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Characterization and function of a group II type I interferon in the perciform fish, large yellow croaker (*Larimichthys crocea*)

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ABSTRACT

Teleost fish possess two groups of type I interferons (IFNs) with two (group I IFNs) or four (group II IFNs) conserved cysteines, which are further classified into seven subgroups. In our previous study, two group I type I IFNs, *Lc*IFNd and *Lc*IFNh (a new subgroup member), were identified in the perciform fish, large yellow croaker (*Larimichthys crocea*). Here, we identified a group II type I IFN, *Lc*IFNc, in this species. The deduced *Lc*IFNc contained six cysteines, four of which are highly conserved (C1: C^{28}, C2:C^{53}, C3: C^{130}, and C4:C^{159}) in the fish group II type I IFNs, and a typical type I IFN signature motif was also found in it. Phylogenetic analysis indicated that *Lc*IFNc belongs to the IFNc subgroup of fish group II type I IFNs. *Lc*IFNc was constitutively expressed in all examined tissues, and was rapidly up-regulated in spleen and head kidney by poly(I:C) and *Aeromonas hydrophila*. Recombinant *Lc*IFNc protein (r*Lc*IFNc) could increase the expression of antiviral genes, Mx1, PKR and ISG15, in large yellow croaker peripheral blood leukocytes (PBLs). The r*Lc*IFNc also exhibited obvious antiviral activity based on less cytopathic effect (CPE) and decreased expression levels of several viral genes in the r*Lc*IFNc-treated grouper spleen (GS) cells following Singapore grouper iridovirus (SGIV) infection. Additionally, r*Lc*IFNc was able to induce the expression of *Lc*IFNc, as well as *Lc*IFNd and *Lc*IFNh in the PBLs and primary head kidney cells (HKCs) from large yellow croaker. These results therefore indicated that *Lc*IFNc not only had antiviral activity, but also mediated the regulation of type I IFN response.

1. Introduction

Type I interferon (IFN) system is the first line of defense against viral pathogens in innate immunity [1]. As in mammals, type I IFNs in teleost fish perform their antiviral effects by inducing a variety of antiviral genes, such as myxovirus resistance (Mx), protein kinase R (PKR), virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible (Viperin), and IFN-stimulated gene (ISG) 15 through JAK-STAT pathway [2–4]. Based on the presence of conserved cysteines in the mature peptide, fish type I IFNs are classified into two groups, with two and four cysteines in group I and group II, respectively [2,5]. Phylogenetically, the two groups can be further divided into seven subgroups, with IFNa, IFNd, IFNe and IFNh belonging to the group I, and IFNb, IFNc and IFNf to the group II [5–7]. Group I IFNs are found in all teleost fish lineages, while group II IFNs only in salmonid, cyprinid, and silurid fish [5,8]. Recently, two group I IFNs, IFNd and IFNh (a new subgroup), have been identified in large yellow croaker (*Larimichthys crocea*) and other perciform species, indicating that the perciform fish, in which only IFNd subgroup was found, possess two subgroups of group I IFNs [6,9,10]. Moreover, identification of three type I IFNs, IFNc (a group II IFN), IFNd and IFNh in two perciform species, meagre (*Argyrosomus regius*) and mandarin fish (*Siniperca chuatsi*), suggested that the group II IFNs also exist in the perciform fish [11,12].

Expression of type I IFN subgroups has been well characterized in different fish species. Overall, teleost group I type I IFNs are ubiquitously expressed in most cell types and tissues, and increased upon viral infection or immune stimulation [5,6,8,13]. Group II type I IFNs appear to show a relatively low constitutive expression in fish tissues, and are inducible only in some specific leukocyte populations except IFNf, which is induced in fibroblasts [5]. For example, rainbow trout IFNc, a group II IFN, was constitutively expressed in various tissues tested at a very low level, and remarkably up-regulated in the lymphoid organs kidney and spleen by viral infection. The similar case was observed in the trout primary head kidney leukocytes, in which trout IFNc

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transcripts were lowly expressed and markedly increased by poly(I:C) [5]. However, recent studies showed that meagre IFNc was highly expressed in both larva and adult tissues tested (except gills), and significantly up-regulated in these tissues by poly(I:C) [11]. IFNc in mandarin fish was the highest expressed IFN in several tissues tested, including skin and muscle, when compared with two group I IFNs, IFNd and IFNh [12]. These findings indicated that expression pattern of IFNc varied greatly among fish species.

As fish group I type I IFNs, group II IFNs also exhibit antiviral activities. Both zebrafish IFNphi1(IFNa, group I) and IFNphi2 (IFNc, group II) can induce the expression of antiviral genes (Viperin and Mx) in the zebrafish larva, and protect the larva against infectious haemorrhagic necrosis virus (IHNV) challenge [14]. Further studies indicated that zebrafish IFNphi2/3 (two IFNcs, group II) actually trigger a rapid and transient induction of antiviral genes [15]. Like IFNa, salmon IFNc also showed powerful antiviral activities by inducing expression of antiviral genes [3]. Recently, IFNc in mandarin fish and turbot (IFN1) was demonstrated to elicit its antiviral effects through JAK-STAT pathway [12,16]. Additionally, IFNc in mandarin fish could induce the expression of itself and IFNh, but not IFNd [12].

In this study, we report the identification of a group II type I IFN, *Lc*IFNc, from large yellow croaker (*Larimichthys crocea*). *Lc*IFNc gene was constitutively expressed in all analyzed tissues, and rapidly up-regulated in spleen and head kidney by poly(I:C), a viral mimic, and *A. hydrophila*. Recombinant *Lc*IFNc protein (r*Lc*IFNc) not only induced the expression of antiviral genes in large yellow croaker peripheral blood leukocytes (PBLs), but also provided enhanced protection of grouper spleen (GS) cells against Singapore grouper iridovirus (SGIV) infection. Additionally, r*Lc*IFNc could induce the expression of *Lc*IFNc and two group I IFNs (*Lc*IFNd and *Lc*IFNh) in the PBLs and primary head kidney cells (HKCs) from large yellow croaker. These data indicated that *Lc*IFNc not only had antiviral activity, but also mediated the regulation of type I IFN response, thus providing further insights into the functional understanding of type I IFNs in teleost fish.

2. Materials and methods

2.1. Fish and challenge experiments

Large yellow croakers (weight: 104 ± 13.6 g; length: 21 ± 1.5 cm) were purchased from a mariculture farm in Ningde district, Fujian province, China. Fish were maintained with a flow-through seawater supply at 25 °C. After acclimating for 5 days, healthy fish were used for the challenge experiments. Two groups of 25 fish each were injected intraperitoneally with poly(I:C) [Sigma-Aldrich, St. Louis, MO, USA; 1 mg/mL in phosphate-buffered saline (PBS)] or *Aeromonas hydrophila* (1×10^9 CFU/mL in PBS) at a dose of 0.2 mL/100 g fish. Third group of 25 fish was injected with sterile PBS at a dose of 0.2 mL/100 g fish as a control. Spleen and head kidney tissues were collected from five fish in each group at 3, 6, 12, 24, and 48 h post-injection, frozen immediately in liquid nitrogen, and stored at 80 °C for RNA extraction.

