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IFNa2 of triploid hybrid of gold fish and allotetraploid is an intracellular antiviral cytokine against SVCV and GCRV

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ABSTRACT

Sterile triploid hybrids (3n = 150) of gold fish (*Carassius auratus red var.*, ♀, 2n = 100) and allotetroploid (♂, 2n = 100) display obviously improved disease resistance and much enhanced growth rate than their parents, which have been cultured widely in China. In this paper, one of the type I IFNs of triploid hybrid (3nIFNa2) has been cloned and characterized. The full-length cDNA of *3nIFNa2* gene consists of 715 nucleotides and the predicted 3nIFNa2 contains 183 amino acids. The transcription of *3nIFNa2* gene was detected in all the examined tissues of triploid hybrid and the mRNA level of 3nIFNa2 was obviously enhanced in response to SVCV and GCRV infection. 3nIFNa2 has been detected in the whole cell lysate of HEK293T cells transfected with plasmids expressing 3nIFNa2 but not in the supernatant media. EPC cells transfected with plasmid expressing 3nIFNa2 at 24 h before SVCV and GCRV infection showed obviously decreased cytopathic effect; and the virus titers in the supernatant media were much lower than those of the control cells. Glycosidase digestion analysis demonstrates that 3nIFNa2 is modified with N-linked glycosylation, which occurs on the asparagine (N) of residue 177 of this cytokine. The un-glycosylated mutant 3nIFNa2-N177Q shows the similar antiviral ability as that of 3nIFNa2, which suggests that the N-linked glycosylation does not contribute directly to its antiviral property. All the above data support the conclusion that 3nIFNa2 is an intracellular cytokine functioning importantly in host antiviral innate immunity.

1. Introduction

Higher vertebrates utilize immune systems to protect them against the invasion of pathogen microbes, which are classified into innate immune system and adaptive immune system [1–3]. Teleost fishes possess both immune systems; but they depend much more on the innate immune system to survive from disadvantage environment [4,5]. The innate immune system provides an immediate, but non-specific response, which exists in all plants and animals. The innate response is usually triggered when microbes are identified by pattern recognition receptors (PRRs), which recognize components that are conserved among broad groups of microorganisms [6,7]. The membrane associated PRRs and cytosolic PRRs trigger downstream signaling to activate the production of interferons (IFNs), which sub-sequentially activate the transcription of diverse interferon-stimulated genes (ISGs) and initiate host antiviral immune responses [8–11]. Many ISGs are widely accepted antiviral proteins and inflammation factors, such as Mx and viperin [12,13].

The interferon system provides a powerful and universal intracellular defense mechanism against viruses, which plays a key role in both the innate and adaptive immunity [8,9]. As to teleost fishes, IFNs have been cloned and characterized from many species, which include zebrafish (*Danio rerio*), catfish (*Ictalurus punctatus*), common carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idella*), crucian carp (*Carassius auratus*), black carp (*Mylopharyngodon piceus*), rainbow trout (*Oncorhynchus mykiss*), salmon (*Salmo salar Linnaeus*), fugu (*Takifugu rubripes*), sea bass (*Dicentrarchus labrax*) etc. [14–22]. Teleost IFNs are referred to be classified into group I and group II type I IFNs, depending on the composition of their cysteine residues [21]. Post-translational modifications such as glycosylation have been found in several fish IFNs although the

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mechanisms of these modifications have not been deeply discussed [22].

Allotetraploid (AT; 4n = 200) has been developed in this laboratory through crossing gold fish (*Carassius auratus red var.*, ♀, 2n = 100) with common carp (*Cyprinus carpio* L., ♂, 2n = 100) and subsequently selective breeding. Both male and female individuals of allotetraploid population are fertile and this allotetraploid population has propagated 22 generations [23]. The triploid hybrids (3n = 150) have been produced in large scale through hybridization between male allotetroploid and female gold fish [24]. Outstanding merits have been developed in this triploid hybrid, which include sterility, fast growth rate and good taste. Especially, these triploid hybrids present obviously stronger disease resistance and stress resistance than their parents [25]. Thus, this species have been cultured widely in China and contributed a lot to the fresh water industrial market. However, the immunity of this triploid hybrid remains unclear.

In our previous study, one of the type I IFNs of triploid hybrid (3nIFNa) has been cloned and characterized as an antiviral cytokine against both spring viremia of carp virus (SVCV) and grass carp reovirus (GCRV) [26]. To further exploit the innate immune system of triploid hybrid, another type I IFN of triploid hybrid (named as 3nIFNa2) has been cloned and characterized in this paper, which is modified with N-linked glycosylation. Our study demonstrates that 3nIFNa2 is an intracellular cytokine and plays an important role in host innate immune response against SVCV and GCRV. The unglycosylated mutant of 3nIFNa2 (3nIFNa2-N177Q) shows similar antiviral ability to that of 3nIFNa2, which suggests that the N-linked glycosylation of 3nIFNa2 does not affect the antiviral ability of this fish cytokine directly.

2. Materials and methods

2.1. Cells and plasmids

HEK293T (293T) cells, HeLa cells, EPC (epithelioma papulosum cyprini) cells and CIK (C. idella kidney) cells were kept in the lab [27]. 293T cells and HeLa cells were cultured at 37 °C; EPC cells and CIK cells were cultured at 25 °C. All the cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 u/ml penicillin and 100 μg/ml streptomycin.

pcDNA5/FRT/TO and pcDNA5/FRT/TO-HA were kept in the lab [28]. The recombinant plasmid pcDNA5/FRT/TO-HA-3nIFNa2 was constructed through inserting the open reading frame (ORF) of 3nIFNa2 into pcDNA5/FRT/TO-HA, in which an HA tag was fused at the N-terminus of 3nIFNa2; for pcDNA5/FRT/TO-3nIFNa2-HA, HA tag was fused at the C-terminus accordingly. The glycosylation mutant pcDNA5/FRT/TO-3nIFNa2-N177Q-HA was generated as previously [22], in which asparagine (N) of residue 177 was alerted to glutamine (Q).

2.2. Cloning of 3nIFNa2 cDNA

Degenerate Primers (Table 1) were designed to amplify 3nIFNa2 cDNA basing on the conserved domains of IFNs of *C. idella* (DQ357216.1), *D. rerio* (AJ544821.1), *C. carpio* (GQ168341.1) and *C. auratus* (AY452069.1). Total RNA was isolated from the spleen of triploid hybrid and the first-strand cDNA were synthesized by using the Revert Aid First Strand cDNA Synthesis Kit (Thermo). Rapid amplification of cDNA ends (RACE) was performed to obtain 5′UTR and 3′UTR of 3nIFNa2 cDNA by using 5′ Full RACE Kit and 3′ Full RACE kit separately (Takara). The full-length cDNA of 3nIFNa2 was cloned into pMD18-T vector and sequenced by Invitrogen.

