Singapore grouper iridovirus (SGIV) encoded SGIV-miR-13 attenuates viral infection via modulating major capsid protein expression

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\begin{abstract}
Singapore grouper iridovirus (SGIV) encodes a number of microRNAs (miRNAs) during infection. Among these, SGIV-miR-13 has robust expression at early stage after SGIV inoculation, raising a huge possibility that it participates in the viral infection. In the present study, we found that SGIV-miR-13 overexpression led to a significant reduction in viral load in cultured fish cells with SGIV infection, as demonstrated by less level of viral transcripts, viral-induced cytopathic effect (CPE) and assembled viral particles. \textit{In silico} analysis showed that SGIV-miR-13 maps antisense to the coding region of SGIV major capsid protein (SGIV-MCP), suggesting it to be a potential target of SGIV-miR-13. Coincidently, SGIV-miR-13 showed an inverted expression profile with SGIV-MCP during SGIV infection, and luciferase reporter assay further demonstrated SGIV-MCP as the direct target of SGIV-miR-13. Functionally, overexpression of SGIV-miR-13 inhibited, whereas knockdown of SGIV-miR-13 restored the expression of SGIV-MCP during viral infection, resulting in altered viral progeny emergences. In conclusion, our data suggest that SGIV-miR-13 functions in a negative regulatory mechanism to restrict early viral replication, and thus prevents excessive cellular antiviral responses during SGIV infection. The detailed investigation of SGIV encoded miRNAs may provide new insights into the mechanism of iridovirus pathogenesis.
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1. Introduction

MicroRNAs (miRNAs) are ~22-nucleotide (nt) noncoding RNA molecules that regulate gene expression at either the post-transcriptional or translational level (Bartel, 2004). Since firstly identified in \textit{Caenorhabditis elegans} in last 1990s, miRNAs are now well known to be encoded by all metazoan eukaryotes and plant species, and modulate a range of fundamental biological processes such as embryonic development, cell differentiation, apoptosis, and oncogenesis (Bushati and Cohen, 2007; Chosh et al., 2009). Given several unique features miRNAs possess, such as minimal genomic space, non-immunogenicity and the capacity to regulate specific but also multiple targets, it is no surprise that viruses also encode miRNAs as ideal mediators to facilitate viral replication (Skalsky and Cullen, 2010).

Recent studies have provided plenty of evidence that the biogenesis of viral miRNA is solely dependent on cellular miRNA biogenesis mechanism, which also initiates from the transcription of primary miRNAs in the nucleus, followed by sequential processing under two endogenous RNase III Drosha and Dicer (Boss and Renne, 2010). Therefore, viral miRNAs are to date investigated and experimentally confirmed to be encoded by DNA viruses of which the genome replication occurs in the nucleus, but not RNA viruses and cytoplasmic DNA viruses like poxviruses (Skalsky and Cullen, 2010). Till now, more than 200 viral miRNAs have been identified, and most of them are herpesvirus origin, including Marek’s disease virus (Bursinse et al., 2006; Yao et al., 2007), herpes simplex virus HSV-1 and HSV-2 (Tang et al., 2009), human and murine cytomegalovirus (hCMV and mCMV) (Dolken et al., 2007; Grey et al., 2005), Kaposi’s sarcoma-associated herpesvirus (KSHV) (Samols et al., 2005) and Epstein-Barr virus (EBV) (Pfeiffer et al., 2004). Apart from herpesviruses, some other dsDNA viruses, such as polyomavirus, adenovirus, ascovirus and baculovirus, are also able to encode miRNAs, suggesting that the encoding of miRNAs...
during replication is a general phenomenon in DNA virus families. (Grundhoff and Sullivan, 2011).

