Multiple isoforms of both HSP70 and HSP90 families required for betanodavirus multiplication in medaka cells

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Research Article

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Abstract

Heat shock proteins (HSPs) are molecular chaperones and have recently been revealed to function as host factors (HFs) for virus multiplication in fish as well as in mammals, plants, and insects. HSPs are classified into some families and have some isoforms in each family. However, no comprehensive studies were performed to clarify the biological importance of multiple isoforms in a certain HSP family for fish virus multiplication. Betanodaviruses are the causative agents of viral nervous necrosis in cultured marine fish and cause very high mortality. Although the viral genome and encoded proteins have been characterized extensively, information on HFs for the virus is limited. In this study, therefore, we focused on HSP70 and HSP90 families to examine the importance of many isoforms in the families for betanodavirus multiplication. We found that HSP inhibitors (17-AAG, radicicol, and quercetin) suppressed viral RNA replication and production of progeny virus in infected medaka (*Oryzias latipes*) cells. A thermal stress or virus infection up-regulated the expression of some isoform genes and facilitated virus multiplication. Furthermore, overexpression and knockdown of some isoform genes revealed that HSP70-1, HSP70-2, HSP70-5, HSP90-α1, HSP90-α2, and HSP90-β isoforms played positive roles in virus multiplication in medaka. Collectively, these results suggest that not a few isoforms in fish HPS families serve as HFs for betanodavirus multiplication.

Introduction

Any viruses require host cell factors for their multiplication. The roles of such host factors (HFs) for virus multiplication have been investigated thoroughly in mammals, insects, and plants. Identification of HFs is important not only for understanding basic mechanisms underlying virus multiplication but also for therapeutics, in which HFs are attractive targets to develop antiviral agents [1–3]. Over the past decade, an increasing number of HFs for fish viruses have been identified. One research trend of fish HFs is the discovery of defense-related factors, many of which are involved in the interferon system. They are categorized into positive [e. g., 4–10] and negative [e. g., 11–15] regulators for fish virus multiplication. Another research trend is the identification of heat shock proteins (HSPs) as fish HFs. Similar to the defense-related factors mentioned above, HSP HFs can be classified into two groups: facilitators [e. g., 16–20] and inhibitory factors [21, 22] for virus multiplication. The identification of the several HSPs as fish HFs is not surprising because in mammals, plants, and insects, many HSPs have been known as HFs for relevant viruses [23, 24]. HSPs comprise a large family of proteins and are known to function as molecular chaperones which are required for correct protein folding and intracellular transport both in prokaryotic and eukaryotic cells [25]. HSPs are classified into some families (e. g., HSP60, HSP70, HSP90) according to their molecular weight, and have some isoforms in each family [26]. However, many of the HSP studies for fish HFs were conducted without specifying which isoform was being studied [16–18, 20, 27]. In addition, although there are several studies of HFs targeting specific isoforms of fish HSPs [22, 28–31], no comprehensive studies were performed to clarify the biological importance of more than one isoforms in a certain HSP family. In this study, we focused on HSP70 and HSP90 families in fish and examined the importance of many isoforms in these families as HFs. In order to improve the efficiency of the study, we used a betanodavirus, a model fish virus, and medaka (*Oryzias latipes*), a model fish, for the study.

Betanodaviruses are the causative agents of viral nervous necrosis of marine fish [32] and belong to the family *Nodaviridae*. Betanodaviruses are small in size [33] and possess a bipartite positive-sense RNA genome, the genome size of which is the smallest among fish viruses thus far identified [34, 35]. The larger genomic RNA

(RNA1) is 3.1 kb in length and encodes an RNA dependent RNA polymerase (protein A) while the smaller one (RNA2) is 1.4 kb and encodes a coat protein. The sub-genomic RNA3, transcribed from RNA1, is 0.4 kb which encodes a suppressor for post-transcriptional gene silencing (protein B2) [36, 37] and an anti-necrotic death factor (protein B1) [38]. The virus can be classified into four genotypes, designated redspotted grouper nervous necrosis virus (RGNNV), striped jack nervous necrosis virus (SJNNV), barfin flounder nervous necrosis virus, and tiger puffer nervous necrosis virus, based on similarities in RNA2 sequences [39, 40]. The biological importance of the viral RNAs and encoded proteins were studied extensively [37, 41–45] using the reverse genetic technology based on a cDNA-mediated infectious RNA transcription strategy [46]. Recently, we established an experimental infection system of betanodaviruses using the model fish, medaka and its cell lines [47, 48].

On the other hand, medaka is an excellent vertebrate model used in various study areas including development, genetics, environmental toxicology and human disease [49–51]. In addition to preferable characteristics as a model animal such as small body size, high fecundity, tolerance to a wide range of environmental conditions, and easiness to rear, the availability of several inbred lines and enormous genetic diversity between those lines [52] are distinct features of medaka not found in other model fish species. Moreover, whole genomic sequencing [53] as well as the establishment of transgenesis and mutagenesis systems was completed in medaka [54–56].

In this study, we performed overexpression and knockdown experiments on many genes of HSP70 and HSP90 isoforms in medaka to evaluate their importance in betanodavirus multiplication. We also examined the inducibility of these isoforms by heat shock or virus infection stress. The elucidation of the isoforms involved or not in virus multiplication should not only help us to understand the mechanism of virus infection, but also provide important information for the development of specific antiviral drugs targeting specific HSP isoforms.

Materials And Methods

Cells and virus

The medaka cell line OLHNI-2 [57] was provided from H. Mitani and cultured at 30°C in Leibovitz's L-15 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 15% fetal bovine serum (FBS) (Nichirei Biosciences, Tokyo, Japan). E-11 cells [58] were cultured at 25°C in L-15 medium supplemented with 5% FBS. The betanodavirus used in this study was RGNNV (SGWak97 strain) [59]. The virus was prepared from the OLHNI-2 cells transfected with in vitro transcribed viral RNAs according to the procedure described previously [43]. The RNA sequences of the obtained progeny virus were verified as described previously [43]. Viral titers were determined based on the $TCID_{50}$ method [60] using E-11 cells.

