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Grouper Atg12 negatively regulates the antiviral immune response against Singapore grouper iridovirus (SGIV) infection

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ABSTRACT

Autophagy is an evolutionarily conserved, multi-step lysosomal degradation process used to maintain cell survival and homeostasis. A series of autophagy-related genes (Atgs) are involved in the autophagic pathway. In mammals, a growing number of studies have attributed functions to some Atgs that are distinct from their classical role in autophagosome biogenesis, such as resistance to pathogens. However, little is known about the functions of fish Atgs. In this study, we cloned and characterized an *atg12* homolog from orange spotted grouper (*Epinephelus coioides*) (*Ecatg12*). *Ecatg12* encodes a 117 amino acid protein that shares 94.0% and 76.8% identity with gourami (*Anabas_testudineus*) and humans (*Homo sapiens*), respectively. The transcription level of *Ecatg12* was lower in cells infected with Singapore grouper iridovirus (SGIV) than in non-infected cells. Fluorescence microscopy revealed that EcAtg12 localized in the cytoplasm and nucleus in grouper spleen cells. Overexpression of EcAtg12 significantly increased the replication of SGIV, as evidenced by increased severity of the cytopathic effect, transcription levels of viral genes, levels of viral proteins, and progeny virus yield. Further studies showed that EcAtg12 overexpression decreased the expression levels of interferon (IFN) related molecules and pro-inflammatory factors and inhibited the promoter activity of IFN-3, interferon-stimulated response element, and nuclear factor-κB. Together, these results demonstrate that EcAtg12 plays crucial roles in SGIV replication by downregulating antiviral immune responses.

1. Introduction

Autophagy is an evolutionarily conserved lysosomal degradation process used to maintain normal intracellular homeostasis. The degraded cytoplasmic components include bulk cytosol, protein aggregates, damaged or superfluous organelles, and invading microbes [1]. The process is tightly regulated by a series of autophagy-related genes (Atgs). Several studies have reported that autophagy and Atgs participate in the immune response to virus infection [2–6]. For example, Atg6 and Atg8 were found to facilitate replication of spring viraemia of carp virus in endothelial progenitor cells [7]. In addition to their autophagy-dependent role, Atgs may also mediate autophagy-independent functions [8]. As a core protein in autophagy, Atg12 is known to play an important role in the formation of autophagosome membrane coupled with Atg5 to form an Atg12-Atg5 conjugate [9]. Interestingly, the unconjugated form of Atg12 can bind and inactivate Bcl2 and directly promote mitochondrial apoptosis [10]. In addition, Atg12 plays a role in microbial infection. For example, researchers studying the vesicular stomatitis virus (VSV) found that Atg12 in association with Atg5 negatively regulated the innate antiviral immune response by impairing the type I interferon (IFN) production pathway [11]. Atg12 also is required for translation of incoming hepatitis C virus (HCV) RNA and for initiation of HCV replication [12].

Singapore grouper iridovirus (SGIV) belongs to the genus *Ranavirus* of the family *Iridoviridae* [13,14]. Fish infected with SGIV have an enlarged spleen as well as hemorrhage and multifocal areas of splenic degeneration [15]. This pathogen usually causes serious systemic diseases, with more than 90% mortality in groupers and seabass [14,16]. Understanding the host response to SGIV might help researchers develop novel means to fight this viral infection. The establishment of susceptible cell lines has promoted research on SGIV multiplication and host immunity [17]. Although multiple genes related to the type I IFN

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and the apoptotic signaling pathway have been identified in groupers and their effects on viral infection have been elucidated [18–26], the role of Atgs in SGIV replication has not been reported.

In the current study, a new *atg12* from orange spotted grouper (*Epinephelus coioides*) (*Ecatg12*) was cloned and characterized. Subcellular localization was observed and the roles of EcAtg12 on SGIV replication were evaluated. In addition, the effects of EcAtg12 on host IFN and the inflammatory response were investigated. Results of this study provide new insights about the role of fish Atg12 during virus infection.

2. Materials and methods

2.1. Fish, cells, and virus

Juvenile orange-spotted grouper (weight 30–40 g) were purchased from Qionghai Marine Fish Farm, Hainan Province, China. Fish were maintained in a laboratory recirculating seawater system at 24–28 °C and fed twice daily for 2 weeks. Cell lines of grouper spleen (GS) was established in our lab [17] and propagated at 28 °C in Leibovitz's L-15 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA). The SGIV (strain A3/12/98) used in this study was originally isolated from diseased grouper (*Epinephelus tauvina*) and the propagation was performed as described previously [13,14]. For SGIV infection, cells were infected with SGIV at a multiplicity of infection (MOI) of 2 in the following experiment.

2.2. Cloning of Ecatg12 and sequence analysis

The *Ecatg12* ORF was amplified by PCR using primers designed based on *Ecatg12* EST from grouper spleen transcriptome [27]. The primers are listed in Table 1. The sequence of EcAtg12 was analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast). Amino acid sequence alignment was carried out using ClustalX1.83 software, and results were edited using the GeneDoc program. A Neighbor-joining (NJ) phylogenetic tree was constructed using MEGA 5.0 software.

2.3. RT-qPCR analysis for the expression patterns of Ecatg12 in grouper

To determine the tissue distribution pattern of *Ecatg12* expression in asymptomatic orange-spotted grouper, total RNA from 12 tissues (spleen, brain, head kidney, kidney, liver, gill, heart, muscle, intestine, fin, skin, and stomach) from three asymptomatic groupers was extracted using the SV Total RNA Isolation Kit (Promega, USA), and total RNA (500 ng) was reverse transcribed to synthesize the first-strand cDNA using the ReverTra Ace kit (Toyobo, Japan). To examine the expression level of *Ecatg12* in response to viral infection, GS cells were infected with SGIV. At indicated time points post infection (h p.i.), GS cells from 3 wells of 24-well plates were collected for RNA extraction. The relative expression level of *Ecatg12* was determined by quantitative real-time PCR (qPCR) according to the manufacturer's instructions. In briefly, it was performed using SYBR® Green Realtime PCR Master Mix (Toyobo, Japan) in an Applied Biosystems QuantStudio 5 Real Time Detection System (Thermo Fisher, USA). The primers for *Ecatg12* (listed in Table 2) was designed in this study according to the obtained sequence. 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 target genes were calculated using the $2^{-\triangle\triangle CT}$ method with *β-actin* or *18S* as internal reference genes. For gene expression normalization, the samples at 0 h was used as the calibrator group. The data are represented as mean ± standard error of the mean (SEM).

