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Identification of Beclin-1 from orange-spotted grouper (*Epinephelus coioides*) involved in viral infection

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ABSTRACT

Beclin-1 is an essential autophagic regulator that plays diverse roles in physiology and disease. However, reports about the function of fish Beclin-1 during pathogen infection are still very limited. In this study, a Beclin-1 homolog (EcBeclin-1) from orange-spotted grouper (*Epinephelus coioides*) was identified and its roles in viral infection were investigated. EcBeclin-1 encoded 447amino acids protein with a BH3 domain, a CCD domain and an ECD domain, which shared high identities (97%–82%) with reported Beclin-1 proteins from mammal to fish. Quantitative real-time PCR (qRT-PCR) analysis revealed that EcBeclin-1 was predominantly expressed in brain and muscle of healthy grouper. Using fluorescence microscopy, we found that EcBeclin-1 was co-localized with endoplasmic reticulum (ER) in grouper spleen cells (EAGS). After red-spotted grouper nervous necrosis virus (RGNNV) infection *in vitro*, EcBeclin-1 transcript was significantly up-regulated, implying that EcBeclin-1 might be involved in viral infection. Furthermore, the *in vitro* studies of EcBeclin-1 overexpression promoted RGNNV induced autophagy, as well as the expression of coat protein (CP) and RNA-dependent RNA polymerase (RdRp). The overexpression of EcBeclin-1 suppressed the expressions of interferon pathway-related factors, inflammatory-related factors and activities of NF-κB and ISRE. Additionally, EcBeclin-1 could interact with EcBcl-xL *in vitro*. These data suggest that EcBeclin-1 affect viral replication through modulating IFN and inflammatory responses, as well as virus-induced cell death, which will help us to further explore the immune response of fish during viral infection.

1. Introduction

Autophagy is a highly conserved metabolic process that mediates the elimination of defective proteins and organelles, as well as the prevention of abnormal proteins accumulation [1–4]. The process of autophagy is mainly regulated by various autophagy-related genes (ATG), which have been identified from yeast to mammals [5]. The autophagy-related proteins also play vital roles in innate or adaptive immunological processes, including pathogen elimination, inflammatory regulation, antigen presentation, pathogen-associated molecular pattern recognition and anti-microbial response [6–9].

Beclin-1 has been identified as the mammalian homolog of yeast ATG6/Vps30 (vacuolar protein sorting 30) and is crucial for initializing autophagy via formatting the complex through interacting with class III phosphatidylinositol 3-kinase (PI3K III) [10,11]. Beclin-1 possesses three conserve domains, including an N-terminal Bcl-2 homology 3 (BH3) domain, a central coiled-coil domain (CCD), and a C-terminal evolutionarily conserved domain (ECD) [12]. Through BH3 domain, Beclin-1 can combine with anti-apoptotic Bcl-2 family members (like Bcl-2 and Bcl-xL) directly to modulate autophagy and apoptosis [13,14]. The ECD domain and CCD domain of Beclin-1 are important for the formation of Beclin1-VPS34 complexes and UVRAG-class-C-Vps complex, which are involved in autophagosomes maturation [10,15].

Extensive evidences indicate that Beclin-1 not only functions in autophagy but also plays vital roles in immune response against pathogen infection. For viral pathogen, overexpression of Beclin-1 can

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reduce the death of mice infected with Sindbis virus [16]. In human T cell leukemia virus type 1 (HTLV-1)-transformed T Lymphocytes, Beclin-1 is critical for maintaining persistent activities of both NF-κB and STAT3 in the pathogenesis of HTLV-1-mediated oncogenesis [17]. Beclin-1 silencing enhances the OAS-1, IFN and IFI27 expression and inhibited viral replication [18]. In the case of bacterial infection, Beclin-1 can restrict bacterial proliferation, bacteria-induced cellular destruction and regulate the bacterial dissemination [19]. To date, Beclin-1 orthologues have been characterized in various species including fish [20–23]. Although the involvement of fish Beclin-1 in the immune response against viral infection has been recorded [21], the exact mechanism of fish Beclin-1 in anti-viral immune response still remains largely unclear.

Groupers, *Epinephelus* spp. are kinds of commercial marine fish species widely cultured in China and other Asian countries. The outbreak of viral disease caused by nervous necrosis virus (NNV) has resulted in serious loss in grouper aquaculture [24]. Recently, numbers of immune genes such as tumor necrosis factor receptor-associated factor 6 (TRAF6), interferon-stimulated gene (ISG)15, myxovirus resistance gene MX, and interferon regulatory factor (IRF) 3 and 7 have been found to be involved in grouper antiviral response [25–29]. However, the information about ATGs during viral infection is very limited. In this study, a Beclin-1 homolog (EcBeclin-1) was isolated and characterized from orange-spotted grouper, *E. coioides*. Its tissue distribution and expression profiles post viral infection *in vitro* were examined through qRT-PCR. Further *in vitro* studies were carried out to clarify the regulatory roles of EcBeclin-1 on virus infection and immune-related factors expressions. These data will contribute to a better understanding of the immune response of fish against virus infection.

2. Materials and methods

2.1. Cloning of EcBeclin-1 and Bioinformatic analysis

The specific primers for the open reading frame (ORF) of EcBeclin-1 (listed in Table 1) were designed according to several expressed sequence tag sequences of EcBeclin-1 in grouper spleen transcriptome library [30]. The ORF sequence of EcBeclin-1 was amplified by PCR amplification and verified with DNA sequencing. The nucleotide and predicted amino acid sequences of EcBeclin-1 (Accession No. MN082044) were analyzed using DNAMAN9.0 software. The identities of EcBeclin-1 with other Beclin-1 proteins were analyzed using the BLASTP search program at the NCBI (http://www.ncbi.nlm.nih.gov/blast). Multiple-sequence alignment of the reported Beclin-1 amino acid sequences was performed using ClustalX2.0 and a phylogenetic tree was constructed using the MEGA 6.0 software.

Table 1
Sequences of primers used in this study.

