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Identification of an IRF8 gene in common carp (*Cyprinus carpio*. L) and its regulatory role in immune responses

Yaoyao Zhu[1,2,3*] and Guiwen Yang[4*]

Abstract

Background Interferon (IFN) regulatory factors (IRF) are the crucial transcription factors for IFN expression and leading host cells response to viral infection. IRF8 in mammals plays vital roles in the innate and adaptive immune systems. In this study, we identified and characterized the common carp (*Cyprinus carpio*. L) *IRF8* gene (cc*IRF8*) to further clarify the function of IRF8 in teleost fish.

Results The complete cDNA sequence of cc*IRF8* was 1431 bp and encodes a polypeptide of 431 amino acids. Analysis of the putative amino acid sequence showed that ccIRF8 encodes structures typical of the IRF family, including a DNA-binding domain (DBD), an IRF-association domain (IAD) and two nuclear localization signals (NLS). Comparison with homologous proteins showed that the deduced protein has the highest sequence identity to grass carp IRF8 (92.7%). Phylogenetic analysis grouped ccIRF8 with other IRF8s of teleosts. Quantitative RT-PCR analysis showed that ccIRF8 transcripts were detectable in all investigated tissues of healthy fish with the highest level in spleen. Following poly I: C and *Aeromonas hydrophila* challenge, cc*IRF8* transcripts were induced significantly in immune relevant tissues. In addition, cc*IRF8* was induced by poly I: C and ipopolysaccharide (LPS), peptidoglycan (PGN) and flagellin in HKLs. Overexpression of cc*IRF8* increased the expression of *IFN* and IFN-stimulated genes (ISGs), and a dual-luciferase reporter assay revealed that cc*IRF8* decreased the activation of *NF-κB* though *TRAF6*.

Conclusions Overall, our findings provide a new perspective on the role of IRF8 in innate immunity in fish, as well as insights that will help the prevention and control of disease in the common carp farming industry.

Keywords IRF8, Common carp (*Cyprinus carpio*. L), Poly I:C, *Aeromonas hydrophila*, IFN, NF-κB

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Background

Interferons (IFNs), a family of multifunctional cytokines, play essential roles in the process of innate immunity in animals. Transcriptional factors of the interferon regulatory factors (IRF) were originally identified in the 1980s, which have been shown to play central roles in regulating the expression of type I IFN genes, IFN-stimulated genes (ISG), and other cytokines and chemokines [1, 2]. It is now recognized that IRFs are vital for innate immune responses elicited by pattern recognition receptors (PRRs), development of various immune cells, cell growth regulation, apoptosis and oncogenesis, and even for sex determination [3–5]. Up to the present time, nine IRF members (IRF1-9) have been identified in mammals, ten members in birds and eleven members in fish [6]. All these eleven IRFs possess a well-conserved amino-terminal DNA-binding domain (DBD), which covers the first 115 amino acids in the N-terminus region. The DBD display a helix-loop-helix structure and 5 tryptophan residues that recognizes the AANNGAAA elements within the promoters of target genes, such as IFN-stimulated response elements (ISRE). All IRFs except IRF1 and IRF2 possess a C-terminal IRF-association domain (IAD), which mediates the recruitment of other IRFs and transcription factors to the promoters of target genes [7–9]. The distinct functional roles of IRFs are determined by the interaction of IRFs with other transcription factors: activators (e.g. IRF1, -3, -7 and – 9) and bi-functional factors that both activate and repress transcription depending on the target gene (e.g. IRF2, -4, -5 and – 8) [10].

IRF8 [also named interferon consensus sequence binding protein (ICSBP)], was originally identified as a nuclear protein binding to interferon consensus sequence (ICS) in the major histocompatibility complex (MHC) class I gene promoter [11]. Expression of IRF8 is predominantly found in immune cells, such as myeloid and lymphoid cell lineages, and its function in the myeloid lineage has been defined [12]. IRF8 deficiency can result in a pathophysiological state called chronic myelogenous leukemia (CML), which is characterized by an uncontrolled growth of myeloid cells in the bone marrow and myeloid accumulation in the blood [13]. IRF8, as a transcriptional regulatory factor, plays a key role between the cross-talk of TLR and IFNγ signaling pathways in regard to poly I: C-TLR3 and LPS-TLR4 ligations [14]. Further, IRF8 together with IRF3 was found to cooperatively regulate rapid IFNβ induction in human blood monocytes [15]. More recently, IRF8 was found to be a critical regulator of NLR family apoptosis inhibitory proteins (NAIPs) and NLR family CARD domain-containing 4 (NLRC4) inflammasome activation for defense against bacterial pathogens [16, 17].

In addition to a large number of studies in mammals, IRF8 in some fish species have been studied, including rock bream (*Oplegnathus fasciatus*) [18], turbot (*Scophthalmus maximus*) [19], Atlantic cod (*Gadus morhua*) [20], Japanese flounder (*Paralichthys olivaceus*) [21], rainbow trout (*Oncorhynchus mykiss*) [22], half-smooth tongue sole (*Cynoglossus semilaevis*) [23], large yellow croaker (*Larimichthys crocea*) [24] and golden pompano (*Trachinotus ovatus*) [25]. These studies have shown that IRF8 can be transcriptionally upregulated by polyinosinic: polycytidylic acid (poly I: C), viruses or bacteria, indicating its vital role in the antiviral and antibacterial immune response of fish. What's more, in marine fishes, golden pompano and miiuy croaker (*Miichthys miiuy*), the studies have been showed that IRF8 can have a positive effect on IFN signaling pathway and a negative effect on NF-κB signaling pathway, respectively [25–27]. Still, more study about IRF8 is need to make better understanding of its regulation role in the immune system of fish.

Common carp (*Cyprinus carpio*. L) is an important freshwater aquaculture species in China and some countries in Europe and Asia. However, this farmed fish is prone to to be infected by virus or bacteria in the breeding process. Adaptive immune system in fish is less well-developed compared with higher vertebrates, in contrast, the innate immune system plays a vital role in lower vertebrates [28]. Thus, insight into the mechanisms of the transcription regulation of common carp IRF genes will help us to prevent and control various infectious diseases. Among the 11 IRFs, IRF1-5, IRF7, IRF9 and IRF10 have been identified and studied in common carp [29–35]. Considering the roles of IRF8 in the regulation of innate immune system, the identification and function studies of common carp IRF8 are important to understand the mechanism against pathogen infection. In this study, we successively identified the full-length cDNA sequence of *IRF8* from common carp (cc*IRF8*) and did the sequence analysis. Apart from investigating its tissue-specific distribution patterns in healthy fish, we also evaluated the expression patterns of cc*IRF8* after intraperitoneal injection with poly I: C and *Aeromonas hydrophila*. Furthermore, we examined its expression in vitro in head kidney leukocytes (HKLs) stimulated with pathogen-associated molecular patterns (PAMPs). We then confirmed the regulatory role of cc*IRF8* in the IFN and NF-κB signaling pathways. These results will help to the further functional study of IRF8 in fish.

