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Transducin β-like 1 X-linked receptor 1 (TBLR1) affects RGNNV infection through negative regulation of interferon immune response in orange-spotted grouper, *Epinephelus coioides*

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ABSTRACT

Transducin β-like 1 X-linked receptor 1 (TBLR1) was identified as an important component of nuclear receptor corepressor (N-CoR) complex, and functionally participated in regulation of transcriptional activation. However, the potential roles of TBLR1 in innate immune response still remain uncertain. In the present work, a novel TBLR1 from orange-spotted grouper, *Epinephelus coioides* (named as EcTBLR1) was cloned and its effect on fish virus infection was characterized. The full length open reading frame (ORF) of EcTBLR1 was 1548 bp and encoded a putative 515-aa polypeptide, which shared 99% and 95% identity with its homologue from large yellow croaker (*Larimichthys crocea*) and human (*Homo sapiens*), respectively. Quantitative PCR (qPCR) analysis revealed a ubiquitous expression of EcTBLR1 in different tissues with remarkable expression in brain, spleen and head-kidney. Subcellular location analysis showed that EcTBLR1 was mainly located in cytoplasm of grouper spleen cells, and partly translocated into nucleus after infection with red spotted grouper nervous necrosis virus (RGNNV). Moreover, RGNNV infection suppressed the protein synthesis of EcTBLR1 in grouper cells. Using RNA interference (RNAi) technology, we found that effective knock-down of EcTBLR1 significantly suppressed the transcription of RGNNV capsid protein (Cp) and RNA-dependent RNA polymerase (RdRp) genes, which implied the crucial role of EcTBLR1 in RGNNV infection. Consistently, overexpression of EcTBLR1 *in vitro* significantly inhibited IFN promoter activity, as well as the transcription of IFN-related downstream effectors, including interferon stimulated gene 15 (ISG15) and interferon regulatory factor 3 (IRF3). Together, our results for the first time demonstrated that fish TBLR1 might exert critical roles during fish RNA virus replication by negatively regulating interferon response.

1. Introduction

It was widely acknowledged that gene transcription underwent precise control regulated by promoter and transcriptional factors [1,2]. Nuclear receptor, one of the most abundant transcriptional factors in metazoan, possessed critical position in physiological development and cellular homeostasis [3,4]. Nuclear receptor superfamily could be activated by sterol, retinoic acid, estrogen, androgen, thyroxine, and established connection between signal molecules and transcriptional activation [5–7]. Transcription regulation mediated by nuclear receptor relied mainly on the ligand-dependent recruitment of cofactors, which were also defined as coactivators and corepressors, and with a dynamic balance between coactivators and corepressors level modulating the threshold of transcriptional activation [8,9]. Transducin β-like 1 X-linked receptor 1 (TBLR1), an F-box/WD40 domain-containing protein, was initially identified as one of an intrinsic component of the nuclear receptor corepressor (N-CoR) complex [10,11], with serving to mediate a required exchange of corepressors for coactivators through recruitment of the ubiquitin-conjugating/19S proteasome, and consecutively induced transcriptional activation [11–13]. Subsequently, TBLR1 was demonstrated to be necessary for NF-κB-mediated transcriptional activation in embryonic stem cells, and depletion of TBLR1 by either antibody neutralizing or siRNA interference resulted in weak activation of NF-κB target genes under TNF-α stimulation [14]. In addition, TBLR1

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also played important role in the recruitment of β-catenin to the Wnt target gene promoter to activate transcription, indicating the crucial function of TBLR1 in canonical Wnt signaling [15]. However, to our knowledge, there was very limited information about the regulatory roles of TBLR1 on immune signaling during virus infection, especially in lower vertebrates.

Grouper, *Epinephelus sp*, as one of the most important maricultured fishes in south China and Southeast Asia, has been in suffering from great damage caused by infectious pathogens, including pathogenic bacteria and virus [16,17]. Red spotted grouper nervous necrosis virus (RGNNV), a two-single-stranded RNA piscine nodavirus that mainly injured the central nervous system and retina of fishes, caused high mortality (near 100%) in juvenile grouper [18,19]. Recently, we have substantially identified several immune genes that deeply involved in RGNNV infection of grouper cells, including IFN-related effectors [20–22]. For example, interferon-stimulated gene 15 (ISG15) and interferon regulatory factor 3 (IRF3) were demonstrated to be dramatically induced by RGNNV infection, overexpression of either ISG15 or IRF3 decreased viral genes transcription or progeny virus production [23,24].