Primers	Sequences (5′-3′)
EcBeclin-1ORF-F	ATGGAGGGCTCCAAGTCGTCGAG
EcBeclin-1ORF-R	CTATCTGTTGTAGAACTGTGAG
GFP-EcBeclin-1-F	CCGCTCGAG ATGGAGGGCTCCAAGTCGTC
GFP-EcBeclin-1-R	ACGCGAATTCC TCTGTTGTAGAACTGTGAGGT
PCDNA-EcBeclin-1-F	GGAATTC ATGGAGGGCTCCAAGTCGTCGAG
PCDNA-EcBeclin-1-R	CCGCTCGAG TCTGTTGTAGAACTGTGAGGT
RT-EcBeclin-1-F	GAGATACCGTCTGGTCCCGTAT
RT-EcBeclin-1-R	CCTTTTTCCACCTCCTCTTTGA
RT-18S-F	ATTGACGGAAGGGCACCACCAG
RT-18S-R	TCGCTCCACCAACTAAGAACGG
RT-RdRp-F	GTGTCCGGAGAGGTTAAGGATG
RT-RdRp-R	CTTGAATTGATCAACGGTGAACA
RT-CP-F	CAACTGACAACGATCACACCTTC
RT-CP-R	CAATCGAACACTCCAGCGACA
RT-EcIRF3-F	ATGGTTTAGATGTGGGGGTGTCGGG
RT-EcIRF3-R	GAGGCAGAAGAACAGGGAGCACGGA
RT-EcIRF7-F	CAACACCGGATACAACCAAG
RT-EcIRF7-R	GTTCTCAACTGCTACATAGGGC
RT-EcISG15-F	CCTATGACATCAAAGCTGACGAGAC
RT-EcISG15-R	GTGCTGTTGGCAGTGACGTTGTAGT
RT-EcMX1-F	CGAAAGTACCGTGGACGAGAA
RT-EcMX1-R	TGTTTGATCTGCTCCTTGACCAT
RT-EcTRAF6-F	CCCTATCTGCCTTATGGCTTTGA
RT-EcTRAF6-R	ACAGCGGACAGTTAGCGAGAGTAT
RT-EcTNFa-F	GTGTCCTGCTGTTTGCTTGGTA
RT-EcTNFa-R	CAGTGTCCGACTTGATTAGTGCTT
RT-EcIL-1β-F	AACCTCATCATCGCCACACA
RT-EcIL-1β-R	AGTTGCCTCACAACCGAACAC

Table 2
GenBank accession numbers of Beclin1 used in this study.

Species and proteins	Accession no.
Epinephelus coioides	MN082044
Maylandia zebra	XP_004567536
Cynoglossus semilaevis	XP_008312022.1
Gasterosteus aculeatus	NP_001254561
Oncorhynchus mykiss	XP_021420056
Stegastes partitus	XP_008290446
Takifugu rubripes	NP_001032963
Oryzias latipes	NP_001098248
Danio rerio	NP_957166
Dicentrarchus labrax	CBN81459
Salmo salar	NP_001133290
Xenopus tropicalis	NP_001029112
Rattus norvegicus	NP_001029289
Gallus gallus	NP_001006332
Homo sapiens	NP_001300927

2.2. Cell lines and virus

Grouper spleen cells (EAGS) and fat head minnow (derived from connective tissue and muscle, FHM) epithelial cells were kindly provided by Prof. Qiwei Qin, which were cultured in Leibovitz's L15 medium containing 10% fetal bovine serum (FBS, Gibco, USA) at 25 °C [31]. RGNNV was kept in the lab of department of aquatic animal medicine, College of Marine Sciences, South China Agricultural University. Virus stocks were stored at −80 °C as previously described [32]. In infection experiments, EAGS cells were subjected to RGNNV infection at a MOI of 0.1 as described in Ref. [32].

2.3. Expression profiles of EcBeclin-1

To clarify the tissue distribution of EcBeclin-1, the tissue samples including spleen, skin, liver, gill, heart, thymus, head kidney, brain and muscle, as well as peripheral blood lymphocyte (PBL) were collected from healthy grouper as previously described [30]. The expression level of EcBeclin-1 was assessed by qRT-PCR as described below.

To analyze the expression patterns of EcBeclin-1 in response to virus infection, EAGS cells were infected with RGNNV at the indicated time points. The cells were harvested for RNA extraction and further qRT-PCR analysis.

2.4. RNA extraction and gene expression analysis

Total RNA from orange-spotted grouper tissues (spleen, skin, liver, gill, heart, thymus, head kidney, brain, muscle), PBL and EAGS cells were isolated using TRIzol reagent (Invitrogen, USA) according to manufacturer's protocol. The total RNA was detected by electrophoresis on 1% agarose gel. The RNA from sampled tissues was used for cDNA synthesis through M-MLV Reverse Transcriptase (TransGen Biotech, China).

qRT-PCR was applied for gene expression analysis using RT primers shown in Table 1. The PCR was performed in a 10 μl reaction volume containing 0.5 μl of each primer (10 mM), 0.5 μl of cDNA, 5 μl of SYBR® Select Master Mix (Applied Biosystems, Foster City, CA, USA), and

3.5 μl of PCR grade water. The PCR conditions were applied as follows: 94 °C for 5 min, followed by 40 cycles of 5s at 94 °C, 10 s at 55 °C and 15 s at 72 °C. 18S was used as control with primers RT-18S-F and RT-18S-R (Table 1). The specificity of the PCR amplification for primers was verified from the dissociation curves. PCR amplification efficiency was identified according to the methods described in Ref. [33]. Relative gene expression was analyzed using $2^{-\triangle\triangle Ct}$ method [34].

2.5. Plasmids construction

To clarify the molecular function of EcBeclin-1 *in vitro*, EcBeclin-1 was subcloned into the eukaryotic vectors pEGFP-N1 and pcDNA3.1(+) using the primers listed in Table 1. Briefly, the ORF of EcBeclin-1 without the stop codon was amplified by PCR with specific primers that contained Xho I (GFP-EcBeclin1-F) and EcoR I (GFP-EcBeclin1-R) restriction site for pEGFP-N1, EcoR I (PCDNA-EcBeclin1-F) and Xho I (PCDNA-EcBeclin1-R) restriction site for pcDNA3.1(+). The PCR products were digested with EcoR I and Xho I, then inserted into pEGFP-N1 and pcDNA3.1(+), respectively. The ligation products were transformed into *Escherichia coli* DH5αand positive clones were verified by DNA sequencing. The recombinant plasmids (designated as pEGFP-EcBeclin-1 and PCDNA-EcBeclin-1) and empty vectors were extracted using E.Z.N.A. Endo-free Plasmid Mini Kit (Promega, USA) in compliance with manufacturer's instructions and then used for further analysis.

