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Molecular cloning of Y-Box binding protein-1 from mandarin fish and its roles in stress-response and antiviral immunity

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ABSTRACT

Mandarin fish (*Siniperca chuatsi*) is a universally farmed fish species in China and has a large farming scale and economic value. With the high-density cultural mode in mandarin fish, viral diseases, such as infectious spleen and kidney necrosis virus (ISKNV) and *Siniperca chuatsi* rhabdovirus (SCRV), have increased loss, which has seriously restricted the development of aquaculture. Y-Box binding protein 1 (YB-1) is a member of cold shock protein family that regulates multiple cellular processes. The roles of mammalian YB-1 protein in environmental stress and innate immunity have been studied well, but its roles in teleost fishes remain unknown. In the present study, the characteristic of *S. chuatsi* YB-1 (*sc*YB-1) and its roles in cold stress and virus infection were investigated. The *sc*YB-1 obtained an 1541 bp cDNA that contains a 903 bp open reading frame encoding a protein of 300 amino acids. Tissue distribution results showed that the *sc*YB-1 is a ubiquitously expressed gene found among tissues from mandarin fish. Overexpression of *sc*YB-1 can increase the expression levels of cold shock-responsive genes, such as *scHsc70a*, *scHsc70b*, and *scp53*. Furthermore, the role of *sc*YB-1 in innate immunity was also investigated in mandarin fish fry (MFF-1) cells. The expression level of *sc*YB-1 was significant change in response to poly (I:C), poly (dG:dC), PMA, ISKNV, or SCRV stimulation. The overexpression of *sc*YB-1 can significantly increase the expression levels of NF-κB-responsive genes, including *scIL-8*, *scTNF-α*, and *scIFN-h*. The NF-κB-luciferase report assay results showed that the relative expression of luciferin was significantly increased in the cells overexpressed with *sc*YB-1 compared with those in cells overexpressed with control plasmid. These results indicate that *sc*YB-1 can induce the NF-κB signaling pathway in MFF-1 cells. Overexpressed *sc*YB-1 can downregulate the expression of ISKNV viral major capsid protein (*mcp*) gene but upregulates the expression of SCRV *mcp* gene. Moreover, knockdown of *sc*YB-1 using siRNA can upregulate the expression of ISKNV *mcp* gene but downregulates the expression of SCRV *mcp* gene. These results indicate that *sc*YB-1 suppresses ISKNV infection while enhancing SCRV infection. The above observations suggest that *sc*YB-1 is involved in cold stress and virus infection. Our study will provide an insight into the roles of teleost fish YB-1 protein in stress response and innate immunity.

1. Introduction

Temperature change is one of the most important environmental stresses for all organisms, profoundly affecting different life activities. Along the history of evolution, organisms have evolved a series of regulations in response to temperature change. At the molecular level, these regulations are predominantly demonstrated by heat or cold shock proteins [1]. As a member of cold shock protein family, Y-box binding protein 1 (YB-1, also termed as nuclease-sensitive element-binding protein 1, NSEP1) is a highly conserved and multifunctional protein that ubiquitously spreads among different organisms [2]. YB-1 consists of three parts: a cold shock domain (CSD), which stays

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Table 1
Primer sequences used in this study.

Primer	Sequence (5′–3′)	Usage
UPM	CTAATAGCACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	RACE PCR
5′ RACE	TTCGCAGTGTTGGAGATGGAG	
3′ RACE	AGGGCAAGCAGTGGTATCAACG	
Myc-*sc*YB-1-R	CGGGGTACCCTACTCATCCCCACCCTGC	cDNA Cloning
Myc-*sc*YB-1-F	GCAGGGTGGGGATGAGTAGGGTACCCCG	
GFP-*sc*YB-1-R	CGGGGTACCCCCTCATCCCCACCCTGC	
GFP-*sc*YB-1-F	CCGGAATTCGGATGAGCAGCGAGGCCGAGACAC	
*sc*YB-1-R	CACAGTTTCTCCATCTCCAACAC	Quantitative real-time PCR
*sc*YB-1-F	GTGTTGGAGATGGAGAAACTGTG	
*sc*Actin-R	ATGCTGTTGTAGGTGGTCTCGT	
*sc*Actin-F	GACATCAAGGAGAAGCTGTGCT	
ISKNV-MCP-R	ACCTCACGCTCCTCACTTGTC	
ISKNV-MCP-F	CAATGTAGCACCCGCACTGACC	
SCRV-MCP-R	TTTGTGGCAGAGTAAGGAGAC	
SCRV-MCP-F	AGTGCTGGATTGGGTGTTC	
*sc*Hsc70a-R	TGAACCTTTGGGCGAGTGTT	
*sc*Hsc70a-F	AGTGAGAGGCTGATCGGAGATG	
*sc*Hsc70b-R	CGACCATTTCCACCACATAACC	
*sc*Hsc70b-F	TCTTCTCAGGAGACTATATTGCGTTC	
*sc*p53-R	TGTCCAGCAACTCCAGACCATCA	
*sc*p53-F	CAGAACGGCACCAAGCAGACTAA	
*sc*IL-8-R	CAGCGTCTTTTGTTGGACATGA	
*sc*IL-8-F	TGCGAGGAGACTGAGATCATTG	
*sc*TNF-α-R	GCTTCTGTTTGGCCTGATTGAG	
*sc*TNF-α-F	TCTCGTTGTGGCCCTTTGTTT	
*sc*IFN-h-R	GGGACTCCACCTCTGCCTTT	
*sc*IFN-h-F	TGCGCTCTGCTGTGATTG	

homologous among the cold shock protein family, an A/P domain rich in Alanine and Proline, and a long C-terminal domain (CTD) [3].

Since YB-1 was first detected and reported in 1970s as a major mRNP in cytoplasm [4], its pleiotropic functions have been gradually uncovered. Most functions of YB-1 underlie in its interaction with nucleotides or other proteins [5]. It takes part in regulating basic life activities, including embryo development, cell proliferation, differentiation, and apoptosis [6–9]. As a stress-responsive gene, YB-1 responds to a variety of stresses in various species and enhances stress tolerance. Different kinds of stresses, such as cold shock, oxidant stress, heavy metal stress, hypoxia, and UV irradiation, have been found to trigger YB-1's response [10–12]. In these processes, YB-1 can act as a transcriptional factor that regulates the transcription of stress-responsive genes or binds to mRNA, modulating translation or forming stress granules [13]. For instance, YB-1 can stabilize hypoxia-inducible factor-1α (HIF-1α) mRNA and regulate HIF-1α-induced gene transcription under hypoxia [14]. It also activates heat shock protein 70 (Hsp70) mRNA translation and controls the formation of stress granules when facing arsenate stress *in vitro* [15].

