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Zebrafish FGFR3 is a negative regulator of RLR pathway to decrease IFN expression

Shu-Bo Liu[a,d], Long-Feng Lu[a], Xiao-Bing Lu[a,d], Shun Li[a,**], Yong-An Zhang[a,b,c,*]

[a] Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China
[b] State Key Laboratory of Agricultural Microbiology, College of Fisheries, Huazhong Agricultural University, Wuhan, China
[c] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China
[d] University of Chinese Academy of Sciences, Beijing, China

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ABSTRACT

Fibroblast growth factor receptor (FGFR) 3 is one of the four distinct membrane-spanning tyrosine kinases required for proper skeletal development. In fish, the role of FGFR3 is still unclear. In this article, we reveal that zebrafish FGFR3 is a negative regulator of interferon (IFN) production in the innate immune response by suppressing the activity of TANK-binding kinase 1 (TBK1) in the process of virus infection. qPCR experiments demonstrate that the transcriptional level of cellular FGFR3 was upregulated by infection with spring viremia of carp virus (SVCV), indicating that FGFR3 might be involved in the process of host cell response to viral infection. Then, overexpression of FGFR3 significantly impeded the IFN promoter activity induced by a stimulator. In addition, the capabilities of a retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) system to activate IFN promoter were decreased during the overexpression of FGFR3. Subsequently, FGFR3 decreased the phosphorylation of interferon regulatory factor 3 (IRF3) and mediator of IRF3 activation (MITA) by TBK1. These findings suggest that zebrafish FGFR3 is a negative regulator of IFN by attenuating the kinase activity of TBK1, leading to the suppression of IFN expression.

1. Introduction

Mammalian interferon (IFN) system is a crucial component of innate immunity, and it is the first line of defense against virus invasion [1]. Upon virus infection, host cellular pattern recognition receptors (PRRs) recognize viral nucleic acids and activate a series of intracellular signaling pathways, especially the retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) axis, eventually resulting in the production of IFN and the establishment of an antiviral state [2–4]. In the RLR system, RIG-I and melanoma differentiation-associated gene 5 (MDA5) appear to recognize and bind viral double-stranded RNA. Then, the downstream adaptor mitochondrial antiviral signaling protein (MAVS, also known as IPS-1, VISA, and Cardif) recruits and activates mediator of IRF3 activation (MITA, also known as STING) [5,6]. MITA is a scaffold protein that migrates in cells and recruits TANK-binding kinase 1 (TBK1), promoting the phosphorylation of interferon regulatory factor 3 (IRF3). The phosphorylated IRF3 forms homo- or hetero-dimers and translocates to the nucleus to induce the expression of IFN and other cytokines. Numerous studies have shown that fish RLR signaling pathways are conserved with those in mammals [5,7–10]. For example, fish RIG-I and MDA5 have been found to strongly induce IFN expression; fish IRF3 and MITA can be phosphorylated by TBK1 and activate IFN production [8,9].

Fibroblast growth factor receptors (FGFRs) are transmembrane tyrosine kinase receptors that regulate a variety of cellular functions, from embryogenesis to adult tissue homeostasis [11]. The FGFR family members contains an extracellular ligand-binding domain, a transmembrane domain, and a split intracellular tyrosine kinase domain. The extracellular region contains two or three immunoglobulin (Ig)-like domains (D1, D2, and D3) and a heparin-binding domain [12,13].

Abbreviations: CPE, cytopathic effect; EPC, epithelioma papulosum cyprini (cell); FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; IFN, interferon; IRF, IFN regulatory factor; ISRE, IFN stimulated response element; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation-associated gene 5; MITA, mediator of IRF3 activation; ORF, open reading frame; poly (I:C), polyinosinic: polycytidylic acid; qPCR, quantitative real-time PCR; PRR, pattern recognition receptor; RIG-I, retinoic acidinducible gene I; RLR, RIG-I-like receptor; STAT, the signal transducers and activators of transcription; SVCV, spring viremia of carp virus; TBK1, TANK-binding kinase 1; TM, transmembrane; ZFL, zebrafish liver (cell)

* Corresponding author. Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China.
** Corresponding author.
E-mail addresses: bob@ihb.ac.cn (S. Li), yzhang@ihb.ac.cn (Y.-A. Zhang).