In this study, we identified a novel TBLR1 homologue from cultured grouper, *Epinephelus coioides*, entitled as EcTBLR1, and explored its regulatory roles in immunity-related transcriptional activation during RGNNV infection. Our results firstly demonstrated that fish TBLR1 was involved in virus infection via regulating the interferon immune response.

2. Materials and methods

2.1. Fish, cell and virus

Orange-spotted groupers, *Epinephelus coioides*, were purchased from Hainan province and kept in our laboratory recirculating seawater system until use. Stable grouper spleen (GS) cell line was established and maintained in our laboratory, and grown in Leibovitz's L-15 medium containing 10% fetal bovine serum (FBS, Gibco, USA) at 25 °C [25]. RGNNV was prepared according to the previous method [26].

2.2. Sequence analysis of EcTBLR1

According to the transcriptome sequencing data and annotation of grouper, *Epinephelus coioides*, we assembled the full length open reading frame (ORF) of a novel TBLR1 homologue named as EcTBLR1. Functional domain analysis was performed using InterPro online tool (http://www.ebi.ac.uk/interpro/). Multiple sequences alignment and phylogenetic tree analysis were performed using MEGA 5.05 and GeneDoc program.

2.3. Expression profile of EcTBLR1

A total of 12 tissues including liver, spleen, kidney, head-kidney, brain, heart, stomach, skin, muscle, intestines, fin and gill from healthy groupers (n = 3) were collected for RNA extraction (Promega, USA). Reverse transcription was performed with random primer (TOYOBO, Japan). The transcription level of EcTBLR1 in different tissues was examined by quantitative PCR (qPCR) with SYBR Green I Master (TOYOBO, Japan) in a LightCycler 480 Detection System (Roche, Switzerland).

2.4. Plasmid construction and siRNA design

To explore the subcellular location of EcTBLR1 *in vitro*, full length ORF was cloned into pEGFP-C1 vector with primers C1-EcTBLR1-F and C1-EcTBLR1-R (provided in Table 1). To evaluate the effects of EcTBLR1 overexpression *in vitro*, EcTBLR1 was subcloned into pcDNA3.1-Flag vector using primers Flag-EcTBLR1-F and Flag-EcTBLR1-R (provided in Table 1). All plasmids were validated by DNA sequencing (Invitrogen, Guangzhou). To knock down the endogenous EcTBLR1 in grouper cells, specific siRNA were designed and synthesized (RiboBio, Guangzhou) for cell transfection.

Table 1
Primers used in this study.

Primer names	Sequence (5′-3′)
C1-EcTBLR1-F	CCAAGCTTCGATGAGCATTAGCAGTGATGA
C1-EcTBLR1-R	GGGGTACCTTTCCGAAGGTCTAGTACAC
Flag-EcTBLR1-F	GGGGTACCGAATGAGCATTAGCAGTGATGA
Flag-EcTBLR1-R	CCGCTCGAGCTATTTCCGAAGGTCTAGTA
EcTBLR1-RT-F	ACTGGCAGAGCAACAATACG
EcTBLR1-RT-R	CTCCACATCCCAAAGACGAA
Actin-RT-F	TACGAGCTGCCTGACGGACA
Actin-RT-R	GGCTGTGATCTCCTTCTGCA
RGNNV CP-RT-F	CAACTGACAACGATCACACCTTC
RGNNV CP-RT-R	CAATCGAACACTCCAGCGACA
RGNNV RdRp-RT-F	GTGTCCGGAGAGGTTAAGGATG
RGNNV RdRp-RT-R	CTTGAATTGATCAACGGTGAACA
EcIRF3-RT-F	GACAACAAGAACGACCCTGCTAA
EcIRF3-RT-R	GGGAGTCCGCTTGAAGATAGACA
EcISG15-RT-F	CCTATGACATCAAAGCTGACGAGAC
EcISG15-RT-R	GTGCTGTTGGCAGTGACGTTGTAGT

2.5. Cell transfection

GS cells were seeded on 24-well plates (Corning, USA) at a concentration of 5×10^5/ml (0.5 ml) overnight prior to cell transfection. In brief, 1 μg of plasmids and 2 μl Lipofectamine 2000 (Invitrogen, USA) were mixed in Opti-MEM and then added to cells in serum-free medium for 6 h. After replacing with fresh complete L-15 medium, transfected cells were sequentially cultured for further analysis. Similarly, si-EcTBLR1 was transfected at a final concentration of 200 nM using Lipofectamine RNAiMAX (Invitrogen, USA).

