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PPAR-δ of orange-spotted grouper exerts antiviral activity against fish virus and regulates interferon signaling and inflammatory factors

Yuxin Wang, Yepin Yu, Qing Wang, Shina Wei, Shaowen Wang, Qiwei Qin**, Min Yang*

Joint Laboratory of Guangdong Province and Hong Kong Region on Marine Bioresource Conservation and Exploitation, College of Marine Sciences, South China Agricultural University, Guangzhou, 510642, China

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ABSTRACT

Peroxisome proliferator-activated receptor δ (PPAR-δ), also called PPAR-β or PPAR-β/δ, is a member of the peroxisome proliferator-activated receptor (PPAR) family, which belongs to the nuclear steroid receptor superfamily. Activated PPARs participate in the regulation of lipid and glucose metabolism and also affect cellular proliferation, differentiation, and apoptosis, and the immune responses. To investigate the roles of PPAR-δ in *Singapore grouper iridovirus* (SGIV) infection, we cloned and characterized the gene encoding a PPAR-δ homologue from the orange-spotted grouper, *Epinephelus coioides* (EcPPAR-δ). EcPPAR-δ encodes a 514-amino-acid polypeptide, with 95.29% and 74.76% homologue to the *Seriola dumerili* and human proteins, respectively. EcPPAR-δ contains a typical DNA-binding domain and a ligand-binding domain. Its expression was induced by SGIV infection *in vitro*. A subcellular localization analysis showed that EcPPAR-δ localizes throughout the cytoplasm and nucleus, with a diffuse intracellular expression pattern. SGIV replication was reduced by EcPPAR-δ overexpression, which was evident in the reduced severity of the cytopathic effect, reduced viral gene transcription, and the reduced expression of the viral capsid protein. The replication of SGIV increased with the knockdown of EcPPAR-δ. The overexpression and silencing of EcPPAR-δ in grouper spleen cells showed that EcPPAR-δ plays a positive role in the regulation of the interferon signaling pathway, but has an anti-inflammatory effect on the inflammatory response. The anti-inflammatory effect of EcPPAR-δ may be related to its function in maintaining cell homeostasis. Because the interferon signaling pathway plays an important role in antiviral immune responses, we speculate that the activation of the interferon signaling pathway by EcPPAR-δ overexpression underlies its inhibitory effect on SGIV replication. Together, our data greatly extend our understanding of the roles of the EcPPAR-δ family members in the pathogenesis of fish viruses.

1. Introduction

Peroxisome proliferator-activated receptor δ (PPAR-δ), also called PPAR-β or PPAR-β/δ, is a member of the PPAR family, which belongs to the nuclear steroid receptor superfamily. PPARs act as transcription factors, forming heterodimers with the retinoid X receptor and then binding to specific PPAR response elements (PPREs) located in the promoter regions of their target genes [1]. Activated PPARs participate in the regulation of lipid and glucose metabolism and also affect cellular proliferation, differentiation, and apoptosis [2]. In addition to these actions, PPARs also have important regulatory functions in the immune and inflammatory responses [3–6]. Research has demonstrated that both the innate and adaptive immune systems are strongly influenced by the activation of PPAR on macrophages and other leukocyte populations, such as lymphocytes and dendritic cells [7,8]. It has recently been reported the host-virus interaction with various responses, such as interference, inflammation, immune evasion, etc., may also be the result of gene expression induced by PPAR proteins after viral infection [9]. Many researchers have also provided valuable insights into the mechanisms of viral immune evasion in aquatic animals [10], which may identify new targets of PPARs. However, only a few studies have focused on PPARs in the immune systems of fish. The teleost PPAR sequences and tissue distributions for *Megalobrama amblycephala* [11,12] and *Salmo salar* [13] have been reported, as has an expression analysis of PPAR-γ in the orange-spotted grouper after challenge with *Vibrio alginolyticus* [14]. Other than those studies, there has been little research into members of the PPAR family in fish.

Because of the considerable economic value of groupers (*Epinephelus*

* Corresponding author.
** Corresponding author.
E-mail addresses: qinqw@scau.edu.cn (Q. Qin), yangmin2016@scau.edu.cn (M. Yang).

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sp.) in tropical and subtropical regions and particularly in southeast Asia, research into these species has received a great deal of attention [15,16]. Groupers are also considered to be an ideal research model for studies of the development and reproduction of the lower vertebrates, especially in terms of sex determination and sexual differentiation [17,18]. However, in recent years, frequent outbreaks of viral diseases have caused huge economic losses in the grouper industry [16]. *Singapore grouper iridovirus* (SGIV) is one of the main viral pathogens involved, and outbreaks of disease caused by SGIV generally occur in fry and adult groupers. The disease lasts for several weeks and the mortality rate is more than 90% [19]. SGIV belongs to the genus *Rana virus* in the family *Iridovirdae*, and is a novel marine fish DNA virus initially isolated from the brown-spotted grouper *E. tauvina.* [19]. Its four genes are usually used as the main indicators of viral replication: *MCP* (major capsid protein), the only capsid protein of SGIV [20]; *LITAF* (lipopolysaccharide-induced TNF-α factor), one of the early-expressed viral genes, which encodes a protein that regulates the transcription of the host's tumor necrosis factor α (TNF-α) gene [21]; *VP19* (envelope protein 19), an envelope protein conserved in iridovirus [22]; and *ICP-18* (infected cell protein 18), an immediate-early gene, which is overexpressed to support the replication of SGIV [23].

To identify the defense mechanisms of the grouper against viral infection, a large number of immune-related genes were cloned, and the roles of these genes during the process of virus infection were studied [24–27]. In our previous study, a transcriptomic analysis of virus-infected groupers suggested that some enriched genes in PPAR pathways, including PPAR-α and PPAR-δ, may be associated with the trait of disease susceptibility or disease resistance in the grouper [28]. In the present study, we characterized the *E. coioides* PPAR-δ gene (EcPPAR-δ) and examined its role in fish viral infections. We investigated the antiviral roles of EcPPAR-δ during the replication of SGIV and its immunomodulatory effects in grouper spleen cells. Our functional studies of EcPPAR-δ in teleosts may have applications in the immunological control of viral diseases.

2. Materials and methods

2.1. Fish, cells, and viruses

Orange-spotted groupers weighing 50 ± 10 g were purchased from a fish farm in Wenchang city, Hainan Province, China. The fish were acclimatized in a seawater recirculation system at 25–30 °C and fed twice daily for 2 weeks before their experimental manipulation. The temporary rearing conditions have been described in previous studies [29,30].

The fish were anesthetized by immersion in 400 mg/L Eugenol (Borui, China). A series of tissue samples, including the liver, spleen, kidney, head kidney, brain, gill, heart, stomach, intestine, muscle, and skin, were collected, immediately frozen in liquid nitrogen, and then stored at −80 °C until use. This study was performed in accordance with the protocols of the Experimental Animal Welfare Ethics Committee of South China Agricultural University, in the Guangzhou city of Guangdong Province, P. R. China.

A grouper spleen cell (GS) line was grown in Leibovitz's L15 medium containing 10% fetal bovine serum (Gibco, USA) at 28 °C. SGIV is maintained in our laboratory and is stored at −80 °C. The viral titer of SGIV used was 1×10^6 median tissue culture infective doses ($TCID_{50}$).

2.2. Challenge experiment in vitro

The GS cells were injected with 10 μL of 1×10^6 $TCID_{50}$ SGIV, and the control group was injected with 10 μL of phosphate-buffered saline (PBS). The GS cell samples were then collected at 0, 6, 12, 18, and 24 h after challenge and stored at −80 °C until further analysis.

2.3. RNA extraction and cDNA library construction

Total RNA was extracted from tissues and cells with TRIzol Reagent (TaKaRa, Japan), according to the manufacturer's instructions. RNA integrity was evaluated by the separation of an aliquot on 1.0% agarose gel. The RNA was then reverse transcribed into cDNA with a PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa).

