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Host defense against DNA virus infection in shrimp is mediated by the siRNA pathway

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The RNA interference (RNAi) system of eukaryotes using siRNAs has been documented as an immune response against invasion by RNA viruses. However, whether the siRNA pathway can be triggered by the infection with DNA viruses in animals remains to be investigated. In the present study, we show that *Marsupenaeus japonicus* shrimp can generate an antiviral siRNA (vp28-siRNA) in response to infection by a double-stranded DNA virus, white spot syndrome virus (WSSV). After challenging with WSSV, vp28-siRNA is detected in all the WSSV-infected organs and tissues of shrimp as early as 24 h postinfection (p.i.). The results indicate that the host Dicer2 and Ago2 proteins are required for the biogenesis and function of vp28-siRNA, respectively. We show further that vp28-siRNA predominates in the cytoplasm of shrimp hemocytes at 48 h p.i. Knockdown of Dicer2 by special siRNA or inhibition of vp28-siRNA with locked nucleic acid antisense oligonucleotides both lead to a significant increase in WSSV copy number at 24–48 h p.i. Our study highlights a novel aspect of the siRNA pathway in the immune response of animals against infection by DNA viruses.

Keywords: Ago2 · Dicer2 · Shrimp · siRNA · WSSV

See accompanying Commentary by Cherry

Introduction

RNA interference (RNAi) is an evolutionarily conserved mechanism that involves the triggering of adaptive immune responses against RNA viruses by small interfering RNAs (siRNAs) in eukaryotes [1–5]. The double-stranded (ds) siRNAs of approximately 21 base pairs in length are generated from dsRNAs, such as those from RNA viruses, endogenous transposons, or other genomic regions that produce transcripts capable of forming dsRNA structures [6–11]. The siRNAs processed from RNA viruses provide antiviral defense in plants and animals through the cleavage and degradation of target viral mRNA [12–15].

The mechanism by which eukaryotic organisms use RNA silencing to combat virus infections was first discovered in plants and invertebrates [16, 17]. In plants, either RNA or DNA virus infection can trigger host Dicer-like proteins to produce virus-derived siRNAs (viRNAs) that destroy the RNAs of invading viruses [18–20]. To further amplify these antiviral molecules, secondary viRNAs are produced by RNA-dependent RNA polymerases (RdRPs) that generate more dsRNAs from these cleaved transcript fragments, suggesting that the formation of dsRNA is necessary to initiate the synthesis of antiviral siRNA [21,22]. Similar amplification of viRNA with RdRPs was also discovered in *Caenorhabditis elegans* [5]. However, no RdRP homologs that convert single-stranded (ss) viral transcripts to dsRNAs have been identified thus far in flies or mammals. Recently, DNA viRNA were identified in mosquitoes using high-throughput sequencing of small RNAs, but the underlying mechanism of viral siRNA biogenesis remains unclear [23]. Although the antiviral activities of siRNAs against RNA viruses have been well documented, it is not clear whether animals can employ siRNAs in defense against DNA virus infections.

In flies and mammals, a new class of endogenous small RNAs, endogenous siRNAs (endo-siRNAs), are generated from dsRNAs

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independent of RdRPs, including long hairpin RNAs embedded in the transcripts of palindromic sequences and overlapping dsRNAs formed by symmetrical transcripts [24, 25]. In mouse oocytes and fruit fly somatic cells, a subset of endo-siRNAs are found to be complementary to overlapping mRNAs that are predicted to form dsRNA in vivo [24, 25]. The biogenesis of endo-siRNAs suggests that antiviral siRNAs may be produced from the dsRNAs that originate from symmetrical transcripts during the replication of animal dsDNA viruses because of the compact nature of virus genomes. However, the biogenesis of antiviral siRNA in response to DNA virus infection remains to be addressed in animals.

To investigate the antiviral RNAi response to DNA virus infection, white spot syndrome virus (WSSV), a dsDNA virus of shrimp, was used to characterize the host response to infection. WSSV contains a ds circular DNA genome of 305 kbp encoding 181 open reading frames (ORFs) [26] and infects shrimp, an economically important marine invertebrate [27]. Recently, 40 distinct miRNAs encoded by WSSV were identified using miRNA microarray and northern blot approaches [28]. In our previous study, the results showed that a specific siRNA targeting the vp28 gene of WSSV that encodes a major viral envelope protein required for WSSV infection [29], possessed strong antiviral activity in *Marsupenaeus japonicus* shrimp [30]. The antiviral capacities of two distinct siRNAs from the vp28 gene of WSSV as well as two control siRNAs were examined, but only vp28-siRNA affected viral replication [30]. It was shown that WSSV was completely eliminated by the administration of vp28-specific siRNA (vp28-siRNA) in the virus-infected shrimp, strongly suggesting that RNAi mediated by siRNA plays an important role in shrimp immunity against infection with DNA virus. In this context, vp28-siRNA was further characterized in this study to address whether dsDNA virus infection could trigger host antiviral siRNA. We show that the host defense against DNA virus infection could be mediated by the siRNA pathway in animals.

Results

Generation of antiviral siRNA in response to WSSV infection in shrimp

We previously showed that in vitro synthesized vp28-siRNA administered to virus-infected shrimp could eliminate WSSV in vivo, indicating the strong antiviral activity of siRNA [30]. To determine whether the host could generate these antiviral siRNAs in response to infection with DNA virus, shrimp were infected with WSSV and vp28-siRNA was detected (Fig. 1A). At 48 h post-infection (p.i.), vp28-siRNA was detected using vp28-siRNA sense and antisense probes in virus-infected shrimp, and not in uninfected shrimp (Fig. 1B). However, when the DIG-labeled sense and antisense probes corresponding to another synthesized siRNA (non-siRNA) targeting vp28 gene employed in our previous study [30] were used for northern blot detection, no positive signal was observed in either WSSV infected or uninfected shrimp hemocytes (Fig. 1B), indicating the specificity and efficiency of vp28-siRNA. During RNAi, it is known that only one strand of ds siRNA is loaded

