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Molecular cloning and characterization of grouper Krüppel-like factor 9 gene: Involvement in the fish immune response to viral infection

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ABSTRACT

Krüppel-like factor 9 (KLF9) is a member of the SP/KL family, which are transcription factors implicated in several biological processes, including cell proliferation, differentiation, development and apoptosis. Studies have focused on the function of KLF9 in mammalian disease and the immune system, such as its regulatory role in the growth of tumors and its impact on interferon-related genes and inflammatory cytokines. In fish, little is known about the role of KLF9, especially its regulatory function in the innate antiviral immune response. In this study, we characterized the grouper KLF9 gene (EcKLF9) and investigated its role in viral infection. Amino acid alignment analysis showed that EcKLF9 was approximately 228 amino acids long and contained a typical three-tandem Krüppel-like zinc fingers. Phylogenetic tree analysis revealed that EcKLF9 clustered with three fish species: *Amphiprion ocellaris, Acanthochromis pollyacanthus* and *Stegastes partitus*. Comparison analyses showed that the three Kruppel-like zinc finger domains of KLF9 were highly conserved in different fish species. Tissue expression analysis showed that EcKLF9 was constitutively expressed in all 12 tissues tested, in the healthy grouper, the highest expression being detected in the gonads. The relative expression levels of EcKLF9 in the head kidney, spleen and brain was significantly increased during red-spotted grouper nervous necrosis virus (RGNNV) and Singapore grouper iridovirus (SGIV) infections. Using fluorescence microscopy, EcKLF9 was primarily localized to the nucleus and cytoplasm. The *in vitro* ectopic expression of EcKLF9 significantly increased the severity of vacuoles induced by RGNNV and the cytopathic effect progression evoked by SGIV infection. Real-time PCR results showed that the transcription levels of viral genes, such as the Singapore grouper iridovirus infection genes, *MCP* (major capsid protein), *LITAF* (lipopolysaccharide induced TNF-α factor), *VP19* (envelop protein) *ICP-18* (infected cell protein-18) and the red-spotted grouper nervous necrosis virus genes, *CP* (coat protein), *RdRp* (RNA-dependent RNA polymerase), were all significantly increased in EcKLF9 overexpressing cells, when compared to control cells. Furthermore, western blotting analyses showed that protein levels of the RGNNV gene, CP and the SGIV gene, MCP were also increased in EcKLF9 overexpressing cells, suggesting EcKLF9 may promote viral activity against iridovirus and nodavirus, *in vitro*. Moreover, the overexpression of EcKLF9 significantly inhibited the expression of several interferon related cytokines and several inflammatory cytokines. Accordingly, we speculate that EcKLF9 may exert stimulatory effects on RGNNV and SGIV replication, through the negative regulation of host immune and inflammation responses.

1. Introduction

The Krüppel-like factor 9 (KLF9), previously referred to as basic transcription element binding protein 1 (BTEB1), was initially identified as a transcriptional inducer of CYP1A1, in hepatocytes [1]. KLF9 is a member of the SP/KL family, which are transcription factors implicated in several biological processes, including cell proliferation, differentiation, development and apoptosis [2,3] The characteristic feature of this protein family is the presence of three Kruppel-like zinc fingers, which bind CACCC elements and GC-rich boxes in the promoters of gene sequences, activating or repressing target gene transcription [4,5].

Target gene activation or repression by KLF9 is determined by the number of GC boxes in the promoter region of the target gene sequence

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[5]. Activation responses often occur on target genes with single GC boxes; however, repression responses often occur on target genes with multi- GC-boxes [6,7]. According to previous studies, for most target genes, KLF9 functions as a transcription activator, whereas for other genes, KLF9 functions as a transcriptional inhibitor [8,9]. To date, several genes have been shown to be regulated by *KLF9*, including *SV40*, *HIV-1*, *P4501A-1*, *Cyclin D1*, *HERV-H, CYP7A, hEF1A-1* and *AP-2a* [10]. Of these genes, several are viral, but most are host genes which have important roles in metabolism, reproduction, immune responses and cell proliferation and differentiation. These varied functions suggest KLF9 has a key regulatory role in many physiological and pathological processes.

Groupers (*Epinephelus* sp.) are widely cultured in China and Southeast Asian countries for their economic value [11,12]. However, diseases caused by viral, bacterial and parasitic pathogens have broken out in recent years, leading to heavy economic losses across the grouper industry [11]. Among these pathogens, the Singapore grouper iridovirus (SGIV) and red-spotted grouper nervous necrosis virus (RGNNV) are two major viral pathogens, causing mass grouper mortality, from fry to juvenile stages [13–15]. Hence, it is necessary to identify grouper host defense mechanisms against viral infections, which may generate effective disease control strategies and long-term sustainable development of grouper aquaculture.

Singapore grouper iridovirus (SGIV) belongs to Iridovirdae family, Ranavirus genus [15]. Four genes in SGIV are usually used as the main measure of viral replication; *MCP* (major capsid protein), the only capsid protein of this virus [16]; *LITAF* (lipopolysaccharide induced TNF-α factor), an early viral gene, a transcription factor, can regulate tumor necrosis factor alpha (TNF-a) transcription [17]; *VP19* (envelop protein), a conserved envelope protein in iridovirus, might contribute greatly to virus assembly during virus infection [18]; *ICP-18* (infected cell protein-18), an immediate-early gene, begins to be transcribed as early as 2 h postinfection and its overexpression contributes to SGIV replication [19].

The Red-spotted Grouper Nervous Necrosis Virus (RGNNV) is one of the four NNV viruses, belonging to Betanodavirus genus. It is a naked icosahedral virus containing only two single-stranded, positive-sense RNA segments (RNA1, 3.1 kb; RNA2, 1.4 kb). The RNA1 encodes RNA-dependent RNA polymerase (*RdRp*), catalyzing the replication of RNA from RNA template. The RNA2 encodes capsid protein (*CP*), the only capsid protein of RGNNV virus [20]. Based on the CP RNA segment, betanodaviruses have been clustered in four genotypes: striped jack NNV (SJNNV), red-spotted grouper NNV (RGNNV), barfin flounder NNV (BFNNV) and tiger puffer NNV (TPNNV) [21]. It has been reported that CP is the structural protein and may induce cellular apoptosis through mitochondria-mediated death pathway [22].

SGIV is a novel marine fish DNA virus firstly isolated from brown-spotted grouper *E. tauvina.* Disease outbreak caused by SGIV generally occurred in fry and adult groupers, lasting several weeks and resulting in more than 90% mortality [15]. RGNNV is the most prevalent genotypes worldwide of the four types NNVs [23]. And the viral nervous necrosis (VNN) disease induced by RGNNV has caused mass mortality in cultured marine fish at the larval stage, resulting in significant economic losses worldwide [20,24].

Many studies have focused on the function of KLF9 in mammalian disease and the immune system, such as its regulatory role in the growth of tumors and its impact on interferon-related genes and inflammatory cytokines [25–33]. However, KLF9 has been rarely reported in fish species, especially in terms of its regulation on innate antiviral immune responses in fish.

In this study, we characterized the *Epinephelus coioides* KLF9 gene (EcKLF9) and highlighted its role in fish viral infections. Our functional studies on KLF9 in teleosts may have applications in the immunological control of viral diseases.

