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MAVS of triploid hybrid of red crucian carp and allotetraploid possesses the improved antiviral activity compared with the counterparts of its parents

Jun Xiao, Yongming Fu, Hui Wu, Xiangding Chen, Shaojun Liu, Hao Feng*

State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Science, Hunan Normal University, Changsha, 410081, China

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ABSTRACT

Triploid hybrid (3n = 150) of red crucian carp (♀, 2n = 100) and allotetraploid (♂, 4n = 200) presents the obviously stronger disease resistance than its parents. To elucidate the innate immunity of triploid hybrid, the MAVS homologues of triploid hybrid (3nMAVS), red crucian carp (2nMAVS) and allotetraploid (4nMAVS) have been identified and characterized separately in this study. 2nMAVS and 4nMAVS were evolutionarily conserved; however, 3nMAVS showed lower amino acid similarity and differently predicted structure to 2nMAVS or 4nMAVS. 3nMAVS transcription increase rate in host cells were obviously higher than 2nMAVS or 4nMAVS in response to different stimuli, which included spring viraemia of carp virus (SVCV), grass carp reovirus (GCRV) and poly (I:C). The reporter assay in EPC cells showed that 3nMAVS owned much stronger ability to induce the production of DrIFNφ1 and eIFN than either 2nMAVS or 4nMAVS. Accordingly, EPC cells transfected with 3nMAVS presented obviously stronger antiviral activity against both GCRV and SVCV than the cells expressing 2nMAVS or 4nMAVS. All the data support the conclusion that 3nMAVS-mediated antiviral signaling during innate immune activation was stronger than those of 2nMAVS and 4nMAVS, which provided us the new insight on the innate immune system of triploid hybrid.

1. Introduction

Hybridization can combine the whole genomes from two different strains or species and bring improved traits to the hybrids, which has been widely used in aquaculture to produce the hybrid fish with positive heterosis [1,2]. By using distant hybridization and subsequently selective breeding, allotetraploid (AT; 4n = 200) population has been developed by crossing red crucian carp (*Carassius auratusred* red var., ♀, 2n = 100) with common carp (*Cyprinus carpio* L., ♂, 2n = 100), of which both male and female individuals are fertile and this allotetraploid population has propagated 27 generations [3–5]. Triploid hybrids (3n = 150) have been produced through the hybridization between the male allotetraploid (4n = 200) and the female red crucian carp (2n = 100), which own many merits, such as outstanding growth rate, good taste and sterility. Especially, triploid hybrids display obviously improved disease resistance and stress resistance compared with their parents [6,7]. However, the immunity of these hybrids remains much unknown.

Teleost fishes possess both innate immunity and adaptive immunity like mammals; however, these lower vertebrates depend more on their innate immune systems to defeat invading pathogenic microbes, such as virus [8]. Hybridization leads to the altered genotype and phenotype of the offspring [9,10], which suggests the improved innate immunity of triploid hybrids based on the genetic recombination. In our previous study, 2nFC, 3nFC and 4nFC cell lines were separately established and characterized, which derived from the primary culture of caudal fin of red crucian carp, triploid hybrid and allotetraploid respectively. 3nFC exhibited stronger antiviral activity against SVCV in plaque assay than 2nFC and 4nFC, which correlated with the disease resistance of their donors [11]. The transcriptome analysis of these cell lines revealed that innate immunity related signaling pathways, such as RIG-I-like receptor (RLR) signaling, were activated remarkably in 3nFC after SVCV infection, which implied that RLR signaling might contribute importantly to the disease resistance of triploid hybrid.

RLRs are a family of molecules that expressed inside cells to sense cytosolic viral RNA and to trigger innate immune responses. RLRs are composed of three structurally homologous members: retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) [12]. Signaling downstream of RIG-I and MDA5 depends on the essential adaptor protein mitochondrial antiviral signaling (MAVS, also known as IPS-1, Cardif, and VISA) [13–16]. Structurally, MAVS comprises a CARD domain at its N terminus, followed by a prolinerich domain, and a transmembrane domain at its C terminus. The mitochondrial

* Corresponding author.
E-mail address: fenghao@hunnu.edu.cn (H. Feng).

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localization of MAVS makes it to become a central adaptor protein for receiving signals from upstream molecules and activating downstream signaling molecules [12]. Teleost MAVS homologues have been identified and characterized in different species, such as grass carp (*Ctenopharyngodon idella*), zebrafish (*Danio rerio*), Atlantic salmon *(Salmo salar*), Japanese flounder (*Paralichthys olivaceus*), rock bream (*Oplegnathus fasciatus*), orange spotted grouper (*Epinephelus coioides*) and black carp (*Mylopharyngodon piceus*). These studies demonstrated that fish MAVS, like their mammalian counterpart, played the important roles in the innate immune response against viral infection [17–23].

In the current study, MAVS homologues from red crucian carp (2nMAVS), triploid hybrid (3nMAVS) and allotetraploid (4nMAVS) have been cloned and characterized. It was interesting that 2nMAVS shared higher amino acid sequence identity with 4nMAVS, while 3nMAVS showed lower sequence identity with either 2nMAVS or 4nMAVS. Both reporter assay and plaque assay demonstrated that 3nMAVS mediated much stronger antiviral signaling in EPC cells than 2nMAVS or 4nMAVS did, which shed a light on the innate immune system of the triploid offspring of allotetraploid and diploid red crucian carp.

2. Materials and methods

2.1. Cells and plasmids

HEK293T cells, NIH3T3 cells, *Epithelioma Papulosum Cyprinid* (EPC) cells, 2nFC, 3nFC and 4nFC cells (cell lines from the primary culture of the caudal fin of red crucian carp, triploid hybrid or allotetraploid accordingly) were kept in the lab. All the cell lines were grown in DMEM supplemented with 10% fetal bovine serum (FBS) (HEK293T, NIH3T3 and EPC) or 15% FBS (2nFC, 3nFC and 4nFC), 2 mM L-glutamine, 100 units/mL penicillin and 100 mg/mL streptomycin. HEK293T and NIH3T3 cells were cultured at 37 °C with 5% CO_2; EPC, 2nFC, 3nFC and 4nFC were culture at 25 °C with 5% CO_2. Transfection was done as previously described, Lipomax (SUDGEN) was used for EPC transfection; calcium phosphate was used for HEK293T transfection [24].

pcDNA5/FRT/TO, pRL-TK, Luci-DrIFNφ1 (for zebrafish IFNφ1 promoter activity analysis) and Luci-eIFN (for fathead minnow IFN promoter activity analysis) were kept in the lab [25]. The recombinant plasmids including pcDNA5/FRT/TO-Flag-2nMAVS, pcDNA5/FRT/TO-Flag-3nMAVS and pcDNA5/FRT/TO-Flag-4nMAVS were constructed by cloning the open reading frame of 2nMAVS, 3nMAVS or 4nMAVS fused with an Flag tag at its *N*-terminus into pcDNA5/FRT/TO between Kpn I and Xho I sites, respectively.