2.6. Subcellular localization of EcBeclin-1

The EAGS cells (1×10^6 per well) were seeded in a 24-well plate. After the cells adhere for 24 h, the EAGS cells were co-transfected with equal amount (400 ng, respectively) pEGFP-N1 or pEGFP-Beclin-1 and pDsRED2-ER (Takara, JAPAN), using Lipofectamine 2000 Reagent (Invitrogen, USA) according to the manufacture's instructions. Briefly, each transfection sample was prepared mixtures as follows: Dilute DNA and Lipofectamine 2000 in 50 μl of Opti-MEM Reduced Serum Medium without serum, respectively. After incubating for 5 min, the diluted DNA and diluted Lipofectamine 2000 (total volume = 100 μl) were mixed for another 20 min of incubation. Then the 100 μl mixture was added into each well. Medium was changed after transfection 6 h. The transfected cells were washed with PBS at 24 h post-transfection and fixed with 4% paraformaldehyde for 30 min, then stained with 6-diamidino-2-pheny-lindole (DAPI) (1 mg/ml) for 15 min. Finally, cells were stained with DAPI (1 mg/ml) and observed under fluorescence microscopy (Leica).

2.7. Effects of EcBeclin-1 on RGNNV infection

To determine the effects of EcBeclin-1 on RGNNV infection, PCDNA-EcBeclin-1 and pcDNA3.1(+) empty (control) vector were transfected into EAGS cells. Briefly, EAGS cells were plated in 24-well plates (for viral gene detection) and 96-well plates (used for Autophagy detection), respectively. Approximately 24 h later, transfection was performed with Lipofectamine 2000 as described in section 2.5. After transfection 24 h, transfected cells were incubated with RGNNV at a MOI of 0.1. The uninfected cells and infected cells were harvested at 24 h after viral incubation. The cells in 24-well plates were used for further analysis of the transcriptions of viral gene coat protein (CP) and RNA-dependent RNA polymerase (RdRp). Autophagic rate of infected cells in 96-well plates were determined using the CYTO-ID® Autophagy detection kit (Enzo Life Sciences, New York, USA). Cells were stained according to manufacturer's instructions and the level of autophagic signal was determined by Fluorescence Microplate Reader (EnSpire 2300, PerkinElmer).

2.8. Luciferase reporter assay

To evaluate the promoter activity regulated by EcBeclin-1, PCDNA-EcBeclin-1 and pcDNA3.1(+) empty vector (applied as control), luciferase reporter plasmids, including interferon-sensitive response element (ISRE)-Luc and nuclear factor (NF)-κB-Luc (Clontech, USA), were co-transfected into FHM cells. Briefly, FHM cells (1×10^6 per well) were seeded into 24-well plates and cultured for 24 h at 25 °C, then 400 ng PCDNA-EcBeclin-1, 400 ng pcDNA3.1(+) (applied as control), 200 ng luciferase reporter plasmids, including interferon-sensitive response element (ISRE)-Luc and nuclear factor (NF)-κB-Luc (Clontech, USA), and 100 ng pRL-TK Renilla (Renilla reniformis) luciferase plasmids were cotransfected using Lipofectamine2000. The transfected cells were harvested with passive lysis buffer (Promega, USA). The luciferase activity of total cell lysates was measured using a luciferase reporter assay system (Promega) as previously described [35].

2.9. Yeast two-hybrid assay

For the yeast two-hybrid analysis, the pGBKT7-EcBcl-xL and pGADT7-EcBeclin-1 plasmids were constructed by cloning the corresponding genes into the pGBKT7 and pGADT7 vectors separately. All constructed plasmids were verified by sequencing. The two plasmids were co-transformed into *Saccharomyces cerevisiae* strain AH109 separately. Simultaneously, we also transformed pGBKT7-53 and pGADT7-T as the positive control and pGBKT7-EcBcl-xL with PGADT7-T, pGADT7 with pGBKT7 as the negative controls. The transformants were tested on SD/-Leu/-Trp and SD/-Leu/-Trp/-His/-Ade media. The yeast two-hybrid assays were performed as described in Ref. [36].

2.10. Statistical analysis

The studies data were expressed as the means ± standard deviation (SD). Statistical comparison and analysis were performed using SPSS 20 software. Significant differences were indicated with p-value < 0.05 (Shown as *).

3. Results

3.1. Sequence analysis of EcBeclin-1

The ORF of EcBeclin-1 (Accession No. MN082044) was 1344 bp, encoding a polypeptide of 447 amino acids with typical APG6 domain (Fig. 1). In addition, the deduced protein sequence of EcBeclin-1 contained three functional domains including a BH3 domain, an ECD domain and a CCD domain, shared high identities with reported Beclin-1 (97%–82%) protein from amphibian to mammal (Fig. 2). Phylogenetic analysis showed that the EcBeclin-1was clustered closest with *Gasterosteus aculeatus* Beclin-1. Fish Beclin-1s were clustered together and separated from amphibian, bird and mammal (Fig. 3).

3.2. Tissue distribution of EcBeclin-1 gene

To detect the tissue distribution of EcBeclin-1, qRT-PCR was carried out to analyze its transcript levels in different tissues. As shown in Fig. 4A, EcBeclin-1 was widely expressed in all examined tissues with the predominantly expression in brain and muscle.

3.3. EcBeclin-1 response to RGNNV infection in vitro

To investigate the expression patterns of EcBeclin-1 following viral infection *in vitro*, EAGS cells were incubated with RGNNV and harvested at various time points. Expression profile of EcBeclin-1was detected through qRT-PCR at various time points. The result of qRT-PCR showed that the transcripts of EcBeclin-1 was induced after viral infection and reached to peak at 4 h p.i compared with mock (Fig. 4B),