YB-1 is also a key modulator of innate immunity in mammals. It can regulate expression of chemokine ligand genes, chemokine (C–C motif) ligand 2 (CCL-2) and CCL-5, at translational level [16]. Impaired cytokine secretion and diminished inflammatory response were observed in heterozygous YB-1 knockout mice (YB-$1^{+/d}$) stimulated with lipopolysaccharides [16]. Extracellular YB-1 can be detected in the serum of sepsis patients, in which it can function as a signaling molecule that recruits immune cells, such as macrophages [17]. In addition, YB-1 can bind to Rel A (p65) subunit in NF-κB pathway and enhance the transcriptional regulatory effect [18]. It attenuates gene transcription mediated by interferon regulatory factor 1, presenting double-edged effects on anti-viral gene expression [19]. Besides modulating immune responses, host YB-1 can directly interact with viral nucleotide or protein. For instance, YB-1 can bind to the 3′-untranslated region (UTR) of dengue virus RNA and suppress translation [20]. It interacts with non-structural proteins encoded by hepatitis C virus (HCV) and promotes viral replication [21]. In retrovirus infections, such as murine leukemia virus, YB-1 can stabilize viral genome RNA and regulate the replication of viral particles [22]. Ribonucleoprotein encoded by influenza A virus can be transported by YB-1 to microtubes [23]. These results indicate the complicated roles of YB-1 in immunity and host-pathogen interactions.

Mandarin fish (*Siniperca chuatsi*) is a universally farmed fish species in China with great farming scale and economic value. Unfortunately, spread and outbreaks of viral diseases, such as infectious spleen and kidney necrosis virus (ISKNV) and *Siniperca chuatsi* rhabdovirus (SCRV), have caused significant loss to mandarin fish culturing industries. To effectively prevent viral infection, studying the immunity of mandarin fish is of great scientific and economic significance. The roles of mandarin fish *S. chuatsi* YB-1 (*sc*YB-1) in stress responses, innate immunity, and host–virus interaction remain unknown. In the present study, *sc*YB-1 was molecularly cloned, and its roles in stress responses and innate immunity were investigated.

2. Materials and methods

2.1. Experimental animals, cells, and virus

Healthy mandarin fish were obtained from Nanhai farm (Guangdong, China) and kept at 27 °C in an aquarium. Before the experiments, the fish were cultured for at least two weeks and assured free from ISKNV or SCRV infection. The mandarin fish fry (MFF-1) cell line was constructed in our laboratory and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) at 27 °C under humidified atmosphere containing 5% CO_2 [24]. ISKNV and SCRV strains were separated from the diseased mandarin fish in 2017 and stored in our laboratory [25]. MFF-1 cells for infection were cultured overnight in 25 cm^2 flasks at 5×10^6 cells prior to further treatment. Each flask was inoculated with the virus suspension [multiplicity of infection (MOI = 1)]. The cells were harvested at different times according to the experimental design. Viral titers were calculated according to the Reed–Muench and Spearman–-Karber methods.

2.2. Antibodies and reagents

Alexa Fluor 488-labeled goat anti-mouse IgG and Hoechst 33342 were obtained from Thermo Fisher Scientific (USA). The chemicals used, including poly (I:C), poly (dG:dC), and phorbol 12-myristate 13-acetate (PMA), were purchased from Sigma–Aldrich (USA).

2.3. Molecular cloning of scYB-1 cDNA

Total RNAs were extracted from the liver of healthy mandarin fish using TRIZOL reagent and Eastep super total RNA extraction kit (Promega, USA). The RACE templates were synthesized by BD SMART RACE cDNA amplification kit (Clontech, USA). The primers for 5′ and 3′ RACE are shown in Table 1. Primers for cloning were designed based on the sequencing result of RACE PCR products. Then the first strand of cDNAs was synthesized by PrimeScript™ reverse transcription (RT) reagent kit (TaKaRa, Japan). Two pairs of primers shown in Table 1 were designed to amplify the cloning sequence of *sc*YB-1 for different vectors. The amplified products were digested and purified before ligation to pCMV-myc (Takara, Japan) or pEGFP-N3 (Takara, Japan) vectors. The plasmids were transformed into *E. coli*, in which positive samples were examined by PCR and then sequenced. The sequencing results were analyzed using DNAStar v6.0 software.

2.4. Sequence analysis

The deduced protein was analyzed using the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/blast) through BLAST function, which estimated its functional domain. Multiple alignment of the deduced *sc*YB-1 sequence was carried out through Clustalx v2.0. The YB-1 proteins of the other species were obtained from GenBank, with numbers as follows: *Carassius auratus* (BAA19849), *Danio rerio* (NP_571695), *Gallus gallus* (AAA02573), *Homo sapiens* (NP_004550), *Lates calcarifer* (XP_018544448.1), *Mus musculus* (AAH49977), *Oncorhynchus mykiss* (NP_001158512), *Oryctolagus cuniculus* (Q28618), *Oryzias latipes* (NP_001098143), *Salmo salar* (ACH71032), *Scophthalmus maximus* (ABH07676), *Larimichthys crocea* (XP_010738566.1), and *Xenopus tropicalis* (NP_001016677). A phylogenic tree was then constructed through MEGA v7.0 by Neighbor–Joining (N-J) method with 1000x replicates of bootstrap.

2.5. Tissue distribution of scYB-1 expression

To detect the tissue distribution of *sc*YB-1 expression, total RNAs were isolated from 12 different tissues of three healthy mandarin fish, including liver, blood, fin, posterior kidney, intestine, brain, fat, gill, head kidney, middle kidney, muscle, and spleen. All RNA samples were synthesized into cDNA using quantitative RT-PCR (qRT-PCR) template immediately after extraction.

2.6. Transcription of scYB-1 in response to stress in mandarin fish

To investigate the transcription of *sc*YB-1 in response to cold stress *in vivo*, qRT-PCR was employed. A total of 15 healthy mandarin fish were kept at 27 °C in an aquarium before the experiments and then gradually cooled down to 15 °C within 24 h. Liver, spleen, and kidney samples from the three fishes were collected at 0, 2, 4, 8, and 12 h post cooling, and then the transcriptions of *sc*YB-1 were conducted by qRT-PCR assays.

2.7. Transcription of scYB-1 in response to immune challenge in MFF-1 cells

To investigate the transcription of *sc*YB-1 regulated by the immune challenge, its expression levels were determined after cells were treated with immune stimulus using qRT-PCR assays. MFF-1 cells were treated with poly (I:C), poly (dG:dC), and PMA at a concentration of 10 μM, and then were harvested at 0, 12, and 24 h post stimulation. To investigate the transcription of *sc*YB-1 in response to virus infection, the expression profiles of *sc*YB-1 were carried out after ISKNV or SCRV challenges. MFF-1 cells were infected with ISKNV (MOI = 1) or SCRV (MOI = 1). Cell samples were collected at 0, 12, 24, 36, and 48 h post infection (hpi), and then the qRT-PCR assays were carried out.

