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Molecular cloning and characterization of TANK of black carp *Mylopharyngodon piceus*

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ABSTRACT

The TRAF family member-associated NF-κB activator (TANK) is linked to the regulation of the transcription of NF-κB in mammals; however, its role in interferon induction is unclear. To elucidate the roles of TANK in teleost, the TANK homologue of black carp (*Mylopharyngodon piceus*) has been cloned and characterized in this paper. The open reading frame (ORF) of black carp TANK (bcTANK) comprises 1050 nucleotides and the predicted bcTANK protein contains 350 amino acids. The transcription of bcTANK in host cells increased in response to the stimulation of LPS, poly (I:C), SVCV and GCRV. bcTANK migrated around 50 KDa in immunoblot assay and was identified as a cytosolic protein by immunofluorescent staining in both EPC and HeLa cells. bcTANK could not induce the activity of IFN promoter in luciferase reporter assay in EPC cells; however, the IFN-activation ability of bcTANK was obviously enhanced when the cells were treated with LPS, poly (I:C) or virus. Both CPE ratio and virus titer in the media of EPC cells expressing bcTANK were obviously lower than those of the control cells, which were examined by violet crystal staining and plaque assay separately. Taken together, our data support the conclusion that bcTANK plays an important role in the antiviral innate immune activation of black carp.

1. Introduction

Vertebrates are immune to disadvantage environments such as viral infection and bacteria invasion, according to which its immunity can be classified into innate immunity and adaptive immunity [1–4]. The innate immune response is triggered right after the invading pathogenic microbes are recognized by pattern recognition receptors (PRRs) and subsequent induction of interferons (IFNs) and interferon stimulated genes (ISGs) through downstream signaling, which include toll-like receptors (TLRs), RIG-I-like receptors (RLRs), nucleotide oligomerization domain-like receptors (NLRs) and so on. The IFN system provides a powerful and universal intracellular defense mechanism against viruses, which plays a key role in both innate and adaptive immunity of vertebrates [5–7].

Rapid induction of type I IFN and inflammatory cytokines are central to the host antiviral responses, which are tightly regulated by extracellular and intracellular signals [8,9]. As to RLR signaling, RIG-I or MDA5 (another RLR member) is activated upon ligand recognition and interacts with MAVS (also known as VISA, IPS-1 or Cardif) through its exposed CARD domain, which is a mitochondrion-anchored adaptor molecule [10,11]. MAVS associates with and activates both the canonical IκB kinase (IKK) complexes (IKKα/β/γ) and two non-canonical IKK-related kinases, TANK-binding kinase 1 (TBK1) and IKKε, which control the regulation of NF-κB transactivation and contribute to IRF3/7 phosphorylation [12,13]. Phosphorylated IRF3/7 and activated NF-κB translocate into nuclear and induce effective type I IFN production and pro-inflammatory cytokines secretion, leading to antiviral response [14,15].

TANK (also known as I-TRAF) was first identified as a TRAF-binding protein with both stimulatory and inhibitory properties in host innate immune activation [16,17]. Among the seven reported TRAF family members (TRAF1 ~ TRAF7), TRAF1, TRAF2, TRAF3, TRAF5 and TRAF6 could interact with TANK [18–21]. A previous study revealed that TANK activated NF-κB signaling in cells overexpressing TRAF2 [16]. However, TANK was also found to negatively regulate NF-κB signaling pathways, which was mediated by TNFα, IL-1 and CD40 [17]. The yeast two-hybrid screen identified the direct association between TANK and TBK1 and TBK1/TANK/TRAF2 signaling complex represented an alternative to the receptor signaling complex for TRAF-mediated activation of NF-κB [22]. TANK was also found to form a complex with the adaptor proteins NAP1 and SINTBAD, which linked TBK1 to virus-activated signaling cascades [23,24]. Some key components of RLR

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signaling, like MAVS, TRAF3, TRIF and IRF3, were also found to be associated with TANK. TANK functioned as a scaffold protein that was assembled into IRF3/TBK1/IKKε complex, where it was recruited into regulating phosphorylation of IRF3 and IFN induction [25–27].

Compared with its mammalian counterpart, teleost TANK remained largely unknown. To our knowledge, there was no report about teleost TANK. As an economical important fresh water species in China, Black carp (*Mylopharyngodon piceus*) is threatened by a bulk of pathogenic microorganisms, such as grass carp reovirus (GCRV) and spring viremia of carp virus (SVCV). The previous studies demonstrate that RLR signaling functions importantly in the antiviral innate immune response of this cyprinid fish. bcTRAF2/6 positively regulates bcMAVS-mediated IFN production, which leads to the exploration of role of bcTANK in the host antiviral innate immune activation initiated by GCRV and SVCV [28–32]. In this study, bcTANK has been cloned and characterized. The transcription of bcTANK in *Mylopharyngodon piceus* kidney (MPK) cells increased in response to the stimulation of LPS, poly (I:C), SVCV and GCRV. Plaque assay demonstrated that EPC cells expressing bcTANK obtained enhanced antiviral activity against both SVCV and GCRV. Thus, our data indicate that bcTANK plays an important role in host antiviral innate immune activation, which is reported for the first time in the teleost.

