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Cloning and characterization of type IV interferon from black carp *Mylopharyngodon piceus*

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ABSTRACT

From mammals to fish, interferons (IFNs) play vital roles in the immune response. In this study, a newly identified type IV interferon (bcIFN-υ) from black carp (*Mylopharyngodon piceus*) has been cloned and characterized. The CDS of bcIFN-υ consists of 489 nucleotides, encoding 163 amino acids, with the first 20 amino acids predicted to be the signal peptide region. The immunoblot and immunofluorescence assays verified that bcIFN-υ was a secreted cytokine. qPCR analysis and reporter assay demonstrated that bcIFN-υ participated in innate immune defense and activated the transcription of fish ISRE promoter under spring viremia of carp virus (SVCV) stimulation. Additionally, compared with control group, EPC cells transfected with bcIFN-υ or incubated with the bcIFN-υ-containing conditioned media before SVCV infection showed greatly enhanced antiviral activity, and the transcription levels of *MX1*, *PKR*, *ISG15* and *Viperin* genes were significantly increased. The subsequential co-immunoprecipitation assay identified the interaction between bcIFN-υ proteins. Collectively, our data conclude that bcIFN-υ is a kind of secretory protein with self-interaction and triggering the expression of downstream ISGs to enhance the antiviral activity of host cells.

1. Introduction

Interferons (IFNs), which are typical class II cytokines, play pivotal roles in innate and adaptive immunity (Pestka et al., 2004; Secombes and Zou, 2017). In mammals, IFNs are classified into three families (type I, type II, and type III) based on sequence/structural homology, receptor types and genomic localization. All of the IFNs are able to regulate the expression of downstream immune-related molecules by binding to specific cell-surface cytokine receptors (Kotenko and Pestka, 2000). IFNs (I, II, and III) can recognize their heterodimeric receptors, such as IFN-αR1/IFN-αR2, IFN-γR1/IFN-γR2, and IFN-λR1/IL-10R2 separately, in both an autocrine and paracrine manner. This lead to the activation of the classical JAK (Janus activated kinase)-STAT (signal transducer and activator of transcription) signaling pathway, which then causes the expression of hundreds of antiviral host effector proteins, known as IFN-stimulated genes (ISGs), such as *mucovirus resistance* (*MX*), *protein kinase R* (*PKR*), *viperin* (*VIP*), and *IFN-stimulated gene 15* (*ISG15*) (Crosse et al., 2018; Negishi et al., 2018; Poynter and DeWitte-Orr, 2016). Studies have shown that different types of interferon have distinct biological functions. Type I and type III IFNs have been shown to have antiviral effects (Lazear et al., 2015, 2019; Teijaro, 2016), while type II IFNs tend to coordinate innate and adaptive immune responses against viral and intracellular bacterial infections, they also promote pathologic inflammatory processes (Alspach et al., 2019; Schoenborn and Wilson, 2007).

IFNs have been extensively identified in vertebrates, from cartilaginous fish to mammals (Gan et al., 2019; Secombes and Zou, 2017; Zou and Secombes, 2011). Fish IFNs have been classified into four types, namely, types I, II, III, and IV (Chen et al., 2022; Stosik et al., 2021). Based on the number of cysteines in their maturation peptides, fish type I IFNs can be categorized into group I (IFNa, d, e, h), group II (IFNb, c) and group III (IFNf) (Su, 2022). Although the expression of group I and group II IFNs varies greatly amongst various cell types, they are similar in function (Zou et al., 2007). Mammals only contain one member of the type II IFN family, IFN-γ, whereas fish have two, IFN-γ and IFN-γrel (Gan et al., 2019). Type III IFNs are only identified in cartilaginous fish but are not reported in teleost fish (Redmond et al., 2019; Venkatesh et al., 2014). It is interesting to note that the type IV interferon system has a highly conserved gene localization in chondrichthyes to primary mammals, whereas higher mammals like Metatheria and Eutheria species

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lack the homologous genes. Up to date, the type IV interferon system was newly identified in zebrafish in 2022, namely zebrafish IFN-υ and its receptor-IFNUR1/IL10R2, functions extremely similar to other IFN subtypes. After grass carp reovirus (GCRV) infection, zebrafish IFN-υ is able to participate in the host antiviral immune response by upregulating ISGs transcription and ultimately inhibit GCRV replication (Chen et al., 2022). However, the specific molecular mechanism of how type IV interferon binds to the receptor and how the signal is transmitted to the nucleus remain to be investigated (Su, 2022).

To our knowledge, fish type IV IFN system has only been reported in zebrafish (*Danio rerio*; a model organism) and has not been identified in any other aquatic organisms (Chen et al., 2022). Black carp, as an economically important fish, is a high-quality protein source for human (Yan et al., 2020). Freshwater cultured fish, such as black carp, are often threatened by viral infectious diseases (Adams, 2019). A better understanding of the black carp IFNs system may provide a theoretical basis for healthy black carp culture. In our previous study, bcIFNa (Huang et al., 2015) and bcIFNb (Wu et al., 2018) have been characterized as vital antiviral cytokines, which belong to type I IFN. In this study, bcIFN-υ has been successfully cloned and its protein expression and antiviral activity have been characterized, respectively. Our data show that bcIFN-υ is a kind of secretory protein with self-interaction, and it can induce the expression of downstream ISGs to enhance the antiviral activity of host cells against SVCV infection.