Plasmids

The mRNA sequences of the five HSP70 isoforms (HSP70-1, HSP70-2, HSP70-5, HSP70-8, HSP70-9) and the five HSP90 isoforms (HSP90-α1, HSP90-α2, HSP90-β, GRP94, TRAP1) of medaka were retrieved from Ensemble Genome Browser (http://www.ensembl.org/index.html) and NBRP medaka (http://www.shigen.nig.ac.jp/medaka/). The nomenclature of HSPs in the present study was according to the guidelines of Kampinga et al [26] and Tavaria et al [61]. Based on the mRNA sequences obtained, the specific primers (Table 1) were designed to amplify DNA that encodes each of the HSP isoforms fused with the HA tag at the N- or C-terminus. PCR was performed using the primers and cDNA prepared from mRNA of the medaka

strain Cab. Amplified fragments were digested with *XbaI* and cloned into the *XbaI* site of the pCI-Neo expression vector (Promega, Madison, WI, USA). The sequence integrity of all the constructs was verified by DNA sequencing using BigDye Terminator v1.1 Cycle Sequencing Kit and Applied Byosystems 3130xl genetic analyzer (Thermo Fisher Scientific).

Table 1
Oligonucleotides used in the present study

Name	Sequence[a] (5' to 3')
Primers for plasmid vector construction	
HSP70-1-pCI F	AGAGAG *TCTAGA*ATGGGCTACCCATACGATGTTCCAGATTACGCGTCTGCGGCCAAGAGCGTG
HSP70-1-pCI R	AGAGAG *TCTAGA*TCAGTCCACCTCCTCAATAGTG
HSP70-2-pCI F	AGAGAG *TCTAGA*ATGGGCTACCCATACGATGTTCCAGATTACGCG GATACAGCAAAGGGAGTTTCCATT
HSP70-2-pCI R	AGAGAG *TCTAGA*CTAGTCCACTTCCTCTATGGTT
HSP70-5-pCI F	AGAGAG *TCTAGA*ATGGGCAAGCTCTTGTGGGTGGTAATG
HSP70-5-pCI R	AGAGAG *TCTAGA*CTACGCGTAATCTGGAACATCGTATGGGTATAATTCATCCTTCTCTGCC
HSP70-8-pCI F	AGAGAG *TCTAGA*ATGGGCTACCCATACGATGTTCCAGATTACGCGTCTAAGGGACCAGCAGTCG
HSP70-8-pCI R	AGAGAG *TCTAGA*TTAATCAACTTCTTCAATGGTTGG
HSP70-9-pCI F	AGAGAG *TCTAGA*ATGGGCTACCCATACGATGTTCCAGATTACGCGTTGAGCGCTGCTAGAACCATTTC
HSP70-9-pCI R	AGAGAG *TCTAGA*TTACTGCTGACCCTCCTTCTT
HSP90-α1-pCI F	AGAGAG *TCTAGA*atgGGCtacccatacgatgttccagattacgcgCCAGAGAATGCTGCACATGTCA
HSP90-α1-pCI R	AGAGAG *TCTAGA*TTAGTCAACTTCTTCCATTCTTG
HSP90-α2-pCI F	AGAGAG *TCTAGA*atgGGCtacccatacgatgttccagattacgcgCCTGAGATGCATGACCAACCAAT
HSP90-α2-pCI R	AGAGAG *TCTAGA*TCAGTCCACCTCCTCCATTCTGGA
HSP90-β-pCI F	AGAGAG *TCTAGA*atgGGCtacccatacgatgttccagattacgcgCCTGAAGAAATGCACCAAGAGGA
HSP90-β-pCI R	AGAGAG *TCTAGA*TTAGTCGACCTCTTCCATGCGTGA

[a]Restriction enzyme sites and HA tag sequences are indicated by italic and underline, respectively.

Name	Sequence[a] (5' to 3')
GRP94-pCI F	AGAGAG*TCTAGA*ATGAAACGGCTGTGGGTGG
GRP94-pCI R	AGAGAG*TCTAGA*CTACGCGTAATCTGGAACATCGTATGGGTACGAAACATTTGCTCCATGCCT
TRAP1-pCI F	AGAGAG*TCTAGA*ATGTCGCTTCCACTTGCTAGAG
TRAP1-pCI R	AGAGAG*TCTAGA*CTACGCGTAATCTGGAACATCGTATGGGTAGTGTTTCTCCAGAGCCTT
Detection primers for quantitative real time RT-PCR	
HSP70-1 F	AGAGGCTGATTGGGCGGAGG
HSP70-1 R	GGCTTCCCTCCTTCTGAAAC
HSP70-2 F	CAAACGTCTCATTGGAAGAAAC
HSP70-2 R	CTCTTCAGGATAGAAGGCTTTAG
HSP70-5 F	CCGTCTGACACCTGAAGACA
HSP70-5 R	AGGCGTAGCTCTCCAATTCA
HSP70-8 F	GAAGTACAAGGCAGAAGATGATG
HSP70-8 R	CTTCATCACTGATCTTGCCAGC
HSP70-9 F	ACACCGAGTCCAAGATGGAG
HSP70-9 R	AGATGTCCCTGACCTTGGTG
HSP90-α1 F	ATGTCATGGAGGAGGAGGTG
HSP90-α1 R	GGAGATGAGCTCTCGAAGGA
HSP90-α2 F	GATCAGTCAGAATACCTGGAG
HSP90-α2 R	TTGTCCTCGTCGTCACTCAC

[a]Restriction enzyme sites and HA tag sequences are indicated by italic and underline, respectively.

Name	Sequence[a] (5' to 3')
HSP90-β F	GAGTACATTGAGGAGAAGAGG
HSP90-β R	TCCTCACCTTCCTCCTTGG
GRP94 F	GCCGACAAGGTCATCGTATC
GRP94 R	GAGGCTTCCTCCTTCAGAAC
TRAP1 F	TGTCGCTTCCACTTGCTAGA
TRAP1 R	AGAAAGACTGCTGGCTCAGG
β-Actin F	GGGAGAAGATGACCCAGATC
β-Actin R	ACCAGAGTCCATGACGATAC

[a]Restriction enzyme sites and HA tag sequences are indicated by italic and underline, respectively.

Northern blot and Western blot analyses

Total RNA and proteins were prepared from cells inoculated with RGNNV using the ISOGEN reagent (Nippon Gene, Tokyo, Japan) according to the manufacture's instructions. Total RNA was subjected to Northern blot analysis as described previously [46]. The coat protein in the protein samples extracted from equal number of cells was detected by Western blotting analysis as described previously [46] except in that a rabbit antiserum, raised against the recombinant RGNNV coat protein, was used as the primary antibody. All images of the blots were photographed by ChemiDoc XR system (Bio-Rad, Hercules, CA, USA) and quantitative data were obtained by analysis using Quantity One software (Bio-Rad).

Treatments of cells with HSP inhibitors

As HSP90 inhibitors, 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) and radicicol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Quercetin, which inhibits both HSP70 and HSP90, was purchased from the same company. All the chemicals were dissolved in dimethyl sulfoxide (DMSO) as the stock solutions (5 mM for 17-AAG and radiciol, 20 mM for quercetin) and stored at − 20°C until use.