2.3. Virus produce and titration

SVCV and GCRV were kept in the lab, which were propagated in EPC cells and CIK cells at 25 °C separately [29]. Virus titers were determined by plaque forming assay on EPC cells separately as previously described [29]. Briefly, the 10-fold serially diluted virus was added onto EPC cells and incubated for 2 h at 25 °C. The supernatant was removed after incubation and DMEM containing 2% FBS and 0.75% methylcellulose (Sigma) was added. Plaques were counted at day-3 post infection.

2.4. Quantitative real-time PCR

Triploid hybrids at the age of six months (120 g) were injected intraperitoneally with GCRV (2×10^6 pfu/fish), SVCV (2×10^6 pfu/fish) or sterile PBS separately and cultured at 25 °C. The injected fish was sacrificed at 41 h post injection and total RNA was isolated from different tissues independently, including kidney, heart, intestine, liver, skin and spleen. Quantitative real-time PCR (q-PCR) was performed to quantify the 3nIFNa2 mRNA expression in triploid hybrid after SVCV or GCRV infection. The primers for q-PCR of 3nIFNa2 were listed in Table 1. The q-PCR program was: 1 cycle of 50°C/2min, 1 cycle of 95°C/10min, 40 cycles of 95°C/15s, 60°C/1min, followed by dissociation curve analysis (60°C-95 °C) to verify the amplification of a single product. The threshold cycle (CT) value was determined by using the manual setting on the 7500 Real-Time PCR System and exported into a Microsoft Excel Sheet for subsequent data analyses where the relative expression ratios of target gene in treated group versus those in control group were calculated by $2^{-\triangle\triangle CT}$ method.

2.5. Immunoblotting

HEK293T cells in six-well plate were transfected with pcDNA5/FRT/TO-HA-3nIFNa2, pcDNA5/FRT/TO-3nIFNa2-HA or the empty vector separately. The transfected cells were harvested at 48 h post-transfection. The whole cell lysate and the supernatant media (20 μl from 2 ml for each sample) were used for immunoblot (IB) separately as previously reported [27]. Briefly, the proteins were isolated by 14% SDS-PAGE and the transferred membrane was probed with monoclonal anti-HA antibody (1:2000; Sigma); then incubated with the goat-anti mouse secondary antibody (1:10000, Sigma). The target proteins were visualized with BCIP/NBT Alkaline Phosphatase Color Development Kit (Sigma).

2.6. Immunofluorescence microscopy

HeLa cells or EPC cells were transfected with pcDNA5/FRT/TO-3nIFNa2-HA or the empty vector separately. Transfected HeLa and EPC cells were fixed with 4% (v/v) paraformaldehyde at 36 h post-transfection. The fixed cells were permeabilized with Triton X-100 (0.2% in PBS) and used for immune-fluorescent staining as previously described [28]. Mouse monoclonal anti-HA antibody (Sigma) was probed at the ratio of 1:300 and Alexa 488-conjugated secondary antibody (Invitrogen) was probed at the ratio of 1:1000; Hoechst 33342 (Sigma) was used to stain the nucleus.

2.7. Glycosidase digestion

HEK293T cells in six-well plate was transfected with pcDNA5/FRT/TO-3nIFNa2-HA and the transfected cells were harvested at 48 h post-transfection. The whole cell lysate was divided into two aliquots; one was used for PNGase F (NEB) digestion according to the manufacturer's instruction and the other aliquot as control. Briefly, the whole cell lysate was denatured and incubated with 500

Table 1
Primers used in the study.

Primer name		Sequence (5'→3')	Amplicon length (nt) and primer information
PM1 (+)		GTGGAGGAYCAGGTGA	ORF (about 275bp)
PM1 (−)		CGGATCTGYTCCCATG	
M13-F		GGAAACAGCTATGACCATGATTAC	Colony PCR
M13-R		CGACGTTGTAAAACGACGGCCAGT	
5' -RACE			
5′GSP1		TGAAGTGCCTTTTTATCCCG	5′UTR 1 s t PCR
5′Race Outer primer		ATGTGACGGCTTTTGGTATTG	
5′GSP2		AGACAAAAAACCTCACCTGG	5′UTR 2nd PCR
5′RACE Inner primer		AGAAACCTCACCTGGTCCTCC	
3' -RACE			
3′GSP1		GTGGAGGACCAGGTGAGGTTTCT	3′UTR 1 s t PCR
UPM	Long primer	CTAATACGACTCACTATAGGGCAAGCAG TGGTATCAACGCAGAGT	
	Short primer	CTAATACGACTCACTATAGGGC	
3′GSP2		CCCAATACCAAAAGCCGTCACAT	3′UTR 2nd PCR
NUP1		AAGCAGTGGTATCAACGCAGAGT	
β-actin mRNA			
ACTIN-F		TGGGCACTGCTGCTTCCT	q-PCR
ACTIN-R		TGTCCGTCAGGCAGCTCAT	
3nIFNa2 q-PCR			
3nIFNa2-F1		GATTGGAGATGCTAAGGTGGAG	q-PCR
3nIFNa2-R1		AAAGTCCTGAAGTGCCTTGTTA	

units of PNGase F at 37 °C for 30 min. The PNGase F digested whole cell lysate and the control whole cell lysate were separated by 14% SDS-PAGE and used for IB to identify if 3nIFNa2 was modified with N-linked glycosylation or not.

