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Isolation and expression of four *Megalobrama amblycephala* toll-like receptor genes in response to a bacterial infection

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ABSTRACT

Toll-like receptors (TLRs) are a category of pattern recognition receptors (PRRs), which recognize pathogen associated molecular patterns (PAMPs) and participate in the immune responses. We identified *tlr5a*, *tlr5b*, *tlr9* and *tlr21* from the genome of blunt snout bream (*Megalobrama amblycephala*). All four *tlrs* were constitutively expressed in all examined tissues. After an immune bacterial challenge with *Aeromonas hydrophila*, their expressionwas up-regulated in lymphoid organs and tissues. Recombinant eukaryotic plasmid pEGFP-N1 was transfected into the common carp (*Cyprinus carpio*) EPC (epithelioma papulosum cyprini) cells for the purpose of subcellular localization. pcDNA3.1(+) recombinant eukaryotic plasmid was used to investigate the effects of overexpression of *tlrs* on the expression of downstream interferon-associated immune factors. The four Tlrs were distributed in the cytoplasm of transfected cells and appeared as filamentous or reticular. The expression of *irf3*, *irf7*, *isg15*, *mx1*, *pkr* and *viperin* at 0, 6, 12, 18, 24, 36, 48 and 72 h post-transfection in transfected EPC cells was quantified by qPCR. Overexpression of *tlrs* upregulated the expression of *viperin*, *isg15*, *irf3*, *irf7*, *mx1* and *pkr* (in that order of magnitude). We also cloned the following promoters of *irfs*: *Irf1-p*, *irf2-p*, *irf6-p*, *irf7-p*, *irf8-p* and *irf9-p*. Results of the dual luciferase reporter assay suggested that *tlr5a*, *tlr5b* and *tlr9* enhanced the activities of *irf7-p*, while *tlr5b* enhanced the activities of *irf1-p* and *irf7-p*. This suggests that they all play a role in the innate immunity. The experiments also indicated that TLRs activate *irf3* or *irf7* signaling to induce IFN secretion and subsequent upregulation of IFN-stimulated genes. These results indicate that *tlrs* and *irfs* play an important immune role in response to *A. hydrophila* infection in blunt snout bream, and pave the way for further studies of immune mechanisms mediated by TLRs in fish.

1. Introduction

Toll-like receptors (TLRs), type I transmembrane proteins that are fundamental sensor molecules of the host's innate immune system, are present in most vertebrates and highly conserved among different species [1]. TLRs are a major class of pattern recognition receptors (PRRs), activated by pathogen associated molecular patterns (PAMPs), such as bacterial cell wall components, and viral genomic DNA and RNA [2]. Upon the recognition of pathogen PAMPs, TLRs induce the expression of transcription factors such as NF-κB and interferon regulatory factors (IRFs) via the activation of myeloid differentiation factor88 (MyD88) or TIR domain-containing adaptor-inducing interferon (TRIF), resulting in the expression of cytokines with anti-pathogenic effects, such as interferon (IFN), tumor necrosis factor (TNF) and interleukin (IL) [2].

Understanding the cellular responses to pathogens (and pathogen antigens), which are coordinated by a complicated gene regulation network that mediates rapid alterations in gene expression patterns within the cell, is critical to understanding the immunity [3]. The regulation is controlled by differential expression of genes, post-translational modification and stimulation of the intracellular transcription factors [4]. The importance of gene-regulatory networks in innate immune responses is relatively well-understood, and genes that encode interferons (IFNs) and proinflammatory cytokines are known to be important elements of these networks [5,6]. Recently, transcription factors from the interferon regulatory factor (IRF) family have gained attention for their regulatory role in the development and their diverse roles in regulating the gene-expression networks comprising the immune system. Many studies have shown that IRF family members can be activated by MyD88-dependent and MyD88-independent pathways; and play a synergistic role in the expression of specific genes induced by TLRs. Therefore, IRFs play an important role both in MyD88-dependent

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Table 1
Primers for construction of overexpression vectors.