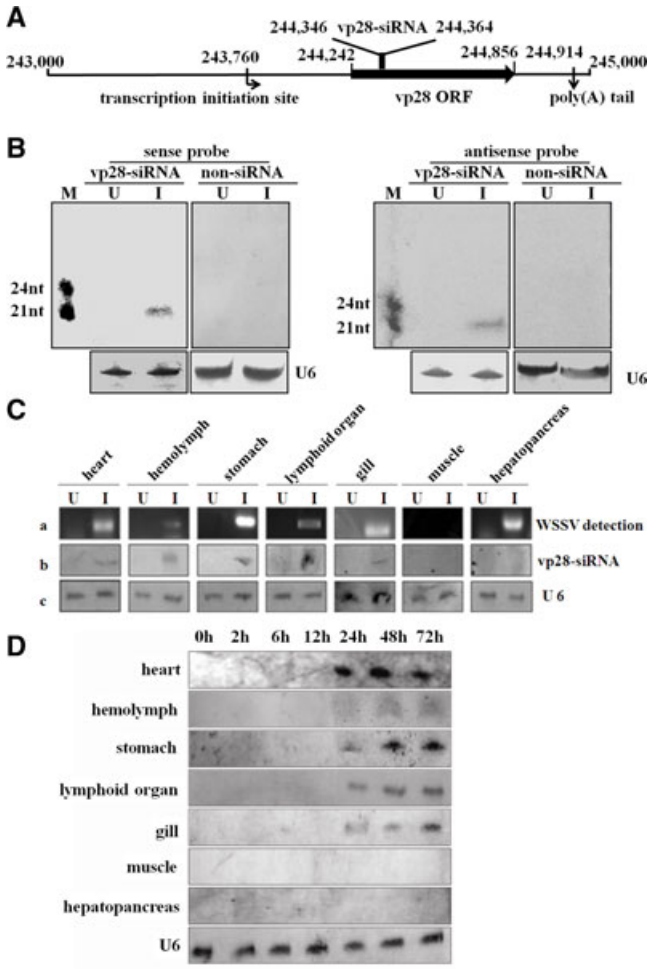

Figure 1. Generation of antiviral vp28-siRNA in WSSV-infected shrimp. (A) Schematic diagram of the location of vp28-siRNA in the WSSV genome. Numbers indicate the locations of vp28 open reading frame, vp28 transcription initiation site, vp28-siRNA, and poly(A) tail in the genome. (B) Detection of siRNAs in WSSV-infected and uninfected shrimp. Shrimp were infected with WSSV and 48 h p.i., the small RNAs were extracted from hemocytes of WSSV-infected (I) and uninfected shrimp (U). The small RNAs (vp28-siRNA and non-siRNA) were detected by northern blots using vp28-siRNA sense probe, non-siRNA sense probe (left), vp28-siRNA antisense probe or non-siRNA antisense probe (right). U6 was used as a control. M, RNA marker. (C) Detection of vp28-siRNA from different organs and tissues of WSSV-challenged shrimp. At 48 h p.i., tissues of WSSV-infected shrimp were collected and subjected to WSSV detection by PCR (top). Small RNAs were extracted from WSSV-infected shrimp at 48 h p.i. and virus-free shrimp and were subsequently probed with the DIG-labeled vp28-siRNA sense probe (middle) or the DIG-labeled U6 probe as a positive control (bottom). The lane headings indicated the uninfected (U) and WSSV-infected (I) shrimp. (D) The time-course generation of vp28-siRNA in response to WSSV infection. At different times after infection of shrimp with WSSV (0, 2, 6, 12, 24, 48, and 72 h p.i.), tissues were collected and subjected to small RNA isolation and northern blot. Small RNAs were detected with the DIG-labeled vp28-siRNA sense probe. As a control, U6 was included in the blotting analysis. The lane headings indicate the time p.i. with WSSV. The data shown are representative of three independent experiments.

in the RNA-induced silencing complex (RISC), as a guiding RNA to regulate the expression of targeted mRNA [16]. Therefore the vp28-siRNA sense probe was used to detect vp28-siRNA in subsequent assays.

After WSSV challenge of virus-free shrimp, PCR detection for WSSV showed that the virus infected different organs and tissues of *M. japonicus* shrimp, including heart, hemolymph, stomach, lymphoid organ, gill, and hepatopancreas, but not muscle (Fig. 1C). The results demonstrated that vp28-siRNA [21 nucleotides (nts)] was detected in the heart, hemolymph, stomach, lymphoid organ, and gill of shrimp at 48 h p.i. (Fig. 1C). However, no positive signal was observed in uninfected shrimp and in the hepatopancreas and muscle of WSSV-infected shrimp. As a control, U6 small nuclear RNA (snRNA) was detected in all organs and tissues of shrimp analyzed (Fig. 1C). Cloning and sequencing of vp28-siRNA from WSSV-infected shrimp confirmed that the siRNA detected by northern blot analysis was indeed vp28-siRNA. These data demonstrated that the generation of vp28-siRNA was induced by WSSV infection. To exclude the possibility that vp28-siRNA was generated from other possible virus infection than WSSV, shrimp were challenged with a ss DNA virus *Penaeus stylirostris* densovirus (PstDNV) and vp28-siRNA was detected. The data showed that there was no vp28-siRNA detected in the PstDNV-infected shrimp (data not shown).

To determine the time course of antiviral siRNA production in shrimp in response to invasion of DNA virus, the expression profile of vp28-siRNA was examined in shrimp at various times p.i. vp28-siRNA was detected as early as 24 h p.i. in various organs or tissues of WSSV-infected shrimp, including the heart, hemolymph, stomach, lymphoid organ, and gill tissue, but not in the shrimp hepatopancreas or muscle (Fig. 1D). From 24 h p.i., antiviral vp28-siRNA was generated in shrimp throughout the time examined (Fig. 1D). The quantitative detection of WSSV virions by real-time PCR determined that there were $\geq 10^6$ virus genomes were present from 24–72 h p.i. (data not shown), indicating that 10^6 virions were sufficient to initiate the production of antiviral vp28-siRNA in shrimp. The above data clearly demonstrated that the siRNA pathway could be triggered in invertebrates by infection with a DNA virus.