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FGFRs bind to their ligands, the fibroblast growth factors (FGFs), with high affinity, and induce their dimerization and the phosphorylation of specific cytoplasmic tyrosine residues [14]. In vertebrates including humans, mice, and zebrafish, four functional FGFR genes, *fgfr1*, *fgfr2*, *fgfr3*, and *fgfr4*, have been identified [12,15]. Among these, FGFR3 serves as high-affinity receptors for at least nine FGFs [16,17]. Many functional studies provide clear evidence that FGFR3 is required for proper skeletal development [18–20]. FGFR3 exerts control over chondrocyte behavior through signaling molecules such as STAT1, MAPK, and PI3K/AKT [21,22]. In zebrafish, the insertion mutation of FGFR3 can result in the thanatophoric dysplasia [23]. Here, we report a novel negative regulation mechanism of zebrafish FGFR3 to modulate IFN expression. Our data show that zebrafish FGFR3 reduces the phosphorylation activity of TBK1, leading to a reduction in the phosphorylation of the downstream effector molecules MITA and IRF3 to decrease IFN expression. This finding suggests that fish FGFR3 plays a potential functional role in the antiviral immunity.

2. Materials and methods

2.1. Cells, viruses and transfection

Epithelioma papulosum cyprinid (EPC) cells were maintained at 28 °C, in 5% CO_2 and cultured in medium 199 (Invitrogen). HEK 293T cells were grown at 37 °C, 5.0% CO_2 in DMEM medium (Invitrogen). Zebrafish liver (ZFL) cells were cultured at 28 °C, 5% CO_2 in Ham's F-12 Nutrient Mixture medium (Invitrogen). All mediums were supplemented with 10% fetal bovine serum (FBS, Invitrogen) and penicillin-streptomycin (100 U/ml). Spring viremia of carp virus (SVCV, strain 741), a negative-sense ssRNA virus, was propagated in ZFL cells until cytopathic effect (CPE) was observed, then the cultured media were stored at −80 °C until use. HEK 293T and EPC cells were seeded in 10 cm^2 dishes or 24-well plates and 24 h later were co-transfected with indicated plasmids by using X-tremeGENE HP DNA Transfection Reagent (Roche) according to the manufacturer's protocol. ZFL cells were seeded in 6-well plates overnight and transfected with 1 mg/ml of poly I:C (transfection efficiency of about 5%) or infected with SVCV (10^5 $TCID_{50}$ (50% tissue culture infectious doses)) for 3, 6, 12, 24 and 48 h.

2.2. Gene cloning and plasmid construction

The sequence of zebrafish FGFR3 (accession No. NM_131606.2) was obtained from the NCBI (National Center for Biotechnology Information) website (http://www.ncbi.nlm.nih.gov/). Using the cDNA of the tissues from adult zebrafish as template, the open reading frame (ORF) of zebrafish FGFR3 was amplified by PCR and cloned into pCMV-Myc vector (BD Clontech). The ORFs of zebrafish MAVS (NM_001080584.2), TBK1 (NM_001044748.2), IRF3 (NM_001143904), IRF7 (NM_200677.2) and MITA (NM_001278837.1) were respectively subcloned into pCMV-HA, pCMV-Myc and pCMV-Tag2C vectors (BD Clontech) as described previously [9,24,25]. The plasmids containing IFNφ1pro-Luc, IFNφ3pro-Luc, and ISRE-Luc in pGL3-Basic luciferase reporter vectors (Promega) were constructed as described previously [9,26,27]. All constructs were confirmed by DNA sequencing. The primers including the restriction enzyme cutting sites used for plasmid construction are listed in Table 1.