Table 1
Primers used in present study.

Primer	Sequence (5′-3′)
KLF9-PF	ATGTCAGAGGTGGATGTCTCTGC
KLF9-PR	TCACACCCCAACAGGGCTTTGG
3HA-KLF9-PF	AAGCTTATGTCAGAGGTGGATGTCTCTGC
3HA-KLF9-PR	GGATCCTCACACCCCAACAGGGCTTTGG
GFP-KLF9-PF	AAGCTTATGTCAGAGGTGGATGTCTCTGC
GFP-KLF9-PR	GGATCCCACCCCAACAGGGCTTTGG
MCP-PF	ACTCGTAAGATCGCCACGGAAGATT
MCP-PR	ACGTTTCTCAAATGCATGTCTGCCAC
ICP18-PF	ATCGGATCTACGTGGTTGG
ICP18-PR	CCGTCGTCGGTGTCTATTC
VP19-PF	GTCAGGGTGGAGAAAACGGGAGTGT
VP19-PR	CTCAAACAGTAACGTTCCGCAAGCG
LITAF-PF	GATGCTGCCGTGTGAACTG
LITAF-PR	GCACATCCTTGGTGGTGTTG
IRF3-PF	GACAACAAGAACGACCCTGCTAA
IRF3-PR	GGGAGTCCGCTTGAAGATAGACA
IRF7-PF	CAACACCGGATACAACCAAG
IRF7-PR	GTTCTCAACTGCTACATAGGGC
IL-1β-PF	AACCTCATCATCGCCACACA
IL-1β-PR	AGTTGCCTCACAACCGAACAC
IL-8-PF	GCCGTCAGTGAAGGGAGTCTAG
IL-8-PR	GCCGTCAGTGAAGGGAGTCTAG
TRAF6-PF	CCCTATCTGCCTTATGGCTTTGA
TRAF6-PR	ACAGCGGACAGTTAGCGAGAGTAT
CP-PF	CAACTGACAACGATCACACCTTC
CP-PR	CAATCGAACACTCCAGCGACA
RdRp-PF	GTGTCCGGAGAGGTTAAGGATG
RdRp-PR	CTTGAATTGATCAACGGTGAACA
MX I-PF	CGAAAGTACCGTGGACGAGAA
MX I-PR	TGTTTGATCTGCTCCTTGACCAT
ISG15-PF	CCTATGACATCAAAGCTGACGAGAC
ISG15-PR	GTGCTGTTGGCAGTGACGTTGTAGT
MDA5-PF	ACCTGGCTCTCAGAATTACGAACA
MDA5-PR	TCTGCTCCTGGTGGTATTCGTTC
TNFα-PF	GTGTCCTGCTGTTTGCTTGGTA
TNFα-PR	CAGTGTCCGACTTGATTAGTGCTT
MyD88-PF	AGCTGGAGCAGACGGAGTG
MyD88-PR	GAGGCTGAGAGCAAACTTGGTC
NF-κB-PF	GGCTAAAGAAATGGACCTCA
NF-κB-PR	CATAGAACCGAACCTGGATA
IFNγ-PF	TTGGCGAGATGTTGAAGC
IFNγ-PR	GGATGTTGGACAGGGAGC
β-actin-PF	TCTTCCAGCCATCCTTCCTTGG
β-actin-PR	CTGCATACGGTCAGCAATGCC

2. Material and methods

2.1. The cloning of EcKLF9 and sequence analysis

Based on the unigene sequence which was announced as a predicted KLF9 in our grouper transcriptome [34], we designed forward and reverse primers for the KLF9 open reading frame. Primer details are shown (Table 1). A PCR was performed using grouper cDNA as an amplification template. Amplification conditions were: 5 min at 94 °C; then 30 cycles at 94 °C for 45s, 55 °C for 45s, 72 °C for 1 min and 72 °C for 5 min. The resulting product (with a length about 684bp), once sequenced and translated, was analyzed by blastx in the Genbank database. The conserved domain was predicted based on human or other mammalian sequences [1]. Multiple amino acid (AA) sequence alignments were performed by ClustalX1.83 software and edited with GeneDoc software. A phylogenetic tree was obtained by MEGA version 6.0 using the boot-strapped neighbor joining method (with 1000 replicates).

2.2. Tissue distribution of EcKLF9 in the grouper

Orange-spotted groupers (weight 30–40 g) were purchased from a local fish farm in Xiamen city, Fujian Province, China. Fish were kept in a seawater recirculation system, the temporary rearing conditions have

been described previously [35,36]. Tissue samples from six fish, containing brain, heart, liver, intestine, stomach, sexual glands, head kidney, kidney, skin, muscle, gills and spleen were dissected and immediately frozen in liquid nitrogen, followed by storage at −80 °C.

2.3. Cells and virus infection

A grouper spleen (GS) cell line was grown and maintained in Leibovitz's L15 culture medium (Gibco, USA), with 10% fetal bovine serum (Gibco, USA) at 28 °C [37].

The genotypes Red-spotted Grouper Nervous Necrosis Virus (RGNNV) is a RNA virus belongs to Betanodavirus genus. And the Singapore grouper iridovirus (SGIV) is a novel marine fish DNA virus belongs to Ranavirus genus. These two viruses were propagated and stored as previously described [38,39]. The viral titers of RGNNV and SGIV were 10^5 and 10^6 $TCID_{50}$, (Tissue Culture Infective $Dose_{50}$), respectively.

For viral infection in vivo, groupers were injected with RGNNV (Volume: 100 μl, Concentration: 10^5 $TCID_{50}$/ml) and SGIV (Volume: 100 μl, Concentration: 10^6 $TCID_{50}$/ml). Briefly, RGNNV injected groupers were collected at 0, 6, 12, 24, 48 h post injection, and SGIV injected groupers were also collected at different time points (0, 6, 12, 24, 48 h). At indicated time points, target tissues, spleen, head kidney and brain, were collected for RNA extraction and further qRT-PCR analysis.

For viral infection *in vitro*, GS cells were infected with RGNNV (Volume: 10 μl, Concentration: 10^5 $TCID_{50}$/ml) or SGIV (Volume: 10 μl, Concentration: 10^6 $TCID_{50}$/ml) for 48 h. Then, cell morphologies were observed or imaged using light microscopy, and RNA from virus infected cells was extracted and stored at −80 °C for further analysis.

2.4. Plasmid construction

A pMD18-T vector containing an EcKLF9 insert was initially constructed as a PCR template. Then, PCR was performed to construct various expression vectors. Primers are listed (Table 1). EcKLF9 PCR products were cloned into the *HindIII* and *BamHI* double digested sites of pcDNA 3.1-3HA and pEGFP-N3 vectors. Recombinant plasmids were confirmed by DNA sequencing and blastx in the Genbank database. NF-κB, IFN and ISRE luciferase reporters (Clontech, USA) were used to analyze NF-κB and IFN signaling activities in GS cells. The pRL-SV40 Renilla luciferase vector (Promega, USA) was used as an internal control.