2. Materials and methods

2.1. Cells and plasmids

HEK293T cells, HeLa cells, *Epithelioma Papulosum Cyprinid* (EPC) cells, *Ctenopharyngodon idella* kidney (CIK) cells and *Mylopharyngodon piceus* kiney (MPK) cells were kept in the lab [28]. All the cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 u/ml penicillin and 100 μg/ml streptomycin. HEK293T and HeLa were cultured at 37 °C with 5% CO_2; EPC, CIK and MPK were cultured at 26 °C with 5% CO_2. Transfection was done as previously described, calcium phosphate was used for HEK293T transfection; Lipomax (SUDGEN) was used for EPC transfection and Lipofectamine®2000 was used for HeLa transfection [28].

pcDNA5/FRT/TO (Invitrogen), pRL-TK, Luci-DrIFNφ1, Luci-DrIFNφ3 (for zebrafish IFNφ1/3 promoter activity analysis accordingly), Luci-eIFN (for fathead minnow IFN promoter activity analysis) and Luci-bcIFNa (for black carp IFNa promoter activity analysis) were kept in the lab [32]. Degenerate Primers (Table 1) were designed to amplify the open reading frame (ORF) of bcTANK. The recombinant vectors expressing bcTANK were constructed by inserting the open reading frame (ORF) of bcTANK into pcDNA5/FRT/TO between KpnI and Xho I restriction sites separately, with a Flag tag at its N-terminus or C-terminus for pcDNA5/FRT/TO-Flag-bcTANK and pcDNA5/FRT/TO-TANK-Flag accordingly.

2.2. Cloning the cDNA of bcTANK

Degenerate Primers (Table 1) were designed to amplify the cDNA of bcTRAF2 based on the sequences of TANK of *D. rerio* (NP_001070068). 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). The coding sequence (CDS) of bcTANK was cloned by using the degenerate primers. The amplified fragments were cloned into pMD18-T vector and sequenced by Invitrogen.

2.3. Virus proliferation and titer

SVCV and GCRV were kept in the lab [32]. SVCV and GCRV were propagated in EPC and CIK separately at 25 °C in the presence of 2% fetal bovine serum. Virus titers were determined by plaque forming assay on EPC cells separately as previously described [32]. Briefly, the 10-fold serially diluted virus supernatants were added onto EPC cells (4×10^5 cells) in 24-well plate 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 the bcTANK mRNA expression in MPK cells. The primers for the q-PCR of bcTANK were bcTANK-Q-F and bcTANK-Q-R and the primers for q-PCR of β-actin 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.

2.5. LPS and poly (I:C) treatment

MPK cells were seeded in 6-well plate (2×10^6 cells/well) at 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. MPK cells were replaced with 1 ml fresh media containing poly (I:C) and harvested at different time points post treatment. For LPS treatment, MPK cells in 6-well plate (2×10^6 cells/well) were treated with LPS at indicated concentration and harvested for q-PCR at different time point post stimulation as above.

Table 1
Primers used in the study.

Primer name	Sequence (5′-3′)	Primer information
ORF		
bcTANK-F	ATGGACAGGAACATCAGTGAGC	ORF cloning
bcTANK-R	CAAGAACAAAGCTGGCGATTGA	
Expression construct		
bcTANK-N-F	ACTGACGGTACCATGGACAGGAACATCAGTGAGC	FRT-TO-FLAG-bcTANK
bcTANK-N-R	ACTGACCTCGAGTCAATCGCCAGCTTTGTTC	FRT-TO-bcTANK-FLAG
bcTANK-C-F	ACTGACGGTACCGCCACCATGGACAGGAACATCAGTGAGC	
bcTANK-C-R	ACTGACCTCGAGATCGCCAGCTTTGTTCTTG	
q-PCR		
bc Q actin-F	TGGGCACCGCTGCTTCCT	*Ex vivo* q-PCR
bc Q actin-R	TGTCCGTCAGGCAGCTCAT	
bcTANK-q-F	ATTCGTCATACTCCGCCCCCTC	*Ex vivo* q-PCR
bcTANK-q-R	CACTTCTCTCGGCTGTTCATCT	