3. Results

3.1. Molecular cloning of 3nIFNa2 cDNA

The full-length cDNA of 3nIFNa2 consists of 715 nucleotides

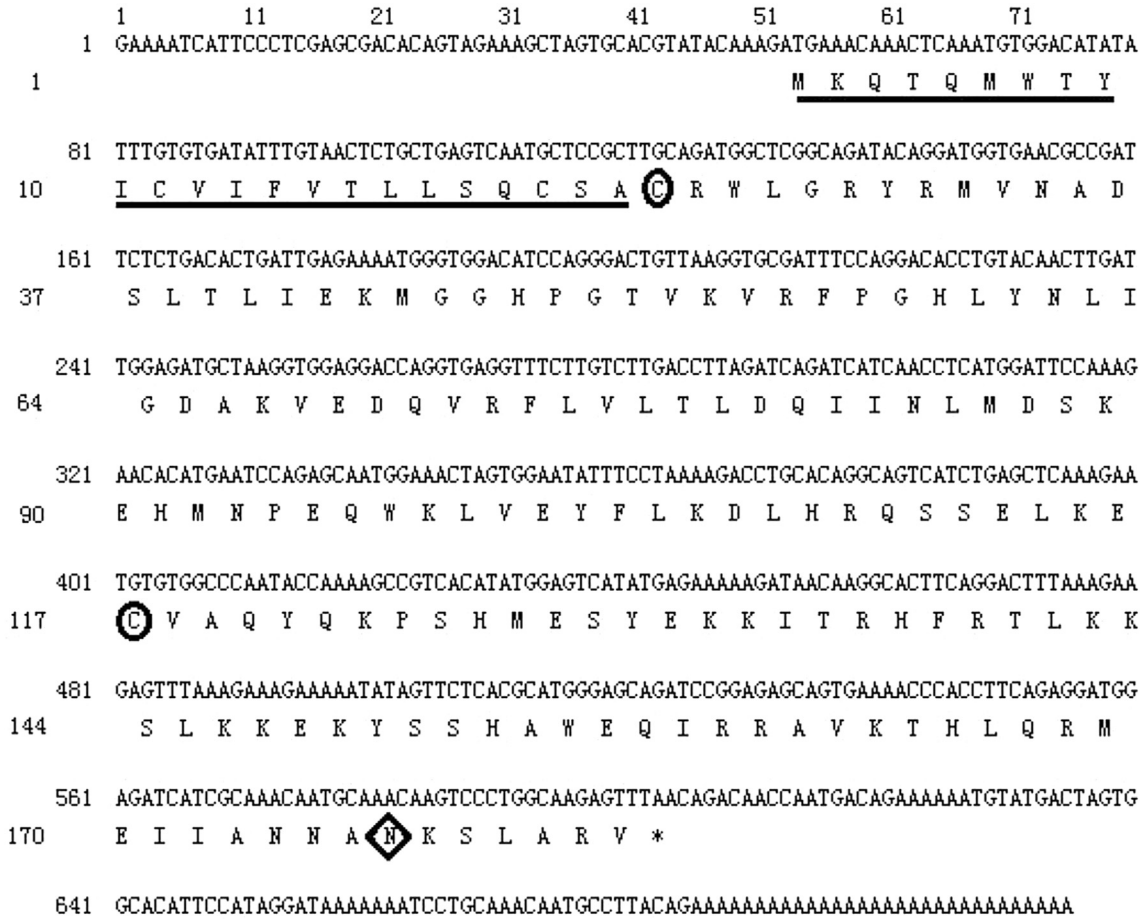

Fig. 1. The full-length cDNA of 3nIFNa2. The nucleotide sequence and the predicted amino acid sequence of 3nIFNa2 are numbered on the left separately. The predicted signal peptide is underlined, two cysteine residues (C) are cycled and the predicted asparagine residue (N) with glycosylation is in the prismatic.

including 5′UTR, coding sequence, 3′UTR and poly (A) tail (NCBI accession number: KX839490). The open reading frame (ORF) of 3nIFNa2 initiates at nucleotide of 53 and terminates at nucleotide of 604. The predicted 3nIFNa2 protein contains 183 amino acids, in which the first 23 amino acids represent a putative signal peptide (predicted by the Signal P program/version 4.1, Center for Biological Sequence Analysis [http://www.cbs.dtu.dk/services/SignalP/]). The calculated molecular weight of 3nIFNa2 is 21.6 kDa and the theoretical isoelectric point of 3nIFNa2 is 9.65. 3nIFNa2 contains two cysteine residues (24 and 117), which potentially form a disulfide bridge in this cytokine molecule and suggest that 3nIFNa2 belongs to group I of type I IFNs [21]. It is interesting that the asparagine residue of 177 is located in the conserved N-linked glycosylation motif (N-X-S/T), which is typically seen in other IFNs (Fig. 1).

To gain insight into 3nIFNa2 evolution, the amino acid (aa) sequence of 3nIFNa2 is aligned with those of vertebrate type I IFNs from different species and the phylogenetic tree has been generated through the Clustal Omega software. Among these type I IFNs, IFNs from *Homo sapiens*, *Felis catus*, *Anas platyrhynchos*, and *Gallus gallus* belong to IFNα subgroup; those from *Carassius auratus*, *Ctenopharyngodon idella*, *Danio rerio*, *Cyprinus carpio* and *Cirrhinus molitorella* belong to the IFN-a subgroup; IFNs from *Epinephelus coioides*, *Sparus aurata* and *Dicentrarchus labrax* belong to the IFN-d subgroup [14,21]. The evolution analysis demonstrated that the selected type I IFNs could be divided into two groups, consisting of mammalian/aves and piscine branches (Fig. 2). 3nIFNa2 is clustered together with other Cyprinidae type I IFNs including crucian carp and grass carp, which correlated with the closed genetic relationship among these three species.

3.2. Transcription of 3nIFNa2 in different tissues

To investigate *3nIFNa2* gene transcription *in vivo*, triploid hybrids were injected with GCRV, SVCV or sterile PBS (as healthy control) separately and sacrificed at 41 h post virus injection. Total RNA was extracted separately from kidney, heart, intestine, gill, liver, skin and spleen of the injected fish. Three fishes were collected for each injected group (PBS, SVCV or GCRV) and tissue total RNA samples of three fishes were combined for cDNA synthesis. The q-PCR data showed that The mRNA of 3nIFNa2 was constitutively detected in all the selected tissues of triploid hybrid under healthy condition, however, the 3nIFNa2 mRNA level in kidney and intestine was much lower than those of other tissues. The 3nIFNa2 mRNA levels in all selected tissues were increased after both GCRV and SVCV infection, especially those in liver and gill (Fig. 3). The data that 3nIFNa2 mRNA levels in different tissues of triploid hybrid increased immediately after viral infection implies that 3nIFNa2 is involved in host innate immune response initiated by pathogen invasion such as GCRV and SVCV.

3.3. 3nIFNa2 is an intracellular cytokine

To study the protein expression of 3nIFNa2, HEK293T cells were transfected with pcDNA5/FRT/TO-HA-3nIFNa2, pcDNA5/FRT/TO-3nIFNa2-HA or the empty vector separately. The transfected cells and the media supernatant of the transfected cells were harvested separately for immunoblot (IB) at 48 h post transfection. For the IB of the whole cell lysate, one clear band around 22 KDa was detected by anti-HA antibody in the lane of HA-3nIFNa2. However, two bands were detected in the lane of 3nIFNa2-HA, one was around 18 KDa and the other band was around 22 KDa (Fig. 4). For the IB of the media supernatant, no band was detected in either the lane of HA-3nIFNa2 or the lane of 3nIFNa2-HA (Fig. 4). The IB data demonstrate that 3nIFNa2 has been well expressed in HEK293T cells but not secreted out of the host cells, which suggests that 3nIFNa2 is an intracellular cytokine. To further investigate the