2.8. qRT-PCR assay

qRT-PCR was performed with SYBR premix Ex Taq™ (Takara, Japan) on a LightCycler 480 instrument (Roche Diagnostics, Switzerland). The primers for qRT-PCR were designed using Primer Express software (Applied Biosystems) (Table 1). For tissue-specific expression analysis, the total RNAs from different tissues were prepared as previously described and were reverse transcribed using PrimeScript™ RT reagent kit (Takara, Japan) following the manufacturer's protocol. The expression levels of *sc*YB-1 and *sc*YB-1 downstream genes (*scHsc70a*, *scHsc70b*, and *scp53*) were detected using the corresponding forward and reverse primers (Table 1). Primers for *scHsc70a*, *scHsc70b*, and *scIL-8* genes were obtained from previous studies [26,27]. All qRT-PCR reactions were performed in triplicate. PCRs were performed on a total reaction volume of 10 μL, containing 0.2 μM primers, 1 μL of cDNA, 5 μL of 2 × SYBR premix ExTaq™, and 3.6 μL of ultrapure water using the following setting: 40 cycles of amplification (5 s at 95 °C, 40 s at 60 °C, and 1 s at 70 °C). The expression level of each transcript was normalized to the expression of the *β-actin* gene, which was used as an internal housekeeping control. The qRT-PCR data of the target genes were analyzed using the Q-gene statistics add-in, followed by unpaired sample *t*-test. Statistical significance was accepted at $p < 0.05$, and high significance was accepted at $p < 0.01$. All data were expressed as mean ± standard deviation.

2.9. Cell transfection

According to standard methods, transient transfection of plasmids was conducted with Lipofectamine™ 2000 (Thermo Fisher Scientific, USA) in accordance with the manufacturer's instructions. Prior to transfection, MFF-1 cells were directly seeded in 24-well cell culture plates for subcellular localization analysis or luciferase reporter assays. Cells were transfected using Lipofectamine™ 2000 in a serum-free culture medium (Opti-MEM, Gibco). After 2–4 h, the mixture was replaced with DMEM supplemented with 10% FBS. For siRNA transfection, the HiPerFect Transfection Reagent (Qiagen, USA) was used. Prior to transfection, MFF-1 cells were directly seeded in six-well cell culture plates with 2 mL of an appropriate culture medium containing FBS and antibiotics. Then 3 μL of HiPerFect Transfection Reagent was added to the diluted si-*sc*-YB-1 (5′ CGTAGAGTTTGATGTGATT 3′) and mixed by vortexing. The control siRNA (siRNA-NC) was obtained from Guangzhou Ribobio Co., Ltd. The samples were incubated for 5–10 min at room temperature (approximately 25 °C) to allow the formation of transfection complexes. The complexes were added dropwise onto the cells, and the plate was gently swirled to ensure uniform distribution of the transfection complexes. The cells were then incubated with the transfection complexes under their normal growth conditions, and gene silencing was monitored after an appropriate time (e.g., 6–72 h after transfection, depending on the experimental setup). The medium was changed as required.

2.10. Subcellular localization of scYB-1 protein

For the subcellular localization analysis of *sc*YB-1 protein, immunofluorescence assay was employed. The endo-free plasmid was transfected into the MFF-1 cells using Lipofectamine™ 2000. At 48 h post-transfection, MFF-1 cells were washed with sterile PBS buffer (pH 7.4) and fixed in PBS containing 3.7% paraformaldehyde for 10 min at room temperature. Fixed cells were permeabilized with 0.5% Triton X-

100, blocked for 1 h with 5% normal goat serum in PBS, and then labeled with the antibodies. Antibody binding was detected using the antibody conjugated with Alexa Fluor 488 (ThermoFisher, UAS). Hoechst 33342 (ThermoFisher, UAS) was used to counterstain the cell nucleus. The samples were observed under a confocal microscope (Zeiss LSM510, Germany).

2.11. Luciferase reporter assay

MFF-1 cells were cultured in 24-well plates for 24 h and then co-transfected with the luciferase reporter plasmid (pGL3-NF-κB-luc, 0.4 μg per well), each tested plasmid (0.4 μg per well), and pRL-TK (40 ng per well) plasmid. The pRL-TK plasmid was transfected as an internal control. After 2 h, the transfection mixture was replaced with 500 μL of DMEM. After 48 h transfection, the total cell lysates were analyzed using Dual Luciferase Reporter Gene Assay Kit (Promega, USA) in accordance with the manufacturer's instructions. Luciferase activity was measured using Glomax (Promega, USA). All experiments were performed in at least three independent experiments with three technical replicates for each experiment.

3. Results

3.1. Molecular cloning and sequence analysis of scYB-1

The cDNA fragment of *sc*YB-1 was obtained by RACE PCR using the universal primer mix (UPM), 5′ RACE and 3′ RACE primers. As shown in Fig. 1A, the full-length cDNA of *sc*YB-1 (MK452250) is 1541 bp, including a 5′-UTR of 219 bp, a 3′-terminal UTR of 419 bp, and a 903 bp open reading frame encoding a protein of 300 amino acids. BLAST homology analysis showed that the deduced amino acid sequences of *sc*YB-1 matched well with that of YB-1 from other species and contained a highly conserved CSD, an A/P domain rich in alanine and proline, and a long CTD (Fig. 1B). Multiple amino acid alignments showed that *sc*YB-1 was highly homologous with other vertebrate YB-1 proteins, sharing a similar architecture with vertebrate YB-1 proteins (Fig. 1C). Phylogenetic analysis results showed that *sc*YB-1 was closest in homology with Sea perch (*L. calcarifer*) YB-1 among the 13 chosen vertebrates, followed by *S. maximus*, *L. crocea*, and *O. latipes* YB-1 proteins. Interestingly, *D. rario* and *C. auratus* YB-1 proteins were distinguished in a different subfamily and displayed lowest homology with *sc*YB-1 among the chosen teleost, which is followed by *O. mykiss* and *S. salar* YB-1 proteins in another subfamily.

3.2. Tissue distribution and subcellular localization of scYB-1

The relative expression levels of *sc*YB-1 mRNA in different tissues were detected by qRT-PCR. As shown in Fig. 2A, the *sc*YB-1 mRNAs were detected in all 12 tissues collected, and the most abundant expression amount was detected in the liver, which is approximately 13-fold that in the spleen. Relatively abundant expression amounts were also found in blood, brain, intestine, gill, and fin; while in the fat, muscle, head, kidney, and spleen, *sc*YB-1 mRNA turned to be deficient, and the least expression amount was detected in the spleen. To investigate the subcellular location of *sc*YB-1, pEGFP-*sc*YB-1 plasmid was transfected into the MFF-1 cells. As shown in Fig. 2B, the green fluorescence was mostly observed in the cytoplasm and very little in the nucleus, indicating that *sc*YB-1 is mainly located in the cytoplasm under normal conditions.