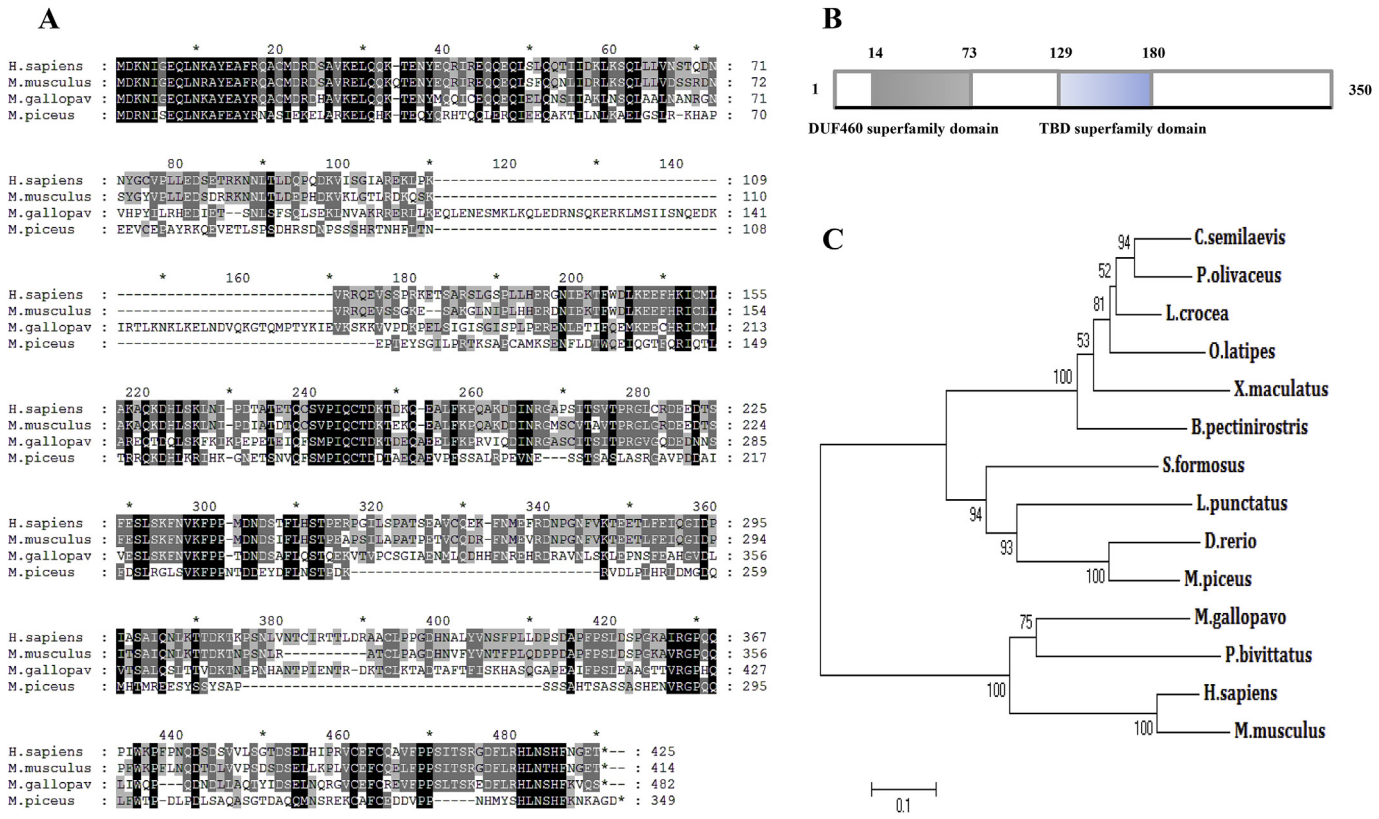

Fig. 1. Homology comparison and evolution analysis of bcTANK. (A) Comparisons of bcTANK with other vertebrate TANK amino acid sequences by using MEGA 6.0 program and GeneDoc program. DUF460 super family domain was underlined (14–73), TBD super family domain was marked double line (129–180). (B) Schematic map of bcTANK. (C) Phylogenetic tree of vertebrate TANK. The amino acid sequence of bcTANK was aligned with TANK from different species by using MEGA 6.0 software, which included (GenBank accession number): C. semilaevis (XP_008326921), *P. olivaceus* (NW_017859656.1), *L. crocea* (XP_010730520), *O. latipes* (XP_004081941), *X. maculatus* (XP_005805397), *B. pectinirostris* (XP_020784622), S. formosus (XP_018597007), *L. punctatus* (XP_017325567), *D. rerio* (NP_001070068), *M. piceus* (MG462752), *M. gallopavo* (XP_010711971), *P. bivittatus* (XP_007427628), *H. sapiens* (NP_004171), *M. musculus* (NP_035659). The bar stands for scale lengthand the numbers on different nodes stand for bootstrap value.

Table 2
Comparison of bcTANK1 with other vertebrate TANK (%).

Species	Full-length sequence of protein	
	Identity	Similarity
Cynoglossus semilaevis	45	60.2
Paralichthys olivaceus	44.5	59.7
Larimichthys crocea	45.7	62.1
Oryzias latipes	43.7	60.4
Xiphophorus maculatus	43.3	58.6
Boleophthalmus pectinirostris	43.7	57.1
Scleropages formosus	47.3	61.7
Lctalurus punctatus	52.2	66.4
Danio rerio	74.7	82.4
Mylopharyngodon piceus	100	100
Meleagris gallopavo	27.4	38.1
Python bivittatus	24.8	39
Homo sapiens	26.2	41.2
Mus musculus	25.5	40.1

2.6. Immunoblotting

HEK293T or EPC cells in 6-well plate were transfected with plasmid expressing bcTANK or the empty vector separately. Transfected cells were harvested at 48 h post-transfection and lysed for immunoblot (IB) assay as previously described [29]. 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).

2.7. Immunofluorescence microscopy

HeLa cells (1×10^5 cells) or EPC cells (2×10^5 cells) in 24-well plate were transfected with the plasmid expressing bcTANK or the empty vector separately. 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 immunofluorescent staining as previously described [31]. Mouse monoclonal anti-Flag 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; DAPI (4, 6-diamidino-2-phenylindole) was used to stain the nucleus.