2.2. Cloning of 2nMAVS, 3nMAVS and 4nMAVS

Degenerate primers were designed to amplify 2nMAVS, 3nMAVS or 4nMAVS basing on the MAVS sequences of Common carp (HQ85044) and EPC (CAX48603) (Table 1). Total RNA was isolated from 2nFC, 3nFC or 4nFC separately, and first-strand cDNA was synthesized by using the Revert Aid First Strand Synthesis Kit (Thermo) independently. Rapid amplification of cDNA ends (RACE) was performed to obtain 5′UTR (untranslated region) and 3′UTR of 2nMAVS, 3nMAVS and 4nMAVS cDNA by using 5′Full RACE Kit and 3′Full RACE Kit (TaKaRa), respectively. The full-length cDNA of 2nMAVS, 3nMAVS or 4nMAVS was separately cloned into pMD18-T vector and sequenced by Invitrogen.

2.3. Virus produce and titration

SVCV (strain: SVCV 741) and GCRV (strain: GCRV 106) were kept in the lab. SVCV and GCRV were propagated in EPC and CIK separately at 25 °C in the presence of 2% FBS. Virus titer was determined by plaque assay on EPC cells separately as previously described [26]. Briefly, the 10-fold serially diluted virus supernatant was added onto EPC cells in 24-well plate (4×10^5 cells/well) and incubated for 2 h at 25 °C. The supernatant was replaced with fresh DMEM containing 2% FBS and 0.75% methylcellulose (Sigma) after incubation. Plaques were counted at day 3 post-infection.

2.4. Quantitative real-time PCR

Quantitative real-time PCR (q-PCR) was performed to quantify MAVS mRNA levels in 2nFC, 3nFC and 4nFC cells. The primers for MAVS were designed based on the homologous sequences of 2nMAVS, 3nMAVS and 4nMAVS and the β-actin was performed as internal control (Table 1). The q-PCR program was: 1 cycle of 95 °C/10 min, 40 cycles of 95 °C/15 s, 60 °C/1 min, 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 (ABI) 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 (8×10^5 cells/well) or EPC cells (1.6×10^6 cells/well) in 6-well plate were transfected with plasmids expressing 2nMAVS, 3nMAVS, 4nMAVS or the empty vector separately. The transfected cells were harvested at 48 h post-transfection and lysed for immunoblot assay as previously described [27]. Briefly, whole cell lysates were isolated by 10% SDS-PAGE and the transferred membrane was probed with mouse monoclonal anti-Flag antibody (1:3000; Sigma), which was followed by incubation with goat-*anti*-mouse IgG (1:30000; Sigma). Target proteins were visualized through BCIP/NBT Alkaline Phosphatase Color Development Kit (Sigma).

2.6. Immunofluorescence microscopy

EPC cells in 24-well plate were transfected with pcDNA5/FRT/TO-HA-2n (3n/4n)MAVS; NIH3T3 cells in 24-well plate were co-transfected with pcDNA5/FRT/TO-HA-2n (3n/4n)MAVS and Mito-EGFP. The transfected cells were fixed with 4% (v/v) paraformaldehyde at 24 h post-transfection. The fixed cells were permeabilized with Triton X-100 (0.2% in PBS) and used for immunefluorescent staining as previously described [27]. For the transfected EPC cells, 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; Mitotracker Deep Red FM (M22426, Invitrogen) was used to stain the mitochondria according the manual of the company. For the transfected NIH3T3 cells, rabbit polyclonal anti-HA antibody (Sigma) was probed at the ratio of 1:200 and Alexa 594-conjugated secondary antibody (Invitrogen) was probed at the ratio of 1:400; DAPI (Sigma) was used for nucleus staining.

2.7. Dual-luciferase reporter assay

EPC cells in 24-well plate (4×10^5 cells/well) were co-transfected with plasmids expressing MAVS (50–200 ng/well), pRL-TK (25 ng/well), Luci-DrIFNφ1 or Luci-eIFN (250ng/well). The empty vector was used in the control group in place of MAVS plasmids. For each transfection, the total amount of plasmid was balanced with the empty vector. The cells were harvested and lysed on ice at 24 h post-transfection. The centrifuged supernatant was used to measure firefly luciferase and renilla luciferase activities according to the instruction of the manufacturer (Promega) as previously [24].

2.8. Statistics analysis

For the statistics analysis of the data of q-PCR, luciferase reporter

Table 1
Primers used in the study.

Primer name	Sequence (5′-3′)	Primer information
PM1-F	ATGTCATTYACNCGNGARC	Partial MAVS CDS cloning
PM1-R	TCATTTGTGNGTRAAYTTCCANGC	
5′-RACE		
5′Race outer primer	CAGGAGATGGTTCTGGGGCTGCAG	5′UTR 1st PCR
5′Race inner primer	CGTTATTTGTGATGCCCCTGATCCTGC	5′UTR 2nd PCR
3′-RACE		
UPM Long primer	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	
UPM short primer	CTAATACGACTCACTATAGGGC	
3′Race outer primer	GCCTGAGGAGGACCACTATGAATCTCTC	
3′Race inner primer1	GTCAGCCGCCAAACATCCTGCAAC	3′UTR 1st PCR
3′Race inner primer2	GGGATTGGTCTGTCTGCTTTGTTCTTGG	3′UTR 2nd PCR
NUP	AAGCAGTGGTATCAACGCAGAGT	
qPCR		
β-actin-F	TGCTATGTGGCTCTTGACT	
β-actin-R	AGGTCCTTACGGATGTCG	
MAVS-F	CTGAGGAGGACCACTAT	
MAVS-R	TGATGGTGGTTGACTTCT	

