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HRI of *Epinephelus coioides* is a critical factor in the grouper immune response to RGNNV infection

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ABSTRACT

Phosphorylation of eukaryotic initiation factor 2 alpha subunit (eIF2α) occurs under a variety of conditions, including viral infection. Heme-regulated inhibitor (HRI) is an eIF2α kinase that modifies this phosphorylation. In this study, a HRI homologue (EcHRI) from the orange-spotted grouper (*Epinephelus coioides*) was cloned and its roles during fish viral infection were characterized. EcHRI encodes a 664-amino acid polypeptide that shares a high degree of similarity with HRIs from other species. Quantitative real-time polymerase chain reaction analysis indicated that EcHRI was distributed in all examined tissues. Expression of EcHRI in the spleen of *E. coioides* was up-regulated when challenged with the synthetic analog of double-stranded RNA (dsRNA) of polyinosine-polycytidylic acid (poly I:C). EcHRI was significantly increased in red-spotted grouper nervous necrosis virus (RGNNV) infected cells. EcHRI was abundantly distributed in the nucleus of grouper spleen (GS) cells. Overexpression of EcHRI inhibited the expression of red-spotted grouper nervous necrosis virus (RGNNV) genes in GS cells. Furthermore, our results showed that EcHRI overexpression significantly increased the expression of interferon (IFN)-related cytokines and enhanced activation of IFN-β, interferon-sensitive response element (ISRE), and nuclear factor κB (NF-κB). Taken together, these results suggest that EcHRI is involved in the fish immune response to virus challenge.

1. Introduction

Eukaryotic initiation factor 2 (eIF2) is an essential eukaryotic initiation factor that mediates initiation of tRNA binding to ribosomes during translation initiation [1]. It is a heterotrimer composed of α, β, and γ subunits. eIF2 was the first eIF discovered that can be regulated by phosphorylation. The α subunit is considered to be the regulatory subunit. Phosphorylation of eIF2α occurs under a variety of conditions, including viral infection, apoptosis, nutrient deprivation, and certain stresses [2]. It also is the main target of phosphorylation because it contains a serine at position 51 (Ser51) that is regulated by phosphorylation [2]. Phosphorylation of eIF2α can result in a severe decline in protein synthesis, which is an important strategy in the cell armory against stressful insults [3]. Phosphorylation of eIF2α is a widely studied mechanism of translation regulation, and it occurs under the influence of eIF2α kinases. The four eIF2α kinases are heme-regulated inhibitor (HRI), double-stranded (ds) RNA-activated protein kinase (PKR), PKR-like endoplasmic reticulum kinase, and general control non-derepressible-2, and they phosphorylate eIF2α under different stress conditions [4–7].

HRI was originally identified as the translation-level regulator (through its eIF2α kinase activity) that couples β-globin synthesis with heme levels during erythropoiesis, and more recently it has been shown to mitigate oxidative stress during erythroid differentiation [8,9]. HRI is also important for various stress responses in mammalian cells and yeast [10,11]. HRI was found to positively regulate specific virulence-related activities of diverse bacterial pathogens [12]. Surprisingly, these HRI effects were independent of its canonical function as a translation regulator via eIF2α, which highlights a novel role for HRI in bacterial pathogenesis [12]. HRI has two regulatory heme binding sites [13]. With the exception of the malaria parasite, *Plasmodium falciparum* [14], HRI genes have been identified in many species. HRIs have been sequenced from some fishes, such as zebrafish, Japanese flounder (*Paralichthys olivaceus*), and so on. *P. olivaceus* HRI homologue (PoHRI) was cloned from cultured flounder

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Table 1
Primers used in the present study.

Name	Sequence (5′–3′)
C1- EcHRI-F	GCGAATTCTATGTTCAGTTCAGCCTCCAACAACG
C1- EcHRI-R	ACGCGTCGACGCTCTTGTCTGACTCAGAGAAATGA
pcDNA3.1-EcHRI-F	GCGAATTCATGTTCAGTTCAGCCTCCAACAACG
pcDNA3.1-EcHRI-R	ATAAGAATGCGGCCGCTCAGCTCTTGTCTGACTCAGAGAAA
RT-EcHRI-F	TCTGGCAGAATGAGTCTGGGATTTT
RT-EcHRI-R	CTTCGGGTGTTGGTACCTTTGTATA
RT-RdRp-F	GTGTCCGGAGAGGTTAAGGATG
RT-RdRp-R	CTTGAATTGATCAACGGTGAACA
RT-CP-F	CAACTGACAACGATCACACCTTC
RT-CP-R	CAATCGAACACTCCAGCGACA
RT-grouper-18S-F	ATTGACGGAAGGGCACCACCAG
RT-grouper-18S-R	TCGCTCCACCAACTAAGAACGG
RT-grouper-Actin-F	TACGAGCTGCCTGACGGACA
RT-grouper-Actin-R	GGCTGTGATCTCCTTCTGCA
RT-EcIFP35-F	TTCAGATGAGGAGTTCTCTCTTGTG
RT-EcIFP35-R	TCATATCGGTGCTCGTCTACTTTCA
RT-EcIRF1-F	AGGGAGCCAGTGGAGTGAATC
RT-EcIRF1-R	GATGCCTGTGCCCAAAGTTAT
RT-EcIRF2-F	ACACGATTGGGTCAAGCAGG
RT-EcIRF2-R	TGGAGCGGGTACGAGTGAGA
RT-EcISG15-F	CCTATGACATCAAAGCTGACGAGAC
RT-EcISG15-R	GTGCTGTTGGCAGTGACGTTGTAGT

embryonic cells (FEC) after treatment with UV-inactivated grass carp haemorrhagic virus (GCHV). The putative PoHRI protein exhibits high identity with all members of eIF2a kinase family. Upon heat shock, virus infection or polyinosine-polycytidylic acid (poly I:C) treatment, PoHRI mRNA and protein are significantly upregulated in FEC cells but show different expression patterns in response to different stresses [15].