2.8. Luciferase reporter assay

EPC cells in 24-well plate were co-transfected with bcTANK, pRL-TK, Luci-DrIFNφ1(Luci-DrIFNφ3, Luci-bcIFNa or Luci-eIFN separately). 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 manufacture (Promega) as previously [29]. Briefly, the ratio of firely luciferase value to renilla luciferase value was calculated. The relative luciferase activity of the transfected group was obtained as the ratio of the control group (set as 1), which was the regulated activity of

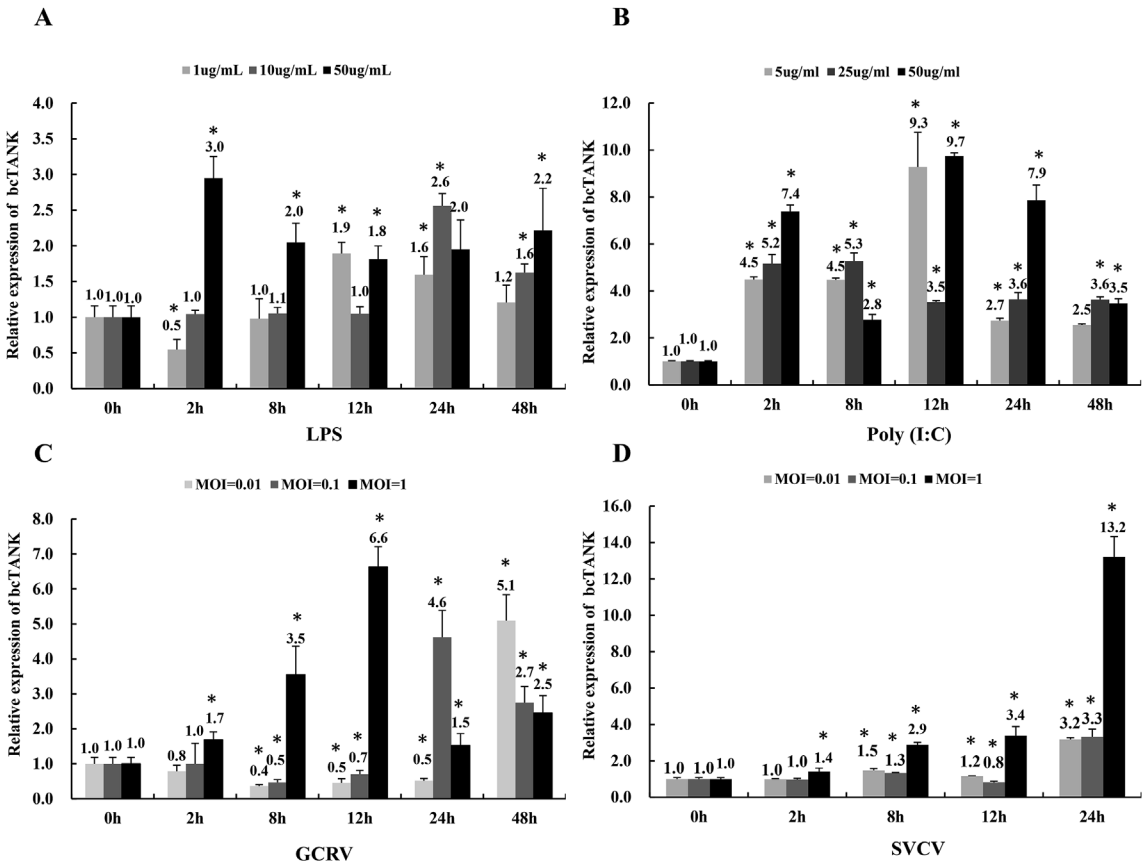

Fig. 2. BcTANK expression in MPK cells in response to different stimuli. (A)&(B) MPK cells were seeded in 6-well plate (2×10^6 cells/well) at 16 h before stimulation. The cells were treated with LPS or poly (I:C) at the indicated concentrations separately and harvested for q-PCR independently at the indicated time points post stimulation. (C)&(D) MPK cells were seeded in 6-well plate (2×10^6 cells/well) at 16 h before viral infection. The cells were infected with GCRV or SVCV at the indicated MOIs separately and harvested for q-PCR independently at the indicated time points post infection. The number above the error bar represents average bcTANK mRNA level compared with that of the control MPK cells (bcTANK mRNA level in the control MPK cells was regarded as 1), error bars denote standard deviation.

gene transcription in the transfected group.

2.9. 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. Asterisk (*) stands for $p < 0.05$. The data were analyzed by two-tailed Student's t-test.

3. Results

3.1. Molecular cloning and sequence analysis of bcTANK

To discern and analyze the role of TANK in the innate immune activation of black carp, the cDNA of TANK orthologue has been cloned from the spleen of black carp. The cDNA of bcTANK (NCBI accession number: MG462752) consists of 1350 nucleotides and the coding sequence (CDS) of bcTANK is composed of 1050 nucleotides (Supplementary Fig. 1). Initial sequence analysis of bcTANK (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) predicts that bcTANK contains 350 amino acid residues, which includes a protein of unknown function (DUF460) super family domain (14–73) and a The TBK1/IKKε binding domain (TBD) super-family domain (129–180). The amino acid sequence comparison among human, mice, wild turkey and black carp showed that both DUF460 super family domain and TBD super family domain were conserved in vertebrates (Fig. 1A&B). The deduced bcTANK protein has a calculated molecular weight of 39.61 KDa and an isoelectric of 5.61 (http://web.expasy.org/protparam/).

To study the evolution of bcTANK, the amino acid sequence of bcTANK was subjected to multiple alignments with those of TANK proteins from different species. Phylogenetic analysis of TANK proteins from the selected species demonstrates that these homologue proteins could be divided into three groups, consisting of mammals, birds and fish branches (Fig. 1 C). In phylogenetic analysis, bcTANK shares high amino acid sequence similarity with that of TANK of zebrafish (*Danio rerio*) (82.4%), which correlates with the closest genetic relationship of these two cyprinid fishes (Table 2).