3.3. Response of scYB-1 expression to cold stress in mandarin fish

To investigate the transcription level of *sc*YB-1 in response to stress, qRT-PCR was used to detect the mRNA level of *sc*YB-1 under cold stress in mandarin fish. As shown in Fig. 3A, the expression levels of *sc*YB-1 were significantly upregulated in the spleen and kidney of mandarin fish after 2–12 h post cold stress. The expression levels of *sc*YB-1 were observably upregulated by 2.11- and 1.69-fold ($p < 0.01$) by cold stress, reaching their peaks at 8 h post cooling and then downregulating at 12 h post cooling; whereas the transcription of *sc*YB-1 exhibited no significant change in the liver. Furthermore, the expression levels of *scHsc70a*, *scHsc70b*, and *scp53* were detected after cold stress in the liver, kidney and spleen. As shown in Fig. 3B, the expression level of *scHsc70a* was upregulated after cold stress either in liver, kidney or spleen; the expression level of *scHsc70b* was significantly downregulated after cold stress either in liver, kidney or spleen; the expression level of *scp53* in the liver and kidney was significantly upregulated, but no significantly change in the spleen after cold stress. Above results suggested that the expression levels of *scHsc70a* and *scp53* were positive correlation with cold stress, but the expression level of *scHsc70b* was negative correlation with cold stress in the tissues of mandarin fish. These results suggested that *sc*YB-1 responds to cold stress in mandarin fish.

3.4. scYB-1 regulates the expressions of cold-stress-responsive gene in MFF-1 cells

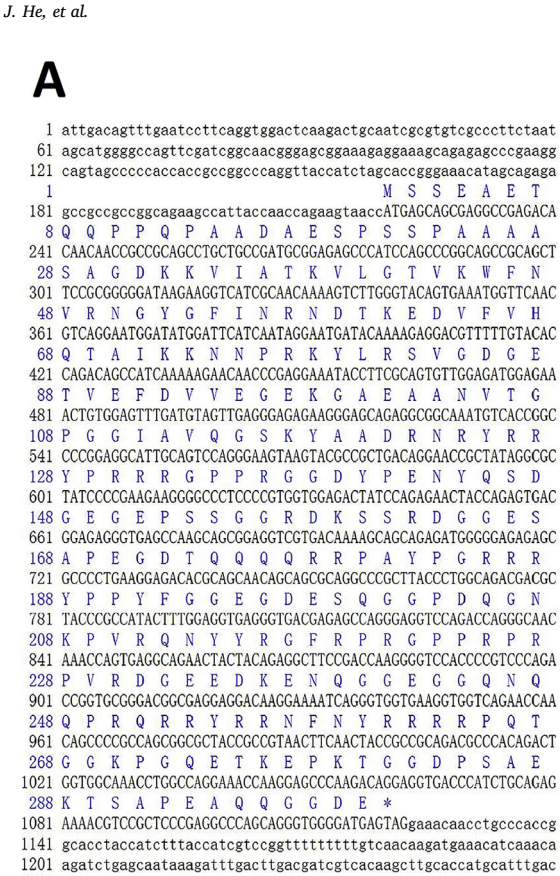
As a stress-responsive protein, YB-1 plays key roles in stress-responsive process through regulation of down-streamed genes, such as *Hsc70a*, *Hsc70b*, and *p53* genes [28,29]. To investigate the *sc*YB-1 regulation of down-streamed genes, the expressions of *scHsc70a*, *scHsc70b*, and *scp53* genes were detected by qRT-PCR. As shown in Fig. 3C, the expression levels of *scHsc70a*, *scHsc70b*, and *scp53* genes were significantly enhanced by 3.07-, 3.11-, and 2.90-fold ($p < 0.01$), respectively, after the cells were transfected with the pCMV-myc-*sc*YB-1 plasmid compared with those in the cells were transfected with pCMV-myc. These results suggested that *sc*YB-1 might be an important regulator when the mandarin fish counter cold stress.

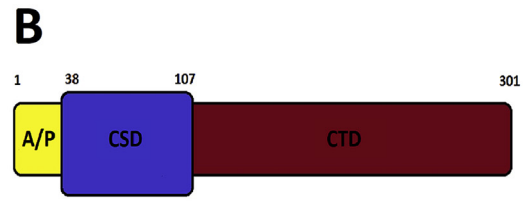
3.5. Response of scYB-1 expression to immune stimulation in MFF-1 cells

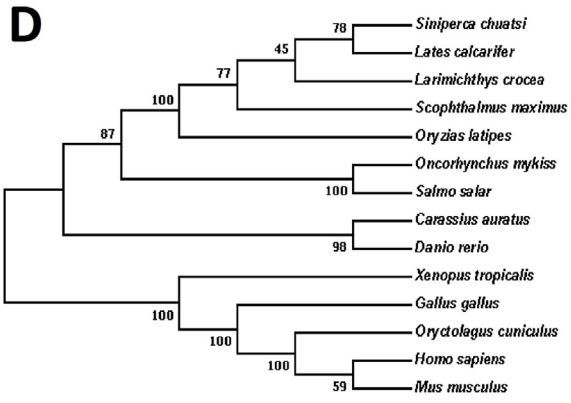
The immune stimulation assay was used on the MFF-1 cells, which were treated with poly (I: C), poly (dG: dC), or PMA. As shown in Fig. 4A, the expression levels of *sc*YB-1 respond to all three kinds of stimuli. For poly (I: C) stimulation, the expression level of *sc*YB-1 was downregulated to 0.58-fold at 12 h post stimulation ($p < 0.01$) and was upregulated to 1.15-fold at 24 h post stimulation ($p > 0.05$, no statistical significance observed compared with that at 0 h). While under both poly (dG: dC) and PMA stimulation, the expressions of *sc*YB-1 showed an upregulating trend until 24 h post stimulation, reaching 1.90- and 1.61-fold ($p < 0.01$). Furthermore, the expression levels of *sc*YB-1 were detected after cells were challenged by DNA virus (ISKNV) or RNA virus (SCRV). As shown in Fig. 4B, the expression levels of *sc*YB-1 fluctuated significantly after cells were infected with ISKNV or SCRV. After cells were challenged by ISKNV, the expression levels of *sc*YB-1 exhibited peaks at 12 hpi (4.38-fold) and gradually downregulated from 24 h to 48 h. While the MFF-1 cells were challenged by SCRV, the expression level of *sc*YB-1 was gradually upregulated from 12 to 36 hpi, presenting a peak at 36 hpi; then the expression level was downregulated at 48 hpi. These results suggested that the transcription of *sc*YB-1 responds to immune stimulation, indicating that *sc*YB-1 might be involved in the innate immune response in mandarin fish.

