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The antiviral signaling mediated by black carp MDA5 is positively regulated by LGP2

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ABSTRACT

Melanoma differentiation-associated gene 5 (MDA5) belongs to RIG-I like receptor (RLR) family, which detects cytosolic viral RNA component in immune response. In this study, MDA5 orthologue of black carp (*Mylopharyngodon piceus*) has been cloned and characterized. The full-length cDNA of black carp MDA5 (bcMDA5) comprises 3244 nucleotides and the predicted bcMDA5 protein contains 984 amino acids. The constitutive transcription of bcMDA5 was extremely low in all the tested tissues, which included gill, skin, muscle, intestine, kidney, spleen, liver and heart. However, bcMDA5 mRNA level was much enhanced in most selected tissues in response to GCRV or SVCV infection. bcMDA5 migrated around 120 KDa in immunoblot and was identified as a cytosolic protein by immunofluorescent staining in both EPC and HeLa cells. Expressing bcMDA5 in EPC cells resulted in the induction of promoter activity of zebrafish IFN3 or fathead minnow IFN. The EPC cells expressing bcMDA5 obtained improved antiviral ability against both SVCV and GCRV. When EPC cells were co-transfected with plasmids expressing bcMDA5 and bcLGP2, the induced IFN expression by bcMDA5 was obviously enhanced. EPC cells expressing both bcMDA5 and bcLGP2 owned much improved antiviral ability than those cells expressing only bcMDA5 or bcLGP2. In general, our data support the conclusion that bcMDA5 plays an important role in the antiviral innate immune response of black carp and bcLGP2 acts as a positive regulator in bcMDA5 mediated signaling.

1. Introduction

Vertebrates utilize immunity to survive them from disadvantaged environment such as viral infection, which is classified into innate immunity and adaptive immunity [1–4]. As to the innate immune system, the invading pathogen microbes are recognized by pattern recognition receptors (PRRs), which include toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), nucleotide oligomerization domain-like receptors (NLRs), C-type lectins (CTLs), AIM2-like receptors (ALRs) and OAS-like receptors in mammals [5]. Interferons (IFNs) are subsequently induce-expressed and activate the transcription of interferon stimulated genes (ISGs) such as viperin, Mx1 and ISG15, which finally initiate host innate immune response [6–8].

RLRs are pivotal cytosolic viral sensors and composed of three major members: retinoic acid-inducible gene I (RIG-I), MDA5 and laboratory of genetics and physiology 2 (LGP2). All these three RLR members share homologous core structural domains, which include a DExD/H box helicase domain, a helicase C-terminal domain and C-terminal domain (CTD). However, caspase recruitment domain (CARD) was found in both RIG-I and MDA5, but not in LGP2 [9]. Both RIG-I and MDA5 possess the ability to induce host antiviral response (via CARD) upon recognition of viral RNA components. RIG-I owns high affinity with short dsRNA (<1 kb) or 5'-ppp uncapped ssRNA, while MDA5 preferentially binds long (>2 kb), capped di- or mono-5'phosphate dsRNA [10,11]. LGP2 lacks the ability to induce signaling alone (due to the absence of CARD), but has been found to be necessary for effective RIG-I/MDA5-mediated antiviral signaling [12].

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MDA5 was discovered first time in human melanoma cells in 2002 as an interferon-inducible putative RNA helicase with dsRNA-dependent ATPase activity and melanoma growth-suppressive properties [13]. After recognition of viral RNA components, RIG-I and MDA5 interact with and activate mitochondrial antiviral signaling protein (MAVS) through CARD, which initiates the activation of IRF3 and NF-κB through different signaling [14]. The activated transcription factors IRF3 and NF-κB translocate into the nucleus and collaboratively trigger the expression of type I IFNs [9]. However, due to the absence of CARD, the role of LGP2 in mediating an antiviral response is contradictory. Some studies showed that LGP2 was unable to interact with MAVS and hence might serve as a negative mediator for RIG-I/MDA5-activated antiviral signaling [15,16]. On contrary, positive regulatory roles of LGP2 in RIG-I/MDA5-activated antiviral signaling have also been reported in LGP2-deficient mice, which lost the ability to synthesize type I IFNs and were unable to mount efficient antiviral responses against encephalomyocarditis virus infection (EMCV) [17]. Further evidence was provided that the ATPase domain of LGP2 was required for its positive regulatory role in mediating RIG-I/MDA5-dependent antiviral response [18].

Fish RLR signaling have been studied extensively in these years and many teleost homologs of RLRs have been characterized in many species, such as zebrafish (*Danio rerio*/RIG-I, MDA5, LGP2) [19–21], grass carp (*Ctenopharyngodon idella*/MDA5, LGP2, RIG-I) [22–24], Japanese flounder (*Paralichthys olivaceus*/MDA5, LGP2) [25,26], rainbow trout (*Oncorhynchus mykiss*/MDA5, LGP2) [12] and sea perch (*Lateolabrax japonicas*/LGP2, MDA5) [27,28]. However, the regulation of MDA5-mediated signaling by LGP2 remains largely unknown in teleost fish immunity.

Black carp (*Mylopharyngodon piceus*) is one of the "four famous domestic fishes" in china. Black carp is subjected to bulk of pathogenic microorganisms, such as grass carp reovirus (GCRV) and spring viremia of carp virus (SVCV), however, the immunity of this cyprinid fish remains much unknown. To elucidate the RLR signaling in the innate immune response of this economical important species, LGP2 and MAVS of black carp (bcLGP2 and bcMAVS accordingly) have been characterized in our previous study [29,30]. EPC cells expressing bcLGP2 owned obviously enhanced antiviral activity when the cells were infected with SVCV or GCRV at low dose (MOI≤0.1). To investigate the mechanism behind how bcMAVS was activated by upstream PRRs, black carp MDA5 (bcMDA5) has been cloned and characterized in this paper, which showed strong IFN-inducing activity and antiviral ability against both SVCV and GCRV. Although bcLGP2 possessed little effect on IFN induction during the reporter assay, the signaling and antiviral activity mediated by bcMDA5 was obviously up-regulated by bcLGP2. Thus our data identified that bcLGP2 was a positive regulator of bcMDA5 in host innate immune response against viral infection.

2. Materials and methods

2.1. Cells and plasmids

HEK293T, HeLa, MPF (*Mylopharyngodon piceus* fin), CIK (*C. idella kidney*) and EPC (*epithelioma papulosum cyprini*) cells were kept in the lab [31]. HEK293T and HeLa cells were cultured at 37 °C; CIK, EPC and MPF cells were cultured at 25 °C. All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cell transfection was done as previously described, calcium phosphate was used for 293T transfection, Lipofectamine®2000 (Invitrogen) was used for HeLa and Fugene 6 (Roche) was used for EPC [30].

pcDNA5/FRT/TO and pcDNA5/FRT/TO-HA were kept in the lab

Table 1
Primers used in the study.

Primer name	Sequence(5′-3′)	Ampcicon length (nt) and primer information
bcMDA5-W1	CTGGACTGTTTCAGAGACAGACT	2739
bcMDA5-W2	TCCARGAGTCKCTCCTGTAGTG	Gene cloning
3'race		
bcMDA5-F2	ACCGAGCAGTTTAGTAAGTTGTT	3′ UTR 1st PCR
bcMDA5-F3	TTGTTTACAGTCCGAGAGAATACG	3′ UTR 2nd PCR
UPM-longer	CTAATACGACTCACTATAGGGCAAGC AGTGGTATCAACGCAGAGT	
UPM-Short primer	CTAATACGACTCACTATAGGGC	
ORF		
bcMDA5-O-F	ATGAGTAGYGATMAGGACGCCGA	2955
bcMDA5-O-R	TCAATCAAAGTCCATGTCTTCCTC	ORF cloning-reverse
5'race		
bcMDA5-A2	TGTCATTCTCGGCCTCTTCTGTGGG	5′ UTR 1st PCR
bcMDA5-A3	GTTTGGCTCTTATTCGGTCCTTTCG	5′ UTR 2nd PCR
5'race out primer	CATGGCTACATGCTGACAGCCTA	
5'race inner primer	CGCGGATCCACAGCCTACTGATG ATCAGTCGATG	
Expression construct		
bcMDA5-N-F1	ACTGACGGTACCGCCACCATGAGTAGTGATAAGG	
bcMDA5-N-R1	ACTGACGGGCCCTCAATCAAAGTCCATG	FRT-To-HA-bcMDA5
bcMDA5-C-F1	ACTGACGGTACCGCCACCATGAGTAGTGATAAGG	
bcMDA5-C-R4	ACTGACCGGCGGCCGCATCAAAGTCCATG	FRT-TO-bcMDA5-HA
q-PCR		
Fish actin F	CATGTTCGAGACCTT	
Fish actin R	AGGCAGCTCATAGCT	*In vivo* Semi-q-PCR
bcMDA5-Semi-F	GTGTAATGTCGTCATACGCTACTGC	
bcMDA5-Semi-R	CCACAAAGTTCTTGATATGGAGGC	*In vivo* Semi-q-PCR
bc Q actin-F	TGGGCACCGCTGCTTCCT	
bc Q actin-R	TGTCCGTCAGGCAGCTCAT	*Ex vivo* q-PCR
bcMDA5-Q-F2	TCGTCATACGCTACTGCCTTGT	
bcMDA5-Q-R2	GGCTTTGCTCATTTTCTCC	*Ex vivo* q-PCR

[31]. The recombinant expression vector pcDNA5/FRT/TO-HA-bcMDA5 was constructed by cloning the open reading frame (ORF) of bcMDA5 fused with an HA tag at its N-terminus into pcDNA5/FRT/TO. pRL-TK, Luci-zIFN3 (for zebrafish interferon-3 promoter activity analysis) and Luci-eIFN (for fathead minnow interferon promoter activity analysis) were kept in the lab [29].