2.3. Luciferase activity assay

EPC cells were seeded in 24-well plates, cultured for 24 h, and then co-transfected with 250 ng of the luciferase reporter plasmids (IFNφ1pro-Luc, IFNφ3pro-Luc or ISRE-Luc) and 25 ng of the *Renilla* luciferase internal control vector (pRL-TK) (Promega). The pRL-TK was used to normalize the transcriptional levels induced by the promoters and the empty vector pCMV-Myc was used to maintain equivalent amounts of DNA in each well. Stimulation with poly I:C was then done 24 h later. At 48 h post-transfection, the cells were washed with PBS and lysed for measuring luciferase activity by Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions and normalized to the amount of *Renilla* luciferase activities. The results were representative of more than three independent experiments, each performed in triplicate.

2.4. RNA extraction, reverse transcription, and quantitative real-time PCR

Total RNAs of ZFL cells and the tissues from adult zebrafish were extracted with Trizol reagent (Invitrogen). Reverse transcription was performed using the random primers within M-MLV reverse system (Promega). Quantitative real-time PCR (qPCR) was performed with Fast SYBR Green PCR Master mix (Bio-Rad) on the CFX96 Real-Time System (Bio-Rad). PCR conditions were as follows: 95 °C for 5 min, then 45 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s. All primers used for qPCR are shown in Table 1 and β-actin was used as an internal control. The relative fold changes were calculated by comparing them to the corresponding controls using the $2^{-\Delta\Delta Ct}$ method. Three independent experiments were conducted for statistical analysis.

2.5. Western blotting

Whole cell extracts were separated by 10% SDS-PAGE and then transferred to PVDF membrane (Bio-Rad). The membrane was blocked for 1 h at room temperature in TBST buffer (25 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.5) containing 5% nonfat dry milk, probed with indicated primary antibodies (Abs) at an appropriate dilution overnight at 4 °C, washed three times with TBST and then incubated with secondary Abs for 1 h at room temperature. After additional three washes with TBST, the membrane was stained with Immobilon TM Western Chemiluminescent HRP Substrate (Millipore) and detected using an ImageQuant LAS 4000 system (GE Healthcare). Abs were diluted as follows: anti-β-actin (Cell Signaling Technology) at 1:1000, anti-Flag/HA (Sigma-Aldrich) at 1:3000, anti-Myc (Santa Cruz Biotechnology) at 1:2000, HRP-conjugated anti-rabbit IgG or anti-mouse IgG (Thermo Scientific) at 1:5000. The results were the representative of three independent experiments.

2.6. In vitro protein dephosphorylation assay

Transfected HEK 293T cells were lysed as above, except that the phosphatase inhibitors (Na_3VO_4 and EDTA) were omitted from the lysis buffer. Protein dephosphorylation was carried out in 100 μl reactions consisting of 100 μg of cell protein and 10 units (U) of calf intestinal phosphatase (CIP, Sigma-Aldrich). The reactions were incubated at 37 °C for 1 h followed by immunoblot analysis.

3. Results

3.1. Induction patterns of zebrafish fgfr3 by SVCV and poly I:C infection

To investigate whether the expression of host *fgfr3* was regulated by viral infection, ZFL cells were stimulated with poly I:C or SVCV. Then, the total RNAs were extracted, and the transcriptional level of *fgfr3* was monitored by qPCR. As shown in Fig. 1A and B, the *ifnφ1* transcription level was upregulated about five-fold 24 h after being stimulated with SVCV. The *ifnφ1* was induced by transfection with poly I:C from 6 h, and it reached a peak at 12 h. Meanwhile, the mRNA levels of *irf3* were also monitored. After stimulation with SVCV, the expression of *irf3* was increased from 6 h; after treatment with poly I:C, the expression of *irf3* was decreased initially, and increased at 12 h (Fig. 1C and D). After stimulation with SVCV, the expression of *fgfr3* was also increased about two-fold at 12 h, while it decreased about 10-fold at 3 h after being stimulated with poly I:C. Considering that poly I:C contains no viral

Table 1
Primers used in this study.