Table 1
Primers used for *Ecatg12* cloning and plasmid construction.

Name	Sequence (5′–3′)	Usage
Ecatg12-ORF-F	ATGTCTGACAATGCAGAG	*Ecatg12* cloning
Ecatg12-ORF-R	ACCCCAGGCTTGAGATTTA	
pEGFP-*Ecatg12*-F	GGGGTACCATGTCTGACAATGCAGAG	pEGFP-C1 cloning
pEGFP-*Ecatg12*-R	CGGGATCCACCCCAGGCTTGAGATTTA	
pHA-*Ecatg12*-F	GGGGTACCATGTCTGACAATGCAGAG	pcDNA3.1-3 × HA cloning
pHA-*Ecatg12*-R	CGGAATTCTTTAACCCCAGGCTTGAG	

Table 2
Primers used for host genes expression analysis.

Name	Sequence (5′–3′)	References
Ecatg12-RT-F	CGACTCAGCGACAAACGATG	Designed in this study
Ecatg12-RT-R	GAGATGAACTGCGACAGGGAT	
β-actin-RT-F	TACGAGCTGCCTGACGGACA	[19]
β-actin-RT-R	GGCTGTGATCTCCTTCTGCA	
EcIRF3-RT-F	ATGGTTTAGATGTGGGGGTGTCGGG	[31]
EcIRF3-RT-R	GAGGCAGAAGAACAGGGAGCACGGA	
EcIRF7-RT-F	CAACACCGGATACAACCAAG	[29]
EcIRF7-RT-R	GTTCTCAACTGCTACATAGGGC	
EcISG15-RT-F	CCTATGACATCAAAGCTGACGAGAC	[30]
EcISG15-RT-R	GTGCTGTTGGCAGTGACGTTGTAGT	
EcMDA5-RT-F	ACCTGGCTCTCAGAATTACGAACA	[19]
EcMDA5-RT-R	TCTGCTCCTGGTGGTATTCGTTC	
EcMXI-RT-F	CGAAAGTACCGTGGACGAGAA	[23]
EcMXI-RT-R	TGTTTGATCTGCTCCTTGACCAT	
EcLGP2-RT-F	TGGTGGTACGCTATGGACTGC	[32]
EcLGP2-RT-R	TTGTAGCTCAGTTATCTTTGTGCGA	
EcTNFα -RT-F	GTGTCCTGCTGTTTGCTTGGTA	[23]
EcTNFα-RT-R	CAGTGTCCGACTTGATTAGTGCTT	
EcIL-1β-RT-F	AACCTCATCATCGCCACACA	[23]
EcIL-1β-RT-R	AGTTGCCTCACAACCGAACAC	
EcIL8-RT-F	GCCGTCAGTGAAGGGAGTCTAG	[19]
EcIL8-RT-R	ATCGCAGTGGGAGTTTGCA	
18S-RT-F	CCTGAGAAACGGCTACCACATCC	[28]
18S-RT-R	AGCAACTTTAGTATACGCTATTGGAG	

2.4. Plasmid construction and cell transfection

The full length of *Ecatg12* (amino acids (aa) 1–117) was cloned into pEGFP-C1 (Clontech, USA) to illustrate the subcellular localization of EcAtg12 in *vitro*. Meanwhile, *Ecatg12* was also cloned into pcDNA3.1-3 × HA (Waryong, China) to assess the effect of EcAtg12 on SGIV infection, and to determine the relative expression level of selected immune-related genes. The primers were listed in Table 1. The recombinant plasmid (named pEGFP-EcAtg12 or pHA-EcAtg12, respectively) was subsequently confirmed by DNA sequencing.

Cell transfection was carried out using Lipofectamine 2000 reagent (Invitrogen, USA). Briefly, GS cells were seeded in 24-well plates or 6-well plates at 70–80% confluence, then plasmid (800 ng recombinant EcAtg12 or the empty plasmid for each of 24-well) and 2 μl of Lipofectamine 2000 were, respectively, diluted in 50 μl of serum-free Opti-MEM (Gibco, USA) in two separate sterile tubes. The Lipofectamine 2000 and diluted plasmid were mixed gently and thoroughly after incubation for 5 min at room temperature. The mixture was further incubated for 25 min at room temperature before being added drop wise to the cells. After 6 h, fresh normal medium was used to replace the incubation medium.

2.5. Fluorescent microscopy

To illustrate the subcellular localization of EcAtg12, pEGFP-C1 or C1-EcAtg12 was transfected into GS cells as described above, respectively. Cells were fixed with 4% paraformaldehyde and stained with 4,6-diamidino-2-phenylindole (DAPI) at 24 h post-transfection. Fluorescence was observed under a fluorescence microscope.

Table 3
Primers used for viral genes expression analysis.

Name	Sequence (5′–3′)	References
MCP-RT-F	GCACGCTTCTCTCACCTTCA	[19]
MCP-RT-R	AACGGCAACGGGAGCACTA	
ICP18-RT-F	ATCGGATCTACGTGGTTGG	[26]
ICP18-RT-R	CCGTCGTCGGTGTCTATTC	
VP19-RT-F	TCCAAGGGAGAAACTGTAAG	[19]
VP19-RT-R	GGGGTAAGCGTGAAGACT	
LITAF-RT-F	GATGCTGCCGTGTGAACTG	[26]
LITAF-RT-R	GCACATCCTTGGTGGTGTTG	

2.6. Virus infection assay and sample collection

To evaluate the effects of EcAtg12 on virus infection, GS cells overexpressing 3×HA tag or the recombinant protein pHA-EcAtg12 were infected with SGIV at 24 h post-transfection. At indicated time points, the cytopathic effect (CPE) was observed under a phase contrast microscope. Meanwhile, cells from 3 wells of 24-well plates were harvested for RNA extraction and RT-qPCR detection, or Western blot analysis.