2.2. Cloning the cDNA of bcMDA5

Degenerate Primers (Table 1) were designed to amplify the cDNA of bcMDA5 based on the sequences of MDA5 of *Carassius auratus* (JF970226.1), *C. idella* (JN986720.1), *Cyprinus carpio* (KM374815.1), *Danio. rerio* (NM001308563.1), *Homo sapiens* (AF095844.1). Total RNA was isolated from the spleen of black carp 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 bcMDA5 cDNA by using the 5′Full RACE Kit and the 3′Full RACE kite separately (TaKaRa). The full-length cDNA of bcMDA5 was cloned into pMD18-T vector and sequenced by Invitrogen.

2.3. Virus produce and titration

SVCV and GCRV were kept in the lab and propagated in EPC or CIK separately at 25 °C in the presence of 2% fetal bovine serum. Virus titers were determined by plaque assay on EPC cells as previously described [30,31]. Briefly, the 10-fold serially diluted virus supernatants were added onto EPC cells and incubated for 2 h at 25 °C. The supernatant was replaced with DMEM containing 2% FBS and 0.75% methylcellulose (Sigma) after incubation. Plaques were counted at day 3 post-infection.

2.4. LPS and poly (I:C) treatment

MPF cells were seeded in 6-well plate (2×10^6 cells/well) 16 h before treatment. Poly(I:C) (Sigma) was used for synthetic dsRNA stimulation, which was heated to 55 °C (in PBS) for 5min and cooled at room temperature before use. MPF cells were replaced with 1 ml fresh media containing poly (I:C) and harvested at different time points post treatment. bcMDA5 mRNA level in the MPF cells was determined by quantitative real-time PCR (q-PCR). For LPS treatment, MPF cells in 6-well plate (2×10^6 cells/well) were treated with LPS and harvested for q-PCR at different time point post stimulation as above.

2.5. Semi-quantitative RT-PCR

Black carp of six months (weight of 120 g) were injected intraperitoneally with GCRV (2.52×10^6 pfu/fish), SVCV (2.43×10^6 pfu/fish) or sterile PBS (as healthy control) separately and cultured at 25 °C. Three fishes were collected for each injected group (PBS, SVCV or GCRV). The injected black carp were sacrificed at 33 h post injection and total RNA was isolated from tissues of heart, liver, spleen, kidney, intestine, muscle, skin and gill independently. The primers of bcMDA5-Semi-F and bcMDA5-Semi-R were used to detect bcMDA5 mRNA in the above tissues (Table 1); and the RT-PCR for β-actin was performed as the internal control. The semi-quantitative RT-PCR program was: 95 °C for 5 min, then 28 cycles

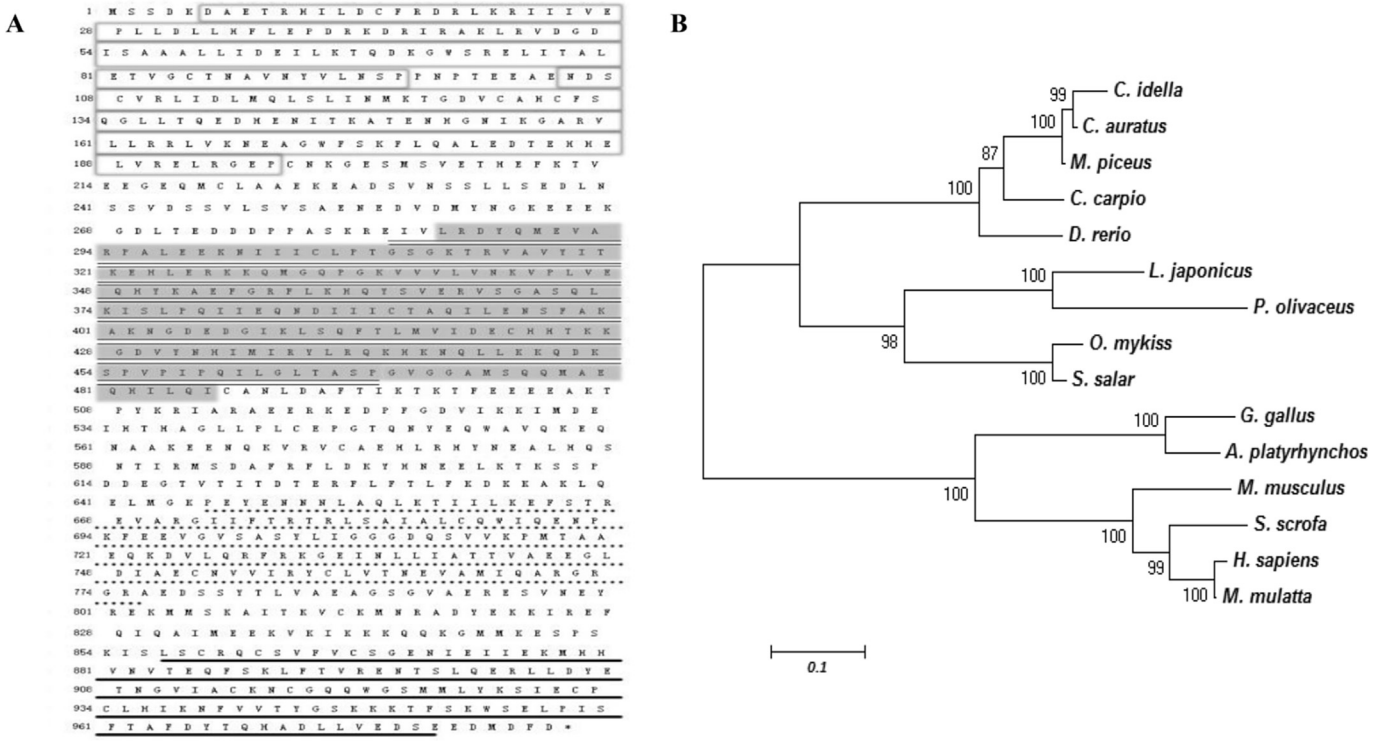

Fig. 1. The evolution study of bcMDA5. A).The predicted bcMDA5 protein. The CARD domains are boxed (6-96,105-196aa); ResIII domain is under double line (282-468aa); DEXDc domain is indicated as shaded residues (285-486aa). HELICc domain is marked with dots (647-776aa); RD domain is under bold line (857-968aa). B). phylogenetic tree of vertebrate MDA5. Maximum likelihood phylogenetic tree has been generated from vertebrate MDA5 of different species by using MEGA 6.0 program, which include (GenBank accession number, unless indicated otherwise): *C. auratus* (JF970226.1), *C. idella* (JN986720.1), *M. piceus* (KX871189), *C.carpio* (KM374815.1), *Lateolabrax japonicus* (KU317137.1), *Macaca mulatta* (DQ875603.1), *D.rerio* (NM001308563.1), *O. mykiss* (NM001195179.1), *Gallus gallus* (AB371640.1), *H.sapiens* (AF095844.1), *Salmo salar* (KU376486.1), *P.olivaceus* (HQ401014.1), *Sus scrofa* (EU006039.1), *Anus platyrhynchos* (KJ451070.1), *Mus musculus* (NM_027835.3).The bar stands for scale length and the numbers on different nodes stand for bootstrap value.

of 95 °C/30 s, 55 °C/30 s and 72 °C/1 min, finally extension at 72 °C for 8 min.

2.6. Quantitative real-time PCR

Quantitative real-time PCR (q-PCR) was performed to quantify the bcMDA5 mRNA expression in MPF cells. The primers for q-PCR of bcMDA5 were bcMDA5-Q-F2 and bcMDA5-Q-R2 and the primers for β-actin q-PCR were bc-Q-actin-F and bc-Q-actin-R (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.

2.7. Luciferase reporter assay

EPC cells in 24-well plate were co-transfected with pcDNA5/FRT/TO-HA-bcMDA5, pRL-TK, Luci-zIFN3 or Luci-eIFN separately. For each transfection, the total amount of plasmid was balanced with the empty vector pcDNA5/FRT-TO-HA. 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 manufacture (Promega) as previously [29].

2.8. Immunoblotting

HEK293T cells were transfected with pcDNA5/FRT-TO-HA-bcMDA5 or the empty vector separately. Transfected cells were harvested at 48 h post-transfection and lysed for immunoblot (IB) assay as previously described [30]. Briefly, whole cell lysates were isolated by 10% SDS-PAGE and the transferred membrane was probed with mouse monoclonal anti-HA antibody (1:3000; Sigma). Target proteins were visualized through BCIP/NBT Alkaline Phosphatase Color Development Kit (Sigma).

2.9. Immunofluorescence microscopy

HeLa cells or EPC cells were transfected with pcDNA5/FRT/TO-HA-bcMDA5 or the empty vector separately. Transfected HeLa and EPC 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 immunofluorescent staining as previously described [30]. Mouse monoclonal anti-HA antibody (Sigma) was probed at the ratio of 1:200 and Alexa 488-conjugated secondary antibody (Invitrogen) was probed at the ratio of 1:400; DAPI were used to stain the nucleus.

2.10. Statistics analysis

For the statistics analysis of the data of q-PCR, luciferase reporter assay and viral titer measurement, all data were obtained from three independent experiments with each performed in triplicate. Error bars represent the standard error of the mean (±SEM) of three independent experiments. * stands for $p < 0.05$ and ** stands for $p < 0.01$. The data were analyzed by two-tailed Student's t-test.

3. Results

3.1. Molecular cloning of the cDNA of bcMDA5

To study the role of MDA5 in the antiviral immune response of black carp, the cDNA of MDA5 orthologue has been cloned from the spleen of black carp. The full-length cDNA of bcMDA5 consists of 3244 nucleotides including 5′UTR, coding sequence, 3′UTR and poly (A) tail (NCBI accession number:KX871189). The open reading frame (ORF) of bcMDA5 cDNA is 2955 bp, which commences at nucleotide of 108 and terminates at nucleotide of 3062. Initial cDNA sequence analysis of bcMDA5 (http://expasy.org/tools/protparam.htm) predicts that bcMDA5 contains 984 amino acid residues (aa). The deduced bcMDA5 protein has a calculated molecular weight of 111.8 KDa and an isoelectric of 5.69. bcMDA5 contains six structure domains: two N-terminnal CARD, a DEXDc (DEAD/DEAH box helicase domain), a Res III (conserved restriction domain of bacterial type III restriction enzyme), a HELICc (helicase superfamily C-terminal domain) and a C-terminal RD (regulatory

Table 2
Comparison of bcMDA5 with other vertebrate MDA5 (%).