The orange-spotted grouper (*Epinephelus coioides*) is one of the main commercial marine aquaculture fish species in South China and Southeast Asia. However, the growing scale of grouper aquaculture and continuous deterioration of the culture environment have led to heavy economic losses to the grouper market due to various infectious diseases, particularly nervous necrosis virus (NNV) and iridovirus, which result in as much as 80% mortality [16,17]. NNVs are non-enveloped RNA viruses, which are the causative agents of viral encephalopathy and retinopathy (VER) of fish [18]. NNV, which consists of two single-stranded positive-sense RNAs, is one of the most devastating fish viruses, with mortality rates > 95% in severe outbreaks [19]. NNV is divided into four genogroups based on the RNA2 sequence: barfin flounder NVV (BFNNV), red-spotted grouper NNV (RGNNV), striped jack NNV (SJNNV), and tiger puffer NNV (TPNNV) [20]. VER outbreaks are mainly caused by the RGNNV [21]. Outbreaks of NNV disease have been a major constraint for grouper culture in China over the past decade [21].

In previous studies, several genes of the orange-spotted grouper that have antiviral function against RGNNV were identified and characterized [22–24]. We previously reported that eIF2α from grouper (EceIF2α) overexpression decreased RGNNV replication in vitro [25]. To elucidate the role of HRI in the interaction between host and virus, HRI from the orange-spotted grouper (EcHRI) was investigated under RGNNV challenge in this study.

2. Materials and methods

2.1. Fish, cells and viruses

Orange-spotted groupers (50–60 g) were purchased from a local fish farm located in Hainan Province, China. They were kept in a laboratory recirculating seawater system for about 3 weeks before use.

Grouper spleen (GS) cells used in this study were established and kept in our laboratory following methods described by Huang et al. [26]. GS cells were cultured in Leibovitz's L15 medium supplemented with 10% fetal bovine serum at 25 °C. RGNNV was isolated in our laboratory and propagated in GS cells as described previously [17]. The viral titers of RGNNV was 10^5 $TCID_{50}$/ml, and GS cells were infected the virus as previously described. Cells were collected for quantitative real-time polymerase chain reaction (qRT-PCR) at 0, 8, 12, 24, 30 and 36 h.

2.2. Cloning of EcHRI and bioinformatic analysis

Based on several expressed sequence tag (EST) sequences of EcHRI from the grouper spleen transcriptome [27], primers (listed in Table 1) were designed to amplify the full-length open reading frame (ORF) of EcHRI. The coding sequence was predicted using the online ORF website (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). The protein molecular weight was deduced using the website (http://www.bio-soft.net/sms/index.html). Sequence alignments and percentage of amino acid conservation were assessed by Clustal-X multiple alignment algorithm. Domains were analyzed with the online CDD tool at NCBI (http://www.ncbi.nlm.nih.gov/) and the SMART program (http://smart.embl-heidelberg.de/). The phylogenetic trees were generated through neighbor-joining (NJ) method with MEGA 4.0.

2.3. Expression patterns of EcHRI in grouper

To determine the tissue distribution of EcHRI in orange-spotted grouper, the relative expression level of EcHRI was examined by qRT-PCR. Liver, spleen, kidney, brain, intestine, heart, skin, muscle, stomach, gill, head kidney and blood were collected for RNA extraction. The expression levels of EcHRI in tissues were measured.

To explore the response of EcHRI after challenge with poly I:C, the expression levels of EcHRI were examined in orange-spotted grouper tissues after the fish were injected with poly I:C at the concentration of 1 μg/ml. At indicated time points, head kidneys and spleens were collected for RNA extraction and further qRT-PCR analysis.

2.4. Plasmid construction

To elucidate the molecular function of EcHRI in vitro, the full length ORF of EcHRI was subcloned into pcDNA3.1 and pEGFP-C1 vectors using the primers listed in Table 1. The target PCR products were digested with *Hin*d III and *Sall*(Takara, Tokyo, Japan) and then subcloned into the *Hin*d III and *SalI*sites of expression vector pEGFP-C1. In the pGFP-HRI plasmid, the EcHRI sequence is at N-terminal, and GFP sequence is at C-terminal. The constructed plasmids were confirmed by DNA sequencing.

2.5. Cell transfection

Cell transfection was performed using transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as described previously [28]. In brief, GS cells were grown to 60–70% confluence in 24-well cell culture plates and then incubated with a mixture of Lipofectamine 2000 and plasmids (0.4 μg) according to the manufacturer's instructions. At 6 h post-transfection, fresh normal medium was added and cells were cultured at 25 °C for further study.