3.2. bcTANK expression ex vivo in response to different stimuli

To learn the profile of bcTANK transcription in host cells during the innate immune response, MPK cells were subjected to LPS/poly (I:C) stimulation or virus infection, then applied to q-PCR analysis. In LPS-treated MPK cells, bcTANK mRNA level increased in all concentrations (1 μg/ml, 10 μg/ml or 50 μg/ml); higher dose of LPS treatment (50μg/ml) induced quicker and stronger bcTANK transcription after stimulation (Fig. 2A). In poly (I:C)-stimulated group, the treatment of all concentrations (5 μg/ml, 25 μg/ml or 50 μg/ml) induced the transcription of bcTANK gene with 48 h post stimulation, in which the induced bcTANK transcription level was higher than that of LPS stimulated group (Fig. 2B). After poly (I:C) stimulation, the transcription of

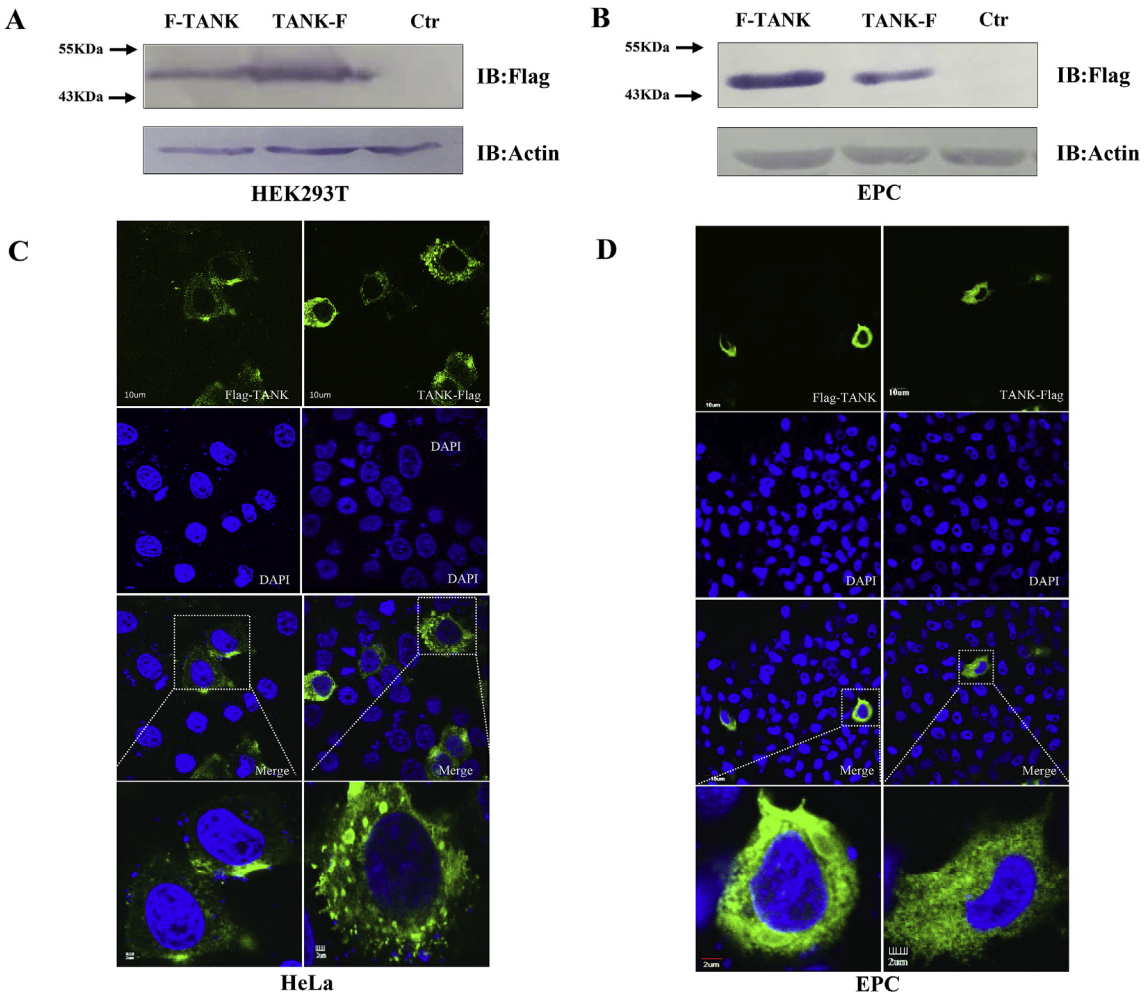

Fig. 3. Protein expression and subcellular distribution of bcTANK. (A)&(B) Immunoblot (IB) assay of EPC and 293 T cells. Ctr: HEK293T cells or EPC cells transfected with empty vector, F-TANK: pcDNA5/FRT/TO-Flag-bcTANK; TANK-F: pcDNA5/FRT/TO-bcTANK-Flag. (C)&(D) Immunofluorescence staining of EPC cells and HeLa cells. Flag-bcTANK: pcDNA5/FRT/TO-Flag-bcTANK; bcTANK-Flag: pcDNA5/FRT/TO-bcTANK-Flag. The bars stand for the scale of 5 μm and 10 μm separately.

bcTANK gene immediately increased for all dose and the highest relative bcTANK mRNA level was up to 9.7 folds (50μg/ml; 12 h point).