Primer name	Sequence (5′-3′)
Matlr5a-G-Xho I	ctaccggactcagatctcgagATGGCAACAAGATGCACTTTATCT
Matlr5a-G-EcoR I	gtaccgtcgactgcagaattcGGTGGCGACCGGTGGATC
Matlr5a-3-EcoR I	tagtccagtgtggtggaattcATGGCAACAAGATGCACTTTATCT
Matlr5a-3-Xho I	aacgggccctctagactcgagTTACACTGCAGTGTCTGCTTGCA
Matlr5b-G-Xho I	ctaccggactcagatctcgagATGGGATTTACATTCATTCTGATCC
Matlr5b-G-EcoR I	gtaccgtcgactgcagaattcCACCATGGTGGCGACCGG
Matlr5b-3-EcoR I	tagtccagtgtggtggaattcATGGGATTTACATTCATTCTGATCC
Matlr5b-3-Xho I	aacgggccctctagactcgagTTATACTGCTGTGTTTGCATGGACA
Matlr9-G-Xho I	ctaccggactcagatctcgagATGTTTGGATACGTGTATTTGATTCTC
Matlr9-G-EcoR I	gtaccgtcgactgcagaattcCACCATGGTGGCGACCGG
Matlr9-3-EcoR I	tagtccagtgtggtggaattcATGTTTGGATACGTGTATTTGATTCTC
Matlr9-3-Xho I	aacgggccctctagactcgagTTAGAAGAAGCTCTCTATGACATTTTTG
Matlr21-G-Xho I	ctaccggactcagatctcgagATGGCAGCTTCTGCATGTCG
Matlr21-G-EcoR I	gtaccgtcgactgcagaattcCACCATGGTGGCGACCGG
Matlr21-3-EcoR I	tagtccagtgtggtggaattcATGGCAGCTTCTGCATGTCG
Matlr21-3-Xho I	aacgggccctctagactcgagTCAGGGCATTAGGTAATACTCTTCC

Table 2
Primers for qPCR.

Target gene	Primer	Sequence (5′-3′)	Length of product (bp)	Gen Bank accession No.
β-actin	β-actin-qF	GGGCACCTGAACCTCTCATT	126	KF844250
	β-actin-qR	CTGCTATGTGGCTCTTGACTTTG		
IRF3	IRF3-qF	GTTTAGAGGGACAATTAACTGGACTA	159	KJ027520
	IRF3-qR	GAGGGTCCACTCTTTGAAAATG		
IRF7	IRF7-qF	CCATTCATTGCTGACATCTACAGT	174	KF844251
	IRF7-qR	GTTCGTCTCAAAGTTGCTCCTC		
ISG15	ISG15-qF	TAATGCCACAGTCGGTGAA	184	KM099174
	ISG15-qR	AGGTCCAGTGTTAGTGATGAGC		
Mx1	Mx1-qF	ATCTGGTGGATAAGGGAAC	153	KM099175
	Mx1-qR	CATCCTCTGTTAATGTGGC		
PKR	PKR-qF	ACCTGAAGCCTCCAAACATA	102	KM099176
	PKR-qR	GCATTCGCTCATCATTGTC		

and TRIF-dependent signaling pathways [7,8].

Toll-like receptor 5 (TLR5), a member of TLR family, plays an important role in the host defense against bacterial pathogens, and is responsible for the recognition of bacterial flagellin in vertebrates. Two types of TLR5 have been identified in some teleost fishes: the membrane form TLR5M and the soluble form TLR5S [9]. TLR9 is a receptor that enables mammalian cells to recognize unmethylated CpG DNA motifs of viruses and bacteria (it also recognizes synthetic CpG oligodeoxynucleotides - ODNs) and induce the production of cytokines [10]. TLR21 is a non-mammalian Toll-like receptor, which was identified in some species of fish and birds [11,12]. In chicken, TLR21 plays similar roles to the mammalian TLR9 [13]. In conclusion, all of these TLRs play an important role in the innate immune responses in teleost fish by identifying certain components of bacteria and activating the nuclear factor (NF)-κB via a MyD88-dependent pathway [14].