Materials and methods

Fish rearing, immune challenge and sampling

Common carp weighing about 200 g were obtained from a local fish farm in Jinan, China. All fish were healthy and acclimatized at 25℃ in aerated tanks for more than one week before process and fed twice a day.

For the immune challenges, fish were challenged by intraperitoneal (i.p.) injection with poly I: C or *Aeromonas hydrophila* according to previously described protocols [33, 35]. In the poly I: C challenge experiments, thirty experimental fish were injected intraperitoneally with 500 μl poly I: C solution (2.6 mg/ml in PBS, Sigma) per fish. Another group of thirty fish were i.p. injected with formalin (overnight at 4 ℃ in 0.5% formalin) inactivated *A. hydrophila* (5×10^7 CFU per fish).

The liver, spleen, head kidney, skin, foregut and hindgut of three infected fish were collected at 3, 6, 12, 24, 48 and 72 h post injection (hpi) in each group, whereas the unchallenged fish served as controls (indicated as 0 h). All resected samples were immediately snap-frozen in liquid nitrogen and stored at -80 ℃ until subsequent real time PCR analysis. All the fish were euthanized by immersion in a solution of tricaine methane sulfonate (MS222, Sigma-Aldrich) at a concentration of 100 mg/L of water, and all efforts were made to minimize fish suffering [30].

Table 1 Primers used in this study

ccIRF8-R	GTAGCTGCACAGGTCCTG	Cloning for *IRF8*
ccIRF8-5Rout	CAACTTCTGTTCCTCCTCTGGCAC	Cloning for *IRF8*
ccIRF8-5Rin	CTGGGATCGGTCAGTAACTTCCTC	Cloning for *IRF8*
ccIRF8-3Fout	CTGTCAGCAGTTGGTGGATGCAG	Cloning for *IRF8*
ccIRF8-3Fin	CTCAGATGACATGGCCAGTGATC	Cloning for *IRF8*
ccIRF8-Frt	TTCTACTATGGAGGTCGGCTGGT	Rea-ltime PCR
ccIRF8-Rrt	AAGTGGATGTTCTGGAGGCTGTC	Rea-ltime PCR
ccS11-F	CCGTGGGTGACATCGTTACA	AB012087
ccS11-R	TCAGGACATTGAACCTCACTGTCT	
ccIRF8-F-ERI	CCGGAATTCATGAATCCGGGTGGTCGCAG	Recombinant plasmid
ccIRF8-R-SII	TCCCCGCGGGACTGGAATCGGCAGGTTGTC	
ccIRF8-F-S1	ATACCTGCAGGAATGAATCCGGGTGGTCGCAG	Recombinant plasmid
ccIRF8-R-NI	CTAGCTAGCTCAGACTGGAATCGGCAGGTTG	
TRAF6-F-EI	CCGGAATTCATGGCTTGCAGTGACATGGAG	Recombinant plasmid
TRAF6-F-SII	TCCCCGCGGAAGTGAAGGTTCTGACCCCCG	
EPC-IFN-F	CGCTAAGGTGGAGGACCAGGTTA	FN178457
EPC-IFN-R	TTAGGTTCCATTGTGCTGCGTTCA	
EPC-PKR-F	TGGAGACTTCGGCCTCGTGACT	KM099176
EPC-PKR-R	TCGCTTGCTCCGGGCTCATGTA	
EPC-viperin-F	AAGACTTCCTGGACCGCCATAAGA	KM099177
EPC-viperin-R	CCTCTCGGCAATCCAAGAAGCG	
EPC-ISG15-F	ACAGTCGGTGAACTCAAGCAAGTC	KM099174
EPC-ISG15-R	CGTAACTGCTGAGGCTTCTGGAAT	
EPC-TNFα-F	TGTGTGCTGCTGCTGCTGTTT	JN412133
EPC-TNFα-R	TCGTAAGCCTGAGCCCAGTTCC	
EPC-IL-1β-F	CCCAGACCAATCTCTACCTCGCT	
EPC-IL-1β-R	GAGGAGGTTGTCATTCTGGTCACC	
EPC-β-actin-F	GCCGTGACCTGACTGACTACCT	KF844250
EPC-β-actin-R	GCCACATAGCAGAGCTTCTCCTTG	

Isolation and stimulation of HKLs

HKLs were isolated by density gradient centrifugation with Percoll (Sigma) according to a previous report [33, 35]. In brief, tissues of freshly killed common carp was used to obtain the head kidney cell suspensions. Subsequently, the resulting suspension was loaded onto a 51/34% non-continuous percoll gradient and separated via centrifugation at 800 *g* for 25 min. HKLs (1×10^6) were maintained at 25 °C in complete L-15 (Gibco) supplemented with 10% FBS, 1% penicillin-streptomycin, and treated with poly I: C (5 μg/ml), LPS (10 mg/ml), PGN (10 mg/ml) and Flagellin (10 ng/ml).

RNA extraction and cDNA synthesis

Total RNA was extracted from various tissues and HKLs using RNAsimple Total RNA Kit (TIANGEN) according to the manufacturer's protocol. The RNA concentration was determined by measuring absorbance at 260 nm (A_{260}), and its quality was monitored by A_{260}/A_{280} ratios > 1.8 (TIANGEN). First-strand cDNA was synthesized from total RNA using the FastQuant RT Kit (with gDNase) (TIANGEN) following the manufacturer's protocol.

Cloning of cc*IRF8*

To obtain the full-length cDNA sequence of cc*IRF8*, degenerate primers were designed by comparing all known *IRF8* sequences. The cDNA was synthesized using spleen-derived RNA. Partial sequence of ccIRF8 was amplified by a pair of degenerate primers IRF8-F/R, then 5′ and 3′ ends were amplified using SMART™ RACE cDNA Amplification Kit (Takara, China) according to the instructions of the manufacturer. All the PCR primers used in this study are shown in Table 1.

Sequence analysis

The structures of the protein sequence were analysed using the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de). Multiple alignment was performed using Clustal W program (http://www.ebi.ac.uk/clustalw/) and phylogenetic analysis was performed by neighbor-joining method using MEGA 6.0 software.