2.7. RT-qPCR analysis for assessing the relative expression level of host genes and viral genes

To examine the transcriptional expression level of host or virus genes, RT-qPCR was performed as described above. The primers for the transcription of host interferon signaling molecules (*IRF3, IRF7,ISG15, MDA5, MXI, MyD88*), proinflammatory factors(*TNFα, IL-1β, IL-8*) and viral genes (*MCP, ICP18, VP19, LITAF*) were designed previously [19,23,26,28–32]. The primers were listed in Tables 2 and 3, respectively. The expression levels of target genes were calculated using the $2^{-\triangle\triangle CT}$ method with *β-actin* or *18S* as internal reference genes. For gene expression normalization, the samples transfected empty vector were used as the calibrator groups. The data are represented as mean ± standard error of the mean (SEM).

2.8. Western blot analysis

Cells were collected and lysed in radioimmunoprecipitation assay buffer. Proteins were separated by 10% SDS-PAGE and transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore, USA). Mouse monoclonal anti-HA antibody (1:1000 dilution) was purchased from Sigma (USA). Mouse monoclonal anti-β-actin antibody (1:1000) was purchased from Proteintech (USA). The polyclonal anti-MCP antibody (1:1000) of SGIV was prepared in our lab. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:5000) or anti-mouse (1:5000) antibodies were purchased from KPL (USA). Immunoreactive proteins were visualized using an Enhanced HRP-DAB Chromogenic Substrate Kit (Tiangen, China). Band intensity was calculated using Quantity-one software and ratios of MCP/β-actin were assessed as described previously [32].

2.9. TCID50 assay for evaluating virus titration

The supernatant and GS cells were harvested at indicated time points post infection. Virus titers were measured based on the median tissue culture infectious dose ($TCID_{50}$). GS cells were inoculated with the diluted samples and further incubated for 7 d. The viral titer was calculated using the Karber method. The statistical significances were determined with Student's t-test. The significance level was defined as $p < 0.05$ (*)

2.10. Dual-luciferase reporter assays

To examine the effects of EcAtg12 on the activity of IFN and the nuclear factor-κB (NF-κB) promoter, luciferase plasmids, including zebrafish IFN-3-Luc [33], human ISRE-Luc and NF-κB-Luc (Promega, USA), were used. In brief, GS cells were co-transfected with 200 ng of ISRE-Luc or IFN-3-Luc or NF-κB-Luc and 600 ng of pcDNA3.1-3×HA or HA-EcAtg12, respectively. A total of 50 ng of pRL-SV40 Renilla luciferase vector (Promega, USA) was used as an internal control. Cells then were harvested to measure the luciferase activities using the Dual-Luciferase® Reporter Assay System (Promega, USA) at 48 h according to the manufacturer's instructions.

2.11. Statistical analysis

Statistical analyses were carried out using SPSS version 17.0. One-way analysis of variance (ANOVA) was used to evaluate the differences in relative expression of host or viral genes, as well as the relative promoter activity of reporter genes. Student's t-test was used to determine significant differences in virus titration. The differences was considered to be statistically significant at $p < 0.05$(*).

2.12. Ethics statement

All animal-involving experiments of this study were approved by the Animal Care and Use Committee of College of Marine Sciences, South China Agricultural University, and all efforts were made to minimize suffering.

2.13. Key resources table

Resource	Source	Identifier
CellLine		
grouper spleen		
GS		
GS cells		
ProteinPeptide		
IFN		

3. Results

3.1. Sequence characterization of Ecatg12

The full-length open reading frame of *Ecatg12* (accession no.MK932876) was obtained using PCR amplification. Sequence analysis indicated that *Ecatg12* encoded a 117 aa protein that shared 94.0% identity with gourami (*Anabas testudineus*, XP_026229172.1), 94.0% identity with *Oreochromis niloticus* (XP_025767264.1), 93.2% identity with *Maylandia zebra* (XP_004550782.1), 92.3% identity with *Oryzias latipes* (XP_004077995.1), 92.3% identity with *Seriola dumerili* (XP_022612454.1), 90.6% identity with *Amphiprion ocellaris* (XP_023117829.1), 88.9% identity with *Cynoglossus semilaevis* (XP_008320831.1), 78.3% identity with *Danio rerio* (NP_001233129.1), 67.5% identity with *Xenopus tropicalis* (XP_002940007.1), 77.8% identity with *Gallus gallus* (XP_424963.1), 76.8% identity with *Mus pahari* (XP_021070549.1), 76.8% identity with *Homo sapiens* (NP_004698.3), respectively. Amino acid alignment indicated that *Ecatg12* contained the APG12 conserved domain (aa 31–117) (Fig. 1A). Phylogenetic analysis indicated that EcAtg12 and other Atg12 from different fish species were clustered into one group which was separated from the amphibians, birds, and mammals in sequence (Fig. 1B).