Blunt snout bream (*Megalobrama amblycephala*) is a commercially important cyprinid fish distributed in the middle reaches of the Yangtze River and accessory lakes [15]. After several decades of intensive human selection for aquaculture purposes, cultured populations began to exhibit growth depression, early maturation and weakened resistance to diseases [16,17]. Therefore, it is important to develop techniques for genetic improvement and disease control to facilitate further development of its aquaculture [18]. As the understanding of TLR-mediated immune responses in blunt snout bream remains limited, here we studied the effects of overexpression of TLRs on the downstream interferon-associated immune factors. This study shall also advance the understanding of the fish immune system and provide theoretical basis for disease prevention in the aquaculture of this and other fish species.

2. Materials and methods

2.1. Experimental materials

The epithelioma papulosum cyprini (EPC) cell line, and pEGEP-N1 and pcDNA3.1(+) vectors, were obtained from the College of Fisheries, Huazhong Agriculture University, Wuhan. RNAiso Plus, PrimerScript II 1st Strand cDNA Synthesis Kit, RNase inhibitor, dNTPs, high-fidelity PrimerSTAR Max DNA polymerase, Ex Taq PCR Mix, DNA molecular weight standards DL5000, agarose gel extraction kit, restriction enzymes *Eco*R I and *Xho* I, T4 DNA ligase, *Escherichia coli* DH5α competent cells, quantitative PCR reagent SYBR Premix Ex Taq TMII (Tli RNaseH Plus), and PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) were purchased from TaKaRa Biotechnology Co., Ltd., Dalian, China. Gel extraction kit and Endo-Free plasmid mini kit were purchased from Omega Bio-Tek Inc., USA. Luria-Bertani medium, kanamycin and ampicillin were purchased from Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China.

2.2. Experimental fish and sample collection

Healthy blunt snout bream weighing about 150 g were obtained from the Huazhong Agriculture University fish hatchery and acclimatized in aerated freshwater at 25 ± 2 °C for one week before the experiment. The fish were fed with commercial feed twice a day, and the water was exchanged daily. All procedures of this study were approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University (HZAU), Wuhan, China.

Aeromonas hydrophila colonies were isolated in our laboratory, and the experimentally determined median lethal concentration (LD50) was 1×10^7 CFU/mL. After a bacterial immune challenge with *A. hydrophila*

Table 3
Primers for cloning the promoter of *Mairfs*.

Primer name	Sequence (5′-3′)
Mairf1-sacl	ctatcgataggtaccgagctcTGACAAATTCTAGAGTTTTGTTTATTGTG
Mairf1-hind	cagtaccggaatgccaagcttAATTGGATAAACATATACACACGCCC
Mairf2-sacl	ctatcgataggtaccgagctc GGTAGTTACATCAAATATTGATTCAGTTAATA
Mairf2-hind	cagtaccggaatgccaagcttGGTTTGATTTCGCTCTTCAGATC
Mairf6-sacl	ctatcgataggtaccgagctcGGGATTCTGTGTCTGCTGCAA
Mairf6-hind	cagtaccggaatgccaagcttCTCTGCTCGAGAGTTTCACCTTC
Mairf7-sacl	ctatcgataggtaccgagctcAACTAGAAGTTAAGCACTTTCTGTTGGT
Mairf7-hind	cagtaccggaatgccaagcttACTTAGATCGCAGATCTCGAGGG
Mairf8-sacl	ctatcgataggtaccgagctcGCTGCACTAGACTGTTAAAGACATTAAT
Mairf8-hind	cagtaccggaatgccaagcttCTTTGACACTCTTAATTCACTCTAATGC
Mairf9-sacl	ctatcgataggtaccgagctcTGTTGATTTACCGCAAGTACCATG
Mairf9-hind	cagtaccggaatgccaagcttATAGTTTCAAATACCTTAGTTTCGGTGC