2.2. Cells and virus

The PBLs and primary head kidney cells (HKCs) were prepared as described previously [17,18]. Briefly, the blood was sampled from the caudal vein sinus of large yellow croaker using a 23-gauge needle and a 2-mL heparinized syringe. After diluted by Leibovitz's medium (L-15 medium, Gibco, USA) containing 0.1% heparin, 200 IU/mL penicillin, and 200 mg/mL streptomycin (Sigma, USA), blood was loaded onto freshly prepared 34%/51% Percoll (GE, USA) density gradients and separated via centrifugation at 650 × *g* for 30 min at 4 °C. After centrifugation, PBLs were taken from the interface between 34%/51% Percoll and washed three times with L-15 medium above. The head kidney tissue was obtained from three freshly killed fish under sterile conditions and gently pushed through a Falcon® 70 μm Cell Strainer (BD, USA). The HKCs were collected by centrifugation at 350 *g* for 10 min. Both PBLs and HKCs were resuspended with L-15 medium at 25 °C and counted using the MOXI Z automated cell counter (ORFLO, USA). Grouper spleen (GS) cells were originated from the spleen of orange-spotted grouper *Epinephelus coioides* and maintained in L-15 medium supplemented with 10% fetal bovine serum (FBS, Life Technologies) at 25 °C. Singapore grouper iridovirus (SGIV) was propagated in GS cells as previously described [19], and the virus stock was stored at −80 °C until use.

2.3. Cloning of complete cDNA of LcIFNc gene

The *Lc*IFNc gene sequence was predicted by blasting large yellow croaker genome (JRPU00000000) using other fish IFNc sequences [20]. Primers (IFNc-ORF-F/-R; Table S1) were designed based on the predicted coding sequence of *Lc*IFNc gene (**NW_017609430.1**), and the complete cDNA of *Lc*IFNc gene was amplified by reverse transcription (RT)—PCR with this primer set. The resulting PCR product was cloned into the pMD18-T simple vector (TaKaRa) and sequenced. Multiple sequence alignment was performed using the ClustalX program, and conserved residues were shaded using the BOX-SHADE server (version 3.21) (http://www.ch.embnet.org/software/BOX_form.htmL). Alignment of the deduced amino acid sequences of fish type I IFNs was performed using ClustalW and the tree was constructed using the Neighbor-joining method using Molecular Evolution Genetics Analysis (MEGA) software version 6.0 with the pairwise deletion option [21]. The tree is boot-strapped 10,000 times, and the bootstrap values of the major branches are shown as percentages. Signal peptides were predicted using Signal P4.0 software, and *N*-glycosylation sites using the Net-NGlyc1.0 server (http://www.cbs.dtu.dk/services/). Amino acid sequence identity and similarity were calculated using the Matrix Global Alignment Tool (Matgat, version 2.0) [22].

2.4. Expression analysis of LcIFNc gene in tissues by real-time PCR

To investigate the tissue expression profile of *Lc*IFNc, various tissues, such as blood, brain, gills, heart, head kidney, intestine, liver, muscle, skin, spleen, and stomach, were sampled and pooled from three healthy fish, and then used for RNA extraction and cDNA synthesis. Real-time PCR was performed on Master-cycler epgradient realplex4 (Eppendorf, Germany) using SYBR® Premix ExTaqTM (TaKaRa) with gene-specific primers (IFNc-F/-R; Table S1). *Lc*β-actin was amplified as an internal control with the Actin-F/Actin-R primers (Table S1). Cycling conditions were 30 s at 95 °C, then 40 cycles of 95 °C for 5s, 55 °C for 15s, and 72 °C for 20s. The expression levels of *Lc*IFNc were normalized by *Lc*β-actin using the $2^{-\Delta\Delta CT}$ method, and expressed as the ratio of *Lc*IFNc expression levels in the blood as previously described [6,23].

To further study the modulation of *Lc*IFNc gene expression upon poly(I:C) or *Aeromonas hydrophila* induction, total RNA was extracted from the pooled head kidney and spleen tissues of five fish collected at different time points post-injection above. The first strand cDNA was synthesized from 0.1 mg of each total RNA. Real-time PCR was then performed using the conditions as aforesaid. The relative expression levels of *Lc*IFNc were normalized by *Lc*β-actin and expressed as fold changes by comparing the normalized gene expression levels of poly (I:C)- or *A. hydrophila*-injected fish with those of the PBS-injected fish (defined as 1) at the same time point [6,23]. The data were obtained from three independent PCR assays with three replicates in each assay.