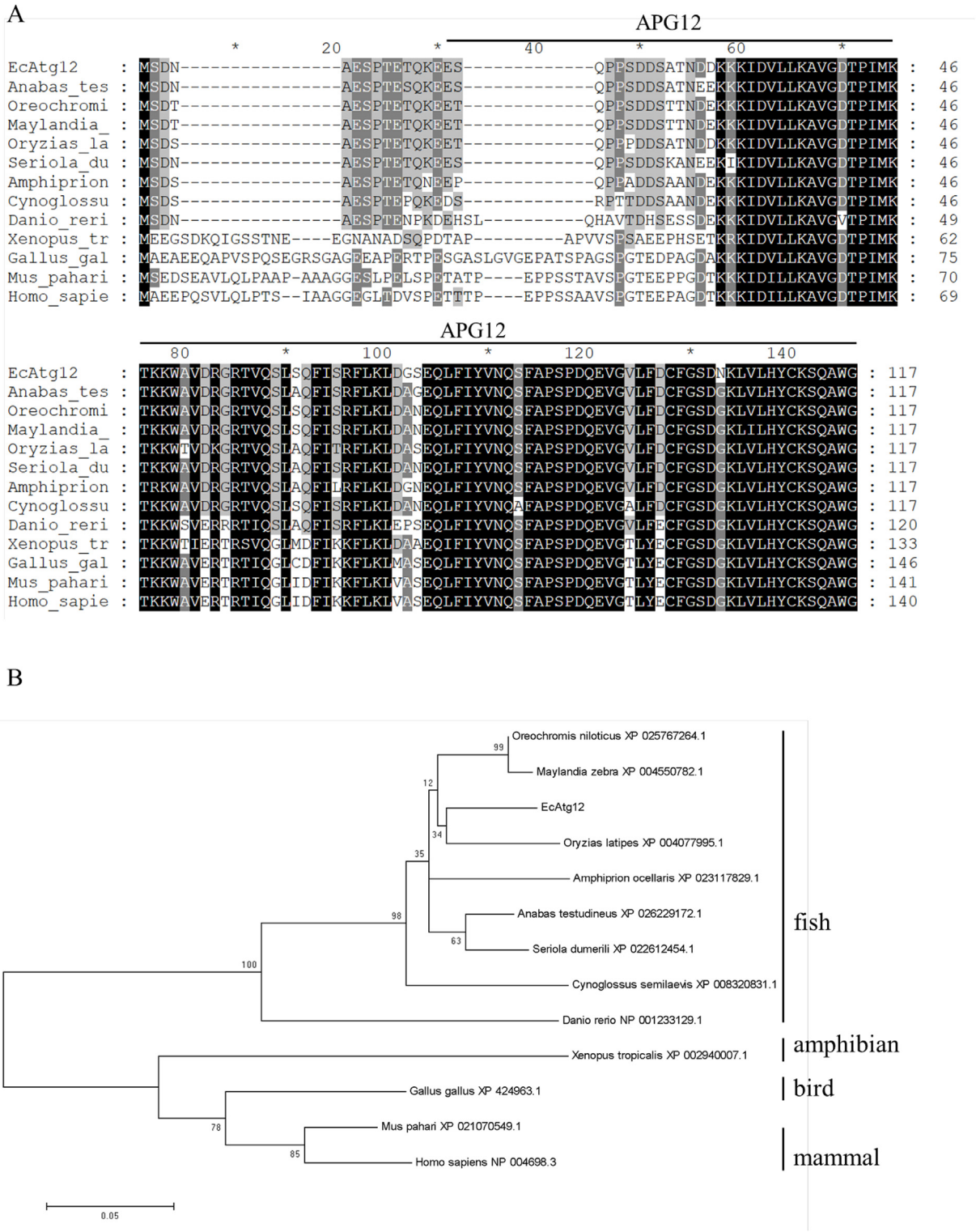

Fig. 1. (A) Multiple sequence alignments of *Ecatg12* amino acid sequences with other Atg12 proteins. The putative APG12 conserved domain is indicated. (B) The phylogenetic tree of the Atg12 proteins. The GenBank accession number for each species is listed to the right of the species name. Numbers at the nodes denote the bootstrap values of 1000 replicates. The scale bar represents 0.05 change per site.

3.2. Tissue distribution and expression patterns of Ecatg12

The *Ecatg12* expression in different tissues from asymptomatic grouper were detected by RT-qPCR with *β-actin* or *18S* as internal reference genes. As shown in Fig. 2, *Ecatg12* was detected in all tissues, with the predominant expression in brain, spleen, and gill. Upon challenge with SGIV infection, the transcription levels of *Ecatg12* in GS cells decreased compared to non-infected (0 h) cells(Fig. 3).

3.3. Subcellular localization of EcAtg12

To demonstrate the subcellular localization of EcAtg12, pEGFP-

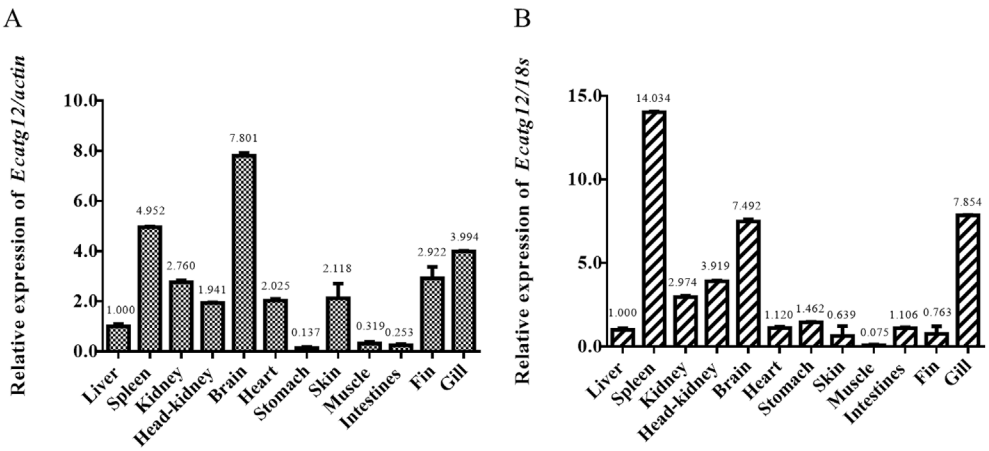

Fig. 2. Expression levels of *Ecatg12* in different tissues from asymptomatic groupers. *β-actin* (A) and *18S* (B) were used as the internal control for the normalization across tissues.