OLHNI-2 cells seeded on a 24-well culture plate (Sumitomo Bakelite, Tokyo, Japan) at the density of 0.5×10^6 cells per well were cultured for 16 h at 30°C. Cells then were inoculated with RGNNV at a multiplicity of infection (MOI) of 1.0 for 30 min and further cultured with medium containing the HSP inhibitors. Control cells were incubated with medium containing the same amount of DMSO. At 12 h post virus inoculation, the culture supernatants were collected and virus particles were purified using the PEG precipitation method [62] to remove the inhibitors from the samples. Viral titers of the samples thus obtained were determined as described earlier.

Total RNA and proteins were also isolated from cells in replicated wells and subjected to Northern blot and Western blot analyses as described earlier. In order to assess the cytotoxic effects of the inhibitors, OLHNI-2 cells were seeded on a 96-well culture plate (Iwaki, Shizuoka, Japan) at the density of 0.5×10^5 cells per well. The cells then were cultured with the medium containing each of the inhibitors and cell viability was determined periodically using WST-1 Cell Proliferation Assay kit (Roche Diagnostics, Basel, Switzerland) according to the manufacture's recommendations.

Thermal stress on the cells

OLHNI-2 cells were seeded on a 6-well culture plate (Sumitomo Bakelite) at the density of 1.0×10^6 cells per well and cultured for 16 h at 30°C. Cells then were thermally stressed by submerging the culture plate in water at 42°C for 1 h and recovered at 30°C for 1 h. The time point when recovery was finished was designated as 0 h after thermal stress. The cytotoxic effect of thermal stress was assessed using WST-1 Cell Proliferation Assay kit according to the manufacture's recommendations.

Overexpression of the HSP genes

OLHNI-2 cells were transfected with the plasmid encoding each of the HSP70 and HSP90 isoforms using Lullaby Transfection Reagent (OZ Biosciences, Marseille, France) according to the procedure recommended by the manufacturer with some modifications. Briefly, 500 ng of plasmid DNA and 1 μl Lullaby Transfection Reagent were diluted separately in 50 μl Opti-MEM-I (Thermo Fisher Scientific). The diluted plasmid DNA and the transfection reagent then were combined and incubated for 20 min at room temperature. Finally, the transfection complex was combined with 400 μl cell suspensions (5×10^5 cells/ml), plated on a 24-well cell culture plate (Sumitomo Bakelite) and cultured for 48 h. Transfected cells then were provided for virus inoculation.

Knock down of the HSP genes

siRNAs targeting each of the medaka HSP70 and HSP90 isoforms were designed using BLOCK-iT RNAi Designer with Stealth modification and purchased from Thermo Fisher Scientific. The Stealth modification is the chemical modification to siRNA, which enhances the potency and stability of siRNA as well as reduces the occurrence of the off-target effect. The target sequences of the siRNAs are listed in Table 2. Stealth RNAi GFP reporter control (Thermo Fisher Scientific), which targets the enhanced green florescent protein (EGFP) gene, was used as the control siRNA (siREGFP). OLHNI-2 cells were transfected with each of the siRNAs using X-treme gene siRNA transfection reagent (Roche Diagnostics) according to the manufacturer's instructions. Total RNA was isolated from cells in replicated wells at 48 h after transfection and target mRNAs in the samples were quantified by real time RT-PCR as described later. Cells at 48 h after transfection were also provided for virus inoculation.

Table 2
siRNAs used in the present study

siRNA	Location[a]	Sequence (5' to 3')
siRHSP70-1	1766–1790	CCGACAAAGACGAGTTCCAGCACAA
siRHSP70-2	786–810	GAGGAGACTGAGAACGGCTTGTGAG
siRHSP70-5	1578–1602	CGACCAGAACCGTCTGACACCTGAA
siRHSP90-α1	213–228	GCTGAAGATTGAAGTCAGACCTGAT
siRHSP90-α2	552–577	CCACCTGAAAGAAGATCAGTCAGAA
siRHSP90-β	581–606	AGAAGAGGGTCAAAGAGATCGTGAA

[a] Adenine residues of the start codons are designated as "1".

Quantitative real time RT-PCR

Total RNA was isolated from cells using the acid guanidinium thiocyanate-phenol-chloroform method [63] and treated with RQ1 RNase-free DNase (Promega), followed by purification using RNeasy mini RNA isolation kit (Qiagen, Venlo, Netherlands). To obtain cDNA, 0.5 μg of the purified total RNA was incubated with 0.5 μl of the oligo dT primer (20 μM; Eurofins Genomics, Val Fleuri, Luxembourg) at 70°C for 10 min and further incubated at 42°C for 60 min supplemented with 2 μl 5× RT reaction buffer (Takara, Otsu, Japan), 2 μl dNTP mix (2.5 mM each dNTP, Takara), 100 unit M-MLV reverse transcriptase (RNase H-, Takara), and 10 unit RNase inhibitor (Toyobo, Osaka, Japan) in a final reaction volume of 10 μl. The synthesized cDNA samples were diluted ten times with double-distilled water and stored at − 20°C until use.

Quantitative real time RT-PCR was performed using Thunderbird SYBR qPCR Mix (Toyobo) and Chromo4 Real-Time PCR Detection System (Bio-Rad). The primer sets used to detect mRNAs of the HSP isoforms and β-actin are listed in Table 1. The reaction mixture for PCR contained 10 μl Thunderbird SYBR qPCR Mix, 0.5 μl each of the primers (10 μM), 3 μl of the cDNA sample, and 6 μl double-distilled water. The amplification procedures included one cycle of 3 min at 95°C, followed by 40 cycles of 30 sec at 95°C and 30 sec at 60°C, with melting curve analysis. The relative mRNA expression was calculated by the standard curve method using the β-actin mRNA expression as the control for normalization.

Immunocytochemistry

Indirect immunofluorescence assay to detect HA-tagged HSP70 and HSP90 was performed as described previously with some modifications (Adachi et al, 2010). Briefly, cells were fixed with 4% paraformaldehyde and permeabilized by treatment with 0.1% NP-40 in PBS. The cells then were treated with a 1:500 dilution of anti-HA monoclonal antibody (Wako Pure Chemical Industries), followed by the treatment with a 1:2000 dilution of Alexa Fluor 555 goat anti-mouse IgG (Thermo Fisher Scientific). Cells were simultaneously stained with 4',6-diamidino-2-phenylindole (DAPI) (Dojindo, Kumamoto, Japan) and were observed under the fluorescence microscope (ORCA-1394 and AQUA-Lite version 1.10 systems; Hamamatsu Photonics, Hamamatsu, Japan).