2.4. Molecular cloning of EcPPAR-δ gene and sequence analysis

To amplify the core sequence of the EcPPAR-δ gene, a gene-specific primer pair was designed with Primer Premier 5.0 based on our previous transcriptome data for the orange-spotted grouper [28]. The primer details are shown in Supplementary Table 1. The PCRs were performed in a total volume of 10 μL, which included 5 μL of premix *Taq* DNA polymerase (TaKaRa), 0.5 μL (20 ng) of cDNA, 0.25 μL (10 μM) of each primer, and 4 μL of ddH_2O. The PCR thermal cycling program was: initial denaturation at 94 °C for 5 min; 30 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min; a final extension step at 72 °C for 10 min; and 16 °C for 5 min.

A sequence similarity analysis was performed with the BLAST program at the National Center of Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi). The conserved domains were predicted based on human and other mammalian sequences. A multiple sequence alignment was constructed with the Clustal X1.83 software and edited with the GeneDoc software. A phylogenetic tree was constructed with the Molecular Evolution Genetics Analysis (MEGA) software version 6.0 using the boot-strapped neighbor-joining method (with 1000 replicates).

2.5. Plasmid construction

To construct the expression vector for EcPPAR-δ, the EcPPAR-δ open reading frame (ORF) sequence was amplified with primers PPAR-δ-F and PPAR-δ-R containing restriction sites *Xho*I and *Kpn*I, respectively. The target amplicon was digested and inserted into the pEGFP-C1 vector. The primer sequences are listed in Supplementary Table 1. The resulting recombinant plasmid was confirmed with DNA sequencing and BLAST in the GenBank database. Clones with the correct sequence were used for the subsequent experiments. The MITA expression vector pcDNA3.1-Flag–MITA was constructed in previous research in our laboratory [31]. Endotoxin-free plasmids were obtained with the Ezgene EndoFree Plasmid Kit (BioMIGA, USA). Luciferase reporter vectors for interferon α (IFN-α), IFN-γ, the interferon-stimulated response element (ISRE), and NF-κB were obtained from Clontech Biotechnology Company (USA), and used to analyze the IFN and NF-κB signaling activities in the GS cells. The pRL-SV40 *Renilla* luciferase vector (Promega, USA) was used as the internal control. The GS cells were transfected with the vectors using Lipofectamine 2000 Transfection Reagent (Invitrogen, USA).

2.6. Cellular localization analysis

GS cells were transfected with recombinant plasmid pEGFP-C1–EcPPAR-δ, and 24 h after transfection, the cell samples were washed twice with PBS and fixed with 4% paraformaldehyde at 4 °C for 1 h. After the cells were stained with 6-diamidino-2-phenylindole (DAPI) for 10 min, they were observed with fluorescence microscopy (Zeiss, Germany).

2.7. Luciferase reporter gene assay

Luciferase reporter gene assays were performed to investigate the functional properties of EcPPAR-δ in the IFN and NF-κB signaling pathways. GS cells were cultured in a 24-well plate and transfected with mixed plasmids (1000 ng/well), which consisted of 800 ng control plasmid/pEGFP-EcPPAR-δ plasmid, 150 ng IFN-α-luc/IFN-γ-luc/ISRE-luc/NF-κB-luc reporter plasmid (Clontech, USA) and 50 ng pRL-SV40 reference plasmids. After transfection for 24 h, the cells were collected and lysed with Passive Lysis Buffer (Promega, USA). The luciferase reporter activity was detected with the Dual-Luciferase Kit (Promega, USA) and the luminescence intensity was measured with a Modulus Microplate Multimode Reader (Thermo Scientific, USA). This experiment was performed in triplicate and the results are expressed as means ± standard errors of the means (SEM). Significant differences were detected with a *t*-test in the SPSS 19.0 software (SPSS, USA).

2.8. Western blotting

The effects of EcPPAR-δ overexpression or silencing on the synthesis of SGIV viral proteins were determined with a western blotting analysis.

An antibody directed against viral protein MCP was prepared in our laboratory [25]. Briefly, a recombinant plasmid expressing MCP was constructed and used to transform *Escherichia coli* BL21 (Tiangen, China). The recombinant MCP protein were expressed in the *E. coli* BL21 cells as a fusion protein and purified. The purified MCP protein was then injected into New Zealand White rabbits to produce polyclonal antibodies (Abs). As an internal reference, the expression level of β-actin was detected with a rabbit *anti*-β-actin antibody (Proteintech, USA).

GS cells were transfected with pEGFP-C1–PPAR-δ or small interfering RNA (siRNA)-PPAR-δ, and the control group was transfected with pEGFP-C1 or the siRNA-negative control. After transfection for 24 h, the GS cells were inoculated with SGIV. At 24 h post infection, the cells were harvested and lysed. The lysates were loaded onto a 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel for electrophoresis (SDS-PAGE), followed by western blotting onto polyvinylidene difluoride (PVDF) membranes, as described previously [25].

The proteins were incubated with *anti*-β-actin or *anti*-MCP primary antibody, diluted 1:1,000, for 2 h at 4 °C, and then treated with peroxidase-conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich, USA), diluted 1:5,000, for 1 h at room temperature. The proteins on the PVDF membranes were visualized with the Enhanced HRP-DAB Chromogenic Substrate Kit (Tiangen, China). The protein blots were quantified with the Quantity One 1-D software (version 4.4.0) (Bio-Rad, USA).

2.9. RNA interference (RNAi) in vitro

Three siRNAs directed against different fragments of the EcPPAR-δ mRNA sequence were synthesized by Ribo Biotechnology Company (Guangzhou, China) to silence the expression of the EcPPAR-δ gene in GS cells. GS cells in 24-well plates were transfected with one or other of the siRNAs (50 ng per well). The efficiency of RNAi was determined with Real time PCR 24 h after siRNA injection.

2.10. Effects of EcPPAR-δ overexpression or knockdown on SGIV infection

To determine the roles of EcPPAR-δ in SGIV infection, GS cells were manipulated with gene overexpression or RNAi. In this experiment, GS cells were transfected with pEGFP-C1–EcPPAR-δ or siRNA-EcPPAR-δ, and the control cells were transfected with pEGFP-C1 or siRNA-negative control. At 24 h posttransfection, the transfected cells were incubated with SGIV at a multiplicity of infection (MOI) of 1. After incubation with SGIV for 24 h, the morphology of the SGIV-infected cells was observed with phase-contrast microscopy and photographed. The mock- and virus-infected cells were collected at 24 h postinfection for RNA extraction and RT-PCR analysis.

A viral titer assay was also performed to determine the effects of EcPPAR-δ on SGIV infection. GS cells were transfected with pEGFP-C1–EcPPAR-δ for 24 h, and the transfected cells were infected with SGIV at an MOI of 1. The virus-infected cells were harvested at 24 h post infection, lysed with three freeze-thaw cycles, and the viral titers determined with a $TCID_{50}$ assay [30]. The cytopathic effect was observed daily with light microscopy (Leica, Germany).

The transcriptions of the SGIV genes *MCP*, *LITAF8*, *VP19*, and *ICP-18* were detected with real-time quantitative PCR (qPCR). β-Actin was used as the reference gene. The primers used in this experiment are listed in Supplementary Table 1. Triplicate Ct values were analyzed with the comparative Ct (ΔΔCt) method. All results are presented as means ± SD. Statistical significance was determined with Student's *t*-test using the SPSS software, version 19 (IBM, USA). Differences in expression were deemed significant when $p < 0.05$.

2.11. Effects of EcPPAR-δ on cytokine expression

To evaluate the effects of EcPPAR-δ on cytokine expression *in vitro*, the expression levels of some cytokines in EcPPAR-δ-overexpressing cells were determined with qPCR. The selected cytokine genes encoded IFN-related proteins (IRF3, IRF7, IFN-γ, ISG15, melanoma differentiation associated protein 5 [MDA5], and myxovirus resistance 1 [MXI]) and inflammatory cytokines (IL-1β, IL-8, TNFR-associated factor 6 (TRAF6), MyD88, TNF-α, and NF-κB). The corresponding primers are listed in Supplementary Table 1. The procedures were performed as described above, in triplicate.