[19] (0.1 mL of bacterial suspension injected intraperitoneally), three specimens were collected from each group (at 6, 12, 24, 48, 72 and 120 h post challenge), and euthanized using 300 mg/L methanesulfonate (MS222; Sigma-Aldrich, USA). We also collected the liver, spleen, kidney, intestines, gills, blood, heart and brain tissues before the bacterial challenge to determine the basal (or constitutive) expression of *tlrs*. The expression of *tlrs* after the *A. hydrophila* challenge was studied in the liver, spleen, kidney, intestine and gills. Quantitative real-time PCR (qPCR) was performed using the LightCycler®480 II (Roche Diagnostics GmbH, USA). *β-actin* was chosen as the reference gene on the basis of zebrafish studies [20] and our previous blunt snout bream studies [21,22], where it was consistently shown to be the most stable reference gene. Gene expression levels were calculated according to the $2^{-\Delta\Delta CT}$ method. The significance of differences among samples was analyzed by one-way analysis of variance (ANOVA) using LSD (Least Significant Difference), with $P < 0.05$ chosen as the statistical significance threshold.

2.3. Construction of pEGFP-N1-tlrs and pcDNA3.1(+) overexpression vectors and Rab5-red and Rab7-red vectors

Primers were designed on the basis of blunt snout bream *tlr5a*, *tlr5b*, *tlr9* and *tlr21* sequences and synthesized by Tian Yi Hui Yuan Biotech (Wuhan) Co., Ltd., Beijing, China (Table 1). Total RNA was isolated from the liver and spleen of blunt snout bream using RNAiso Plus according to the manufacturer's instructions. Reverse transcription reactions were performed using PrimerScript II 1st Strand cDNA Synthesis Kit. The amplification of Tlrs was performed using high-fidelity DNA polymerase, with blunt snout bream cDNA as the template. PCR products were digested with corresponding restriction enzymes and analyzed by agarose gel electrophoresis. After enzyme digestion reaction, fragments of Tlrs were purified and isolated using gel extraction kit according to the manufacturer's instructions. The vectors were linearized with the same restriction enzymes and purified using gel extraction kit and ligated with the *tlrs* fragments. The recombinant plasmid was transfected into competent *E. coli* DH5α cells to obtain pEGFP-N1-Matr5a, pEGFP-N1-Matlr5b, pEGFP-N1-Matlr9, pEGFP-N1-Matr21, pcDNA 3.1(+)-Matr5a, pcDNA 3.1(+)-Matr5b, pcDNA 3.1(+)-Matr9 and pcDNA 3.1(+)-Matr21 overexpression plasmids.

To investigate the subcellular localization of blunt snout bream Tlr5a, Tlr5b, Tlr9 and Tlr21, DNA fragments that contained complete ORFs of *tlr5a, tlr5b, tlr9* and *tlr21* were amplified from the blunt snout bream cDNA. All fragments were inserted into the pEGFP-N1 vector, and the obtained plasmids were named pEGFP-N1-MaTlr5a, pEGFP-N1-MaTlr5b, pEGFP-N1-MaTlr9 and pEGFP-N1-MaTlr21. EPC cells were transfected with plasmid pEGFP-N1-Tlrs and Rab5-red/Rab7-red and subjected to fluorescence observation at 48 h post-transfection. Simultaneously, empty plasmid pEGFP-N1 was transfected as a negative control. The transfection efficiency was calculated by measuring the proportion of cells in fluorescence field and white light field.