Compared to the well studied miRNAs in metazoan eukaryotes and plant species, information about the exact functions of most viral miRNAs is still imperfect. Generally, viral miRNAs are thought to favor the virus propagation and pathogenesis by evading immune response or promoting cell survival to support persistent infection (Chosh et al., 2009; Gottwein and Cullen, 2008). To accomplish this, viral miRNAs may target cellular or viral transcripts with full-length or seed sequence match through Watson-Crick base-pairing mechanisms, resulting in gene repression at post-transcriptional level. For example, the major histocompatibility complex class I-related chain B (MHC) was selectively targeted by hCMV miR-UL112-1, and its repression benefits the persistent viral infection via preventing infected cells from killing by natural killer (NK) cells (Stern-Ginossar et al., 2007). Two subsequent studies lent support to this finding that both KSHV miR-K7 and EBV miR-BART2 also suppressed MHC expression by targeting its 3’UTR but at different sites (Nachmani et al., 2009). Besides, the role of viral miRNAs in regulating viral functional genes has also begun to be appreciated. To date, most of the well-defined viral targets recognized by viral miRNAs are predominantly involved in persistent/viral infection. One of the best examples is the discovery that EBV miR-BART2 conducted negative regulation of a lytic gene—DNA polymerase BALF5 and hence led to enhanced latent infection (Barth et al., 2008). Other instances arose from the studies on HSV-1 and HSV-2, which utilized their miRNAs such as miR-H2, miR-H3 and miR-H4 to downregulate the expression of two immediate early genes, ICP0 and ICP34.5 in a siRNA-like manner to establish the latent viral infection state (Tang et al., 2008, 2009; Umbach et al., 2008). These findings suggest that viral miRNAs play indispensable roles during viral infection. An in-depth and systematic study of the interaction of viral miRNAs with cellular or viral targets will help us better understand the molecular mechanism of viral pathogenesis.

Singapore grouper iridovirus (SGIV) was firstly isolated from diseased brown-spotted grouper, Epinephelus tauiwana, and subsequently characterized as a novel member of the family Iridoviridae, genus Ranavirus (Qin et al., 2003, 2001). In recent years, SGIV has gained increasing attentions because of the high mortality and huge economic losses it posed to aquaculture in China and Southeast Asia (Song et al., 2004). In the past decade, the SGIV genome has been sequenced (Song et al., 2004), the whole genome transcriptional profiles of SGIV in virus-infected grouper cells and tissues, as well as the viral-infection induced cellular transcriptome alteration have been broadly studied (Teng et al., 2008). However, a comprehensive understanding of SGIV still remains largely unknown due to the limited information about defined roles of SGIV-encoded genes and molecular mechanisms of viral pathogenesis (Yan et al., 2013).

Recently, by employing Illumina/Solexa deep-sequencing, we identified 16 viral miRNAs in SGIV infected grouper cells. These viral miRNAs are dispersed throughout the SGIV genome, and some showed marked sequence and length heterogeneity at their 3’ end or 5’ end that could modulate their functions (Yan et al., 2011). Moreover, we found that 11 of these miRNAs possess potential biological activities, and one of our recent studies provides experimental evidence that SGIV encoded miR-homoHSV, which shares 57% sequence identity with HSV2-miR-H4-5p, was able to attenuate virus-cell death through targeting the pro-apoptotic viral gene SGIV-LITAF (Yan et al., 2011; Guo et al., 2013).

Among the SGIV miRNAs with potential biological activities, SGIV-miR-13, which maps antisense to the transcripts of SGIV major capsid protein gene (SGIV-MCP), also raised our great interest. In the present study, we found that SGIV-miR-13 could directly target SGIV-MCP. Acting like siRNAs, SGIV-miR-13 promotes the cleavage/degradation of SGIV-MCP transcripts and thus attenuates SGIV propagation in the early stage after viral infection. Our data suggest that iridovirus encoded miRNAs could function in a negative regulatory mechanism to fine-tune the process of viral assembly during the course of infection.

2. Material and methods

2.1. Cells and virus

Two fish cell lines, Grouper spleen cells (GS) and fathead minnow cells (FHM) were cultured as previously described (Cui et al., 2011; Gravell and Malsberg, 1965). SGIV (strain A3/12/98) was originally isolated from diseased brown-spotted grouper, E. tauiwana, and the propagation of SGIV was performed as reported previously (Qin et al., 2003).