2. Materials and methods

2.1. Cell culture

Epithelioma Papulosum Cyprini (EPC) cells were purchased from ATCC (CRL-2872). *Mylopharyngodon piceus* kidney (MPK) cells were kindly provided by Dr. TianSheng Chen (College of Aquatic Sciences, Jimei University) (Xue et al., 2018). Human embryonic kidney 293T (HEK293T) cells were maintained in our lab. All the cell lines were cultured in DMEM (Gibco, USA) medium containing 10% fetal bovine serum, 2 mmol/L glutamine, 100 mg/L penicillin, and 100 mg/L streptomycin. Mammalian cells were cultured in an incubator with 5% CO_2 at 37 °C. EPC and MPK cells were cultured in an incubator with 5% CO_2 at 26 °C.

2.2. Cloning of bcIFN-υ cDNA

Total RNA was extracted from MPK cells, which were infected with SVCV at a multiplicity of infection (MOI) 0.01. The first-strand cDNA was then synthesized using the PrimScript RT Reagent kit (Takara). The open reading frame (ORF) of bcIFN-υ was amplified by 3′ RACE PCR, which was then cloned into the pMD18-T vector and inserted between the KpnI and XhoI digestion sites of the pcDNA5/FRT/TO-Flag and pcDNA5/FRT/TO-Myc. The primers used for cloning the CDS of bcIFN-υ were listed in Table 1.

2.3. Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was programmed as 1 cycle of 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by dissociation curve analysis (60°C–95 °C). Transcription levels of relevant genes were normalised according to the expression level of β-actin in each sample (as an endogenous control). All sample values after analyzed by $2^{-\triangle\triangle CT}$ were homogenized with the control values as 1. which has been described previously (Wang et al., 2022). Primers used in qPCR were listed in Table 1.

2.4. Viral infection

SVCV (strain: SVCV741) and GCRV (strain: GCRV106) were kept in the lab. The SVCV was propagated in EPC while the GCRV was in CIK (*Ctenopharyngodon idella* kidney) cells at 26 °C in the presence of 2% fetal bovine serum. EPC or CIK cells were infected with SVCV or GCRV accordingly; the cells and the supernatant media were collected together when the cytopathic effect (CPE) was about 50% and stored at −80 °C.

Table 1
Primers used in the study.

Primer name	Sequence (5′-3′)	Primer information
3′ RACE		
3′ GSP1 (bcIFN-υ-F)	ATGGTCTGGATTCGAATCATC	3′ RACE and CDS cloning
3 ‘RACE outer primer	TACCGTCGTTCCACTAGTGATTT	3′ UTR 1st PCR
3′ RACE inner primer	CGCGGATCCTCCACTAGTGATTTCACTATAGG	3′ UTR 2nd PCR
CDS		
bcIFN-υ-R	CTAACGGCGCCTTCTGAGTGA	CDS cloning
Expression vector		
C-bcIFN-υ-F	ACTGACGGTACCGCCACCATGGTCTGGATTCGAATC	Expression construct
C-bcIFN-υ-R	ACTGACCTCGAGACGGCGCCTTCTGAGTGA	
Q-bc actin-F	TGGGCACCGCTGCTTCCT	*ex vivo* q-PCR
Q-bc actin-R	TGTCCGTCAGGCAGCTCAT	
Q-bcIFN-υ-F	ATGGTCTGGATTCGAATC	
Q-bcIFN-υ-R	CCTCAATAAACGCCTGTGGGT	
Q-bc viperin-F	CCAAAGAGCAGAAAGAGGGACC	
Q-bc viperin-R	TCAATAGGCAAGACGAACGAGG	
Q-bc PKR-F	GAGCGGACTAAAAGGACAGG	
Q-bc PKR-R	AAAATATATGAGACCCAGGG	
Q-bc Mx1-F	TGAGCGTAGGCATTAGCAC	
Q-bc Mx1-R	CCTGGAGCAGCAGATAGCG	
Q-EPC actin-F	AAGGAGAAGCTCTGCTATGTGGCT	
Q-EPC actin-R	AAGGTGGTCTCATGGATACCGCAA	
Q-EPC viperin-F	GCAAAGCGAGGGTTACGAC	
Q-EPC viperin-R	CTGCCATTACTAACGATGCTGAC	
Q-EPC PKR-F	ACCTGAAGCCTCCAAACATA	
Q-EPC PKR-R	GCATTCGCTCATCATTGTC	
Q-EPC Mx1-F	TGGAGGAACCTGCCTTAAATAC	
Q-EPC Mx1-R	GTCTTTGCTGTTGTCAGAAGATTAG	
Q-EPC ISG15-F	TGATGCAAATGAGACCGTAGAT	
Q-EPC ISG15-R	CAGTTGTCTGCCGTTGTAAATC	
Q-SVCV-G-F	GATGACTGGGAGTTAGATGGC	
Q-SVCV-G-R	ATGAGGGATAATATCGGCTTG	

After freezing and thawing for three times, the mixture was used for virus titer mensuration.

Virus titers were examined by the plaque assay on EPC cells as previously described (Wu et al., 2018). Briefly, the virus supernatant was serially diluted 10-fold and added to EPC cells, incubated for 2 h at 26 °C. The supernatant was discarded and fresh DMEM containing 2% FBS and 0.75% methylcellulose (Sigma, USA) was added. And the plaques were measured at day-3 post-infection.