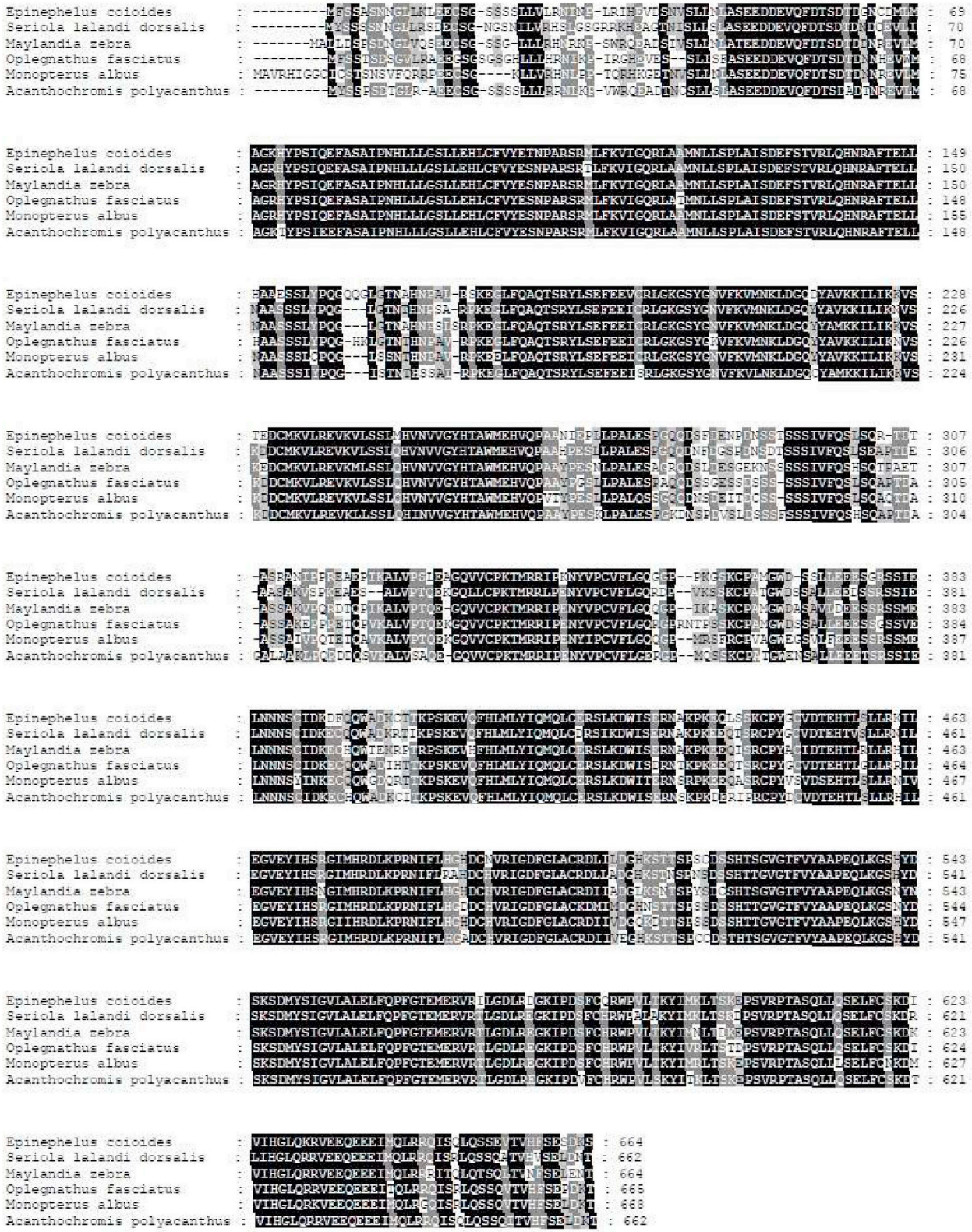

Fig. 1. Characterization of EcHRI. (A) Multiple sequence alignment of the EcHRI proteins. (B) Phylogenetic analysis of EcHRI and other EcHRI homologs. All sequences of HRI homologs from different species were obtained from the NCBI database. The phylogenetic tree was constructed with MEGA 4.0 using the neighbor-joining method and 1000 bootstrap replicates. The GenBank accession number of each species is listed to the right of the species name.