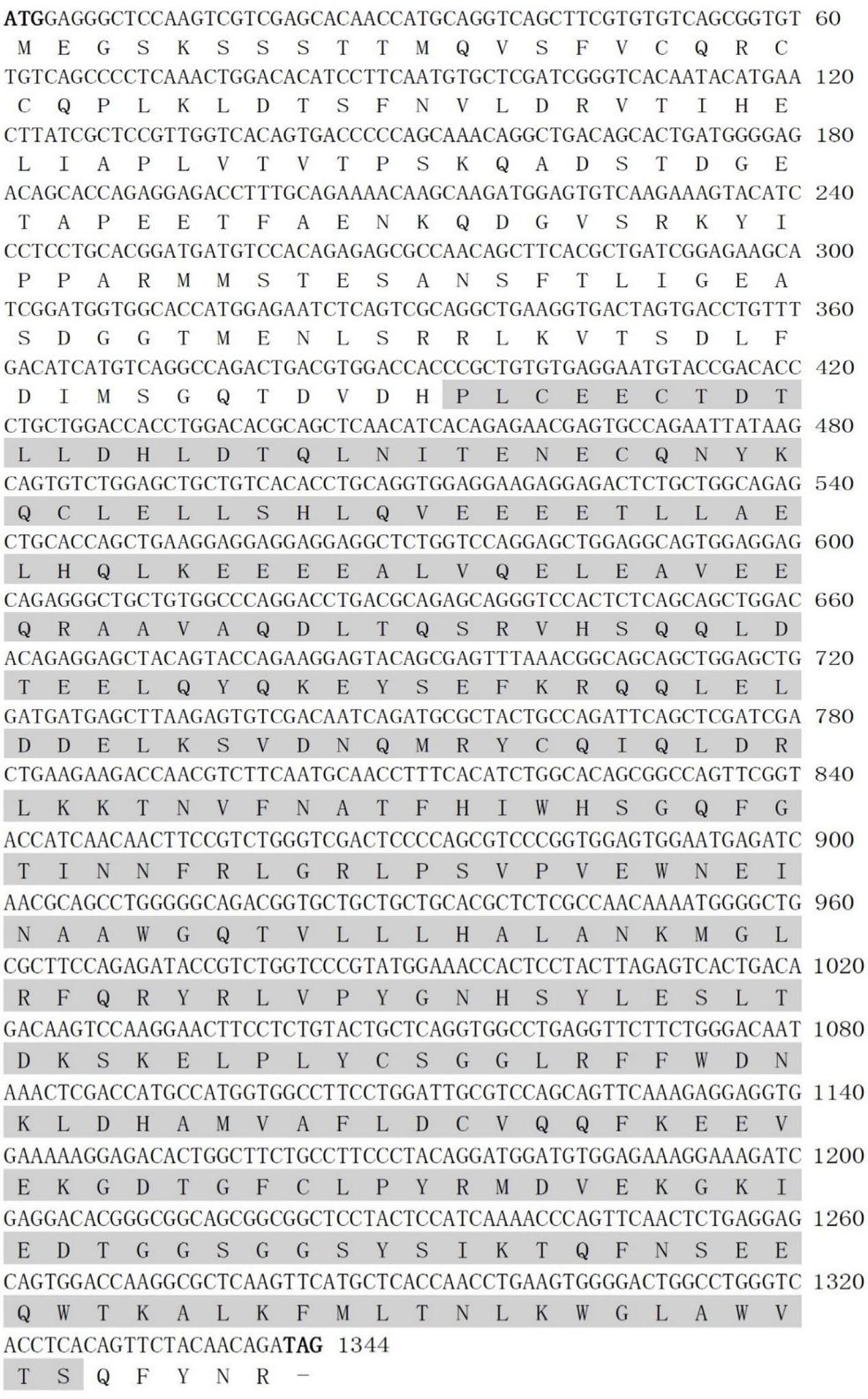

Fig. 1. The nucleotide and deduced amino acid sequences of EcBeclin-1. The start (ATG) and stop (TAA) codons are in bold. The nucleotide is numbered along the right margin. The dash (−) indicates the stop codon. APG6 domain is shadowed in grey.

then gradually declined to the basal level till the end of experiment.

3.4. Subcellular localization of EcBeclin-1

To determine the intracellular localization of EcBeclin-1, pEGFP-EcBeclin-1 or pEGFP-N1 and pDsRED2-ER plasmids were co-transfected into EAGS cells. As shown in Fig. 5, the green fluorescence distributed throughout the cytoplasm and nucleus in pEGFP-N1 transfected cells (upper row), and the green fluorescence was mainly focused in cytoplasm in pEGFP-EcBeclin-1 transfected cells. The red fluorescence in