Species	Full-length sequence of protein	
	Identity similarity	
M. piceus	100.0	100.0
C. auratus	98.8	99.4
C. idella	93.0	94.0
C. carpio	86.0	90.7
D. rerio	83.2	89.8
L. japonicus	58.3	72.7
O. mykiss	62.4	75.3
S. salar	63.0	75.7
P. olivaceus	55.4	69.5
G. gallus	47.3	65.2
A. platyrhynchos	48.5	66.3
M. musculus	47.2	63.0
Sus scrofa	47.8	65.0
H. sapiens	47.7	63.7
M. mulatta	48.0	64.2

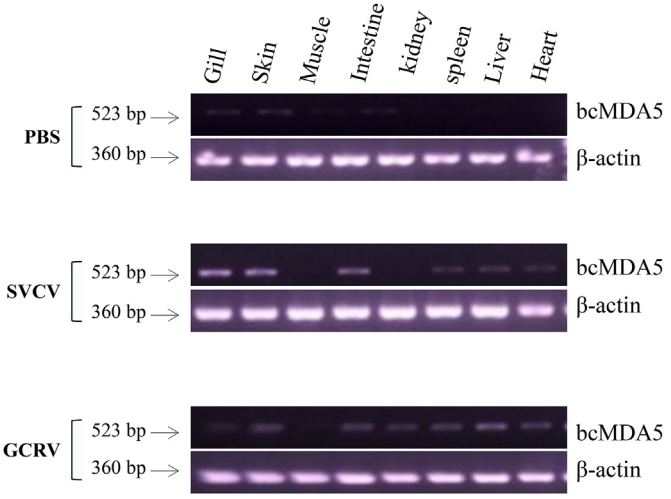

Fig. 2. Tissue specific mRNA expression of bcMDA5. Black carps were injected with GCRV, SVCV or PBS separately and total RNA was isolated from the indicated tissues independently according to the methods. Three injected (PBS, GCRV or SVCV) fishes were collected for one group; three total RNA samples of each group for each tissue were combined and used for cDNA synthesis. β-actin was recruited as an internal control; the transcription of bcMDA5 after virus challenge or in healthy condition (PBS injection) was detected by RT-PCR separately.

domain). (Fig. 1 A).

To study the evolution of bcMDA5, the protein sequence of bcMDA5 was subjected to multiple alignments with those of MDA5 proteins from different species (Table 2). Phylogenetic analysis of MDA5 from the selected species demonstrates that these homologue proteins could be divided into three groups, consisting of mammalian, fish and birds branches (Fig. 1 B & Supplementary Fig. 1). bcMDA5 shares high protein sequence similarity with grass carp MDA5 (94%) and gold fish MDA5 (99.4%) and is clustered tightly with grass carp and gold fish MDA5, which correlates with the closest genetic relationship of these cyprinid fishes (Fig. 1 B).

3.2. The mRNA expression of bcMDA5 in vivo

To investigate the transcription of bcMDA5 *in vivo*, total RNA was extracted separately from heart, liver, spleen, kidney, intestine, muscle, skin and gill of the black carp injected with GCRV, SVCV or sterile PBS (as healthy control). bcMDA5 transcription in different tissues was examined by semi-quantitative RT-PCR, in which the RT-PCR of β-actin was recruited as the inside parameter. In the control group, an extremely faint band of 523 bp representing bcMDA5 could be detected in all selected tissues, which meant bcMDA5 transcription level was very low in these tissues under healthy condition (Fig. 2, **upper panel**). However, bcMDA5 mRNA level was obviously increased in most tested tissues except kidney and muscle after SVCV infection (Fig. 2, **middle panel**). Similar to that of SVCV infected group, bcMDA5 transcription was fiercely enhanced in most tested tissues except gill and muscle post GCRV infection (Fig. 2, **lower panel**). The data implies that bcMDA5 is involved in the host innate immune response initiated by both GCRV and SVCV infection.

3.3. bcMDA5 mRNA expression ex vivo in response to different stimulations

MPF cells were treated with LPS or poly (I:C) at different concentrations independently to analyze bcMDA5 mRNA variation in response to these stimulations. In the group of LPS treatment, bcMDA5 transcription level did not increase obviously during the first 24 h post stimulation for all doses compared with that without LPS stimulation. It was interesting that bcMDA5 mRNA level was decreased right after LPS treatment at all doses and was recovered at 24 h post stimulation (Fig. 3 A). However, after poly (I:C) stimulation, bcMDA5 mRNA level was immediately up-regulated and reached the peak value at 24 h post stimulation for all dose (Fig. 3 B). These data suggest that bcMDA5 is an important component of host defense mechanism responsible for virus infection; however, whether this teleost RLR member is involved in the recognition of bacteria invasion or not needs further investigation.

To depict bcMDA5 mRNA profile in host during viral infection, MPF cells were infected with SVCV or GCRV at various MOIs and harvested for q-PCR at different time points post infection. After challenge with GCRV, the highest mRNA level of bcMDA5 was up to 18.1 fold at 24 h in 1 MOI group. For 0.1 group, the bcMDA5 mRNA level was up-regulated at 24 h and reached its maximum (17.1 fold) at 48 h. However, bcMDA5 transcript level was hardly increased until 72 h post GCRV infection in 0.01 MOI group (Fig. 4 A & B). For SVCV infected MPF cells, bcMDA5 mRNA level increased immediately after viral infection (2 h) and reached the peak value at 72 h in both 0.1 MOI and 1 MOI group; however, the mRNA expression of bcMDA5 was fiercely up-regulated from 48 h in 0.01MOI group (Fig. 4 C & D). The q-PCR data showed that this fish RLR member was involved in the host innate immune response initiated by both SVCV and GCRV.

3.4. Protein expression and intracellular distribution of bcMDA5

HEK293T cells were transfected with pcDNA5/FRT-TO-HA-bcMDA5 or the empty vector separately to investigate the *ex vivo* expression of bcMDA5. A specific band of ~120 KDa was detected in lane of bcMDA5 and matched the predicted molecular weight of bcMDA5 (Fig. 5 A). The small weaker band might be the degraded

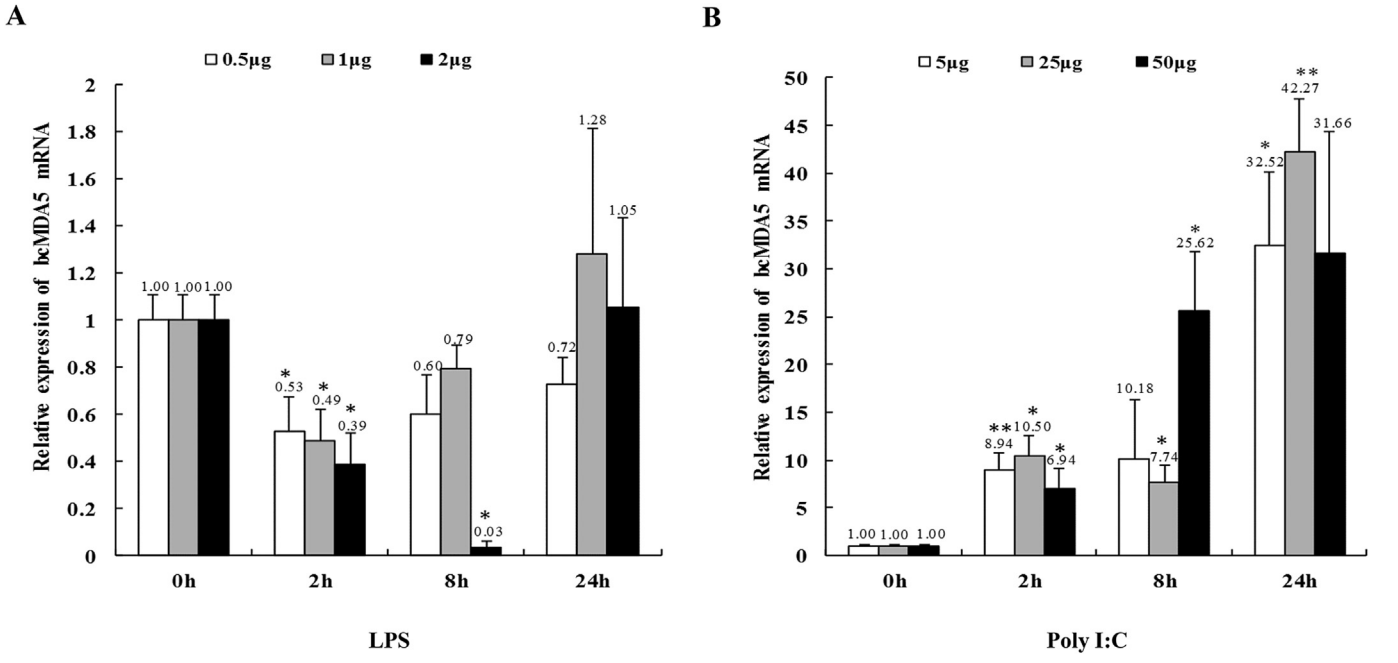

Fig. 3. bcMDA5 transcription in response to LPS or poly (I:C) stimulation. MPF cells in 6-well plate (2×10^6 cells/well) were treated with LPS or poly (I:C) at the indicated concentration separately. The cells were harvested at 2, 8 or 24 h post stimulation separately and used for RNA isolation. The relative bcMDA5 mRNA level was examined by q-PCR. Error bars represent the standard error of the mean (±SEM) of three independent experiments. Asterisks (*) on the pillar to mark the significant difference between experimental data and control data(*$p < 0.05$; **$p < 0.01$). The numbers above the error bars stand for average bcMDA5 mRNA level.