Construction of overexpression vector

To construct the overexpression vector of cc*IRF8* (abbreviation, p*IRF8*), the ORF of cc*IRF8* was amplified with

primers IRF8 -ERI-F /IRF8 -SII-R (Table 1) using Phusion HighFidelity DNA polymerase (PrimeSTAR). The purified fragments were ligated into the pcDNA3.1-EGFP at the EcoRI and SacII sites. The resulting overexpression vector pIRF8 was confirmed by sequencing. Likewise, the overexpression vector of cc*TRAF6* (p*TRAF6*) was constructed using the same method and ligated into the FUGW-2flag vector at the EcoRI and SacII sites for the luciferase activity assay.

Transfection and expression profiles of immune molecules

Epithelioma papulosum cyprini (EPC) cells were cultured at 25 ℃ in medium 199 (HyClone) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco), and seeded in 24-well plates with 500 µl in each well at a concentration of 4×10^5 cells/ml for 1 day in order to reach 80% confluency. Cells were transfected with 1 µg pIRF8 using 1 µl X-tremeGENE HP DNA Transfection Reagent (Roche) following the manufacturer's protocols. After 48 h transfection with pIRF8 or empty vector, EPC cells were collected for quantification of associated immune molecule expressions (IFN, PKR, Viperin, ISG15, TNFα and IL-1β). The primers are shown in Table 1.

Luciferase activity assay

293T cells were kindly supplied by Guangxun Meng from the Institut Pasteur of Shanghai, University of Chinese Academy of Sciences in China and cultured at 37 ℃, 5.0% CO_2 in DMEM (HyClone) supplemented with 10% FBS and 1% penicillin-streptomycin (Gibco). Cells were co-transfected in 96-well plates with reporter gene plasmids, pGL-Renilla-luc and pGL-NF-κB-luc, and the pIRF8 vector using Lipofectamine 2000 (Invitrogen). After transfection for 48 h, the cells were washed with PBS and lysed with lysis buffer (Promega). Subsequently, Firefly and Renilla luciferase activities were measured with enzyme-labelling measuring instrument (FilterMax F3). The firefly luciferase activity was normalized to that of Renilla. Data were calculated from three independent replicates.

Real-time PCR analysis

Real-time PCR was performed in a Rotor-Gene Q PCR instrument (Qiagen) with TransStart Tip Green qPCR SuperMix (TransGen). Reaction was initiated at 94 °C for 30 s, followed by 40 cycles of 5 s at 94 °C and 30 s at 60 °C. All samples were analyzed in triplicates and the expression levels of all genes were calculated relative to those of the housekeeping gene 40 S ribosomal protein *S11* or the *β-actin* gene with the $2^{(-\Delta\Delta Ct)}$ method [36]. The primers used are listed in Table 1.

Statistical analysis

Statistical analysis was carried out using the GraphPad Prism 6.0 software. Differences between control and treatment groups were assessed by one-way analysis of variance (ANOVA) or T-test. The results of three independent experiments are expressed as means ± SD. *P* values of less than 0.05 were considered statistically significant.

Results

Molecular characterization of cc*IRF8*

The full-length of cDNA of common carp *IRF8* was found to consist of 1431 bp and has an open reading frame (ORF) of 1272 bp (GenBank accession no. OP759641). It encodes for a protein with 423 amino acid residues with an expected molecular weight of 48.3 kDa and an isoelectric point of 6.27. The 5 and3 untranslated regions are 67 bp and 92 bp, respectively. Smart analysis showed that the deduced protein exhibited a DBD (G4-P112) and an IAD (F194-T374) domain, with five tryptophan residues (Trp11, 26, 38, 58 and 76) and two nuclear localization signals (NLS) in the DBD.

Sequence alignments showed that IRF8 amino acids sequences were conserved in all vertebrates, and significant homology was found in DBD and IAD (Fig. 1). What's more, ccIRF8 is 57.3–92.7% identical to homologous proteins from mammals, chicken, amphibia and fish, and shares the highest identity to grass carp IRF8 (Table 3). The high similarity of ccIRF8 to known homologous proteins was further supported by a phylogenetic tree, which was built with IRF8 proteins from mammals, birds, amphibians, fish, appendicularia and hydrozoan (Fig. 2).

Tissue distribution

The *IRF8* gene was expressed in all tissues collected from common carp (liver, spleen, head kidney, foregut, hindgut, gills, gonad, skin, muscle, buccal epithelium and brain) analyzed in this study. The cc*IRF8* transcript showed the highest expression in spleen, followed by gills, brain and head kidney, with the lowest expression observed in the foregut (Fig. 3).

Expression profiles of cc*IRF8* after poly(I: C) and *A. hydrophila* injection

To understand the immune function of ccIRF8, gene expression pattern was examined in the immune-related tissues of common carp after i.p. injection with poly I: C and *A. hydrophila*. The expression profile of cc*IRF8* upon poly (I: C) stimulation was shown in Fig. 4, significant up-regulation of ccIRF8 was observed in all the tissues examined. The expression level of cc*IRF8* was increased and peaked at 6 hpi in the liver (11.5 folds), spleen (3.5 folds) and skin (8.5 folds). In the foregut and hindgut,