2.5. Expression and purification of recombinant LcIFNc protein in E. coli

Recombinant *Lc*IFNc protein (r*Lc*IFNc) was expressed as a fusion

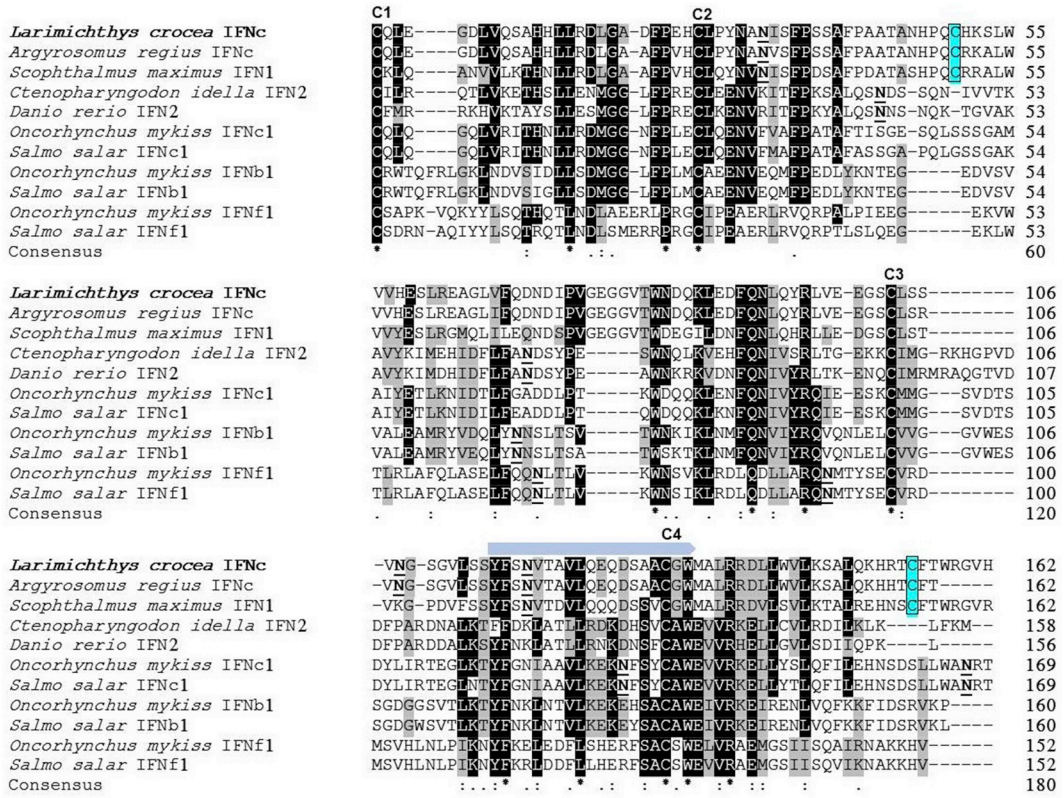

Fig. 1. Multiple alignment of mature peptide sequences of *Lc*IFNc with type I IFNs in other fish species. Sequence alignments were obtained using ClustalX program, and the conserved residues are shaded using BOXSHADE (v3.21). The conserved cysteine residues which may be involved in the formation of intramolecular disulphide bonds were indicated by paired lines (C1−C3 and C2−C4). The typical signature motif of fish type I IFNs is marked with the light blue arrow above. Identical residues are indicated by stars. A: (colon) indicates conservation between groups of strongly similar properties, scoring > 0.5 in the Gonnet PAM 250 matrix, while A. (period) indicates conservation between groups of weakly similar properties scoring ≤ 0.5. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

protein with the *N*-utilization substance A (Nus) tag and 6 × His tag using pET-43.1a vector (Novagen, Germany). Briefly, *Lc*IFNc gene fragment without the signal peptide sequence was amplified with gene-specific primers (rIFNc-F/-R; Table S1), and inserted into the *Eco*R I/*Hin*d III-digested vector pET-43.1a. The resulting recombinant plasmid pET-43.1a-*Lc*IFNc was transformed into component cells of *E. coli* BL21 (Novagen). The r*Lc*IFNc was expressed by 0.1 mM IPTG induction at 16 °C overnight. The r*Lc*IFNc was purified by NI-NTA nitrilotriacetic acid (NI-NTA) affinity chromatography under native condition using the ProBondTM Purification System (Life Technologies, USA). Purified protein was dialyzed against PBS at 4 °C, filtered with a sterile 0.2 mm filter, and stored at −80 °C for next use. The recombinant Nus (rNus) was also expressed and purified in the same system as a control.

2.6. Antiviral activity assays in grouper spleen cells

The GS cells (1×10^6 cells/well) were seeded onto the 6-well plates (Thermo Fisher Scientific) at 28 °C for 18 h. The cells were pretreated with r*Lc*IFNc or rNus (as a control) at a final concentration of 10 ng/mL for 2 h; then, the cells were infected with SGIV at a multiplicity of infection (MOI) of 2. After 24 h infection, the cells were observed microscopically for the cytopathic effect (CPE) (Leica Microsystems, Wetzlar, Germany).

The expression of four SGIV genes, major capsid protein (MCP), envelope protein VP19 and VP136, and infected cell polypeptide (ICP18), was detected by real-time PCR. Briefly, the infected cells were harvested at 12, 24, and 48 h post-infection. Total RNA was extracted using the SV total RNA Isolation System (Promega), and reverse-transcribed into the first-strand cDNA using an Oligo dT-Adaptor primer (TaKaRa, Dalian, China). Real-time PCR was performed with gene-specific primer sets (Table S1). *E. coioides* β-actin (*Ec*β-actin) gene was amplified as an internal control with the *Ec*actin-F/-R primers (Table S1). Real-time PCR was performed on the Mastercycler ep gradient realplex4 system (Eppendorf, Germany) using SYBR® Premix ExTaq™ (TaKaRa). Cycling conditions were 3 min at 94 °C, then 40 cycles at 94 °C for 5 s, 60 °C for 10 s, and 72 °C for 10 s. The fluorescence output for each cycle was analyzed upon the completion of the entire run. The expression levels of four viral genes, MCP, VP19, VP136 and ICP18, were normalized by *Ec*β-actin using the $2^{-\Delta\Delta CT}$ method [24]. Each experiment was repeated three times.

2.7. Bioactivity analysis of rLcIFNc in vitro

To investigate the bioactivity of *Lc*IFNc, the PBLs and HKCs were plated in 6-well plates at a density of 1×10^6 cells/well. Then PBLs and HKCs were incubated with r*Lc*IFNc or rNus (as a control) at a final concentration of 10 ng/mL for 2, 4 and 8 h, respectively. Total RNA was extracted from these cells using ReliaPrepTM RNA Cell Miniprep System Kit (Promega, USA). Real-time PCR was performed to determine the expression levels of antiviral genes (Mx1, PKR and ISG15) and IFN genes (*Lc*IFNc, *Lc*IFNd, and *Lc*IFNh) at different time points post-treatment with the respective primers (Table S1). The relative expression levels of these genes were normalized by *Lc*β-actin, and expressed as fold changes by comparing the normalized gene expression levels in