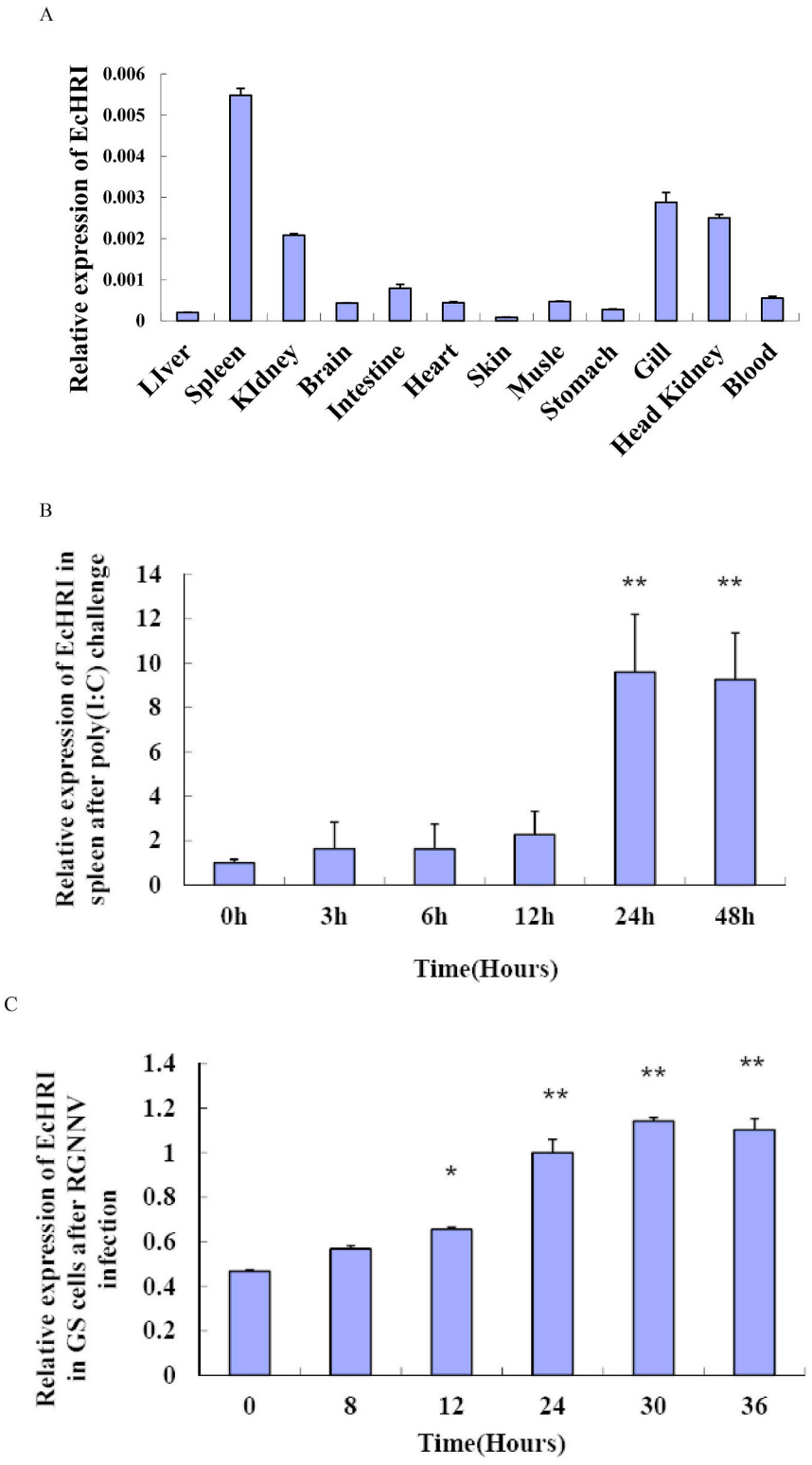

Fig. 2. Expression profiles of EcHRI. (A) Expression patterns of EcHRI in different tissues from healthy grouper. (B) Expression patterns of EcHRI in the spleen from groupers after challenging with poly I:C. (C) Expression patterns of EcHRI in GS cells after infection with RGNNV.

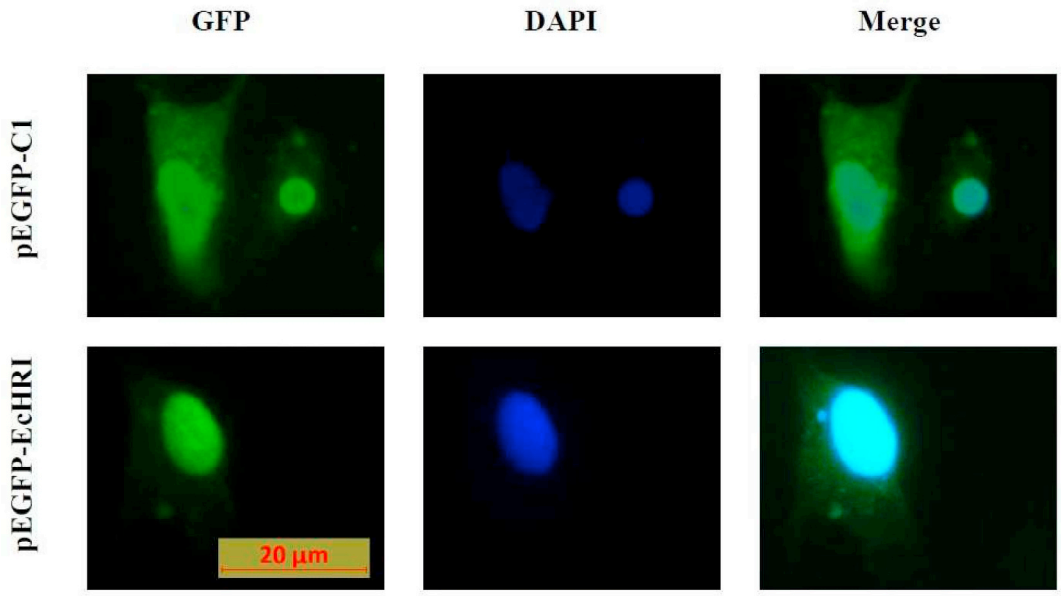

Fig. 3. Subcellular localization of EcHRI in GS cells. GS cells were seeded into 6 well plates and then transfected with EcHRI and the control vector pEGFP-C1. At 48 h post-transfection, cells were fixed, stained with DAPI, and then observed under fluorescence microscopy.

2.6. Fluorescent microscopy

To analyze the subcellular localization of EcHRI, plasmids (0.4 μg) including pEGFP-C1 and pEGFP-EcHRI were transiently transfected into GS cells as described above. At 48 h post-transfection, cells were fixed with polyformaldehyde and then stained with 4,6-diamidino-2-phenylindole (DAPI). Finally, samples were imaged under fluorescence microscopy.

2.7. Virus infection

To evaluate the effects of EcHRI on virus replication, EcHRI or empty vector transfected cells were infected with RGNNV at an MOI of 0.1. Virus infected cells were collected at 12 h and 24 h for RNA extraction and further qRT-PCR analysis.

2.8. Reporter gene assay

At 1 day pre-transfection, GS cells were seeded into a 24-well culture plate in 500 μl of L15 with 10% fetal calf serum at 28 °C. The pcDNA3.1 and pcDNA3.1- EcHRI vectors (0.4 μg) as well as the interferon (IFN)-β, interferon-sensitive response element (ISRE) (Stratagene, USA), and nuclear factor (NF)-κB luciferase reporters (Stratagene, USA) (0.1 μg) were co-transfected with Lipofectamine 2000 according to the manufacturer's instructions. The pRL-SV40 Renilla luciferase vector (0.05 μg) was used as an internal control. The plasmid IFN-β luciferase reporter was saved in our own laboratory. At 48 h post-transfection, GS cells were harvested and lysed to examine the luciferase activity using a dual-luciferase reporter assay system (Promega, USA) [22].

2.9. RNA extraction and qRT-PCR analysis

Total RNA isolation was performed using the SV Total RNA Isolation System (Promega) according the manufacturer's instructions, and reverse transcription was carried out with ReverTra Ace (Toyobo, Osaka, Japan).

qRT-PCR was performed using a SYBR® Green Realtime PCR Master Mix (Toyobo, Japan) in an Applied biosystems QuantStudio 5 Real Time Detection System (Thermofisher, USA). Each assay was carried out in triplicate with the following cycling conditions: 95 °C for 1 min for activation, followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s and 72 °C for 45 s. The expression levels of viral genes, including RGNNV CP and RdRp, and host immune genes, including IFN regulatory factor 1 (IRF1), IRF2, IFN stimulated gene 15 (ISG15), IFN induced protein 35 (IFP35), and MXII, were all detected. The used primers were listed in Table 1. The expression levels of target genes were normalized to 18S ribosomal RNA and calculated with the $2^{-\triangle\triangle CT}$ method. The data were represented as mean ± SD. Statistical analysis was carried out using Non-parametric test of SPSS Version 13. One-way analysis of variance (ANOVA) was used to evaluate the variability between treatment groups (*$p < 0.05$, **$p < 0.01$).