In MPK cells infected with GCRV, bcTANK mRNA level varied within 48 h post infection (hpi). Higher dose of virus treatment (1 MOI) induced quicker and stronger bcTANK transcription after infection (2hpi) and the highest relative bcTANK mRNA level (24hpi) was up to 6.6 folds. However, in lower dose GCRV-infected MPK cells (0.1 or 0.01 MOI), bcTANK mRNA level increased at 24 hpi and 48 hpi separately (Fig. 2 C). Similar data were seen in SVCV-infected group, higher dose of virus treatment (1 MOI) induced quicker and stronger bcTANK transcription after infection (2hpi) and the highest relative bcTANK mRNA level (24hpi) was up to 13.2 folds (Fig. 2 D). Increased transcription of bcTANK in MPK cells infected with GCRV/SVCV implied this fish TANK homologue functioned importantly in host antiviral innate immune activation initiated by these two viruses.

3.3. Protein expression and intracellular distribution of bcTBK1

To study the function of bcTANK, HEK 293 T and EPC cells were transfected with plasmid expressing bcTANK to investigate the protein expression of bcTANK. In the immunoblot assay of both HEK293T and EPC cells, a specific band of ~50 KDa was detected in the lane of Flag-bcTANK or bcTANK-Flag, which demonstrated that the TANK homologue of black carp were well expressed in both mammalian and fish system and the location of Flag tag did not impact the protein expression of bcTANK (Fig. 3A&B). bcTANK migrated around ~50 KDa in immunoblot assay, which was larger than its predicted molecular weight of 39.61 KDa. It is speculated that bcTANK is modified with post-translation, which is most likely to be phosphorylation, ubiquitination and sumoylation like its mammalian counterpart [26,33,34]. In our previous study, TRAF2/6 and TBK1 of black carp (bcTRAF2/6, bcTBK1) had been identified as cytosolic proteins, which assumed to be associated with bcTANK as their mammalian counterparts [28,29,35]. To further examine the subcellular distribution of bcTANK, both Hela cells and EPC cells were used for immunofluorescence staining. In the immunofluorescence staining data of both fish cells and mammalian cells, bcTANK-expressing region (green) surrounded the nucleus (blue), which demonstrated that bcTANK was a cytosolic protein (Fig. 3C&D).

3.4. IFN-inducing activity of bcTANK

To investigate the role of bcTANK in IFN production, EPC cells were transfected with bcTANK and used for reporter assay to see if it could induce the transcription of zebrafish IFNφ1/3 (DrIFNφ1/3), fathead minnow IFN (eIFN) or bcIFNa. It is clear that the overexpressed bcTANK in EPC cells showed little activity to induct the transcription of DrIFNφ1/3, eIFN or bcIFNa (Fig. 4 A, B, C, D). LPS, poly (I:C), GCRV or SVCV were used to stimulate the above transfected EPC cells to see whether these stimuli impact the IFN-inducing activity of bcTANK or not. The reporter assay of DrIFNφ3 results demonstrated that the IFN-