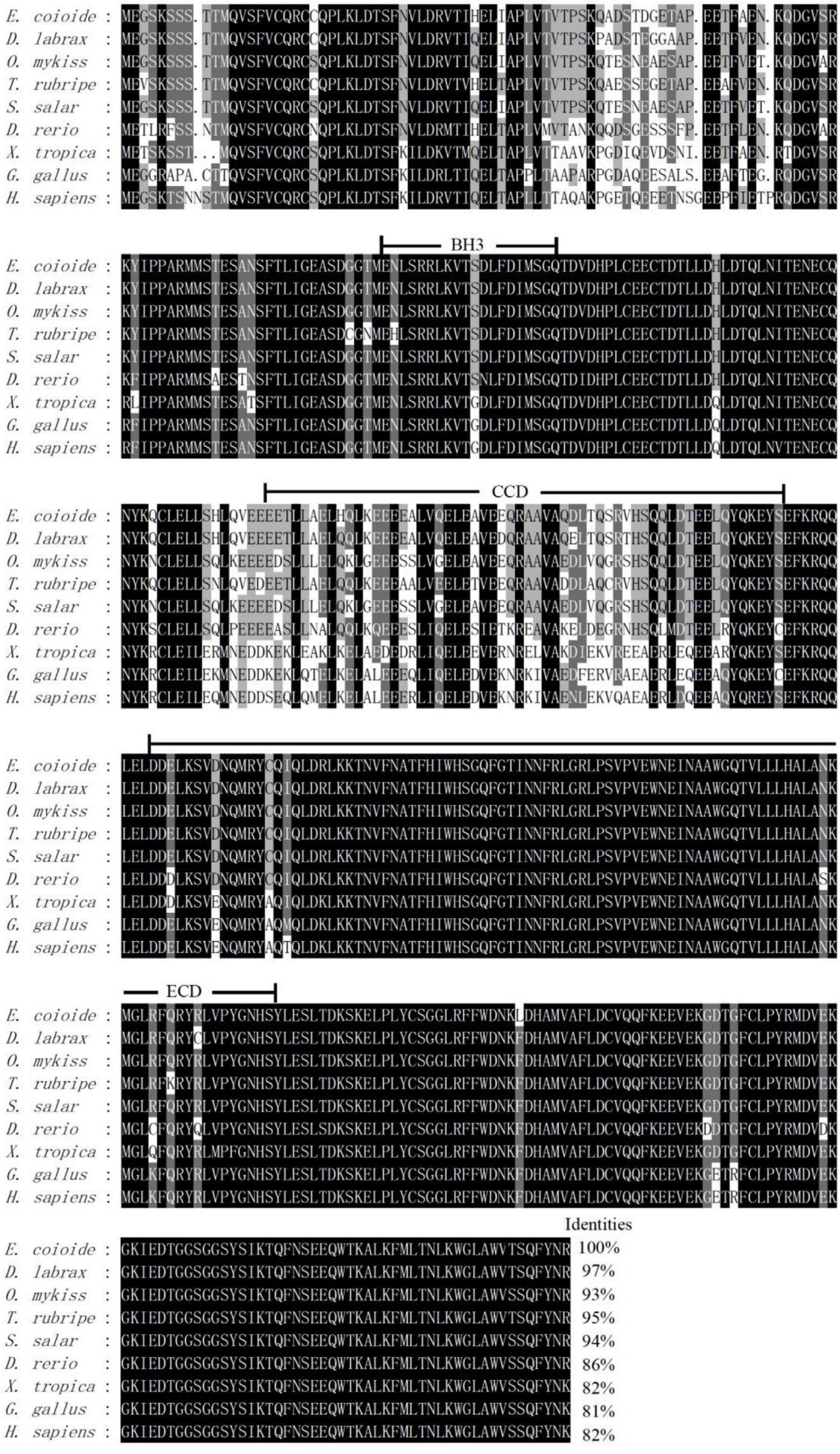

Fig. 2. Multiple sequence alignment of Beclin-1 proteins. The accession numbers and similarities of different proteins used in this study are listed in Table 2. The BH3 domain, central coiled coil domain (CCD) and evolutionarily conserved domain (ECD) were marked with continuous line.

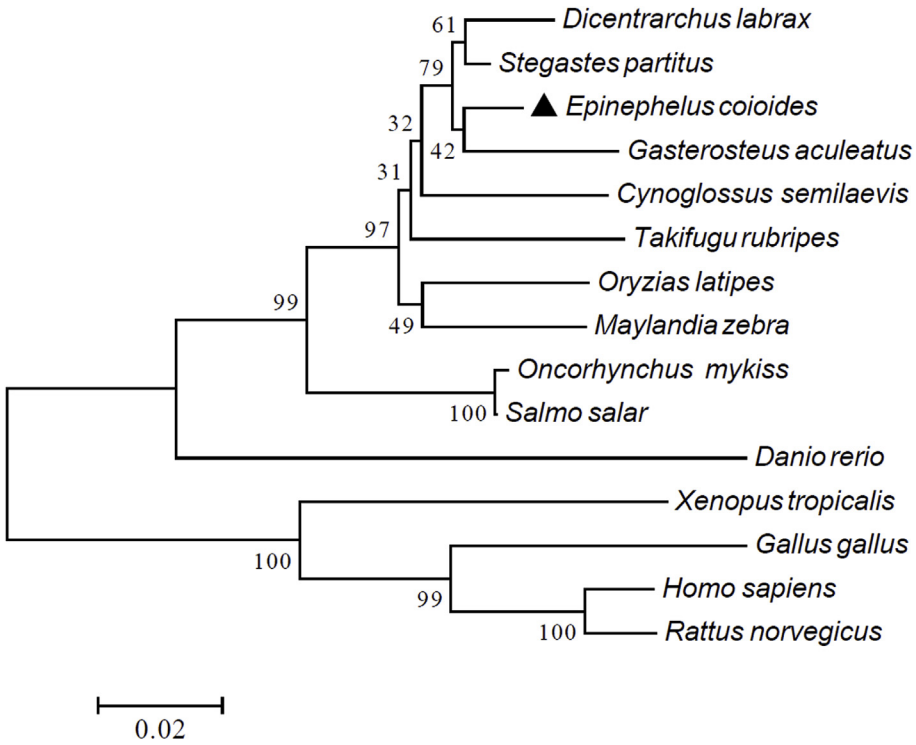

Fig. 3. Phylogenetic relationship of EcBeclin-1 with known Beclin-1 proteins constructed based on amino acid sequences. The GenBank accession numbers of selected Beclin-1 sequences are listed in Table 2.

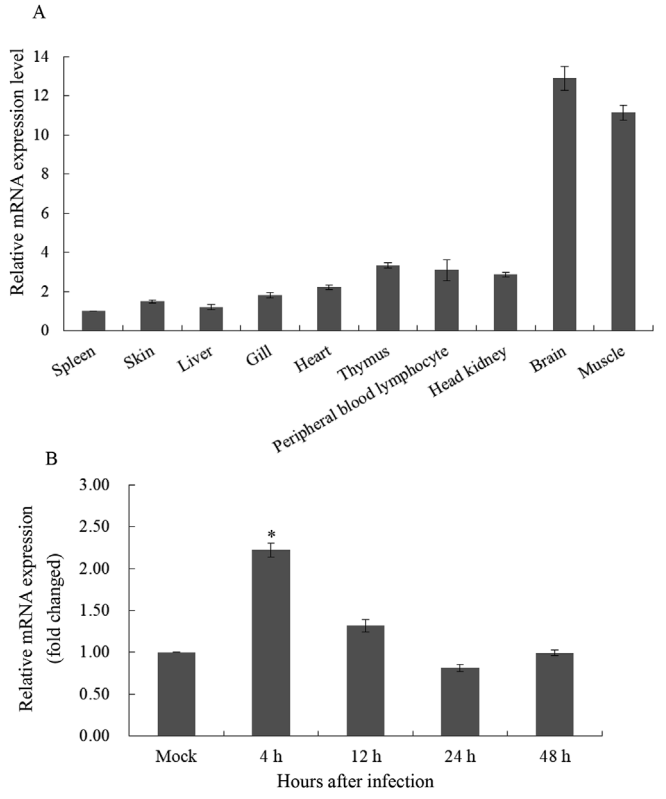

Fig. 4. (A) Tissue distribution of EcBeclin-1. Data are presented as a ratio to EcBeclin-1 mRNA expression in spleen. (B) The expression profile of EcBeclin-1 in EAGS cells after RGNNV incubation. EcBeclin-1 expression was normalized to the mock cells. Vertical bars represented the means ± SD (n = 3), and significant differences of EcBeclin-1 expression between the infected and mock samples were indicated with an asterisk (*) at $p < 0.05$.

pDsRED2-ER transfected cells represented the localization of endoplasmic reticulum (ER). Moreover, the green fluorescence in EcBeclin-1 expression cells was predominately overlapped with red fluorescence (lower row), suggesting that EcBeclin-1 was an ER-localized protein.

3.5. Overexpression of EcBeclin-1 promoted RGNNV replication

To clarify the effects of EcBeclin-1 overexpression on virus infection, EcBeclin-1transfected cells were infected with RGNNV. The autophagic rate of infected-cells and viral genes expressions were investigated by CYTO-ID® Autophagy Detection Kit and qRT-PCR, respectively. As shown in Fig. 6, the more obvious vacuoles (Fig. 6A) and higher autophagy rate (Fig. 6B) induced by RGNNV infection were found in EcBeclin-1 overexpressing cells compared to the empty vector transfected cells. At the transcription level, the expression levels of RdRp and CP were significantly increased in virus infected EcBeclin-1-transfected cells compared with vector-transfected cells (Fig. 6C).