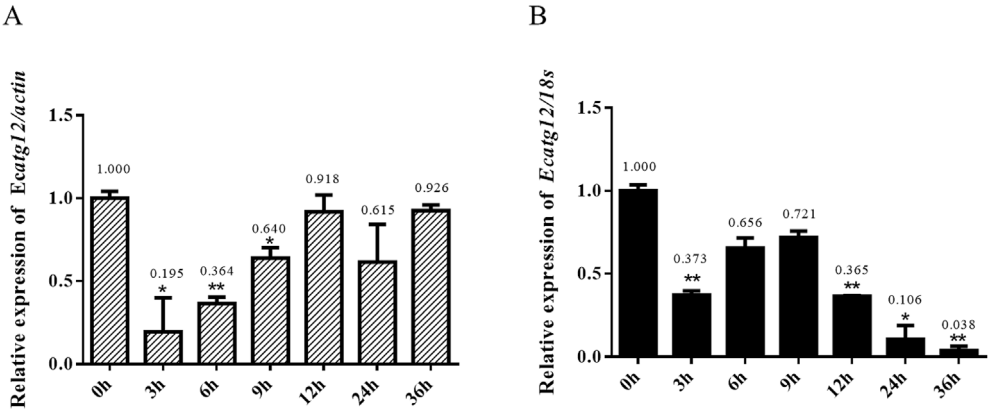

Fig. 3. Expression levels of *Ecatg12* in GS cells at different time points post SGIV stimulation. The samples at 0 h was used as the calibrator group. *β-actin* (A) and *18S* (B) were used as the internal control. Asterisk denotes a significant difference (*p < 0.05, **p < 0.01).

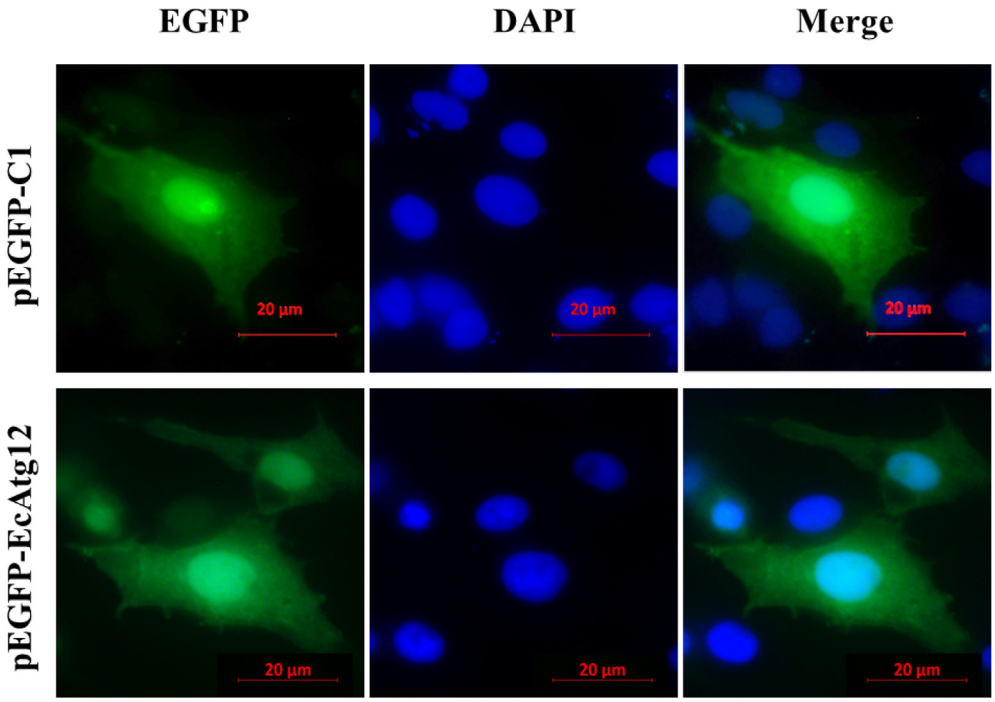

Fig. 4. Subcellular localization of EcAtg12 in grouper cells. GS cells were transfected with the plasmids of pEGFP-C1 and pEGFP-EcAtg12 and then stained with DAPI. Samples were observed under fluorescence microscopy.