To further clarify the role of EcPPAR-δ in the upstream IFN signaling pathway, a co-transfection experiment with EcPPAR-δ and MITA was performed. In brief, GS cells were transfected with pEGFP-C1 (800 ng) or pEGFP-C1–MITA (400 ng) + pEGFP-C1 (400 ng) or pEGFP-C1–MITA (400 ng) + pEGFP-C1 (200 ng) + pEGFP-C1–EcPPAR-δ (200 ng). At 24 h posttransfection, the mock-transfected and transfected cells were collected for RNA extraction and qPCR analysis. The expression levels of the IFN-related genes (*IFP35*, *IRF3*, *IRF7*, *ISG15*, and *ISG56*), induced by MITA, were determined with qPCR.

3. Results

3.1. Sequence characterization of EcPPAR-δ

Using PCR assays, the ORF sequence of EcPPAR-δ was obtained from the orange-spotted grouper and deposited in GenBank (accession number: MN103549). The EcPPAR-δ ORF cDNA was 1,545 bp in size, encoding a protein of 514 amino acids (Fig. 1) with a deduced molecular weight of 57.85 kDa and a predicted isoelectric point of 6.13.

Based on the SMART program, a DNA-binding domain (DBD; residues 148–218) and a ligand-binding domain (LBD; residues 328–487) were predicted in the middle part of the amino acid sequence of EcPPAR-δ (Fig. 1).

A phylogenetic tree was constructed with the neighbor-joining method to analyze the evolutionary relationships between PPAR-δ homologues. As shown in Supplementary Fig. 1, the fish PPAR-δ sequences clustered on one main branch of the phylogenetic tree, the bird and mammal PPAR-δ sequences clustered together on another main branch, and PPAR-δ of *Xenopus laevis* occurred alone on another branch. Of the fish PPAR-δ sequences, EcPPAR-δ was most closely related to the PPAR-δ proteins of *Labrus bergylta* and the large yellow croaker *Larimichthys crocea*.

Using an NCBI BLAST analysis, we compared the amino acid sequences of the PPAR-δ proteins from multiple species. EcPPAR-δ shared relatively high identity with *Seriola dumerili* PPAR-δ (95.29%), 92.2% identity to the *L. crocea* homologue, 75.0% identity with the *Danio rerio* homologue, 70.2% identity with the *X. laevis* homologue, 73.4% identity with the *Mus musculus* homologue, and 74.8% identity with the *Homo sapiens* homologue.

The sequence alignment suggested that the amino acid sequences included in the DBD and LBD were conserved, and the amino acid sequence in the C-terminal region was highly conserved.

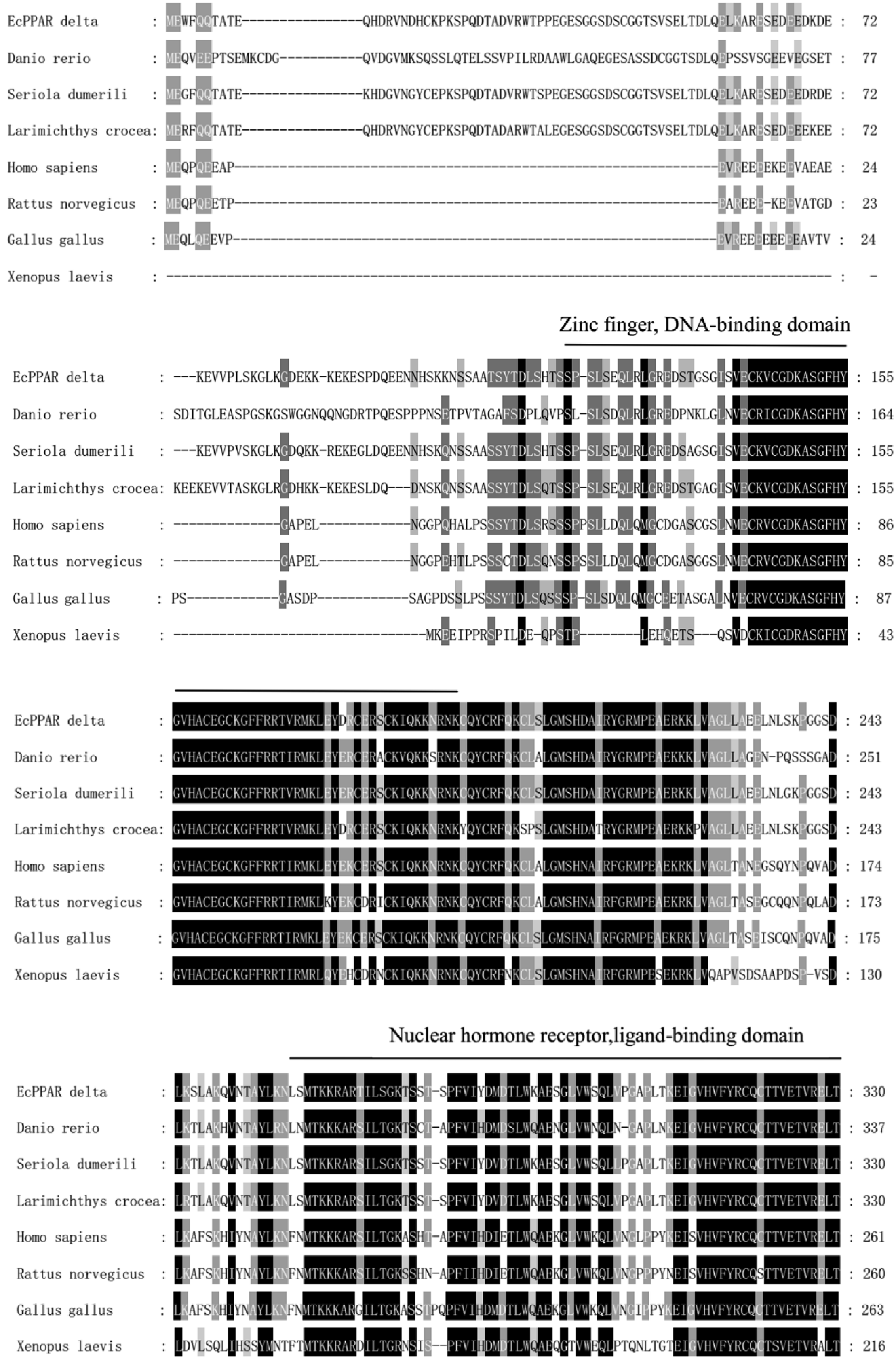

Fig. 1. Multiple sequence alignment of EcPPAR-δ and other PPAR-δ homologues from different species. Alignment of these PPAR-δ protein sequences were performed using ClustalX. The containing two domains, Zinc finger, DNA binding domain and Nuclear hormone receptor, ligand-binding domain, were over lined.