Requirements of host Dicer2 and Ago2 proteins for the siRNA pathway

To reveal the pathway for the biogenesis of siRNA in the host in response to DNA virus infection, key genes (Drosha, Dicer1, and Dicer2) required in RNAi were silenced by administration of sequence-specific siRNAs in shrimp. Real-time PCR data showed that the mRNA levels of Drosha, Dicer1, and Dicer2 genes were significantly decreased by gene-specific siRNAs at 24 and 48 h after the injection of siRNA (Fig. 2A–C, left). However, the mRNA levels of these three genes were not significantly changed when treated with the mutation-siRNAs (Fig. 2A–C, left), suggesting that the effect of siRNA was highly sequence specific. Thus, when Drosha, Dicer1, or Dicer2 genes were sufficiently silenced, vp28-siRNA expression in shrimp was detected. Northern blot analysis indicated that vp28-siRNA could not be detected only for Dicer2-siRNA treatment, while vp28-siRNA was generated for the Drosha-siRNA and Dicer1-siRNA treatments (Fig. 2, right). Therefore, the biogenesis of vp28-siRNA was dependent on the expression of Dicer2, but not Drosha or Dicer1.

To determine whether vp28-siRNA was associated with Ago1 or Ago2 proteins during RISC assembly, the ds vp28-siRNA or the ds control siRNA were incubated with Ago1 or Ago2 proteins tagged with GST and GST control protein, respectively. Electrophoretic mobility shift assay (EMSA) showed that the GST-Ago2 protein interacted with vp28-siRNA, whereas the GST-Ago1 and GST proteins did not bind to vp28-siRNA (Fig. 3). The control siRNA was shown to interact with Ago1, but not Ago2 (Fig. 3). These data show that host Ago2 was essential for the function of siRNA in RISC. Overall, these findings revealed that the host Dicer2 and Ago2 play essential roles in the pathways of vp28-siRNA biogenesis and function.

The localization of siRNA in hemocytes

In an attempt to examine the generation and localization of siRNA in response to DNA virus infection, WSSV-challenged shrimp hemocytes were stained with FITC-labeled vp28-siRNA sense probe or FITC-labeled vp28-siRNA-mutation sense probe. Northern blot analysis showed that no signal was produced in WSSV-infected or uninfected shrimp when the vp28-siRNA-mutation sense probe was used (data not shown). Confocal microscopy showed that vp28-siRNA was detected at 48 h p.i. in the WSSV-challenged shrimp using the vp28-siRNA sense probe (Fig. 4A and B). No signal was detected in the WSSV-challenged shrimp (0 and 48 h p.i.) when the FITC-labeled vp28-siRNA-mutation sense probe was used (Fig. 4C), showing that the generation of vp28-siRNA was in response to the WSSV infection and the probes were highly sequence specific. At 48 h p.i., the vp28-siRNA was distributed in the cytoplasm of shrimp hemocytes, suggesting that vp28-siRNA might be generated and execute its antiviral function in the cytoplasm in response to DNA virus infection. However, when the expression of host Dicer2 gene was inhibited by the corresponding sequence-specific siRNA, vp28-siRNA could not be detected using the FITC-labeled vp28-siRNA sense probe at 48 h p.i. (Fig. 4D), showing that the host Dicer2 was essential for the biogenesis of vp28-siRNA.

Antiviral role of vp28-siRNA in shrimp

Our data show that the vp28-siRNA was generated in shrimp in response to the WSSV infection. To assess the roles of vp28-siRNA in the WSSV-infected shrimp, the expression of host Dicer2 gene was inhibited by Dicer2-specific siRNA. The results indicate that the knockdown of Dicer2 expression led to a significant increase of WSSV copies at 24 and 48 h p.i. compared with that of WSSV only (the positive control), while the control Dicer2-mutation-siRNA had no effect on virus replication (Fig. 5). These data indicated that vp28-siRNA played important roles in shrimp antiviral immunity.