2.10. Overexpression of EcHRI on eIF2α phosphorylation

GS cells were seeded into a 24-well culture plate in 500 μl of L15 with 10% fetal calf serum at 28 °C. The pcDNA3.1 and pcDNA3.1-EcHRI vectors (0.4 μg) were transfected with Lipofectamine 2000 according to the manufacturer's instructions. Cells were collected at 24 h post transfection. eIF2α Phosphorylation was analyzed by Western blot as follows [29]. Proteins were fractionated by electrophoresis through 12.5% SDS-PAGE and electrophoretic transferred onto a PVDF membrane (Millipore). Membranes were blocked with 5% nonfat milk and PBST at room temperature for 2 h and washed three times with PBST. Anti-eIF2α (Immunoway, USA) and Phospho-eIF2α-S51 (Immunoway, USA) or Actin (Immunoway, USA) antibodies were diluted 1/1000 in 5% nonfat milk and incubated with the membranes overnight at 4 °C. After washing

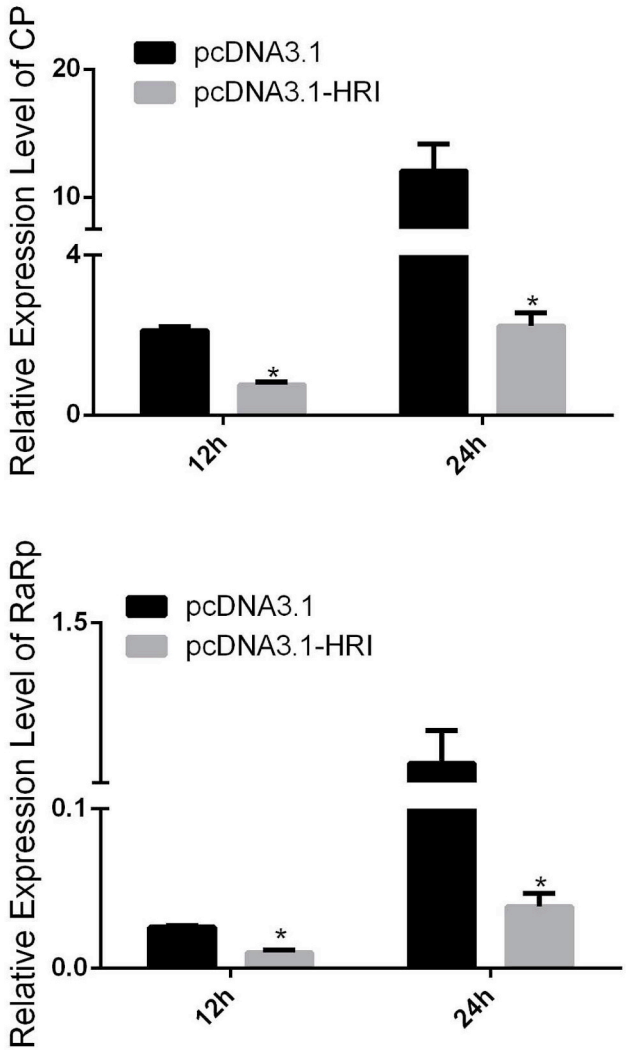

Fig. 4. EcHRI overexpression decreased viral gene transcription. Viral gene expression of RGNNV was detected using qRT-PCR. After transfection with EcHRI and the control vector, GS cells were infected with RGNNV for the indicated length of time and then collected for RNA extraction. Expression levels of RGNNV RdRp and CP were detected using qRT-PCR. Bars represent means ± SD (n = 3). *, $p < 0.05$.

three times with PBST, membranes were incubated for 1 h with HRP-labeled anti-Rabbit IgG Ab diluted 1/1000 in 5% nonfat milk. The membranes were washed three times with PBST and detected with DAB.

3. Results

3.1. Characterization of EcHRI

Using the EST sequences from the transcriptome data (accession number is SRA040065.1), we obtained the full-length cDNA of the EcHRI using PCR amplification and DNA sequencing. The full-length ORF of EcHRI is 1995 base pairs, and it encodes a 664-amino acid polypeptide that displays high homology with other known HRI proteins (Fig. 1A). Multiple sequence alignments were carried out using Clustal X multiple-alignment software. Phylogenetic analysis shows that EcHRI shares a close relationship with HRI from *Stegastes partitus* (Fig. 1B).

3.2. Expression patterns of EcHRI

To analyze the gene expression profiles, qRT-PCR was conducted in different tissues of healthy juvenile orange-spotted grouper. EcHRI was distributed in all examined tissues, and it was relatively high mRNA levels in the spleen, gill, and head kidney, and lower mRNA levels in liver, skin and stomach (Fig. 2A).

To determine the gene expression profiles of EcHRI in grouper cells, the transcription levels of EcHRI were examined in the spleen of groupers and GS cells after poly I:C challenge or RGNNV infection. The mRNA expression level of EcHRI in the spleen was up-regulated after poly I:C stimulation, and it reached the maximal level at 24 h compared with the blank group (Fig. 2B). EcHRI was significantly increased in RGNNV infected cells, and it reached the maximal level at 36 h compared with the blank (Fig. 2C).

3.3. Subcellular localization of EcHRI in vitro

To detect the subcellular localization of EcHRI, the plasmids of pEGFP-C1 and pEGFP-EcHRI were transfected to GS cells, and fluorescence were observed under fluorescence microscopy. As shown in Fig. 3, the green fluorescence was mainly located in the nucleus in EcHRI transfected grouper cells. In pEGFP-C1 transfected cells, fluorescence was distributed throughout both the cytoplasm and nucleus. Thus, EcHRI was proposed to encoded a nucleus protein.