2.5. Cell transfection

Cell transfections were performed using the transfection reagent (TA), Lipofectamine 2000 (Invitrogen). Briefly: GS cells were seeded in 24-well cell culture plates at 60%–70% confluence. After 24 h, cells were transfected with relevant plasmids and Lipofectamine 2000. Approximately 6 h after transfection, the cell transfection medium was replaced with fresh medium. And then cells were collected at 24 h post transfection for RNA extraction and qRT-PCR.

2.6. Cellular localization analysis

Cells were transfected with the pEGF-N3-EcKLF9 plasmid according to the cell transfection method described above. At 24 h post transfection, cells were washed with PBS and fixed with 4% paraformaldehyde at 4 °C, for 1 h. Cells were stained with 6-diamidino-2-pheny-lindole (DAPI) for 10 min, after which they were observed using fluorescence microscopy (Zeiss, Germany).

2.7. Dual-luciferase reporter assays

To detect activated patterns of interferon and NF-κB promoter by EcKLF9, luciferase plasmids including IFN-Luc, ISRE-Luc and NF-κB -Luc (Clontech, USA) were used for co-transfection. Luciferase activity in total cell lysates was measured by luciferase reporter assay (Promega, USA). The Renilla luciferase vector, pRL-SV40, was used as an internal control.

In this case, GS cells cultured in a 24-well plate were transfected with mixed plasmids (900 ng/well). The combinations of these mixed plasmids are: IFN-luc/ISRE-Luc/NF-κB-Luc (200 ng) + pRL-SV40 (100 ng) + pcDNA3.1-3HA (600 ng), IFN-luc/ISRE-Luc/NF-κB-Luc (200 ng) + pRL-SV40 (100 ng) + pcDNA3.1-3HA (300 ng) + pcDNA3.1-3HA-KLF9 (300 ng) and IFN-luc/ISRE-Luc/NF-κB-Luc (200 ng) + pRL-SV40 (100 ng) + pcDNA3.1-3HA-KLF9 (600 ng), respectively.

At 24 h post-transfection, GS cells were harvested and lysed to detect luciferase activity using a microplate reader (Thermo, USA).

2.8. RNA extraction and cDNA synthesis

Total RNA was extracted using the SV Total RNA Isolation System (Promega, USA) according to manufacturer instructions. RNA quality was visually assessed on 1.5% agarose gels. The reverse transcription of total RNA was performed using ReverTra Ace kit (Toyobo, Japan) according to manufacturer instructions. The qRT-PCR was performed in the Roche 480 Real Time Detection System (Roche, Germany). The conditions were: 95 °C for 5 min, then 45 cycles at 95 °C for 5s, 60 °C for 10s and 72 °C for 15s. The SGIV genes, *MCP* (major capsid protein), *LITAF* (lipopolysaccharide induced TNF-α factor), *VP19* (envelop protein) *ICP-18* (infected cell protein-18) and the RGNNV genes, *CP* (coat protein) and *RdRp* (RNA-dependent RNA polymerase), were assessed for expression profiles. Fourteen interferon related genes (including *IRF3, IRF7, IFN-γ, ISG15, MDA5* and *MXI*) and inflammatory cytokine genes (including *IL-1β, IL-8, TRAF6, MyD88, TNFα*and *NF-κB*) were also selected for expression profile assessment. The beta-actin gene was used as an internal control. The primers for these genes are shown (Table 1) and each assay was performed in triplicate. Triplicate Ct values were analyzed using the comparative Ct (ΔΔCt) method. All results were presented as mean ± SD values. Statistical significances were detected with Student *t*-tests using the SPSS software, version 19 (IBM, USA). Expression difference were categorized as significant when $p < 0.05$.

2.9. Western blot analysis

Western blotting analyses were performed to determine the effects of KLF9 overexpression on viral protein synthesis. The process of antibody preparation briefly is: firstly, the recombinant proteins of CP and MCP were expressed in *E. coli* BL21 as a fusion protein and purified. Then purified proteins were injected into the white New Zealand rabbits to raise polyclonal Abs.

The GS cells were transfected with pcDNA3.1-3HA-KLF9 over expression vector for 12 h and then inoculated with RGNNV or SGIV. After 24 h post infection, cells were collected for Western blotting assay. Results are presented as ratios of CP/β-actin or MCP/β-actin. Lysates from KLF9 over-expressed GS cells were incubated with anti-beta-actin (Proteintech, 60008-1-Ig, USA), MCP or CP antibodies at a dilution of 1:1000 for 2 h, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich, AP132, USA) or horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich, AP124, USA) at a dilution of 1:5000 for 1 h at room temperature. After this, the PVDF membranes were visualized with the Enhanced HRP-DAB Chromogenic Substrate Kit (Tiangen China). Quantification of protein blots was performed using Quantity One 1-D software (version 4.4.0) (Bio-Rad Laboratories).