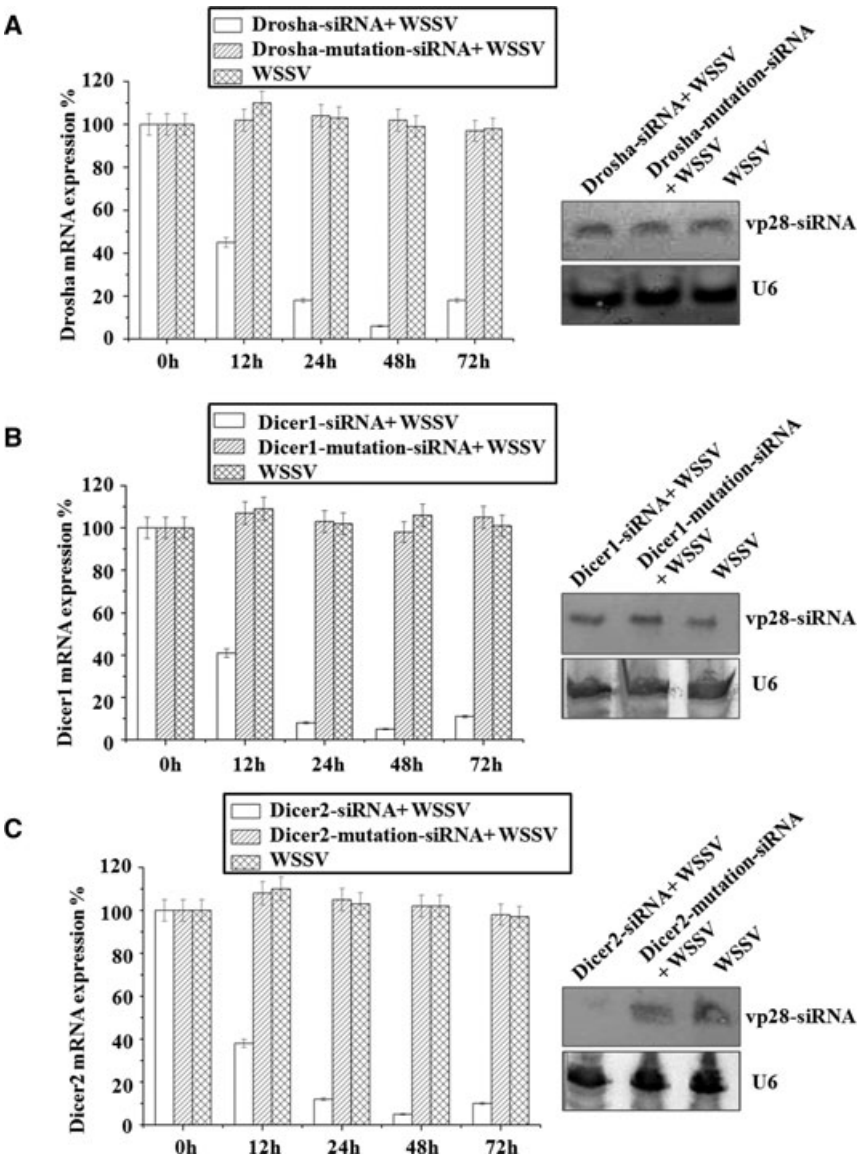

Figure 2. Requirement of Dicer2 in the biogenesis of vp28-siRNA. (A–C) The sequence-specific (A) Drosha-siRNA, (B) Dicer1-siRNA, or (C) Dicer2-siRNA, as well as the randomly mutated siRNAs (Drosha-mutation-siRNA, Dicer1-mutation-siRNA, or Dicer2-mutation-siRNA) were simultaneously injected into shrimp with WSSV. WSSV was also injected alone. At the indicated times after injection, lymphoid organs were collected and subjected to real-time PCR to quantify gene expression (left). At 48 h after injection, the collected lymphoid organs were analyzed by northern blot using the vp28-siRNA sense probe or the control U6 probe (right). Data are shown as mean ± SD of triplicate assays within 1% SD and are representative of three independent experiments performed. Probes used for analyses are shown on the right.

To block the function of vp28-siRNA, a locked nucleic acid (LNA)-modified anti-siRNA oligonucleotide (anti-vp28-siRNA LNA inhibitor, vp28-siRNA-inhibitor) was used. The quantitative PCR results demonstrated that the inhibition of vp28-siRNA by vp28-siRNA-inhibitor resulted in significant increases in virus copies at 24 and 48 h p.i. ($p < 0.05$) compared with the WSSV controls. However, the control anti-vp28-siRNA LNA inhibitor-mutation (vp28-siRNA-inhibitor-mutation) had no effect on virus replication (Fig. 5). These analyses indicated that vp28-siRNA was involved in shrimp antiviral responses, but failed to provide a lasting protective effect at 72 and 96 h p.i. ($p > 0.05$; Fig. 5).

Discussion

The siRNA pathway is one of the most efficient strategies employed by multicellular organisms to fight RNA virus infection [16]. It has been reported that the siRNAs (24 nts) derived from plant DNA viruses (Cauliflower mosaic virus and Geminivirus), seem to confer resistance against DNA viruses in plants through epigenetic modifications of the corresponding DNA in the nucleus [31–33]. Recently, siRNAs derived from DNA viruses were identified in mosquitoes using small RNA high-throughput sequencing [23]. However, it remains unclear whether the siRNA pathway can be triggered to defend against infections with DNA viruses in animals. In this study, it was revealed for the first time that infection with a dsDNA virus (WSSV) could trigger the production of DNA viRNA in the host (shrimp) to prevent virus infection. In our study, vp28-siRNA was detected in WSSV-challenged shrimp, but not in virus-free shrimp or PstDNV-infected shrimp. Using small RNA cloning and sequencing analyses, the antiviral siRNA (vp28-siRNA) was cloned and identified in WSSV-infected shrimp. These findings indicated that the vp28-siRNA was specifically generated in shrimp in response to WSSV infection. In this context, RNAi played