3.4. Ectopic expression of EcHRI decreased RGNNV replication in vitro

To clarify the effects of EcHRI overexpression on virus infection, EcHRI transfected cells were infected with RGNNV, and then viral replication was investigated. Quantitative analysis indicated that transcription of RGNNV CP and RdRp genes was significantly decreased in EcHRI overexpressing cells compared to control cells (Fig. 4). Accordingly, our results demonstrated that EcHRI functioned as a critical antiviral factor during RGNNV infection.

3.5. Overexpression of EcHRI enhanced the IFN immune response

To explore the potential mechanism of the antiviral function of EcHRI, the regulatory effect of EcHRI on host immune factors was evaluated using qRT-PCR. The expression levels of IFN-related cytokines or effectors, including IFP35, IRF1, IRF2, ISG15, and MXII, were all significantly higher in EcHRI overexpressing cells compared to the control vector transfected cells (Fig. 5). Thus, EcHRI appears to positively regulate the IFN immune response in vitro.

3.6. Overexpression o EcHRI enhanced the IFN-β-, ISRE-, and NF-κB-mediated immune response

Activations of reporter genes were investigated to reveal the

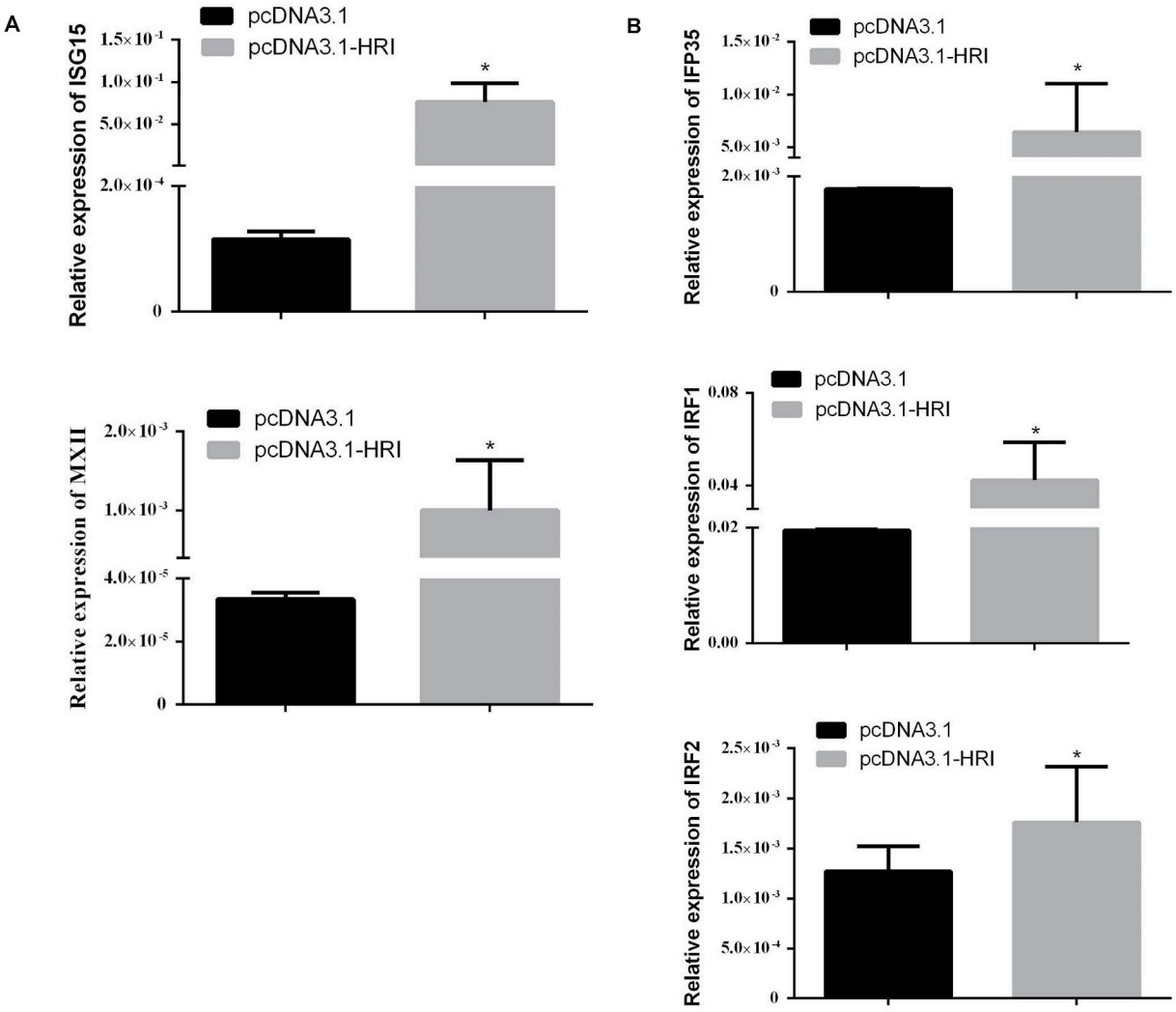

Fig. 5. EcHRI overexpression increased the expression of IFN-related genes. GS cells were transfected with EcHRI, cells were harvested at 48 h post-infection, and the expression levels of host IFN-associated genes were determined using qRT-PCR. Bars represent the means ± SD (n = 3). *, $p < 0.05$.

molecular mechanism by which EcHRI functions. Overexpression of EcHRI significantly increased the luciferase activity of IFN-β, ISRE, and NF-κB promoters compared to the control vector. The fold changes of IFN-β-Luc, ISRE-Luc, and NF-κB-Luc were 13.6, 19.1, and 71.6 in cells transfected with pcDNA3.1 and 114.3, 7.2, and 9.7 in cells transfected with pcDNA-EcHRI, respectively (Fig. 6). These data show that EcHRI likely regulates IFN-β, ISRE, and NF-κB, which are involved in the IFN immune response.