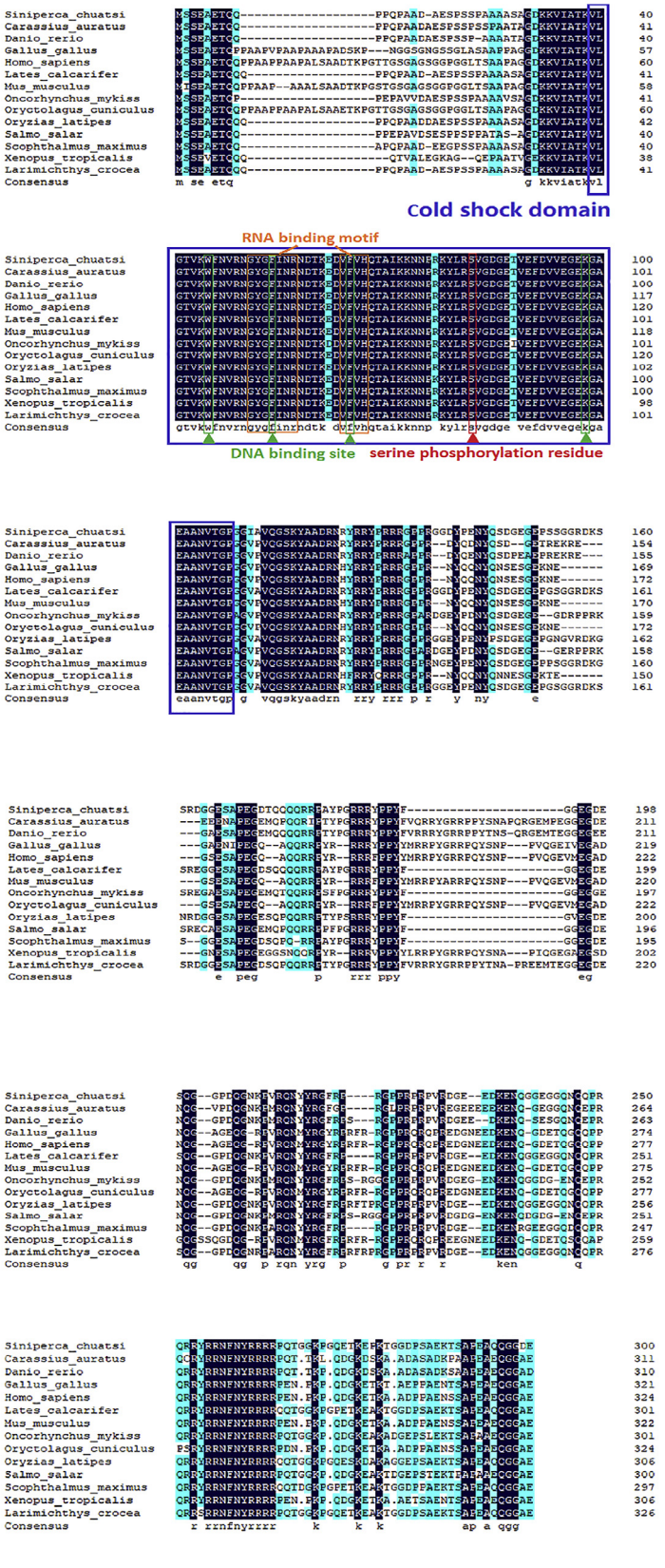
3.6. Overexpression of scYB-1 can activate the NF-κB pathway in MFF-1 cells

To further investigate the role of *sc*YB-1 in innate immune response *in vitro*, the NF-κB pathway activity was detected by dual luciferase reporter assays when cells were overexpressed with *sc*YB-1. The plasmids of pGL3-NF-κb-luc, pCMV-myc-*sc*YB-1, and pRL-TK were transiently co-transfected into MFF-1 cells, as control cells were transfected with pGL3-NF-κb-luc, pCMV-myc, and pRL-TK. The luciferase report









(caption on next page)

Fig. 1. Bioinformatics analysis of *sc*YB-1 sequence. (A) Cloned cDNA sequence and deduced amino acid sequence of *sc*YB-1. The amino acid sequence is shown in blue letters, and the open reading frame is shown in capital. The start and ending codons are marked by M and an asterisk, respectively. (B) Domain organization of *sc*YB-1 protein was predicted by NCBI website. CSD indicated CSD, A/P indicated a domain rich in alanine and proline, and CTD indicated C-terminal domain (C) Multiple alignment of *sc*YB-1 and known YB-1 protein sequences from other species. Identical and similar aa residues are shown in white character + red background and red character + white background, respectively. The conserved CSD is indicated by the blue box, in which the RNA binding motif and DNA binding site are indicated by yellow and black boxes, respectively. (D) Phylogenetic analysis of *sc*YB-1 and other known YB-1 protein sequences constructed by Neighbor–Joining method with 1000 bootstraps. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

assay results showed that the relative expression of luciferin was increased 2.7 times in the cells transfected with the pCMV-myc-*sc*YB-1 plasmid compared with those in the cells were transfected with pCMV-myc (Fig. 5A). To further confirm these results, the expressions of NF-κB-regulated downstream genes were detected by qRT-PCR. The relative expression levels of *scIL-8*, *scTNF-α*, and *scIFN-h* genes were significantly enhanced 4.22-, 1.81-, and 4.75-fold ($p < 0.01$), respectively, when cells were transfected with the pCMV-myc-*sc*YB-1 plasmid, compared with those in the cells were transfected with pCMV-myc (Fig. 5B, C, and D). These results suggested that *sc*YB-1 can induce the NF-κB pathway activation in MFF-1 cells.

3.7. Role of scYB-1 in virus infection

To investigate the roles of *sc*YB-1 in virus replication, cells were transfected with pCMV-*sc*YB-1 or siRNA-*sc*YB-1 and then infected with ISKNV or SCRV. The expression levels of viral major capsid protein (*mcp*) gene were determined by qRT-PCR. As shown in Fig. 6A, the relative expression level of ISKNV *mcp* gene in the cells were transfected with pCMV-myc-*sc*YB-1 was significantly lower than the control group at 72 h post ISKNV infection ($p < 0.01$). While knockdown of *sc*YB-1 leads to reverse result, the relative expression levels of ISKNV *mcp* gene in the cells transfected with siRNA-*sc*YB-1 was significantly higher than that of the control group at 72 h post ISKNV infection ($p < 0.01$). These results indicated that *sc*YB-1 plays an important role in the antiviral effects against ISKNV infection. However, *sc*YB-1 exhibited contrary effects on SCRV replication. At 36 h post SCRV infection, the relative expression level of SCRV *mcp* gene in the cells transfected with pCMV-*sc*YB-1 was significantly higher than that of the control group ($p < 0.01$) (Fig. 6B). Knockdown assay showed that the relative expression level of SCRV *mcp* gene in the cells transfected with siRNA-*sc*YB-1 was significantly lower than that of the control group ($p < 0.05$). Above observations suggested that *sc*YB-1 might inhibit ISKNV replication but promote SCRV replication, indicating that *sc*YB-1 might play a complicated role in host–virus interactions.