Name	Sequence (5'→3')	Application
pCMV-Myc-FGFR3-F	CCGCTCGAGGGATGTCTGAGTCTGCTCTCCG	Eukaryotic expression
pCMV-Myc-FGFR3-R	ATAAGAATGCGGCCGCTTATGTTCGTATGACCCCG	
pCMV-Myc-MAVS-F	AAGCTAGCATGTCACTGACACGTGAGC	
pCMV-Myc-MAVS-R	AACTCGAGTTAATGATTGAGCTTCCAG	
pCMV-Myc/Tag2C-TBK1-F	CCGGAATTCCGATGCAGAGTACGGCCAAT	
pCMV-Myc/Tag2C-TBK1-R	AACTCGAGTCACATCCGCTCCACTG	
pCMV-Myc/HA-MITA-F	CCGGAATTCCGATGTCTGTGATGGGAGAA	
pCMV-Myc/HA-MITA-R	CCGCTCGAG TTAGTTTTGTTTCATTGC	
pCMV-Myc/HA-IRF3-F	CCGGAATTCCGATGACTCAAGCAAAACCG	
pCMV-Myc/HA-IRF3-R	AACTCGAGTTAGCAGAGCTCCATCA	
pCMV-Myc-IRF7-F	CCGGAATTCCGATGCAGAGCACAAATGC	
pCMV-Myc-IRF7-R	AACTCGAGTTATTCCACTGAAGGCA	
qFGFR3-F	CCTACGTCAATGTTCTTAAGACT	Real-time PCR
qFGFR3-R	TCTCTCCATCTCCACCGCTG	
qIRF3-F	AAGGTGCTGTCGGTGGTTTG	
qIRF3-R	ATGTGGTTGAGTGGCAGTCT	
qIFN1-F	AAGTTTTTAGTCCTGACATTGGATCA	
qIFN1-R	TCCCAGTTCACCGAGTTCATG	
β-actin-F	CACTGTGCCCATCTACGAG	
β-actin-R	CCATCTCCTGCTCGAAGTC	