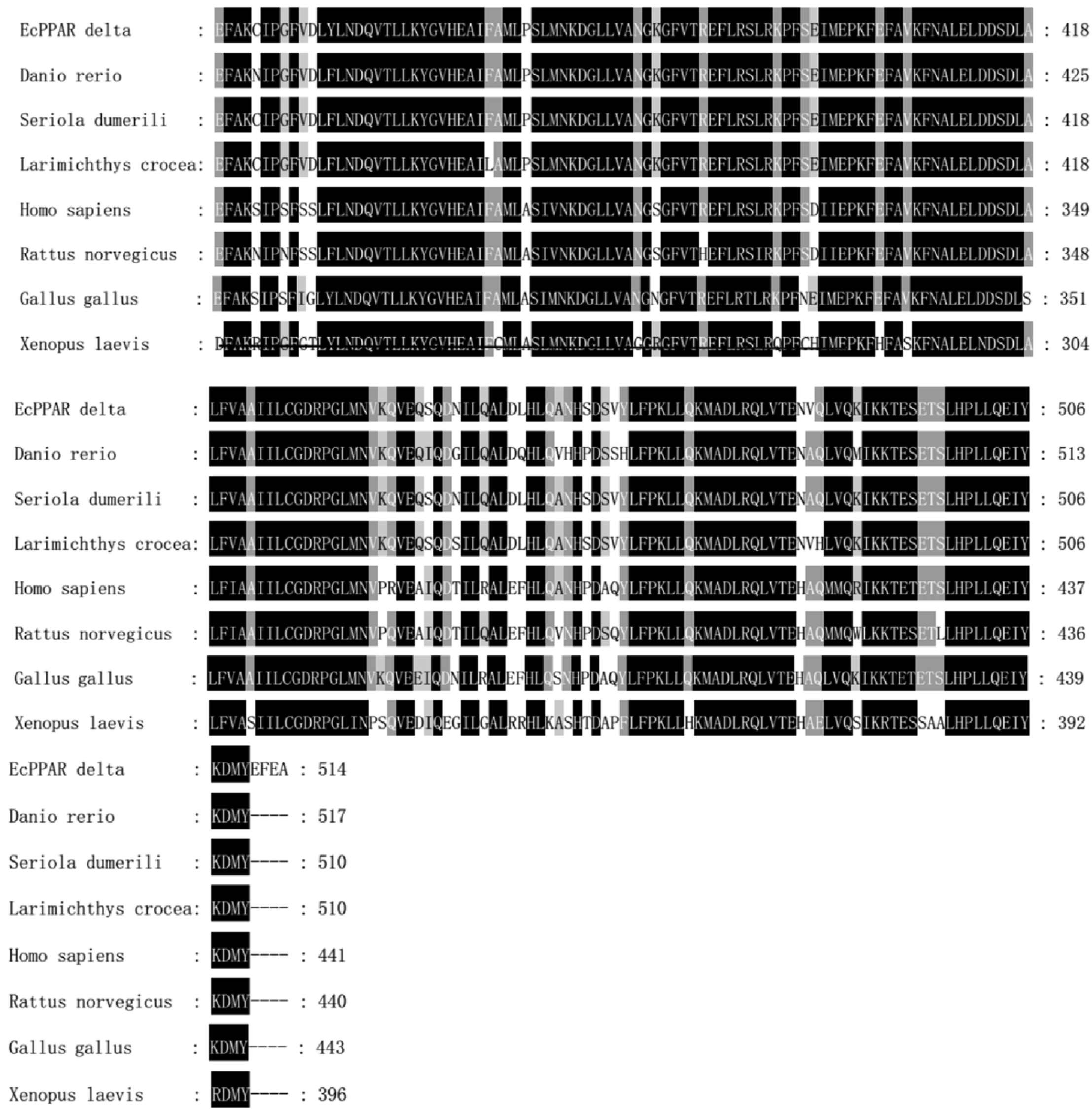

Fig. 1. (*continued*)

3.2. Expression of EcPPAR-δ in vivo and in vitro

The expression patterns of EcPPAR-δ in different tissues, including the liver, spleen, kidney, head kidney, brain, gill, heart, stomach, intestine, muscle, and skin, under normal physiological conditions were determined with a qPCR analysis. EcPPAR-δ was constitutively expressed in all the selected tissues of healthy groupers, with highest expression in the liver (Fig. 2A).

In GS cells, experimental infection with SGIV caused a significant increase in EcPPAR-δ expression at 6, 12, 18, and 24 h postinfection, with peak induction at 24 h post infection (Fig. 2B).

3.3. Subcellular localization of EcPPAR-δ

The appropriate subcellular localization of a protein can be an important indicator of its function. To determine the subcellular localization of EcPPAR-δ, a fusion protein was expressed from pEGFP-C1–EcPPAR-δ in GS cells. The green fluorescent protein (GFP) and DAPI signals were detected with fluorescence microscopy. As shown in Supplementary Fig. 2, free GFP was detected throughout the entire cell in the pEGFP-C1-transfected control cells, whereas the fusion protein GFP–EcPPAR-δ was also detected throughout both the cytoplasm and nucleus. The localization of EcPPAR-δ and GFP in the cells was quite similar, indicating that EcPPAR-δ had a whole cell distribution.

3.4. Antiviral effects of EcPPAR-δ on SGIV replication in vitro

To explore the effects of EcPPAR-δ overexpression on viral replication in fish, GS cells were transfected with pEGFP-C1 or pEGFP-C1–EcPPAR-δ, and then infected with SGIV for a further 24 h. A comparative analysis of the cell morphologies showed that the cytopathic effect (CPE) induced by SGIV was weakened in the EcPPAR-δ-overexpressing cells relative to that in the control vector-transfected cells (Fig. 3A). Consistent with this, the transcription levels of the SGIV *MCP*, *LITAF8*, *VP19*, and *ICP-18* genes were significantly reduced when EcPPAR-δ was overexpressed (Fig. 3B). A western blotting analysis showed that at 24 h postinfection with SGIV, the MCP protein levels were also suppressed in the EcPPAR-δ-overexpressing cells (Fig. 3C). A viral titer assay revealed that the overexpression of EcPPAR-δ significantly inhibited virus production compared with that in the vector-transfected cells (Fig. 3D). These results suggested that the overexpression of EcPPAR-δ inhibited the replication of SGIV *in vitro*.

To investigate whether the knockdown of EcPPAR-δ promoted SGIV replication, three siRNAs were designed to target EcPPAR-δ, and the efficiency of their interference was examined in GS cells with qPCR. Of the three siRNAs, siRNA3 showed the highest silencing efficiency, with

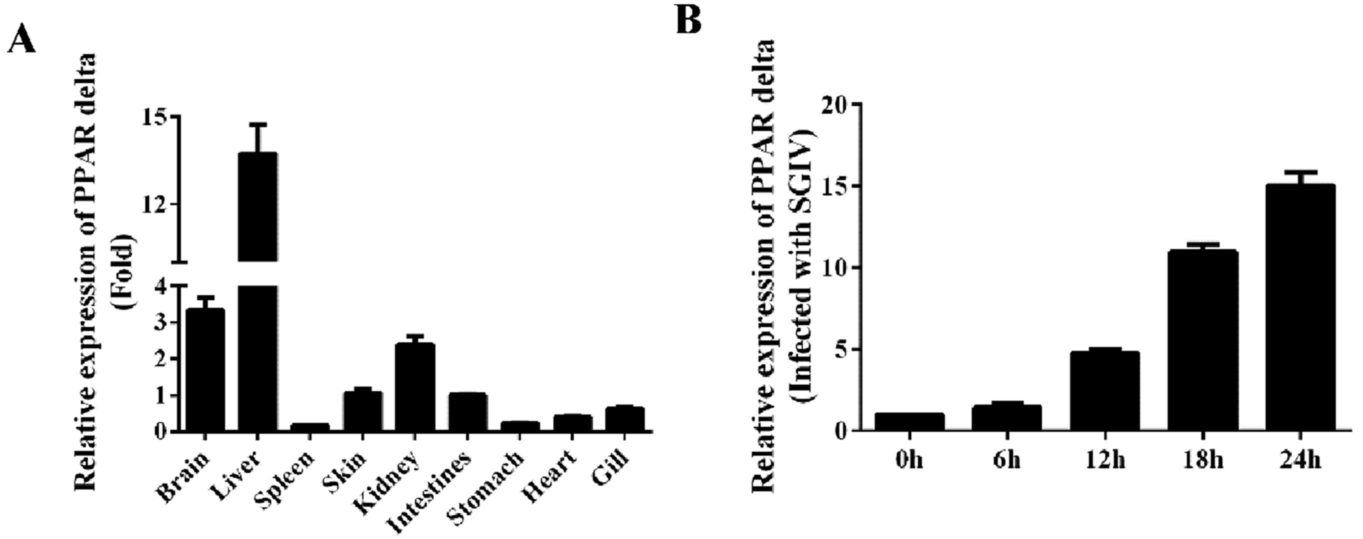

Fig. 2. Expression profiles of EcPPAR-δ. (A)The tissue distribution patterns of EcPPAR-δ in healthy grouper. Total RNAs were extracted from three healthy fish. The expression levels of EcPPAR-δ were examined using qRT-PCR, setting mRNA expression level in skin as 1-fold. (B)Expression changes of EcPPAR-δ in response to challenge with SGIV for the indicated length of time, at which time cells were collected for RNA extraction and qRT-PCR analysis.