2.6. Subcellular location analysis of EcTBLR1

To identify the subcellular location of EcTBLR1, recombinant pEGFP-EcTBLR1 and pEGFP-C1 (control) plasmids were transfected as described above. Transfected cells were fixed with 4% paraformaldehyde and nucleus were strained with Hochest 33342 at 48 h after transfection (Thermo, USA). Then fluorescence was observed under a fluorescence microscope (Leica, Germany).

To further clarify whether subcellular location of EcTBLR1 could be affected by virus infection, transfected cells were subjected to RGNNV infection at an MOI of ~0.1, and the fluorescence was observed as described at 24 h post infection (p.i.).

2.7. Western blotting

To evaluate the possible effect of virus infection on protein expression level of EcTBLR1, RGNNV infected cells at indicate time points (12 h, 24 h, 36 h and 48 h p.i.) were collected and lysed in Pierce IP Lysis Buffer (Thermo, USA). Rabbit TBLR1 polyclonal antibody (Abcam) was used for western blotting assay according to our previously published method [27].

2.8. Virus infection assay

To evaluate the effect of si-EcTBLR1 on virus infection, cells were infected with RGNNV at 24 h after siRNA transfection. At indicate time points (24 h and 48 h p.i.), cells were collected for RNA extraction and qPCR analysis as described in the following section. The transcription levels of RGNNV Cp and RdRp were detected using the primers CP-RT-F/CP-RT-R and RdRp-RT-F/RdRp-RT-R listed in Table 1.

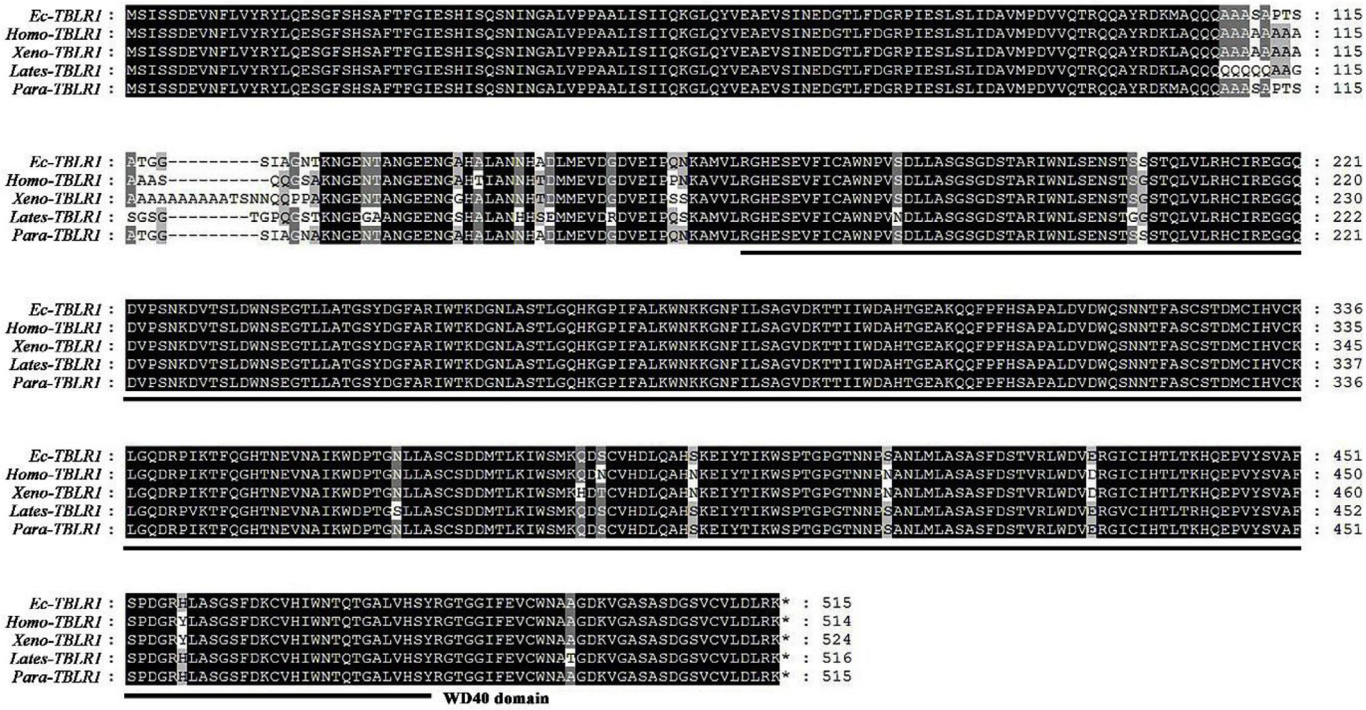

Fig. 1. Amino acid alignment of EcTBLR1 and its homologs from other species. The sequences of TBLR1 homologs used in this study were obtained from GeneBank. The characteristic and conservative WD40 domain of TBLR1 was underlined.