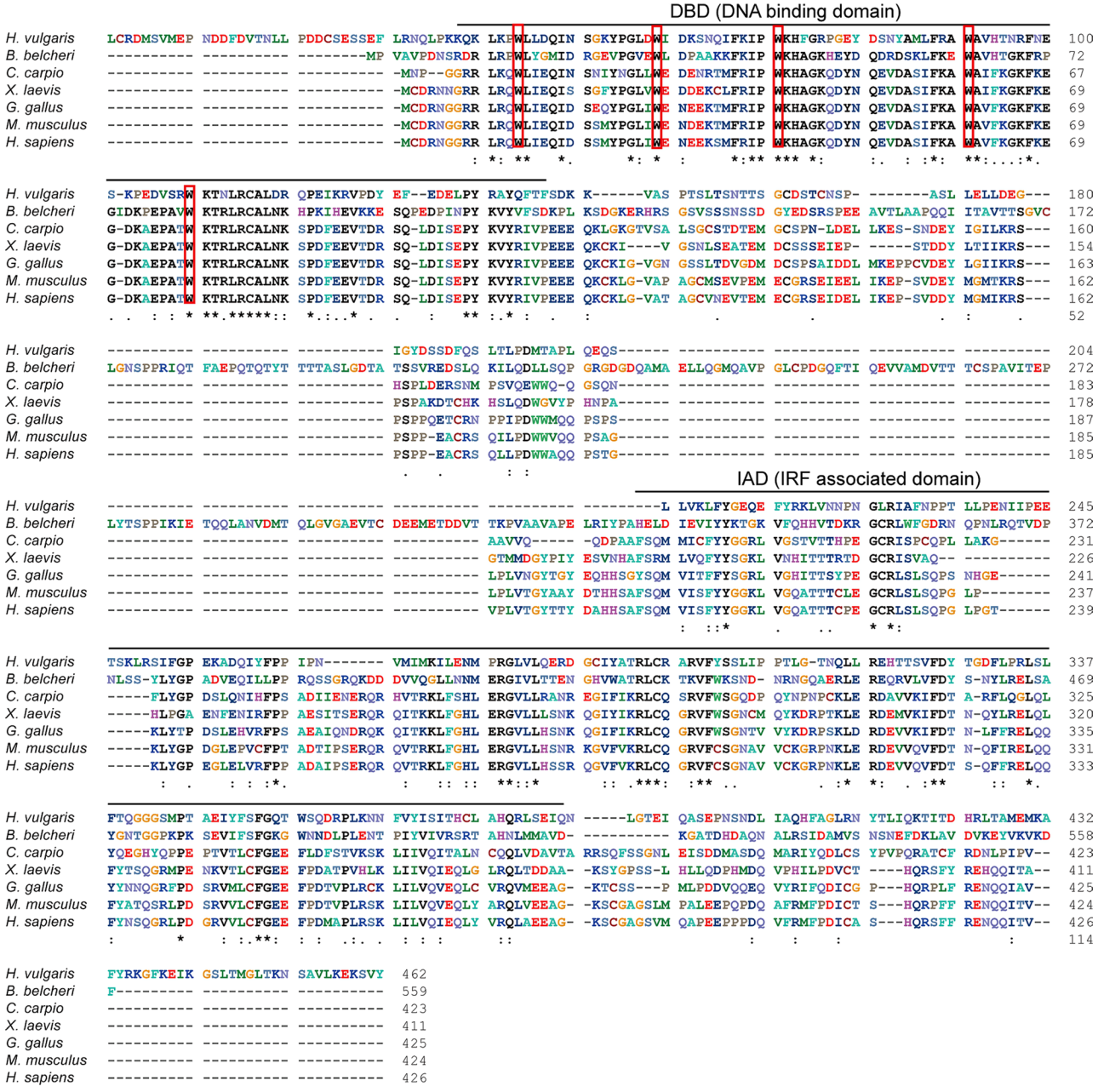

Fig. 1 Multiple alignments of IRF8 protein sequences in different species. The sequences were aligned using the Clustal W method. The identical, conservative and highly conservative substituted amino acid residues are indicated in (*), (.) and (:), respectively. The DBD and IAD domains are indicated by black lines. Five tryptophan (W) residues of the DBD domain are boxed in red. The GenBank accession numbers for these genes are listed in Table 2

cc*IRF8* expression peaked at 3 hpi (7.2 folds and 5.7 folds, respectively). The peak expression of cc*IRF8* was observed at 48 hpi in the head-kidney with a 8.6 folds induction (Fig. 4).

The above results indicated that ccIRF8 might be involved in the antiviral immune response. Whether ccIRF8 participates in antibacterial immunity was also investigated. The fish were injected intraperitoneally with *A. hydrophila*, and the mRNA expression level of cc*IRF8* was detected at 3, 6, 12, 24, 48 and 72 h post injection. It was shown that significant upregulation of cc*IRF8* was observed in the spleen (8.1 folds), head kidney (2.2 folds) and hindgut (2.5 folds) at 6 hpi. In the foregut, cc*IRF8* reached its peak at 3 hpi, with a 4.7 folds induction (Fig. 5).

Inductive expression of cc*IRF8* upon immune stimulation in HKLs

We isolated leukocytes from head kidney of common carp and real-time PCR was used to examine the

Table 2 Protein length and Genbank accession numbers of the IRF8 sequences in different species

Species	Protein length (aa)	GenBank accession numbers
Paralichthys olivaceus	421	AFE18695
Scophthalmus maximus	421	AFE88897
Monopterus albus	422	XP_020460872
Miichthys miiuy	423	AHB59740
Oplegnathus fasciatus	423	AFU81292
Labrus bergylta	422	XP_020516083
Ictalurus punctatus	433	AHH39223
Danio rerio	424	AAH75963
Ctenopharyngodon idella	429	*AMT92197*
Xenopus laevis	412	NP_001087097
Gallus gallus	426	NP_990747
Mus musculus	425	AAH05450
Castor canadensis	425	JAV43171
Homo sapiens	427	AAI26248
Branchiostoma belcheri tsingtauense	560	AJA02103
Hydra vulgaris	463	CDG70387

Table 3 Amino acid identities (%) of ccIRF8 to other vertebrate IRF8 proteins

Species	Identities (%)
Ctenopharyngodon idella	92.7
Danio rerio	86.3
Miichthys miiuy	71.9
Scophthalmus maximus	70.2
Oplegnathus fasciatus	71.6
Ictalurus punctatus	75.4
Paralichthys olivaceus	69.5
Xenopus laevis	57.8
Gallus gallus	57.9
Mus musculus	57.6
Homo sapiens	57.3

expression levels of cc*IRF8* upon stimulation with poly I: C, LPS, PGN and flagellin. As shown in Fig. 6, cc*IRF8* expression was induced by poly I: C (2.1 folds) and LPS (1.2 folds) at 24 h post stimulation. After challenge with PGN, the expression was up-regulated at 3 h and peaked at 12 h (2.1 folds). After flagellin treatment, the expression of cc*IRF8* was increased and peaked at 3 h (2.0 folds).

Effect of ccIRF8 on the cytokines in EPC cells

To investigate the role of ccIRF8 in the IFN signaling pathway, real-time PCR was used to analyse the mRNA expression of *IFN*, *PKR*, *Viperin*, *ISG15*, *TNFα* and *IL-1β* after overexpression of cc*IRF8* in EPC cells. As shown in Fig. 7, the expression level of all above genes was significantly increased with a 75.2 folds, 1.4 folds, 5.7 folds, 3.7 folds, 6.4 folds and 27.6 folds of the control group, respectively.

Effect of ccIRF8 on NF-κB activation

In mammals, NF-κB could be activated by IRF8 in a tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6)-dependent manner [37]. Therefore, we tested if cc*IRF8* could activate *NF-κB*. As shown in Fig. 8, *NF-κB* was significantly activated by *TRAF6* overexpression, but not cc*IRF8*. What's more, co-transfection of cc*IRF8* and *TRAF6* inhibited activity of *NF-κB* compared to transfection of *TRAF6* alone (Fig. 8).