2.5. Luciferase reporter assay

For luciferase reporter assay, EPC cells were seeded overnight in 24-well plates. Then pRL-TK (0.025 μg), Luci-fish ISRE (0.25 μg) and pcDNA5 (0.2 μg) or bcIFN-υ (0.2 μg) plasmids were co-transfected into EPC cells. At 24 h post-transfection, cells were either left untreated or stimulated with SVCV (MOI 0.01 for 8 h). The cells were then collected, washed with PBS, and lysed on ice with PLB (Passive Lysis Buffer) for 15 min. The firefly and renilla luciferase activities were measured by Dual-Luciferase Repoter Assay system (Promega, USA).

2.6. Immunoblot assay

Immunoblot assay was performed as previously described (Wang et al., 2022). Briefly, HEK293T and EPC cells seeded in 6-well plates were transfected with bcIFN-υ-Flag (3 μg) recombinant vectors or empty vector (3 μg) (as control). The whole cell lysate and supernatant media were harvested at 48 h post-transfection and separated by 14% SDS-PAGE. The transferred PVDF membranes were blotted with anti-Flag (1:3000, Abmart) and goat-anti mouse secondary antibody (1:30000, Sigma). Target proteins were visualized with NBT/BCIP Alkaline Phosphatase Color Development Kit (Sigma, USA).

2.7. Co-immunoprecipitation

HEK293T cells seeded in 10 cm plates were co-transfected with bcIFN-υ-Flag vectors (7.5 μg) and bcIFN-υ-Myc vectors (7.5 μg). After 48 h transfection, the cells and supernatant media were collected, respectively. The cells were lysed in 1% NP40 buffer and then incubated with protein A/G agarose besds at 4 °C for 1 h before incubated with anti-Flag-conjugated protein A/G agarose besds. After washing with 1% NP40 buffer for five times, the 5 × SDS PAGE loading buffer were added in beads-containing buffer and used for immunoblot assay as above. The supernatant media were treated the same as described above but without lysed in 1% NP40 buffer.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The bcIFN-υ-containing conditioned media was measured through ELISA assay as previously described (Huang et al., 2015). Briefly, bcIFN-υ-containing conditioned media was blocked with carbonate buffer (pH 9.6) at 37 °C for 3 h in 96-well plate. After three times of wash, each well was probed with anti-Flag antibody (1:1000, Abmart) at different concentration and cultured at 37 °C for 1 h. After three times of wash, each well was probed with goat-anti mouse secondary antibody (1:30000, Sigma) at 37 °C for 1 h. Then, each well was added with 200 μl PNPP for 30 min. The OD value at 405 nm was measured by Synergy-2 Multi-mode reader (BioTek).

2.9. Statistics analysis

For the statistics data analyzed of qPCR, luciferase reporter assay and viral titration, all date were obtained from three experiments independently. Significant different were measured by two-tailed Student's t-test. Error bars represented the standard error of the mean (+SEM) of three independent experiments. The asterisk represents the significant difference, (*) stands for $p < 0.05$, (**) stands for $p < 0.01$.

3. Results

3.1. Molecular cloning and sequence analysis of bcIFN-υ

The bcIFN-υ cDNA was cloned from the MPK cells and deposited in GenBank with accession number, ON675580. Five exons encode the cDNA of *bcIFN-υ* gene. The open reading frame (ORF) of *bcIFN-υ* gene consists of 489 nucleotides, which encodes 162 amino acids, with the first 20 amino acids are predicted to be the signal peptide region (using SignalP program/version 5.0, Center for Biological Sequence Analysis) (Fig. 1A). The molecular weight of bcIFN-υ is estimated to be 19.1 kDa by EXPASy Compute PI/Mw analysis, and its theoretical isoelectric point is 9.05. The amino acid sequence of bcIFN-υ was compared with that of zebrafish IFN-υ, green anole IFN-υ, African clawed frog IFN-υ using MEGA-X and GeneDoc software. According to the sequence alignment results, the sequence homology between bcIFN-υ and zebrafish IFN-υ was 82.1%, implying that IFN-υ is conserved during the evolution of teleost fish (Fig. 1B).

3.2. Transcription of bcIFN-υ under different stimuli in vivo and ex vivo

To better investigate the transcription levels of bcIFN-υ post stimulation *in vivo* and *ex vivo*, black carp and MPK cells were infected with SVCV or GCRV, respectively. Then the immune-related tissues and MPK cells were collected for RNA extraction. According to qPCR results, the up-regulation levels of bcIFN-υ mRNA after the stimulation of the two viruses are different. In SVCV infection group, the transcription levels of bcIFN-υ increased gradually up to 23.18-fold (MOI 0.1) (Fig. 2A). In contrast, the mRNA level of *bcIFN-υ* gene was up-regulated most significantly in lower MOI infection group, which reached to 7.67-fold (Fig. 2B). As shown in Fig. 2C, the bcIFN-υ transcription levels in spleen, kidney and intestine were increased after viral infection compared to the control group, with the transcription in kidney showing the most robust expression (61.55-fold). Based on these results, we can speculate that bcIFN-υ participates in the immune response against virus infection *in vivo* and *ex vivo*, which is consistent with the previous findings of zebrafish IFN-υ. As a class of cytokines, interferons not only help host cells to resist viruses, but also participate in the host cell immune response induced by bacteria (Gan et al., 2019). However, it is unclear whether IFN-υ has any regulatory activities against other pathogens, such as bacteria (Su, 2022).