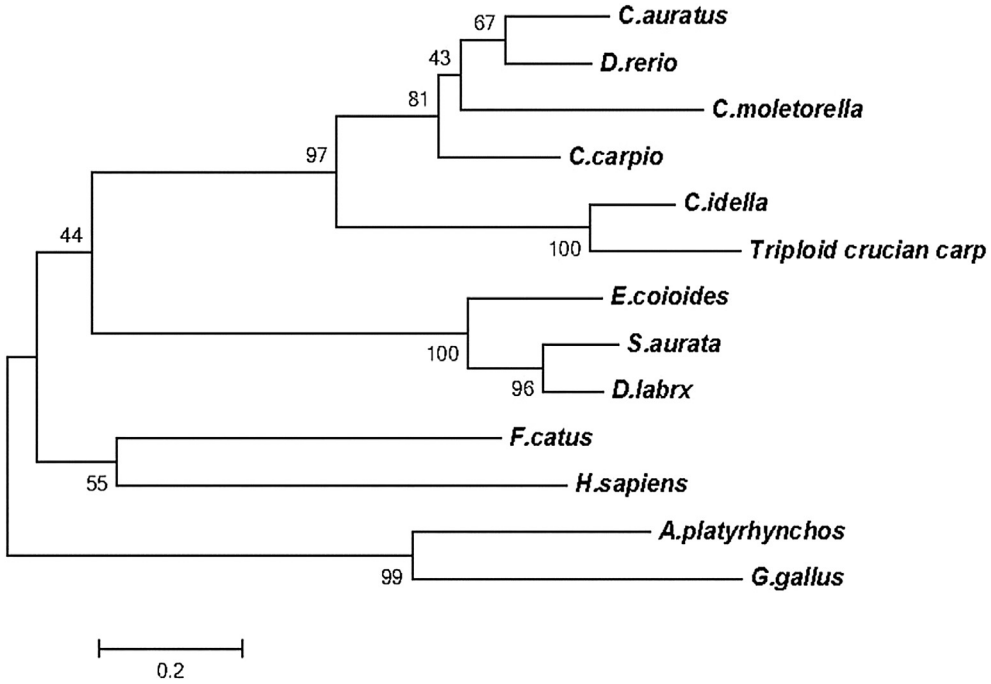

Fig. 2. Phylogenetic tree analysis of 3nIFNa2 with other type I IFNs. The amino acid sequence of 3nIFNa2 is aligned with those of selected type I IFNs from different species by using MEGA 6.0 software. IFN sequences from the following organisms (GenBank accession number, unless indicated otherwise) include: *Homo sapiens* (AAA59181.1);*Anas platyrhynchos* (CAA59235);*Gallus gallus* (AAA50213);*Felis catus* (AAB27160);*Cyprinus carpio* (BAG68521.1);*Danio rerio* (CAD67754.1);*Labeo rohita* (ADX30615.1);*Ctenopharyngodon idella* (ACZ36480.1);*Cirrhinus molitorella* (AAY56128);*Epinephelus coioides* (AGL21770);*Sparus aurata* (CAT03224);*Dicentrarchus labrax* (CAO78741)The bar stands for scale length and the numbers on different nodes stand for bootstrap value.

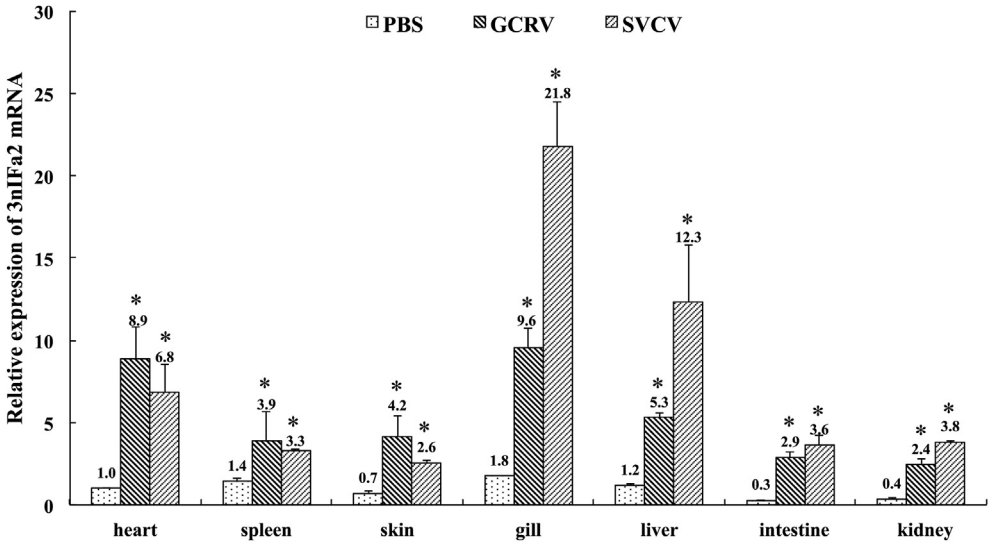

Fig. 3. Tissue-specific mRNA expression of 3nIFNa2. Triploid hybrids were injected with GCRV, SVCV or PBS separately and total RNA was isolated from the indicated tissues independently according to the methods. The transcription of 3nIFNa2 after viral challenge or in healthy condition (PBS injection) was examined by q-PCR separately. Error bars denote standard deviation and data represent three independent experiments. The number above each error bar stands for the averaged 3nIFNa2 mRNA level. Asterisks (*) mark the significant difference between experimental data (GCRV and SVCV) and control data (PBS) ($P < 0.05$).

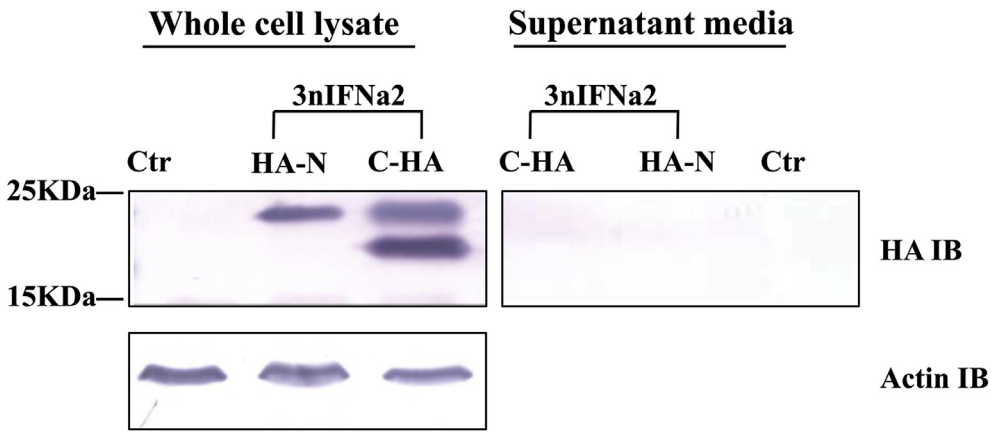

Fig. 4. *Ex-vivo* expression of 3nIFNa2. HEK293T cells were transfected with pcDNA5/FRT/TO-HA-3nIFNa2, pcDNA5/FRT/FRT/TO-3nIFNa2-HA or the empty vector separately and cells were harvested at 48 h post-transfection. The whole cell lysate (WCL) and conditioned media were used for western blot separately. IB: immunoblot; HA-N: pcDNA5/FRT/FRT/TO-HA-3nIFNa2; C-HA: pcDNA5/FRT/FRT/TO-3nIFNa2-HA; Ctr: control.

intracellular distribution of 3nIFNa2, both EPC cells and HeLa cells were transfected with pcDNA5-FRT/TO-HA-3nIFNa2 separately and used for immunofluorescence (IF) staining, in which 3nIFNa2 was detected by mouse monoclonal anti-HA antibody and nucleus were labeled with Hoechst. The IF data of both HeLa cells and EPC cells show clearly that the nucleus (blue) are surrounded tightly by 3nIFNa2-expressing region (green), which demonstrates that 3nIFNa2 is a cytosolic protein (Fig. 5).