4. Discussion

In the present study, the full-length cDNA of mandarin fish *S. chuatsi* YB-1 (*sc*YB-1) was cloned. The *sc*YB-1 was an ubiquitously-expressed gene found in the tissues of mandarin fish. As a member of cold shock protein family, the *sc*YB-1 could also respond to cold stress. Overexpression of *sc*YB-1 could increase the expressions levels of *scHsc70a*, *scHsc70b*, and *scp53* genes. Furthermore, the roles of *sc*YB-1 in innate immunity were also investigated. The expression of *sc*YB-1 could respond to the stimulation of poly (I:C), poly (dG:dC), PMA, ISKNV, or SCRV, and its overexpression could significantly induce the NF-κB signaling pathway activation. The relationship between the *sc*YB-1 and mandarin fish virus was presented; *sc*YB-1 suppressed ISKNV infection but enhanced SCRV infection. The above observations suggested that *sc*YB-1 was involved in cold stress and virus infection. Our results will provide an understanding of the roles of teleost fish YB-1 protein in innate immunity.

As a stress-responsive protein, previous studies indicate that YB-1 can respond to stress. In oysters (*Crassostrea hongkongensis*), YB-1 responds to heat stress by positively regulating the transcription of *Hsc70* gene [28]. In bees (*Apis cerana*), YB-1 can respond to a series of stresses by regulating the transcriptions of stress-tolerant genes and activities of related enzymes [10]. In turbots (*Scophthalmus maximus*), YB-1 responds to viral and bacterial infection both *in vivo* and *in vitro* [30]. These results suggest that YB-1 can respond to different stresses in diverse approaches. In our study, *sc*YB-1 was shown to respond to cold stress *in vivo*. Cold stress (15 °C) significantly upregulated the mRNA amount of *sc*YB-1 in the spleen and kidney of mandarin fish. However, no significant transcriptional response of *sc*YB-1 was presented in the liver, implicating that *sc*YB-1 might function through different approaches in different organs under cold stress. Both *Hsc70* and *p53* were identified as stress-responsive genes in previous studies. Hsc70 is a multi-functional protein that can respond to a series of environmental stress, in which it can act as a molecular chaperone that maintains protein homeostasis [31]. Upregulation of p53 expression is another vital part of cold stress response *in vivo* in Japanese pufferfish (*Takifugu rubripes*) [32]. *In vitro* assay similarly suggests that modulation of p53 expression is a key approach in response to cold stress following recovery in mammalian cell lines [33]. In the present study, overexpression of *sc*YB-1 could also upregulate the expressions of *scHsc70* and *scp53* genes. This result implied that the *sc*YB-1 might play a role in regulating cold stress.

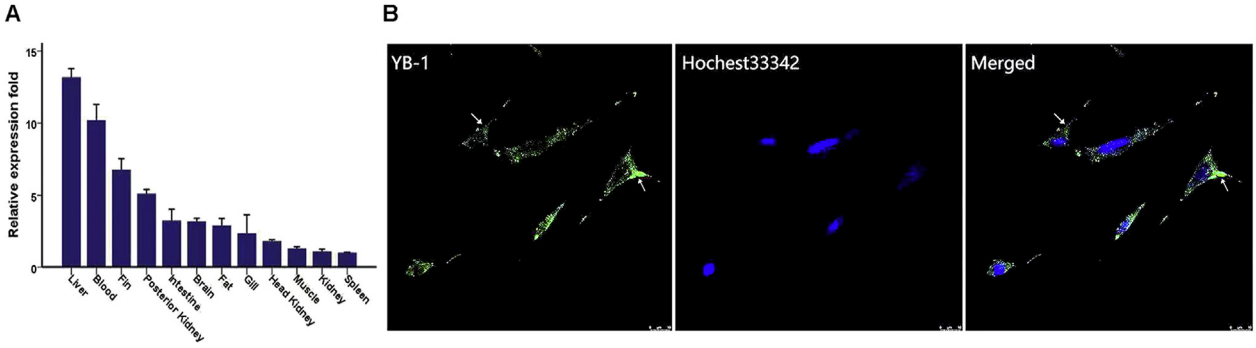


Fig. 2. Tissue distribution and subcellular localization of *sc*YB-1. (A) Tissue distribution of *sc*YB-1 expression in 12 different tissues. Samples were harvested from three healthy mandarin fish and tested through qRT-PCR. The β-actin gene was used as internal control. Raw data were analyzed by $2^{-\Delta\Delta Ct}$ method, and the expression in the spleen (lowest) was utilized as a calibrator. Vertical bars represent ± SD ($n = 3$). (B) Subcellular localization of *sc*YB-1 protein. MFF-1 cells were transfected with pEGFP-*sc*YB-1 plasmid. After allowing the cells to adhere for 48 h in 24-well plates, the nucleus was stained with Hoechst 33342, and fluorescent signals were observed under a fluorescence microscope. Magnification: × 630.