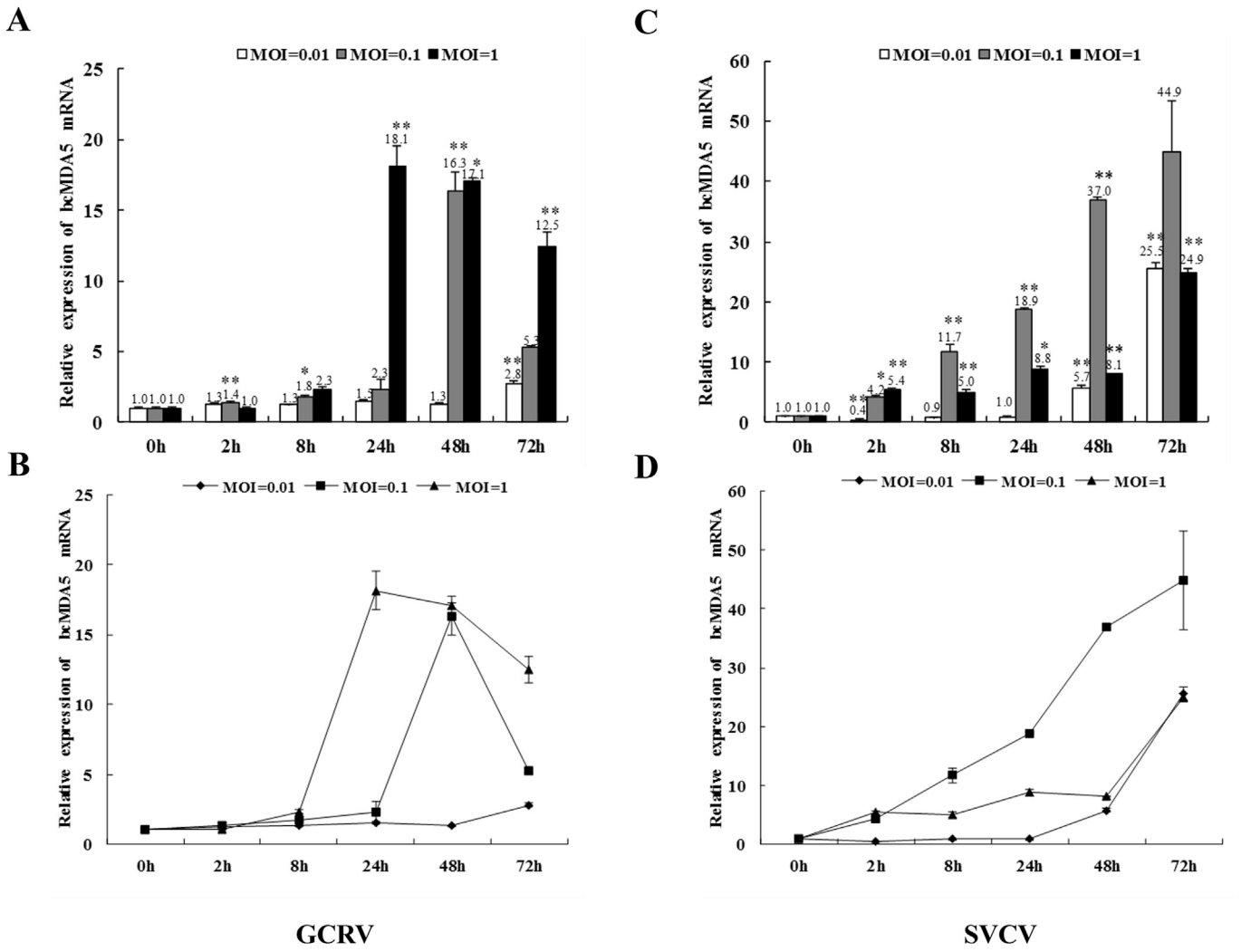

Fig. 4. bcMDA5 transcription in response to viral infection. MPF cells in 6-well plate (2×10^6 cells/well) were infected with SVCV and GCRV at the indicated MOI separately and the cells were harvested at the indicated time point post infection independently. bcMDA5 mRNA level was examined by q-PCR. (**A & C**): The bar chart of relative bcMDA5 mRNA level in MPF cells. (**B & D**): The trend chart of relative bcMDA5 mRNA level in MPF cells. Error bars represent the standard error of the mean (±SEM) of three independent experiments. Asterisks (*) on the pillar to mark the significant difference between experimental data and control data(*$p < 0.05$; **$p < 0.01$).The numbers above the error bars stand for average bcMDA5 mRNA level.

bcMDA5 and the larger faint band might be the modified form of this protein, which is most likely glycosylation. Actually, six asparagine (N) of bcMDA5 have been found in the conserved motif (N-X-S/T) for N-linked glycosylation (N_{98},N_{105},N_{144},N_{231},N_{882},N_{896}). It was interesting that we could not detect bcMDA5 through western blot in the whole cell lysate of HEK293T cells transfected with pcDNA5/FRT-TO-bcMDA5—HA (Supplementary Fig. 2), which implied that HA tag fusing at the C terminus suppressed the expression of this fish RLR member in 293T cells.

In the data of immunofluorescence (IF) staining of both EPC cells and HeLa cells, bcMDA5 expression region (green) surrounded the nucleus (blue), which demonstrated that bcMDA5was a cytosolic protein (Fig. 5 B). It is interesting that the subcellular location of bcMDA5 in HeLa cells was different with that in EPC cells. The distribution of bcMDA5 in EPC cells was most cytosolic area and that in HeLa cells was majorly beneath the cell membrane (Fig. 5 B).

3.5. Signaling and antiviral ability of bcMDA5

To investigate whether bcMDA5 could induce the IFN expression, EPC cells were transfected with pcDNA5/FRT/TO-HA-bcMDA5 at the dose of 50 ng, 100 ng or 200 ng respectively to study the signaling mediated by this fish RLR member. Over-expressing bcMDA5 led to the obvious transcription of the fathead minnow IFN in EPC cells, in which the highest fold induction level was up to 89 folds for 100 ng bcMDA5 transfection group (Fig. 6 A). Similarly, exogenous bcMDA5 in EPC cells strongly activated the transcription of zebrafish IFN3, in which the highest fold induction level was up to 112 folds for 100 ng bcMDA5 transfection group (Fig. 6 B).

To investigate the role of bcMDA5 in host antiviral immune response, EPC cells were transfected with pcDNA5-FRT-TO-HA-bcMDA5 and used for plaque assay. For GCRV infection, complete cytopathic effect (CPE) was found in control group at 24 h post infection (hpi) through crystal violet staining, however, EPC cells expressing bcMDA5 presented much reduced CPE. (Fig. 7 A). Consistently, viral titer in the supernatant media of EPC cells expressing bcMDA5 was much lower relative to the control group(Fig. 7 B). The data in SVCV infection group was similar to that of the GCRV group, both CPE rate and viral titer in the supernatant

media of the EPC cells transfected with pcDNA5/FRT/TO-HA-bcMDA5 were obviously decreased (Fig. 7 C, D). These data indicated that bcMDA5 mediated the host IFN signaling and functioned importantly in the host antiviral innate immunity.

3.6. Association of bcMDA5 and bcLGP2 in IFN signaling

EPC cells over-expressing bcLGP2 showed enhanced antiviral ability against both GCRV and SVCV, when the host cells were infected with virus at low MOI [30]. The reporter assay of this study demonstrated that bcLGP2 alone presented little ability to activate the expression of zebrafish IFN3 and eIFN, in which the highest induction level was less than 5 folds (Fig. 8). These data suggested that exogenous bcLGP2 in EPC cells associated with endogenous RIG-I or MDA5 to activate IFN signaling and initiate host antiviral innate response after viral infection. To determine if bcLGP2 associate with bcMDA5 and regulate bcMDA5 mediated signaling, EPC cells were co-transfected with pcDNA5-FRT-TO-HA-bcMDA5 and pcDNA5-FRT-TO-HA-bcLGP2 and applied to reporter assay. The reporter assay demonstrated that the fold induction of both zIFN3