Results

Effects of the HSP inhibitors on virus multiplication in medaka cells

To confirm whether medaka HSP70 and HSP90 include any HFs for RGNNV, we tested the effects of the three well-known chemical inhibitors for HSPs on virus multiplication in medaka cells. Although the concentrations of inhibitors used in this study (5 μM for 17-AAG and radicicol, 25 μM for quercetin) were suggested to be the most effective and the least toxic to the insect cells [64], a modest cytotoxic effect was observed in medaka cells at 24 post treatment with these inhibitors (Fig. 1A). Accordingly, hereafter, effects of these inhibitors were investigated within 12 h post treatment.

First, effects of these inhibitors on the production of progeny virus were examined. Viral titers in the supernatants at 12 h post virus inoculation were reduced by the ratio of 0.28, 0.03, and 0.07 in the cells treated with 17-AAG, radicicol, and quercetin, respectively, when compared with cells without any treatments (Fig. 1B). Slight reduction in viral titer was observed in control cells treated with vehicle only. To further elucidate the steps of virus multiplication affected by the HSP inhibitors, the viral RNA and protein were analyzed quantitatively by Northern blotting and Western blotting, respectively. Production of the viral RNA and protein was suppressed in cells treated with each of the inhibitors, the effect of which was the most significant in cells treated with radicicol (Fig. 1C, D). No obvious difference was found in replication competence between the positive and negative strand RNAs. Interestingly, the production of the coat protein was diminished by treatment with the inhibitors, the level of which was lower than that observed for RNA2 replication. These results suggest that inhibition of HSPs affected viral protein production as well as viral RNA replication. Alternatively, HSP inhibition might affect exclusively the production of viral proteins including protein A, which hence reduces viral RNA replication.

Effect of the thermal stress on virus multiplication

Since the inhibition of HSPs activity suppressed virus multiplication, we next examined the effect of artificial induction of HSPs on virus multiplication by subjecting cells to non-lethal thermal stress. We found that the treatment of cells at 42°C for 1 h was the minimum requirement to up-regulate HSPs (data not shown) though a slight cytotoxic effect on the cells was also observed at 12 h after the treatment (Fig. 2A). When cells were inoculated with RGNNV immediately after the thermal stress, from 4.64 to 6.12 times more progeny virus was produced compared with control cells (Fig. 2B). Similarly, production of the viral RNA and protein was increased by the heat treatment (Fig. 2D, E). On the other hand, when cells were inoculated with RGNNV at 12 h after the thermal stress, from 1.45 to 2.82 times more virus was produced (Fig. 2C).

We examined the effect of the thermal stress on the expression of major HSP70 isoforms (HSP70-1, HSP70-2, HSP70-5, HSP70-8, HSP70-9) and all of the HSP90 isoforms (HSP90-α1, HSP90-α2, HSP90-β, GRP94, TRAP1) of medaka. Among the ten HSPs tested, HSP70-1, HSP70-2, HSP70-5, HSP90-α1, and HSP90-α2 was greatly up-regulated immediately after the thermal stress (Fig. 3). The up-regulation of HSP70-2 and HSP90-α2 was sustained at least for 48 h post thermal stress while the expression of HSP70-1 and HSP90-α1 was returned to the control level at 48 h post thermal stress. The up-regulation of HSP70-5 was observed only immediately after the thermal stress and then down-regulated to basal level at 12 h post thermal stress.

Effect of the virus infection on the expression of the HSP70 and HSP90 isoforms

We next examined whether virus infection itself induces the expression of the HSPs. Among the ten HSP isoforms tested, HSP70-1, HSP70-2, HSP90-α1, and HSP90-α2 were up-regulated strongly by virus infection and the levels were increased with time (Fig. 4). In contrast, no up-regulation was observed for HSP70-5, HSP70-9, GRP94, or TRAP1. HSP70-8 and HSP90-β were slightly up-regulated at 6 h post virus infection (Fig. 4). Nevertheless, the levels of up-regulation by virus infection were relatively low compared with those obtained after the thermal stress (Fig. 3).

Effects of overexpression of the HSP isoform genes on virus multiplication

In order to identify the HSP isoforms which support virus multiplication, we overexpressed each of the ten HSP isoforms in medaka cells prior to virus inoculation. HA-tagged HSPs were successfully expressed in cells transfected with each of the expression vectors (Fig. 5A). When compared with the control, the levels of virus multiplication were elevated in cells transfected with the vector expressing HSP70-1, HSP70-2, HSP70-5, HSP90-α1, HSP90-α2, or HSP90-β (Fig. 5B). While the levels of virus multiplication were reduced to less than those of the control when cells were transfected with the vector expressing HSP70-8, HSP70-9, GRP94, or TRAP1. These results suggest that HSP70-1, HSP70-2, HSP70-5, HSP90-α1, HSP90-α2, and HSP90-β serve as positive factors for betanodavirus multiplication. Conversely, HSP70-8, HSP70-9, GRP94, and TRAP1 might function as negative components.

Effects of knockdown of the HSP isoform genes on virus multiplication

Based on the results of the overexpression experiments, we further examined the positive roles of HSP70-1, HSP70-2, HSP70-5, HSP90-α1, HSP90-α2, and HSP90-β in virus multiplication via knockdown experiments. The treatments of siRNAs, targeting HSP70-1, HSP70-2, HSP70-5, HSP90-α1, HSP90-α2, and HSP90-β, reduced successfully the expression levels of corresponding mRNA to 42.1, 46.2, 49.7, 48.5, 42.7, and 38.9% of the control level, respectively (Fig. 6A). When the cells, transfected with each of the six siRNAs, were inoculated with RGNNV, progeny virus was decreased in all the treatments compared to the control (Fig. 6B).