3.6. Overexpression of EcBeclin-1 impaired the expression of interferon pathway-related factors and inflammatory-related factors

To explore the potential mechanism involved in the action of EcBeclin-1in fish virus infections, the roles of EcBeclin-1 on the host interferon immune and inflammation response were evaluated. The GFP-EcBeclin-1 and the empty vector were transfected into EAGS cells, and cells were harvested at 24 h following transfections. The transcription levels of host immune factors were detected using qRT-PCR. As shown in Fig. 7, expression levels of interferon pathway-related cytokines or effectors, including IRF3, IRF7, ISG15, MX1 were all decreased in EcBeclin-1 overexpressing cells compared with control vector transfected cells. In addition, we also found that the expressions of inflammatory-related factors such as IL-1β, TNFα, and TRAF6, were all significantly decreased in EcBeclin-1 overexpressing cells.

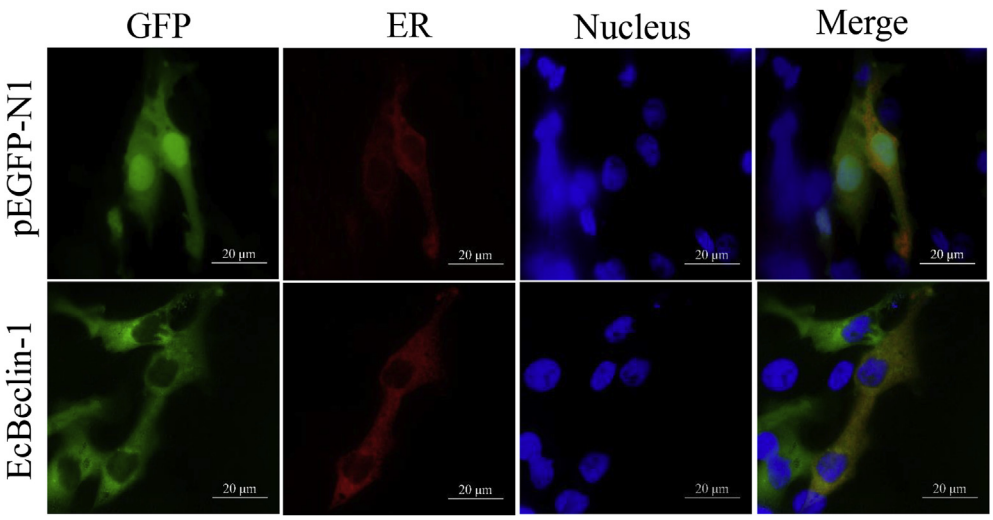

Fig. 5. Subcellular localization of EcBeclin-1 in EAGS cells. Cells were transfected with plasmids (pEGFP-EcBeclin-1 and pEGFP-N1) and subjected to fluorescence observation at 24 h post-transfection. Green fluorescence showed the distribution of GFP or GFP-tagged proteins, and blue fluorescence shows the nucleus that is stained by DAPI. Cells were co-transfected with pDsRed2-ER and pEGFP-EcBeclin-1 under normal condition. Green fluorescence shows the distribution GFP-tagged proteins, and red fluorescence shows the distribution of ER-DsRed2 protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.7. EcBeclin-1 inhibited ISRE and NF-κB promoter activities

To further explore the regulatory roles of EcBeclin-1 during fish virus infection, the promoter activity of reporter genes in typical antiviral pathways including ISRE and NF-κB were examined using the plasmids ISRE-Luc and NF-κB-Luc. As shown in Fig. 8, EcBeclin-1overexpression significantly suppressed the promoter activity of these factors.

3.8. EcBeclin-1 interacted with EcBcl-xL protein

Yeast two hybrid assay was performed to confirm the interaction of EcBeclin-1and EcBcl-xL. The EcBeclin-1 CDS was cloned into pGADT7, the EcBcl-xL were cloned into pGBKT7, respectively. Yeast containing vectors combination of empty pGBKT7 and pGADT7, did not activate the expression of the reporter gene and these two vectors were served as negative controls. Positive *in vitro* interactions were observed between pGBKT7-53 and pGADT7-T. Only yeast containing the vector combination of pGBKT7-EcBcl-xL and pGADT7-EcBeclin-1 exhibited strong galactosidase activity (Fig. 9), indicating that EcBeclin-1 protein physically interacted with EcBcl-xL *in vitro*.

4. Discussion

Beclin-1 is a key regulator in autophagy initiation and autophagosomes maturation, which also play crucial roles in multiple biological processes including immunity [37]. Until now, Beclin-1 orthologs have been well-described in various species, but the functions of fish Beclin-1 still require further investigation. In present study, a Beclin-1 homolog (EcBeclin-1) was isolated and characterized from orange-spotted grouper, *E. coioides*. EcBeclin-1 had a BH3 domain, a CCD domain and an ECD domain, exhibited high identities with other Beclin-1 from fish