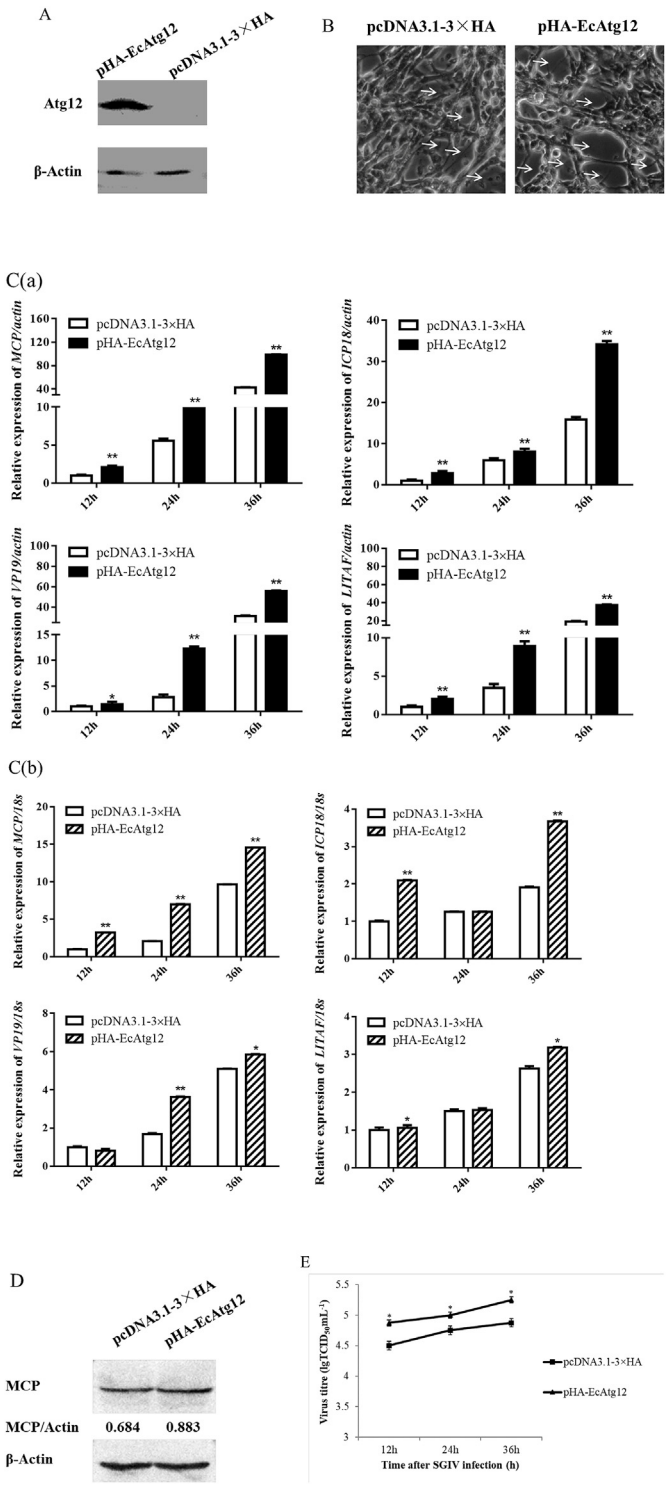

Fig. 5. Effect of *Ecatg12* overexpression on virus replication. (A) Detection of recombinant HA-Atg12 protein in pcDNA3.1-3×HA-EcAtg12-transfected GS cells by Western blot. (B) CPE progression induced by SGIV infection in EcAtg12 overexpressing cells. The white arrows indicated round and aggregated cells evoked by SGIV infection. (C) Expression levels of viral genes in transfected GS cells. Asterisk denotes a significant difference (**$p < 0.01$). (D) Detection of SGIV-MCP protein in transfected cells by Western blot. β-actin was used as the internal control. Band intensity was calculated using Quantity-one software and ratios of MCP/β-actin were assessed. (F) Progeny virus production of SGIV after transfection with EcAtg12. Viral titers were measured as $TCID_{50}$ and calculated using the Karber method (*$p < 0.05$).

EcAtg12 was transfected into grouper cells, and the fluorescence was observed under a fluorescence microscope. Fluorescent imaging of the pEGFP-EcAtg12 fusion protein showed that the green fluorescence was distributed in both the cytoplasm and nucleus in GS cells, which was similar to the green fluorescence in pEGFP-C1 transfected cells (Fig. 4).

3.4. EcAtg12 overexpression increased SGIV replication

We investigated the effects of EcAtg12 on SGIV infection from different levels, including the CPE progression, the viral gene transcription, the MCP protein synthesis, and viral yield. Firstly, the recombinant plasmid successfully expressed Atg12 protein after being transfected into GS cells (Fig. 5A). EcAtg12-transfected cells were infected with SGIV. The extension of CPE produced by SGIV infection at 24 h p.i. was significantly higher in EcAtg12-overexpressing cells compared to control cells (Fig. 5B). Furthermore, quantitative analysis indicated that the transcription levels of SGIV genes (*MCP*, *ICP18*, *VP19*, and *LITAF*) in EcAtg12 overexpressing cells were significantly higher ($p < 0.01$) at 12, 24, and 36 h p.i. (Fig. 5C). Consistently, the MCP protein level was increased relative to the control in EcAtg12-overexpressing cells (Fig. 5D). Finally, we explored the function of overexpressed EcAtg12 in the generation of progeny virus using the $TCID_{50}$ assay. A high level of EcAtg12 promoted increased viral yields (Fig. 5E, $p < 0.05$). These results indicate that EcAtg12 promotes virus replication in GS cells.

3.5. EcAtg12 overexpression negatively regulated the IFN immune response and the inflammatory response

To explore the potential mechanism by which EcAtg12 promotes SGIV replication, we evaluated the regulatory effect of EcAtg12 overexpression on host immune factors. Expression levels of IFN-related molecules, including *IRF3*, *IRF7*, *MDA5*, *ISG15*, *MyD88*, and *MXI*, were significantly decreased in EcAtg12-overexpressing cells compared with control vector-transfected cells (Fig. 6). Expression levels of cytokines, including interleukin -1β (*IL-1β*), *IL-8*, and tumor necrosis factor alpha (*TNFα*), were also significantly decreased in EcAtg12-overexpressing cells (Fig. 7). Thus, ectopic expression of EcAtg12 in *vitro* negatively regulated the IFN and inflammatory responses.

3.6. EcAtg12 overexpression suppressed the NF-κB response promoter and IFN and ISRE promoter activities

Using the dual-luciferase reporter assay system, we evaluated the promoter activity of IFN, ISRE, and NF-κB in EcAtg12-overexpressed cells. Overexpression of EcAtg12 significantly increased the luciferase activity of IFN, ISRE, and NF-κB promoters (Fig. 8). Thus, EcAtg12 decreased IFN and NF-κB promoter activities and regulated gene transcription.

4. Discussion

Autophagy, as a cell steward, aims to eliminate useless or harmful substrates to maintain cell homeostasis [34]. It is orchestrated by a series of specific autophagy proteins that mediate the formation and elongation of a double-membrane structure, cargo engulfment, phagophore closure, and autophagosome fusion with the lysosome [35–37]. Conjugation of Atg12 to Atg5 is essential for Atg8 lipidation and autophagosome formation. Additionally, Atg12 plays Atg5-independent roles in diverse processes such as mitochondrial fusion and mitochondrial-dependent apoptosis [38]. It also has been implicated in the IFN immune response, inflammation response, and virus replication [8]. However, little was known about the function of fish Atg12. Here, we cloned an *atg12* homolog from orange-spotted grouper (*Ecatg12*). Sequence analysis showed that *Ecag12* encodes a 117 aa protein and shares the highest identity with gourami. Amino acid alignment