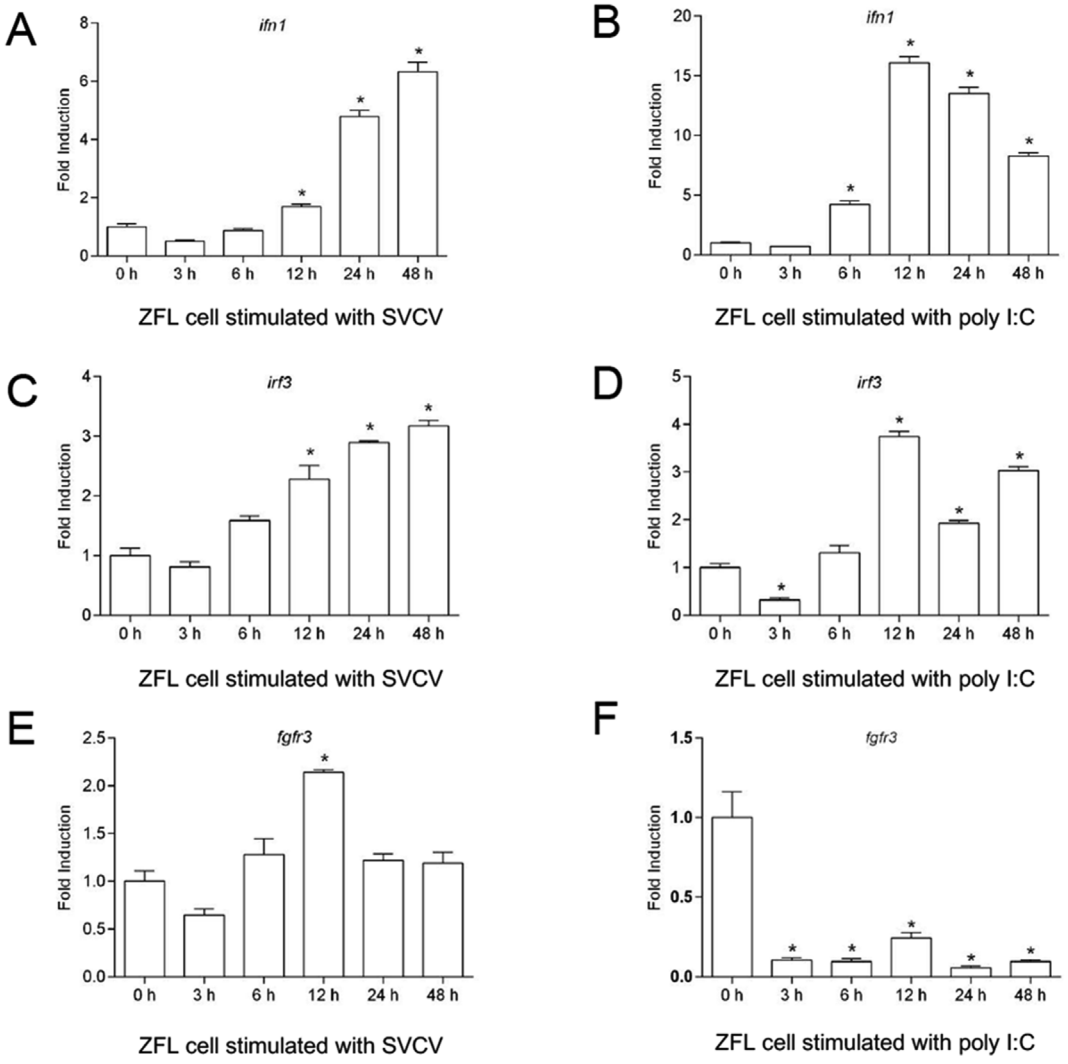

Fig. 1. Inducible expression patterns of FGFR3. ZFL cells were seeded in 6-well plates at 5×10^5 cells per well overnight and transfected with SVCV (10^5 $TCID_{50}$) (A, C and E) or poly I:C (1 μg/ml) (B, D, and F) for 3, 6, 12, 24 and 48 h. Then total RNAs were extracted to examine the mRNA levels of IFNφ1 (A and B), IRF3 (C and D) and FGFR3 (E and F) by qPCR. β-actin was introduced as endogenous control. Error bars represent the standard deviations (SDs) obtained by measuring each sample in triplicate. Asterisks indicate significant differences from control ($^*p < 0.05$).

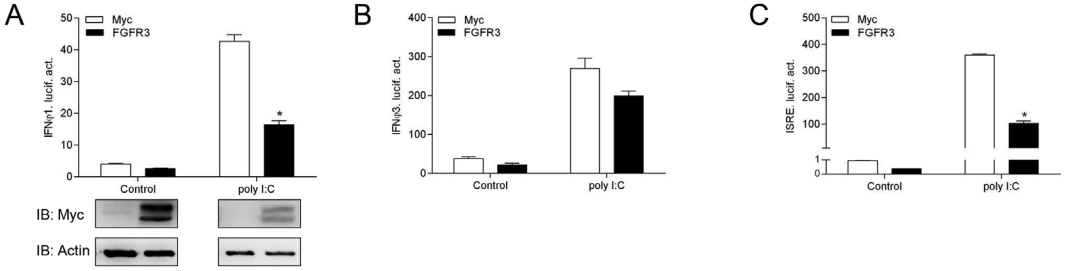

Fig. 2. Effects of FGFR3 on IFNφ1, IFNφ3 and ISRE promoter activities induced by poly I:C. EPC cells were seeded in 24-well plates at 10^5 cells per well overnight and co-transfected with 250 ng IFNφ1pro-Luc (A), IFNφ3pro-Luc (B) or ISRE-Luc (C) and 25 ng pRL-TK, plus 250 ng pCMV-Myc or pCMV-Myc-FGFR3. At 24 h post-transfection, cells were left untreated (null) or transfected with poly I:C (1 μg/ml). After 24 h stimulation, the cell lysates were harvested for Western blotting with the indicated antibodies and for luciferase assay. Error bars are the SDs obtained by measuring each sample in triplicate. Asterisks indicate significant differences from control (*$p < 0.05$).

proteins, the induction of *fgfr3* might be caused by the viral proteins of SVCV. These results suggest that FGFR3 might be involved in the process of virus infection.