2.2. Plasmids, oligoribonucleotides and transfection

Plasmids used in this study were constructed and stored in our laboratory as reported previously (Yan et al., 2011; Ou-Yang et al., 2012). Briefly, to generate SGIV-miR-13 overexpression plasmids, a 418-bp fragment that is antisense to SGIV ORF072 (genomic position: c66748-67165), comprising the SGIV-miR-13 stem-loop structure was subcloned into pLL3.7 modification vector and termed as pLL-miR-13. A green fluorescent protein (GFP) tag carried by pLL3.7 facilitates the monitoring of transfection efficiency by fluorescence microscopy (Leica Microsystems, Germany). To perform luciferase reporter assay, a partial sequence of SGIV ORF072R (SGIV-MCP) containing the predicted SGIV-miR-13 binding site was inserted into the 3’ untranslated region (UTR) of the Renilla gene in psiCHECK-2M (termed as psiC-miR-13). Besides, ORF072 expressing plasmid, pcDNA-72, created based on pcDNA-3.1, was generated previously by our laboratory (Ou-Yang et al., 2012). The RNA duplex mimicking SGIV-miR-13 duplex intermediate (mimic-miR-13), the antagonist with sequence perfectly complementary to SGIV-miR-13 (anti-miR-13), as well as their negative controls (mimic-NC and anti-NC) were obtained from RiboBio (RiboBio, China).

Lipofectamine™ 2000 or RNAiMAX reagent (Life technologies, USA) was used to perform transient transfection with plasmids and RNA Oligoribonucleotides individually, according to the manufacture’s instructions.

2.3. Stem-loop quantitative RT-PCR and real-time PCR

To assess the expression of SGIV-miR-13 during SGIV infection, stem-loop quantitative RT-PCR was performed as described previously (Yan et al., 2011). Briefly, mature miRNAs were extracted from SGIV infected GS cells (triplicate wells per time point; MOI = 0.1) using mirVana™ miRNA Isolation Kit (Life technologies, USA), and followed by reverse transcriptase reactions and stem-loop quantitative RT-PCR using customized TaqMan® microRNA Assays kit (Life technologies, USA) on the LightCycler® 480 Detection System (Roche, Switzerland). Small nuclear RNA U6 (RNU6-1) was used as a reference gene. Experiments were performed at least three times independently to verify the data.

Real-time PCR was performed to determine the expression of regular genes. In brief, total RNA was extracted using TRIzol reagent (Invitrogen, USA) and digested with RNase-free DNase I (TaKaRa, Japan). The first-strand cDNA was synthesized with ReverTra Ace® qPCR RT Kit (TOYOBO, Japan), and real-time PCR was performed using specific RT primer pairs (Supplemental Table 1). β-actin was used as a reference gene. For all experiments, triplicate wells were included in each group, and experiments were repeated at least three times.
2.4. Western blotting

To examine the expression of SGIV-MCP at the protein level, Western blotting was performed using mouse polyclonal antiserum against SGIV-MCP (1:2000). HRP-conjugated goat anti-mouse IgG was used as second antibody at a dilution of 1:2000 (Pierce, USA). Mouse anti-β-actin antibody (Sigma, USA) was used to confirm comparable loading. All experiments were repeated at least three times independently.

2.5. Generation of stable cell lines

To obtain higher and continuous expression of SGIV-miR-13 in vitro, GS cells in 24-well plates were transfected with 800 ng pLL-miR-13 or empty vector and selected with 2 μg puromycin ml⁻¹ (Sigma–Aldrich, USA) for 3 weeks to generate stable cell lines. The purity of established cell lines was monitored by detecting green fluorescence derived from pLL3.7, and the expression of SGIV-miR-13 was confirmed by stem-loop quantitative RT-PCR. These established stable GS lines were termed as sta-miR-NC and sta-miR-13, respectively.

2.6. Luciferase reporter assay

To ascertain whether SGIV-miR-13 directly targets SGIV-MCP, FHM cells cultured in 96-well plates were transfected with 40 ng sensor vector psiC-miR-13. 24 h after transfection, cells were re-transfected with 100 nM control mimics or mimics for SGIV-miR-13, 200 nM control antagomirs or SGIV-miR-13 antagomirs, for an additional 24 h. Then renilla/firefly luciferase activities were measured as reported previously (Yan et al., 2011). Experiments were performed three times with sample analyzed in triplicate.