Discussion

Non-lethal stressors including thermal stresses activated the synthesis of HSPs and protected fish from bacterial infections [65, 66]. For example, treatments of the chemical compound TEX-OE and its variant induced HSPs non-lethally in salmon and gilthead sea bream (*Sparus aurata*), which protected these fish species from *Vibrio anguillarum* infection [66]. Bacterial infections up-regulated HSP70 in rainbow trout (*Oncorhynchus mykiss*) and sea bream (*Sparus sarba*), which were suggested as defense responses of the hosts against the bacterial pathogens [67–71]. However, these inhibitory functions of HSPs in bacterial infections are not necessarily true for viral infections. Relatively more fish HSPs are thus far described as positive HFs for the relevant viruses [e. g., 16–20] compared to those designated as negative factors [21, 22]. As for HSPs involved in

betanodavirus infection, HSP70-5 supports RGNNV multiplication in the grouper (*Epinephelus coioides*) cells GF-1 [29] and HSP90-β also serves as a positive HF for the virus in GF-1 cells [72] and in the marine medaka *(Oryzia melastigma*) cells hMMES1 [31], which are consistent with our overexpression and knockdown experiments using OLHNI-2 cells (Figs. 5 and 6). In contrast, HSP27 in sea perch (*Lateolabrax japonicus*) exerts anti-RGNNV activities by regulating the apoptosis signaling pathway [21]. In the present study, HSP70-1, HSP70-2, HSP90-α1, and HSP90-α2 as well as HSP70-5 and HSP90-β facilitated RGNNV multiplication in medaka cells (Figs. 5 and 6). In contrast, HSP70-8, HSP70-9, GRP94, and TRAP1 suppressed virus multiplication though further studies including knockdown each of the four genes are required to confirm this activity. One curious observation is that overexpression of HSP70-8 suppressed RGNNV multiplication in medaka cells, which is apparently contradict to the precedent report by Chang and Chi [28]. A possible explanation for the discrepancy is that our data were obtained using OLHNI-2 cells derived from medaka, the host specificity of which is different from that of grouper used by Chang and Chi [28]. Medaka is susceptible to SJNNV as well as RGNNV though the levels of SJNNV infections are less than those of RGNNV infections [48]. Conversely, groupers are susceptible to RGNNV but entirely resistant to SJNNV [42, 43]. Chang and Chi [28] indicated that HSP70-8 functions as an RGNNV receptor or coreceptor which probably distinguishes the difference between SJNNV and RGNNV. Therefore, some protein other than HSP70-8 might function as a RGNNV receptor in medaka. In any event, more studies are required to know the whole picture of HSP isoforms involved in fish virus multiplication.

Among the three inhibitors used in this study, radicicol and 17-AAG inhibit specifically HSP90 activity through the inhibition of ATP binding to HSP90 [73]. Quercetin inhibits the activity of the transcription factor, heat shock factor 1, resulting in down-regulation of broader members of HSPs including HSP70 and HSP90 [74, 75]. In this study, radicicol, 17-AAG, and quercetin had inhibitory effects on the RNA replication and progeny virus production of RGNNV (Fig. 1). These results are consistent with the previous studies that the treatment of the marine medaka cells hMMES1 with ganetespib or NVP-AUY922, the inhibitors of HSP90ab1, significantly decreased RGNNV entry into the cells [31]. Furthermore, geldanamycin (GA, a prototype of 17-AAG) and radicicol negatively affected the replicative and multiplicative competence of frock house virus (FHV) belonging to the same family *Nodaviridae* as RGNNV [64]. Similar negative effects of the HSP inhibitors were found for other virus species such as hepatitis viruses [76–79], influenza virus [80], Ebola virus [81], and herpes simplex virus [51]. However, in contrast, the treatment of GF-1 cells with GA facilitated the multiplication of RGNNV [72]. One possible reason for these contradictory findings is the difference in the dose of inhibitors used. While we treated the 5 μM concentration of 17-AAG, Chen et al [72] used the relatively low concentration of GA (1.5 uM). Since inhibition of HSP90 by GA is known to trigger simultaneously up-regulation of HSP90 [82], the concentration of GA used by Chen et al [72] might be sufficient to up-regulate HSP90 but not to inhibit HSP90 activity. Alternatively, a treatment of GF-1 cells with GA could give some unexpected effects on virus multiplication. Although the cytotoxic effect of GA on GF-1 cells was not tested by Chen et al [72], we confirmed that GA gave a harmful effect on medaka cells at the concentration of as little as 1 μM within 12 h, which prompted us to use 17-AAG instead of GA in this study.

In this study, overexpression and knockdown experiments on the HSP70 and HSP90 isoforms revealed that HSP70-1, HSP70-2, HSP70-5, Hso90-α1, Hso90-α2, and HSP90-β facilitated virus multiplication. Overexpression of these six isoforms increased the levels of viral multiplication from 1.69 to 3.63 times more than that of the control (Fig. 5B). However, the increasing rates were less than those observed in the previous study [29]. One possible explanation for this discrepancy is that the expression levels of the exogenous HSP genes were not

high enough to give good increasing rates in viral titer in this study because of the low transfection efficiencies (Fig. 5A). Similarly, knockdown of the endogenous genes encoding the six HSP isoforms decreased the levels of viral multiplication at the rates from 0.26 to 0.55 of that of the control (Fig. 6B), which is less efficient than the previous results reported by Su et al [29]. Since the knockdown levels of the isoform mRNAs obtained in this study were around 40% of the control (Fig. 6A), more efficient knockdown of the HSPs should impair more efficiently virus multiplication.

We showed that HSP70-1, HSP70-2, HSP70-5, HSP90-α1, HSP90-α2, and HSP90-β played positive roles for betanodavirus multiplication. Surprisingly, all of these but HSP70-5 were up-regulated by virus infection exclusively among the ten HSP isoforms tested, though the ten HSP isoforms are known to be up-regulated by various physiological or pathological stimuli in human [26, 61, 82]. Su et al [29] reported that up-regulation of HSP70-5 occurred in GF-1 cells at 48 and 72 h post inoculation with RGNNV (MOI = 5). We could not examine the expression of HSP70-5 in medaka cells at 48 h post inoculation because almost all of the cells were disrupted by a cytopathic effect at this period (data not shown). HSP90-β in GF-1 cells was up-regulated by inoculation with RGNNV (MOI = 0.1) at as early as 6 h post inoculation [72], which is well consistent with our data that HSP90-β was up-regulated in medaka cells at 6 h post RGNNV inoculation (Fig. 4). Collectively, these results imply that RGNNV actively induces those six HSP isoforms to facilitate its multiplication. The viral manipulation of host cells via alteration of the gene expression has been reported in various virus species [83–86]. Nevertheless, we still can not rule out the possibility that induction of some or all of the virologically important HSP isoforms were just results of host stress responses against virus infection and were incidentally utilized for virus multiplication.

Declarations

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Author contributions

KZ performed the experiments and prepared the manuscript. YO prepared the manuscript and supervised the experiments described in the article.

Compliance with Ethical Standards

Disclosure of potential conflicts of interest:

All authors declare no conflict of interest.

Research involving Human Participants and/or Animals:

No humans or animals were used in this study.

Informed consent:

All authors approved the final draft.