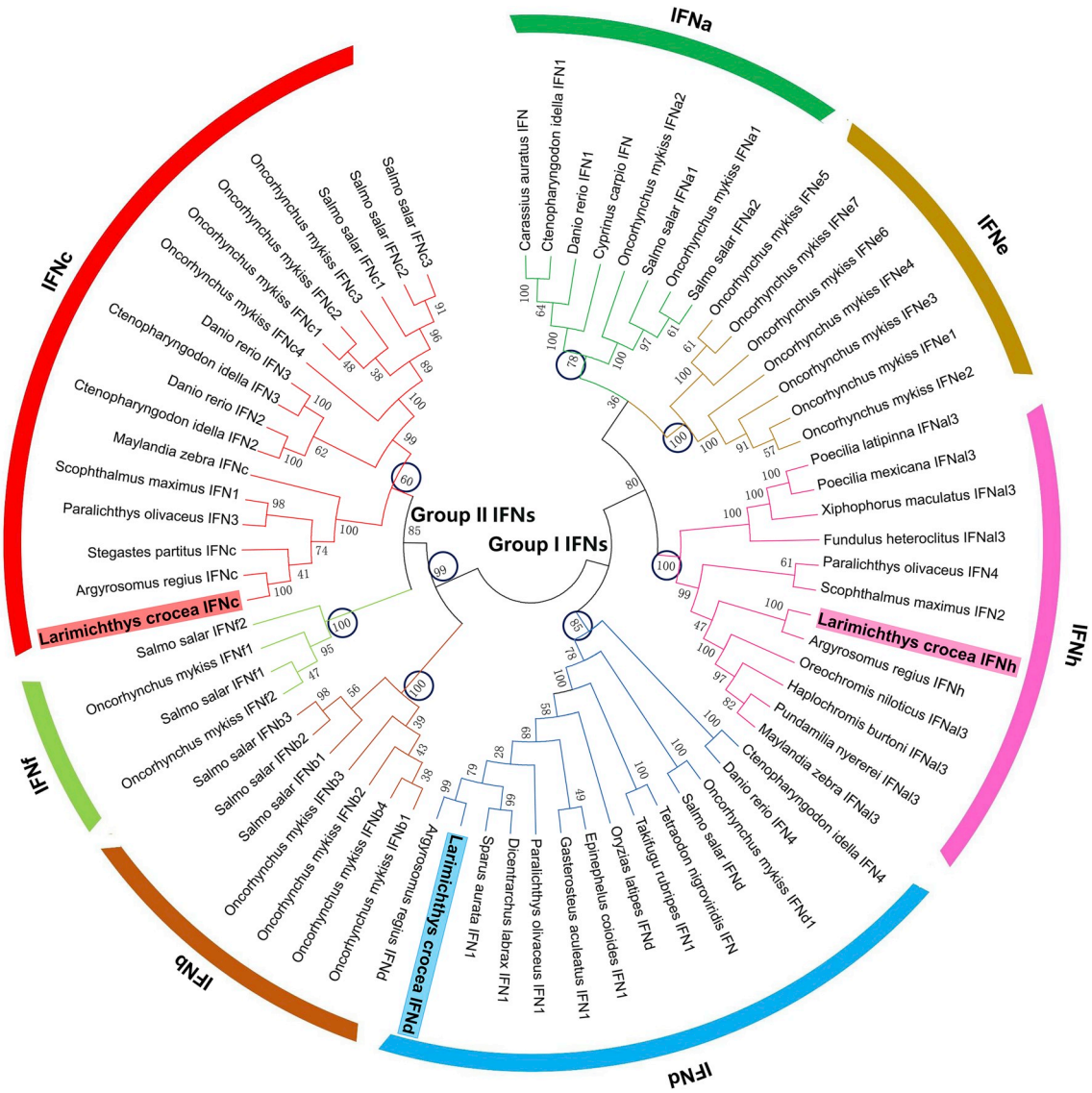

Fig. 2. Phylogenetic tree of fish type I IFN family members based on the genetic distances of deduced amino acid sequences. Deduced amino acid sequences of type I IFN family members were aligned, and the tree was constructed with the Neighbor-Joining method using the MEGA (version 6) software package. The tree is boot-strapped 10,000 times, and the bootstrap values of the major branches are shown as percentages. The *Lc*IFNs are shown in bold and distinguished by different colors. The GenBank accession numbers for fish type I IFN amino acid sequences used here are as follows: *Argyrosomus regius* (*Ar*), AVD96636 (IFNc), AVD96637 (IFNd), AVD96638 (IFNh); *Carassius auratus* (*Ca*), AAR20886; *Ctenopharyngodon idella* (*Ci*), ABC87312 (IFN1), AMT92190 (IFN2), AMT92191 (IFN3), AMT92192 (IFN4); *Cyprinus carpio* (*Cc*), ADI81047; *Danio rerio* (*Dr*), AAM95448 (IFNphi1), NP_001104552 (IFNphi2), NP_001104553 (IFNphi3), NP_001155212 (IFNphi4); *Dicentrarchus labrax* (*Dl*), CAQ17043 (IFN1); *Epinephelus coioides* (*Ec*), AGL21770 (IFN1); *Fundulus heteroclitus* (*Fh*), XP_012711611 (IFNal3); *Gasterosteus aculeatus* (*Ga*), CAM31706 (IFN1); *Haplochromis burtoni* (*Hb*), XP_005950669 (IFNal3); *Larimichthys crocea*, API68651 (IFNd), API68650 (IFNh); *Maylandia zebra* (*Mz*), XP_013771349 (IFNal3), XP_013771529 (IFNc); *Oncorhynchus mykiss* (*Om*), CAM28541 (IFNa1), NP_001153977 (IFNa2), CCV17397 (IFNa3), CCV17398 (IFNa4), NP_001153974 (IFNb1), NP_001158515 (IFNb2), CCV17399 (IFNb3), CCV17400 (IFNb4), CCV17401 (IFNb5); CCV17402 (IFNc1), CCV17403 (IFNc2), CCV17404 (IFNc3), CCV17405 (IFNc4), CAV07949 (IFNd1), CCV17406 (IFNe1), CCV17407 (IFNe2), CCV17408 (IFNe3), CCV17409 (IFNe4), CCV17410 (IFNe5), CCV17411 (IFNe6), CCV17412 (IFNe7), CCV17413 (IFNf1), CCV17414 (IFNf2); *Oreochromis niloticus* (*On*), XP_005469255 (IFNal3); *Oryzias latipes* (*Ol*), BAU25609 (IFN1); *Paralichthys olivaceus* (*Po*), BAA02372 (IFN1, BBA46271 (IFN3) BBA46272 (IFN4); *Poecilia latipinna* (*Pl*), XP_014869968 (IFNal3); *Pundamilia nyererei* (*Pn*), XP_013771349 (IFNal3); *Salmo salar* (*Ss*), ABD39320 (IFNa1), ABD39321 (IFNa2), ACE75687 (IFNa3); ACE75691 (IFNb1), ACE75693 (IFNb2), ACE75689 (IFNb3), ACE75692 (IFNc1), XP_014048249 (IFNc2), ACE75688 (IFNc3), DAA64377 (IFNd); *Scophthalmus maximus* (*Sm*), AID59461 (IFN1), AID59462 (IFN2); *Sparus aurata* (*Sa*), CAT03221 (IFN1); *Stegastes partitus* (*Sp*), XP_008298153 (IFNc) *Takifugu rubripes* (*Tr*), CAM82750 (IFN1); *Tetraodon nigroviridis* (*Tn*), CAD67779. *Xiphophorus maculatus* (*Xm*), XP_023204970 (IFNal3). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the r*Lc*IFNc-treated cells with those in the rNus-treated cells at the same time point as described previously [6].

2.8. Statistical analysis

All data were analyzed using GraphPad Prism 5 software and expressed as the standard error of the mean (SEM) of three repeated experiments. The data were subjected to analysis of one way ANOVA by using IBM SPSS Statistics 19, and the *P* values smaller than 0.05 were considered statistically significant.

Table 1
Amino acid (aa) similarity (bottom, left) and identity (top, right) of large yellow croaker IFNc, IFNd and IFNh with other known aa sequences of teleost fish type I IFNs.