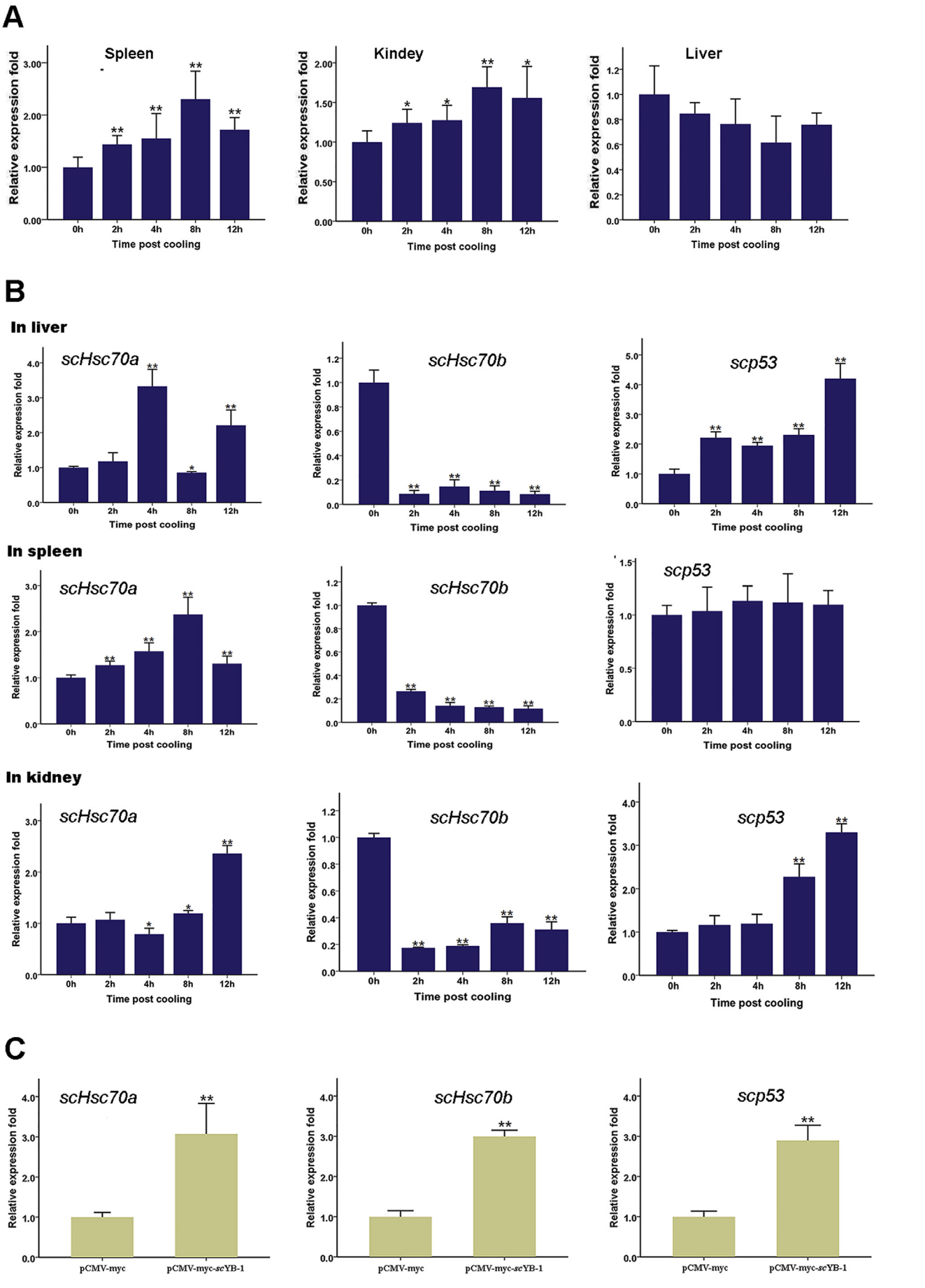


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Fig. 3. Transcription of *sc*YB-1 in response to cold stress stimulation. (A) Expression profiles of *sc*YB-1 in cold stress in the spleen, kidney, and liver of mandarin fish. (B) Expression levels of the *scHsc70a, scHsc70b and scp53* genes in the spleen, kidney and liver of mandarin fish after 2–12 h post cold stress. Vertical bars represent ± SD ($n = 3$). Statistical significance was indicated by asterisks, with * referring to $p < 0.05$ and ** referring to $p < 0.01$. (C) Expression profiles of *scHsc70a*, *scHsc70b*, and *scp53* genes were detected by qRT-PCR after the cells were transfected with pCMV-myc-*sc*YB-1. Cells transfected with pCMV-myc served as control. Vertical bars represent statistical significance as indicated by asterisks, with * referring to $p < 0.05$ and ** referring to $p < 0.01$.

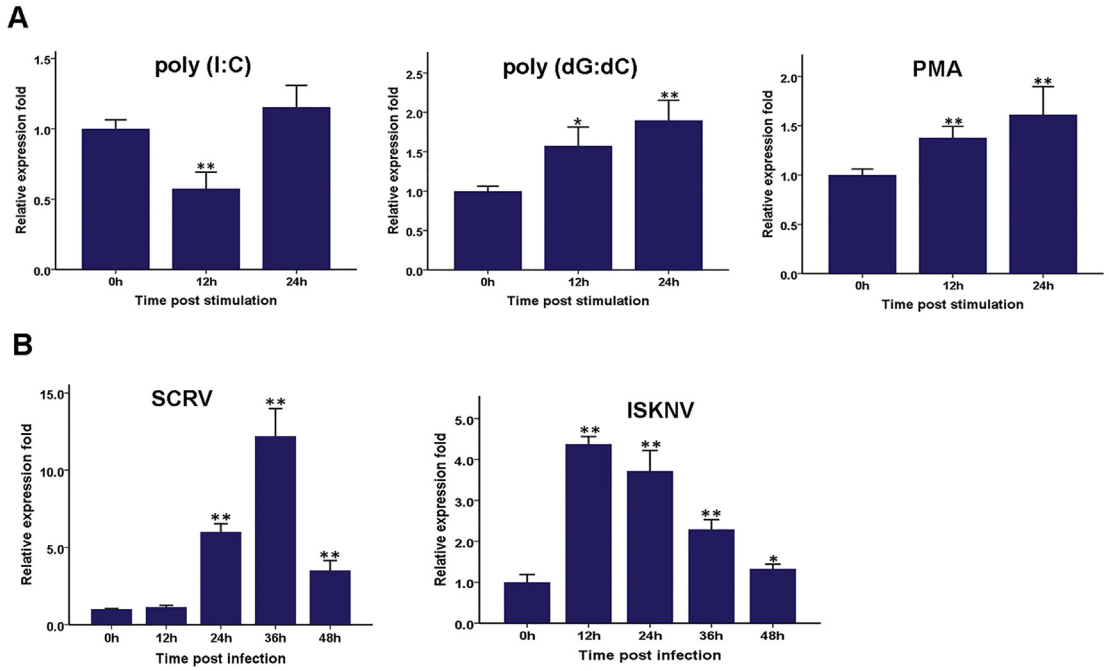

Fig. 4. Expression profiles of *sc*YB-1 in response to immune stimulations. (A) The expression levels of *sc*YB-1 were detected after cells were treated with poly (I: C), poly (dG: dC), or PMA at indicated times. (B) Expression profile of *sc*YB-1 upon ISKNV or SCRV infection at the indicated time. Vertical bars represent ± SD ($n = 3$). Statistical significance was indicated by asterisks, with * referring to $p < 0.05$ and ** referring to $p < 0.01$.