2.7. Viral replication kinetics assay

To determine the effect of SGIV-miR-13 on viral replication in grouper cells, GS cells with stable SGIV-miR-13 overexpression or control cells, and GS cells with or without SGIV-miR-13 antagomirs, were cultured in 24-well plates and infected by SGIV at an MOI of
~0.1 for different time. Then the cell lysate in each well was collected and viral titers were determined using 50% tissue culture infectious dose (TCID50) assay (Reed and Muench, 1938). Cytopathic effect (CPE) was observed daily under a light microscope (Leica, Germany). Triplicate wells were included in each group per time point and experiments were repeated three times independently.

2.8. Transmission electron microscopy (TEM)

To determine the effect of SGIV-miR-13 on virus assembly, GS cells with stable SGIV-miR-13 overexpression or control cells were infected with SGIV. At 24 h after infection, cells were collected and fixed with 2.5% glutaraldehyde (v/v) at 4°C for overnight, and then post-fixed in 1% osmium tetroxide (w/v) for 1 h. After dehydrating in graded ethanol, embedding and sectioning, sections were double stained with uranyl acetate and lead citrate. Electron micrographs were recorded with a JEM-1340 electron microscopy (JEOL, Japan) at 120 kV. Experiments were performed three times to verify the data.

2.9. Statistical analysis

The two-tailed Student t test was used to compare between control and treatment groups throughout. Results are expressed as means ± SD, and a p value <0.05 was considered statistically significant.

3. Results

3.1. SGIV-miR-13 was implicated in SGIV infection

To ascertain whether SGIV-miR-13 possesses biological functions in SGIV infection, we employed vector-based overexpression of SGIV-miR-13 (pLL-miR-13) in FHM cells followed by SGIV infection for 28 h. Comparing with control group, pLL-miR-13 transfected cells exhibited weak cytopathic effect (CPE) phenotypically, with obvious reduction in the amount of detached cells and viral plaques (Fig. 1A). Furthermore, we also examine the transcriptional kinetics of SGIV genes, including immediate early gene ICP18, early gene LITAF as well as late gene MCP, VP16, VP19 and VP088 in cells treated as above. We found all these genes had lower expression levels in the presence of SGIV-miR-13 interventions. In particular, the expression of SGIV-MCP, VP088 and ICP18 were significantly reduced by 51% (p < 0.01), 40% (p < 0.05) and 33% (p < 0.05) respectively compared to those in the control group (Fig. 1B). These data suggested that SGIV-miR-13 is involved in the regulation of SGIV infection, which urgently prompted us to unravel the exact mechanism.

3.2. SGIV-miR-13 is expressed at an early stage after viral infection

The function of a miRNA is mainly executed through regulating the expression of its target gene(s) (Bartel, 2004). To explore the mechanism by which SGIV-miR-13 attenuates SGIV propagation, we firstly searched for the putative targets of SGIV-miR-13. In silico analysis showed SGIV-miR-13 is encoded by sequences found within the SGIV-MCP gene and is complementary to the MCP transcript (Fig. 2A), suggesting SGIV-MCP transcript may be a potential target of SGIV-miR-13.

To test our hypothesis, initially, we investigated the temporal expression profile of these two genes during viral infection. Interestingly, we found a high-level expression of SGIV-miR-13 in GS cells at early stage after SGIV infection (6 hpi). However, the expression of SGIV-miR-13 dropped dramatically at 12 hpi and maintained at a relative lower level till to 48 hpi (Fig. 2B). Conversely, the expression of SGIV-MCP remained undetectable at 6 hpi, and started to gradually accumulate from 12 to 48 hpi (Fig. 2B). These data suggested an inverted expression profile relationship between SGIV-miR-13 and SGIV-MCP. Meanwhile, SGIV-miR-13 may be involved in the SGIV replication since it is expressed in an early stage.