Discussion

IRFs, as transcription mediators, play vital roles in immune response, especially against viral or bacterial infections, in apoptosis and in cell growth. In this study, we cloned the full-length cDNA of *IRF8* from spleen of common carp. The cc*IRF8* gene is predicted to encode a protein of 423 amino acids which harbors two conserved domains, an N-terminal DBD and an IAD at the C-terminus, and two NLSs in the DBD. All these domains/motifs are shared by other IRF8s, indicating that IRF8 is a highly conserved gene from lower to higher in vertebrate evolution. The DBD is typical in all IRFs and mediates binding with the IRF element or ISRE consensus sequence in the target promoters [3]. Similar to other IRF8s, the DBD domain is characterized by a cluster of five well-spaced tryptophan residues, which are located at the 11, 26, 38, 58 and 76 positions of ccIRF8. The IAD domain was initially found in IRF8, which is another conserved domain among IRFs, except for IRF1 and IRF2. It is essential for the formation of IRF homo/hetero-dimers and associations with other transcription factors [8]. The NLS is related to nuclear translocation and preservation of IRFs [38]. All IRFs, except for IRF6, have 1 or 2 NLRs in terminal regions. In the present study, 2 NLSs were located in the DBD region, similar to those of golden pompano IRF8 [25].

Multiple alignments of the different IRF8 revealed that a high sequence homology existing in the DBD and IAD, indicating that the action pattern of vertebrate IRF8s is probably evolutionarily conserved. In addition, phylogenetic analysis revealed that ccIRF8 was well clustered into the teleosts branch and exhibited the closest relatedness to grass carp. This matches a close genetic relationship between the two cyprinid fishes.

In vivo, *IRF8* shows broad and constitutive expression patterns in various tissues of teleost fish, although the expression profiles vary among different species. In this study, the highest mRNA expression of cc*IRF8* has been observed in the spleen, which was similar to that in turbot, Japanese flounder, rainbow trout, half-smooth tongue sole and rock bream [18, 19, 21–23], suggesting a potential role of IRF8 in fish systematic immunity. However, the tissue distribution of cc*IRF8* was somewhat different to that of golden pompano,

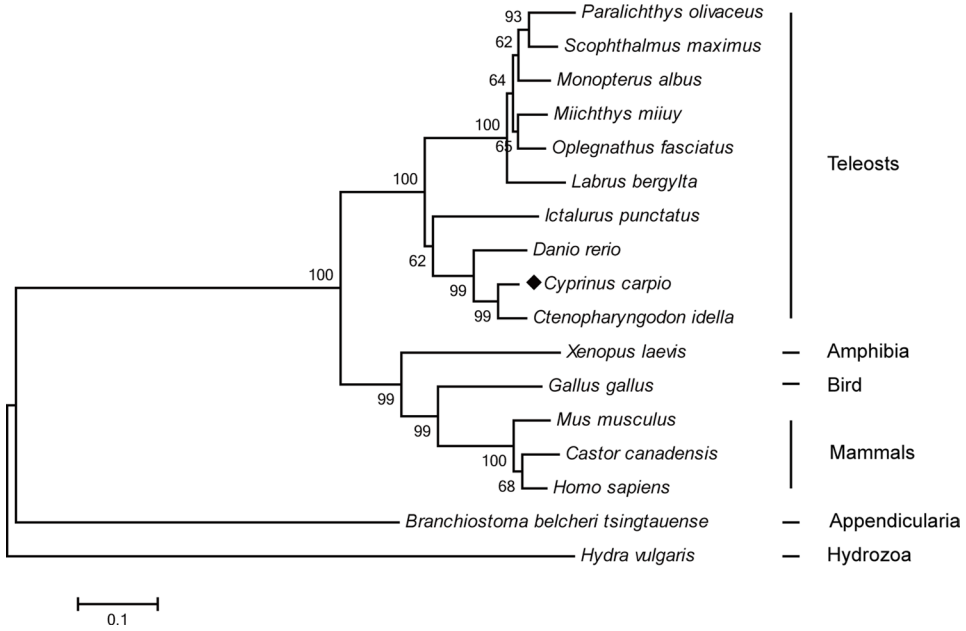

Fig. 2 Phylogenetic analysis between ccIRF8 and other IRF8 proteins. Phylogenetic tree was produced by the neighbor-joining method in MEGA 6.0. The numbers at tree nodes indicate the boot-strap percentage of 1000 bootstrap samples. The ccIRF8 is marked with rhombus (◆). The GenBank accession numbers for these genes are listed in Table 3

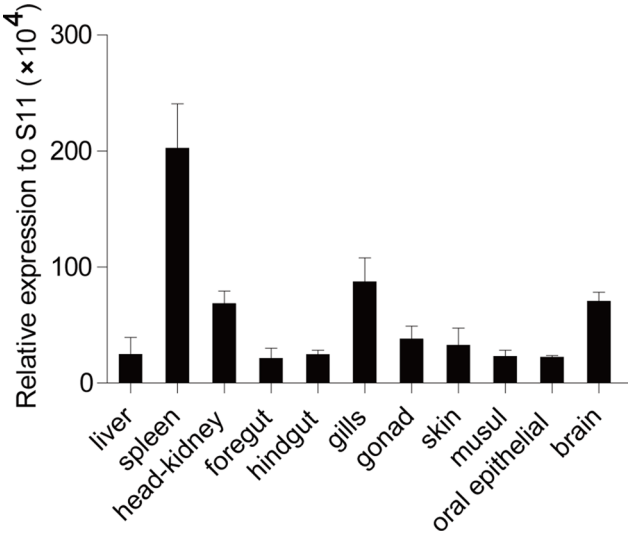

Fig. 3 Tissue expression of *IRF8* in normal common carp. The mRNA expression of cc*IRF8* in the liver, spleen, head kidney, foregut, hindgut, skin, gills, buccal epithelium, muscle, gonad and brain was detected by real-time PCR. The 40 S ribosomal protein *S11* in each tissue was amplified as internal control, $n = 3$

large yellow croaker and Atlantic cod *IRF8*. For example, in golden pompano, *IRF8* was highly expressed in the kidney but weakly expressed in the liver [25]. Large yellow croaker *IRF8* transcripts were more abundant in the heart and liver, but less abundent in the intestine [24]. While, in Atlantic cod, *IRF8* appeared to be expressed at a moderate level in all tissues [20]. These results indicated that fish IRF8 might play a potential role in both immune and nonimmune tissue compartments.