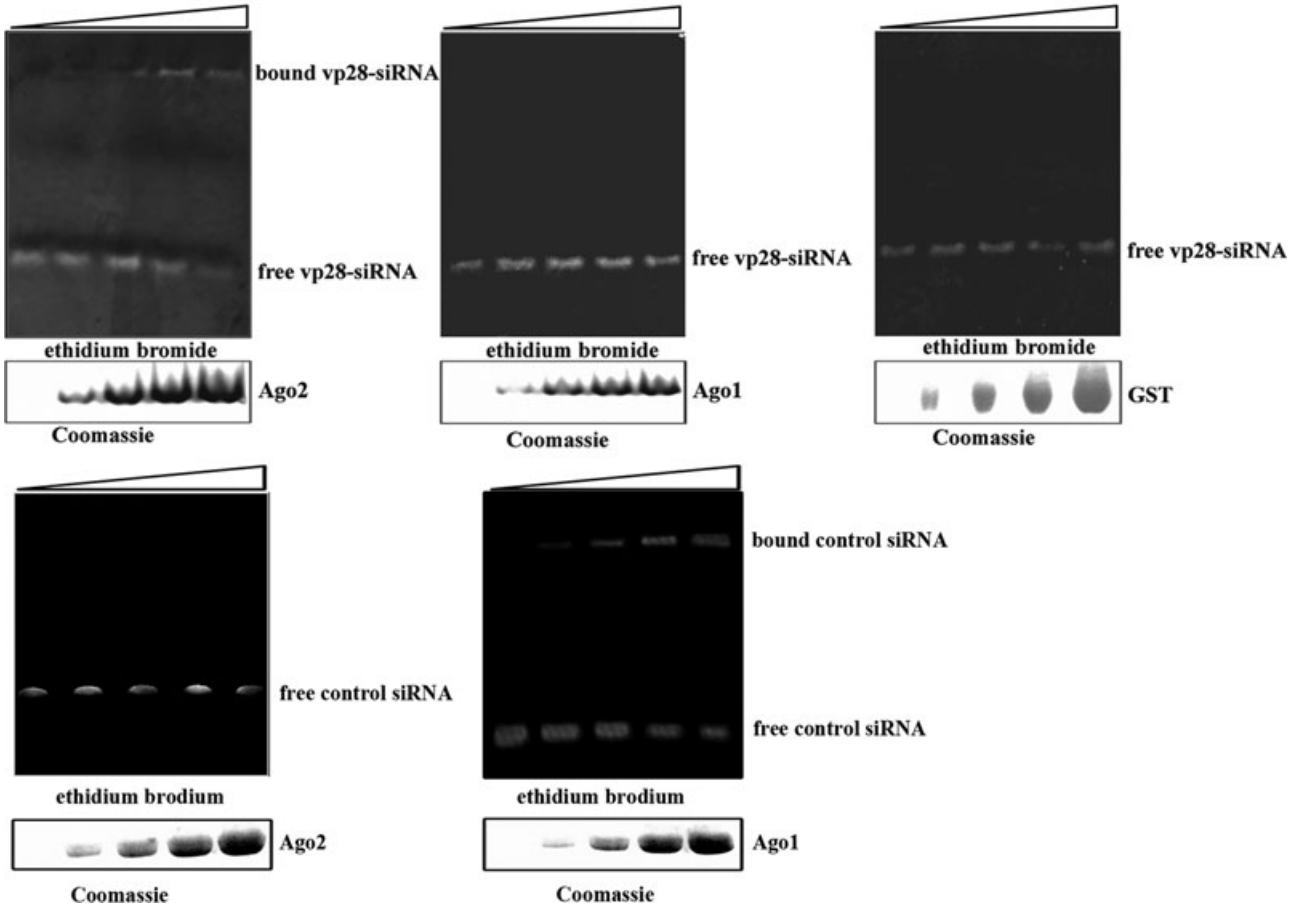

Figure 3. Preferential interaction of vp28-siRNA with Ago2 protein by EMSA. The ds vp28- siRNA (2 μg) or the ds control siRNA (2 μg) were incubated with recombinant GST-Ago1, GST-Ago2, or GST proteins at different concentrations. Subsequently, the mixture was separated by native polyacrylamide gel and stained with ethidium bromide to show the siRNA (top), followed by staining with Coomassie blue (bottom). The wedges show the concentration gradients of recombinant proteins used. Data shown are representative of three experiments performed.

an important role in animal immune responses against virus infection.

In plants, it is inferred that the generation of siRNA in response to infection with DNA virus may result from the replication of the virus in the host cell's nucleus, leading to epigenetic modifications of the virus genome to render it similar to the host genome [31–33]. However, the current study indicates that the biogenesis of vp28-siRNA in WSSV-infected shrimp was Dicer2-dependent, indicating that the host siRNA pathway in response to RNA virus infection was employed in defense against the DNA virus infection, which is very different from the biogenesis of DNA viRNA in plants. It was previously documented that dsRNAs were commonly generated from a range of DNA viruses during their replication cycle [34], and these dsRNAs derived from DNA viruses might be processed by Dicer into siRNA and interact with Ago for the restriction of DNA virus replication. Despite the common production of dsRNA during DNA virus infection of animals, it is surprising that the siRNAs derived from DNA viruses have been rarely demonstrated in animals; this is especially surprising in invertebrates that are thought to rely on the ancient RNAi immune system for antiviral defense because of the lack of a true adaptive immune response system and the typical interferon response system such as that found in vertebrates [13]. Our study shows that the silencing of shrimp Dicer2 or the inhibition of vp28-siRNA leads to significant increases in WSSV copies during infection, demonstrating that the DNA viRNA is involved in the host antiviral response. The identification of the siRNA pathway in response to DNA virus invasion in this study suggests a novel aspect of siRNA in the innate immunity of animals against virus infection: siRNA not only plays an important role in the immune response against RNA virus infection, but also for DNA virus infection. Considering the key role of vp28-siRNA in the shrimp immune response, it was proposed that WSSV might have evolved to encode some unknown RNA-silencing suppressors to counteract the antiviral function of vp28-siRNA. Indeed, this might be the reason that vp28-siRNA seemed to fail to provide a lasting protective effect [35].

In this study, the results showed that vp28-siRNA was predominantly found in the cytoplasm of shrimp hemocytes at 48 h p.i., indicating the possible sites of production and function of siRNA. Furthermore, vp28-siRNA was not detected when Dicer2 was knocked down by RNAi. The presence of vp28-siRNA in the cytoplasm might lead to the inhibition of WSSV infection