3.7. Effects of EcHRI on eIF2α phosphorylation

HRI is an eIF2α kinase, which modifies the phosphorylation of eIF2α. To examine the effects of EcHRI on eIF2α phosphorylation, GS cells were transfected with EcHRI expression vectors, and 24 h after transfection, the phosphorylation levels of eIF2α were determined by western blot using an antiphospho-eIF2α (Ser51) antibody. Compared with the control group (cells transfected with an empty vector), the EcHRI-expressing cells display increases in the phosphorylation levels of eIF2α of approximately 1.6-fold (Fig. 7).

4. Discussion

Much attention has been paid to HRI due to it is a crucial role in modifying the phosphorylation of eIF2α, which occurs under a variety of conditions, including viral infection [8–12]. However, few studies have focused on the function of fish HRI, especially its role in virus infection. By using the EST sequences of EcHRI in the grouper spleen transcriptome, we cloned the full-length EcHRI and characterized its

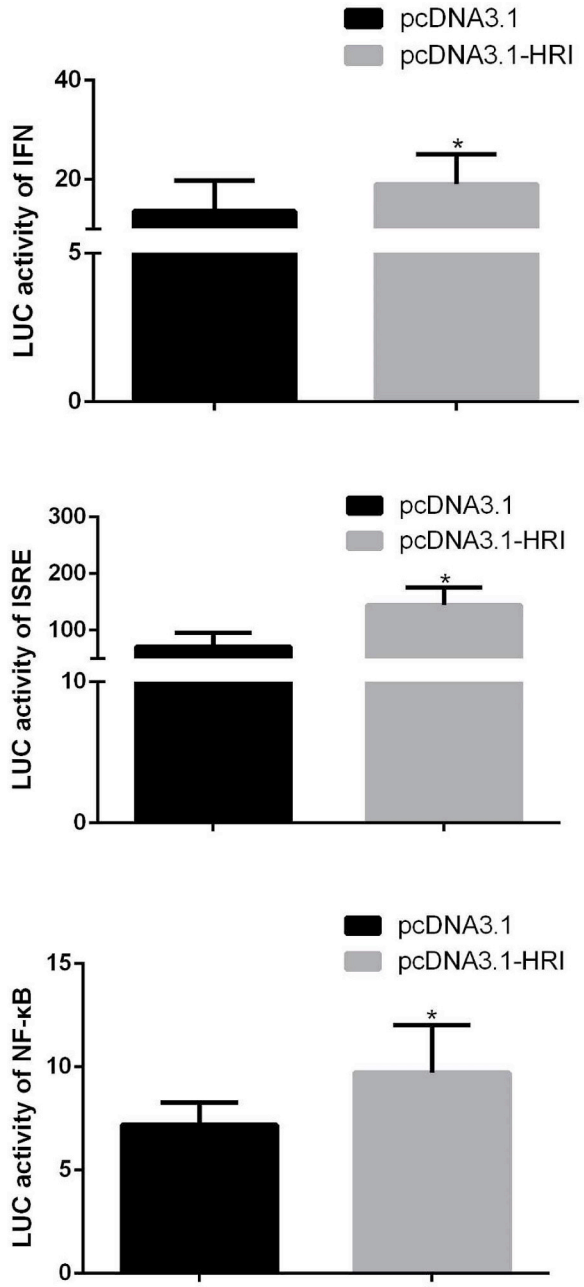

Fig. 6. Activation of reporter genes by EcHRI in GS cells. EcHRI was co-transfected with IFN-β, ISRE, and NF-κB promoter plasmids, and promoter activity was detected using the luciferase reporter gene assay. Bars represent means ± SD (n = 3). *, $p < 0.05$.

roles in grouper virus infection. We found that EcHRI shares high homology with HRIs of other species, suggesting that the function of EcHRI might be conservative.

eIF2α plays an important role in viral infection, especially for RNA viruses such as Newcastle disease virus and *Scophthalmus maximus* rhabdovirus [30–32]. In our previous study, overexpression EceIF2α decreased RGNNV replication in vitro, and replication of RGNNV was inhibited after EceIF2α was phosphorylated [25]. In Mouse, HRI positively affects the cell-level infection dynamics of three dissimilar bacterial pathogens. The extracellular pathogen *Yersinia*, the vacuole-bound pathogen *Chlamydia*, and the cytosolic pathogen *Listeria*, all require HRI to efficiently complete their respective cellular infection cycles [12]. In fish, *PoHRI* mRNA and protein are significantly upregulated in FEC cells but show different expression patterns under heat shock, virus infection or Poly I:C treatment [15]. Those results showed that HIR may have difference mechanisms to bacteria and viruses. In the present study, overexpression EcHRI resulted in antiviral activity against RGNNV infection, as evidenced by the reduced transcription levels of viral genes. Therefore, we speculate that HRI functions as a host restriction factor in RGNNV infection in fish.

eIF2α kinase is the enzyme that modifies eIF2α. PKR and HRI belong to the eIF2α protein kinases [4–7]. Both PKR and HRI can modify the phosphorylation of eIF2α. In *Danio rerio*, DrPKZ-A and DrPKZ-B functionally interact with eIF2α and inhibit protein synthesis. DrPKZ-A and DrPKZ-B effectively phosphorylate eIF2α [33]. In this study, EcHRI increases in the phosphorylation levels of eIF2α of approximately 1.6-fold. The results indicated that both PKR and HRI modify the phosphorylation of eIF2α, and they may have the same mechanisms.