3.3. Protein expression of bcIFN-υ

In order to further investigate the function of bcIFN-υ, recombinant vectors for the expression of bcIFN-υ-Flag were constructed and transfected into HEK293T and EPC cells. Whole cell lysates and supernatant media were collected 48 h post transfection and used for immunoblotting (IB). Interestingly, in both the IB of whole cell lysates and supernatant, there are two clear bands of about 17 kDa and 34 kDa detected by anti-Flag antibody, indicating that bcIFN-υ is a secretory protein (Fig. 3A and B).

3.4. bcIFN-υ enhances the antiviral state of cells by promoting the expression of ISGs

Previously study has found that zebrafish IFN-υ is an antiviral cytokine and plays a role in the immune response of zebrafish against viral infestation (Chen et al., 2022). To further investigate the function of bcIFN-υ in host antiviral response, plasmids encoding bcIFN-υ or pcDNA5 (as control) were transfected into MPK cells. At 24 h post-transfection, the mRNA levels of downstream ISGs were detected by qPCR. As expected, overexpression of bcIFN-υ in host cells increased the expression of ISGs such as *MX1*, *PKR* and *Viperin* (Fig. 4A). Moreover, bcIFN-υ together with fish ISRE promoter were co-transfected into EPC cells, stimulated with SVCV or left untreatment, and then subjected to

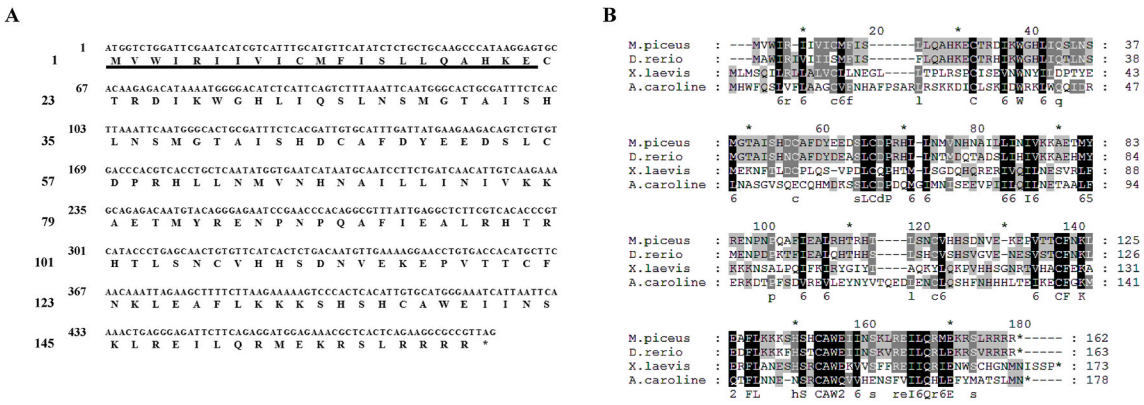

Fig. 1. Cloning and Sequence analysis of bcIFN-υ. (A) The nucleotide sequence and the amino acid sequence of bcIFN-υ. The first 20 amino acids are predicted to be the signal peptide region, which was underlined (SignalP program/version 5.0, Center for Biological Sequence Analysis).
(B) Comparisons of amino acid sequence of bcIFN-υ (*Mylopharyngodon piceus*, ON675580), zebrafish IFN-υ (Danio *rerio*, MW547062), Green anole IFN-υ (*Anolis carolinensis*, OK104795.1), African clawed frog IFN-υ (*Xenopus laevis*, MW924834.1) by using MEGA-X program and GeneDoc program.