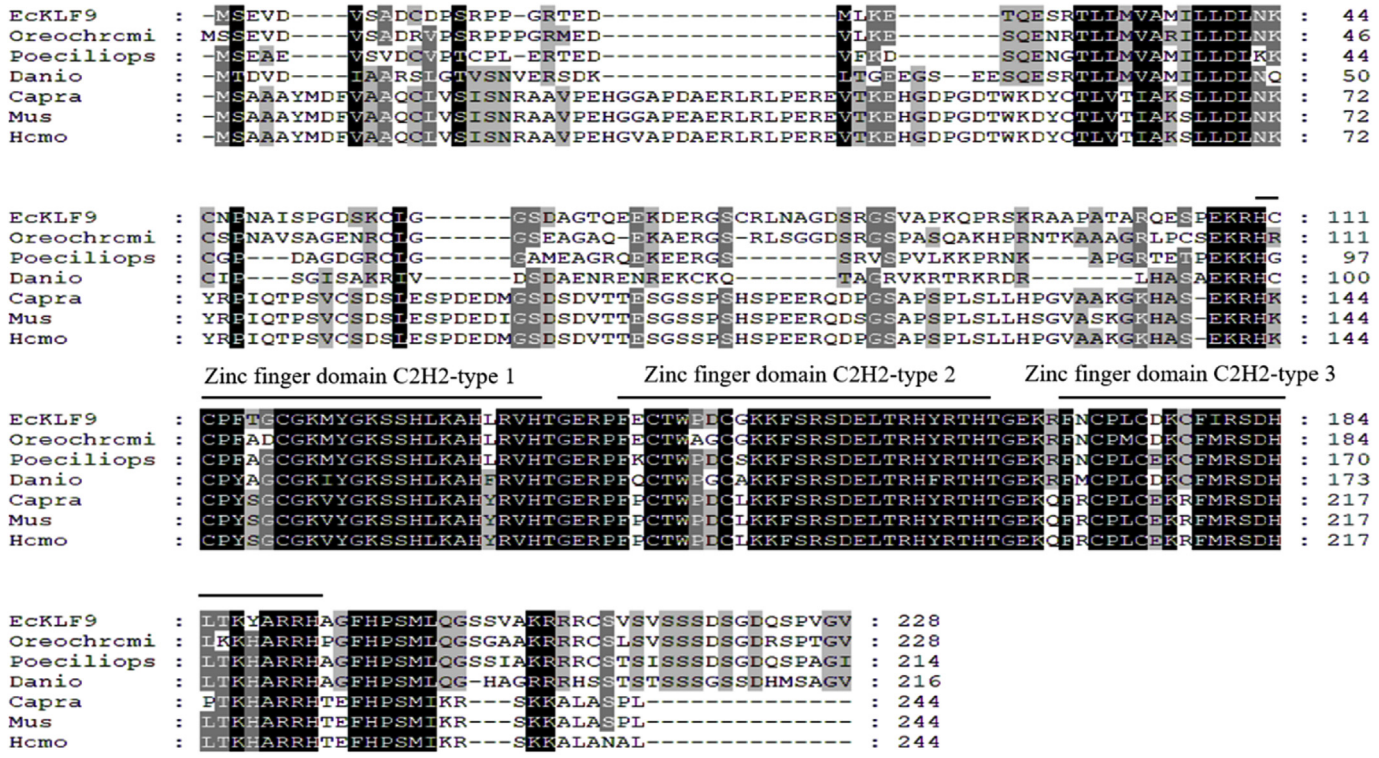

Fig. 1. Multiple sequence alignment of EcKLF9 and other KLF9 homologs from different species. Alignment of these KLF9 protein sequences were performed using ClustalX. The conserved Zinc finger domains were over lined.

3. Results

3.1. Sequence characterization of EcKLF9

Based on the transcriptome database in our laboratory, we amplified the open reading frame of grouper KLF9 (EcKLF9) using a PCR approach. The EcKLF9 open reading frame encoded a 228 amino acid protein (Fig. 1). Bioinformatics analyses demonstrated the protein had 57% identity with its human homolog, 56% identity with the *Mus musculus* homolog, 56% identity with the *Capra hircus* homolog, 59% identity with the *Danio rerio* homolog, 72% identity with the *Poeciliopsis prolifica* homolog and 77% identity with the *Oreochromis niloticus* homolog. Phylogenetic tree analyses showed that EcKLF9 was clustered with *Amphiprion ocellaris, Acanthochromis pollyacanthus* and *Stegastes partitus* (Fig. 2).

It has been reported that a characteristic feature of the KLF family is the presence of three Kruppel-like zinc fingers, which bind to DNA regions to mediate transcription activation or repression [5]. In this study, we analyzed these three Kruppel-like zinc finger domains of KLF9 from human, *M. musculus, C. hircus, D. rerio, P. prolifica* and *O. niloticus*. The comparison analysis showed the three Kruppel-like zinc finger domains from KLF9 were highly conserved across species, especially across different fish species.

These results suggest that, although the grouper EcKLF9 gene is not highly homologous with mammals, birds and even other fish, its core functional domains are quite conservative. Therefore, the regulation function of EcKLF9 on DNA transcription is similar to that of KLF9 in other species.

3.2. Expression patterns of the EcKLF9 gene

EcKLF9 expression profiles in different tissues from healthy groupers were analyzed using a real-time PCR approach. The results showed that EcKLF9 was constitutively expressed in all selected tissues in healthy groupers, with the highest expression detected in the gonads (Fig. 3). There was no significant difference in expression level between the tissues. Therefore, the mRNA of grouper EcKLF9 is widely expressed in various tissues like that in human [1,48].

To characterize KLF9 gene expression during viral infection, the temporal expression profiles of EcKLF9 in head kidney and spleen tissue, after SIGV and RGNNV stimulation, were determined by qRT-PCR. Results showed that the expression level of EcKLF9 in head kidney, spleen and brain at 6, 12, 24, 48 h after SGIV stimulation were all significantly upregulated compared that expression level at 0 h. And the expression level of EcKLF9 reached maximum levels at 12 h post-infection (Fig. 4A, B and 4E). Then at different time points (6, 12, 24, 48 h) after RGNNV stimulation, the expression profiles of EcKLF9 in head kidney, spleen and brain tissues were also all significantly upregulated compared that expression level at 0 h. And the maximum expression occurs at 24 h post-infection (Fig. 4B, D and 4F). This result suggests EcKLF9 play a regulatory role in the virus-stimulated immune response in grouper.

3.3. Subcellular localization of EcKLF9

To explore the *in vitro* localization of EcKLF9, pEGFP-EcKLF9 was constructed and transfected into GS cells and fluorescence observed using fluorescence microscopy. As shown (Fig. 5), green fluorescence was mainly observed in cytoplasm, surrounding the nucleus. This result suggests EcKLF9 is mainly a nuclear localized protein. As a control, the fluorescence of pEGFP-N3 transfected cells was found throughout the cytoplasm and nucleus. This subcellular localization result is similar to those in human, suggesting the sites where EcKLF9 functions in GS cells are consistent with those in human cells [40].

3.4. EcKLF9 overexpression promotes RGNNV and SGIV replication in vitro

To evaluate the potential role of EcKLF9 overexpression on fish virus replication, we transfected pcDNA3.1-3HA-EcKLF9 into GS cells, and then infected these cells with RGNNV or SGIV, separately for 48 h.

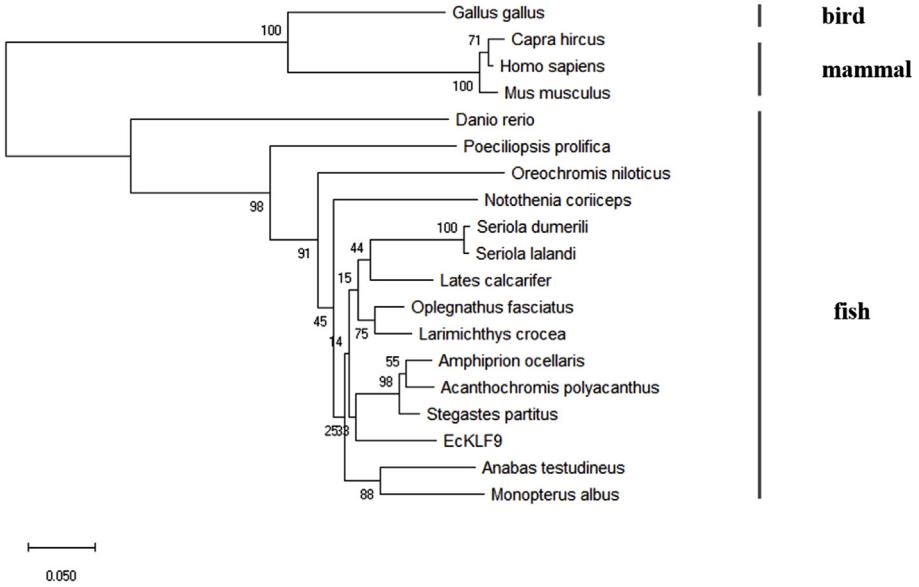

Fig. 2. Phylogenetic tree constructed based on the KLF9 homologs from different species. The tree was constructed with KLF9 gene sequences by using the neighbor-joining method in MEGA 6.0.

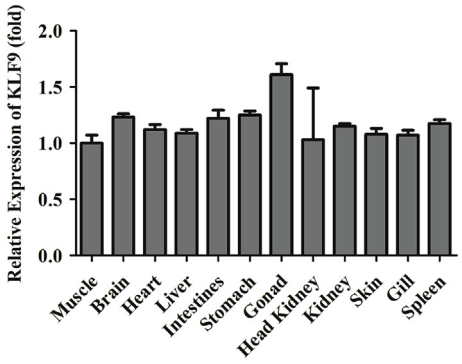

Fig. 3. The tissue distribution patterns of EcKLF9 in healthy grouper. Total RNAs were extracted from six healthy fish. The expression levels of EcKLF9 were examined using qRT-PCR. Setting mRNA expression level in muscle as 1-fold, mRNA expression in other tissues is relative to the expression in head kidney. Error bars indicate standard error (n = 5). There was no significant difference in expression level between the tissues.