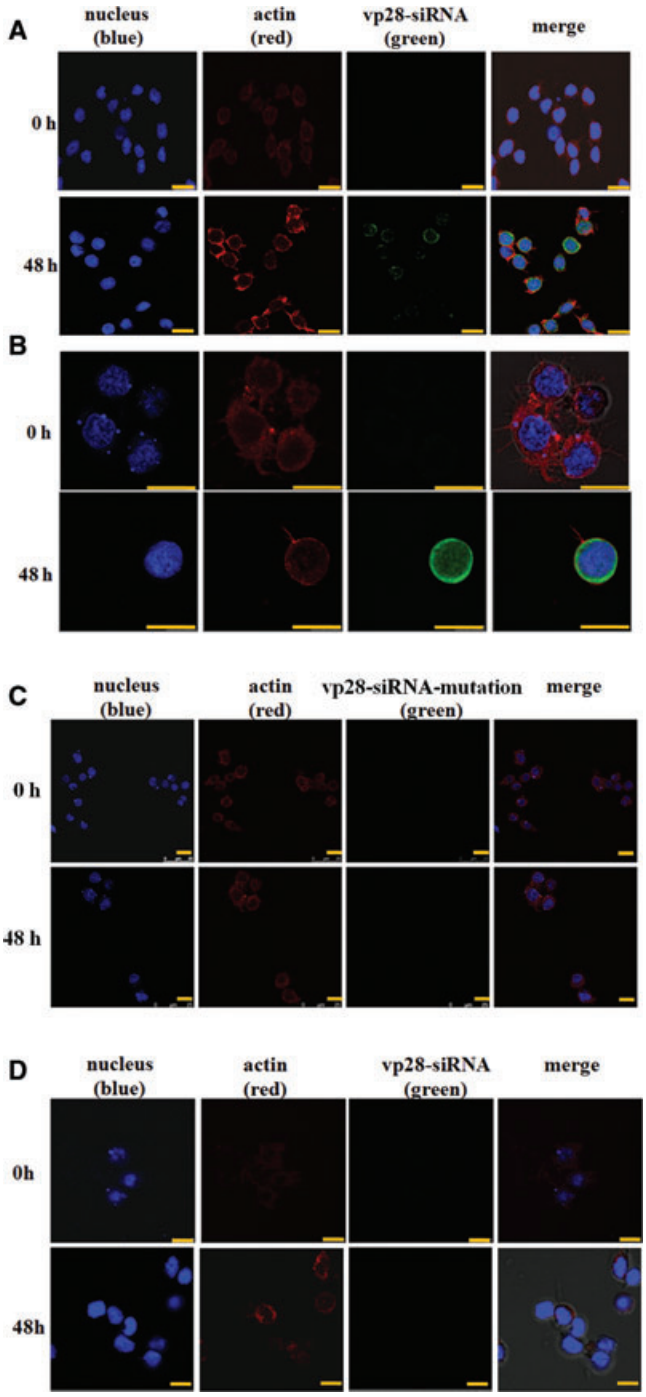

Figure 4. Localization of vp28-siRNA in hemocytes. Shrimp were infected with WSSV. At 0 and 48 h p.i., hemocytes were collected and vp28-siRNA, actin and nucleus were stained with the FITC-labeled vp28-siRNA sense probe (green), rhodamine-phalloidin (red), and 4′ 6-diamidino-2-phenylindole (blue). As controls, the FITC-labeled vp28-siRNA-mutation sense probe (green) was used in the vp28-siRNA localization assays. Samples were examined by confocal microscopy. The lane headings indicate the targets for labeling. The numbers show the times p.i. (A) Distribution of vp28-siRNA in hemocytes with the vp28-siRNA sense probe. (B) The enlarged hemocytes at 0 and 48 h p.i. with the vp28-siRNA sense probe. (C) Detection of vp28-siRNA using the vp28-siRNA-mutation sense probe. (D) Detection of vp28-siRNA using the vp28-siRNA sense probe when shrimp were treated with WSSV + Dicer2-siRNA. Scale bar, 10 μm. Data shown are representative of three experiments performed.

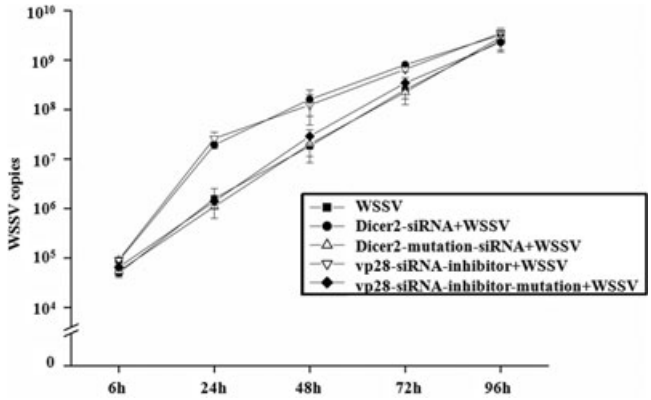

Figure 5. Antiviral role of vp28-siRNA in shrimp. Shrimp were injected with WSSV+Dicer2-siRNA, WSSV + Dicer2-mutation-siRNA, WSSV + vp28-siRNA-inhibitor, WSSV + vp28-siRNA-inhibitor-mutation, and WSSV only, respectively. At different times p.i. (6, 24, 48, 72, and 96 h), randomly selected specimens were subjected to real-time PCR to quantify WSSV copies. Data are shown as mean ± SD of triplicate assays and are representatives of three independent experiments.

through cleavage and degradation of target viral RNA. It is likely that this mechanism of virus restriction is complex and requires further investigation.

The finding that the siRNA pathway is responsible for the restriction of DNA virus replication in this study is novel discovery, expanding our knowledge of the innate immunity of animals. Moreover, siRNA not only plays a pivotal role in the immune response against RNA virus infection, but for defense against DNA viruses.

Materials and methods

Shrimp culture and WSSV infection

Marsupenaeus japonicus shrimp (approximately 10 g/shrimp) were reared in groups of 20 individuals in artificial seawater at 20°C. The shrimp were cultured temporarily for 2–3 days, and then 2–3 shrimp were chosen at random for the detection of WSSV using WSSV-specific primers to ensure that the experimental shrimp were virus free before WSSV challenge. The virus-free shrimp were infected with WSSV, as previously described [36]. Briefly, the WSSV-infected shrimp were homogenized in Tris-NaCl (TN) buffer (20 mM Tris–HCl, 400 mM NaCl, pH 7.4) at 0.1 g/mL, followed by centrifugation at 2000 × *g* for 15 min. Then the supernatant was diluted with 0.85% NaCl to 1:100 and filtered using a 0.45 μm filter. Subsequently, 0.1 mL of filtrate (10^5 virus copies/mL) was administrated intramuscularly into shrimp by injection. After WSSV challenge, various organs and tissues of virus-infected shrimp, including heart, hemolymph, stomach, lymphoid organ, gill, muscle, and hepatopancreas were collected at different times p.i. (0, 2, 6, 12, 24, 48, and 72 h p.i.). The collected organs and tissues were homogenized and used for

PCR detection of WSSV and small RNA isolation. PCR detection for WSSV was performed as previously described [36].

Isolation of small RNAs

Small RNAs were isolated from various shrimp organs or tissues (heart, hemolymph, stomach, lymphoid organ, gill, muscle, and hepatopancreas) at different times p.i. (0, 2, 6, 12, 24, 48, and 72 h p.i.) using a mirVana™ miRNA isolation kit according to the manufacturer's protocols (Ambion, USA). Briefly, the collected shrimp organs or tissues were homogenized in lysis/binding buffer. After centrifugation, the supernatant was mixed with 0.1 volume of miRNA Homogenate Additive solution, followed by incubation on ice for 10 min. Then, equal volume of acid-phenol:chloroform was mixed. The aqueous phase was extracted, followed by two sequential filtrations with different ethanol concentrations. After rinses with washing buffer three times, the small RNAs (<200 nts) were eluted using 50 μL of preheated (95°C) RNase-free water. The total small RNAs were treated with RNase-free DNase I (Roche, Switzerland) for 30 min at 37°C to remove the possibly contaminated DNA. The nucleic acid concentration of the extracted small RNAs was measured and immediately frozen at −80°C. RNA quality was analyzed by electrophoresis in a denaturing 15% polyacrylamide gel containing 7 M urea.