PKR is one of the key players involved in the type I interferon (IFN) inducible innate immune system of vertebrates. Upon viral infection, PKR is up-regulated through type I IFN signaling and bound by virally derived dsRNA. The activated PKR then phosphorylates alpha subunit of eukaryotic initiation factor 2 (eIF2α) [34,35]. To explore the potential mechanism of the action of EcHRI on virus replication, we evaluated the effect of EcHRI on the IFN immune response. Overexpression of EcHRI in grouper cells significantly increased the expression of IFN regulation factors such as IRF1 and interferon induced/stimulated genes, including IFP35, ISG15, and MXII. Considering that ISG15 and MX have significant antiviral activity against RGNNV infection [28,36], these immune cytokines induced by EcHRI might contribute directly to an inhibitory effect on RGNNV replication. Moreover, ectopic expression of EcHRI significantly increased the IFN-β- and ISRE-mediated immune response. PKR was shown to be responsible for NF-κB activation in herpes simplex virus infected cells. This activation participates in the control of virus replication as the virus yields in PKR or NF-κB deficient cells were 10-fold higher than in PKR and NF-κB sufficient cells [37]. In this study, EcHRI significantly increased the NF-κB-mediated immune response. The results indicated that HRI may have the similar functions with PKR in fish, and may be involved in the regulation of the translation of many viruses and is regulated by viral and cellular factors.

In summary, we have demonstrated the function of EcHRI in response to RGNNV infection. We found that EcHRI was localized in the nucleus of grouper cells. The transcription level of EcHRI was changed in response to viral pathogens. Furthermore, overexpression of EcHRI significantly inhibited RGNNV replication by enhancing the host IFN immune response. Our results will contribute greatly to understanding the roles of fish HRIs during RNA virus infection.

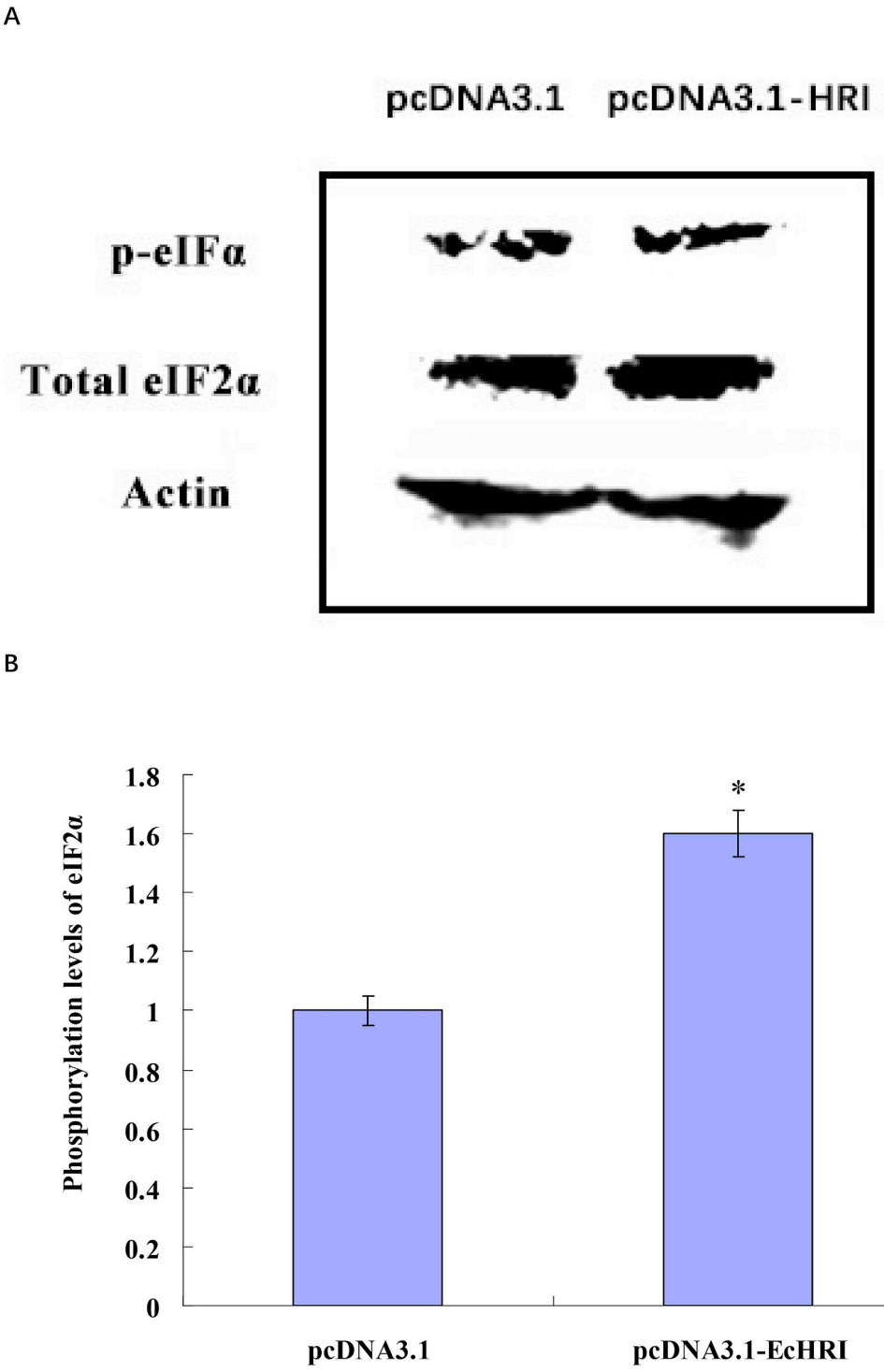

Fig. 7. Effects of EcHRI on eIF2α phosphorylation. (A) The phosphorylation levels of eIF2α are shown in western blot, which was blotted with Anti-eIF2α and Phospho-eIF2α-S51 or Actin antibodies. (B) Quantification of the phosphorylated eIF2α levels versus actin levels. Compared with the control group (cells transfected with an empty vector) (column 1), the EcHRI-expressing cells display increases in the phosphorylation levels of eIF2α of approximately 1.6-fold (columns 2). Data are presented as the means ± SD (n = 3 replicates). *, $p < 0.05$.

Acknowledgements

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