3.2. FGFR3 inhibits poly I:C-stimulated IFNφ1pro and ISRE activities

Previous studies demonstrated that only IFNφ1 and IFNφ3 of the four type I IFNs (IFNφ1 to IFNφ4) in zebrafish were activated by poly I:C, indicating that IFNφ1 and IFNφ3 should be related to the antiviral process [9,25]. In the current study, poly I:C infection resulted in the loss of a small number of cells, but still induced the activation of IFNφ1pro; however, the IFNφ1pro induction stimulated by poly I:C was significantly impeded by the overexpression of FGFR3 (Fig. 2A). Similarly, FGFR3 also suppressed poly I:C-induced ISRE activity (Fig. 2C), suggesting that FGFR3 inhibits the activation of IFNφ1pro and ISRE upon infection with poly I:C. Interestingly, the overexpression of FGFR3 did not affect poly I:C-stimulated IFNφ3pro activity (Fig. 2B). These results demonstrate that the expression of IFNφ1 was suppressed by FGFR3.

3.3. FGFR3 blocks RLR-mediated activation of IFN promoter

Fish RLR factors are efficient for triggering IFN production [8,28]; consequently, these constructs were used in the following luciferase reporter gene assay. In light of the results demonstrating that FGFR3 inhibited IFNφ1 expression, the relationship between FGFR3 and the RLR pathway was investigated. As shown in Fig. 3A, the components of the zebrafish RLR system that activated IFNφ1pro acted as the positive control, while these inductions were decreased by the overexpression of FGFR3; Western botting showed that overexpression of FGFR3 led to partial degradation of the RLR molecules. Similarly, the capabilities of MAVS, MITA, IRF3, and IRF7 for activating IFNφ3pro were also decreased by overexpression of FGFR3, while TBK1-activated IFNφ3pro was unaffected (Fig. 3B). These results indicate that zebrafish FGFR3 suppresses IFNφ1 and IFNφ3 production via the negative regulation of the components of RLR system.

3.4. FGFR3 decreases activity of TBK1 kinase

Previous studies demonstrated that fish TBK1 is able to phosphorylate MITA and IRF3 via its functional kinase domain to activate IFN expression [24,29].In the current study, when IRF3-Myc or MITA-Myc was cotransfected with TBK1-Flag, shifted bands with higher molecular weights could be detected by the anti-Myc Ab. Furthermore, to confirm whether the shifted bands represented the phosphorylated IRF3 and MITA, a dephosphorylation assay was performed *in vitro*, and the shifted bands partially disappeared after being treated with CIP (Fig. 4A and B). To investigate whether the antagonization of IFN expression activated by the RLR pathway was regulated by FGFR3 on TBK1, 293T cells were co-transfected with the appropriate plasmids. The result