Northern blotting

After separation in a denaturing 15% polyacrylamide gel containing 7 M urea, small RNAs extracted from shrimp were transferred to a Hybond N^+ nylon membrane (Amersham Biosciences) for 1 h at 400 mA, followed by crosslinking under UV light (Ultra-Violet Products). Then the membrane was prehybridized in DIG Easy Hyb granules buffer (Roche) for 0.5 h, and further hybridized with the DIG-labeled-vp28-siRNA sense probe or the vp28-siRNA antisense probe overnight at 42°C. After rinses two times for 5 min in washing buffer (0.1 M Tris-HCl, 0.15 M NaCl, 0.3% Tween 20, pH 7.5) at room temperature, the membrane was incubated in blocking buffer (Roche) for 30 min. Subsequently, the membrane was incubated in the alkaline phosphatase conjugated antibody against DIG (Roche) for 30 min, followed by washing for three times at room temperature. The membrane was incubated in the detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) with nitroblue tetrazolium NBT/bromo-chloro-indolyl phosphate substrates. The vp28-siRNA sense probe (5′-GACCATCGAAACCCACACA-3′, the same sequence as vp28 mRNA) and the vp28-siRNA antisense probe (5′-TGTGTGGGTTTCGATGGTC-3′, antisense to the vp28 mRNA) were used to detect the antisense and sense strand of vp28-siRNA, respectively. As controls, the DIG-labeled vp28-siRNA-mutation sense probe was included in northern blots. The sequence of vp28-siRNA sense probe was randomly mutated at two nucleotides resulting in the vp28-siRNA-mutation sense probe (5′-GACCATCGGGACCCACACA-3′). According to our previous study [30], the non-siRNA (5′-ATGGATCTTTCTTTCACTC-3′) was also used as a control. U6 was detected in the northern blot analyses using the DIG-labeled-U6 probe (5′-GGGCCATGCTAATCTTCTCTGTATCGTT-3′). Probes were synthesized and labeled with DIG at the 5′ end using the DIG High Prime DNA Labeling and Detection Starter Kit II (IDT) according to the manufacturer's protocol. The synthetic RNA oligonucleotides of 21 and 24 nts were used as small RNA markers.

Cloning and sequencing of cDNA corresponding to vp28-siRNA

The cloning of cDNA corresponding to vp28-siRNA was conducted using a small RNA cloning kit (Takara, Japan) following the manufacturer's instructions. The small RNAs of 18–24 nts were recovered using the small RNA gel extraction kit (Takara). After ligation with a 5′ adaptor (5′-TCTAGCCTGCAGGATCGATG-3′) and a 3′ adaptor (5′-AGATCCTGCAGGTGCGTCA-3′), the small RNAs were used for the synthesis of cDNAs. The cDNA corresponding to vp28-siRNA was amplified using primers 5′-TAGCCTGCAGGATCGATGAATGTG-3′ (forward; vp28 gene sequence, underlined) and 5′-CCTGCAGGTGCGTCAGACCAT-3′ (reverse; vp28 gene sequence, underlined). The bands of interest approximately 60 bp were ligated to pCR4-TOPO TA vector (Invitrogen, USA) and used to transform OneShot Top10 competent *Escherichia coli* (Invitrogen). The recombinant plasmid was subjected to DNA sequencing.

Quantitative analysis of WSSV copies by real-time PCR

Real-time PCR was performed to quantify genome copies of WSSV in shrimp. The WSSV genome was extracted and subjected to real-time PCR, as previously described [36]. The primers, 5′-CCACCAATTCTACTCATGTACCAAA-3′ (forward) and 5′-TCCTTGCAATGGGCAAAATC-3′ (reverse), were used to amplify a region from 260075 to 260138 of the WSSV genome (GenBank accession number AF332093.1). The sequence of TaqMan probe was 5′-FAM-CTGGGTTACGAGTCTAA-TAMRA-3′. As an internal standard, the plasmid containing a DNA fragment of 1400 bp from the WSSV genome was digested with EcoRI [36]. The linear plasmid was quantified and serially diluted at tenfold to generate standard curves. Threshold cycle numbers were converted to copy number of virus genome. The PCR reaction mixture (25 μL) contained 1.5 U Premix Ex Taq (Takara), 800 mM dNTPs, 40 pmol of each primer, and 40 pmol of probe. PCR was performed using an initial denaturation at 94°C for 4 min, 45 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s. The assays were performed three times.

In vivo RNAi assays

To reveal the pathway for the biogenesis of antiviral siRNA, RNAi assays were conducted. Based on the available sequences of shrimp

Drosha, Dicer1, and Dicer2 genes, the sequence-specific siRNA oligonucleotides were synthesized with in vitro transcription T7 kit for siRNA synthesis (Takara, Japan) according to the manufacturer's instructions. The sequences of siRNAs were randomly mutated at one nucleotide to generate the control mutation-siRNAs. The siRNAs were as follows:

Drosha-siRNA 5′-GCATCAGAATACTTATATA-3′
Drosha-mutation-siRNA 5′-GCATCAGAAGACTTATATA-3′
Dicer1-siRNA 5′-GCAACTTTCCCTCCTTTAA-3′
Dicer1-mutation-siRNA 5′-GCAACTTTCACTCCTTTAA-3′
Dicer2-siRNA 5′-GCTTCCCAGAATAATGGGT-3′
Dicer2-mutation-siRNA 5′-GCTTCCCAGAGTAATGGGT-3′

The synthesized siRNAs, dissolved in siRNA buffer (50 mM Tris–HCl, 100 mM NaCl, pH 7.5), were examined by electrophoresis and quantified by spectrophotometry.

To conduct RNAi assays, the siRNA (Drosha-siRNA, Dicer1-siRNA, or Dicer2-siRNA; 30 μg/shrimp) and WSSV (10^4 copies/shrimp) were simultaneously administrated into shrimp by injection. As negative controls, the mutation-siRNA (Drosha-mutation-siRNA, Dicer1-mutation-siRNA, or Dicer2-mutation-siRNA; 30 μg/shrimp) and WSSV (10^4 copies/shrimp) were injected into shrimp at the same time. As a positive control, WSSV only (10^4 copies/shrimp) was also delivered into shrimp. In each treatment, 20 individuals were used. At different time points p.i. of WSSV (0, 12, 24, 48, and 72 h), the lymphoid organs of three randomly selected shrimp from each treatment were collected and subjected to real-time PCR to quantify gene expressions and WSSV genome copies. At 48 h p.i., the extracted RNAs from the collected lymphoid organs were blotted with the vp28-siRNA sense probe or the control U6 probe. The above assays were performed three times.