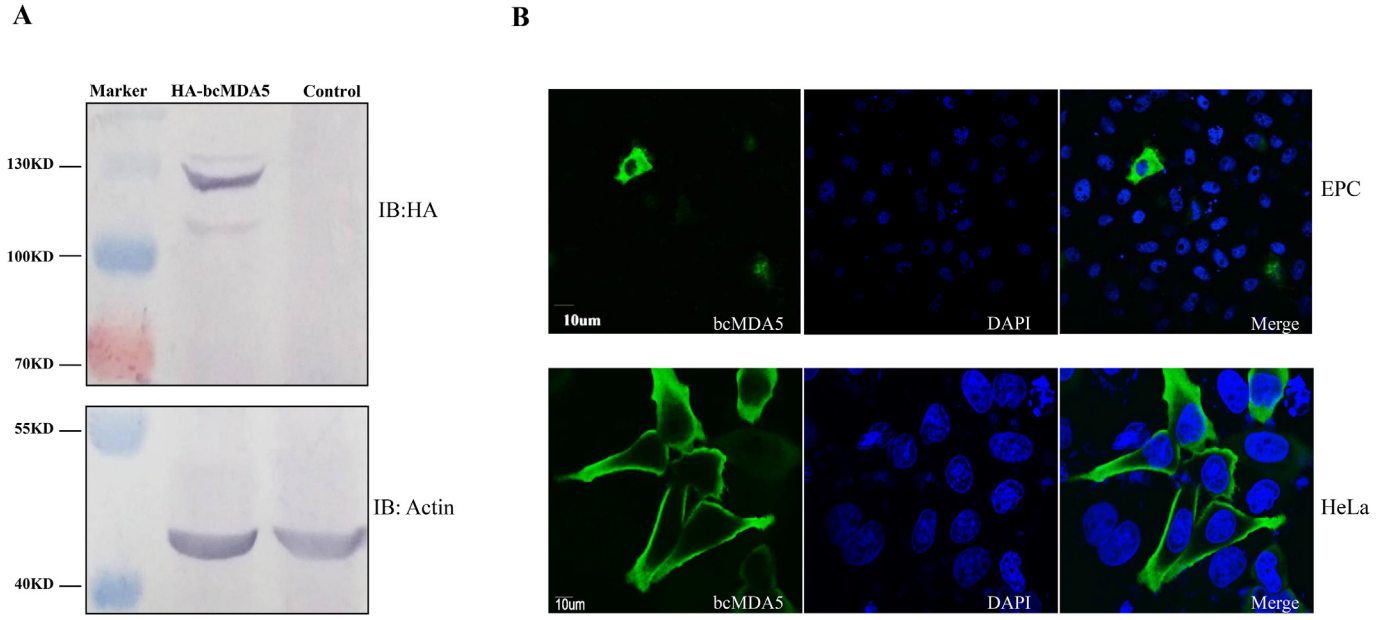

Fig. 5. Protein expression and intracellular localization of bcMDA5. **A**).HEK293T cells were transfected with pcDNA5/FRT/TO-HA-bcMDA5 or pcDNA5/FRT/TO-HA (control) separately. The transfected cells were harvested and lysed at 48 h post transfection. The whole cell lysate was used for western blot in which bcMDA5 was detected by anti-HA antibody. HA-bcMDA5: pcDNA5/FRT/TO-HA-bcMDA5. **B**).Both EPC cells and HeLa cells were transfected with 500 ng pcDNA5/FRT/TO-HA-bcMDA5 or the empty vector separately; the transfected cells were fixed at 24 h post transfection and used for immunofluorescence staining according to the methods. MDA5 (green) indicates intracellular expression of bcMDA5, DAPI (blue) indicates nucleus of EPC and HeLa. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

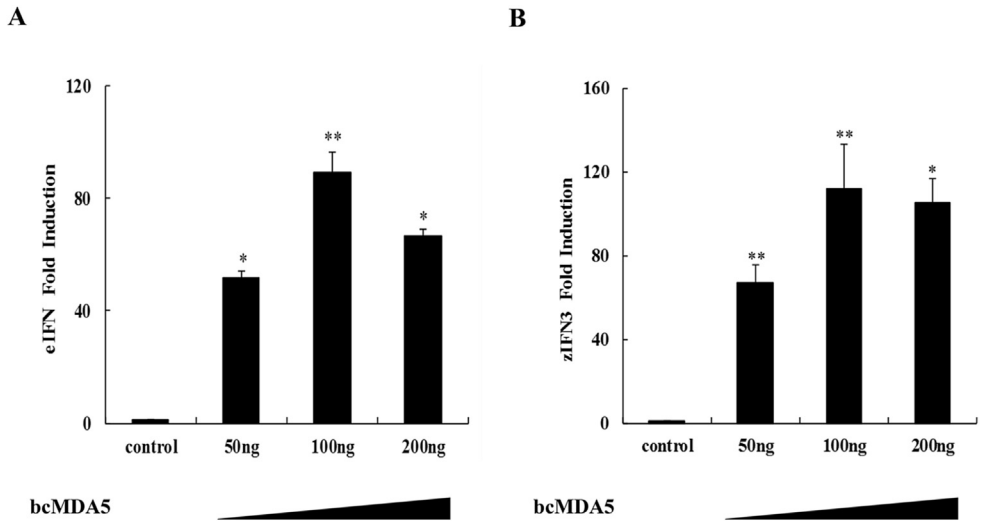

Fig. 6. IFN Induction by bcMDA5. EPC cells in 24-well plate were co-transfected with pcDNA5/FRT/TO-HA-bcMDA5, pRL-TK (25 ng), Luci-zIFN3 or Luci-eIFN (200 ng). For each transfection, the total amount of DNA was balanced with pcDNA5/FRT-TO-HA. Transfected cells were harvested and used for dual-luciferase reporter assay at 24 h post transfection. **A**: Fold induction of fathead minnow IFN (eIFN). **B**: Fold induction of zebrafish IFN3 (zIFN3). Error bars represent the standard error of the mean (±SEM) of three independent experiments. Asterisks (*) on the pillar to mark the significant difference between experimental data and control data(*$p < 0.05$; **$p < 0.01$).

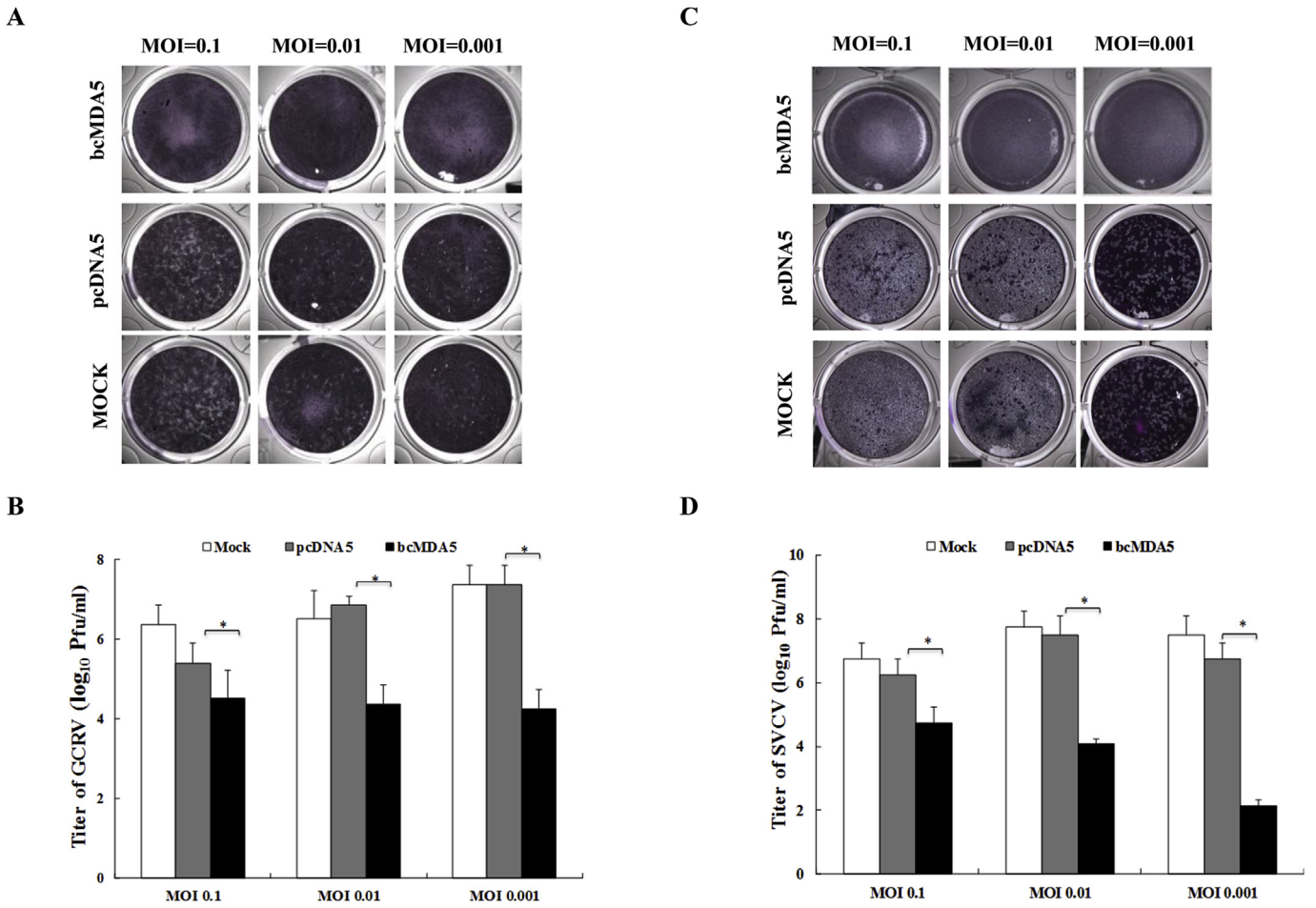

Fig. 7. EPC cells expressing bcMDA5 presented enhanced antiviral ability. EPC cells in 24-well plate were transfected with 300 ng of pcDNA5/FRT/TO-HA-bcMDA5 or pCDNA5/FRT/TO-HA separately and infected with SVCV or GCRV at the indicated MOIs at 24 h post transfection. The cell monolayers were stained with crystal violet (**A&C**) and the virus titers in the supernatant media were determined by plaque assay at 48 h post-infection (**B&D**). Mock: EPC cells without transfection; bcMDA5: pcDNA5/FRT/TO-HA-bcMDA5; pcDNA5: pcDNA5/FRT/TO-HA. Error bars represent the standard error of the mean (±SEM) of three independent experiments. Asterisks (*) on the pillar to mark the significant difference between experimental data and control data (*$p < 0.05$; **$p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and eIFN mediated by bcMDA5 and bcLGP2 was much higher than that mediated by bcMDA5 alone; and obviously higher than that combined value of bcMDA5 alone and bcLGP2 alone (Fig. 9). As the input of bcLGP2 increased, the fold induction of zIFN3/eIFN mediated by bcMDA5 was increased (Fig. 9), which indicated clearly that IFN signaling mediated by bcMDA5 was up-regulated by bcLGP2.