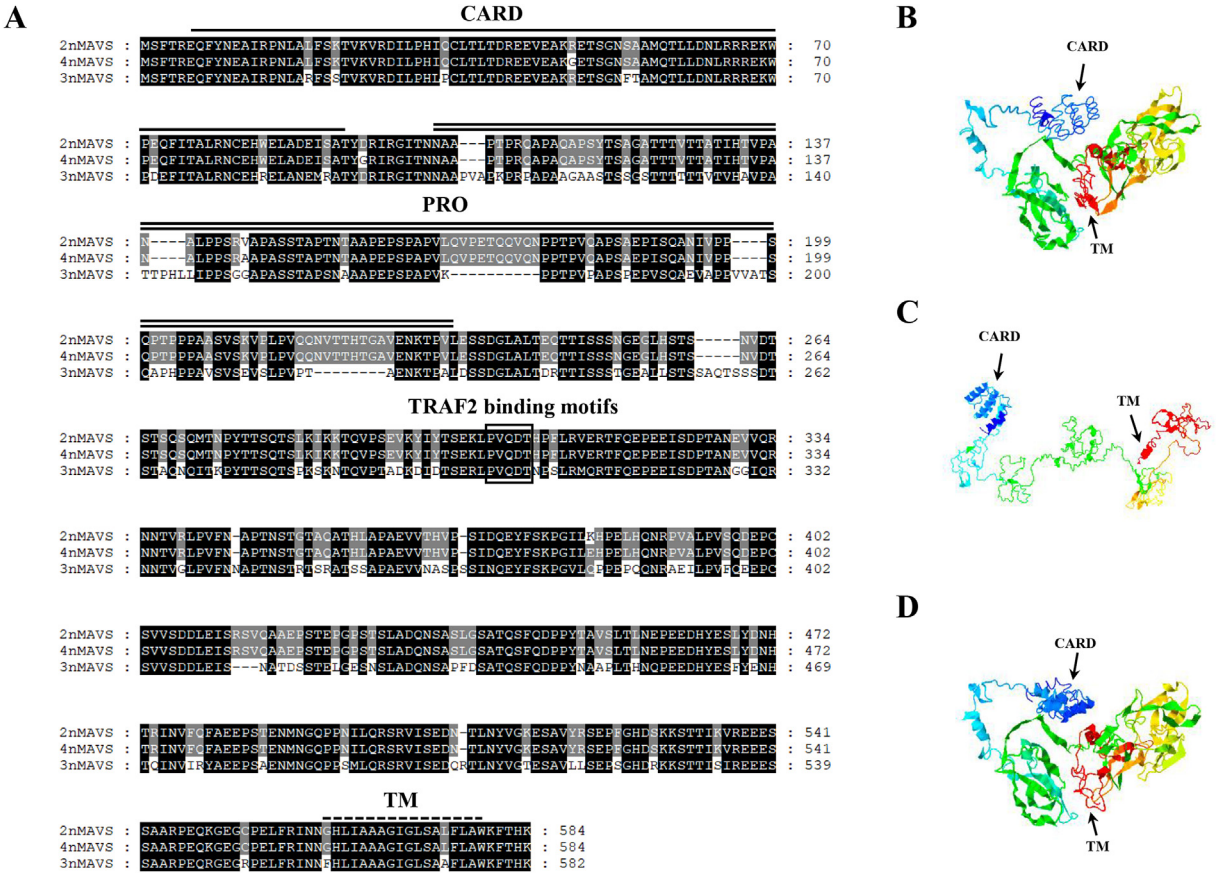

Fig. 1. Sequence comparison and spatial structures of 2nMAVS, 3nMAVS and 4nMAVS.
(A) Comparisons of 2nMAVS, 3nMAVS and 4nMAVS amino acid sequences by using MEGA 7.0 program and GeneDoc program. *N*-terminal caspase-recruitment domain (CARD) is indicated by single line; Proline-rich domain (PRO) is marked by double line and *C*-terminal transmembrane domain (TM) is indicated by dash line; TRAF2 binding motifs were boxed. **(B ~ D)** Three-dimensional structures of 2nMAVS (B), 3nMAVS (C) and 4nMAVS (D). The predicted protein structures were generated by I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) based on the amino acid sequences of 2nMAVS, 3nMAVS and 4nMAVS. CARD and TM domains are indicated with arrows.

assay and viral titration, all data were obtained from three independent experiments with each performed in triplicate (Two-tailed Student's t-test was used for all statistical analysis). Error bars represent the standard error of the mean (+SEM) of three independent experiments. Asterisk (*) stands for $p < 0.05$ and asterisks (**) stand for $p < 0.01$.

3. Results

3.1. Molecular cloning and sequence analysis of 2nMAVS, 3nMAVS and 4nMAVS

The full-length cDNA of 2nMAVS is composed of 3441 nucleotides, which includes an open reading frame (ORF) of 1755 bp, a 5′ UTR of

Table 2
Comparison of vertebrate MAVS homologues (%).

Species	Red crucian carp		Triploid hybrid		Allotetraploid	
	Identity	Similarity	Identity	Similarity	Identity	Similarity
Red crucian carp	100	100	71.3	78.4	99.3	99.5
Triploid hybrid	71.3	78.4	100	100	71	78.1
Allotetraploid	99.3	99.5	71	78.1	100	100
Common carp	70.6	77.9	97.4	98.1	70.3	77.6
Crucian carp	64.2	72.5	68.6	75.2	63.9	72.2
Grass carp	64.4	72.7	68.8	75.5	64.1	72.4
Black carp	65.6	73.9	68.4	75.4	65.3	73.6
EPC	59.4	68.6	62.5	72.1	59	58.3
Zebrafish	53.5	63.8	55.1	66.2	53.3	63.5
Atlantic salmon	27.1	41	28.7	46.4	26.9	41.9
Rainbow trout	27.2	42.5	29.1	44.1	27.3	42.2
Human	22.8	34.1	23	36.5	22.8	33.9
House mouse	22.5	32.9	21.3	34.1	22.5	32.9
Chicken	22.1	34.1	20.4	31	22	33.6
Duck	20.6	33.0	23.1	34.1	20.6	32.8
Pig	23.5	34.2	24.3	36	23.6	34.2

The proteins IDs of MAVS in the table were the same as those in Fig. 2.