Similarity/Identity	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1. trout IFNa1		39.1	30.3	22.2	21.6	24.4	19.3	19.8	19.5	26.4	26.8	30.6	31.7	34.9	34.4	32.1	36.5	27.1	27.6	23	33	32.5	36.4	27.1	28.4
2. grass carp IFN1	59.4		25.8	18.6	19.6	19.8	17.1	19.2	19.4	25.9	24.7	31.1	31.6	30.9	33.3	29.2	34.2	22.7	30	21.5	30.5	31	30.5	25.9	28.8
3. trout IFNb1	51.4	47.5		26.2	25.7	26.2	24	22.5	24.8	35.2	32.1	28.6	28.8	27.2	24.9	27.8	32.1	23.3	21.3	24.9	23.4	23.6	22.8	22.5	21.2
4. croaker IFNc	44.4	41.3	45.5		94.7	64.7	63	57.9	49.5	32.7	28.1	21.2	20.7	19.8	22.2	18.5	21.1	19.7	18.1	24.5	21.6	23.7	19.4	17.1	16.6
5. meagre IFNc	42.9	41.8	44.4	96.3		66.8	65.1	58.9	48.6	35.2	28	20.9	20.4	20.3	23.2	20.2	20.6	20.5	17.5	21.4	20.3	21.4	18	17.1	17.6
6. damselfish IFNc	44.6	41.3	49.5	79.4	80.4		62.8	56.8	54.1	31.6	25.6	23.2	22.7	18.8	23.7	20.9	22.4	20.1	19.6	19.3	22.8	23.9	21.2	22.1	19.5
7. turbot IFN1	43.1	40.3	48.1	80.4	82	78.3		72.8	47.6	34.4	28.1	21	21.5	18.5	24.1	20	20.6	21.8	19.1	20.6	17.2	18.7	21.6	18.5	18.9
8. flounder IFN3	39.7	40.2	48.4	74.6	75.7	76.1	82.6		45.9	27.9	25.3	18.2	17.7	17.9	20.8	16.8	22.6	17.8	17.5	17.3	14.7	15.8	21.1	18.2	17.6
9. cichlid IFNc	38.9	40.4	40.4	66.3	65.9	67.3	60.1	60.6		25	26.8	20.5	19.5	18.5	24.3	17.7	21.5	19.2	16.8	20.7	19.6	21.1	20.9	21.4	17.1
10. trout IFNc1	46.5	46.5	56.7	59.3	59.3	56.1	57.2	52.9	47.6		42.6	24	24.2	23.6	22.6	25.4	27.6	24	21.9	22.8	25.2	25.4	27.2	20.5	20.3
11. grass carpIFN2	48.6	47	56.8	48.1	50.8	50	53	51.1	45.7	62.6		22.8	22.8	23.3	24.6	24	20.7	20.3	21.2	19.9	22.9	23.9	22.7	20.9	18.1
12. croaker IFNd	52.4	53	51.4	42.9	41.8	44.3	43.8	39.5	38.9	44.9	44.3		95.7	82.8	73	68.4	45.7	34.1	23.7	17.7	27.7	27.2	28.3	25.8	22.9
13. meagre IFNd	54.1	53.5	49.7	43.9	42.9	44.3	44.3	40.5	39.4	45.5	44.9	98.4		85.5	72.4	72.2	44.7	33.5	24.2	17.6	27.7	28.2	29.8	26.3	24.5
14. sea bass IFN1	55.1	49.7	49.7	46	44.4	43.2	47	40	38.5	43.9	46.5	90.3	91.4		71.4	71.1	41.2	34.6	25.8	21.2	28.6	30.5	28.3	24.4	25.5
15. grouper IFN1	55.1	57.2	45.9	44.4	44.4	44.6	45.3	41.8	41.8	44.9	48.1	85.9	85.9	84.9		59.7	39.9	32	24.2	19.8	27.5	27.5	28.4	25.8	23.8
16. flounder IFN1	54.8	52.7	52.2	45.5	47.1	44.1	46.2	41.4	40.4	48.1	51.1	82.8	83.9	82.3	78		40	35.8	28	22.9	27.6	28.1	30.2	27	25.7
17. trout IFNd1	59.6	55.6	55.2	43.9	42.9	46.2	42.5	40.2	38.9	46	45.9	64.9	64.9	61.6	65.7	64		31.1	32.5	22.8	27.4	28.9	30.6	25.8	25.3
18. grass carpIFN4	46.9	47.2	48.6	40.2	42.3	46.2	41.4	37.5	35.6	46.5	44.3	53	54.6	50.3	52.8	54.3	55.1		23.8	22.9	25.9	25.4	25.3	22.7	24
19. trout IFNe1	51.6	55.9	43.5	41.3	41.3	43	39.8	37.1	35.1	41.7	43	47.8	50	52.7	48.4	53.2	51.1	47.8		19.1	24.6	25.6	27	22.4	22.2
20. trout IFNf1	40.1	42.2	43.7	46.6	42.9	46.2	43.1	43.5	39.9	43.3	47	40.5	36.8	40	41	43.5	44.9	45.8	40.3		22.1	21.8	21.6	19.9	20.3
21. croaker IFNh	55.8	53.7	49.5	43.2	40	46.8	41.6	37.4	32.7	46.3	47.9	48.4	48.9	48.9	46.3	50.5	47.4	46.8	47.4	43.7		89.5	55.3	52.7	48.4
22. meagre IFNh	56.3	53.2	48.4	45.3	41.1	47.9	42.6	35.8	36.1	44.2	44.2	47.9	48.9	48.9	45.3	50	47.4	46.8	47.9	42.1	93.2		56.3	54.1	48.4
23. flounder IFN4	57.7	55	44.3	38.6	34.9	41.3	44.2	40.8	36.1	46.5	42.6	49.2	50.3	47.6	50.6	51.6	48.9	53.1	48.9	41.8	66.3	69.5		45.1	50.3
24. Tilapia IFNa3	49.5	49	45.1	38.8	36.9	39.3	35.9	36.4	33.7	41.3	38.8	46.6	47.1	44.2	43.7	48.1	41.3	42.7	41.3	37.4	66	66.5	55.8		39.3
25. turbot IFN2	51.4	45	39.9	33.9	34.4	36.4	38.1	35.9	27.9	39.6	37.7	41.1	42.2	41.6	41	39.8	40.4	45	41.9	42.4	61.6	63.2	66.9	48.1	

The similarity and identity of IFNc aa sequences between fish species are shaded in yellowish color. The aa sequence identity of *Lc*IFNc and other fish type I IFNs is shaded in bluish color.

3. Results

3.1. Sequence analysis of LcIFNc gene and cDNA

The determined genomic DNA sequence of *Lc*IFNc gene (**MH636810**) is 1893 nucleotides (nt) in length, consisting of 5 exons and 4 introns (Fig. S1). The open reading frame (ORF) of *Lc*IFNc cDNA (**MH636809**) is 570 nt, encoding a protein of 172 amino acids (aa), where a 27-aa signal peptide is predicted and three putative *N*-glycosylation sites are found (Fig. S1). The deduced *Lc*IFNc protein contains six cysteines, four of which are highly conserved (C1: C^{28}, C2:C^{53}, C3: C^{130}, and C4:C^{159}) in the fish group II type I IFNs, and extra two only present in perciform fish (Fig. S1, Fig. 1). *Lc*IFNc also has a typical signature motif of fish type I IFNs ([FYH]-[FY]-X-[GNRCDS]-[LIVM]-X2-[FYL]-L-X7-[CY]-[AT]−W) at the C-terminus (Fig. 1), although it has some amino acid variations compared with other group II IFNs.