81% knockdown of EcPPAR-δ expression compared with the negative control siRNA (Supplementary Fig. 3). The siRNA1 and siRNA2 showed 63% and 74% knockdown, respectively (Supplementary Fig. 3). After GS cells were transfected with siRNA-EcPPAR-δ for 24 h, they were infected with SGIV for a further 24 h. A cell morphology analysis showed that the CPE induced by SGIV was more obvious in the EcPPAR-δ-knockdown cells than in the control vector-transfected cells (Fig. 4A).

At 24 h postinfection, the SGIV-infected siRNA-EcPPAR-δ-transfected cells were harvested to examine the transcription of the SGIV genes with qPCR. The knockdown of EcPPAR-δ by siRNA promoted SGIV replication compared with that in cells transfected with the negative control siRNA (Fig. 4B). A western blotting analysis showed that

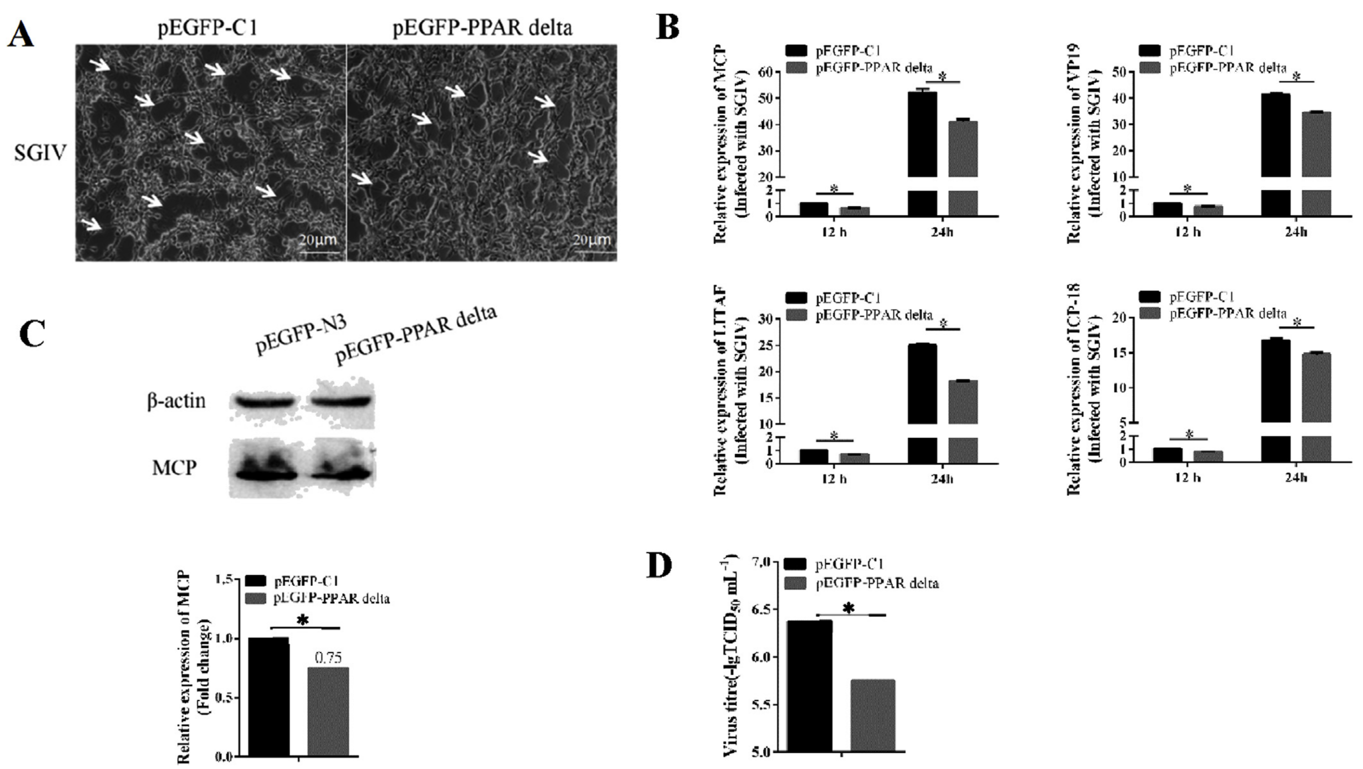

Fig. 3. The effect of EcPPAR-δ overexpression on virus replication. (A)The small white arrows showed that CPE progression induced by SGIV infection. GS cells were transfected with EcPPAR-δ, and then infected with SGIV. The morphology of CPE inhibited virus infection at 24 h post infection were imaged under light microscopy. (B)The viral gene transcription of SGIV in EcPPAR-δ overexpressing cells. After transfection with EcPPAR-δ, GS cells were infected with SGIV for 24 h. Infected cells were collected for RNA extraction, and the relative expression of viral genes, including MCP, VP19, ICP-18 and LITAF were examined using qRT-PCR.(n = 3, means ± SD).*P < 0.05. (C)Virus protein level after the transfection with EcPPAR-δ. The level of SGIV-MCP was detected by Western blot, and β-actin was used as the internal control. Band intensity was calculated using Image J software and then the ratio of MCP/β-actin was assessed. (D)The virus production of SGIV infected cells at 24 h.Viral titer was determined using the $TCID_{50}$ method.