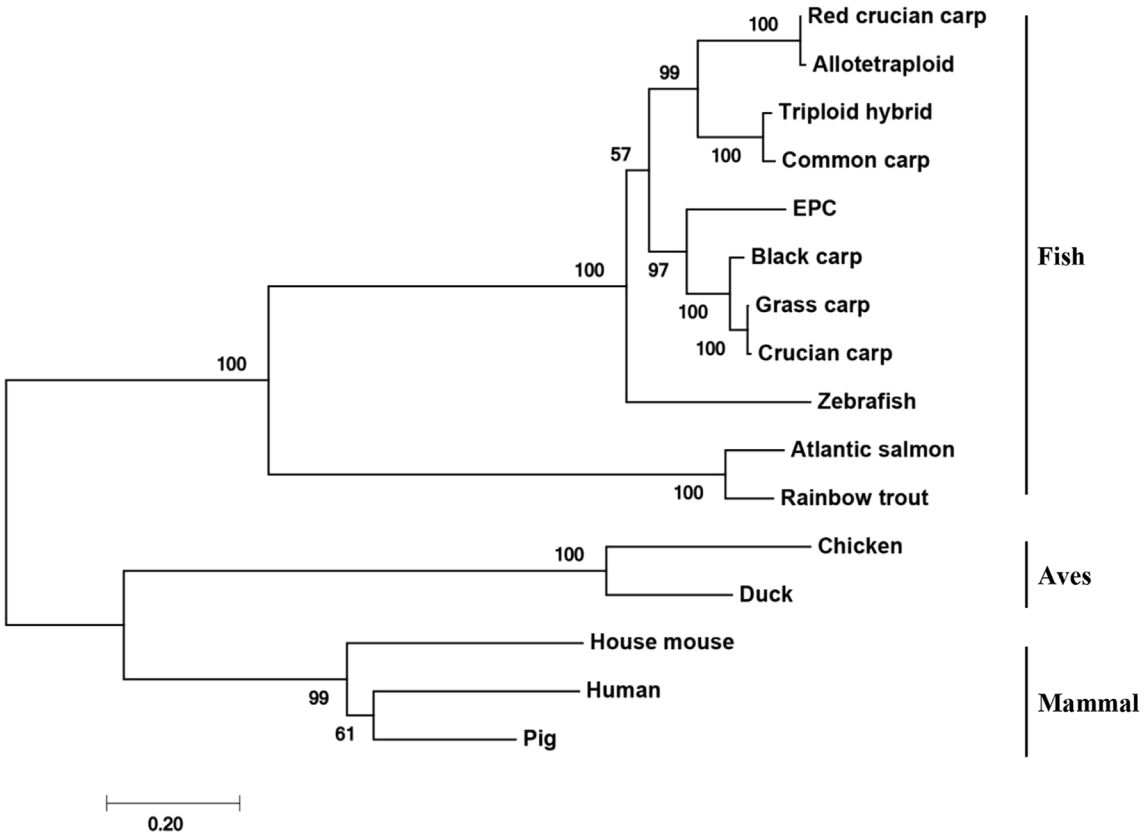

Fig. 2. Phylogenetic tree of vertebrate MAVS from different species.
The amino acid sequences of 2nMAVS, 3nMAVS and 4nMAVS were aligned with MAVS from different species by using MEGA 7.0 software, which included (GenBank accession number): Common carp (ADZ55453), EPC (CAX48603), Black carp (AGP75919.1), Grass carp (AHW04051), Crucian carp (AIR08566.1), Zebrafish (CAX48608), Atlantic salmon (NP_001161824.1), Rainbow trout (CAZ27722), Chicken (BAO25513), Duck (APB08796.1), House mouse (AAZ80418), Human (AAZ8417), Pig (BAF42542). The bar stands for scale length and the numbers on different nodes stand for bootstrap value. The Latin names of these species are: Common carp (*Cyprinus carpio*); EPC (*Epithelioma Papulosum Cyprinid*); Black carp (*Mylopharyngodon piceus*); Grass carp (*Ctenopharyngodon idella*); Crucian carp (*Carassius carassius*); Zebrafish (*Danio rerio*); Atlantic salmon (*Salmo salar*); Rainbow trout (*Oncorhynchus mykiss*); Chicken (*Gallus gallus*); Duck (*Anas platyrhynchos*); House mouse (*Mus musculus*); Human (*Homo sapiens*); Pig (*Sus scrofa*).

129 bp and a 3′UTR of 1557 bp (GeneBank accession number: MK251472). The ORF of 2nMAVS encodes a polypeptide of 584 amino acids with a predicted molecular mass of 63.6 kDa and an isoelectric point (pI) of 5.22. The full-length cDNA of 3nMAVS consists of 3244 nucleotides and includes a 5′UTR of 94 bp, a 3′UTR of 1401 bp and an ORF of 1749 bp (GeneBank accession number: MK251473). The predicted 3nMAVS protein contains 582 amino acids with a predicted molecular mass of 62.6 kDa and a pI of 5.05. The full-length cDNA of 4nMAVS consists of 3364 nucleotides with an ORF of 1755 bp encoding a polypeptide of 584 amino acids, a 136 bp 5′UTR and a 1473 bp 3′UTR

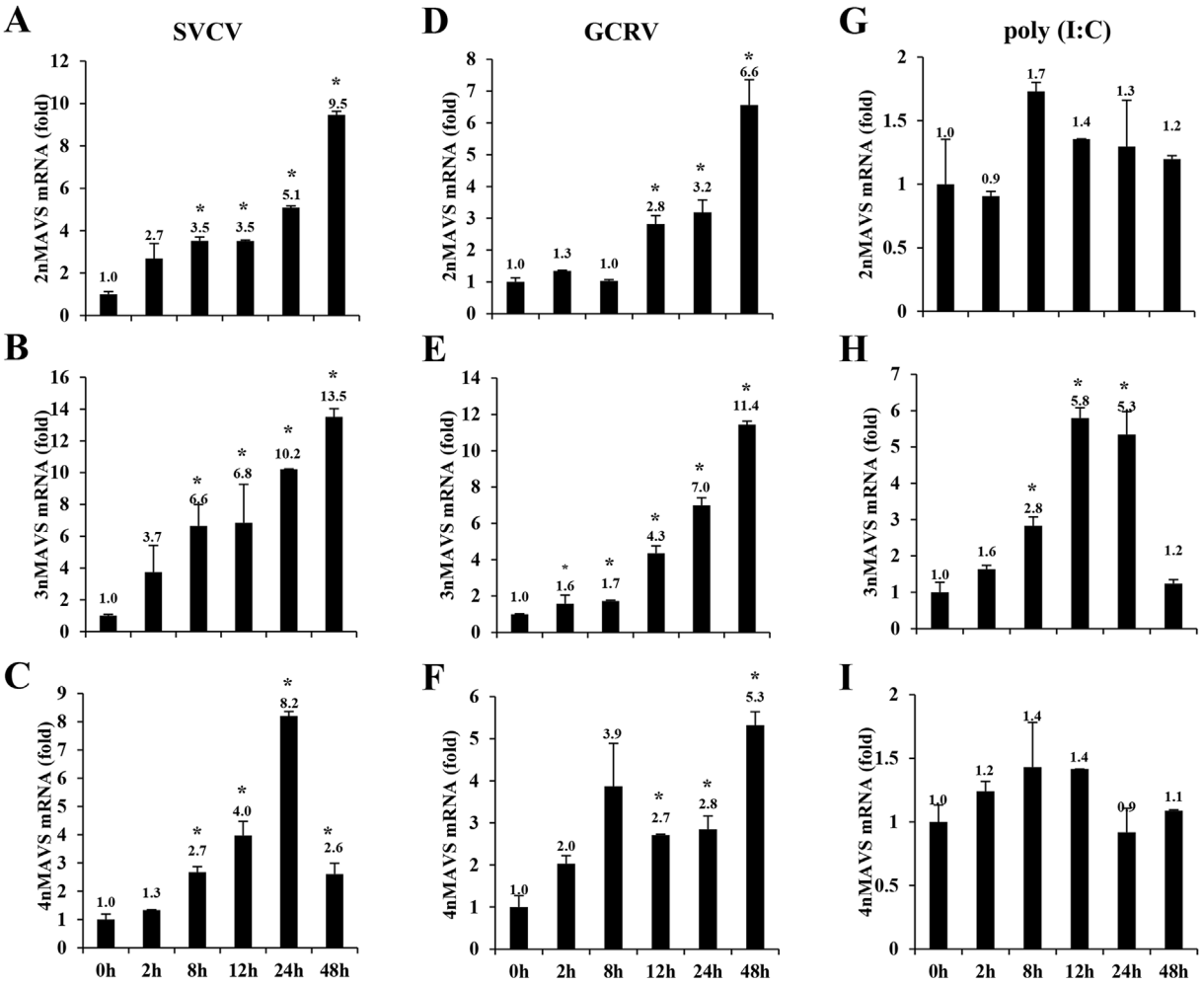

Fig. 3. Transcription of 2nMAVS, 3nMAVS and 4nMAVS in response to different stimuli.
2nFC, 3nFC and 4nFC cells in 12-well plates were infected with SVCV **(Ã C)** (MOI 0.003), GCRV **(D ~ F)** (MOI 0.003) or treated with poly (I:C) **(G ~ I)** (25 μg/mL) and harvested independently at indicated time points post-infection. Total RNA was extracted and MAVS transcript was analyzed by qPCR as mentioned in methods. The number above the bar represents the average MAVS mRNA level.