3.3. SGIV-MCP is a direct target of SGIV-miR-13

Next, we employed a series of independent strategies to experimentally determine whether SGIV-MCP is a direct target of SGIV-miR-13. Firstly, we transfected FHM cells with SGIV-miR-13 overexpression plasmids (pLL-miR-13) or control vector (PLL3.7), followed by SGIV infection for 18 and 28 h. We found the abundance of SGIV-MCP expression obviously declined in the presence of SGIV-miR-13 overexpression at both mRNA and protein levels, especially at 28 hpi by an approximate 50% decline (p < 0.05) compared to empty vector control group (Fig. 3A and B). Meanwhile, we also examined the effect of SGIV-miR-13 on the vector-driven expression of SGIV-MCP by co-transfection of pLL-miR-13 and pcDNA-72 into FHM cells. As shown in Fig. 3C and D, transfection of pLL-miR-13 resulted in a significantly decreased level of SGIV-MCP transcripts as well as huge declines in protein levels.

To determine whether mature SGIV-miR-13 oligos possess the same function with vector-driven miRNA precursors, we transfected FHM cells with synthetic SGIV-miR-13 mimics, followed by either SGIV infection or pcDNA-72 transfection. Similar to the results observed in pLL-miR-13 transfection experiments, we found that 100 nM and 200 nM SGIV-miR-13 mimics were able to dramatically repress the endogenous expression of SGIV-MCP by more than 50% (Fig. 3E and F). In addition, when co-transfected with SGIV-MCP expression plasmids, mimics for SGIV-miR-13, but not for another reported SGIV miRNA—SGIV-miR-homolHSV, could specifically repress the expression of SGIV-MCP (Fig. 3G and H).

Subsequently, we examined the effect of SGIV-miR-13 in SGIV host cell line-GS cells. Considering the poor transfection efficiency in GS cells, we established stable GS cell line expressing...
SGIV-miR-13 (sta-miR-13) and control line (sta-miR-NC). As shown in Fig. 4A, both lines displayed high and homogeneous fluorescence signals with no obvious morphological difference, suggesting a high purity of each cell line. The overexpression of SGIV-miR-13 in sta-miR-13 cells was also confirmed by TaqMan® MicroRNA Assay (Fig. S1). Similar to the results observed in FHM cells, SGIV-miR-13 overexpression dramatically diminished the endogenous expression of SGIV-MCP after viral infection in GS cells (Fig. 4B and C). Furthermore, SGIV-miR-13 was also able to efficiently repress the pCDNA-72 precursor expression of SGIV-MCP in GS cells (Fig. 4D and E).

Moreover, we found that synthetic SGIV-miR-13 mimics were able to inhibit the luciferase activity (>30% decrease, p < 0.05) arising from the SGIV-miR-13 sensor vectors (Fig. 5A). Collectively, these data demonstrate that SGIV-miR-13 directly targets and directs the cleavage of SGIV-MCP mRNA in both non host cells and host cells of SGIV.

3.4. Blocking SGIV-miR-13 elevated the endogenous and exogenous SGIV-MCP levels

To further confirm the findings observed above, we next employed antagonors against SGIV-miR-13 (anti-miR-13) to conduct the loss-of-function analysis. In contrast to SGIV-miR-13 mimics which could inhibit the luciferase activity of pSIC-miR-13 sensor plasmids, overexpression of anti-miR-13 in FHM cells could rescue the decline of luciferase activities caused by co-transfection with pLL-miR-13 (Fig. 5A and B). To investigate if anti-miR-13 has any effect on the expression of SGIV-MCP during viral infection, we transfected FHM cells with anti-miR-13 or anti-miR-NC followed by SGIV infection. We found inhibition of SGIV-miR-13 during SGIV infection moderately elevated SGIV-MCP levels compared to anti-miR-NC group (Fig. 5C). In addition, anti-miR-13 also showed the ability to rescue the inhibition of SGIV-MCP by pLL-miR-13-driven SGIV-miR-13 precursors in pCDNA-72 transfected cells (Fig. 5D).
These data provide further evidence that SGIV-MCP is a direct target of SGIV-miR-13 during viral infection. Meanwhile, manipulation of SGIV miRNAs may serve as a novel tool to regulate the expression of viral functional genes.