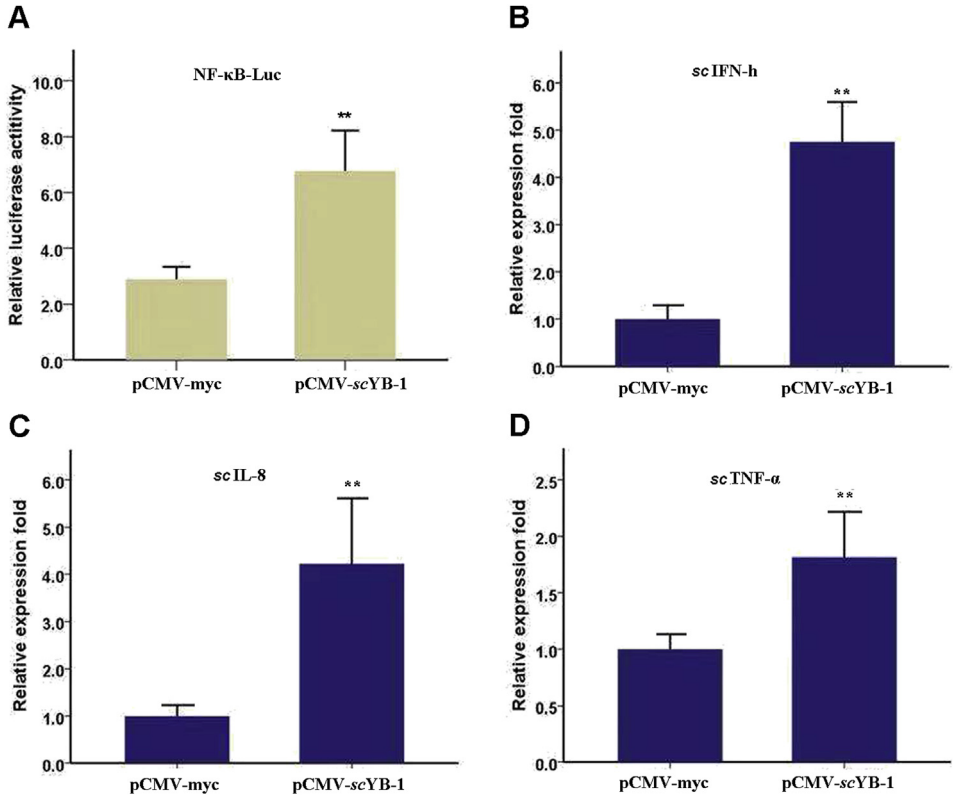

Fig. 5. Overexpression of *sc*YB-1 activates NF-κB pathway. (A). Relative luciferase activity of dual luciferase was detected after MFF-1 cells were transfected with pCMV-myc-*sc*YB-1. Cells transfected with pCMV-myc served as a control. (B–D) The expression fold change of NF-κB-targeted genes (*scIFN-h*, *scIL-8*, and *scTNF-α*) following *sc*YB-1 overexpression. Cells transfected with pCMV-myc served as control. The expression or luciferase activity level of the control group was used as calibrator. Vertical bars represent ± SD ($n = 3$). Statistical significance is indicated by asterisks, with * referring to $p < 0.05$ and ** referring to $p < 0.01$.

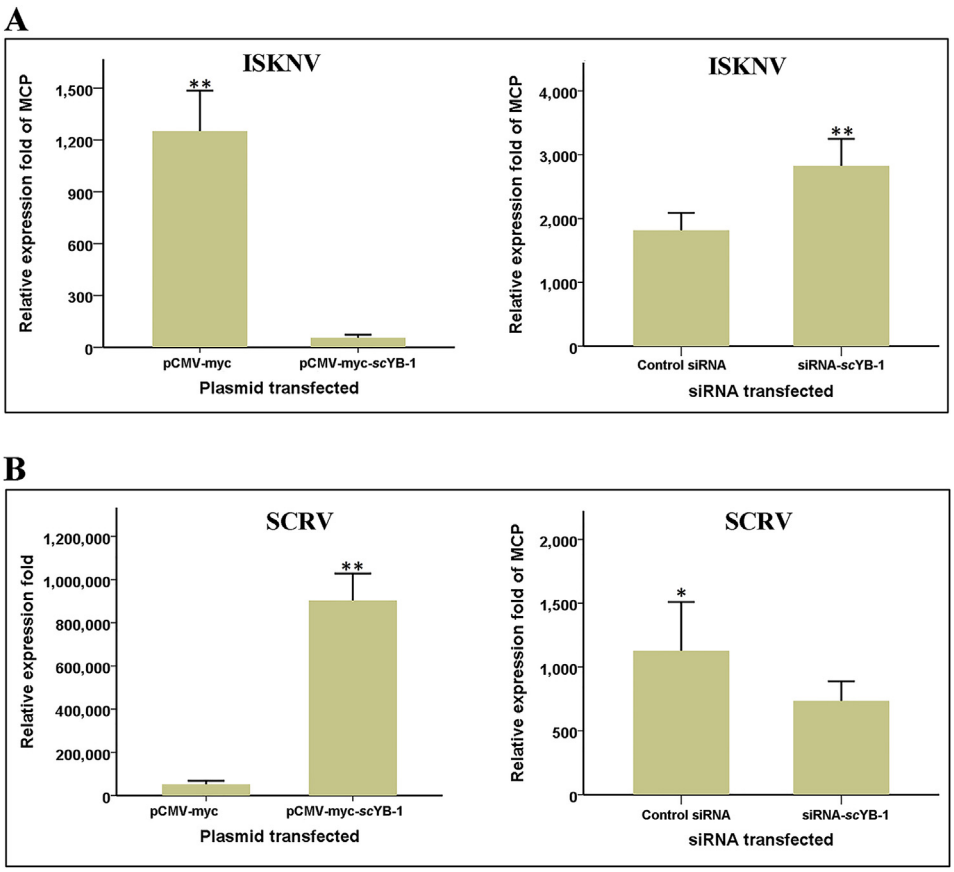

Fig. 6. *sc*YB-1 mediates different effects on ISKNV and SCRV infections. (A) Overexpression and knockdown of *sc*YB-1 lead to inhibition and enhancement of ISKNV replication in MFF-1 cell lines, respectively. (B) Overexpression and knockdown of *sc*YB-1 lead to enhancement and inhibition of SCRV replication in MFF-1 cells, respectively. The replication level of the virus is indicated by the relative expression amount of viral *mcp* gene and detected by qRT-PCR. Samples of ISKNV and SCRV challenge groups were collected at 72 and 36 hpi following different replication rates, respectively. Expression level at 0 h of each group was used as calibrator. Vertical bars represent ± SD ($n = 3$). Statistical significance is indicated by asterisks, with * referring to $p < 0.05$ and ** referring to $p < 0.01$.

YB-1 plays an important role in the innate immunity of mammals; however, studies on the effect of YB-1 on fish are few. In our study, we observed that *sc*YB-1 responds to immune challenge, implicating that it may participate in immune response. Furthermore, we revealed that *sc*YB-1 could activate NF-κB pathway, which is a very important immune signal pathway that plays pivotal roles in immune response and is crucial for pro-inflammatory cytokines and interferon release [34]. Knockout of *RelA* (p65) gene significantly attenuates the production of IFNs following viral infection [35]. Our findings suggested that *sc*YB-1 might participate in anti-viral response, at least in part, through NF-κB pathway. Furthermore, the expression level of *sc*YB-1 was elevated by NF-κB pathway activator PMA. Wang et al. [36] revealed that NF-κB inhibitor PDTC can attenuate YB-1 expression induced by anti-microbiome peptide LL-37, implicating that NF-κB might participate in YB-1 expression. We speculated that the NF-κB pathway and *sc*YB-1 might form a positive feedback loop, which was crucial for immune responses.

In addition, YB-1 plays an important role in pathogen infections in mammals. YB-1 can affect viruses in different ways. Its interaction with various viral nucleotides and its complicated roles have been gradually uncovered. For example, in JCV infection, YB-1 shows high affinity to C/T rich domains and binds to viral DNA promoters, thus promoting the transcriptional activity of viral DNA [37]. As for some RNA viruses, such as HCV [38], and retroviruses like HIV-1 [39], host YB-1 can be hijacked, enhancing viral RNA stability and sustaining viral RNA replication and particle propagation. In the present study, overexpression of *sc*YB-1 was shown to inhibit ISKNV infections, while the corresponding opposite results showed that knockdown the *sc*YB-1 could promote ISKNV replication. However, opposite results were presented for SCRV replication. These observations suggested that *sc*YB-1 participated in the virus infection in mandarin fish and had a complicated function counter with different viruses. Based on our results and those of previous reports, we speculated that the different effects of *sc*YB-1 might be attributed to its different approaches of interaction with viral nucleotides. However, the detailed mechanisms would be investigated in further research.

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