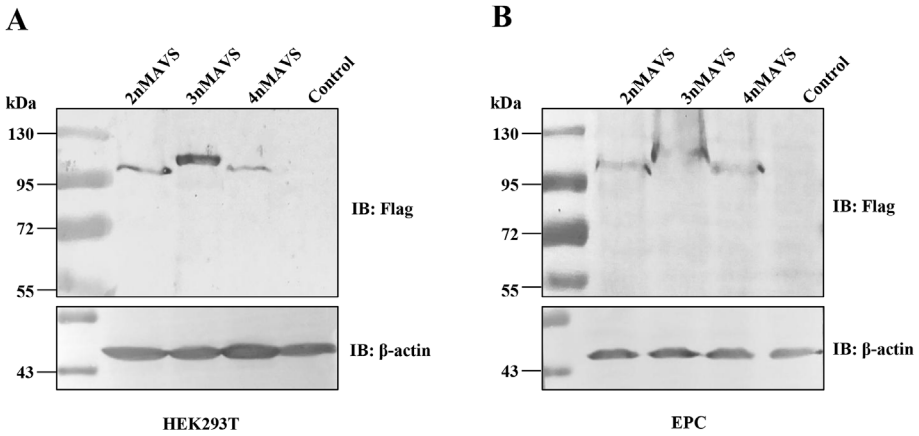

Fig. 4. Protein expression of 2nMAVS, 3nMAVS and 4nMAVS.
HEK293T cells **(A)** or EPC cells **(B)** were transfected with pcDNA5/FRT/TO-Flag-2nMAVS, pcDNA5/FRT/TO-Flag-3nMAVS, pcDNA5/FRT/TO-Flag-4nMAVS or the empty vector separately. The cells were harvested at 48 h post-transfection and the whole cell lysate was used for immunoblot assay. Control: 293 T or EPC cells transfected with the empty vector; 2nMAVS: pcDNA5/FRT/TO-Flag-2nMAVS; 3nMAVS: pcDNA5/FRT/TO-Flag-3nMAVS; 4nMAVS: pcDNA5/FRT/TO-Flag-4nMAVS.

(GeneBank accession number: MK251474). The deduced 4nMAVS protein has a calculated molecular weight of 63.4 kDa and a pI of 5.13 (Supplementary Fig. 1Ã C).

Analysis of 2nMAVS, 3nMAVS and 4nMAVS amino acid sequences revealed that all of them possessed three characteristic domains: a CARD domain, a proline-rich domain and a transmembrane domain (Fig. 1A). It was interesting that there existed a higher sequence homology between 2nMAVS and 4nMAVS (99.3%); however 3nMAVS exhibited lower identity with either 2nMAVS (71.3%) or 4nMAVS (70.9%) (Table 2). Through tertiary structure prediction, it was found that 2nMAVS showed the high structure similarity with 4nMAVS; however, the spatial structure of 3nMAVS was different with that of 2nMAVS or 4nMAVS (Fig. 1B ~ D). To further gain insights into MAVS evolution, amino acid sequences of 2nMAVS, 3nMAVS and 4nMAVS