3.5. **SGIV-miR-13 attenuated SGIV replication and production**

MCP is one of the major structural components which are essential to the assembly of intact viral particles. We next determined if the association between SGIV-miR-13 and SGIV-MCP has biological significance during the process of viral infection. Firstly, we selectively transfected FHM cells with pLL-miR-13 or control vector, SGIV-miR-13 mimics or control mimics, followed by SGIV infection (MOI=0.1) for 18 and 28 h. Then the viral loads were titrated and compared between each group. As shown in Fig. 6A, the viral titers yielded from SGIV-miR-13 expression cells were markedly lower (~2.6–3.5 folds; p < 0.05) than that of control group (Fig. 6A, left and middle panel). Concordant results were obtained in stable GS cells with or without SGIV-miR-13 overexpression (Fig. 6A, right panel).
In addition, we also examined the impact of SGIV-miR-13 on viral particle assembly in stable GS cells by employing transmission electron microscopy (TEM). As expected, sta-miR-NC cells displayed high-orderly arranged hexagonal virions in the cytoplasm of SGIV infected cells (Fig. 6B, upper lane). However, the total number of assembled viral particles is much lower in sta-miR-13 cells, which only showed a scattered pattern of virions accumulation (Fig. 6B, lower lane). Meanwhile, we examined the transcripts levels...
of SGIV functional genes in these two stable lines after SGIV infection. We found all the tested genes had relatively lower expression in sta-miR-13 cells (Fig. 6C), which is in striking congruence with the results observed in pLL-miR-13 transfected FHM cells (Figs. 1B and 6C). Moreover, blocking SGIV-miR-13 expression by specific antagonirs resulted in higher expression levels of SGIV functional genes, as well as elevated virus titers (∼2.8–5.2 folds, p < 0.05) after SGIV infection (Fig. 6D and E). Taken together, these data suggest the endogenous expression of SGIV-miR-13 during viral infection has biological significance, which may serve as a negative-regulatory mechanism to fine tune the expression of SGIV major capsid protein.

4. Discussion

In the past decade, miRNAs have attracted tremendous attentions due to their regulatory roles in almost all biological processes in eukaryotes (Bartel, 2004). Of note, since the first discovery of viral origin miRNA in EBV, mounting evidence illustrates divergent viral families possess the ability to encode miRNAs which share similar biogenesis with cellular origin miRNAs (Cullen, 2006; Umbach and Cullen, 2009). However, compared to the well studied miRNAs of cellular origin, the information regarding virus-encoded miRNAs is still much limited.

Compared to miRNAs of cellular origin, it is assumed to be more straightforward to identify putative viral target genes of viral miRNAs when a miRNA is encoded antisense to viral transcripts in the virus genome. There has been plenty of evidence that viral miRNAs could functionally target their corresponding antisense viral transcripts. One elegant example is the studies on Simian Virus 40 (SV40) miR-S1, which was reported to degrade the antisense-transcribed mRNA of viral T antigen, helping the viruses to avoid being attacked by host immune system (Sullivan et al., 2005). Such functional relevance of miRNA–viral target pairs were also observed in other virus families, including EBV, murine Polyomavirus, HSV and hCMV (Grundhoff and Sullivan, 2011). Of note, unlike mammalian miRNAs which inhibit targets expression primarily through promoting the degradation of target mRNAs, most viral miRNAs in cases above downregulate their viral targets through a translational repression mechanism, with less efficacy on the cleavage of target transcripts (Umbach et al., 2008). By employing bioinformatic analysis, we found SGIV-miR-13 gene and SGIV ORF072R, which encodes the SGIV-MCP, lie in the same SGIV genome locus, and SGIV-miR-13 mature sequence is completely complementary antisense to SGIV-MCP transcripts, suggesting a high possibility that SGIV-MCP is a direct target of SGIV-miR-13. In the present study, we found SGIV-miR-13 overexpression attenuated, whereas inhibition of SGIV-miR-13 enhanced SGIV-MCP expression during viral infection. Moreover, consistent with our previous finding that vector-driven SGIV-miR-13 precursors could downregulate the luciferase activities in the SGIV-miR-13 sensor system (Yan et al., 2011), luciferase reporter assay using synthetic SGIV-miR-13 mimics further demonstrated SGIV-MCP as the direct target of this viral miRNA. Intriguingly, unlike most reported viral miRNAs in diverse virus families, SGIV-miR-13 could promote the degradation of target mRNA as demonstrated by less SGIV-MCP transcripts in the absence of SGIV-miR-13, suggesting SGIV-miR-13 regulates gene expression through the most common manner like cellular miRNAs.