3.4. 3nIFNa2 is an antiviral cytokine

IFNs are cytokines with antiviral properties in both innate immunity and adaptive immunity [30]. To examine the function of 3nIFNa2 during host innate immune response, EPC cells were transfected with pcDNA5/FRT/TO-3nIFNa2-HA or the empty vector separately at 24 h before viral infection. For the EPC cells infected with SVCV at different MOIs (0.1, 0.01 & 0.001), both the CPE rate and the viral titer in the supernatant media of the EPC cells over-expressing 3nIFNa2 were much lower than those of the controls, which were determined by crystal violet staining and classic plaque assay separately (Fig. 6). For the EPC cells infected with GCRV at different MOIs (0.1, 0.01 & 0.001), both the CPE rate and the viral titer in the supernatant media of the EPC cells over-expressing 3nIFNa2 were much lower than those of the controls, which were similar to those of SVCV infected EPC cells (Fig. 7). These data demonstrate clearly that 3nIFNa2 is an antiviral cytokine against both SVCV and GCRV.

3.5. 3nIFNa2 is modified with N-linked glycosylation

The asparagine (N) of residue 177 of 3nIFNa2 is located in the conserved N-linked glycosylation motif (N-X-S/T), which is typically seen in other IFNs **[ ]**. Two bands have been detected in the whole cell lysate of 293T cells overexpressing 3nIFNa2-HA, in which there exist 2–3 KDa difference in molecular weight (Fig. 4). The IB data suggest that the larger band of 3nIFNa2 is the post-translation modified form, most likely glycosylation. The whole cell lysate of HEK293T cells over-expressing 3nIFNa2-HA was

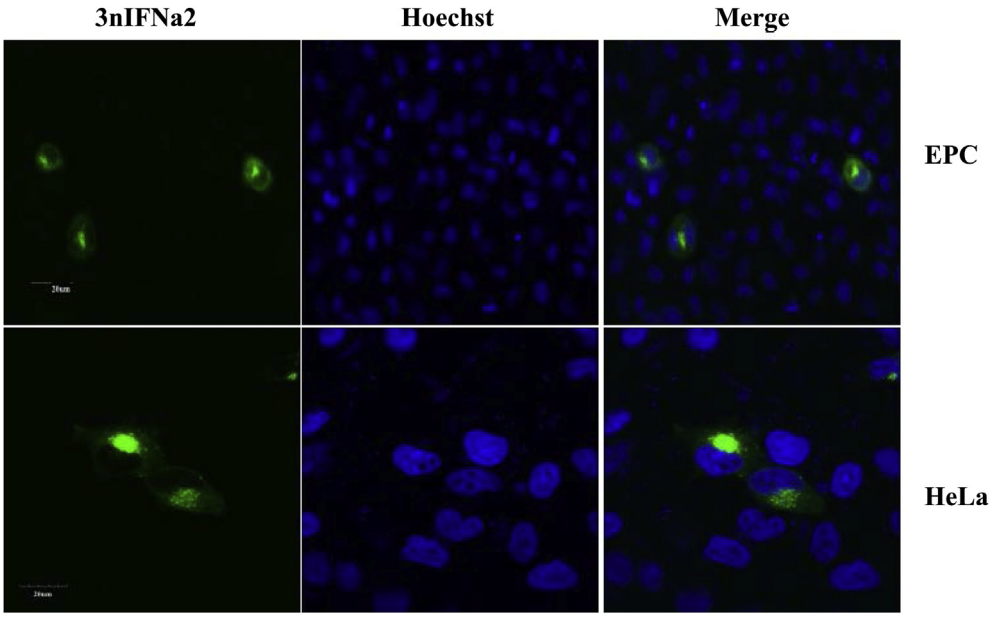

Fig. 5. Intracellular distribution of 3nIFNa2. EPC cells and HELA cells were transfected with pcDNA5/FRT/FRT/TO-3nIFNa2-HA separately. The transfected cells were fixed at 48 h post transfection and used for immunofluorescence staining according to the methods. 3nIFNa2 (green) indicates intracellular location of 3nIFNa2, Hoechst (blue) indicates the nucleus of EPC and HELA; the bar stands for the scale of 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