Results showed that when compared to control transfected cells, the severity of vacuoles induced by RGNNV was significantly increased in EcKLF9 overexpressing cells and the severity of CPE induced by SGIV was also significantly increased (Fig. 6A).

Real-time PCR results showed that transcription levels of *CP* and *RdRp* (RGNNV genes) were significantly upregulated in RGNNV-infected EcKLF9-overexpressing cells (Fig. 6B). The transcription levels of *MCP*, *LITAF*, *ICP-18* and *VP19* (SGIV genes) were also significantly increased in SGIV infected EcKLF9 overexpressing cells, when compared to control no-transfected cells (Fig. 6C). Furthermore, western blot analysis showed that after 48 h post infection with RGNNV, CP protein expression was increased in EcKLF9 overexpressing cells (Fig. 7A). MCP protein levels were also increased in EcKLF9 overexpressing cells, upon infection with SGIV (Fig. 7B). These results suggested that the regulatory effect caused by the overexpression of EcKLF9 promoted the replication of both RNA and DNA viruses *in vitro*.

3.5. The ectopic expression of EcKLF9 inhibits interferon immune responses

To assess the effects of EcKLF9 overexpression on host interferon immune responses, the expression profiles of several interferon related cytokines or effectors were examined. The results showed that the interferon related genes, including *IRF3, IRF7, IFN-γF ISG15, MDA5* and *MXI* were all significantly decreased in EcKLF9 overexpressing cells, when compared to control no-transfected cells. Hence, EcKLF9 was speculated to negatively regulate the interferon immune response *in vitro* (Fig. 8A).

3.6. The ectopic expression of EcKLF9 inhibited several inflammatory cytokines

To further dissect the potential mechanism of EcKLF9 involvement in antiviral activities, we examined the effects of EcKLF9 on inflammatory responses. The expression levels of several inflammatory cytokines, including, *IL-1β*, *IL-8*, *MyD88, TRAF6*, *TNFα* and *NF-κB* were significantly decreased in EcKLF9 overexpressing cells, when compared to control transfected cells. Together, EcKLF9 was speculated to negatively regulate these inflammatory cytokines *in vitro* (Fig. 8B).

3.7. EcKLF9 inhibits the promoters of IFN, ISRE and NF-κB genes

To assess the effect of EcKLF9 overexpression on the promoter activity of interferon related genes and inflammatory factors, we evaluated the promoter activity of *IFN*, *ISRE* and *NF-κB* genes using transfection and luciferase reporter assays in EcKLF9 overexpressing cell lines. Results showed that EcKLF9 significantly inhibited the promoters of *IFN*, *ISRE* and *NF-κB* genes (Fig. 9).

4. Discussion

KLF9 is a member of the Krüppel-like factor (KLFs) family, which are transcription factors implicated in several biological processes, including cell proliferation, differentiation, development and apoptosis [2]. In mammals, Krüppel-like transcription factors (KLFs) are detect to consist of 17 zinc finger domain transcription factors which bind to GC- or GT-rich DNA regions of target genes to regulate transcription and thereby influence multiple biological events [4,5].

Krüppel-like factor-9 (KLF9), a relatively unexplored member of the large KLF family, has emerged as a regulator of oncogenesis, cell differentiation, and neural development. Many Studies have revealed that KLF9 performs important functions in human disease, especially during the occurrence and development of tumors, which suppresses the

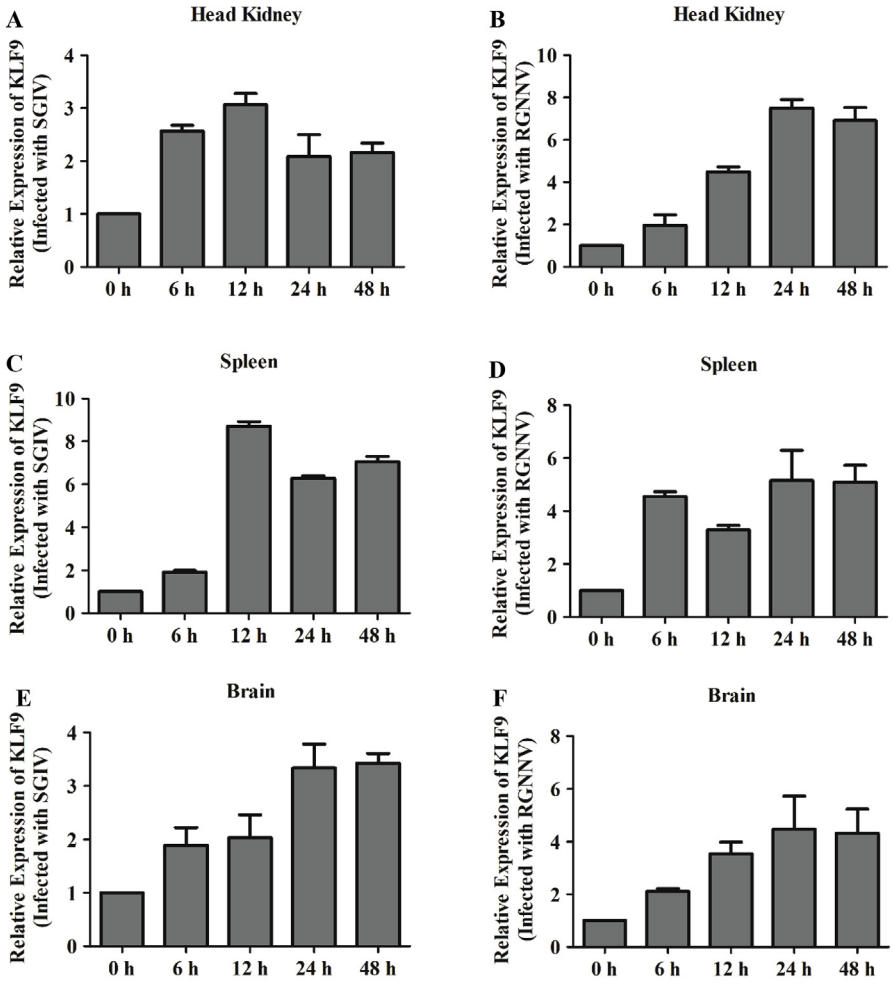

Fig. 4. Expression profiles of EcKLF9 in challenged grouper. (A), (C) and (E): expressions of EcKLF9 in head kidney (A), Spleen (C) and brain (E) at different time points post SGIV stimulation. (B), (D) and (F): expressions of EcKLF9 in Head Kidney (B), spleen (D) and brain (F) at different time points post NNV stimulation. The beta-actin gene was employed as an internal control. mRNA expression level in 0 h was set as 1-fold. The expression level of EcKLF9 in head kidney and spleen at 6, 12, 24, 48 h after SGIV stimulation were all significantly upregulated compared that expression level at 0 h ($P < 0.05$) (A and C). At different time points (6, 12, 24, 48 h) after RGNNV stimulation, the expression profiles of EcKLF9 in head kidney and spleen tissues were also all significantly upregulated compared that expression level at 0 h ($P < 0.05$) (B, D and E).

growth of tumors cells [41,42,47]. Whereas, the molecular basis for the diverse functions of KLF9 remains unclear. The genome-wide gene targets of KLF9 have not been identified, and the molecular mechanism underlying the functional contributions of KLF9 to many biological and cellular processes remains elusive.