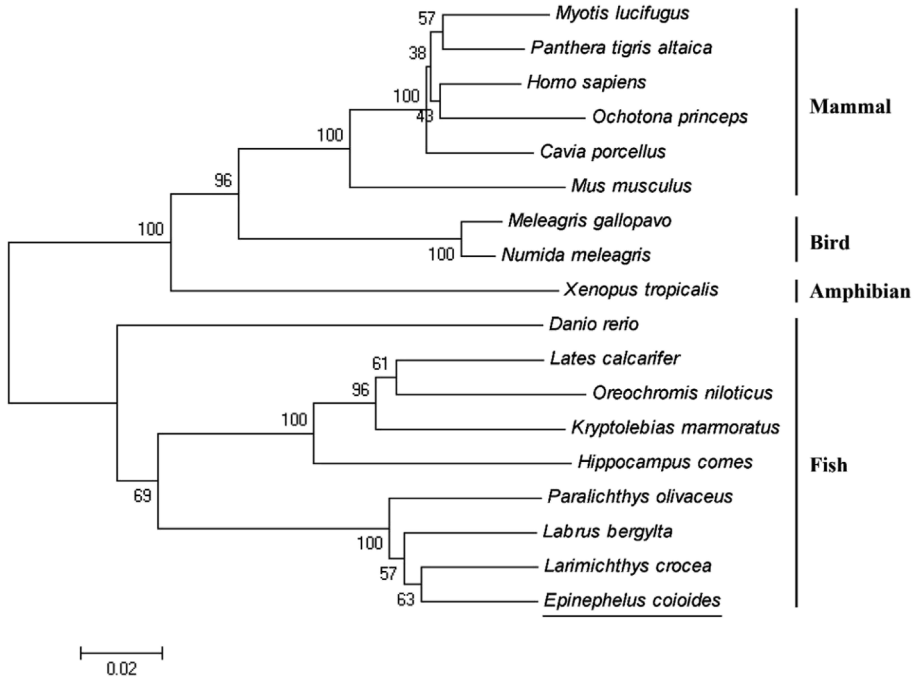

Fig. 2. Phylogenetic analysis of EcTBLR1. A neighbor-joining tree was constructed based on the amino acid sequences of TBLR1 homologs from different species. The bootstrap values were indicated at the branch points.

2.9. Luciferase activity

To assess effects of EcTBLR1 on IFN promoter activity, EcTBLR1 was co-transfected with IFN promoter-contained luciferase reporter plasmid, ranilla luciferase plasmid was co-transfected as internal reference. 24 h after transfection, Dual-Luciferase Reporter assay system (Promega, USA) was used to measure the luciferase activities according to the manufacturer's instructions.

2.10. qPCR analysis

To detect the transcript of viral or host genes in cells or tissues, qPCR was carried out in a Roche 480 Real Time Detection System (Roche, German). In brief, each assay was performed with the following cycling conditions: 95 °C for 5 min for activation, followed by 45 cycles at 95 °C for 5 s, 60 °C for 10 s and 72 °C for 15 s. All the primers were listed in Table 1. Samples were carried out in triplicates and the

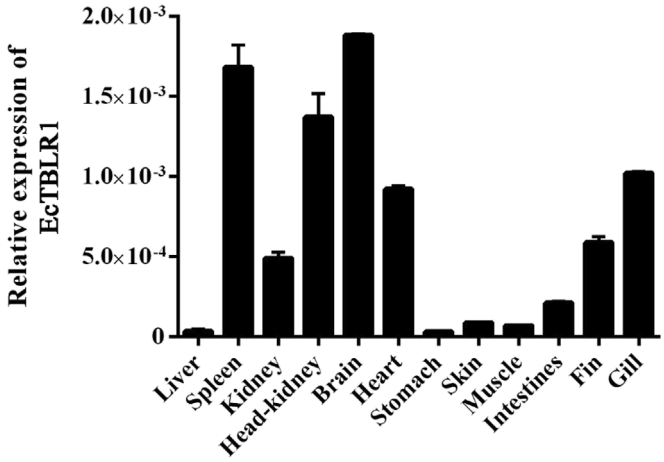

Fig. 3. The expression pattern of EcTBLR1 in different tissues from healthy grouper. The relative expression levels of EcTBLR1 were detected using qPCR.

expression level of target genes normalized to β-actin was calculated with the $2^{-\triangle\triangle CT}$ method.