The phylogenetic tree based on the deduced amino acid sequences of fish type I IFNs showed that type I IFNs in teleost fish can be divided into two groups, which are further classified into seven obvious subgroups (Fig. 2). It was clear that *Lc*IFNc fell into a major clade formed by known fish IFNc sequences, which belonged to group II type I IFNs, while *Lc*IFNd and *Lc*IFNh were clustered with their respective orthologues belonging to group I type I IFNs (Fig. 2). In IFNc cluster, *Lc*IFNc had a closer phylogenetic relationship with meagre IFNc (**AVD96636**), turbot IFN1(IFNc subgroup;**AID59461**) and Japanese flounder (*Paralichthys olivaceus*) IFN3 (IFNc subgroup; **BBA46271**) (Fig. 2). Homology comparison showed that *Lc*IFNc exhibits a higher identity of 49.5–97.4% and similarity of 66.3–96.3% with IFNc sequences in pleuronectiform and perciform fish, and a relatively low identity of 25.6–35.6% and similarity of 48.1–59.3% with IFNc sequences in cypriniform and salmoniform species, respectively (Table 1). However, *Lc*IFNc shares a very low sequence identity of 17.1–24.0% and similarity of 33.9–46.6% with the other subgroup members of fish type I IFNs (Table 1).

3.2. Tissue expression analysis of LcIFNc gene

Tissue expression analysis showed that *Lc*IFNc gene was constitutively expressed in all examined tissues, with the highest levels in spleen and the lowest levels in blood (Fig. 3A). Induction of poly(I:C) and *A. hydrophila* resulted in significant up-regulation of *Lc*IFNc expression in spleen and head kidney. The expression of *Lc*IFNc peaked at 3 h after induction with poly(I:C) in both spleen and head kidney, with 9.4- and 104.5-fold increases, respectively (Fig. 3B), while its expression reached the highest levels at 6 h in spleen or 12 h in head kidney after bacterial challenge, with 14.9-fold or 37.9-fold increases (Fig. 3C).

3.3. Antiviral activity of LcIFNc

To investigate whether *Lc*IFNc was able to induce the expression of antiviral genes, r*Lc*IFNc was expressed and purified for the in vitro induction experiments (Fig. 4A). The large yellow croaker PBL*s* were treated with r*Lc*IFNc for 2, 4 and 8 h. As expected, the expression of three major antiviral genes, Mx1, PKR and ISG15, was significantly increased in the PBL*s* by r*Lc*IFNc, and reached the highest levels at 8 h post-treatment (Fig. 4B).

The antiviral activity of r*Lc*IFNc was analyzed using a grouper spleen cell line (GS), where a viral infection model was established [19]. GS cells pretreated with r*Lc*IFNc prior to SGIV infection showed a less CPE compared to the control cells (GS cells pretreated with rNus) (Fig. 4C), suggesting that r*Lc*IFNc could effectively protect GS cells against SGIV challenge. Furthermore, r*Lc*IFNc could significantly

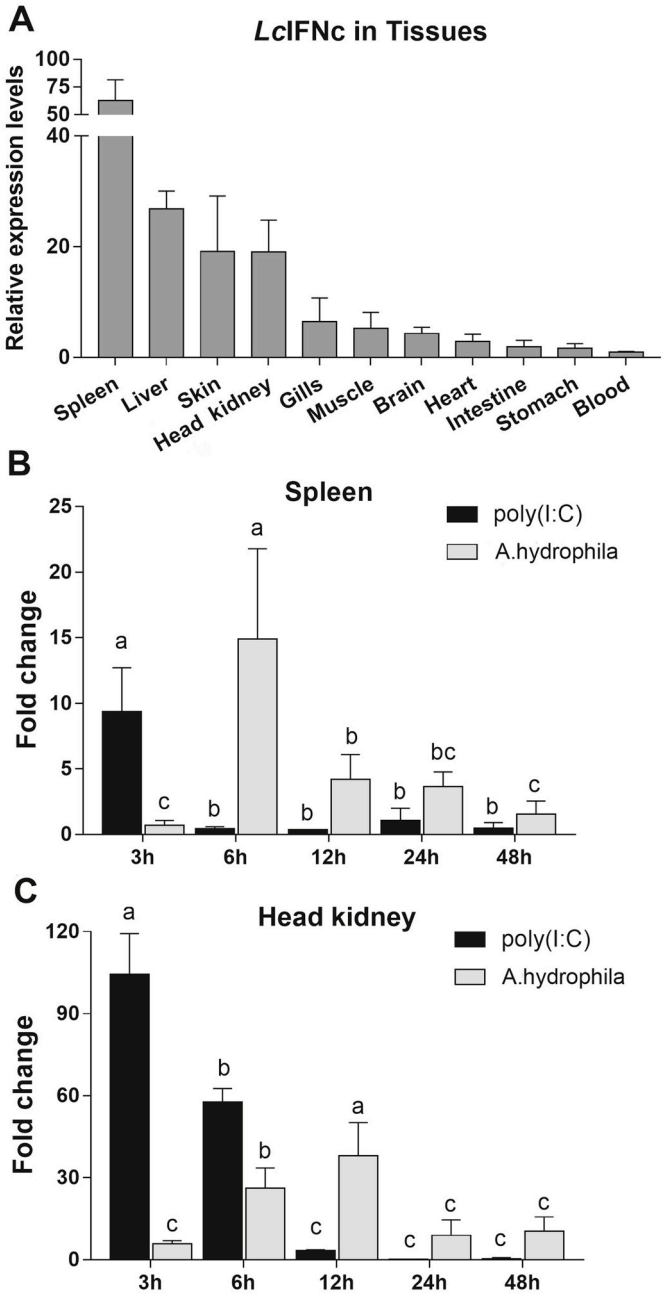

Fig. 3. Expression analysis of *Lc*IFNc. (A) Tissue expression profile of *Lc*IFNc. Total RNA was extracted from various tissues of five healthy fish and used for real-time PCR analysis. The relative expression levels of *Lc*IFNc were normalized by β-actin using the $2^{-\Delta\Delta CT}$ method and expressed as the ratio of its expression levels in the blood. The tissues were ordered according to the relative expression levels from the highest to the lowest. (B, C) Modulation of *Lc*IFNc gene expression in the spleen (B) and head kidney (C) tissues after poly(I:C) or *A. hydrophila* stimulation. Spleen and head kidney tissues were collected from five fish at different time points post-injection for real-time PCR analysis. The relative expression levels of *Lc*IFNc gene were normalized by β-actin and expressed as fold changes by comparing the normalized gene expression levels of poly(I:C)- or *A. hydrophila*-injected fish with those of the PBS-injected fish at the same time point. Error bars represent the standard error of the mean (± SEM) of three independent PCR assays. Data in the same treatments with a, b, c are significant difference ($p < 0.05$) among different time points.

decrease the expression levels of four viral genes (MCP, VP19, VP136 and ICP18 genes) within 48 h (Fig. 4D), further supporting its antiviral activity.