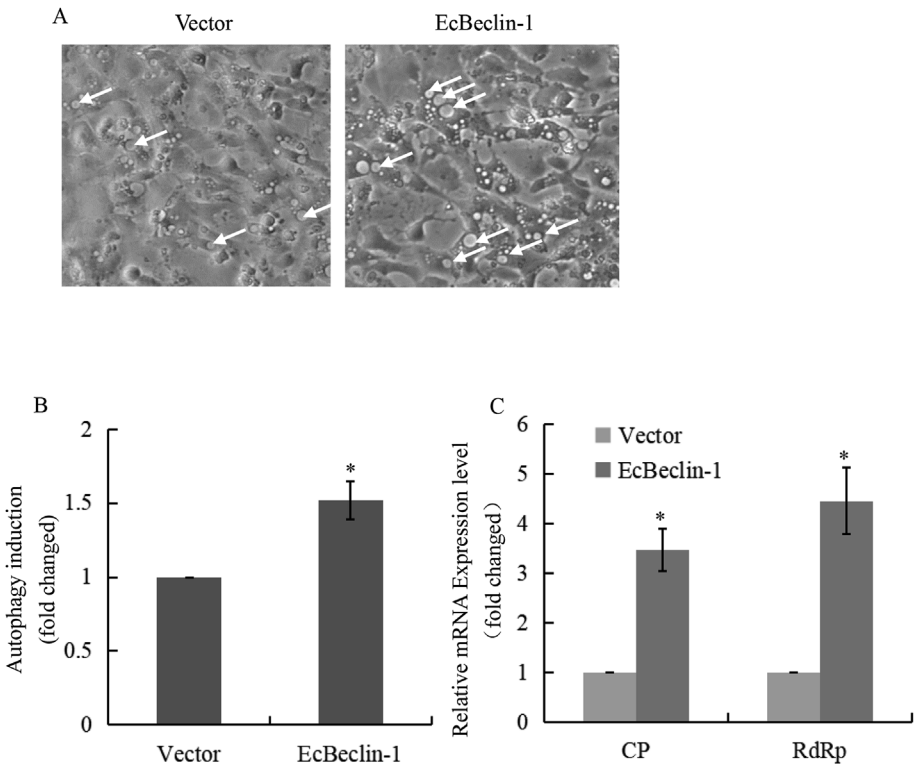

Fig. 6. Effects of EcBeclin-1 during RGNNV infection. (A) Phase microscopy observation of RGNNV infection in EcBeclin-1 transfected cells. (B) Autophagy rates of vector and EcBeclin-1 transfected cells after RGNNV incubation. (C) qRT-PCR analysis of viral genes RdRp and CP genes. Data represent the means for three independent experiments, error bars indicate SD. (*$p < 0.05$).

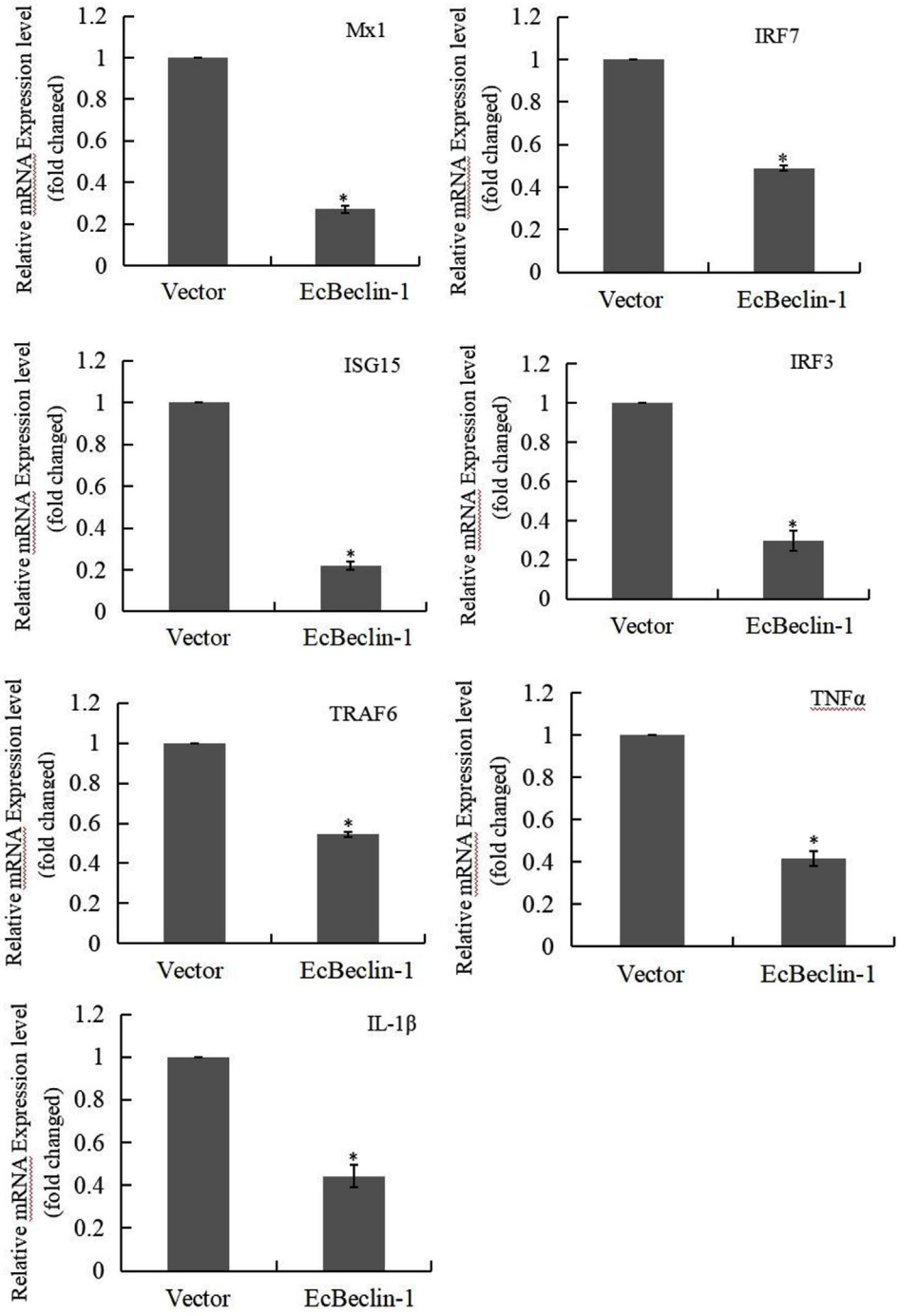

Fig. 7. EcBeclin-1 overexpression decreased expression of interferon pathway-related genes and inflammatory-related factors. EAGS cells were transfected with EcBeclin-1, then cells were harvested 24 post-transfections. Expression levels of selected genes were determined using qRT-PCR. Data represent the means for three independent experiments, error bars indicate SD. (*$p < 0.05$).

to mammals (97%–82%), suggesting that EcBeclin-1 probably have similar function as reported Beclin-1.

Previous studies have reported that Beclin-1 is ubiquitously expressed in different tissues. The Beclin-1s of human, mouse and tongue sole are extensively detected in various tissues, with the highest level in muscle [16,20]. The expression of flounder Beclin-1 is ubiquitous and is present predominantly in brain [21]. Like flounder Beclin-1, EcBeclin-1 was also distributed in all detected tissues with highest levels in brain. Additionally, mammalian Beclin-1 can be induced by various stimulations such as physical injuries, environment pressure and pathogen invasion [20,38–40]. Similarly, fish Beclin-1 homologs are also up-regulated after bacterial infection, viral infection and Cd treatment [20–23]. Our data showed that the expression level of EcBeclin-1 was significantly increased following RGNNV incubation *in vitro*, suggesting an involvement of EcBeclin-1 in immune response against viral infection.