In the current study, we investigated whether ccIRF8 is involved in the antiviral and antibacterial response by real-time PCR. Poly I: C, a synthetic analog of virus dsRNAs, is a well-known inducer of fish type I IFNs and ISGs [39]. After i.p. injection with poly I: C, cc*IRF8* was induced in the liver, spleen, head kidney, skin, foregut and hindgut (Fig. 4). Similarly, in rock bream, turbot, Japanese flounder and large yellow croaker, the expression of *IRF8* was found to be up-regulated in the head kidney and spleen upon poly I: C and viruses stimulation [18, 19, 21, 24]. These results revealed that ccIRF8, like mammalian IRF8, might play a critical role in the antiviral immune response [15].

In addition to the anti-virial immune function, fish IRF8 was found to take part in the antibacterial immune response in a few fish species. For instance, in large yellow croaker, the *Vibrio anguillarum* induced the expression of *IRF8* in the liver, spleen and kidney [24]. What's more, in half-smooth tongue sole and rock bream, significant upregulation of *IRF8* was detected in the spleen and head kidney upon infection with *Edwardsiella tarda*, *Streptococcus iniae* or LPS [18, 23]. In the present study, when common carp were injected with *A. hydrophila*, cc*IRF8* was up-regulated in the spleen, head kidney, foregut and hindgut in an early phase of the stimulation.

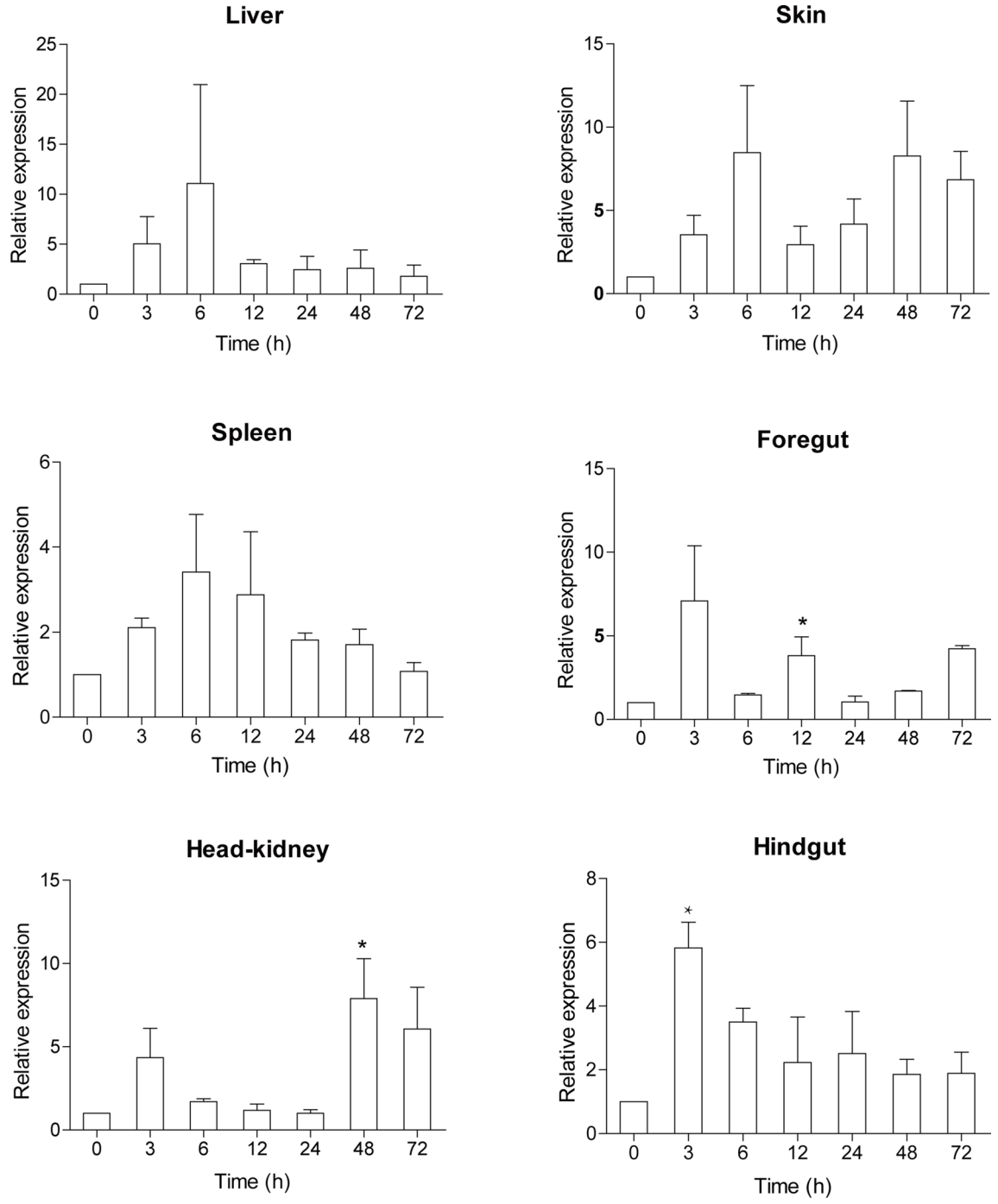

Fig. 4 Expression analysis of cc *IRF8* in various tissues of common carp after intraperitoneal injection with poly(I: C). The mRNA expressions of cc*IRF8* in liver (**A**), spleen (**B**), head kidney (**C**), skin (**D**), foregut (**E**) and hindgut (**F**) at different time points are shown. Gene expression results were calculated relative to the expression of 40 S ribosomal protein *S11*. The Y-axis represents the fold changes based on unstimulated control group (denoted by 0 h). ($n = 3$, mean ± SD, $^*P < 0.05$)