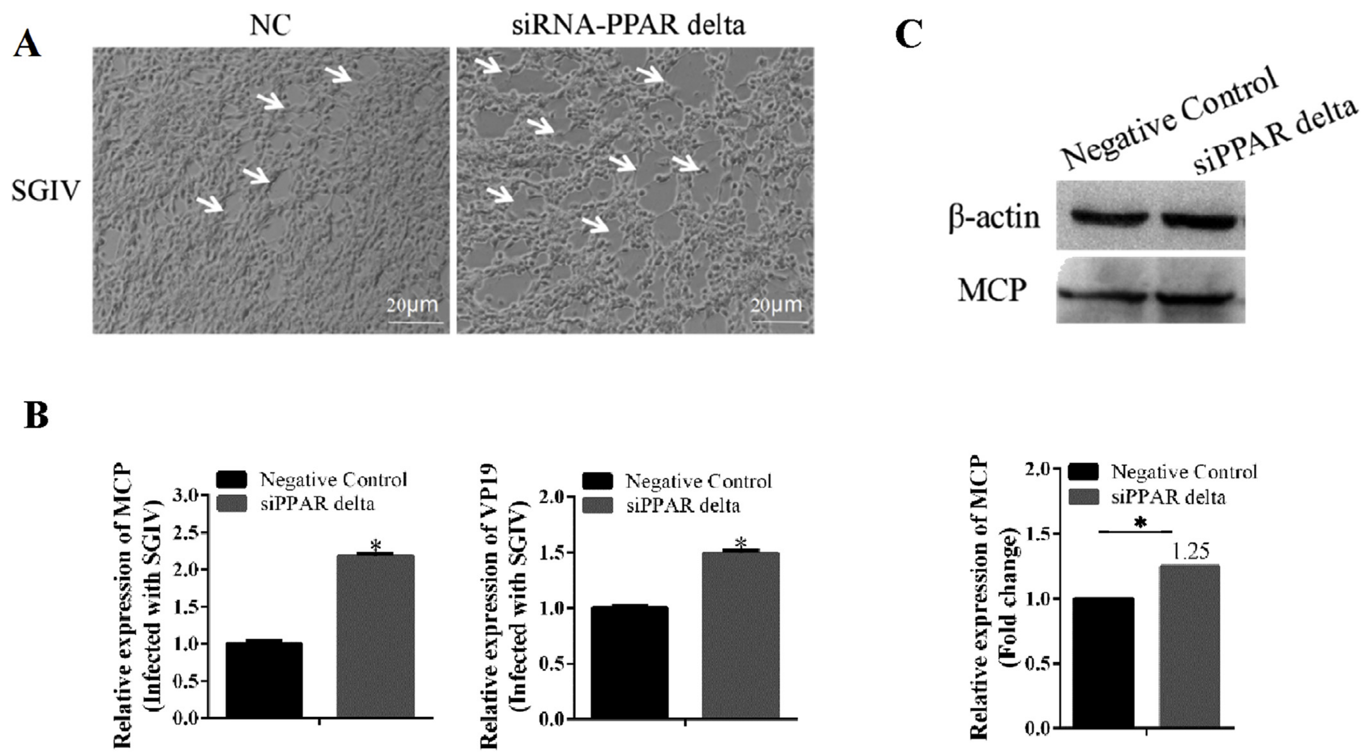

Fig. 4. Knockdown of EcPPAR-δ by siRNA promoted SGIV replication *in vitro*. (A)The small white arrows showed that CPE progression induced by SGIV infection. GS cells were transfected with EcPPAR-δ, and then infected with SGIV. The morphology of CPE induced virus infection at 24 h post infection were imaged under light microscopy. (B)The viral gene transcription of SGIV in siRNA-EcPPAR-δ and siRNA negative control (NC) overexpressing cells. After the transfection with siRNA-EcPPAR-δ and siRNA negative control (NC), GS cells were infected with SGIV at 24 h, and were collected at 24 h. p.i. to determine expression of MCP and VP19 of SGIV (n = 3, means ± SD).*P < 0.05. (C)Virus protein level after transfection with siRNA-EcPPAR-δ. The level of SGIV-MCP was detected by Western blot, and β-actin was used as the internal control. Band intensity was calculated using Image J software and then the ratio of MCP/β-actin was assessed.

SGIV MCP protein levels were increased in the EcPPAR-δ-knockdown cells (Fig. 4C). Taking these findings together, we proposed that EcPPAR-δ exerts an antiviral effect during SGIV infection.

3.5. EcPPAR-δ plays different roles in the regulation of cytokines in different signaling pathways

To analyze the effects of EcPPAR-δ on cytokine expression, we examined the expression of IFN signaling molecules and proinflammatory cytokines in EcPPAR-δ-overexpressing GS cells (Fig. 5), and the promoter activities of the genes encoding IFN-α, IFN-γ, ISRE, and NF-κB (Fig. 6). As shown in Fig. 5A, compared with the control cells, the expression levels of several IFN-related genes, including IFN-γ, ISG15, ISG56, MDA5, MXI, and MXII, was significantly increased in the EcPPAR-δ-overexpressing cells. Thus, the overexpression of EcPPAR-δ clearly upregulated the expression of IFN signaling molecules. A reporter gene analysis showed that the luciferase activity from the IFN-α, IFN-γ and ISRE promoters was significantly increased in the EcPPAR-δ-overexpressing cells compared with that in the control cells (Fig. 6), indicating that EcPPAR-δ enhances the promoter activities of IFN-α, IFN-γ and ISRE in GS cells. Therefore, we proposed that EcPPAR-δ positively regulated the IFN-based immune response.

After GS cells were cotransfected with plasmids encoding EcPPAR-δ and MITA, the expression levels of several IFN signaling genes (*IRF3*, *IRF7*, *ISG15*, and IFN-induced 35-kDa protein [*IFP35*]) induced by MITA was significantly increased compared with that in the MITA-only-transfected cells (Fig. 7), suggesting that EcPPAR-δ overexpression activated the MITA-dependent IFN immune response.

To further investigate whether the knockdown of EcPPAR-δ suppressed the IFN immune response, we transfected GS cells with siRNA-EcPPAR-δ to investigate the promoter activities of IFN-α, IFN-γ and ISRE. The reporter gene analysis showed that the luciferase activities from the IFN-α, IFN-γ and ISRE promoters were significantly reduced by the knockdown of EcPPAR-δ compared with that in the control cells (Fig. 8). Therefore, the knockdown of EcPPAR-δ inhibited the promoter activities of IFN- α, IFN- γ and ISRE in GS cells.

The effects of EcPPAR-δ overexpression on the expression of proinflammatory cytokines were also examined. A qPCR analysis showed that the mRNA levels of IL-1β, IL-6, IL-8, TNF-α, TRAF6, and MyD88 were significantly reduced in the EcPPAR-δ-overexpressing cells compared with those in the control cells (Fig. 5B). Because these inflammatory factors act in the NF-κB signaling pathway, we infer that the overexpression of EcPPAR-δ plays an important role in enhancing the expression of NF-κB-pathway-related genes. A reporter gene analysis showed that the luciferase activity from the NF-κB promoter was also significantly reduced in EcPPAR-δ-overexpressing cells (Fig. 6). Conversely, in the EcPPAR-δ gene knockdown experiment, the activity of the NF-κB promoter was significantly enhanced (Fig. 8). Together, these two experiments showed that EcPPAR-δ inhibited the promoter activity of NF-κB, whereas EcPPAR-δ knockdown enhanced it. Therefore, we propose that EcPPAR-δ played an anti-inflammatory role by negatively regulating the NF-κB signaling pathway.

Thus, the exogenous expression of EcPPAR-δ *in vitro* differentially regulated the IFN and inflammatory responses.

4. Discussion

PPARs are lipid-ligand-inducible transcription factors. Initially, these receptors were identified as critical controllers of several key enzymes that catalyze the oxidation of fatty acids [33]. Recent evidence indicates that PPARs also play critical roles in the immune and inflammatory responses [3–6]. In recent years, increasing attention has been paid to the immunomodulatory roles of PPARs in mammals. However, there have been few reports of the roles of PPARs in the