2.11. Statistical analysis

Data were shown as mean ± SD (n ≥ 3), and the statistical differences between groups were analyzed using the Student's *t*-test. $p < 0.05$* was considered statistically significant.

3. Results

3.1. Sequence characterization of EcTBLR1

According to the EST sequences from grouper spleen transcriprome, we confirmed the full length of EcTBLR1 using PCR amplification and DNA sequencing. EcTBLR1 was composed of 1548 bp and encoded a 515-aa protein which shared 99% and 95% identity with its homologue from large yellow croaker (*Larimichthys crocea*) and human (*Homo sapiens*), respectively. Sequence alignment showed that EcTBLR1 contained the conserved WD40 domain at 166–480 amino acids (Fig. 1) which functioned as a site for protein-protein interaction or platform for the assembly of protein complexes [28,29]. Phylogenetic analysis also indicated that EcTBLR1 shared the closest relationship to large yellow croaker, followed by other fishes, amphibians, birds and mammals (Fig. 2).

3.2. Expression pattern of EcTBLR1 in different tissues

We also examined the mRNA expression level of EcTBLR1 in different tissues from healthy grouper. As shown in Fig. 3, EcTBLR1 was detected in the selected 12 tissues and was most abundant in the brain, followed by spleen, head-kidney, gill and other tissues.

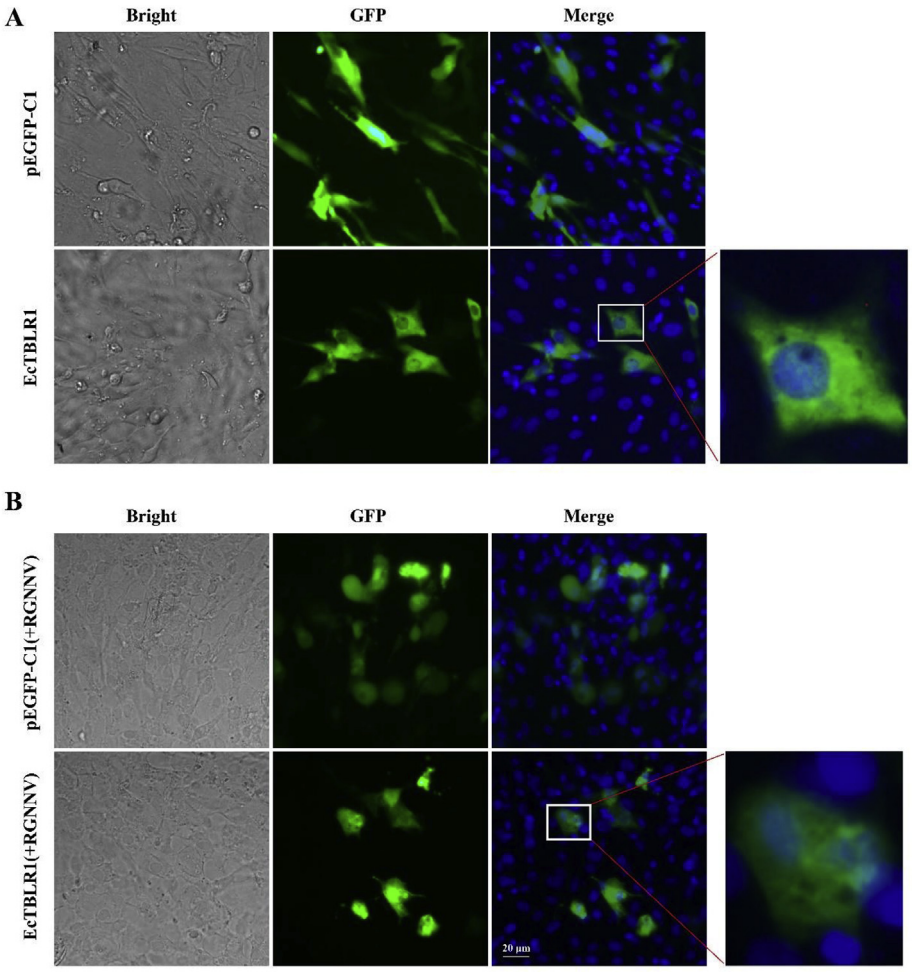

Fig. 4. Subcellular location of EcTBLR1 *in vitro*. (A) EcTBLR1 encoded a specific cytoplasmic protein. (B) RGNNV infection altered the location of EcTBLR1 in grouper cells. After transfection with pEGFP-C1 and pEGFP-EcTBLR1, GS cells were infected with RGNNV, and the fluorescence was observed under fluorescence microscope.

