIV (siR-MCP) was designed and tested for anti-RSIV activity. Transfection with siR-MCP efficiently silenced the expression of the target gene and 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 the introduction of naked siR-MCP into a fish cell line can effectively and specifically inhibit the expression of the target gene and hider RSIV replication during an *in vitro* infection, providing a potential approach for the control of viral diseases in aquaculture.

Anti-RSIV activity of viral-encoded miRNAs was investigated in a fish cell line by using plasmid-based pre-miRNA expression system. By incorporating sequences encoding miRNAs specific to the MCP gene of RSIV into a murine miR-155 pre-miRNA backbone (miR-MCPs) under control of Pol II promoter, we were able to intracellularly express miR-MCPs in cells transfected with plasmids capable of expressing pre-miRNAs (pcDNA-miRs). miR-MCPs reduced the expression of MCP gene, resulting in inhibition of RSIV replication. However, expression of miR-MCPs was found to activate the antiviral IFN-related pathways in transfected cells, correlating with the upregulation of the antiviral IFN-induced Mx protein. The observed anti-RSIV effects of virus-encoded miRNAs were partly the result of the antiviral miRNA-related pathways and partly the result of the antiviral IFN-related pathways. In terms of the potential use of miRNA-related pathways for development of antiviral therapeutics, the possibility of activation of RNAi and IFN-related pathways by pre-miRNAs must be of great interest to the control

of diseases because these two pathways interact functionally to mount immunity to a viral pathogen.

Despite DNA microarray technology has been successfully applied in the field of molecular virological research, its enormous potential still faces several challenges. The biggest challenge in DNA microarrays is the challenge of data handling and informatics (Choudhuri, 2004). This is due to the large volume of data generated in each experiment. In addition, differential gene expression analysis is not a stand-alone technique; results must be confirmed through direct examination of selected genes using more sensitive assays, such as RT-PCR, real-time PCR. A second major limitation is the high cost associated with the technology itself. These costs render repeat measures very expensive, and thus often only limited experimental data are available.

Similarly, the potential for use of RNAi as antiviral tool also has several limitations that should be taken into account when designing RNAi-based experiments. Short dsRNAs (21-23 bp) or siRNAs are commonly used in vertebrates because they are able to bypass this general non-specific response and achieve gene target-specific silencing via RNAi. However, one shortcoming of the introduction of naked siRNAs into cells is transient or short-term effect. To get around this problem, plasmid-based expression systems for endogenously producing pre-miRNAs have been developed (Tuschl and Borkhardt, 2002). Although the use of plasmid-based expression systems is an easy and inexpensive way to generate miRNAs, the expression of long hairpin RNA in some cases have been shown able to trigger sequence-nonspecific interferon responses in the cells, thereby leading to a global inhibition of mRNA translation (Dykxhoorn et al., 2003; Samuel, 2001). Thus, caution must be exerted in the interpretation of data from experiments using miRNA-related pathways. The side effects elicited by miRNAs are of concern for the use of RNAi technology as antiviral approaches, where gene silencing is often taken as indicative of successful specific-sequence manner.

Overall, a better understanding of the molecular pathogenesis of RSIV, as revealed by the DNA microarrays, is enormous contribution to the thorough knowledge of RSIV infection and control iridoviral diseases in aquaculture. Additionally, *in vitro* RNAi

studies have demonstrated that RNAi-related pathways are involved in antiviral defenses and could be evoked by introduction of both viral-specific siRNAs and miRNAs into fish cells. Thus the antiviral potency of RNAi can be applied *in vivo*, for instance, by using transgenic technology. The use of the trangene technology will generate transgenic fish that can induce stable gene silencing, resulting in long-lasting protective potency against viral infections. However, efficiency and stability of RNAi are influenced by many factors, including tissue-targeted types, route of administration and delivery vehicles for generating RNAi. Therefore, *in vivo* RNAi studies require careful considerations, especially for the use of delivery system for generating RNAi.

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