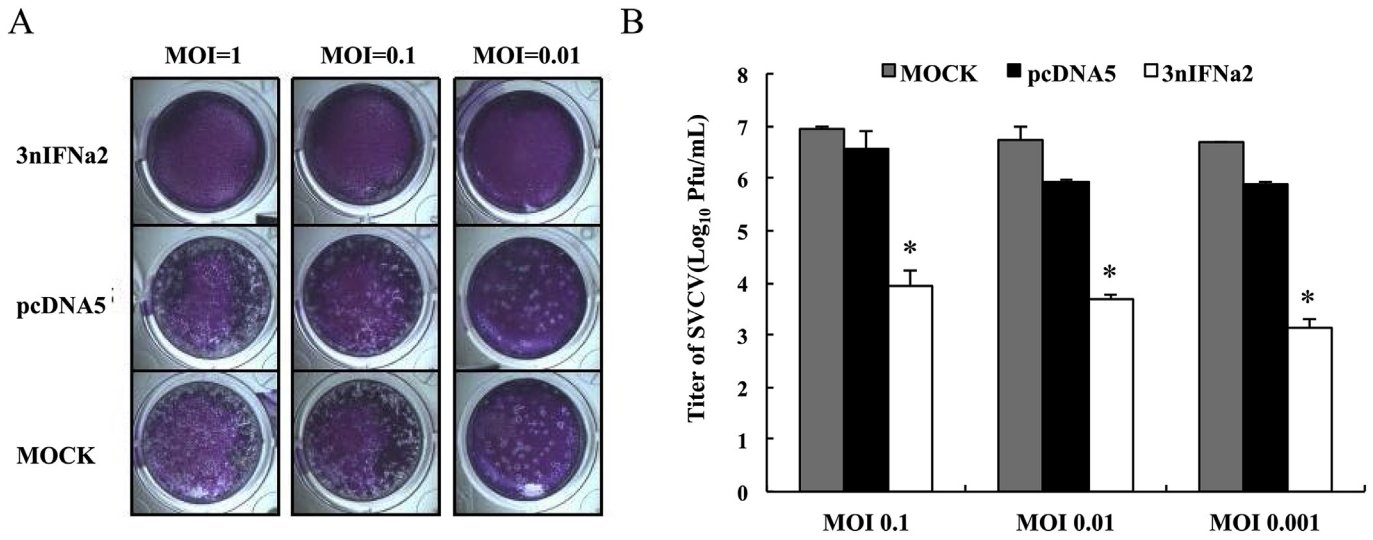

Fig. 6. EPC cells over-expressing 3nIFNa2 present enhanced antiviral ability against SVCV. EPC cells in 24-wells plate were transfected with 250 ng of pcDNA5/FRT/TO-3nIFNa2-HA or pcDNA5/FRT/TO-HA (as control) separately and infected with SVCV at indicated MOIs at 24 h post transfection. The cell monolayers were stained with crystal violet (A) and virus titers in the supernatant media were examined by plaque assay at 24 h post-infection (B). Mock: EPC cells without transfection; 3nIFNa2: pcDNA5/FRT/TO-3nIFNa2-HA; pcDNA5: pcDNA5/FRT/TO-HA. Error bars denote standard deviation and data represent three independent experiments. Asterisks (*) mark the significant difference between experimental data (3nIFNa2) and control data (pcDNA5) ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

digested with PNGase F and used for IB to investigate if this fish cytokine is glycosylated or not. Only one band was detected in the lane of 3nIFNa2 digested with PNGase F, which migrated identically to the smaller band of 3nIFNa2 without digestion (Fig. 8A). The PNGase F digestion data clearly demonstrates that 3nIFNa2 possesses N-linked glycosylation, the larger band stand for the glycosylated 3nIFNa2 and the smaller band is the un-glycosylated form of this fish cytokine.

3.6. Glycosylation is not crucial to the antiviral ability of 3nIFNa2

To investigate if the glycosylation of 3nIFNa2 is recruited into its antiviral function or not, the un-glycosylated mutant 3nIFNa2-N177Q has been generated as previously [22], in which asparagine (N) of 177 has been replaced with glutamine (Q). Only one band was detected by anti-HA antibody in the whole cell lysate of the HEK293T cells transfected with pcDNA5/FRT/TO-3nIFNa2-N177Q-HA, which migrated identically to the smaller band of 3nIFNa2 (the un-glycosylated form). The IB data demonstrate

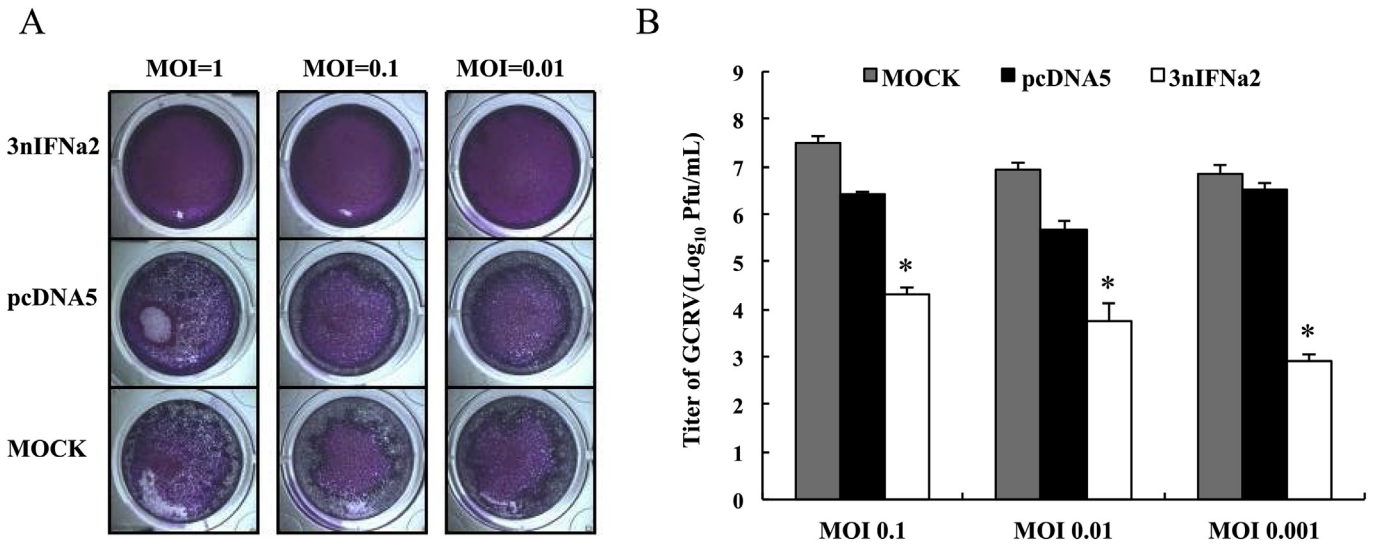

Fig. 7. EPC cells over-expressing 3nIFNa2 present enhanced antiviral ability against GCRV. EPC cells in 24-wells plate were transfected with 250 ng of pcDNA5/FRT/TO-3nIFNa2-HA or pcDNA5/FRT/TO-HA (as control) separately and infected with GCRV at indicated MOIs at 24 h post transfection independently. The cell monolayers were stained with crystal violet (A) and virus titers in the supernatant media were determined by plaque assay at 24 h post-infection (B). Mock: EPC cells without transfection; 3nIFNa2: pcDNA5/FRT/TO-3nIFNa2-HA; pcDNA5: pcDNA5/FRT/TO-HA. Error bars denote standard deviation and data represent three independent experiments. Asterisks (*) mark the significant difference between experimental data (3nIFNa2) and control data (pcDNA5) ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