3.4. Activation of type I IFN response by LcIFNc

To investigate whether *Lc*IFNc could trigger the type I IFN response, the PBLs and HKCs from large yellow croakers were treated with r*Lc*IFNc for 8 h, and then the expression changes of three IFNs, *Lc*IFNc, *Lc*IFNd and *Lc*IFNh genes, were examined by real-time PCR. The results showed that r*Lc*IFNc not only could induce the expression of *Lc*IFNc in the PBLs and HKCs (with 9.3- and 11.6-fold increases, respectively; Fig. 5A and B), but also up-regulate the expression of *Lc*IFNd and *Lc*IFNh in these two cell types in varying degrees (Fig. 5 A and B), indicating that *Lc*IFNc could induce the expression of itself and two group I IFNs in the large yellow croaker PBLs and HKCs.

4. Discussions

Recently, three type I IFNs, IFNc, IFNd and IFNh, were identified in two perciform species, meagre and mandarin fish [11,12]. In our previous study, two group I type I IFNs, *Lc*IFNd and *Lc*IFNh, were found in large yellow croaker [6]. Here, we identified a group II type I IFN, *Lc*IFNc, in this species. These data suggested that three subgroups of type I IFNs (IFNc, IFNd and IFNh) may be commonly present in the perciform fish, contrasting with the previous proposal that only IFNd subgroup existed in the perciform species [5,6]. Interestingly, the IFNc sequences known in the perciform species not only contain four highly conserved cysteines, as found in the fish group II IFNs, but also two extra cysteines (Fig. 1). However, whether these two additional cysteines are involved in the formation of intramolecular or intermolecular disulfide bond requires further investigations.

*Lc*IFNc was constitutively expressed in all analyzed tissues, with the highest levels in spleen (Fig. 3A), which was similar with the findings in meagre and mandarin fish where constitutive IFNc expression was observed in various tissues tested, with the higher levels in spleen [11,12]. High constitutive expression in the spleen of IFNc in three perciform species suggests that the cell types expressing IFNc are widespread at this organ, possibly due to the function of the spleen in protecting splenic cells from infection. Induction of IFNc by poly(I:C) or virus in spleen and head kidney was observed in salmon and brown trout [3,5]. Administration of poly(I:C) or virus also increased the expression levels of IFNc in two perciform species, meagre and mandarin fish [11,12], suggesting an important role in antiviral immune response of fish IFNc. In the present study, the expression levels of *Lc*IFNc were significantly increased in spleen and head kidney by both poly(I:C) and *A. hydrophila* (Fig. 3B), implying that *Lc*IFNc may be involved in host immune responses against both viral and bacterial infections. It was found that induction of *Lc*IFNc by *A. hydrophila* was stronger than that by poly(I:C) in spleen (Fig. 3B), whereas up-regulation of *Lc*IFNc by poly(I:C) was much higher than that by *A. hydrophila* in head kidney (104.5- versus 37.9- fold increases, Fig. 3C), suggesting that *Lc*IFNc induction may be differentially regulated by *A. hydrophila* and poly(I:C) in different tissues. The IFN-regulatory factor (IRF)3 and IRF7 are two key transcription factors that regulate the expression of fish type I IFNs [6,25]. Both large yellow croaker IRF3 (*Lc*IRF3) and *Lc*IRF7 could be induced by three pathogenic bacteria including *A. hydrophila* [26]. Therefore, up-regulation of *Lc*IFNc expression by *A. hydrophila* might be due to induction of *Lc*IRF3 and/or *Lc*IRF7 by this bacterium.

Accumulating data show that IFNc is a potent IFN in fish and exert antiviral activity by inducing the expression of antiviral genes [3,12,14,27]. Zebrafish IFNphi2 (IFNc) can induce the expression of antiviral genes (Viperin and Mx) in the larva, and protect the larva against IHNV challenge [14]. In juvenile salmon, injection with IFNc producing constructs dramatically up-regulates antiviral genes in several tissues, and these fish have a significant increase in survival when exposed to infectious salmon anemia virus compared with control fish [27]. In mandarin fish fry cell line (MFF-1 cells), overexpression of mandarin fish IFNc increased the expression levels of antiviral genes Mx