3.7. Association of bcMDA5 and bcLGP2 in host antiviral ability

Since bcLGP2 up-regulated bcMDA5 mediated IFN signaling in EPC cells, we further look into if this association would enhance the host antiviral ability or not, comparing with those EPC cells only expressing bcMDA5. EPC cells were co-transfected pcDNA5/FRT-TO-HA-bcMDA5 and/or pcDNA5/FRT-TO-HA-bcLGP2, and infected with GCRV or SVCV at 1 MOI at 24hr post transfection, which was much higher than previous MOIs (Fig. 7 and [30]). Confirming our speculation, as the input of bcLGP2 increased, both CPE rate and viral titer in the supernatant media of the EPC cells co-expressing both bcMDA5 and bcLGP2 were much decreased compared with those cells overexpressing only bcMDA5 or bcLGP2 (Figs. 10 and 11). These data demonstrated clearly that the antiviral ability mediated by bcMDA5 was up-regulated by bcLGP2.

4. Disscussion

In recent years, significant progress has been achieved in the field of fish immunity. MDA5 in teleost was first cloned in grass carp, which was induced *in vivo* at mRNA level in several tissues or organs when healthy fish were challenged with GCRV [22]; and grass carp MDA5 was found to induce a stronger interferon response than RIG-I to GCRV infection through the phosphorylation and dimerization of IRF3 and IRF7 in CIK cells recently [32]. Subsequently, MDA5 of other teleost have been identified and characterized. For example, (i) Janpanese flounder MDA5 was found to be involved in the induction of antiviral response against VHSV, hirame rhabdovirus (HIRRV) and infectious pancreatic necrosis virus (IPNV) infection [25]. (ii) Two splicing forms of zebrafish MDA5, MDA5a and MDA5b, were identified and up-regulated following the infection of SVCV and an intracellular Gram-negative bacterial, *Edwardsiella tarda*. Meanwhile, overexpression of MDA5a and MDA5b both induced type I IFN promoter activity in zebrafish and protected the transfected cells against SVCV infection [20]. (iii) The overexpression of grouper MDA5 not only increased IFN and ISRE promoter activities in a dose dependent manner obviously, but also enhanced the expression of IRF3, IRF7 and TRAF6. In addition, the overexpression of grouper MDA5 differently

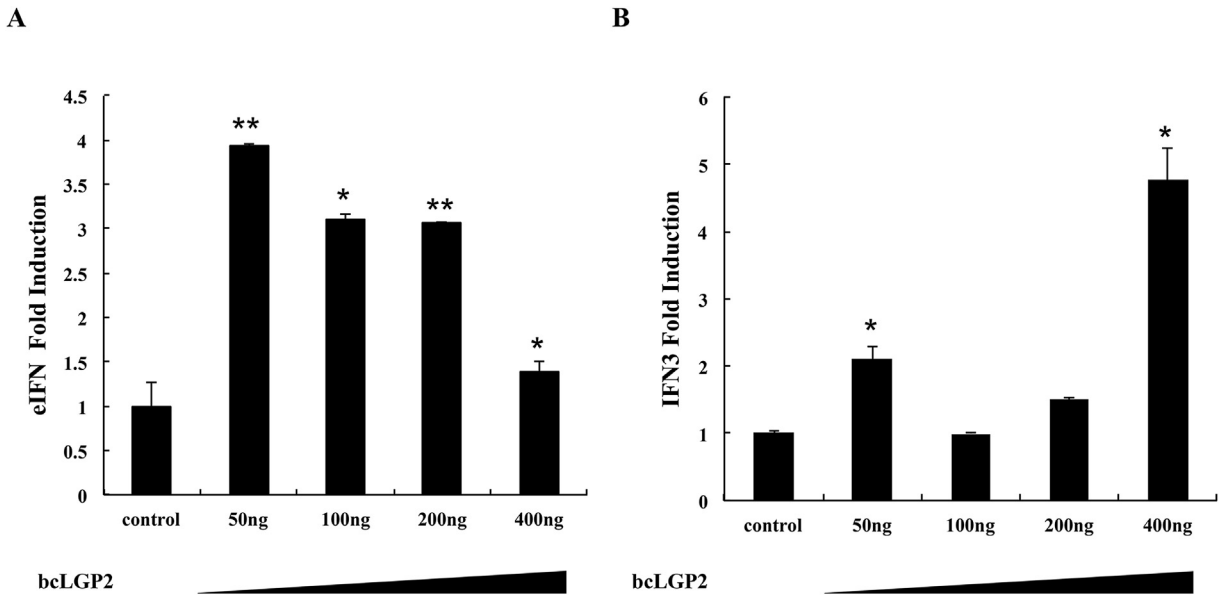

Fig. 8. IFN induction by bcLGP2. EPC cells in 24-well plate were co-transfected with pcDNA5-FRT-TO-HA-bcLGP2, pRL-TK(25 ng), Luci-zIFN3 or luci-eIFN (200 ng). For each transfection, the total amount of DNA was balanced with pcDNA5-FRT-TO-HA. Transfected cells were harvested and used for dual-luciferase reporter assay at 24 h post transfection. (A). Fold induction of fathead minnow IFN (eIFN). (B). Fold induction of zebrafish IFN3 (zIFN3). Error bars represent the standard error of the mean (±SEM) of three independent experiments. Asterisks (*) on the pillar to mark the significant difference between experimental data and control data(*p < 0.05; **p < 0.01).

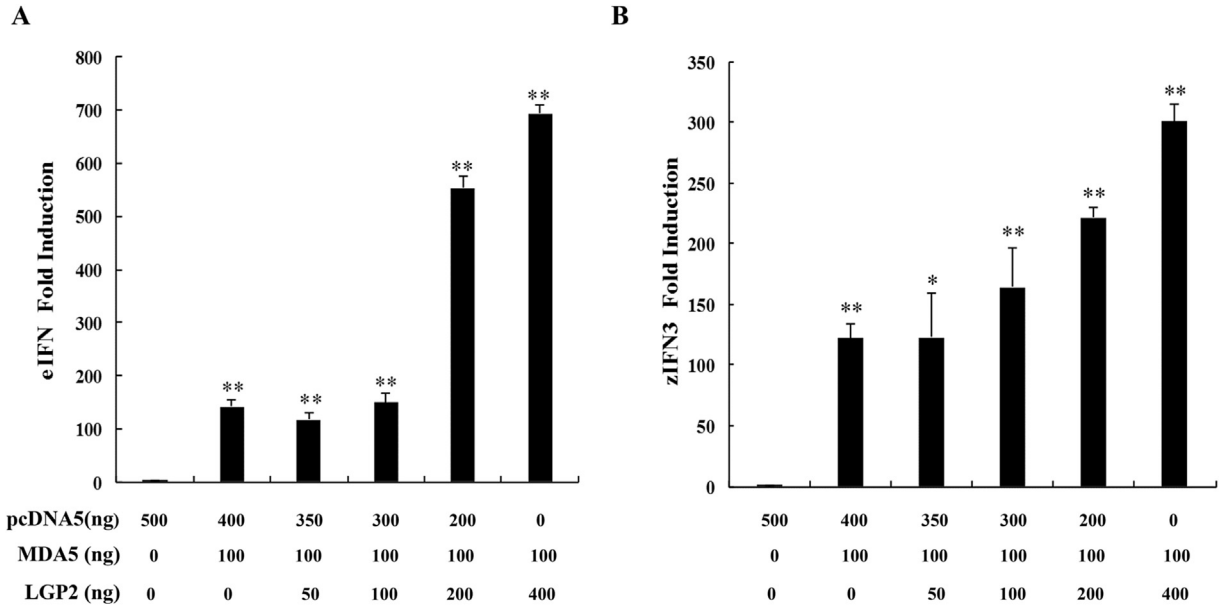

Fig. 9. IFN Induction by bcMDA5 is up-regulated by bcLGP2. EPC cells were co-transfected with pRL-TK (25 ng), pcDNA5-FRT-TO-HA-bcMDA5, pcDNA5-FRT-TO-HA-bcLGP2, luci-zIFN3 or luci-eIFN (200 ng). For each transfection, the total amount of DNA was balanced with pcDNA5/FRT-TO-HA. Transfected cells were harvested and used for dual-luciferase reporter assay at 24 h post transfection. (A).Fold induction of fathead minnow IFN (eIFN). (B).Fold induction of zebrafish IFN3 (zIFN3) in EPC cells. Error bars denote standard deviation and data represent three independent experiments. Error bars represent the standard error of the mean (±SEM) of three independent experiments. Asterisks (*) on the pillar to mark the significant difference between experimental data and control data(*p < 0.05; **p < 0.01).

altered the transcription level of the pro-inflammatory factors, including TNF-α, IL-6 and IL-8 during SGIV or RGNNV infection [33]. In our study, we have identified MDA5 homologue from black carp, and characterized its function in antiviral defense against RNA virus (Fig. 7 A&B). Although bcMDA5 transcription was not obviously enhanced in MPF cells during the first 24 h post LPS stimulation, this fish RLR member still possesses the potential sensitivity to the invading bacteria (Fig. 3A). Higher dose LPS input might initiate stronger transcription of bcMDA5 in host cells since 250 μg/ml LPS triggered obviously enhanced transcription of MDA5 of Japanese flounder (*P. olivaceus*) in both kidney leukocytes and peripheral blood leukocytes [25]. And the time of LPS treatment should be taken into consideration, because slightly increased mRNA level of bcMDA5 was seen at 24 h post stimulation for the dose of 1ug/ml and 2ug/ml (Fig. 3A). This implies that longer time of treatment might enhanced the transcription of bcMDA5 since it was found that Japanese flounder MDA5 mRNA level at 48 h post LPS stimulation was much higher than that of 24 h [25].