Overexpression plasmids were verified via PCR and sequenced at Tian Yi Hui Yuan Biotech (Wuhan) Co. Ltd. Beijing, China. Comparison of the pEGFP-N1/pcDNA3.1(+) and recombinant pEGFP-N1-Tlrs/pcDNA3.1(+)-Tlrs overexpression plasmid vectors revealed that cloning *tlr5a/5b/9/21* into multiple cloning sites did not lead to a frame

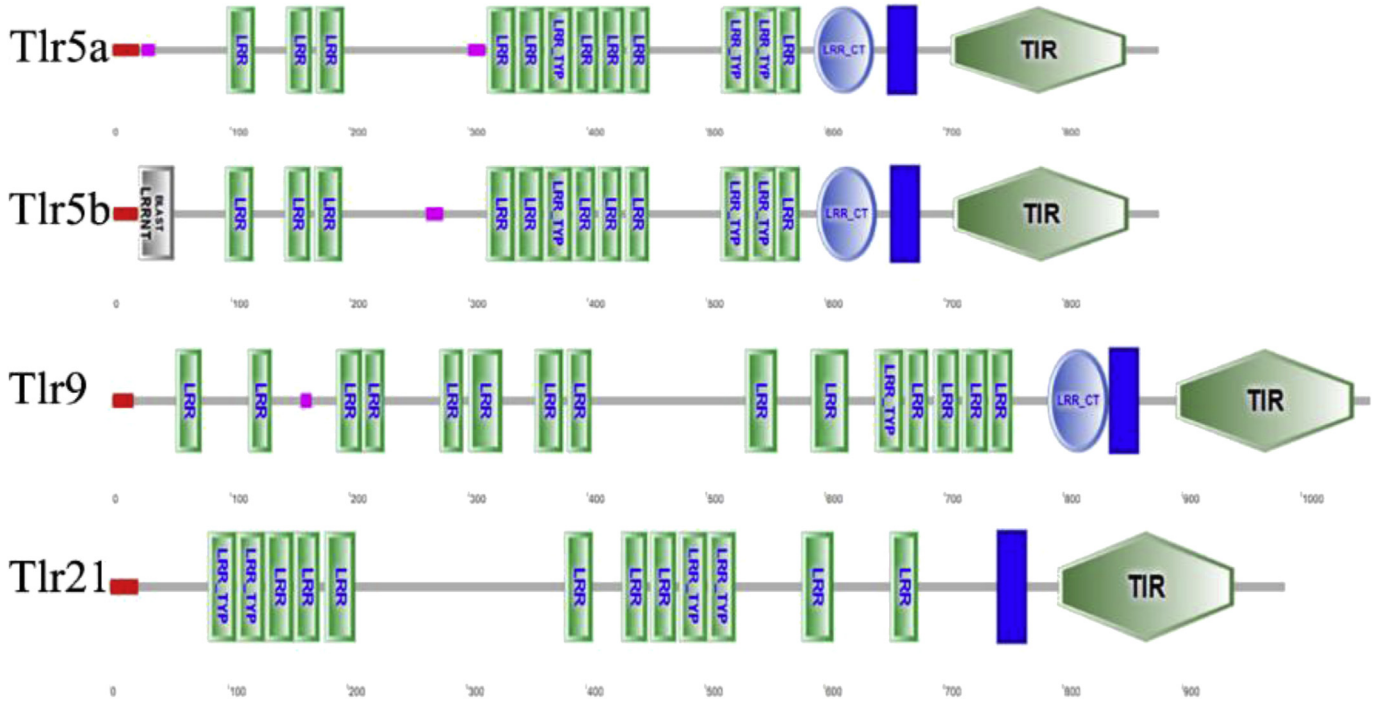

Fig. 1. Domain architectures of *M. amblycephala* Tlrs. The green box represents the LRR domain, the blue box the TM domain, the blue circle the LRRCT, and the hexagonal box the TIR domain. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