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Figures

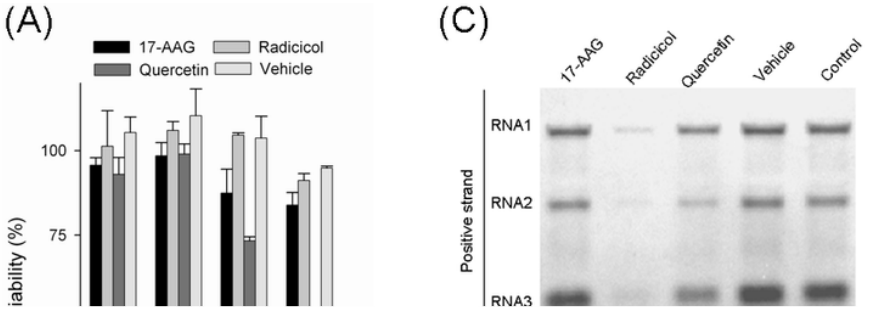

Figure 1

Effect of the HSP inhibitors on virus multiplication.

OLHNI-2 cells were incubated with 5 mM radiociol, 5 mM 17-AAG, or 20 mM quercetin after inoculation with RGNNV (MOI =1) for 30 min. Cells incubated with DMSO only were indicated as “vehicle”. Cells incubated with normal culturing medium were used as the control. (A) The cytotoxic effects of the inhibitors were assessed by the WST-1 assay. The relative cell viability against the control was shown. (B) The production of progeny virus in the culture supernatants was determined by the $TCID_{50}$ method at 12 h post virus inoculation. The numbers above the bars indicate the relative viral titers against the control cells. (C) Northern blot analysis of the viral

RNAs and Western blot analysis of the coat protein were performed at 12 h post inoculation with RGNNV. Representative data are shown here. Positions of RNA1, RNA2, and RNA3 are indicated to the right. Expression of β-actin was detected as the loading control. (D) Quantitative analysis of the Northern blot and Western blot images in Fig. 1C. The relative expression was calculated against the control cells after normalization with the β-actin expression. Data are shown as mean values of three independent experiments with standard deviation.

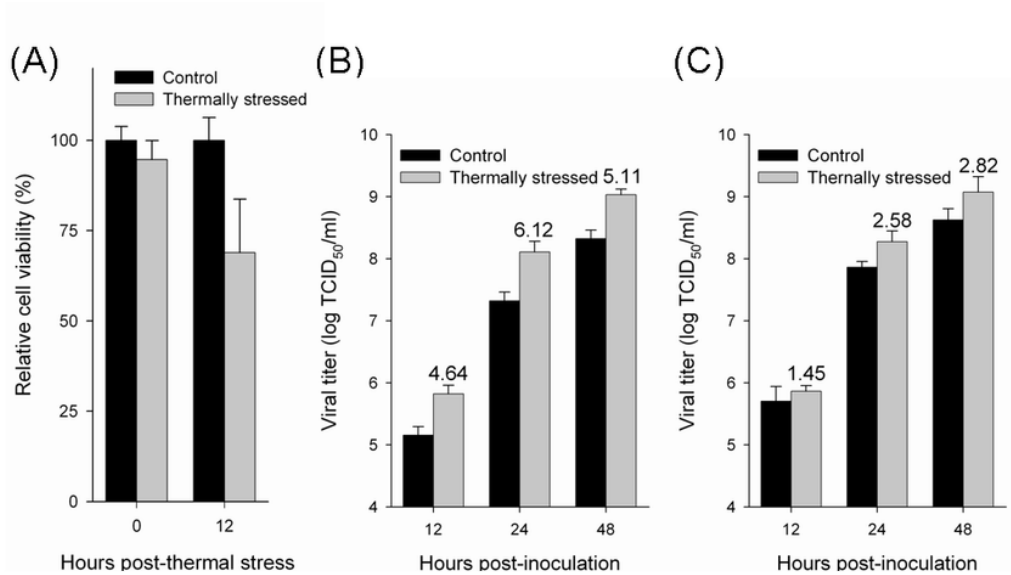

Figure 2

Effect of the thermal stress on virus multiplication.

OLHNI-2 cells were thermally stressed at 42°C for 1 h, and recovered at 30°C for 1 h (designated as 0 h post thermal stress). Cells continuously incubated 30°C was used as the control. (A) The cytotoxic effect of the thermal stress was assessed by the WST-1 assay. The relative cell viability against the control was shown. Thermally stressed cells then were inoculated with RGNNV (MOI=1) for 1 h at 0 (B), or 12 h (C) post thermal stress. The progeny virus in the culture supernatants was determined by the $TCID_{50}$ method at 48 h post virus inoculation. The numbers above the bars indicate the relative viral titers against the control. (D) Northern blot analysis of the viral RNAs and Western blot analysis of the coat protein were performed at 12 h post inoculation with RGNNV. Representative data are shown here. Positions of RNA1, RNA2, and RNA3 are indicated to the left. Expression of β-actin was detected as the loading control. (E) Quantitative analysis of the Northern blot and Western blot images in Fig. 2D. The relative expression was calculated against the control cells after normalization with the β-actin expression.