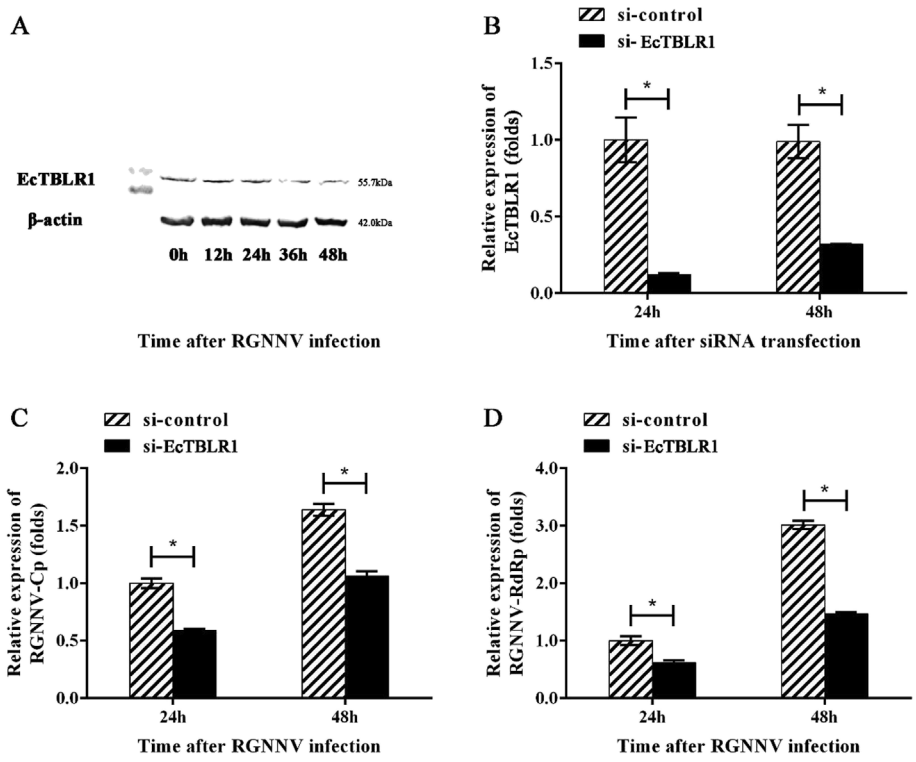

Fig. 5. The roles of EcTBLR1 during RGNNV infection. (A) RGNNV infection down-regulated the protein expression level of EcTBLR1. (B) The endogenous expression of EcTBLR1 was effectively decreased by siRNA interference. The fold changes in y-axis was calculated as the ratio of EcTBLR1 expression in EcTBLR1 overexpressing cells versus that in control vector transfected cells at indicate time points. Transcription levels of RGNNV-Cp (C) and RGNNV-RdRp (D) were decreased by siRNA interference of EcTBLR1. The expression level of viral genes normalized to β-actin were calculated with the $2^{-\triangle\triangle}$CT method. The fold changes in y-axis was calculated as the ratio of target gene (Cp or RdRp) expression in indicate time points versus that in mock-transfected cells at 24 h p.i.. Data were shown as mean ± SD, $^{*}p < 0.05$.

3.3. EcTBLR1 encoded a cytoplasmic protein

To determine the subcellular location of EcTBLR1 *in vitro*, pEGFP-C1, pEGFP-EcTBLR1 was transfected into GS cells, respectively. Under the fluorescence microscope, we found that the green fluorescence in pEGFP-EcTBLR1 transfected cells was mainly distributed in the cytoplasm but not nucleus in GS cells (Fig. 4A). In pEGFP-C1 transfected cells, fluorescence was observed in both cytoplasm and nucleus. Thus, we speculated that EcTBLR1 encoded a cytoplasmic protein.