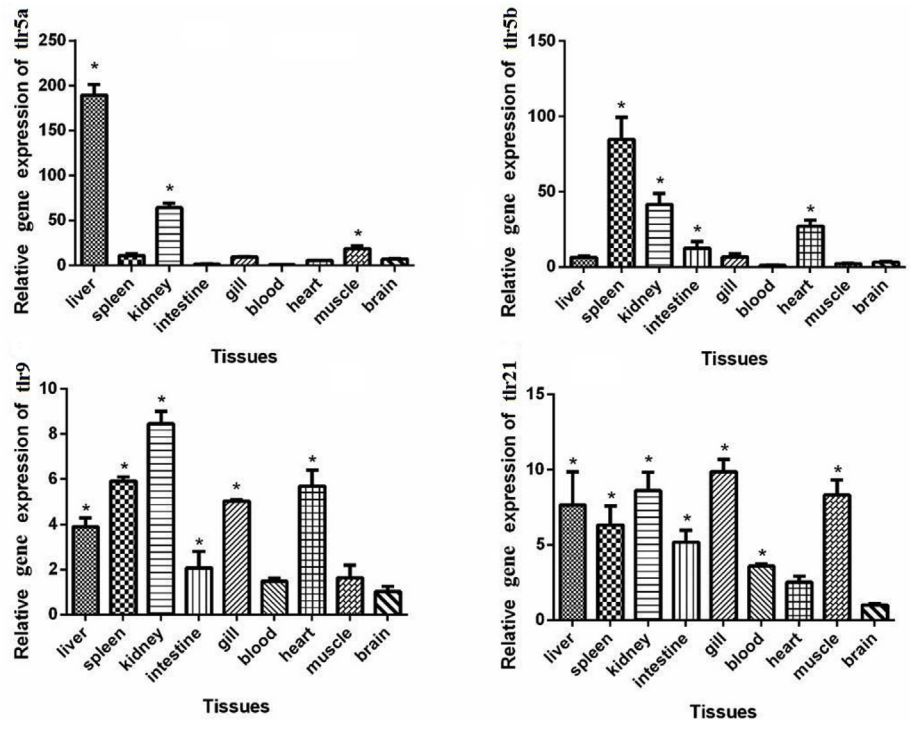

Fig. 2. Constitutive expression of four *tlrs* (mRNA) in nine tissues of blunt snout bream. The expression was detected by qPCR, with *β-actin* as the reference gene, and expression in all tissues normalized to the expression in blood (*tlr5a* and *tlr5b*) and brain (*tlr9* and *tlr21*). Vertical bars represent mean ± SD of three technical replicates, and asterisks "*" above the bars represent statistically significant differences from the control samples at the $P < 0.05$ threshold.