In human cancer cells, Ying et al. [49] used RNA-Seq and ChIP-Seq (chromatin immunoprecipitation followed by deep sequencing) to investigate genome-wide KLF9 binding sites and KLF9-regulated genes. In this test, a total of 31,261 possible KLF9 targets genes were detected, and among them, 1849 gene targets were identified to be directly

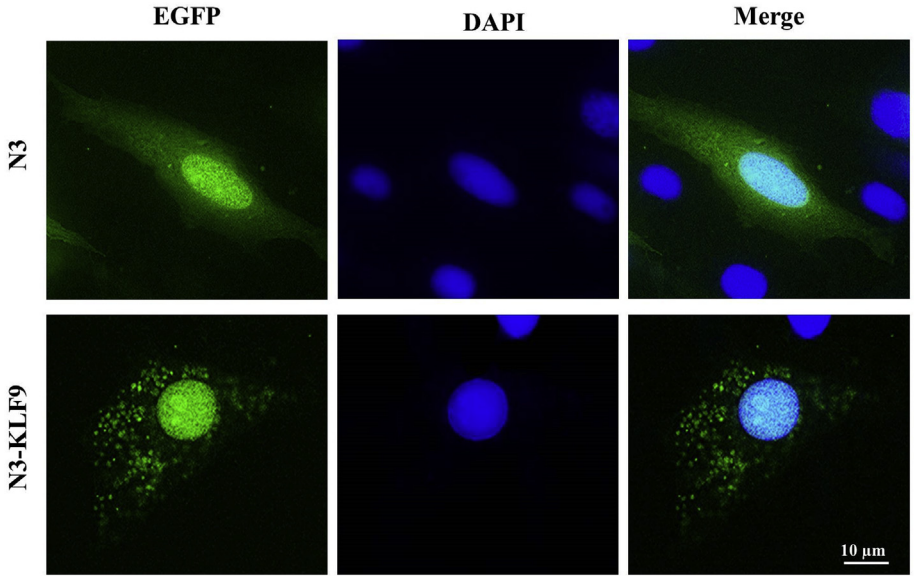

Fig. 5. Cellular localization of EcKLF9. EcKLF9 was localized to the specific cytoplasmic site in GS. A total of 5 × 105 GS cells were seeded onto the coverslips in 24-well plates the day before the transfection. Cells were transfected with the pEGFP-N1-KLF9 plasmid using Lipofectamine 2000. After 24 h, fixed cells on the coverslips were stained with PI and imaged by fluorescence microscopy. The subcellular localization of N3 is showed in the upper line, and the subcellular localization of EcKLF9 is showed in the bottom line.

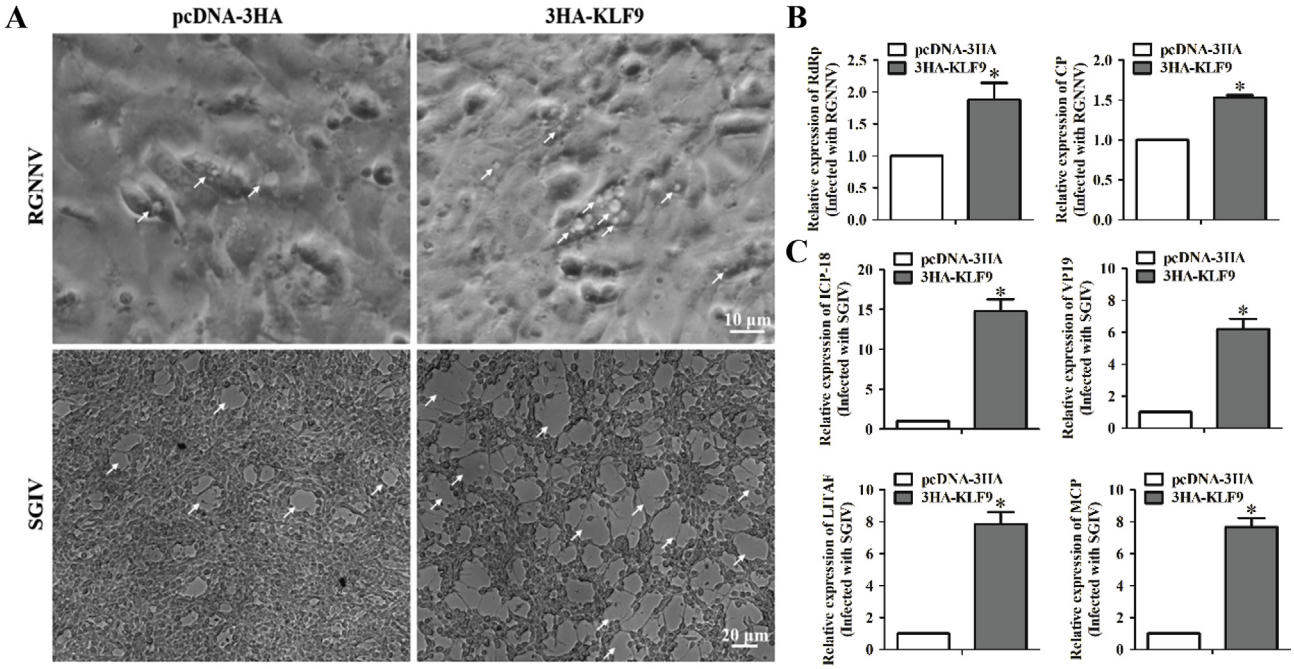

Fig. 6. The effect of EcKLF9 overexpression on virus replication. (A)The small white arrows showed that CPE progression induced by RGNNV and SGIV infection. GS cells were transfected with EcKLF9, and then infected with RGNNV or SGIV. The morphology of CPE induced virus infection at 48 h post infection were imaged under light microscopy. (B and C) The viral gene transcription of RGNNV or SGIV in EcKLF9 overexpressing cells. After transfection with EcKLF9, GS cells were infected with RGNNV or SGIV for 48 h. Infected cells were collected for RNA extraction, and the relative expression of viral genes, including RdRp, CP, ICP-18, VP19, LITAF and MCP were examined using qRTPCR.

down-regulated by KLF9. Gene function annotation and pathway analyses revealed that signaling pathways relevant to cancer signaling, stem cell regulation, and neural cell function are enriched in KLF9 downregulated gene targets. It shows that KLF9 has a wide range of regulatory functions, and its regulatory role is dominated by negative regulation.