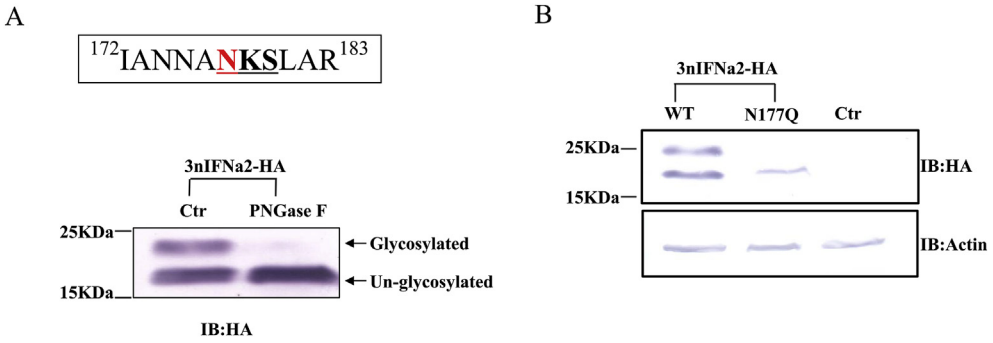

Fig. 8. 3nIFNa2 possesses N-linked glycosylation. (A) Upper panel: N-X-S/T consensus sequence (underlined) for N-linked glycosylation in 3nIFNa2. Lower panel: Glycosidase digestion of 3nIFNa2:HEK293T cells were transfected with pcDNA5/FRT/TO-3nIFNa2-HA and the transfected cells were harvested at 48 h post-transfection. The whole cell lysate was divided into two aliquots, one was digested with PGNase F and the other aliquot treated only with the enzyme buffer (as control). (B) HEK293T cells were transfected with pcDNA5/FRT/TO-3nIFNa2-HA, pcDNA5/FRT/FRT/TO-3nIFNa2-N177Q-HA or the empty vector separately and the cells were harvested at 48 h post-transfection. The whole cell lysate was isolated by 14% SDS-PAGE and applied to IB assay. IB:immunoblot; WT:pcDNA5/FRT/FRT/TO-3nIFNa2-HA; N177Q: pcDNA5/FRT/FRT/TO-3nIFNa2-N177Q-HA; Ctr: pcDNA5/FRT/FRT/TO-HA.

clearly that the mutant of N177Q has been well expressed in HEK293T cells without N-linked glycosylation (Fig. 8B). EPC cells were transfected with pcDNA5/FRT/TO-3nIFNa2-N177Q-HA, pcDNA5/FRT/TO-3nIFNa2-HA or the empty vector separately at 24 h before GCRV or SVCV infection. Both the cytopathic effect (CPE) and the viral titer of the supernatant media of the EPC cells transfected with pcDNA5/FRT/TO-3nIFNa2-HA and pcDNA5/FRT/TO-3nIFNa2-N177Q-HA were obviously decreased compared with those of the control, which was determined by crystal violet staining and classic plaque assay separately (Fig. 9 and Fig. 10). These data demonstrate that 3nIFNa2-N177Q possesses the similar antiviral ability against both SVCV and GCRV to that of 3nIFNa2, which suggests that N-linked glycosylation is not crucial for the antiviral property of this fish cytokine.

4. Discussion

Host immune defense combat virus invasion through coordinating an intracellular innate immune response with the adaptive immune response [31]. The signaling required to coordinate the successful induction of the intracellular immune response to virus infection relies on the activation of interferon (IFN) genes [32]. IFNs are cytokines characterized with antiviral property, which are induce-expressed in host cells after viral infection. IFNs are usually small and secreted proteins, which stimulate host cells' immune response through autocrine or paracrine [8,31]. It is interesting that functioning intracellular IFN system was found in rainbow trout (*Oncorhynchus mykiss*), which might act as a novel defense to counteract viral infection [33].

Teleost fish with different ploidy present variated immune activities. The studies on rainbow trout and turbot (*Scophthalmus maximus*) demonstrated that diploid fish and triploid fish were at the similar immunity level [34,35]. However, data generated from Amazon molly (*Poecilia formosa*) and Chinook salmon (*Oncorhynchus tshawytscha*) showed that triploid fish presented stronger immunizing activity against pathogen invasion [36,37]. Triploid

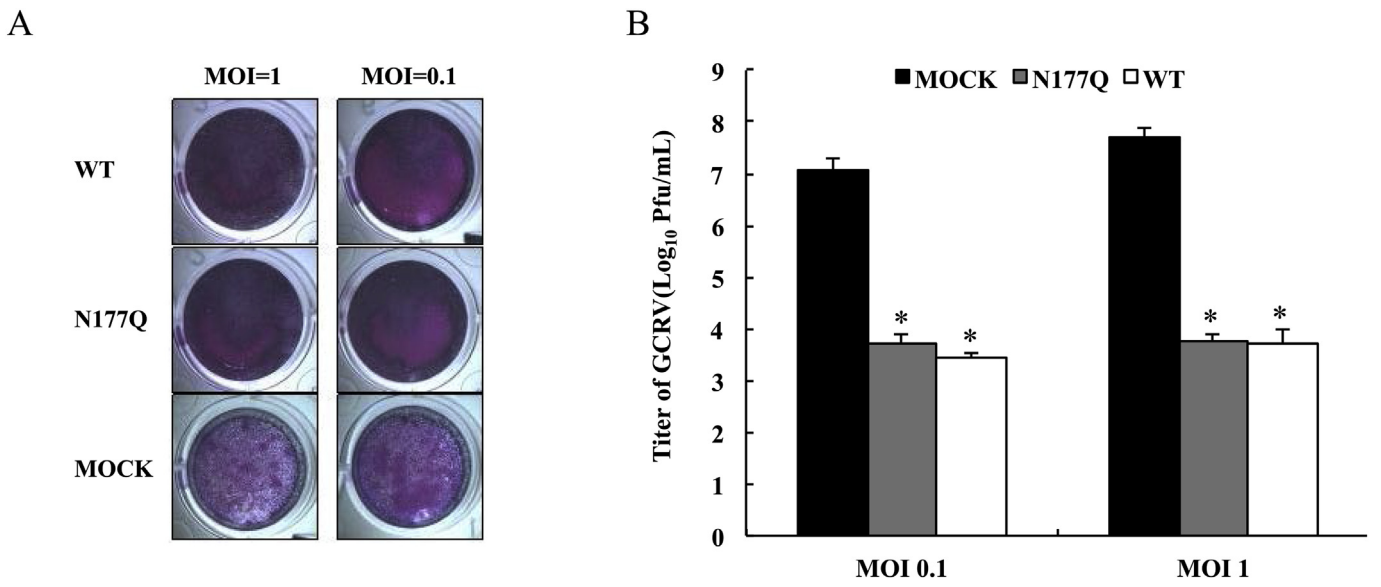

Fig. 9. Glycosylation in 3nIFNa2 is not crucial for its antiviral ability against GCRV. EPC cells in 24-wells plate were transfected with 250 ng of pcDNA5/FRT/TO-3nIFNa2-HA, pcDNA5/FRT/TO-3nIFNa2-N177Q-HA or the empty vector separately and infected with GCRV at indicated MOIs at 24 h post transfection. The cell monolayers were stained with crystal violet (A) and virus titers in the supernatant media were determined by plaque assay at 24 h post-infection (B). Mock: pcDNA5/FRT/TO-HA; WT: pcDNA5/FRT/TO-3nIFNa2-HA; N177Q: pcDNA5/FRT/TO-3nIFNa2-N177Q-HA. Error bars denote standard deviation and data represent three independent experiments. Asterisks (*) mark the significant difference between experimental data (WT and N177Q) and control data (mock) ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