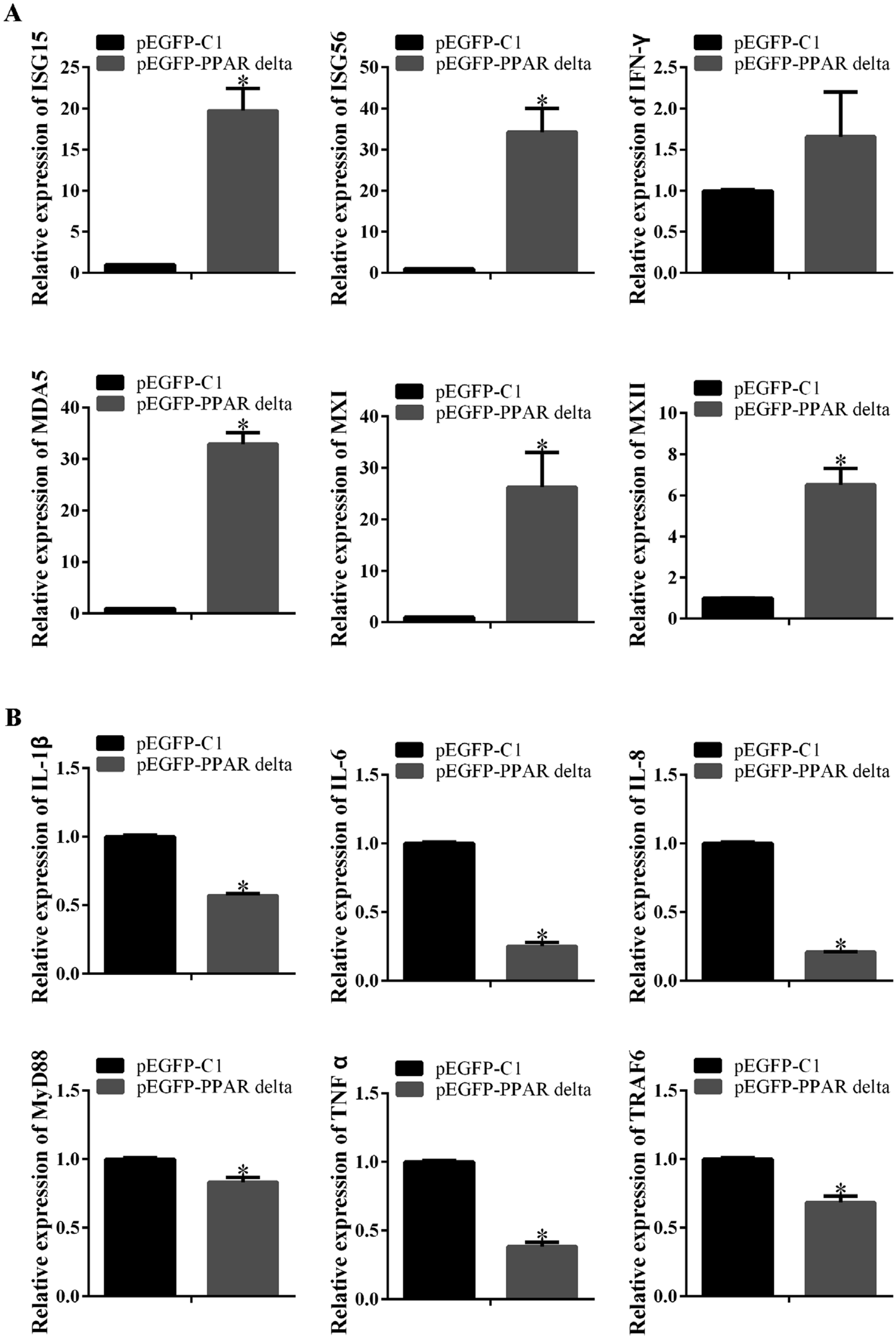

Fig. 5. The ectopic expression of EcPPAR-δ increased the expression of interferon related immune genes but decreased the expression of inflammatory factors genes. (A)The expression levels of interferon signaling molecules, including ISG15, ISG56, IFN-γ, MDA5, MXI and MXII were examined. (B)The expression levels of different inflammatory cytokines including IL-1β, IL-6, IL-8, MyD88, TNFα and TRAF6 were examined. GS cells were transfected with EcPPAR-δ 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 ($P < 0.05$).

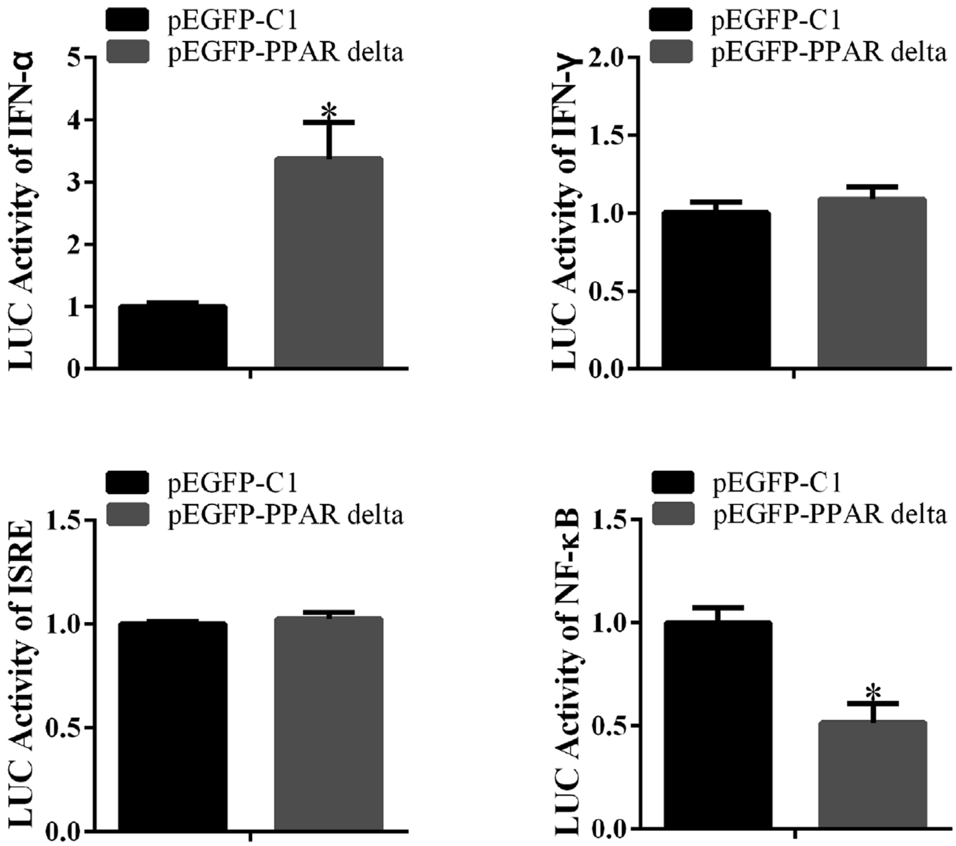

Fig. 6. Overexpression of EcPPAR-δ increased IFN-α promoter, IFN-γ promoter, ISRE promoter but inhibited NF-κB response promoter. In this case, the GS cells cultured in a 24-well plate and transfected with mixed plasmids (1000 ng/well), which consisted of 800 ng control plasmid/pEGFP-EcPPAR-δ plasmid, 150 ng IFN-α-luc/IFN-γ-luc/ISRE-luc/NF-κB-luc reporter plasmid (Clontech, USA) and 50 ng pRL-SV40 reference plasmids. 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.

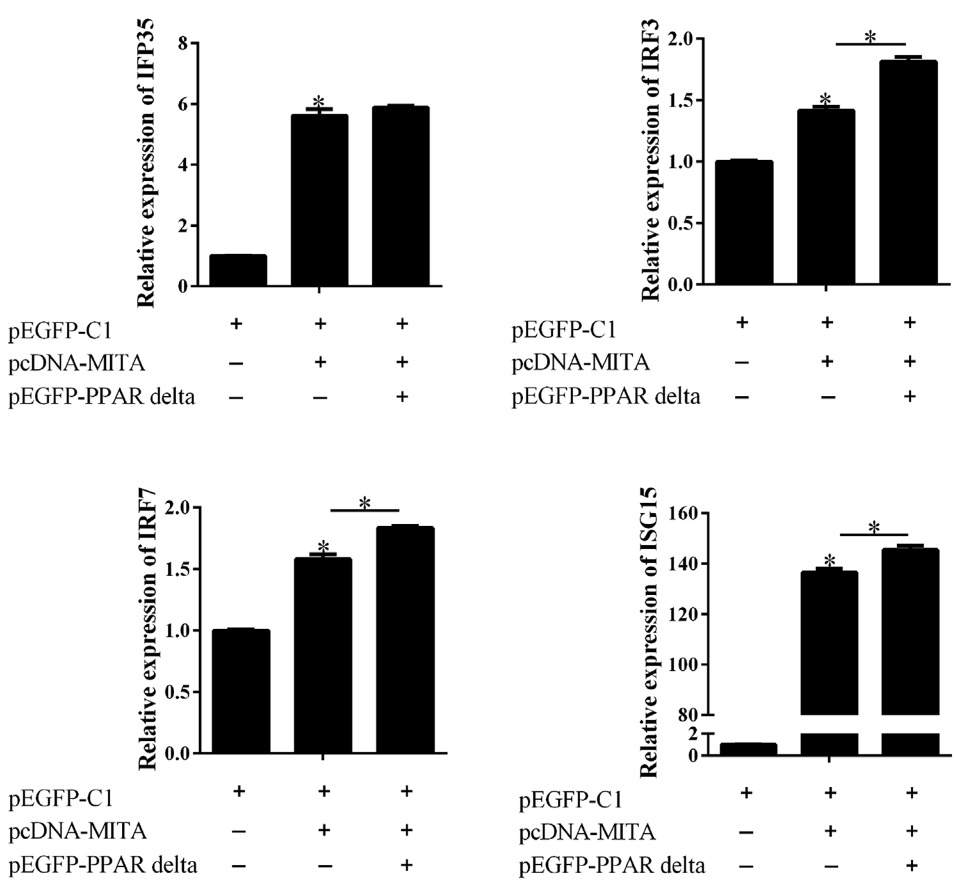

Fig. 7. Overexpression of EcPPAR-δ increased MITA-induced interferon response. GS cells were co-transfected with EcMITA and EcPPAR-δ. The transcript of interferon related genes, including IFP35, IRF3, IRF7, and ISG15 were examined using qRT-PCR. mRNA expression level in GS cells which were transfected with empty vector was set as 1-fold. Asterisks (*) mark the significant difference between experimental and control groups ($P < 0.05$).