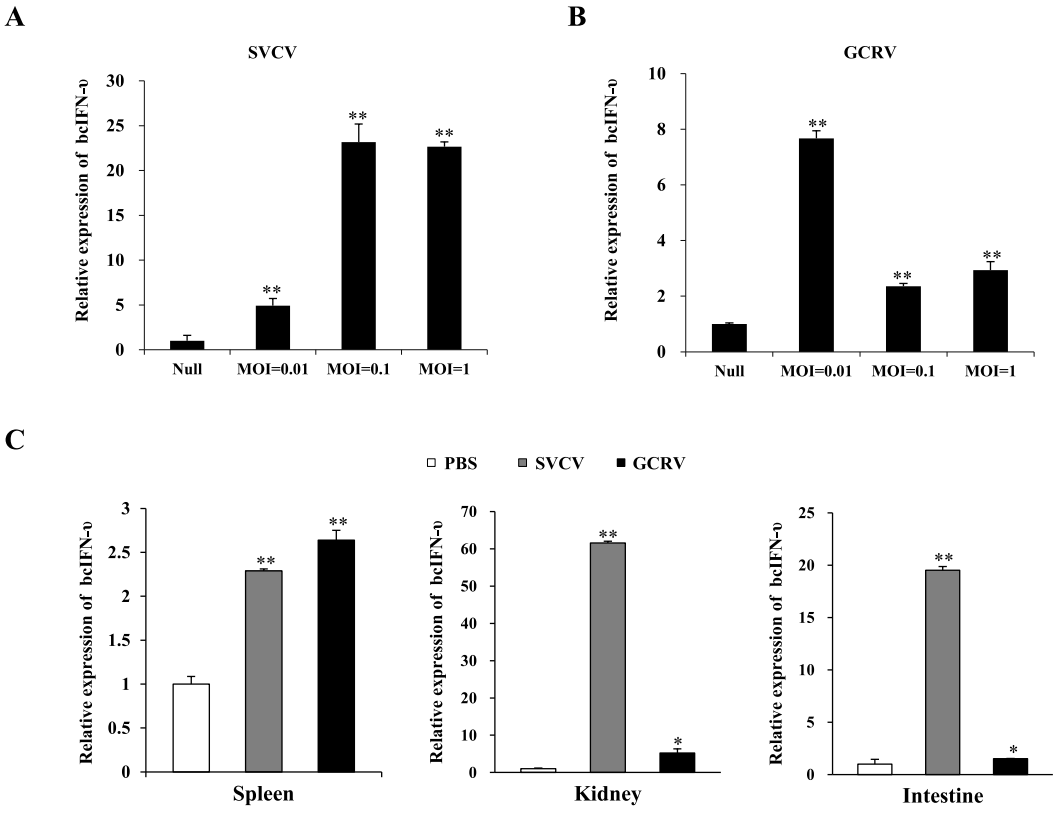

Fig. 2. Transcription of bcIFN-υ under different stimuli *in vivo* and *ex vivo*.
MPK cells in 6 well-plates were treated with SVCV (A) or GCRV (B) at indicated MOI for 48 h respectively; (C) Black carps were injected intraperitoneally with GCRV, SVCV or sterile PBS separately. Fishes were collected for each injected group at 33 h post injection and total RNA was isolated from the indicated tissues independently. All the relative mRNA expression level of bcIFN-υ was examined by qPCR. The significant difference analysis was conducted between the values of the control and the stimulated groups. Null: no treatment.

reporter assay. The data show that overexpression of bcIFN-υ could remarkably activate the transcription of fish ISRE promoter (Fig. 4B).

Subsequently, EPC cells seeded in 12-well plate were transfected with bcIFN-υ or empty vector and infected with SVCV at indicated MOI. The results of plaque assay showed that the viral titer of the EPC cells over-expressing bcIFN-υ were lower than those of the control groups. Particularly in low MOI infection group, IFN-mediated antiviral action is more obvious (Fig. 4C). In addition, the transcription of SVCV-G and ISGs were examined by qPCR. The results were consistent with the plaque assay: compared with control group, the mRNA level of *SVCV-G* gene was markedly reduced in bcIFN-υ-overexpressing EPC cells (Fig. 4D), while the transcription levels of *MX1*, *PKR*, *ISG15* and *Viperin* genes were significantly increased (Fig. 5). All results above collectively confirm that bcIFN-υ could enhance host cell antiviral activity by up-

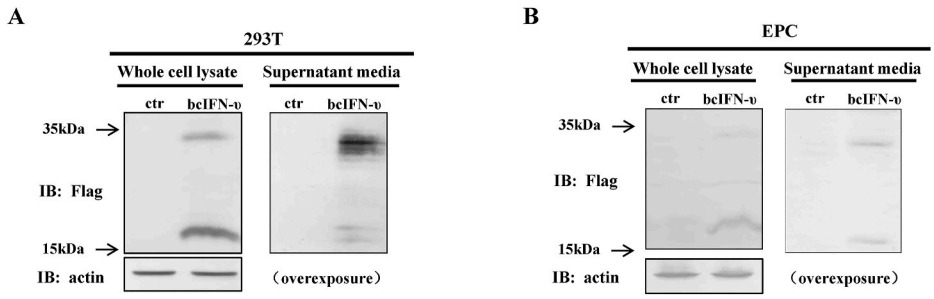

Fig. 3. Protein expression of bcIFN-υ. (A)&(B) Immunoblot assay of bcIFN-υ in HEK293T cells and EPC cells. HEK293T cells and EPC cells in 6-well plates were transfected with pcDNA5 or bcIFN-υ (3 μg). At 48 h post-transfection, cells were collected for Immunoblot assay. CTR: cells transfected with empty vector. CTR: cells transfected with empty vector.