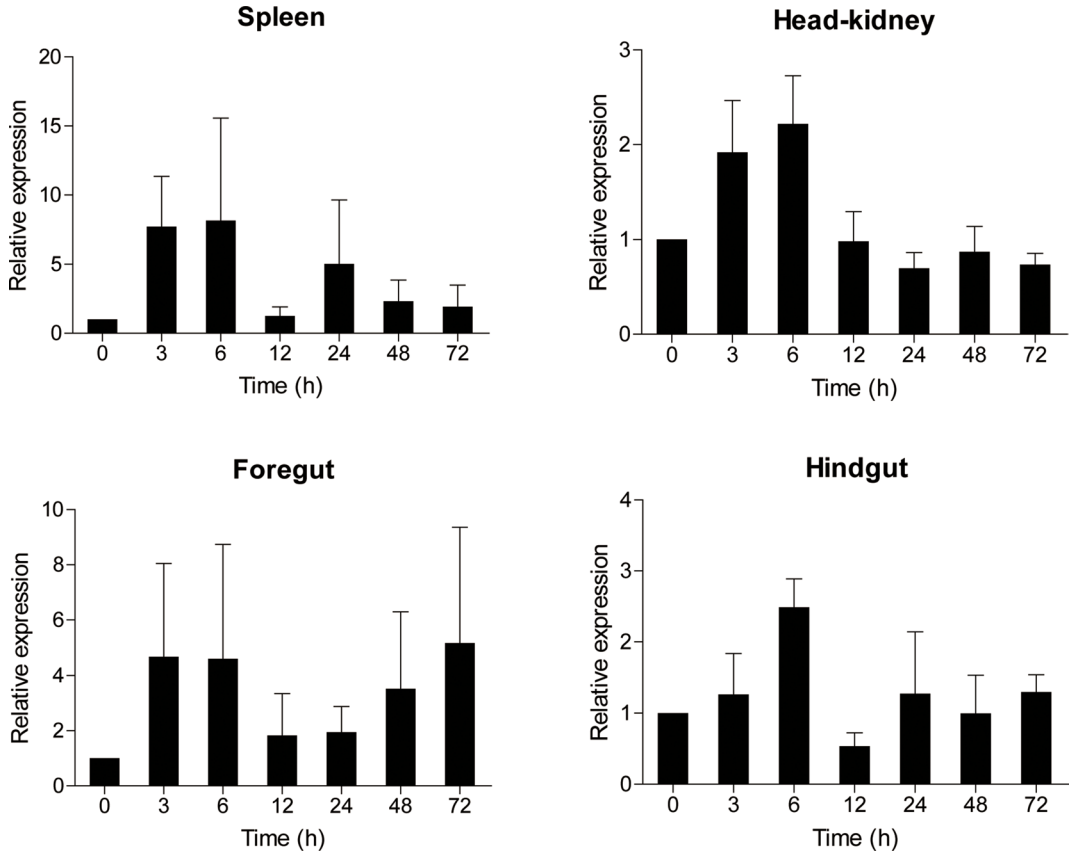

Fig. 5 Expression analysis of cc*IRF8* in various tissues of common carp after i. p. injection with *A. hydrophila*. The expression of cc*IRF8* in the spleen (**A**), head kidney (**B**), foregut (**C**) and hindgut (**D**) at different time points is shown. The results were calculated relative to the expression of the 40 S ribosomal protein *S11*. The Y-axis represents a fold increase to the unstimulated control group (denoted by 0 h). ($n = 3$, mean ± SD, $^*P < 0.05$)

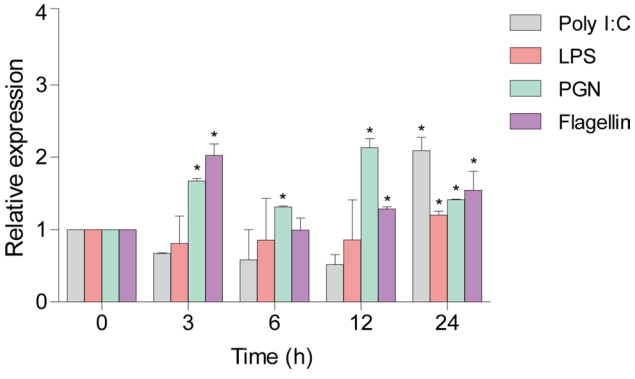

Fig. 6 The relative expression of cc *IRF8* in the HKLs of common carp after treatment with poly(I: C), LPS, PGN and flagellin at different time points. The results were calculated relative to the expression of the 40 S ribosomal protein *S11*. The Y-axis represents a fold increase to the unstimulated control group (denoted by 0 h). ($n = 3$, mean ± SD, $^*P < 0.05$)

Further, in mammals, IRF8 was shown to act as a key regulator of host defenses against bacteria, such as, *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Salmonella typhimurium* [40, 41]. Therefore, our findings, together with these analogous results, suggest that ccIRF8 may play vital roles in the antibacterial defense as reported for mammalian IRF8.

Subsequently, leukocytes from head kidney were isolated for the in vitro experiments, which was used to get a better understanding of the mechanisms of ccIRF8. The expression of cc*IRF8* was determined in HKLs upon stimulation with poly I: C, LPS (a component of the outer membranes of gram-negative bacteria), PGN (a unique and essential component of gram-positive bacterial cell walls) and Flagellin (a principal component of bacterial flagella) [42–44]. The expression of cc*IRF8* was upregulated in HKLs by all the four stimulants (Fig. 6), which further confirmed the in vivo results.

Previous studies have shown that IRF8 binds to the ISRE domains and upregulates type I IFN expression in dendritic cells [45]. Moreover, IRF8 has also been verified as a downstream target of the IFN-γ/STAT1-signaling pathway in mammals [46]. IRF8-deficient mice is more susceptible to infection by virus due to impaired dendritic cell development and defective production of IFNγ [47]. The EPC cell line was initially established from