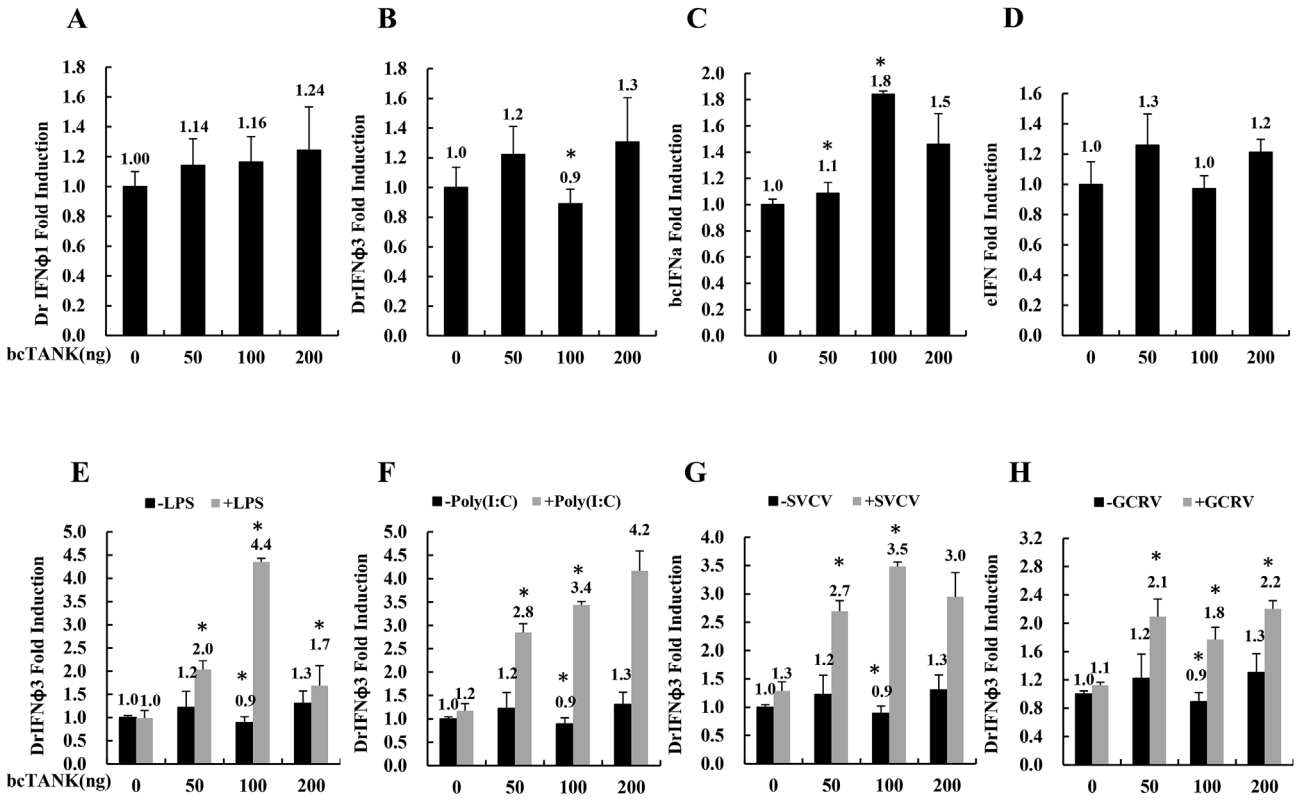

Fig. 4. BcTANK-mediated antiviral signaling. (**A**～**D**) EPC cells in 24-well plate were co-transfected with pRL-TK, Luci-DrIFNφ1 (Luci-DrIFNφ3, Luci-eIFN, Luci-bcIFNa), bcTANK or the empty vector separately and applied to luciferase reporter assay. The error bars represent the standard deviation; bcTANK: pcDNA5/FRT/TO-Flag-bcTANK. (**E**～**H**) EPC cells in 24-well plate were transfected co-transfected with pRL-TK, Luci-DrIFNφ3, bcTANK or the empty vector separately and treated with LPS or poly (I:C) at the concentration of 50 μg/ml; or infected with GCRV/SVCV at MOI = 1 at 24 h post transfection; then applied to luciferase reporter assay at 24 h after treatment according to methods. Error bars denote standard deviation and data represent three independent experiments. The numbers above the error bars stand for average IFN folds induction. bcTANK: pcDNA5/FRT/TO-Flag-bcTANK.

inducing ability of bcTANK was enhanced in response to all these stimuli (Fig. 4 E, F, G, H). Thus, it implied that pathogen invasion (both bacteria and virus) improved the IFN-inducing activity of bcTANK.

3.5. Antiviral activity of bcTANK against SVCV and GCRV

To interpret the role of bcTANK during the innate immune response, EPC cells were transfected with bcTANK and applied to virus infection at 24 h post transfection separately. In SVCV-infected group, both the CPE ratio and the virus titer in the supernatant media of the EPC cells expressing bcTANK were obviously lower than those of the empty vector transfected cells or the un-transfected cells (Fig. 5A&5C). The virus titer in the media of bcTANK-expressing cells showed the biggest reduction (> 1000 times) when the cells were infected with SVCV at the dose of 0.1 MOI; and the smallest reduction happened at the dose of 0.01 MOI. The data of GCRV group was similar to that of SVCV group; both the CPE ratio and the virus titer in the media of the EPC cells expressing bcTANK obviously lower than those of control cells (Fig. 5B and D). The virus titer in the media of bcTANK-expressing cells showed the biggest reduction (～1000 times) when the cells were infected with GCRV at the dose of 0.1 MOI. Thus, our data presented clearly that exogenous bcTBK1 enhanced the antiviral ability of EPC cells against both SVCV and GCRV, which demonstrated that that this fish TANK ortholog functioned as an important factor in host antiviral innate immune response.

4. Discussion

In both fish and mammals, induction of type I IFN can be triggered by microbial components through TLR or RIG-I pathways [36,37]. In mammalian RIG-I signaling pathway, a critical step is TBK1- and IKKε-induced phosphorylation and activation of IRF3/7 [10,38]. Some studies demonstrated that TANK was not involved in IFN responses and was a negative regulator of pro-inflammatory cytokine production induced by TLR signaling, which led to the debate about how this protein functions in IFN induction and NF-κB activation [39]. Since TBK1 and IKKε are able to interact with TANK in mammals, it is possible that TANK may participate in IFN induction pathways by organizing TBK1/IKKε complexes [40–42]. In the innate immune response following RIG-I activation by invaded virus, TANK may serve as an adaptor connecting MAVS with TRAF3/TBK1/IKKε, which promotes phosphorylation and activation of IRF3/7, and subsequently leads to effective type I IFN production [10,24,26].

Recent findings on the role of TBK1/IKKε as well as IRF3 in IFN induction shed new light on the function of TANK, in which Encephalomyocarditis virus 3C protease attenuates type I IFN production through disrupting the TANK/TBK1/IKKε/IRF3 complex [43]. Results from these studies suggest that TANK might be an important modulator of type I IFN induction in both TLR signaling and RLR signaling during viral infections; however, the potential mechanism needs to be further explored. As plenty of signaling molecules including MAVS, TRAF2/3, IKKγ, TRIF, TBK1and IKKε were found to be associated with TANK and TANK was recruited into various signaling such as RLR pathway and TLR pathway, it is very interesting to explore the specific role of TANK in different signaling pathways [21,24,33].