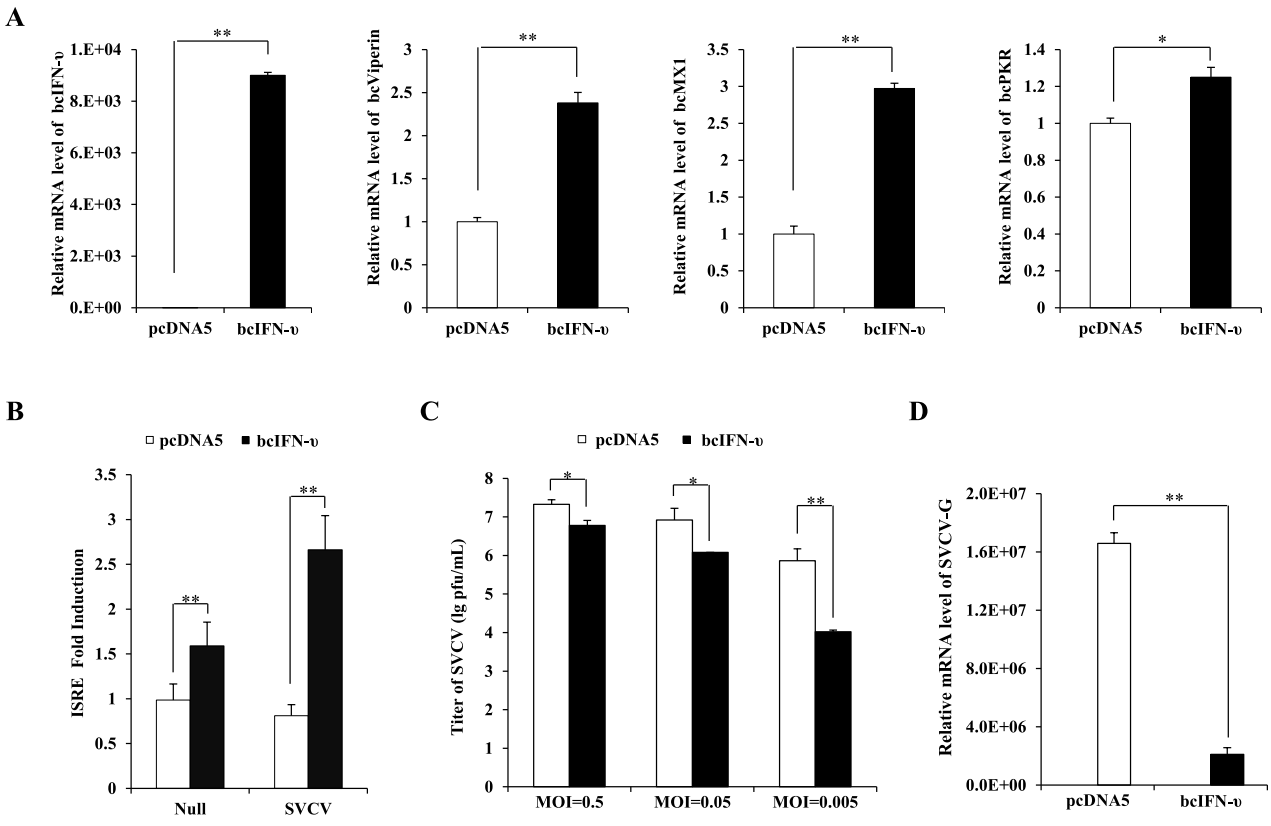

Fig. 4. bcIFN-υ enhances the antiviral state of cells.
(A) MPK cells in 6-well plates were transfected with pcDNA5 or bcIFN-υ (3 μg). At 48 h post-transfection, cells were collected for RNA extraction and the relative mRNA level of *bcViperin*, *bcMx1*, *bcPKR* gene were detected by qPCR. (B) EPC cells in 24-well plates were co-transfected with PLR-TK (0.025 μg), Luci-fish-ISRE (0.25 μg), pcDNA5 or bcIFN-υ (0.2 μg). The cells were infected with SVCV (MOI = 0.01) or left untreatment at 24 h post-transfection, then used for report assay. (C) EPC cells in 12-well plates were transfected with pcDNA5 or bcIFN-υ (1 μg). After 24 h post-transfection, the cells were infected with SVCV at indicated MOI and the virus titers of supernatant media were examined by plaque assay at 24 h post-infection. while the remaining EPC cells in MOI = 0.005 group were used for RNA extraction and the relative mRNA level of *SVCV-G* gene (D). bcIFN-υ: pcDNA5/FRT/TO-bcIFN-υ-Flag. Null: no treatment.

regulating ISG expression.

3.5. *bcIFN-υ is a secretory cytokine and capable of self-interaction*

Since IFNs belong to a class of secretory cytokines which can help adjacent cells establish an antiviral state. To test this, the supernatant of EPC cells over-expressing bcIFN-υ was collected. Then EPC cells were treated with supernatant media before SVCV infection. After 2, 4, 8, 12, or 24 h of incubation, the supernatant was collected for plaque assay respectively. The results showed that either a long- or short-term incubation with bcIFN-υ-containing media could increase the cells' antiviral activity (Fig. 6A). But 24 h-incubation group exhibited the strongest effect, with a large reduction in the mRNA level of *SVCV-G* gene (Fig. 6B). Since we detected two bands in the IB assay of whole cell lysates and supernatant media of bcIFN-υ. In order to elucidate the potential mechanism, we collected bcIFN-υ-overexpressing HEK293T cells and bcIFN-υ-containing supernatant media, and used them for co-immunoprecipitation (co-IP), respectively. The results of whole cell