3.4. RGNNV infection altered the localization of EcTBLR1 and its protein synthesis

To dissect whether EcTBLR1 was involved in fish virus infection, we firstly detected the effect of RGNNV infection on EcTBLR1 localization in grouper cells. As shown in Fig. 4B, the distribution of green fluorescence was obviously altered in RGNNV-infected EcTBLR1-transfected cells. In detail, the fluorescence was observed both in the nucleus and cytoplasm after RGNNV infection. Moreover, we also assessed the expression profile of the endogenous EcTBLR1 during RGNNV infection. Western blotting results showed that the protein expression level of EcTBLR1 was gradually down-regulated with the infection time increased (Fig. 5A). Thus, we proposed that RGNNV infection was able to alter the localization of EcTBLR1 and regulate its protein synthesis.

3.5. EcTBLR1 knock-down inhibited virus infection in vitro

To assess the effect of EcTBLR1 on RGNNV infection *in vitro*, specific siRNA was designed to decrease the endogenous expression of EcTBLR1. As shown in Fig. 5B, transcription of EcTBLR1 was significantly reduced up to ~12% and ~32% at 24 h and 48 h after si-EcTBLR1 transfection compared with the siRNA control. Correspondingly, the transcription of Cp gene was decreased up to ~59% and 65% at 24 h and 48 h p.i., respectively (Fig. 5C). Consistently, the transcription of RdRp gene was decreased up to ~62% and ~49% at 24 h and 48 h p.i., respectively (Fig. 5D). Together, our results demonstrated that knock-down of EcTBLR1 significantly inhibited RGNNV infection in grouper cells.

3.6. Overexpression of EcTBLR1 negatively regulated host interferon response

To explore the potential mechanism underlying the effect of EcTBLR1 on host immune-related signal pathway, we detected the effect of EcTBLR1 overexpression on interferon response. Firstly, we evaluated the IFN promoter activity in EcTBLR1 overexpressing cells. As shown in Fig. 6A, the transcription of EcTBLR1 was significantly increased in EcTBLR1 overexpressing cells compared to the control vector transfected cells. The ectopic expression of EcTBLR1 in grouper cells inhibited IFN promoter activity to ~40% compared to control vector transfected cells (Fig. 6B). After RGNNV infection, the inhibitory effect of EcTBLR1 overexpression on IFN promoter activity was almost distorted (Fig. 6C). Furthermore, we also found that EcTBLR1 overexpression significantly inhibited the transcription of ISG15 (up to 16%) and IRF3 (up to 24%) compared to control cells (Fig. 6D and E). Thus, we proposed that overexpression of EcTBLR1 negatively regulated host interferon response.

4. Discussion

Although great progress has been made in understanding the roles of TBLR1 in cancer, few literatures focused on the effect of TBLK1 on innate immune response, especially during virus infection. In this work, we identified a novel TBLR1 homologue, EcTBLR1 from cultured grouper *Epinephelus coioides*, and explored its regulatory role in interferon immune response during RGNNV infection. We found that EcTBLR1 shared highly sequence similarity with TBLR1 from other species, suggesting that the ability of TBLR1 on transcriptional activation might be highly conserved during evolution [30,31]. In un-stimulated GS cells, EcTBLR1 was observed to be mainly in cytoplasm, which was in accordance with previous report that human TBLR1 was primarily cytoplasmic without physiological stimulation [32]. While when RGNNV infection was proceeded, we found the migration of EcTBLR1 from cytoplasm to nucleus. Moreover, RGNNV infection