To further determine the roles of EcBeclin-1 during RGNNV infection, we investigated the effects of EcBeclin-1 on virus-induced cell death and viral genes transcriptions. Our data found that overexpression of EcBeclin-1 promoted RGNNV replication, evidenced by the vacuoles and severity of CPE, the higher levels of autophagic activity and the increased transcription levels of viral genes, which were

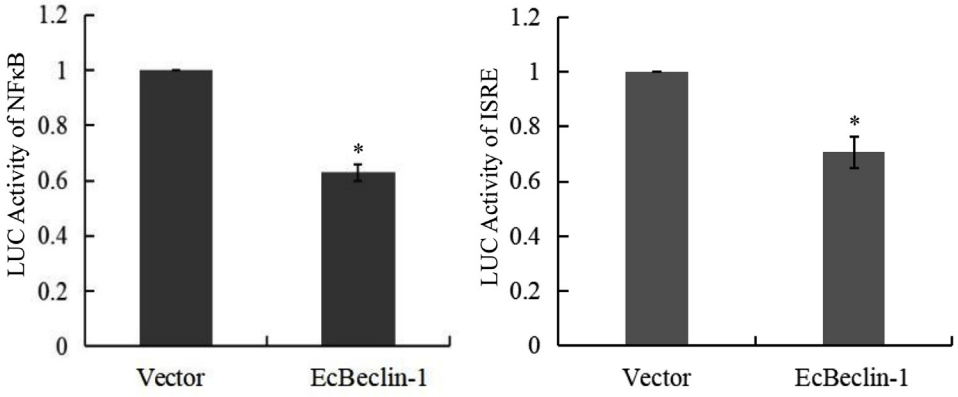

Fig. 8. Effects of EcBeclin-1 on the activities of NF-κB promoter and ISRE promoter. The values are shown as mean ± SD. Significant difference was indicated by asterisks, *p < 0.05.

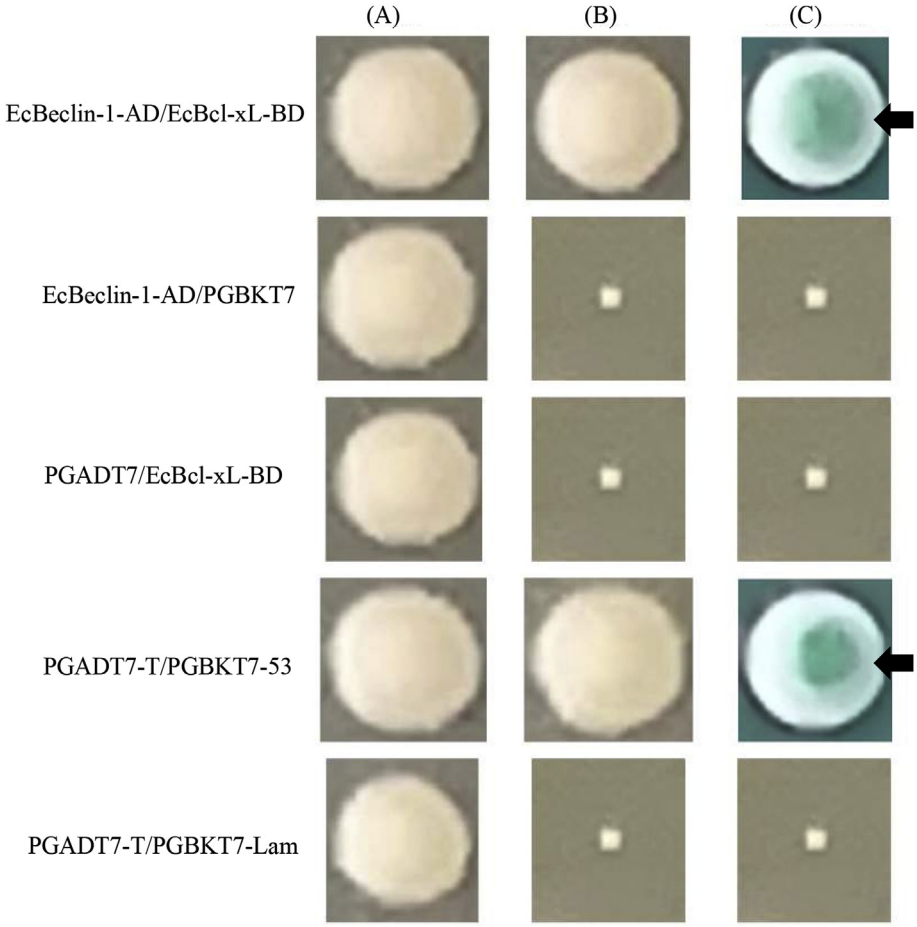

Fig. 9. EcBeclin-1 interacted with EcBcl-xL in yeast two-hybrid assay. Interaction between EcBeclin-1 and EcBcl-xL were verified by culturing the transformed AH109 cells on selective medium SD/-Leu/-Trp (A) and SD/-Leu/-Trp/-His/-Ade. (B) SD/-Leu/-Trp/-His/-Ade with X-gal. (C) was used to detect the activity of the interactions. The positive clone has been indicated with arrow.

consistent with human Beclin-1 during viral infection [41,42]. Given Beclin-1 functions as a negative regulator of IFN-I production through blocking RIG-I-MAVS interaction [43], the impacts of EcBeclin-1 on several related cytokines or effectors were further investigated. Overexpression of EcBeclin-1 in grouper cells impaired the expression levels of several interferon pathway-related factors and inflammatory factors. Moreover, the ectopic expression of EcBeclin-1 significantly decreased ISRE and NF-κB promoter activities. Also, EcBeclin-1 could also interacted with EcBcl-xL *in vitro*. It has been recorded that interaction of Bcl-xL and Beclin-1 can inhibit Beclin-1-dependent autophagy and facilitated anti-apoptotic function of Bcl-xL [44,45], thus the current result implied a potential role of EcBeclin-1 in connecting autophagy and apoptosis, further studies were required to clarify the mechanism of EcBeclin-1 and EcBcl-xL during viral infection.

In summary, a key autophagy-related gene Beclin-1 (EcBeclin-1) was cloned and characterized from orange-spotted grouper (*E. coioides*), encoding an ER-localized protein. EcBeclin-1 modulated virus infection via promoting autophagy, suppressing antiviral IFN responses and inflammation response. Moreover, EcBeclin-1 could interact with EcBcl-xL *in vitro*. The present data will improve our understanding of the cross-talk between fish Beclin-1, autophagy and viral infection.

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