The final outcome of a viral miRNA expression is largely dependent on the endogenous function of its target gene during viral infection. In most cases, inhibition of viral transcripts by viral miRNAs leads to attenuated viral infection. For example, hCMV miR-UL112-1 was reported to directly target and repress the expression of immediate-early gene IE72, a key transactivator of multiple viral early genes, resulting in significantly decreased viral replication (Grey et al., 2007). Another elegant example came from the studies on Heliothis virescens ascovirus (HvAV) encoded miR-1, which was reported to negatively regulate HvAV replication through downregulating virus-encoded DNA polymerase (Hussain et al., 2008). However, this is not always the case. A recent study from Zhang’s group revealed that two miRNAs from white spot syndrome virus (WSSV), named WSSV-miR-66 and WSSV-miR-68, could promote viral infection through targeting four viral genes during the WSSV infection in shrimp in vivo (He et al., 2014).

Although the molecular mechanisms by which these four viral genes negatively regulate viral infection are still unclear, these findings added another layer of complexity into the regulatory mechanisms of viral miRNAs during viral infection. In the present study, we found SGIV-miR-13 overexpression led to a less severe virus-induced CPE, decreased viral titers, less density and scattered arrangement of viral particles, as well as reduced global transcriptional activity of viral genes after SGIV infection in fish cells. These effects could be presumably attributed to SGIV-miR-13–mediated inhibition of SGIV-MCP, the predominant structural component of the iridovirus particles. Meanwhile, to the best of our knowledge, our study provides the first evidence that viral miRNAs could directly target viral structural proteins to participate in the regulation of viral infection.

Like protein coding genes, both cellular miRNAs and viral miRNAs are subjected to regulations at the transcriptional level, giving rise to the expression profiles with cell, tissue or particular developmental stage preference (Bartel, 2004; Kim and Nam, 2006). In general, the expression of miRNA is strictly controlled in certain biological events, where it displays an inverse relationship with the expression pattern of its target genes (Kim and Nam, 2006). This inverted correlation also applies to viral miRNAs and their targets. For examples, HvAV-miR-1 can be detected from 96 hpi up to 144 hpi in virus infected S99 cells. In contrast, its target gene–HvAV DNA polymerase I, showed a rapidly declined expression since 96 h after infection, the same time as HvAV-miR-1 starts to emerge in the cells (Hussain et al., 2008). In this study, we found a very-high expression of SGIV-miR-13 at early stage after SGIV infection (6 hpi) in GS cells, suggesting it is an early transcribed gene. However, the level of SGIV-miR-13 dropped dramatically from 12 hpi up to 48 hpi. Conversely, SGIV-MCP started to accumulate stably from 12 hpi, demonstrating an inverted expression profile with SGIV-miR-13. Presumably, during the term of SGIV infection, the higher expression of SGIV-miR-13 at early stage may help to suppress viral assembly through targeting SGIV-MCP to achieve a reduced cellular immune surveillance, a commonly used strategy by a number of viruses to benefit sufficient viral replication. While at the late state of viral infection, a reduced SGIV-miR-13 expression may release its inhibition on SGIV-MCP and ultimately facilitates viral production. Of note, the expression profiles regarding SGIV-miR-13 and SGIV-MCP are quite similar with that observed with SGIV encoded miR-homoHSV and its target SGIV-LITAF (Guo et al., 2013), suggesting a tightly controlled kinetics of viral miRNA-mediated gene silencing exists during the course of SGIV infection.

Taken together, we characterized a recently identified viral miRNA SGIV-miR-13. We found SGIV-miR-13 is highly expressed at early stage after SGIV infection and serves as a negative regulator of SGIV replication. Moreover, we identified SGIV-MCP as a direct target of SGIV-miR-13, which could mediate downregulation of SGIV-MCP in a siRNA-like mechanism. Our data provide further evidence that SGIV encoded miRNAs contribute to another layer of complexity in the self-regulation of SGIV replication during viral infection. A further and systematic investigation of SGIV encode miRNAs may help us better understand the pathogenesis of SGIV infection, and employing viral miRNA may serve as a novel therapeutic strategy to combat this aquatic animal virus.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virosis.2015.05.010

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