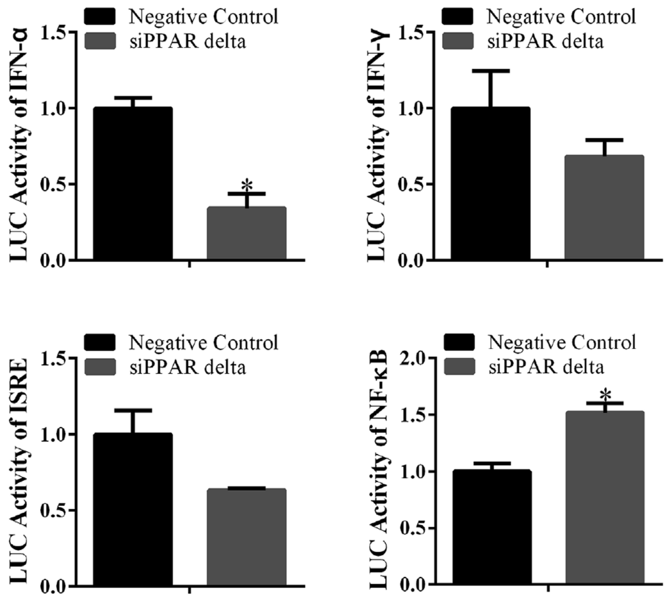

Fig. 8. The co-transfection of siRNA-EcPPAR-δ respectively with IFN-α-Luc, IFN-γ-Luc, ISRE-Luc and NF-κB-Luc, which compared with siRNA negative control (NC), inhibited IFN-α promoter, IFN-γ promoter, ISRE promoter but increased NF-κB response promoter. (n = 3, means ± SD).*$P < 0.05$.

immune responses of lower vertebrates [11–13].

In this study, we examined the expression, biological activity, and antibacterial effects of the orange-spotted grouper EcPPAR-δ. Based on a bioinformatic analysis, we found that PPAR-δ of *E. coioides* shared 95.3% identity with that of *S. dumerili*. An amino acid alignment showed that EcPPAR-δ had a typical LBD and a typical DBD. The LBD is responsible for its interaction with ester compounds, including long-chain unsaturated fatty acids and their metabolites, lipophilic drugs (fibrates), and antidiabetic drugs (glitazones). The DBD interacts with the PPREs on its target genes [34,35]. The two domains are highly conserved among different species, suggesting that the activation ligands and interaction elements on the target genes of the PPAR-δ proteins are similar from lower vertebrates to mammals.

An RT-PCR analysis showed that the EcPPAR-δ gene was expressed widely in multiple tissues under normal physiological conditions. PPAR-δ expression has not been studied in teleosts, but studies in human and mouse have shown that PPAR-δ expression is constitutive in a wide range of tissues [33,36], suggesting that EcPPAR-δ plays a regulatory role in different tissues, as in mammals.

In GS cells, EcPPAR-δ increased significantly during SGIV infection. This differs from previous studies in human cells, in which PPAR expression was repressed in response to *Hepatitis C virus* (HCV) infection [37,38]. This discrepancy may be attributable to the infection characteristics of the different types of virus. However, both findings suggested that PPAR-δ played a crucial role in the innate immune response to viral infection.

A subcellular localization analysis showed that EcPPAR-δ localizes throughout the cytoplasm and nucleus, with a diffuse intracellular expression pattern. This is the same as the localization patterns of human PPARs [39].

Few studies have addressed the roles of PPARs in the antiviral immune response, although the relationship between PPARs and HCV replication has been the focus of recent studies [37,38,40,41]. In these studies, researchers have established that the human PPAR-α and PPAR-δ pathways are involved in HCV RNA replication, and the knockdown of PPAR-δ effectively inhibited HCV RNA replication. They speculated that the inhibition of HCV replication may result from the disruption of the membranous web in which HCV replicates [37,39].

In contrast, we found that the overexpression of PPAR-δ suppressed the replication of SGIV, whereas the knockdown of PPAR-δ promoted the replication of SGIV, based on the detection of the mRNA levels of viral genes, western blotting of MCP protein, and morphological CPEs. These differences may be attributable to the different virus types and the functional nuances of the PPAR-δ proteins in different species.

A growing number of studies have shown that PPAR-δ has significant regulatory roles in the cells of the immune system [42,43]. In different human tissues, PPAR-δ overexpression reduces the inflammatory response, and the deletion of PPAR-δ aggravates the inflammatory state [44]. Moreover, in cardiomyocytes and microglial cells, PPAR-δ regulates inflammation by controlling NF-κB activity, and increased PPAR-δ expression reduces NF-κB activity by physically interacting with the p65 subunit of NF-κB [45,46]. In some immune cells, PPARs also suppress NF-κB and signal transducer and activator of transcription (STAT), thus activating the activator protein 1 (AP-1) signaling pathway and TNF-α production [47,48].

In addition to maintaining the stability of the inflammatory response, PPAR-δ also affects the IFN signaling pathway. In both mouse and human immune cells, PPAR-δ reduces the production of the IFN-γ and IL-12 family cytokines [49]. In malignant B cells, PPAR-δ increases the strength of IFN signaling, specifically altering the immunostimulatory IFN signaling response to an immunosuppressive response [50]. An analysis of the PPAR-δ-regulated transcriptome showed that among the target genes regulated by PPAR-δ, there were not only genes related to metabolism but many genes involved in immunoregulation, especially those related to the NF-κB and IFN signaling pathways [51].

In the present study, we examined the expression of IFN signaling molecules in EcPPAR-δ-overexpressing cells to clarify the effects of EcPPAR-δ on the fish IFN and inflammatory responses. The expression levels of several IFN-related cytokines, including IFN-γ, ISG15, ISG56, MDA5, MXI, and MXII, were significantly increased. The luciferase activity from the IFN-α, IFN-γ and ISRE promoters was also significantly increased in the EcPPAR-δ-overexpressing cells. The exogenous expression of both EcPPAR-δ and MITA also showed that the expression of IFN signaling genes (*IRF3*, *IRF7*, *ISG15*, and *IFP35*) induced by MITA was significantly increased by EcPPAR-δ. After the knockdown of EcPPAR-δ in GS cells, the promoter activities of IFN-α, IFN-γ and ISRE were significantly reduced. The expression of inflammatory cytokines was also examined in EcPPAR-δ-overexpressing cells. Contrary to the expression patterns of the IFN-related genes, the mRNA levels of IL-1β, IL-6, IL-8, TNF-α, TRAF6, and MyD88 were significantly reduced. The luciferase activity from the NF-κB promoter was also significantly reduced in EcPPAR-δ-overexpressing cells. However, when the EcPPAR-δ gene was silenced, the promoter activity of the NF-κB gene was significantly enhanced.

Based on these results, we suggest that EcPPAR-δ plays an immunomodulatory role in GS cells, and that its regulatory effects on the IFN and inflammatory responses differ. EcPPAR-δ plays a positive role in the regulation of the IFN signaling pathway, but has an inhibitory effect on the inflammatory response. The anti-inflammatory effect of EcPPAR-δ may be related to its function in maintaining cell homeostasis. Considering the important role of the IFN signaling pathway in the antiviral immune response, we speculate that the activation of the IFN signaling pathway caused by EcPPAR-δ overexpression underlies its inhibitory effect on SGIV replication.

In summary, we have demonstrated the roles of EcPPAR-δ in SGIV replication and cytokine regulation. A fish PPAR-δ homologue from the orange-spotted grouper (EcPPAR-δ) was cloned and characterized. EcPPAR-δ encodes a protein located in both the cytoplasm and nucleus and its expression is induced by SGIV infection. The overexpression of EcPPAR-δ *in vitro* significantly suppressed the replication of SGIV by stimulating the host IFN immune response, which was contrary to the results of RNAi. EcPPAR-δ also exerted an anti-inflammatory effect in GS cells, which may be related to its maintenance of cell homeostasis. Together, our data extend our understanding of the roles of the

EcPPAR-δ family members in viral pathogenesis in fish.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.08.068.

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