In our study, we cloned the grouper *KLF9* gene (*E. coioides KLF9* gene, EcKLF9 for short), which encoded a protein of 228 amino acids. The length of EcKLF9 was slightly different to its human, mouse, zebra fish and *O. niloticus* homologs (Genbank number are, respectively: NP_001197.1, AAI38725.1, NP_001122201.1 and XP_003452699.1). Blastp analysis showed that EcKLF9 shared 57%, 56%, 56%, 59%, 72% and 77% identity with human, *M. musculus*, *C. hircus*, *D. rerio*, *P. prlifica* and *O. niloticus,* respectively. Domain analysis showed that EcKLF9 contained a conserved DNA-binding region (three tandem zinc fingers) at the carboxyl terminal (Fig. 1), suggesting EcKLF9 also possess transcriptional activation or inhibition function in grouper as its homologies of other vertebrate animals. Moreover, localization analysis showed that EcKLF9 was primarily co-localized with the cell nucleus and cytoplasm. These results were consistent with the human localization [40], indicating EcKLF9 sharing similar activity place with mammals.

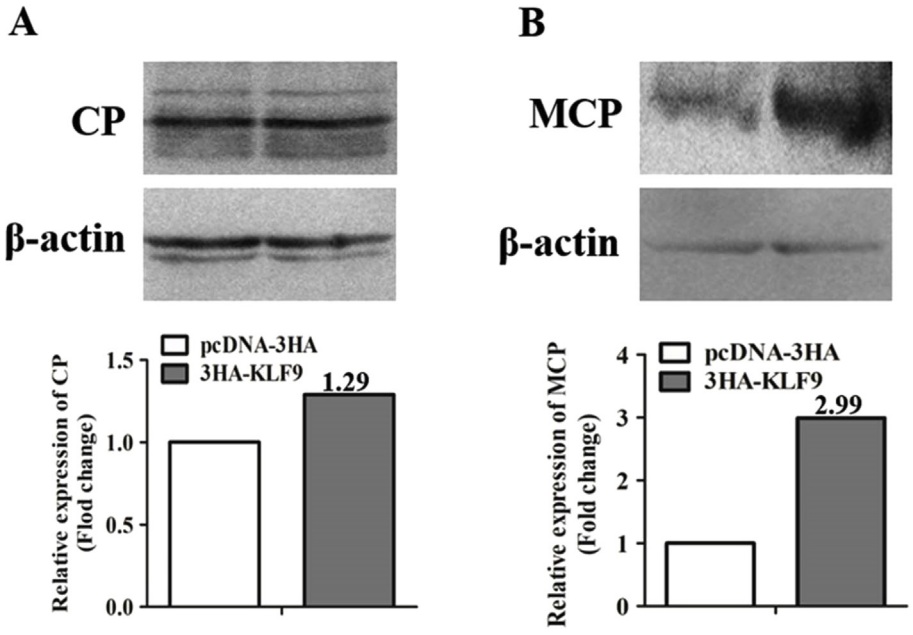

Fig. 7. Determination of the viral yields in EcKLF9 over expression GS cells using Western blotting analysis and plaque assay. The GS cells were transfected with EcKLF9 over expression vector and then infected with SGIV or RGNNV. After 24 h post infection, cells were collected for Western blotting assay. Then, MCP/CP conversions were evaluated using Western-blotting (WB)-based quantitation of the MCP/beta-actin (A) or CP/beta-actin (B) intensity ratio.

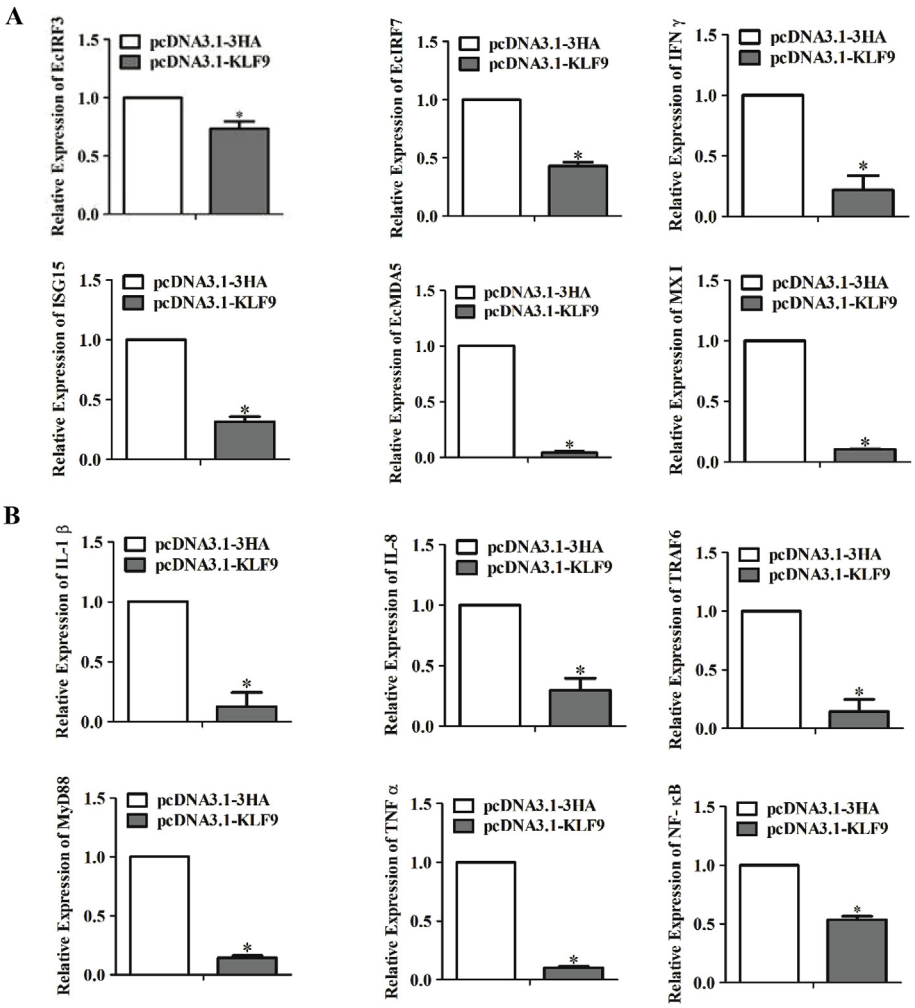

Fig. 8. The ectopic expression of EcKLF9 decreased the expression of interferon related immune genes and inflammatory factors genes. (A)The expression levels of interferon signaling molecules, including IRF3, IRF7, IFN-γ, ISG15, MDA5 and MXI were examined. (B) The expression levels of different inflammatory cytokines including IL-1β, IL-8, TRAF6, MyD88, TNFα and NF-κB were examined. GS cells were transfected with EcKLF9 and empty vector, and then cells were collected at 24 h post infection for RNA extraction and qRT-PCR. The beta-actin gene was employed as an internal control. mRNA expression level in GS cells which were transfected with empty vector was set as 1-fold. Asterisks (*) mark the significant difference between experimental and control groups ($P < 0.05$).