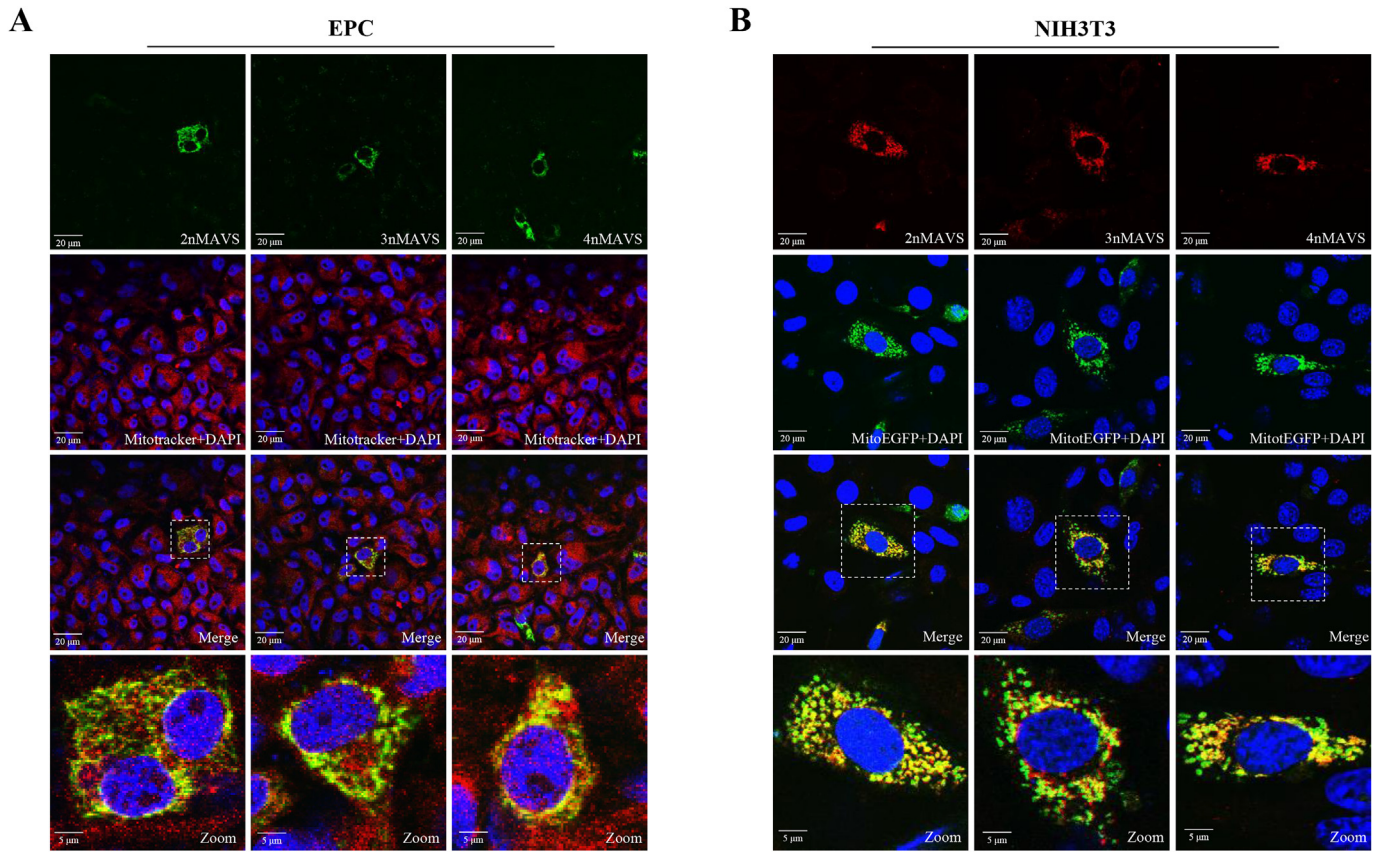

Fig. 5. Subcellular distribution of 2nMAVS, 3nMAVS and 4nMAVS.
(A) EPC cells were transfected with 2nMAVS/3nMAVS/4nMAVS and the transfected cells were stained with Mitotracker before used for immunofluorescence staining. **(B)** NIH3T3 cells were co-transfected with 2nMAVS/3nMAVS/4nMAVS and Mito-EGFP and used for immunofluorescence staining. 2nMAVS: pcDNA5/FRT/TO-HA-2nMAVS; 3nMAVS: pcDNA5/FRT/TO-HA-3nMAVS; 4nMAVS: pcDNA5/FRT/TO-HA-4nMAVS.

were subjected to multiple alignments with those of MAVS from other fishes and vertebrates. Phylogenetic analysis demonstrated that the selected MAVS homologues were classified into three groups: fish, aves and mammals. 2nMAVS, 3nMAVS and 4nMAVS were clustered among the fish MAVS group. Particularly, 2nMAVS was clustered closely with 4nMAVS, while 3nMAVS was clustered together with MAVS of common carp (*Cyprinus carpio*) (Fig. 2).

3.2. MAVS expression ex vivo in response to different stimuli

To investigate the transcription profiles of 2nMAVS, 3nMAVS and 4nMAVS during innate immune response, 2nFC, 3nFC and 4nFC were infected with SVCV (0.003 MOI) or GCRV (0.003 MOI) or treated with poly (I:C) (25 μg/mL) respectively and MAVS transcription post-infection were examined by q-PCR. In SVCV infected cells, 2nMAVS, 3nMAVS and 4nMAVS mRNA level increased from 2 h post-infection (hpi) (Fig. 3Ã C). The highest relative mRNA levels of 2nMAVS and 3nMAVS were 9.5-fold of the control and 13.5-fold of the control at 48 hpi respectively, while the 4nMAVS mRNA level reached its maximum (8.2 folds) at 24 hpi followed by a decline of mRNA level within 48 hpi. In GCRV infected group, the trends of 2nMAVS and 3nMAVS mRNA level in host cells were similar to those of SVCV group. The highest relative 2nMAVS mRNA level (48h point) was 6.6-fold of the control and the highest relative 3nMAVS mRNA level (48h point) was 11.4-fold of the control (Fig. 3D&E). 4nMAVS mRNA level increased immediately after GCRV infection and decreased slightly; then increased again from 12 hpi to reach its highest level (5.3 folds) at 48 hpi (Fig. 3F). In poly (I:C) treated group, 2nMAVS and 4nMAVS mRNA level were not obviously undulated (Fig. 3G&I). However, 3nMAVS transcription in host cells was obviously increased right after poly (I:C) stimulation and the maximum relative mRNA level of 3nMAVS (12h point) was 5.8-fold of the control (Fig. 3H). The q-PCR analysis results demonstrated 3nMAVS transcription increase rate in response to viral infection was obviously higher than 2nMAVS or 4nMAVS.

3.3. Protein expression and intracellular distribution of 2nMAVS, 3nMAVS and 4nMAVS

Both HEK293T cells and EPC cells were transfected with plasmids expressing 2nMAVS/3nMAVS/4nMAVS or the empty vector separately and used for immunoblot assay to investigate the protein expression of 2nMAVS, 3nMAVS and 4nMAVS. The specific bands of ~100 kDa were detected in the lane of 2nMAVS and 4nMAVS. Meanwhile, a band of ~110 kDa was detected in the lane of 3nMAVS (Fig. 4A&B). The different migration of 2nMAVS, 3nMAVS and 4nMAVS were probably related to their different amino acid sequences. The immunoblot data showed that the molecular weight of 2nMAVS (~100 kDa), 3nMAVS (~110 kDa) or 4nMAVS (~100 kDa) was much higher than the predicted molecular weight of them (63.6 kDa for 2nMAVS; 62.6 kDa for 3nMAVS; 63.4 kDa for 4nMAVS), which was seen the immunoblot assay of black carp MAVS [23]. The increased molecular weight of 2nMAVS/3nMAVS/4nMAVS and black carp MAVS might be explained by the proline-rich domain of these proteins [28].

To further examine the subcellular distributions of 2nMAVS, 3nMAVS and 4nMAVS, EPC cells were transfected with plasmids expressing 2nMAVS/3nMAVS/4nMAVS and used for immunofluorescence staining (IF). The IF data showed that green fluorescence representing 2nMAVS, 3nMAVS or 4nMAVS was co-localized with the red fluorescence (Mitotracker), which demonstrated that 2nMAVS, 3nMAVS and 4nMAVS were located on the mitochondria (Fig. 5A). Similar