Analysis of the expression of shrimp Drosha, Dicer1, and Dicer2

Total RNAs were extracted from shrimp lymphoid organs using a mirVana™ miRNA isolation kit according to the manufacturer's instructions (Ambion). To analyze the expressions of Drosha, Dicer1, and Dicer2, total RNAs were analyzed by TaqMan quantitative real-time PCR. The expression level of shrimp β-actin was served as a normalization control. The primers were 5′-CTCTTGCAGCTGGCCCTTAC-3′ and 5′-GCTGCCGTATTCCACAATTTG-3′ for Drosha, 5′-GACAGCGAACATTACTCACTGGTT-3′ and 5′-AGTGTAAG TGGTCACAGCTAGATTGG-3′ for Dicer1, 5′-ACCGGGTCAGG CAAAACTT-3′ and 5′-TCTCGAATCTGATGCCCTAGCT-3′ for Dicer2, and 5′-CGAGCACGGCATCGTT ACTA-3′ and 5′-TTGTAGAAAGTGTGATGCCAG ATCT-3′ for β-actin. The TaqMan probes were 5′-FAM-TTTGGCACGAATCCCGAT- TAMRA-3′ (Drosha), 5′-FAM-AGGGCTAGTGCGCGC-TAMRA-3′ (Dicer1), 5′-FAM-ATCTCAATCCTGCTAATCA- TAMRA-3′ (Dicer2), and 5′-FAM-CTGGGACG ACATGGA-TAMRA-3′ (β-actin). The real-time PCR was performed as described above.

Electrophoretic mobility shift assay

EMSA was carried out as previously described [37]. The recombinant glutathione S-transferases (GST)-Ago1, GST-Ago2, and GST proteins were purified using glutathione agarose resin according to the instructions provided by manufacturers (Amersham Biosciences, USA). Then 2 μg of vp28-siRNA (ds siRNA) or the control siRNA (ds siRNA) were incubated with 10 μL of the recombinant proteins at different concentrations (0, 20, 40, 75, and 150 μM). According to the sorting mechanism of small RNA in fly and worm [38–41], the sequence of sense strand of vp28-siRNA duplex was mutated at 10–11 nts, yielding the control siRNA (5′-GACCATCGGGACCCA CACA-3′) specifically bound to Ago1 instead of Ago2. After incubation in the reaction buffer (0.1 M KCl, 1 mM DTT, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.6) for 15 min at 37°C, the mixture was electrophoresed by a 8% native polyacrylamide gel at 200 V for 2 h. RNA bands were visualized by staining with ethidium bromide and subsequently the proteins were stained with Commassie blue.

Examination of vp28-siRNA in shrimp hemocytes

Shrimp were infected with WSSV and at different time points p.i., shrimp hemocytes were collected and fixed on glass slides with 4% paraformaldehyde and 0.16 M l-ethyl-3-(3-dimethylaminopropyl) carbodiimide in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4). Slides were incubated for 30 min at 37°C, followed by washing three times with TBS. For hybridization, 40 nM FITC-labeled oligodeoxynucleotide vp28-siRNA sense probe (5′-GACCATCGAAACCCACACA-3′) and the FITC-labeled vp28-siRNA-mutation sense probe (5′-GACCATCGGGACCCACACA-3′) were added onto the slides in a sealed humidified chamber for 3 h at 45°C. After washing twice with 0.5 mL 0.2 × sodium chloride/sodium citrate (SSC) for 15 min and twice with TBS containing 0.1% Tween 20, the cytosolic F-actin was stained using rhodamine-phalloidin (Invitrogen), according to the manufacturer's protocols. The slides were washed three times with TBS and subsequently incubated with 4′ 6-diamidino-2-phenylindole (Vector Laboratories, USA) to label the nucleus. After washes with TBS, the slides were examined by confocal microscopy.

Effects of vp28-siRNA on WSSV infection

To assess the effects of vp28-siRNA on virus infection, shrimp were injected with Dicer2-siRNA (30 μg/shrimp) or Dicer2-mutation-siRNA (30 μg/shrimp). Twenty-four hours later, WSSV (10^4 copies/shrimp) and Dicer2-siRNA (30 μg/shrimp) or Dicer2- mutation-siRNA (30 μg/shrimp) were simultaneously injected into shrimp. To block the function of vp28-siRNA, LNA-modified anti-siRNA oligonucleotide (antisense oligonucleotide), designated as vp28-siRNA-inhibitor, was used as previously described [42, 43]. The vp28-siRNA-inhibitor (5′-GACCATCGAAACCCACACA-3′) was synthesized with LNA and

2′-O-methoxyethyl modifications as described in our previous study [28]. As a negative control, two nucleotides of vp28-siRNA-inhibitor sequence were mutated at random, resulting in the corresponding vp28-siRNA-inhibitor-mutation (5′-GACCATCGGGACCCACACA-3′) with the same modifications as vp28-siRNA-inhibitor. Shrimp were injected with vp28-siRNA-inhibitor (15 μg/shrimp) or vp28-siRNA-inhibitor-mutation (15 μg/shrimp). Twenty-four hours later, shrimp were challenged with the mixture containing WSSV (10^4 copies/shrimp) and vp28-siRNA-inhibitor (15 μg/shrimp) or WSSV (10^4 copies/shrimp) and vp28-siRNA-inhibitor-mutation (15 μg/shrimp). The positive control injected with WSSV only (10^4 copies/shrimp) was included in these experiments.

At different times p.i. (6, 24, 48, 72, and 96 h), three random shrimp from each treatment group were selected and subjected to nucleic acid extraction and real-time PCR analysis to quantify the number of WSSV copies. All experiments were performed three times.

Statistical analysis

One-way analysis of variance was used for statistical analysis of data from three independent experiments and all data were expressed as means ± SD. Statistical significances between treatments were carried out using Student's *t*-test and p values < 0.05 were considered statistically significant.

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Abbreviations: **DIG**: digoxigenin · **LNA**: locked nucleic acid · **p.i.**: post-infection · **RdRP**: RNA-dependent RNA polymerase · **RNAi**: RNA interference · **WSSV**: white spot syndrome virus

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