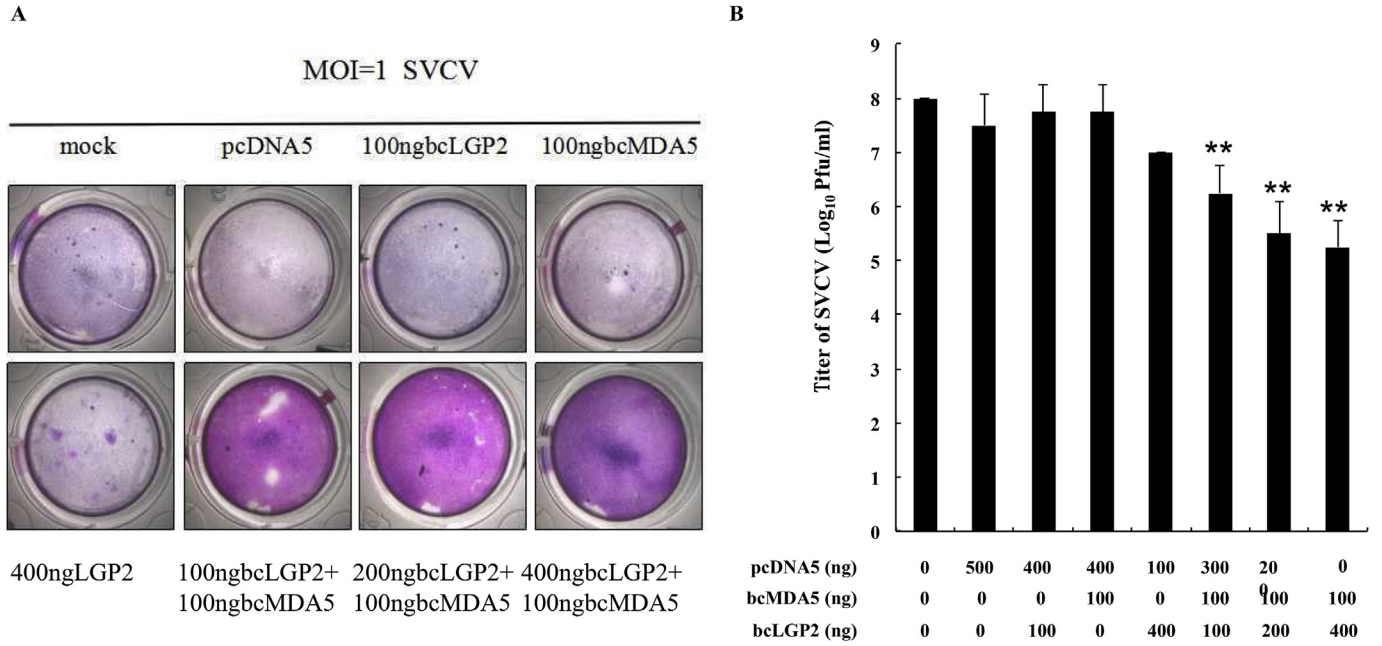

Fig. 10. Up-regulated antiviral ability of bcMDA5 against SVCV by bcLGP2. EPC cells in 24-well plate were co-transfected with pcDNA5/FRT-TO-HA-bcMDA5 or/and pcDNA5/FRT-TO-HA-bcLGP2 and infected with SVCV (MOI = 1) at 24 h post transfection. The cell monolayers were stained with crystal violet (**A**) and the virus titers in the supernatant media were determined by plaque assay at 48 h post-infection (**B**). Error bars represent the standard error of the mean (±SEM) of three independent experiments. Asterisks (*) on the pillar to mark the significant difference between experimental data and control data(*p < 0.05; **p < 0.01). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

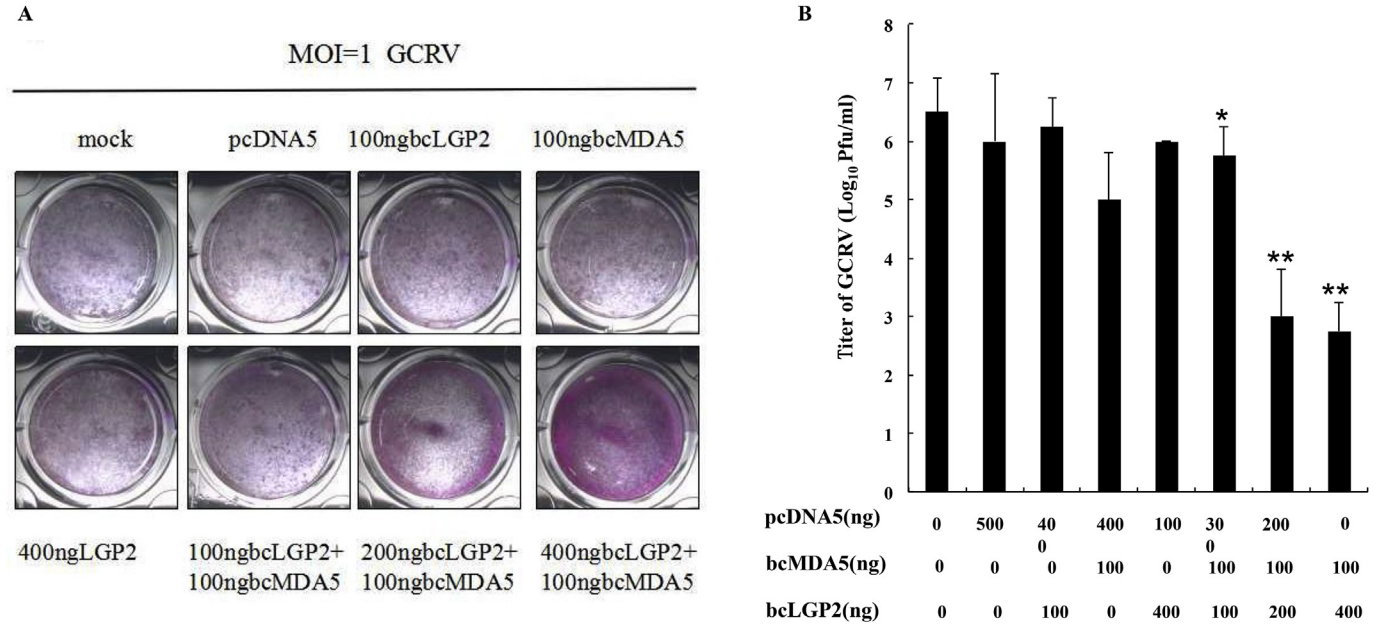

Fig. 11. Up-regulated antiviral ability of bcMDA5 against GCRV by bcLGP2. EPC cells in 24-well plate were co-transfected with pcDNA5/FRT-TO-HA-bcMDA5 or/and pcDNA5/FRT-TO-HA-bcLGP2 and infected with SVCV (MOI = 1) at 24 h post transfection. The cell monolayers were stained with crystal violet (**A**) and the virus titers in the supernatant media were determined by plaque assay at 48 h post-infection (**B**). Error bars represent the standard error of the mean (±SEM) of three independent experiments. Asterisks (*) on the pillar to mark the significant difference between experimental data and control data(*p < 0.05; **p < 0.01). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

LGP2, also as a crucial sensor of RLRs, was first cloned in Atlantic cod (*Gadus morhua*) in teleost [34]. Rainbow trout LGP2 acted as a positive regulator for IFN production; however, LGP2 of crucian carp functioned as a negative regulator of both RIG-I and MDA5 in cytosolic dsRNA-induced IFN signaling [12,35]; and grass carp LGP2 served as a negative regulator in RIG-I- and MDA5-mediated antiviral signaling in resting state and early stage of GCRV infection [36]. In our study, we amazedly found both zIFN3 and eIFN mediated by bcMDA5 and bcLGP2 were much higher than that mediated by bcMDA5 alone (Fig. 9), when EPC cells co-transfected with bcMDA5 and bcLGP2. Additionally, over-expressing of bcLGP2 in EPC cells had little effect to activate the expression of zIFN3 and

eIFN (Fig. 8). Subsequently, we found that EPC cells co-transfected with bcMDA5 and bcLGP2 obtained much enhanced resistance against GCRV and SVCV infection compared with those cells transfected with bcLGP2 or bcMDA5 alone (Figs. 10 and 11).

To explain the mechanism how LGP2 functions in a MDA5-mediated manner, some researchers have found that LGP2 forms a physical association in response to poly(I:C) and proposed a model in which a heterodimer or heterooligomer of MDA5 and LGP2 representing a PRR for poly(I:C) [37]. In addition, a notable feature of MDA5 activation was the formation of long filaments in which MDA5 dimers co-operatively bound along the length of dsRNA molecule [38–40]. However, the affinity of MDA5 for dsRNA such as poly(I:C) was low compared than RIG-I and LGP2, it is possible that LGP2 binds poly(I:C) and helps to recruit MDA5 [41]. Hence, LGP2 plays a role in formation or the stability of these filaments and becomes incorporated into the structure. To elucidate which domain of LGP2 has this function, amount of work have been done and it has been demonstrated that the helicase domain is essential for the ability that LGP2 promotes signaling through MDA5 [42,43], which is helpful for us to study the interaction between bcMDA5 and bcLGP2. In our study, bcLGP2 positively regulated MDA5-mediated IFN signaling and host antiviral response. The mechanism that how bcLGP2 regulate the bcMDA5 mediated signaling may be different from previous studies in mammals and other teleost, which is worth studying further more. Besides, bcMAVS has been cloned and characterized in our lab [29], which provides theoretical help for us to study the interaction between bcMDA5 and bcMAVS.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.05.035.