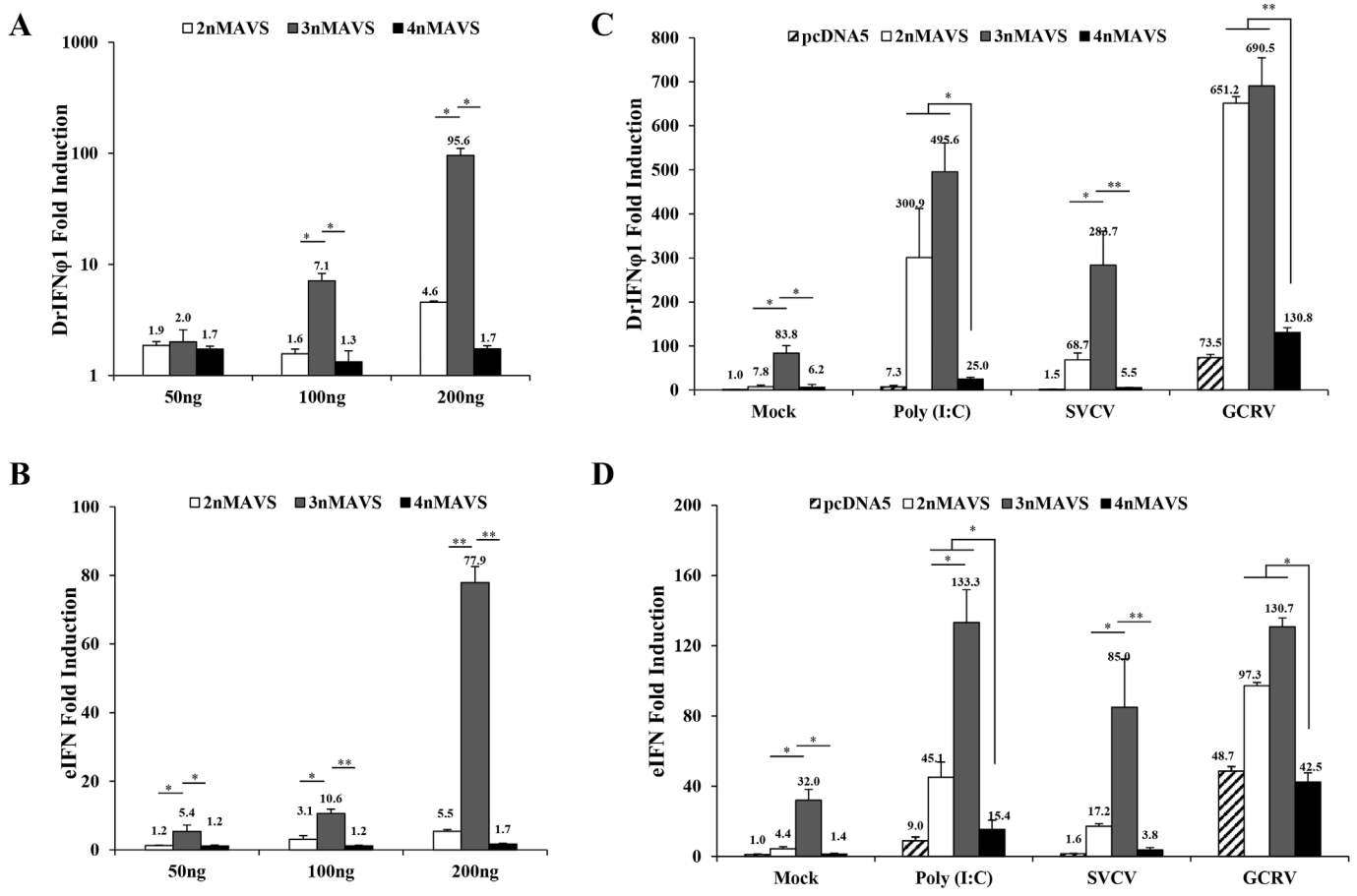

Fig. 6. IFN-inducing abilities of 2nMAVS, 3nMAVS and 4nMAVS.
(A) Zebrafish IFN1 (DrIFNφ1) promoter activity induced by 2nMAVS, 3nMAVS and 4nMAVS. **(B)** Fathead minnow IFN (eIFN) promoter activity induced by 2nMAVS, 3nMAVS and 4nMAVS. **(C&D)** EPC cells were incubated with poly (I:C) (25 mg/mL) or infected with SVCV (MOI 0.1) or GCRV (MOI 0.1) at 24h post-transfection, then cells were harvested at 24h post-stimulation and the luciferase activity was measured. 2nMAVS: pcDNA5/FRT/TO-Flag-2nMAVS; 3nMAVS: pcDNA5/FRT/TO-Flag-3nMAVS; 4nMAVS: pcDNA5/FRT/TO-Flag-4nMAVS. Mock: EPC cells without stimulation. The numbers above the error bars stand for the average IFN fold induction.

phenomena were seen in the IF of NIH3T3 cells, in which the red fluorescence representing 2nMAVS, 3nMAVS or 4nMAVS was clearly merged with the green fluorescence (Mito-EGFP) (Fig. 5B). These data collectively identified that 2nMAVS, 3nMAVS and 4nMAVS were mitochondrial proteins.

3.4. IFN-inducing activities of 2nMAVS, 3nMAVS and 4nMAVS

To investigate whether 2nMAVS, 3nMAVS or 4nMAVS were involved in type I IFN production or not, EPC cells were transfected with plasmids expressing 2nMAVS, 3nMAVS or 4nMAVS separately at different doses and used for luciferase reporter assay. The luciferase reporter assay results showed that overexpression of 2nMAVS and 3nMAVS activated both DrIFNφ1 and eIFN promoter transcription. Notably, the promoter induction activity of 3nMAVS was much higher than that of 2nMAVS. However, overexpression of 4nMAVS showed little effect on the induction of neither DrIFNφ1 nor eIFN promoter activity (Fig. 6A&B). To clear up the interferon promoter induction activities of 2nMAVS, 3nMAVS and 4nMAVS under different stimulation conditions, EPC cells transfected with the above plasmids were stimulated with poly (I:C), or infected with SVCV or GCRV and used for luciferase reporter assay. The results showed that the induced IFN transcription by 2nMAVS and 3nMAVS were enhanced in response to poly (I:C), SVCV and GCRV stimulation. The interferon-inducing activity of 3nMAVS was much higher than that of 2nMAVS; however, 4nMAVS only moderately increased DrIFNφ1 and eIFN promoter activity upon poly (I:C) stimulation or SVCV infection (Fig. 6C&D). Together, these data indicated that 3nMAVS exhibited much stronger IFN-inducing ability than 2nMAVS and 4nMAVS, whether upon viral stimulation or not.

3.5. Antiviral activities of 2nMAVS, 3nMAVS and 4nMAVS

To investigate the roles of 2nMAVS, 3nMAVS and 4nMAVS in the antiviral innate immune response, EPC cells were transfected with plasmids expressing 2nMAVS, 3nMAVS, 4nMAVS or the empty vector separately at 24 h before SVCV or GCRV infection. The crystal violet staining assay showed that the cytopathic effect (CPE) ratio of the EPC cells overexpressing 2nMAVS or 3nMAVS was obviously decreased compared with the control (Fig. 7A). In addition, the viral titer in the media of 3nMAVS-expressing cells presented the biggest reduction to that in other transfected cells (Fig. 7B). Similar results were observed in GCRV infected group, both the CPE ratio and the viral titer in the supernatant media of the EPC cells expressing 3nMAVS were the lowest (Fig. 7C&D). Our data showed clearly that 3nMAVS was a potent antiviral protein, which induced the strongest antiviral signaling against both SVCV and GCRV among these three MAVS homologues.