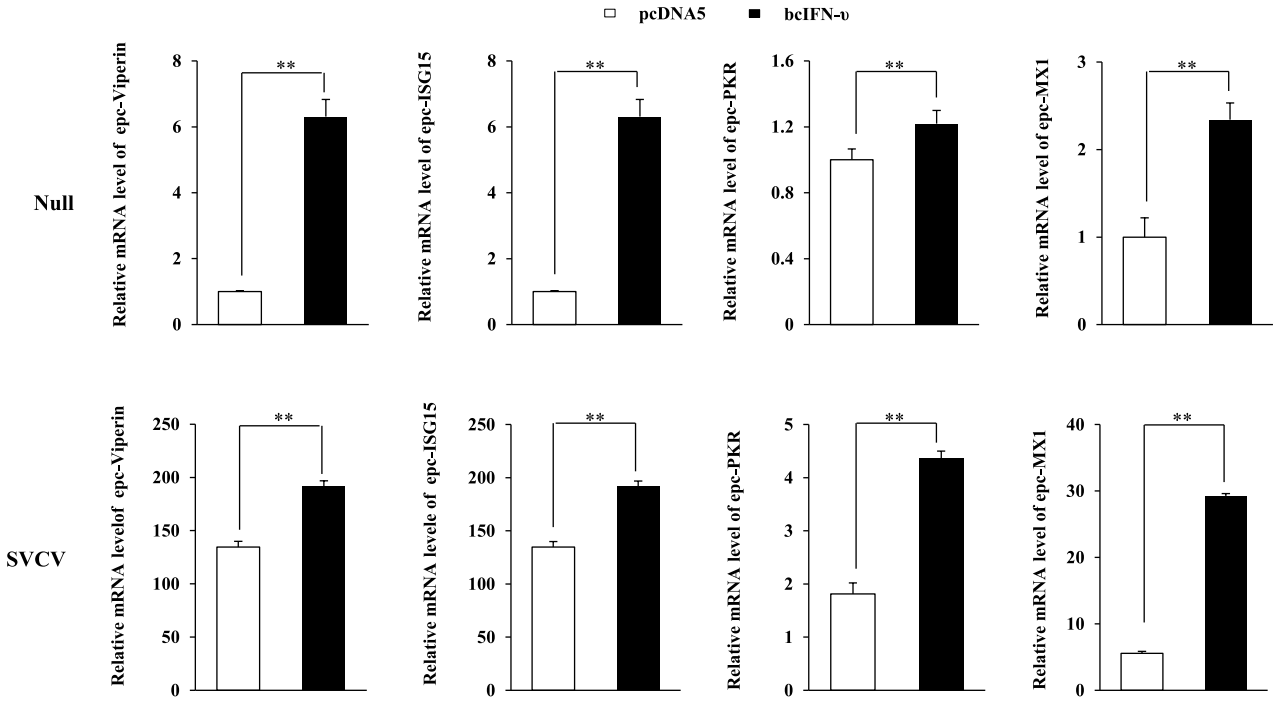

Fig. 5. bcIFN-υ attenuates viral replication by regulating the expression of ISGs.
EPC cells in 12-well plates were transfected with pcDNA5 or bcIFN-υ (1 μg). After 24 h post-transfection, the cells were infected with SVCV at indicated MOI and the virus titers of supernatant media were examined by plaque assay at 24 h post-infection. while the remaining EPC cells in MOI = 0.005 group were used for RNA extraction and the relative mRNA level of *epc-Viperin*, *epc-Mx1*, *epc-ISG15*, *epc-PKR* gene were detected by qPCR.