References

[1] O. Takeuchi, S. Akira, Pattern recognition receptors and inflammation, Cell 140 (6) (2010) 805–820.
[2] T. Boehm, N. Iwanami, I. Hess, Evolution of the immune system in the lower vertebrates, Annu. Rev. Genomics Hum. 13 (2012) 127–149.
[3] B. Magnadottir, Immunological control of fish diseases, Mar. Biotechnol. (NY) 12 (2010) 361–379.
[4] B. Robertsen, The interferon system of teleost fish, Fish. Shellfish Immunol. 20 (2) (2006) 172–191.
[5] C.A. Thaiss, M. Levy, S. Itav, E. Elinav, Integration of innate immune signaling, Trends Immunol. 37 (2) (2016) 84–101.
[6] P. Broz, D.M. Monack, Newly described pattern recognition receptors team up against intracellular pathogens, Nat. Rev. Immunol. 13 (8) (2013) 551–565.
[7] J.W. Schoggins, C.M. Rice, Interferon-stimulated genes and their antiviral effector functions, Curr. Opin. virology 1 (6) (2011) 519–525.
[8] J.W. Schoggins, Interferon-stimulated genes: roles in viral pathogenesis, Curr. Opin. virology 6 (2014) 40–46.
[9] J. Wu, Z.J. Chen, Innate immune sensing and signaling of cytosolic nucleic acids, Annu. Rev. Immunol. 32 (2014) 461–488.
[10] A. Pichlmair, O. Schulz, C.P. Tan, T.I. Näslund, P. Liljeström, F. Weber, e Reis, C. Sousa, RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates, Science 314 (5801) (2006) 997–1001.
[11] M. Yoneyama, M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, T. Fujita, The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviralresponses, Nat. Immunol. 5 (7) (2004) 730–737.
[12] M. Chang, B. Collet, P. Nie, K. Lester, S. Campbell, C.J. Secombes, J. Zou, Expression and functional characterization of the RIG-I-like receptors MDA5 and LGP2 in Rainbow trout (Oncorhynchus mykiss), J. Virol. 85 (16) (2011) 8403–8412.
[13] D.C. Kang, R.V. Gopalkrishnan, Q. Wu, E. Jankowsky, A.M. Pyle, P.B. Fisher, mda-5: an interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties, Proc. Natl. Acad. Sci. 99 (2) (2002) 637–642.
[14] F. Hou, L. Sun, H. Zheng, B. Skaug, Q.X. Jiang, Z.J. Chen, MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response, Cell 146 (3) (2011) 448–461.
[15] S. Goodbourn, R.E. Randall, The regulation of type I interferon production by paramyxoviruses, J. Interferon Cytokine Res. 29 (9) (2009) 539–547.
[16] S. Liu, X. Cai, J. Wu, Q. Cong, X. Chen, T. Li, F. Du, J. Ren, Y.T. Wu, N.V. Grishin, Z.J. Chen, Phosphorylation of innate immune adaptor proteins MAVS, STING, and TRIF induces IRF3 activation, Science (6227) (2015) 347.
[17] T. Venkataraman, M. Valdes, R. Elsby, S. Kakuta, G. Caceres, S. Saijo, Y. Iwakura, G.N. Barber, Loss of DExD/H box RNA helicase LGP2 manifests disparate antiviral responses, J. Immunol. 178 (2007) 6444–6455.
[18] T. Satoh, H. Kato, Y. Kumagai, M. Yoneyama, S. Sato, K. Matsushita, T. Tsujimura, T. Fujita, S. Akira, O. Takeuchi, LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 1512–1517.
[19] P.F. Zou, M.X. Chang, Y. Li, S. Huan Zhang, J.P. Fu, S.N. Chen, P. Nie, Higher antiviral response of RIG-I through enhancing RIG-I/MAVS-mediated signaling by its long insertion variant in zebrafish, Fish. Shellfish Immunol. 43 (1) (2015) 13–24.
[20] P.F. Zou, M.X. Chang, N.N. Xue, X.Q. Liu, J.H. Li, J.P. Fu, S.N. Chen, P. Nie, Melanoma differentiation-associated gene 5 in zebrafish provoking higher interferon-promoter activity through signalling enhancing of its shorter splicing variant, Immunology 141 (2) (2014) 192–202.
[21] W. Wang, M. Asim, L. Yi, A.M. Hegazy, X. Hu, Y. Zhou, T. Ai, L. Lin, Abortive infection of snakehead fish vesiculovirus in ZF4 cells was associated withthe RLRs pathwayactivation by viral replicative intermediates, Int. J. Mol. Sci. 16 (3) (2015) 6235–6250.
[22] J. Su, T. Huang, J. Dong, J. Heng, R. Zhang, L. Peng, Molecular cloning and immune responsive expression of MDA5, gene, a pivotal member of the RLR gene family from grass carp Ctenopharyngodon idella, Fish Shellfish Immunol. 28 (4) (2010) 712–718.
[23] T. Huang, J. Su, J. Heng, J. Dong, R. Zhang, H. Zhu, Identification and expression profiling analysis of grass carp Ctenopharyngodon idella LGP2 cDNA, Fish. Shellfish Immunol. 29 (2) (2010) 349–355.
[24] C. Yang, J. Su, T. Huang, R. Zhang, L. Peng, Identification of a retinoic acid-inducible gene I from grass carp (Ctenopharyngodon idella) and expressionanalysis in vivo and in vitro, Fish. Shellfish Immunol. 30 (3) (2011) 936–943.
[25] M. Ohtani, J. Hikima, H. Kondo, I. Hirono, T.S. Jung, T. Aoki, Characterization and antiviral function of a cytosolic sensor gene, MDA5, in Japanese flounder, Paralichthys olivaceus, Dev. Comp. Immunol. 35 (5) (2011) 554–562.
[26] M. Ohtani, J. Hikima, H. Kondo, I. Hirono, T.S. Jung, T. Aoki, Evolutional conservation of molecular structure and antiviral function of a viral RNA receptor, LGP2, inJapanese flounder, Paralichthys olivaceus, J. Immunol. 185 (12) (2010) 7507–7517.
[27] P. Jia, J. Zhang, Y. Jin, L. Zeng, K. Jia, M. Yi, Characterization and expression analysis of laboratory of genetics and physiology 2 gene in sea perch, Lateolabrax japonicus, Fish. Shellfish Immunol. 47 (1) (2015) 214–220.
[28] P. Jia, K. Jia, L. Chen, Y. Le, Y. Jin, J. Zhang, L. Zhu, L. Zhang, M. Yi, Identification and characterization of the melanoma differentiation - associated gene 5 in sea perch, Lateolabrax japonicus, Dev. Comp. Immunol. 61 (2016) 161–168.
[29] W. Zhou, J. Zhou, Y. Lv, Y. Qu, M. Chi, J. Li, H. Feng, Identification and characterization of MAVS from black carp Mylopharyngodon piceus, Fish. Shellfish Immunol. 43 (2) (2015) 460–468.
[30] J. Xiao, J. Yan, H. Chen, J. Li, Y. Tian, H. Feng, LGP2 of black carp plays an important role in the innate immune response against SVCV and GCRV, Fish Shellfish Immunol. 57 (2016) 127–135.
[31] Z. Huang, S. Chen, J. Liu, J. Xiao, J. Yan, H. Feng, IFNa of black carp is an antiviral cytokine modified with N-linked glycosylation, Fish. Shellfish Immunol. 46 (2) (2015) 477–485.
[32] Q. Wan, C. Yang, Y. Rao, Z. Liao, J. Su, MDA5 induces a stronger interferon response than RIG-I to GCRV infection through a mechanism involving the phosphorylation and dimerization of IRF3 and IRF7 in CIK Cells, Front. Immunol. 8 (2017) 189.
[33] Y. Huang, Y. Yu, Y. Yang, M. Yang, L. Zhou, X. Huang, Q. Qin, Antiviral function of grouper MDA5 against iridovirus and nodavirus, Fish Shellfish Immunol. 54 (2016) 188–196.
[34] M.L. Rise, J. Hall, M. Rise, T. Hori, A. Gamperl, J. Kimball, S. Hubert, S. Bowman, S.C. Johnson, Functional genomic analysis of the response of Atlantic cod (Gadus morhua) spleen to the viral mimic polyriboinosinic polyribocytidylic acid (pIC), Dev. Comp. Immunol. 32 (8) (2008) 916–931.
[35] H. Kato, O. Takeuchi, S. Sato, M. Yoneyama, M. Yamamoto, K. Matsui, S. Uematsu, A. Jung, T. Kawai, K.J. Ishii, O. Yamaguchi, K. Otsu, T. Tsujimura, C.S. Koh, C. Reis e Sousa, Y. Matsuura, T. Fujita, S. Akira, Differential roles ofMDA5 and RIG-I helicases in the recognition of RNA viruses, Nature 441 (7089) (2006) 101–105.
[36] Y. Rao, Q. Wan, C. Yang, J. Su, Grass carp laboratory of genetics and physiology

2 serves as a negative regulator in retinoic acid-inducible gene I- and melanoma differentiation-associated gene 5-mediated antiviral signaling in resting state and early stage of grass carp reovirus Infection, Front. Immunol. 8 (2017) 352.
[37] K.S. Childs, R.E. Randall, S. Goodbourn, LGP2 plays a critical role in sensitizing mda-5 to activation by double-stranded RNA, PLoS One 8 (5) (2013) 759–762.
[38] A. Peisley, C. Lin, B. Wu, M. Orme-Johnson, M. Liu, T. Walz, S. Hur, Cooperative assembly and dynamic disassembly of MDA5 filaments for viral dsRNA recognition, Proc. Natl. Acad. Sci. U. S. A. 108 (52) (2011) 21010–21015.
[39] I.C. Berke, X. Yu, Y. Modis, E.H. Egelman, MDA5 assembles into a polar helical filament on dsRNA, Proc. Natl. Acad. Sci. U. S. A. 109 (45) (2012) 18437–18441.
[40] I.C. Berke, Y. Modis, MDA5 cooperatively forms dimers and ATP-sensitive filaments upon binding double-stranded RNA, Embo J. 31 (7) (2012) 1714–1726.
[41] M. Yoneyama, M. Kikuchi, K. Matsumoto, T. Imaizumi, M. Miyagishi, K. Taira, E. Foy, Y.M. Loo, M. Gale Jr., S. Akira, S. Yonehara, A. Kato, T. Fujita, Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity, J. Immunol. 175 (5) (2005) 2851–2858.
[42] K. Childs, R. Randall, S. Goodbourn, Paramyxovirus V proteins interact with the RNA Helicase LGP2 to inhibit RIG-I-dependent interferon induction, J. Virology 86 (7) (2012) 3411–3421.
[43] D. Bamming, C.M. Horvath, Regulation of signal transduction by enzymatically inactive antiviral RNA helicase proteins MDA5, RIG-I, and LGP2, J. Biol. Chem. 284 (15) (2009) 9700–9712.