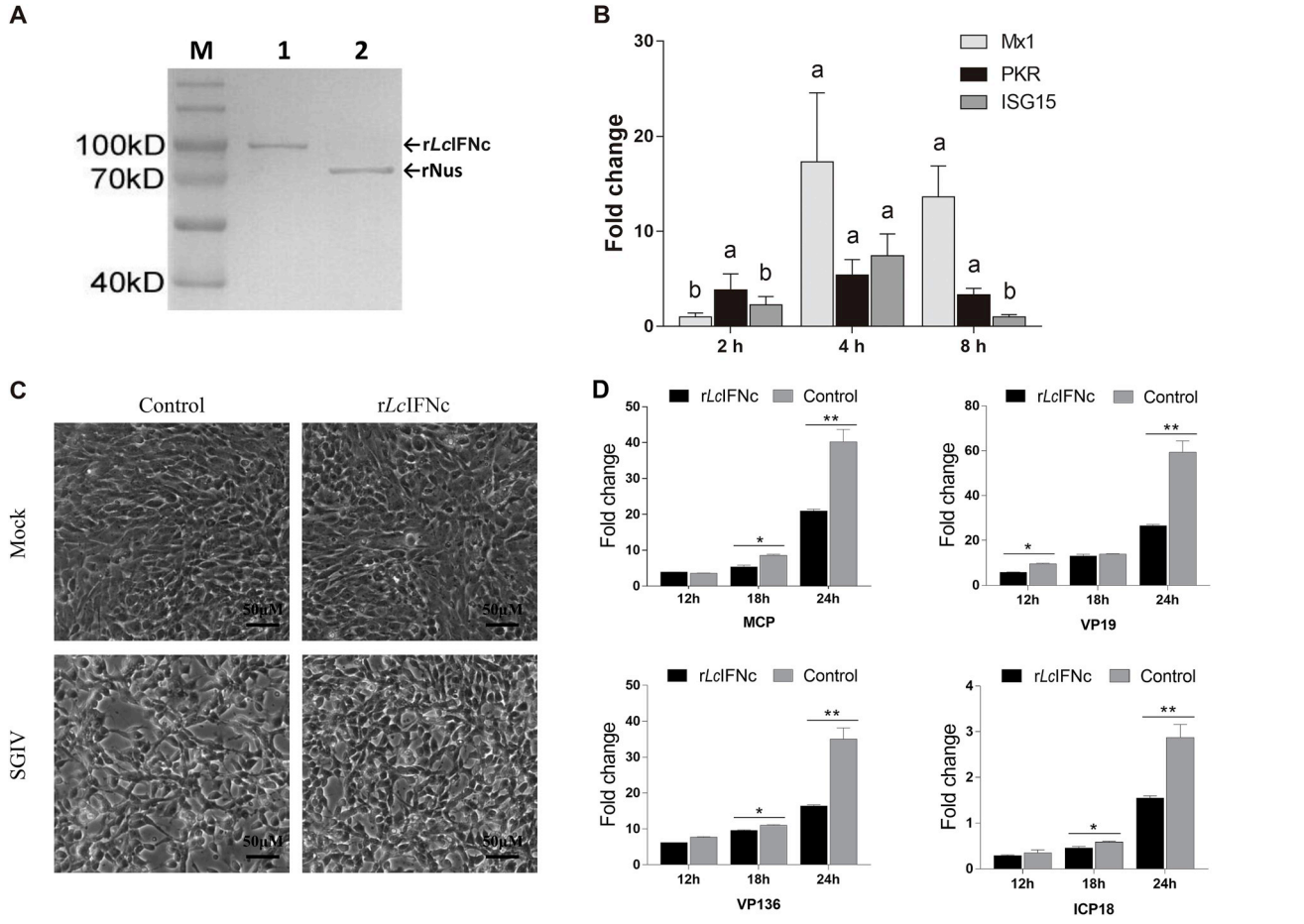

Fig. 4. Antiviral activity of recombinant *Lc*IFNc protein (r*Lc*IFNc). (A) Purification of r*Lc*IFNc and rNus. The r*Lc*IFNc was expressed as a fusion protein with the Nus tag and 6 × His tag in *E. coli* BL21. Two recombinant proteins were analyzed by SDS-PAGE. M: Standard protein molecular weight marker. 1: purified r*Lc*IFNc; 2: purified rNus. (B) Induction of large yellow croaker antiviral genes (Mx1, PKR and ISG15) by r*Lc*IFNc in the PBLs. The PBLs were plated in 6-well plates (1×10^6 cells/well) and then treated with r*Lc*IFNc at a final concentration of 10 ng/mL or rNus (control). Treated cells were sampled at the indicated time points and used for the expression analysis of Mx1, PKR and ISG15 genes by real-time PCR. The relative expression levels of these three genes were normalized by *Lc*β-actin, and fold induction was calculated by comparing the relative expression levels of these genes in the r*Lc*IFN-treated cells with those in rNus-treated cells at the same time point. All data were obtained from three independent experiments with each performed in triplicate. Error bars represent the standard error of the mean (± SEM) of three independent experiments. Data for each gene expression with a or b are significant difference ($p < 0.05$) among different time points. (C) Observation of cytopathic effect (CPE). GS cells were pretreated with r*Lc*IFNc or rNus (control) at a final concentration of 10 ng/mL for 2 h; then, the cells were infected with SGIV at MOI 2. At 24 h post-infection, GS cells were observed for the CPE using microscopy. (D) Expression analysis of viral genes in the r*Lc*IFNc-treated GS cells following SGIV infection. At 12 h, 24 h and 48 h post-infection, the expression levels of four SGIV genes were detected by real-time PCR as described in the Materials and methods. All data were obtained from three independent experiments with each performed in triplicate. Error bars represent the standard error of the mean (± SEM) of three independent experiments. *$p < 0.05$; **$p < 0.01$.

and Viperin, and significant reduction in viral titre was observed when these cells were infected with infectious spleen and kidney necrosis virus [12]. In this study, r*Lc*IFNc not only significantly induced the expression of three antiviral genes, Mx1, PKR and ISG15 in the PBLs (Fig. 4B), but also protected GS cells against SGIV infection (Fig. 4C and D), showing the antiviral activity of *Lc*IFNc. Our previous studies confirmed that both *Lc*IFNd and *Lc*IFNh could induce the expression of antiviral genes, MxA and PKR, in GS cells, and protected GS cells against SGIV infection [6]. These findings therefore lead us to propose that the antiviral activity of *Lc*IFNc may result from induction of host antiviral genes, which then caused the inhibition of SGIV gene expression. It is known that mammalian type I IFNs cannot directly induce their own expressions [28]. In contrast, fish type I IFNs can induce the expression of themselves and other IFNs [6,12]. For example, *Lc*IFNd can induce the expression of itself and *Lc*IFNh [6]. IFNc in mandarin fish also induces the expression of itself and IFNh [12]. Here, *Lc*IFNc was demonstrated to induce the expression of itself, as well as *Lc*IFNd and *Lc*IFNh in two large yellow croaker primary cells (Fig. 5). These observations therefore suggested the presence of a positive feedback regulation mediated by type I IFNs, which may facilitate the amplification of IFN response in fish. However, further researches are needed to clarify the details on the positive feedback regulation mediated by type I IFNs in fish.

In summary, we report the identification of a group II type I IFN, *Lc*IFNc, from the perciform fish large yellow croaker. *Lc*IFNc was constitutively expressed in all analyzed tissues, and rapidly up-regulated in spleen and head kidney by poly(I:C) and *A. hydrophila*. Functional studies indicated that *Lc*IFNc not only had antiviral activity, but also mediated the regulation of IFN response by inducing the expression of itself and other IFNs (IFNd and IFNh), thus providing further insights

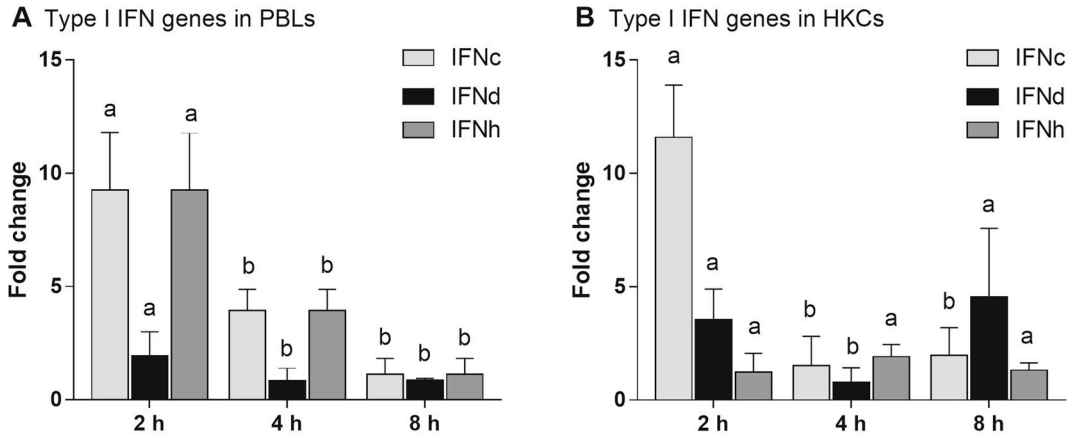

Fig. 5. Expression analysis of *Lc*IFNc, *Lc*IFNd and *Lc*IFNh genes in the r*Lc*IFNc-treated PBLs and HKCs. The PBLs and HKCs were plated in 6-well plates (1×10^6 cells/well) and then treated with r*Lc*IFNc or rNus (control), respectively. Then the cells were sampled at the indicated time points and used for real-time PCR analysis of *Lc*IFNc, *Lc*IFNd and *Lc*IFNh expression. The relative expression levels of these genes were normalized by β-actin, and fold change was obtained by comparing the normalized gene expression levels in the r*Lc*IFNc-treated cells with those in the rNus-treated cells at the same time point. All data were obtained from three independent experiments with each performed in triplicate. Error bars represent the standard error of the mean (± SEM) of three independent experiments. Data for each gene expression with a or b are significant difference ($p < 0.05$) among different time points.

into the functional understanding of type I IFNs in teleost 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.2018.11.036.

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