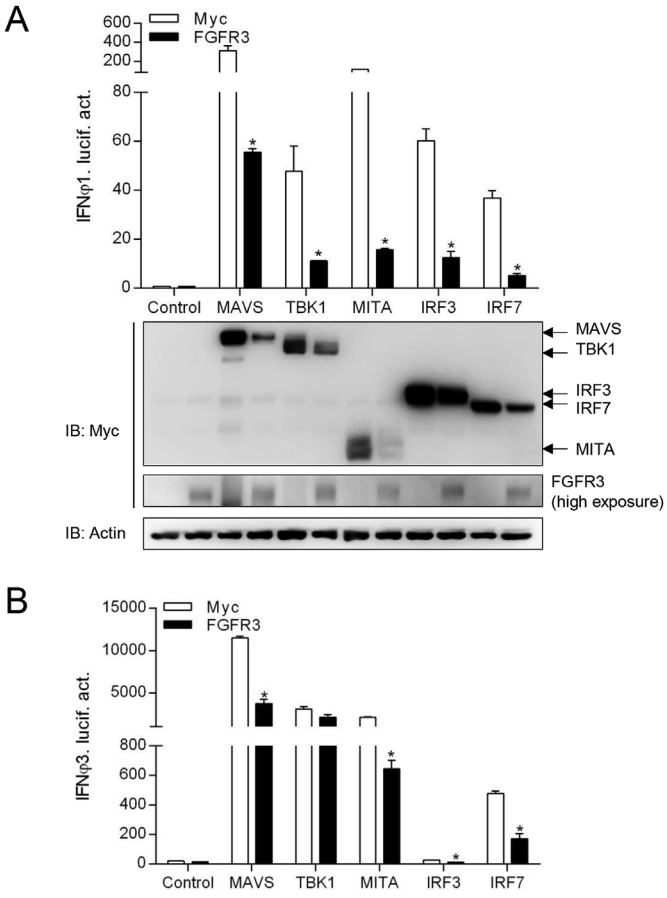

Fig. 3. FGFR3 blocks RLR-mediated activation of the IFNφ1 promoter. EPC cells were seeded in 24-well plates at 10^5 cells per well overnight and co-transfected with MAVS-, TBK1-, MITA-, IRF3- or IRF7-expressing plasmids and pCMV-Myc-FGFR3 plus IFNφ1pro-Luc (A) or IFNφ3pro-Luc (B) at a ratio of 1:1:1. pRL-TK was used as a control. At 24 h post-transfection, cells were collected for Western blotting with the indicated antibodies and for detection of luciferase activities. Error bars are the SDs obtained by measuring each sample in triplicate. Asterisks indicate significant differences from control (*$p < 0.05$).

showed that TBK1-induced phosphorylation of IRF3 and MITA declined because of FGFR3 overexpression (Fig. 4C and D). These data demonstrate that FGFR3 reduced the phosphorylation of IRF3 and MITA activated by TBK1.

4. Discussion

Growth factors and their receptors are natural proteins that stimulate cell growth, proliferation, and differentiation; few studies have

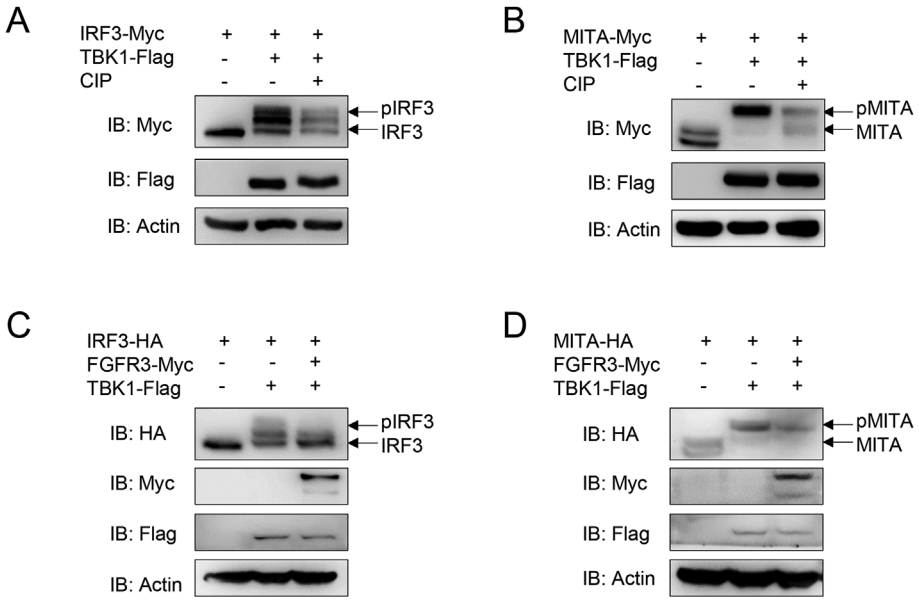

Fig. 4. FGFR3 abolishes the phosphorylation of MITA and IRF3 mediated by TBK1. (A and B) TBK1 mediates the phosphorylation of IRF3 and MITA. HEK 293T cells were seeded in 6-well plates at 10^7 cells per well overnight and transfected with the indicated plasmids (1 μg each) for 24 h. The cell lysates (100 μg) were treated with or without CIP (10 U) for 1 h at 37 °C. Then the lysates were detected by immunoblotting (IB) with the anti-Myc, anti-Flag, and anti-β-actin Abs. (C and D) Overexpression of FGFR3 inhibits TBK1-mediated phosphorylation of IRF3 and MITA. HEK 293T cells were seeded in 6-well plates overnight and transfected with the plasmids indicated (1 μg each) for 24 h. Whole-cell lysates were subjected to immunoblotting with the anti-Flag, anti-HA, anti-Myc, anti-Flag and anti-β-actin Abs.