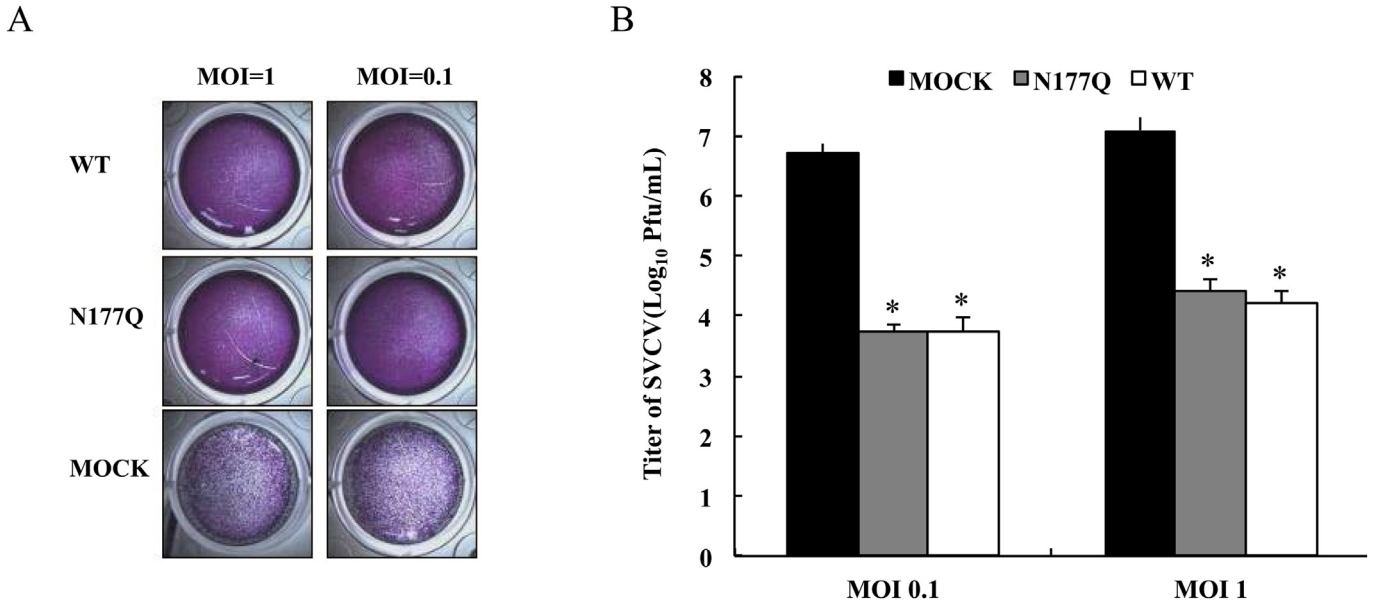

Fig. 10. Glycosylation in 3nIFNa2 is not crucial for its antiviral ability against SVCV. EPC cells in 24-wells plate were transfected with 250 ng of pcDNA5/FRT/TO-3nIFNa2-HA, pcDNA5/FRT/TO-3nIFNa2-N177Q-HA or the empty vector separately and infected with SVCV at indicated MOIs at 24 h post transfection. The cell monolayers were stained with crystal violet (A) and virus titers in the supernatant media were determined by plaque assay at 24 h post-infection (B). Mock: pcDNA5/FRT/TO-HA; WT: pcDNA5/FRT/TO-3nIFNa2-HA; N177Q: pcDNA5/FRT/TO-3nIFNa2-N177Q-HA. Error bars denote standard deviation and data represent three independent experiments. Asterisks (*) mark the significant difference between experimental data (WT and N177Q) and control data (mock) ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hybrid of gold fish and allotetroploid demonstrates much higher survival rate in aquaculture environment compared with its parents, which suggests this triploid hybrid owns evolved immunity to support its enhanced disease-resistance and stress-resistance [25].

To understand the innate immune system of this triploid hybrid, one of the type I IFNs from triploid hybrid (3nIFNa2) was cloned and characterized in this paper. The full-length cDNA of 3nIFNa2 gene consists of 715 nucleotides and the predicted 3nIFNa2 contains 183 amino acid residues. 3nIFNa2 has been cloned and identified as a group I of type I IFNs, which is induced *in vivo* and *ex vivo* in response to both SVCV and GCRV infection. 3nIFNa2 has been detected in the whole cell lysate of HEK293T cells transfected with pcDNA5/FRT/TO-3nIFNa2-HA but not in the supernatant media of the transfected media, which clearly demonstrates that 3nIFNa2 is an intracellular protein (Fig. 4). In the IB data, one band (~22 KDa) was detected by anti-HA antibody in the lane HA-

3nIFNa2 and two bands were detected in the lane of 3nIFNa2-HA (~18 KDa and ~22 KDa). The bigger band and smaller band of 3nIFNa2-HA lane represent the glycosylated form and un-glycosylated form of this cytokine, which has been identified by the glycosidase digestion assay (Fig. 8). Thus, it is reasonable that the band of HA-3nIFNa2 lane represents the pre-matured peptide, which contains a signal peptide at its N-terminus. The alternative splicing of rainbow trout IFN1 leads to synthesis of intracellular cytokines (iIFN1a and iIFN1b), which contain no signal peptide [33]. It is interesting why 3nIFNa2 is not secreted out of host cells? Like intracellular iIFN1a and iIFN1b, 3nIFNa2 presents strong antiviral ability. However, the antiviral mechanisms behind these intracellular IFNs might be different.

As one of the most common posttranslational protein modification, glycosylation serves various functions such as to help protein folding correctly in eukaryotic cells. N-linked glycosylation is the most common glycosylation type, in which the glycan chain is added to the asparagine in the conserved motif of N-X-S/T [38]. The asparagine of 177 site of 3nIFNa2 is in the conserved domain of N-linked glycosylation and two bands (2–3 KDa different in molecular weight) have been detected through IB, which suggests that 3nIFNa2 is a glycoprotein (Fig. 4). The glycosidase digestion verified that 3nIFNa2 is modified with N-linked glycosylation and the glycan chain is attached to the asparagine of 177, which was determined by the analysis of mutant N177Q (Fig. 8). N-linked glycosylation plays an important role in the protein trafficking to the membrane or out of the cell [38]. Apparently, N-linked glycosylation of 3nIFNa2 has nothing to do with its secretion. Several studies showed that N-linked glycosylation does not affect the antiviral property of fish IFNs, such as IFNs from Atlantic salmon and black carp [22,39]. The data of both CPE and viral titer show that 3nIFNa2 and un-glycosylated mutant 3nIFNa2-N177Q possess similar ability against SVCV and GCRV, which suggests that N-linked glycosylation of 3nIFNa2 does not contribute directly to its antiviral property (Figs. 9 and 10). However, the functional mechanism of glycosylation of this fish antiviral cytokine needs further exploitation.

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