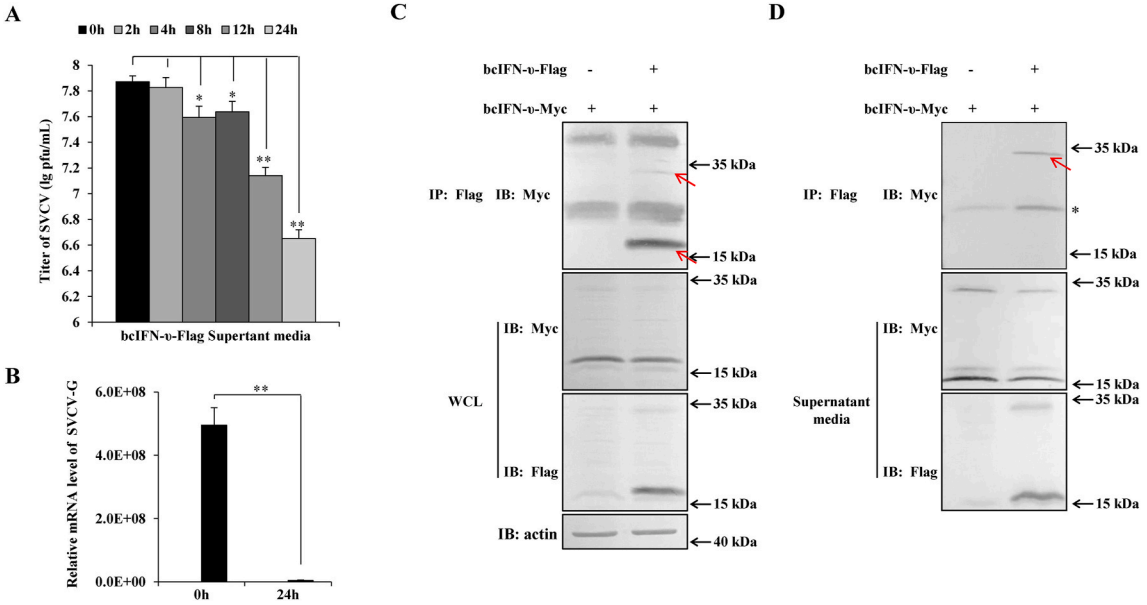

Fig. 6. bcIFN-υ is capable of being secreted extracellularly as a monomer or homodimer to perform biological functions.
EPC cells in 12-well plates were treated with the bcIFN-υ-containing media (500μ, 2.79 ng/μl). The cells were harvested at 0, 2, 4, 8, 12, 24 h post-stimulation separately before infected with SVCV (MOI = 0.005). The supernatant media were subjected to virus titers by plaque assay at 24 h post-infection (A), and the remaining EPC cells in 24 h group were used for RNA extraction. The relative mRNA level of *SVCV-G* gene was detected by qPCR (B). The concentration of bcIFN-υ in the media was 2.79 ng/μl, which was measured by ELISA. (C) Co-immunoprecipitation assay of bcIFN-υ-Flag and bcIFN-υ-Myc in HEK293T cells. (D) Co-immunoprecipitation assay of supernatant media in HEK293T cells between bcIFN-υ-Flag and bcIFN-υ-Myc. IP: immunoprecipitation; IB: immunoblot; bcIFN-υ-Flag: pcDNA5/FRT/TO- bcIFN-υ-Flag; bcIFN-υ-Myc: pcDNA5/FRT/TO-bcIFN-υ-Myc; *: non-specific band.

lysates showed that two bands of bcIFN-υ-Myc (~17 kDa and ~34 kDa, arrows indicated) were detected in the proteins immunoprecipitated by bcIFN-υ-Flag, indicating that bcIFN-υ exist protein self-interaction. Besides, a band of bcIFN-υ-Myc (~34 kDa, arrow indicated) was also detected in the co-immunoprecipitation of supernatant media. In conclusion, these results collectively showed that both intracellular and extracellular bcIFN-υ were capable of self-interaction (Fig. 6C and D).

4. Discussion

Interferons (IFNs), which play significant roles in innate immunity, have been reported in teleost fish as class II cytokines against pathogen infections (Zhang and Gui, 2012; Zou et al., 2014). Compared with adaptive immunity, innate immunity of fish is more sophisticated (Lieschke and Trede, 2009; Zou and Secombes, 2016). Three IFN systems in teleost fish have been separated and identified: type I, type II, and type IV (Chen et al., 2022; Gan et al., 2019). A paper published in February 2022 reported that type IV interferon system exists in cartilaginous fish to primary mammals (Chen et al., 2022). It is hypothesized that the loss of the IFN-υ gene in higher mammals may be due to chromosomal rearrangements resulting in the lack of IFNUR1. The functional receptors of IFN-υ are IFNUR1 and IL-10R2/CRFB4, which are different from those of type I and type II IFNs. However, IL-10R2/CRFB4 may be shared by IFN-υ and type III IFNs as a chain of their receptor complexes.

In the present study, the IFN-υ homologue from black carp has been identified. In terms of function, bcIFN-υ was similar to bcIFNa and bcIFNb (Huang et al., 2015; Wu et al., 2018) (Figs. 5A and 6A). SVCV or GCRV infection substantially increased the transcription levels of bcIFN-υ (Fig. 2A and B), but the increase of bcIFN-υ mRNA levels in SVCV infection group was more obvious than that in GCRV group, suggesting that bcIFN-υ might more sensitive to SVCV. This was further confirmed by qPCR detection of bcIFN-υ mRNA levels in different tissues under viral infection status (Fig. 2C).

In previous study, knocking down of zebrafish IFN-υ resulted in a significant reduction in transcription levels of downstream ISGs following GCRV infection (Chen et al., 2022). In this work, it was found that both bcIFN-υ-overexpression and bcIFN-υ-containing supernatant incubation are capable of boosting the antiviral activity of host cells by promoting the expression of ISGs (Figs. 5B and 6B), indicating that the mechanisms by which zebrafish IFN-υ and black carp IFN-υ function in the host antiviral innate immune response are similar.

Type II interferons are mainly produced by mitogen-stimulated T lymphocytes and tend to play a more coordinated role in innate and adaptive immunity compared to other types of interferons (Billiau and Matthys, 2009; Schoenborn and Wilson, 2007). The only known type II interferon is IFN-γ (IFN-γ and IFN-γrel in fish) (Zahradnik et al., 2018). In human and zebrafish, the type II interferon is a non-covalently linked homodimer composed of two identical 17 kDa monomers. The individual polypeptides bind in a helical, antiparallel fashion to form a compact, spherical molecule. Their homodimerization is necessary for their biological activity (He et al., 2017; Langer et al., 1994; Lunn et al., 1992; Yoon et al., 2016), and dissociation of IFN-γ dimers in human requires treatment with strong denaturing agents or lowering the pH to below 2.0. In zebrafish, dissociation of IFN-γ and IFN-γrel homodimers also requires boiling at higher concentrations of β-mercaptoethanol or high temperatures for 30 min (Samudzi et al., 1991; Yoon et al., 2016). Co-immunoprecipitation experiments on the supernatant medium revealed that bcIFN-υ was capable of self-interaction (Fig. 6C). Moreover, the SDD-AGE result suggested bcIFN-υ might undergo oligomerization (Supplementary figure). However, it is interesting to note that there was another band of ~34 kDa in the western blot results (Fig. 3A and B). Since our immunoblot assays were performed under denaturing conditions, which should destroy the protein-protein interaction. Therefore, this band should not be the dimerized IFN-υ, which needs to be further identified by mass spectrometry.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2022.104614.

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