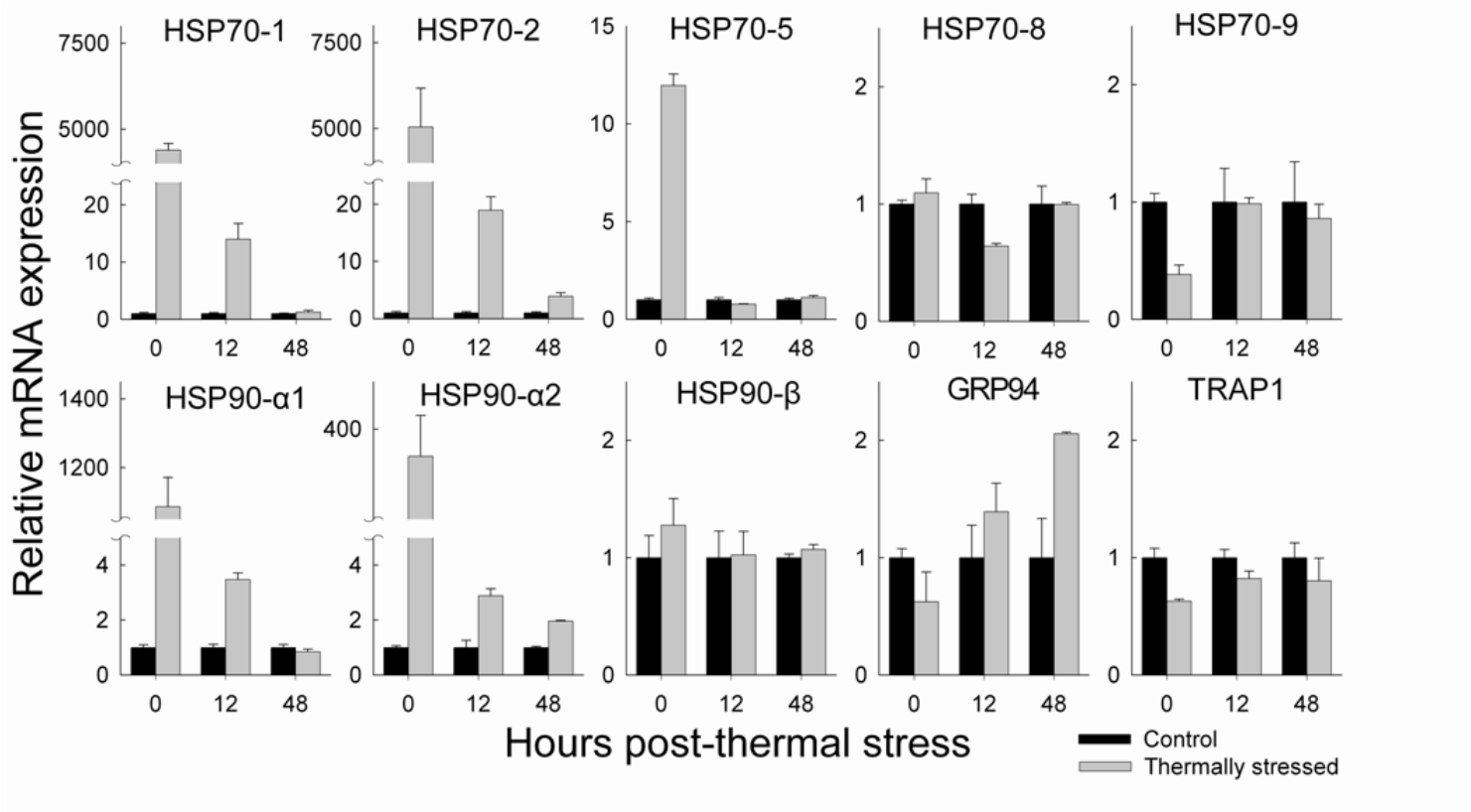

Figure. 3

Figure 3

Effect of the thermal stress on the expression of the HSP70 and HSP90 isoforms.

OLHNI-2 cells were thermally stressed at 42°C for 1h and recovered at 30°C for 1 h (designated as 0 h post thermal stress). Cells continuously incubated 30°C was used as the control. Total RNA was isolated from cells at

0, 12, and 48 h post thermal stress and subjected to the quantitative real time RT-PCR analysis. The relative mRNA expression was calculated against the control after normalization with β-actin expression.

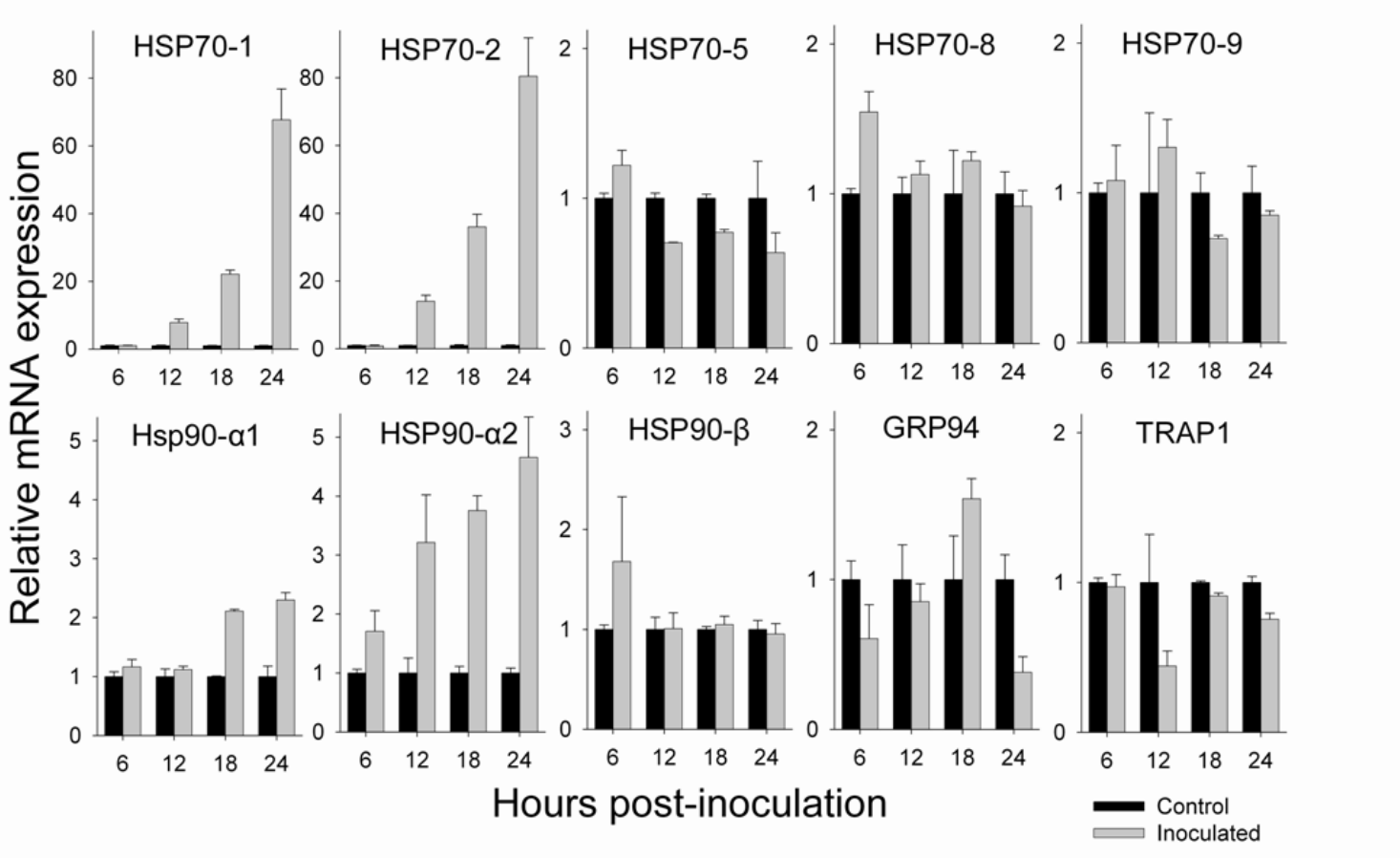

Figure. 4

Figure 4

Effect of the virus infection on the expression of the HSP70 and HSP90 isoforms.