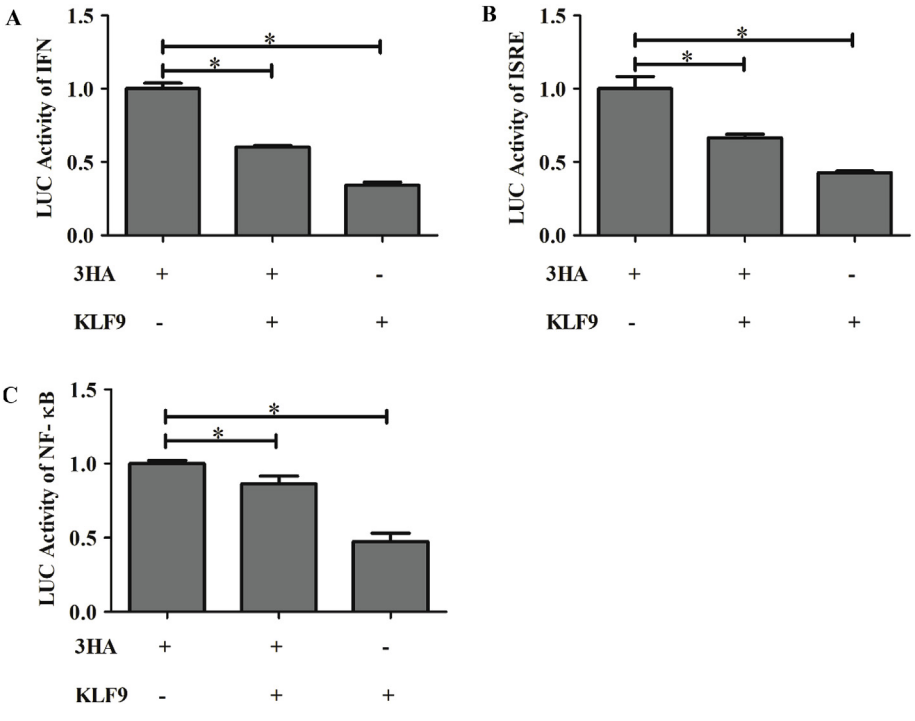

Fig. 9. EcKLF9 overexpression inhibited IFN promoter, ISRE promoter and NF-κB response promoter. (A) EcKLF9 expression vector with different amounts (0, 300 and 600 ng) or empty vector was cotransfected with the IFN luciferase reporter vector (200 ng) and pRL-SV40 Renilla luciferase vector (100 ng) into GS cells. (B) EcKLF9 expression vector with different amounts (0, 300 and 600 ng) or empty vector was cotransfected with the ISRE luciferase reporter vector (200 ng) and pRL-SV40 Renilla luciferase vector (100 ng) into GS cells. (C) EcKLF9 expression vector with different amounts (0, 300 and 600 ng) or empty vector was cotransfected with the NF-κB luciferase reporter vector (200 ng) and pRL-SV40 Renilla luciferase (100 ng) vector into GS cells. After 24 h, luciferase vs Renilla luciferase activities in cell lysates were measured and expressed as the fold stimulation. All data are representative of three independent experiments.

In grouper cells, the over-expression of EcKLF9 significantly decreased the expression of interferon related genes, including interferon gene (*IFN-γ*), interferon regulation factors (*IRF3*, *IRF7*) and interferon induced/stimulated genes (*ISG15, MDA5* and *MXI*). Similarly, the ectopic expression of EcKLF9 significantly decreased interferon promoter activity. Considering the critical role of KLF9 in transcription regulation, we speculate that there were EcKLF9 target genes existed in those detected interferon related genes. Actually, such deduction had ever been identified in mammals. To detect the effect of KLF9 level on intestinal tumorigenesis in humans, Brown et al. [26] found that a number of IFN-stimulated genes, including *Isg15*, *Usp 18*, *Oasl2*, *If47*, *Ift3*, *Cd274*, *Cxcl9*, *Trim 30* and *Igtp* were significantly upregulated in *ApcMin/ + /Klf9 ±* and/or *ApcMin/ + /Klf9 −/−* compared to *ApcMin/ + /Klf9 + / +* mice (the *ApcMin/ +* mouse has served as the most widely used model of intestinal neoplasia). Further analysis in human HT29 colorectal cancer cells showed that the reduction of KLF9 expression with siRNAs also caused a similar increase in expression of IFN-responsive genes, suggesting KLF9 down-regulates interferon signaling pathway in cancer cells. Then, Chromatin Immunoprecipitation test demonstrated KLF9 recruitment to the *ISG15* promoter.

Moreover, the overexpression of EcKLF9 significantly decreased the expression of inflammatory factor related genes, comprising *IL-1β*, *IL-8*, *TRAF6*, *MyD88, TNFα* and *NF-κB*. The ectopic expression of EcKLF9 also significantly decreased *NF-κB* promoter activity. This may suggest that EcKLF9 may play a regulatory role in inflammatory signaling networks like its homology in human. Research on human KLF9 found that myometrium from women with late-term pregnancy showed lower KLF9 expression compared with term pregnancy myometrium. Moreover transcript levels of several chemokines/cytokines were up- (*CSF3*, *IL1*, *IL12A*, *TGFB2*) and down- (*CCL3*, *CCL5*, *CXCL1*, *CXCL5*, *IL15*) regulated in late-term relative to term myometrium. To determine whether KLF9 directly regulates induction of inflammatory signals, Human myometrial cells transfected with nontargeting or KLF9 small interfering RNAs (siRNA) were treated with the progesterone antagonist RU486 and analyzed for pro-inflammatory chemokine/cytokine gene expression. The results showed that loss of KLF9 *in vitro* attenuated the expression of a subset of proinflammatory cytokines, including *CCL3*, *CXCL1*, *CXCL5* and *IL6*. Hence, these collective findings suggest that many proinflammatory cytokines may be the direct targets of KLF9, and moreover KLF9 play different regulation role on various proinflammatory cytokines.

To investigate the effects of EcKLF9 on fish viruses infection, we evaluated the CPE progression and detected the viral gene transcription of SGIV and RGNNV in infected EcKLF9 overexpressing cells, respectively. The results showed EcKLF9 also promoted the expression of the SGIV related genes, *MCP, LITAF, ICP-18* and *VP19* and the RGNNV related genes *CP* and *RdRp in vitro*. The protein expression levels of the SGIV related gene, MCP and the RGNNV related gene, CP were also promoted by EcKLF9. Therefore, we believe that EckLF9 could promote the replication of SGIV and NNV in GS cells. Since our experiments also showed that KLF9 inhibits interferon and inflammatory signaling pathways, and both inflammatory factors and interferon have a great influence on viral infection and replication, then the antiviral effect of these detected interferon-related gene and inflammatory cytokines has already been confirmed in our previous studies in grouper [28,29,38,39,43–46]. Therefore, we consider that the promotion effect of KLF9 on SGIV and NNV replication is caused by its down-regulation on interferon and inflammatory response.

In summary, we identified KLF9 gene characteristics from the grouper, and demonstrated EcKLF9 function in response to grouper viral infection. Our data indicated that EcKLF9 contained a conserved DNA-binding region (three tandem zinc fingers) and encoded a nucleus and cytoplasm localized protein, suggesting conserved functions from fish to mammals. Transcription levels of EcKLF9 in immune tissues were upregulated in response to SGIV and RGNNV infection. Furthermore, the ectopic expression of EcKLF9 significantly promoted SGIV and RGNNV replication, due to its inhibitory effects on grouper interferon immune responses and inflammatory responses. Our results revealed that KLF9 functions in the innate immune systems of teleosts.

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