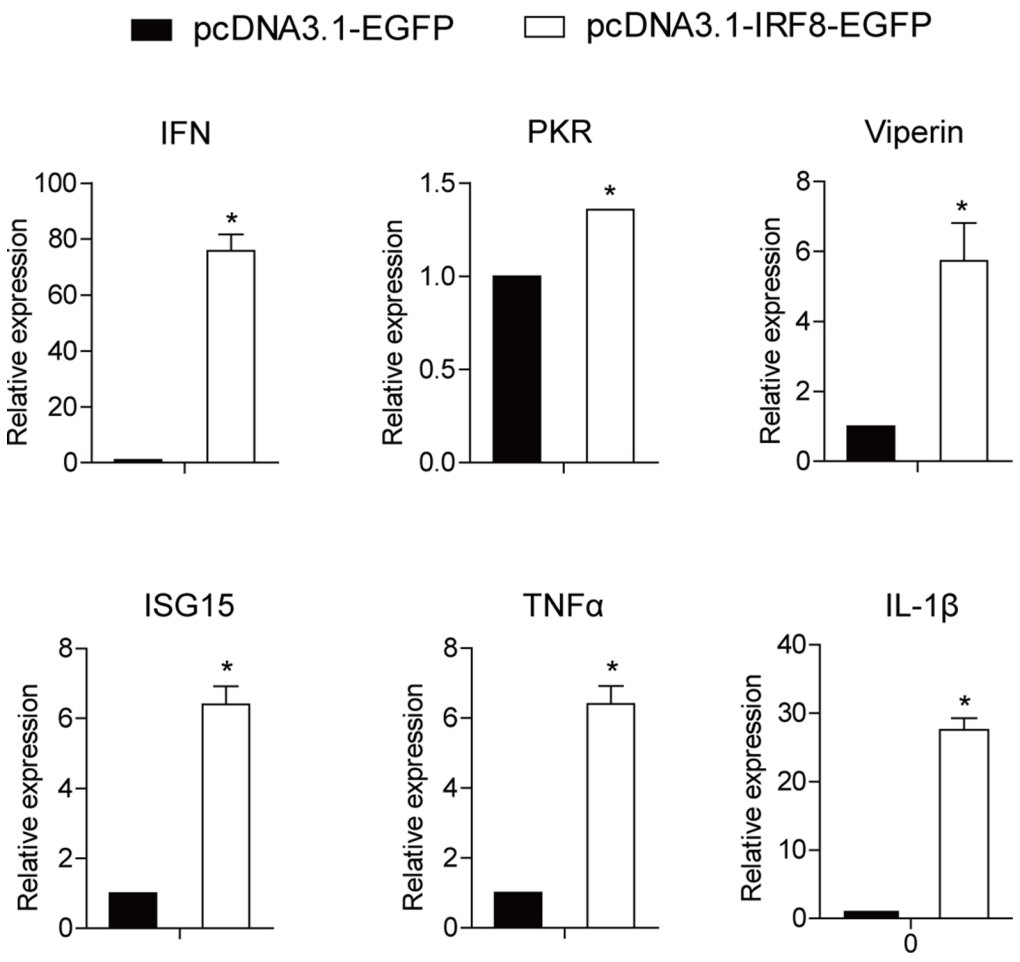

Fig. 7 The relative expression of *IFN*, *PKR*, *Viperin*, *ISG15*, *TNFα* and *IL-1β* in cc *IRF8*-transfected EPC cells. The results were calculated relative to the expression of the *β-actin* using real-time PCR. ($n = 3$, mean ± SD, $^*P < 0.05$)

proliferative skin lesions of common carp in the 1970s [48]. The temperature growth range, good splitting ratio (1/10), easy culture, much higher transfection efficiency and virus susceptibility make EPC cells become one of the most widely used tools for research on the immune response in fish and the diagnosis of fish viral diseases [49–54]. Although current EPC lineages are contaminated with cells from another Cyprinidae family member, fathead minnow (*Pimephales promelas*) [55], EPC remains a current subject for the study of functional assay of immune genes in common carp [32, 34, 35, 56–61]. In this study, the overexpression of IRF8 in common carp upregulated the expression of *IFN*, *PKR*, *Viperin*, *ISG15*, *TNFα* and *IL-1β* in EPC cells (Fig. 7). This finding is consistent with the study in golden pompano, which suggested the positive regulatory function of IRF8 on IFNγ expression [25]. Thus, ccIRF8 may also play a positive role in regulating the expression of IFN and related factors as reported for other fish and mammalian IRF8s.

NF-κB, a fast-response transcription factor that mediates the production of a great deal of pro-inflammatory cytokines, plays vital roles in many signaling pathways of the innate immune response [62]. In mammals, IRF8 interacts with TRAF6 to participate in the activation of NF-κB in an MyD88-dependent way [14]. In the present study, the dual-luciferase reporter assay showed that cc*IRF8* failed to activate *NF-κB* and inhibited the NF-κB signaling pathway mediated by *TRAF6* (Fig. 8). Similarly, in miiuy croaker, IRF8 negatively regulate both of the MyD88- and RLR-mediated NF-κB signaling pathways [26, 27]. Thus, we can conclude that fish IRF8 is a negative regulator in the NF-κB signaling pathway as mammalian IRF8.

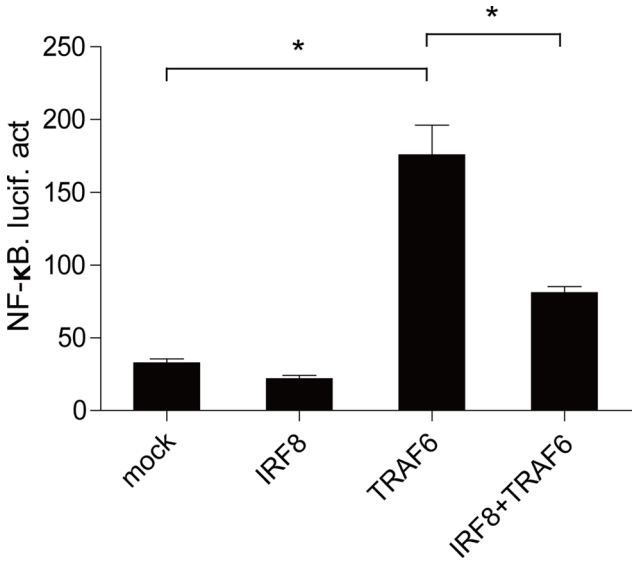

Fig. 8 Activation of *NF-κB* mediated by cc *IRF8*. 293T cells were co-transfected with expression vectors for firefly luciferase reporter gene with *NF-κB* promoter, Renilla luciferase gene and target gene for 48 h. The relative activity of *NF-κB* was the ratio of firefly fluorescence to Renilla fluorescence. Data are presented as a fold increase relative to the mock (vector without target gene). ($n = 3$, mean ± SD, $^*P < 0.05$)

Conclusions

In the present study, we have cloned the full-length cDNA of *IRF8* from common carp, and found that its expression was significantly induced in immune relevant tissues and HKLs following pathogen invasion. Our data imply that ccIRF8 may play a pivotal role in the innate antiviral and antibacterial immune response in common carp. Meanwhile, its positive regulatory function in the IFN signaling pathway and negative regulatory function in the NF-κB signaling pathway was also determined. Our findings may help further understanding of the regulatory functions of IRF8 in fish and exploring effective methods for fish disease control.

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Author contributions
Y.Z. performed the experiments, analyzed the data and wrote the paper. G.Y. conceived and designed the experiments. All authors read and approved the final manuscript.

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Data availability
The dataset supporting the conclusions of this article is available in the GenBank (https://www.ncbi.nlm.nih.gov/nuccore/OP759641) and the accession number is OP759641.

Declarations

Ethics approval and consent to participate
We obtained informed consent from the owners to use the animals. The protocol was approved by the Animal Experimental Ethics Committee of Shandong Normal University, and all methods were performed in accordance with the relevant guidelines and regulations. The study was carried out in compliance with the ARRIVE guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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