identified that they are involved in innate immune regulation. Recently, mammalian FGF2 was shown to be related in innate immunity; that is, it affects the production of type I IFN by interacting with RIG-I [30]. For FGFR3, it has been reported that it is involved in bone remodeling and osteoclastogenesis and that osteoclasts of adult mice are positive for FGFR3 expression [31–33]. Interestingly, in the current study, we demonstrated for the first time, to our knowledge, that zebrafish FGFR3 negatively regulates the expression of IFN for a balanced immune response.

In the luciferase reporter gene assays we performed, zebrafish FGFR3 reduced IFNφ1 and ISRE transcription stimulated by poly I:C (a mimic of viral RNA). We speculate that FGFR3 may play multiple functional roles in fish; that is, besides participating in the progress of cell life, it may also be a regulator of IFN expression. The production of IFN activated by RLR molecules is necessary for the host to defend against a virus infection. However, the capabilities of the RLR system for activating IFNφ1pro and IFNφ3pro decreased during the overexpression of FGFR3. Indeed, the suppression of FGFR3 on IFNφ3pro was lower than that on IFNφ1pro, and FGFR3 could blunt IFNφ3pro activity induced by IRF3/7, but not poly I:C. The possible reason is that on the progress of the activation of IFNφ3pro by poly I:C, IRF3/7 are parts of the RLR pathway which response to the stimulation. Besides the RLR pathway, several signaling pathways are also activated by poly I:C, such as TLR and NOD pathways. The role of FGFR3 on these pathways is unclear, that might lead to the different effect on IFNϕ3 promoter activity induced by poly I:C or IRF3/7. Actually, we failed to observe the interaction between FGFR3 and any of the RLR molecules, while FGFR3 indeed decreased the activation of TBK1 and partially degraded the RLR molecules. That might be because FGFR3 was a receptor that mediated the signaling transduction to trigger some downstream gene transcription, and the productive protein(s) were associated with and sequestered TBK1, leading to the reduction of IFN production.

In mammals, a number of diseases are caused by uncontrolled IFN expression. Therefore, in order to control the excessive production of IFN, it is essential to investigate the host inhibiting molecules in the RLR pathway [34]. For example, mammalian IRF2 acting as an antagonist for IRF1 reduces IFN expression by binding to the promoter region of IFN [35–37]. Similarly, mammalian IRF4, as a negative regulator of IFN transcription, functions by competing with IRF5 for MyD88 interaction [38]. In zebrafish, several factors are also considered negative regulators for IFN expression. For example, zebrafish IRF10 inhibits IFN production by the obstruction of MITA-mediated IFN activation or competitive binding to the ISRE site of the IFN promoter [25]; STAT6 interacts with and dephosphorylates TBK1, which reduces IFNφ1 expression [39]; and MAVS-tv2 blocks the activation of IRF7 to inhibit IFN transcription [40]. In fish and mammals, TBK1 is essential for the phosphorylation of MITA and IRF3, which are transferred to the nucleus to activate the expression of IFN [9]. In our study, FGFR3 decreased the phosphorylation of IRF3 and MITA phosphorylated by TBK1, displaying that multiple negative regulation mechanisms exist in lower vertebrates.

In summary, our results provide insight into the relationship between FGFR3 and the RLR system in the innate immune response. This study has identified FGFR3 as a novel negative regulator of IFN transcription that functions by decreasing the phosphorylation of IRF3 and MITA. For similar ligands of the FGFR family, it is also conceivable that other FGFR family members might play roles in immune responses to certain pathogens, and further studies should be carried out to confirm this hypothesis.

Conflicts of interest

The authors have no conflicting commercial or financial interest in publishing this paper.

Acknowledgments

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