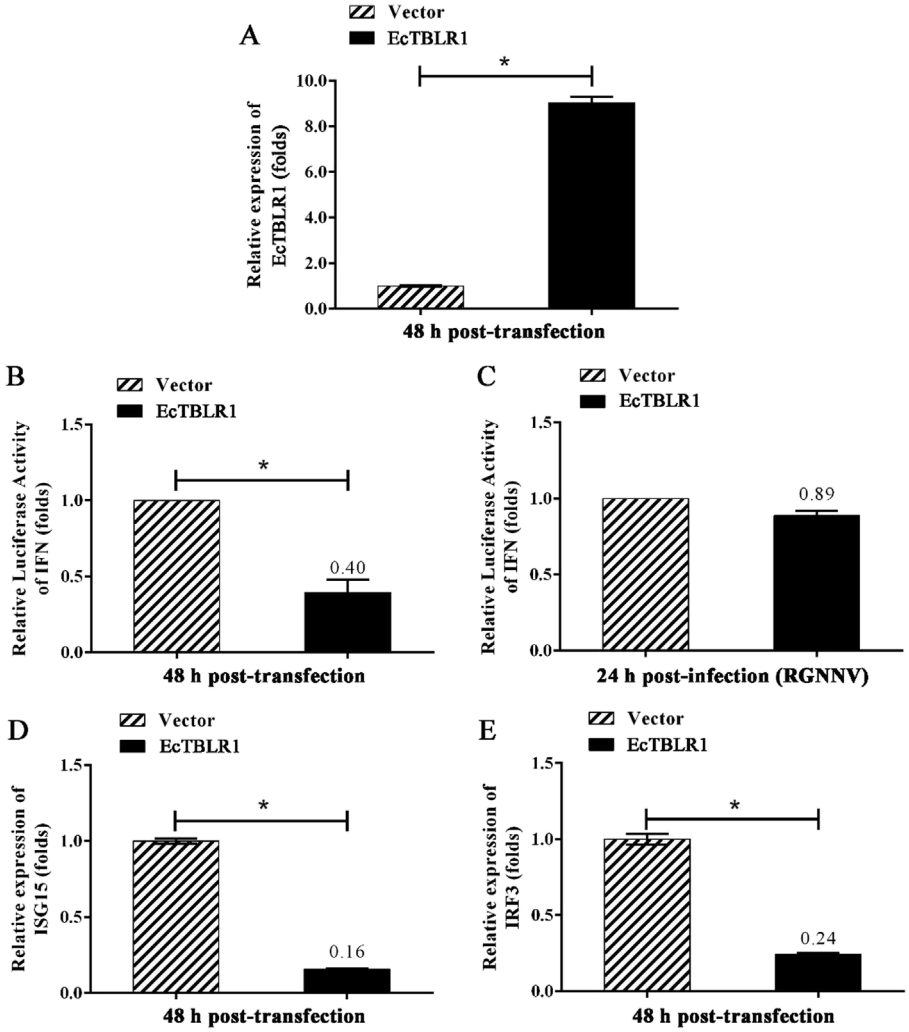

Fig. 6. Overexpression of EcTBLR1 suppressed host interferon immune response. (A) The expression levels of EcTBLR1 in EcTBLR1 overexpressing cells. (B) IFN promoter activity was significantly inhibited by ectopic expression of EcTBLR1. (C) RGNNV infection distorted the inhibitory effect of EcTBLR1 overexpression on IFN promoter activity. Overexpression of EcTBLR1 also significantly inhibited the transcriptional levels of ISG15 (D) and IRF3 (E). Data were shown as mean ± SD, $^{*}p < 0.05$.

dramatically suppressed the protein expression level of EcTBLR1 *in vitro*. Thus, we speculated that EcTBLR1 might be involved in RGNNV infection via its translocation to nucleus. Interestingly, in EcTBLR1 known-down cells, RGNNV replication was remarkably inhibited, demonstrated by reduced transcriptional levels of viral Cp and RdRp genes. Together, our results suggested that EcTBLR1 served as a pro-viral factor during RGNNV infection.

To further explore the potential mechanism underlying the action of EcTBLR1 on RGNNV replication, we evaluated the regulatory roles of EcTBLR1 on interferon immune response, which was crucial for RGNNV infection [24,33]. Our data showed that overexpression of EcTBLR1 not only significantly suppressed IFN promoter activity, but also reduced the transcriptional levels of IFN-related downstream effectors ISG15 and IRF3. These two effectors have been proved to be critical antiviral factors during RGNNV infection [23,24]. In addition, the inhibitory effect of EcTBLR1 on interferon promoter activity was significantly abolished during RGNNV infection. Thus, we speculated that RGNNV infection decreased the expression level of EcTBLR1 *in vitro*, and EcTBLR1 might act as a pro-viral factor via negative regulation on the interferon immune response.

In summary, we cloned a novel fish TBLR1 and characterized its roles during virus infection in this study. Our results showed that EcTBLR1 mainly located in cytoplasm in un-stimulated cells, and its distribution was obviously altered during RGNNV infection. Moreover, the expression level of EcTBLR1 was significantly decreased during RGNNV infection, and knock-down of EcTBLR1 inhibited viral genes transcription. In addition, overexpression of EcTBLR1 *in vitro* attenuated interferon immune response. Taken together, EcTBLR1 was proposed to negatively regulate host interferon immune and promote viral replication *in vitro*. Our results will contribute greatly to understanding the roles of fish TBLR1 in innate immune response against virus infection.

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