4. Discussion

Disease has been a major threaten to the aquaculture industry, which make the strong disease resistance to be one of the most

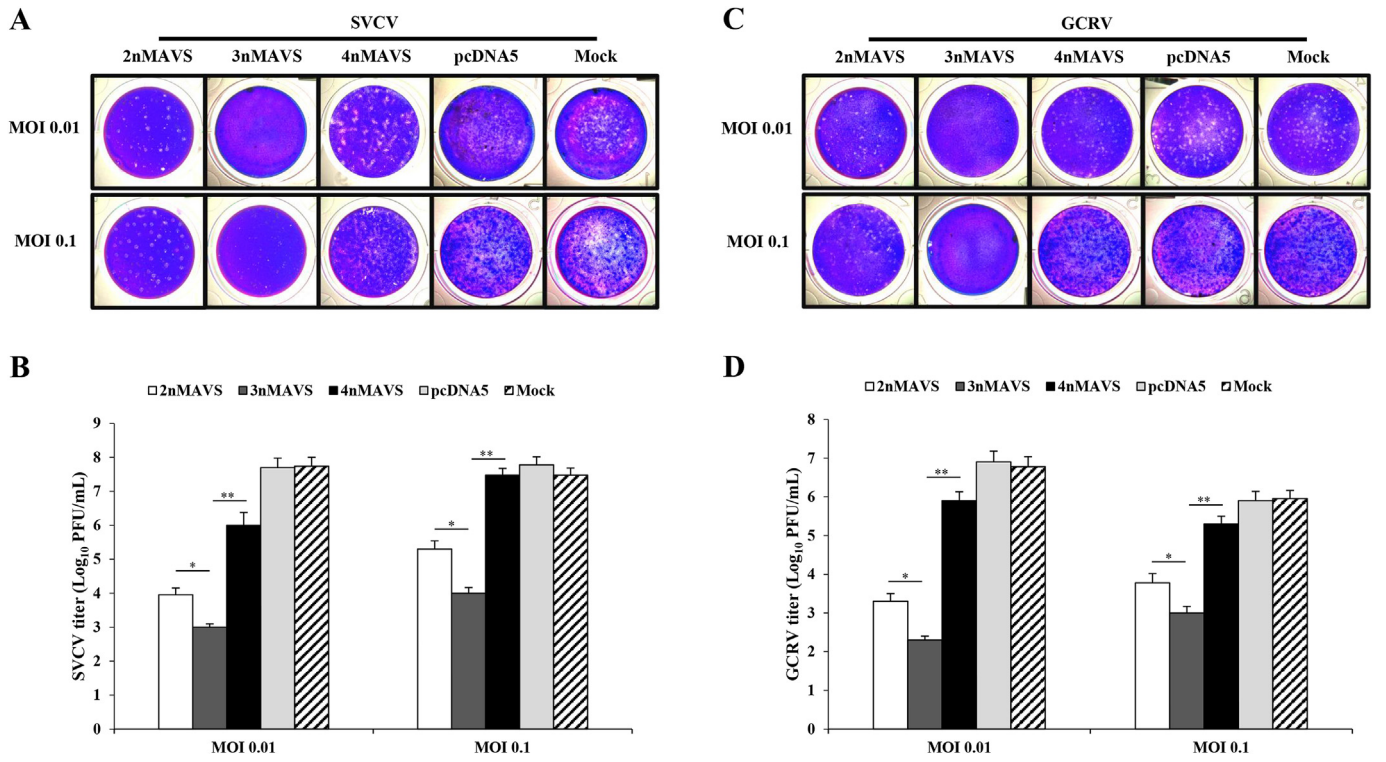

Fig. 7. Antiviral activities of 2nMAVS, 3nMAVS and 4nMAVS.
EPC cells in 24-well plate were transfected with the plasmids expressing 2nMAVS/3nMAVS/4nMAVS or the empty vector separately, and infected with SVCV or GCRV at indicated MOI at 24 h post-transfection. The cell monolayers were stained with crystal violet **(A&C)** and the virus titers in the supernatant media were determined by plaque assay at 24h post-infection **(B&D)**. 2nMAVS: pcDNA5/FRT/TO-Flag-2nMAVS; 3nMAVS: pcDNA5/FRT/TO-Flag-3nMAVS; 4nMAVS: pcDNA5/FRT/TO-Flag-4nMAVS; pcDNA5: pcDNA5/FRT/TO; Mock: EPC cells without transfection.

important traits of economic aquaculture species. Thus, to develop aquaculture species with improved disease resistance is crucial for the aquaculture industry, which can be reached through different ways, such as hybridization [1,2,29,30]. Distant hybridization combines the genomes of different species and leads to the changes of the offspring in phenotypes and genotypes [9,10]. Triploid hybrids have been developed through the interspecies crossing between female diploid red crucian carp and male allotraploid, which have boosted their market in China by the improved merits: fast growing, good flavor, strong disease resistance and stress resistance [6].

The disease resistances of teleosts with different ploidy have been analyzed in numbers of studies, in which several reports suggested that both diploid and triploid possessed equal disease resistance. For example, triploid rainbow trout (*Oncorhynchus mykiss*) showed similar immune response in saltwater adaptation to diploids [31]. The activities of the humoral components of the innate immune system tested were similar in diploid and triploid turbot (*Scophthalmus maximus* L) [32]. However, other studies demonstrated that triploid fish presented improved immunity compared with its diploid counterpart. For instance, triploid Amazon molly (*Poecilia formosa*) possessed more type I MHC alleles than diploid fish [33]. Triploid cell lines of *Misgurnus anguillicaudatus* had higher intracellular content of detoxification enzymes than their diploid counterparts [34]. The triploid hybrids of rainbow trout × coho salmon showed a significant increase in IHNV resistance when compared to pure-species rainbow trout groups [35]. However, the molecular mechanisms underlying the immune system of these mentioned triploid species are largely unclear.

Since hybridization and ploidy changes occurred in the process of the formation of triploid hybrid of red crucian carp and allotraploid, the molecular basis of disease resistance heterosis in triploid hybrid could be complex. Polyploidy also show changes in gene expression, such as gene silencing, up or down-regulation of gene expression, subfunctionalization, and generation of new gene functions [36–39]. Therefore, triploid hybrid, compared with its parents, possessed reconstituted genome and different gene expression patterns, which was suggested to be a potential reason for its improved disease resistance. To explore the underlying mechanism, in our previous studies, 3nIFNa and 3nIFNb, which were two of the type I IFNs of triploid hybrid, have been cloned and characterized as antiviral cytokines against GCRV and SVCV separately [40,41]. However, the antiviral activities of these triploid hybrid IFNs had not been systematically compared with that of the IFNs from red crucian carp and allotetraploid. The subsequent study in this lab reported that 3nFC cells exhibited obviously stronger antiviral activity than 2nFC and 4nFC cells, in which the transcriptome analysis of these three cell lines revealed that RLR signaling pathway might be a key factor for the improved antiviral ability of 3nFC cells [11]. As a key component of RLR signaling, MAVS was chosen to investigate the “probably improved” RLR signaling of triploid hybrid and the data generated in this study demonstrated clearly that 3nMAVS actually “obtained” the stronger antiviral activity than the counterparts of its parents.

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Appendix A. Supplementary data

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