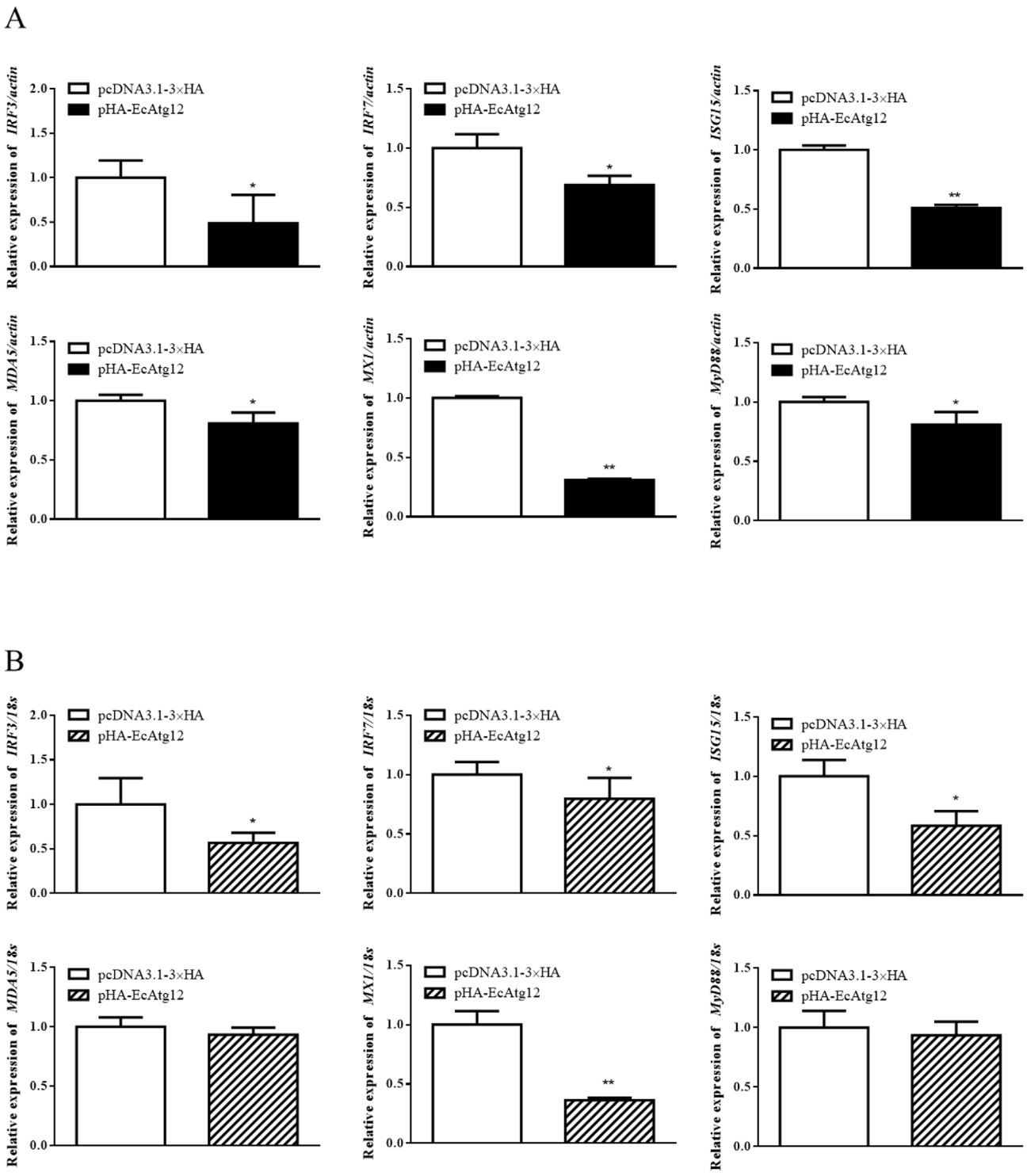

Fig. 6. The relative expression levels of IFN-signaling molecules in EcAtg12 overexpressing cells, Asterisk denotes a significant difference (*$p < 0.05$, **$p < 0.01$).

analysis indicated that EcAtg12 contains a APG12 conserved domain.

As a preliminary step to unravel the physiological role of EcAtg12, the mRNA tissue distribution was determined. The results indicated that the mRNA expression of *Ecatg12* was ubiquitous within all the tested tissues, which suggested that autophagy was implicated in many metabolic pathways among the tissues. It is reported that autophagy can be induced by a variety of factors, including starvation, reactive oxygen species, endoplasmic reticulum stress, microbial invasion and so on [39]. Based on this, we detected the expression of *Ecatg12* in GS cells under the stimulation of SGIV. The expression level of *Ecatg12* significantly decreased after SGIV infection. While, whether autophagy was inhibited after SGIV infection need further study.

Similar to localization of human Atg12 [40], EcAtg12 was found in both the cytoplasm and nucleus of GS cells; this finding suggests that Atg12 in fish and mammals might exert its physiological function in the same cellular compartments. Studies of mammals suggested that Atg5-Atg12 promotes viral replication by negatively regulating the IFN response [11]. In the reported study, we also found that EcAtg5 contributes to SGIV replication by inhibition of the host antiviral responses [32]. Here, we investigated the impact of EcAtg12 overexpression on