OLHNI-2 cells were inoculated with RGNNV (MOI=1) for 1 h. Cells without virus inoculation were used as the control. Total RNA was isolated from cells at 6, 12, 18, and 24 h post inoculation and subjected to the quantitative real time RT-PCR analysis. The relative mRNA expression was calculated against the control after normalization with β-actin expression.

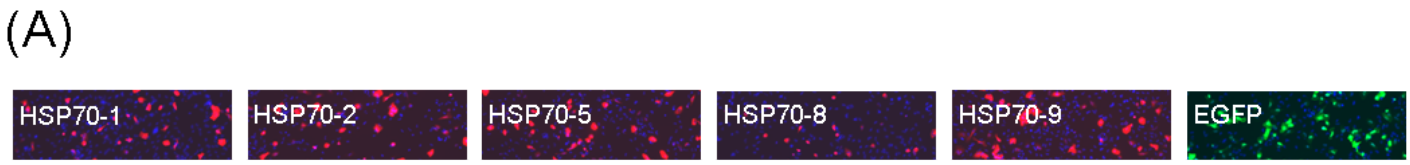

Figure 5

Effects of overexpression of the HSP isoforms on virus multiplication.

OLHNI-2 cells were transfected with the expression plasmid vector encoding each of the HSP70 and HSP90 isoforms. Cells transfected with the expression vector encoding EGFP was used as the control. Cells then were inoculated with RGNNV (MOI=10) at 48 h after transfection. (A) Cells expressing each of the HA-tagged HSP70 and HSP90 isoforms were detected by immunocytochemistry at 48 h after transfection. The red fluorescence images of HSP-expressing cells were merged with the blue fluorescence images of DAPI-stained nuclei. Representative data from three independent experiments are shown here. The green fluorescence micrograph indicates the expression of EGFP in the control cells. (B) The viral titers in the culture supernatants were determined by the $TCID_{50}$ method at 48 h post virus inoculation. The numbers above the bars indicate the

relative viral titers against the control. Data are shown as mean values of three independent experiments with standard deviation.

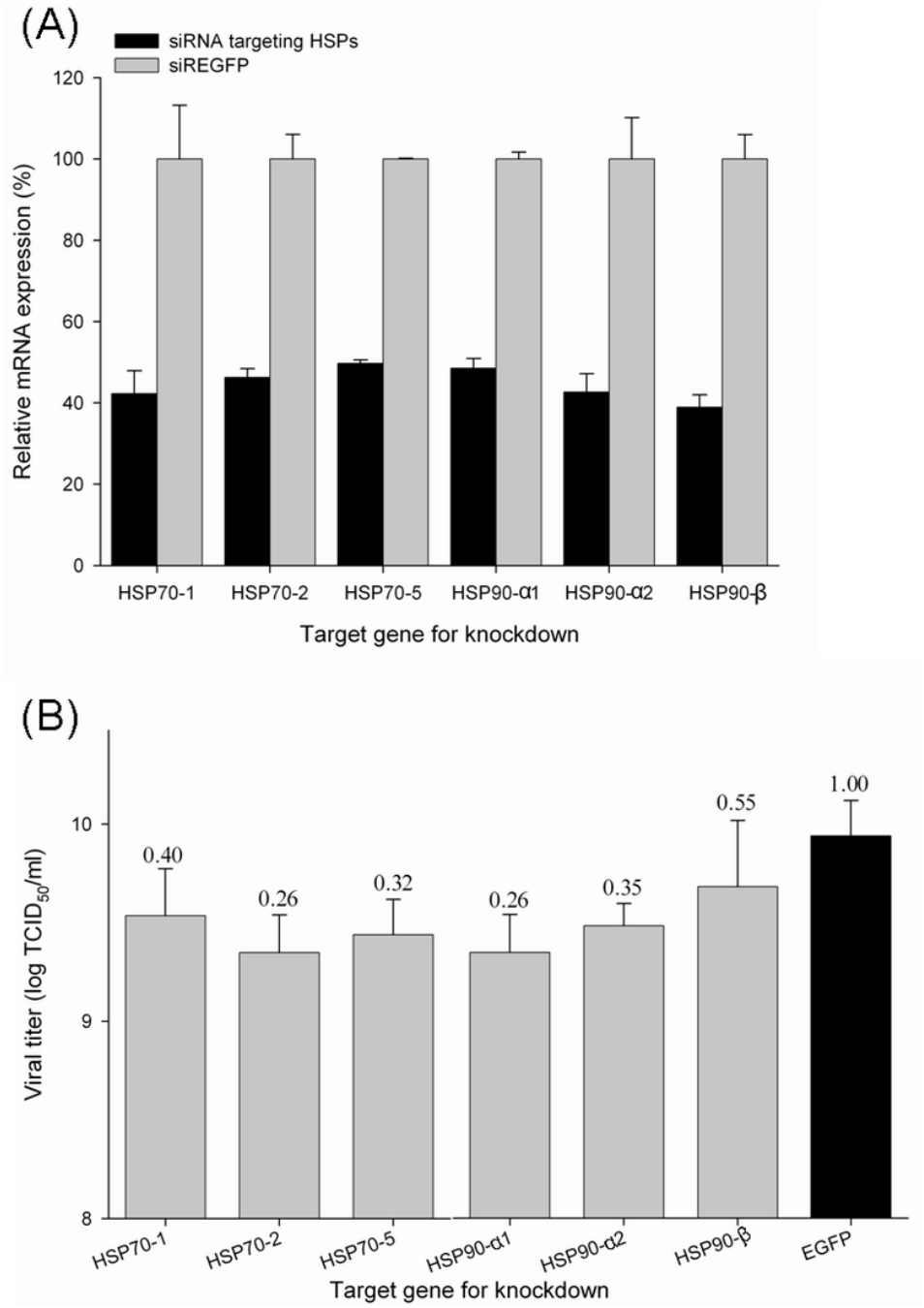

Figure. 6

Figure 6

Knock down effects of the HSP isoform genes on virus multiplication.

OLHNI-2 cells were transfected with the siRNA targeting each of the HSP70 and HSP90 isoforms. Cells transfected with the siRNA targeting EGFP (siREGFP) were used as the control. Cells then were inoculated with RGNNV (MOI=1) at 48 h after transfection. (A) The knockdown efficiencies of the siRNAs were assessed by the

relative mRNA expression of the isoforms. Total RNA was isolated from cells at 48 h post transfection and subjected to the quantitative real time RT-PCR analysis. The relative mRNA expression was calculated against the control after normalization with β-actin expression. (B) The progeny virus in the culture supernatants was titered by the $TCID_{50}$ method at 48 h post virus inoculation. The numbers above the bars indicate the relative viral titers against the control.