In this study, bcTANK has been identified and characterized, which is aimed to elucidate the role of the teleost TANK homologue in host antiviral innate immunity. The amount of TANK expression has been proved to differ following different stimuli treatment and time treatment [25,26,34,44]. A previous study showed a number of signaling

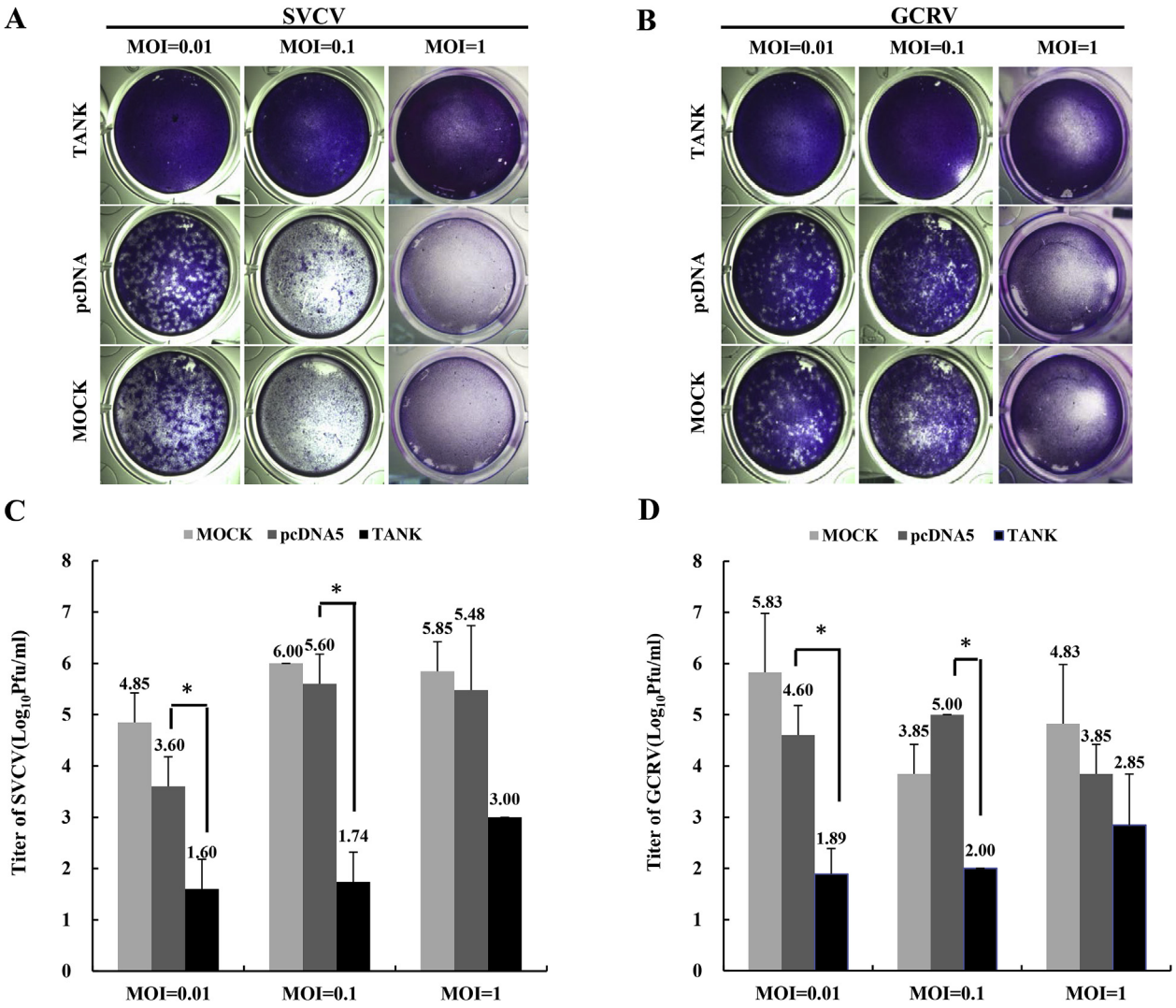

Fig. 5. Antiviral activity of bcTANK. EPC cells in 24-well plate were transfected with the plasmid expressing bcTANK or the empty vector separately and infected with GCRV/SVCV at the indicated MOIs at 24 h post transfection. The cell monolayers were stained with crystal violet (**A&B**) and the virus titers in the supernatant media were determined by plaque assay at 24 h post infection (**C&D**). The numbers above the error bars stand for average virus titers. Mock: EPC cells without transfection; pcDNA5: EPC cells transfected with pcDNA5/FRT/TO; TANK: EPC cells transfected with pcDNA5/FRT/TO-Flag-bcTANK.

molecules including MAVS, TRAF3 and TBK1 were found to be associated with TANK and the interaction among those signaling molecules were increased in a time-dependent manner [25]. Researches also demonstrated that anti-TANK immunoprecipitates caused IRF3 phosphorylation after stimulation by LPS and poly (I:C) and TANK enhanced poly (I:C)-mediated IRF3 phosphorylation [26]. To further elucidate the mechanism of TANK-mediated IFN induction by viral infection, the involvement of TANK in RIG-I pathways has been examined. It was found that overexpressed TANK itself did not induce type I IFN production, which was consistent with previous study [39]. However, the finding is contradictory to the study that overexpressed TANK alone promoted type I IFN activation [25]. What' more, the data generated in this paper demonstrate that bcTANK showed IFN-inducing ability when treated with different stimuli in reporter assay (Fig. 4) and presented antiviral activity against SVCV and GCRV in EPC cells (Fig. 5). It was reasonable that bcTANK functions as a positive modulator in host IFN signaling initiated by GCRV and SVCV. In our previous study, bcMAVS, bcTBK1, bcIFNb and bcIRF7 had been identified as crucial members of RLR signaling during black carp antiviral innate immune response [27,28,35,45]. It is speculated that bcTANK bridges bcMAVS with bcTBK1/bcIKKε through direct association or by recruiting other molecules such as bcTRAF2/3/6 like its mammalian counterpart, and leads to effective phosphorylation and activation of bcIRF3/7. However, the mechanism of bcTANK-regulated IFN signaling post GCRV/SVCV infection still needs to be extensively explored.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fsi.2018.07.019.

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