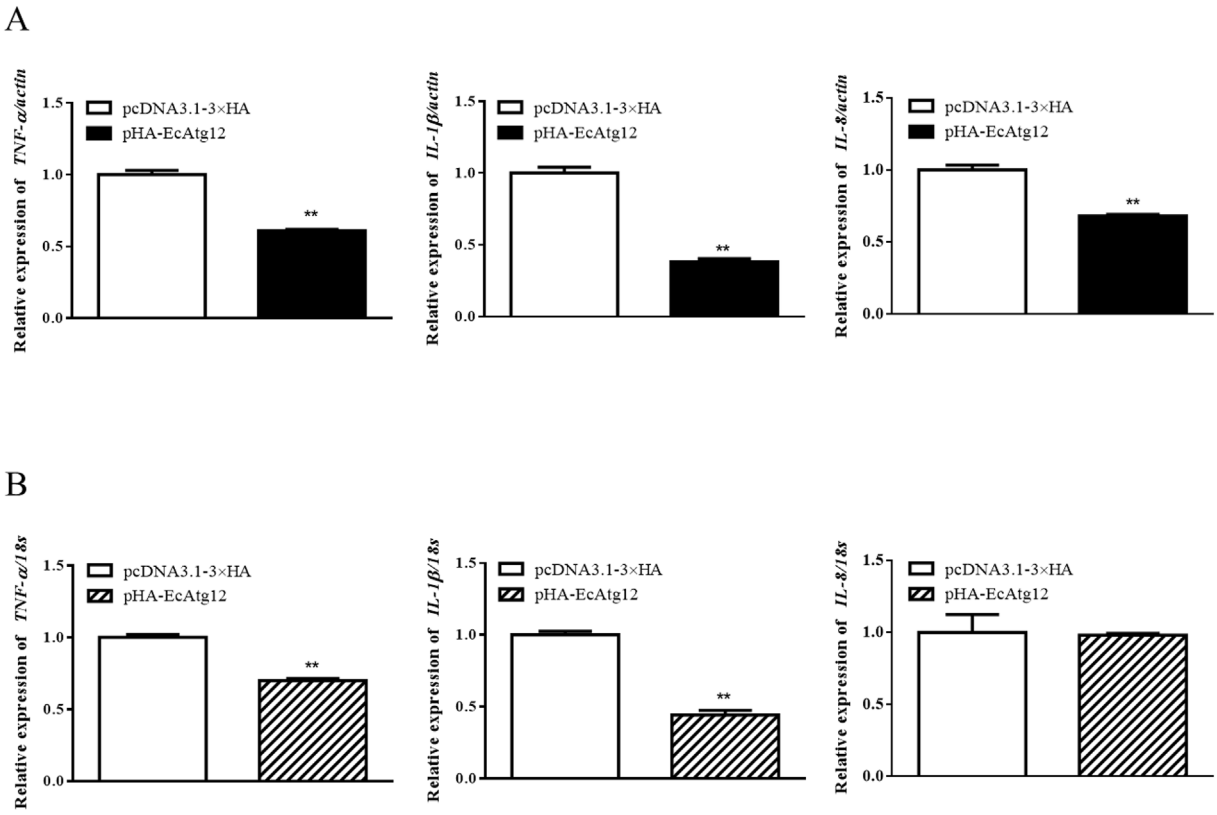

Fig. 7. The relative expression levels of proinflammatory cytokines in EcAtg12 overexpressing cells. Asterisk denotes a significant difference (**p < 0.01).

SGIV multiplication. The results showed overexpression of EcAtg12 promoted SGIV replication, evidenced by severity of CPE, the increased transcription levels of viral genes(*MCP, ICP18, VP19*, and *LITAF*), the increased levels of viral proteins (MCP), and progeny virus yield. These results indicate that EcAtg12 acts as a pro-viral factor in GS cells upon SGIV infection. Additionally, EcAtg12 overexpression significantly decreased the transcription level of several IFN-related cytokines (*IRF3*, *IRF7*, *ISG15*, *MDA5*, *MXI*, and *MyD88*) and pro-inflammatory cytokines (*IL-1β*, *IL-8*, and *TNFα*). Further analysis showed that EcAtg12 reduced IFN-3, ISRE, and NF-κB promoter activity. Together, the ectopic expression of EcAtg12 in *vitro* could negatively regulate the interferon immune response and the expression of pro-inflammatory cytokines to facilitate SGIV multiplication. Virus infection may trigger innate immune response through the retinoic acid-inducible gene I(RIG-I)-IFN-β promoter stimulator 1 (IPS-1)-mediated interferon response [41]. It has been reported that Atg5-Atg12 conjugate interacts directly with the caspase recruitment domain (CARD) of IPS-1 and RIG-I, resulting in inhibition of type I IFN production and promoting VSV replication [11]. Is there a similar phenomenon between EcAtg12-Atg5 conjugate and CARD domain is worth exploring. In addition, whether the unconjugated form of EcAtg12 or EcAtg12 combined with EcAtg5 affects SGIV replication requires further study. However, the opposite was found for foot-and-mouth disease virus (FMDV) [42]. Atg5-Atg12 increased the IRF3 activity through stabilization of TRAF3, thereby increasing the phosphorylation of TBK and IRF3. It thus appear that Atg12 may affect innate immune signaling in virus-specific and cell type-dependent manners.

In summary, an important autophagy related gene (*atg12*) from orange-spotted grouper (*E. coioides*) (*Ecatg12*) was cloned and characterized. The response of EcAtg12 to pathogen challenges was investigated in *vitro*. Intracellular localization was analyzed for EcAtg12 and the effect of EcAtg12 overexpression on SGIV multiplication was investigated. EcAtg12 negatively regulated the IFN and inflammatory responses to achieve its pro-viral functions. These results contribute to understanding the link between the Atg12 and innate immune signaling in response to virus.

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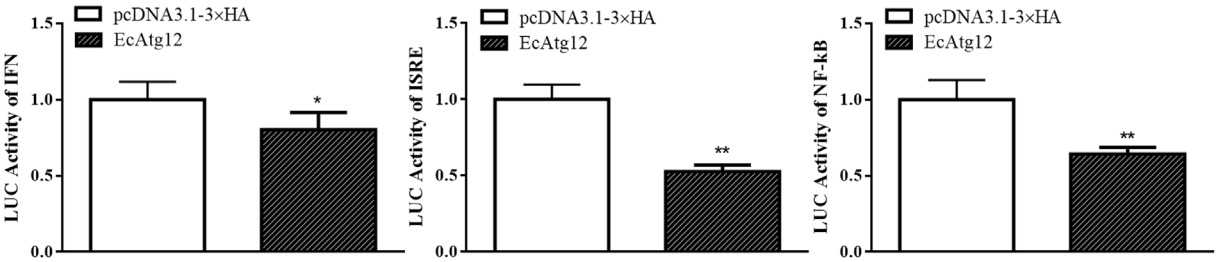

Fig. 8. The relative luciferase activity of NF-κB and IFN promoter in EcAtg12 overexpressing cells. GS cells were co-transfected with IFN-3-Luc/ISRE-Luc/NF-κB-Luc and pHA-EcAtg12/pcDNA3.1-3×HA, then the IFN-3, NF-κB, and ISRE promoter activity were detected using the luciferase reporter gene assay (*p < 0.05, **p < 0.01).

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