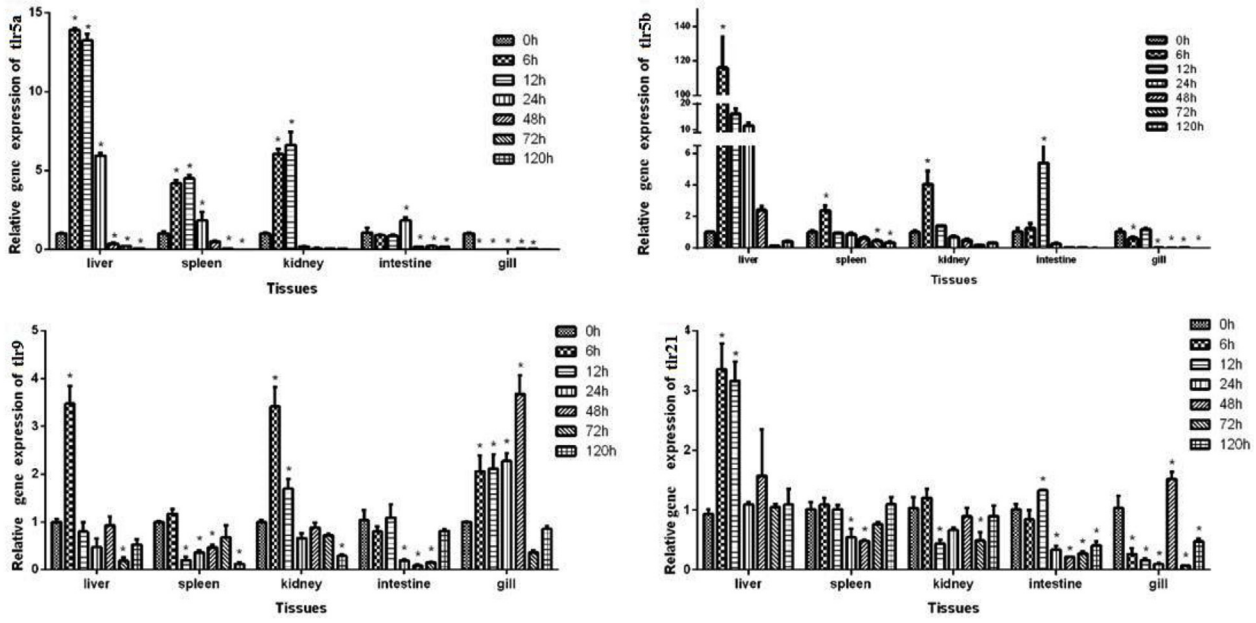

Fig. 3. Transcript expression responses of four *tlrs* to *A. hydrophila* challenge in five tissues of blunt snout bream. The expression was detected by qPCR and normalized to *β-actin*. Vertical bars represent mean ± SD of three technical replicates, and asterisks "*" above the bars represent statistically significant differences from the control samples at the $P < 0.05$ threshold.

shift in the reading frame of either *tlr* genes or the green fluorescent protein (GFP) gene, indicating a successful construction of pEGFP-N1-Tlrs/pcDNA3.1(+)-Tlrs vectors for the overexpression of Tlrs. The same manufacturer's instructions were used for EPC cells, where Rab5 and Rab7 fragments were insert into pdsred1-c1.

2.4. Transfection of EPC cells with pcDNA 3.1(+) and pEGFP-N1 overexpression plasmid

pcDNA 3.1(+)-Matlrs overexpression plasmid and control vector pcDNA3.1(+) plasmid were extracted using an endotoxin-free plasmid isolation kit following the manufacturer's instructions. The extracted plasmids were dissolved by sterile ultrapure water, concentrations were quantified by NanoDrop 2000 spectrometer (Thermo Scientific, USA), and preserved at −20 °C. One day prior to transfections, EPC cells were seeded in 6-well plates with the concentration of 1×10^5 cells/mL. Cells were transfected once they reached 70–90% confluency. Plasmids were transfected into cells at 4μg/well using Lipofectamine2000 Transfection Reagent at 8μL/well following the manufacturer's instructions. The EPC cells transfected with an empty overexpression plasmid vector pcDNA 3.1(+) were used as the control, whereas five recombinant plasmids, pcDNA 3.1(+)-Matlr5a, pcDNA 3.1(+)-

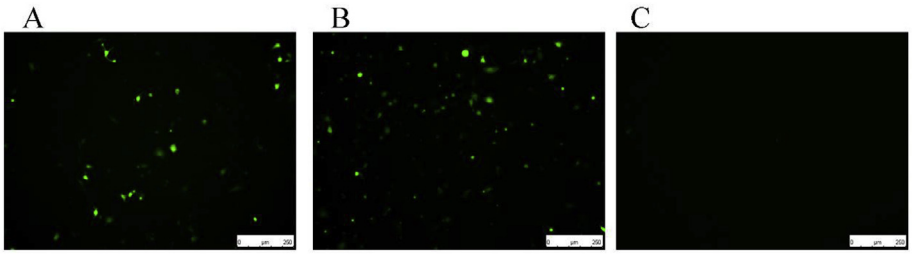

Fig. 4. Transfection efficiency of the pEGFP-N1 plasmid. Transfection fluorescence map at 24 h (A) and 48 h (B) after transfection. (C): fluorescence of the non-transfected plasmid (control).

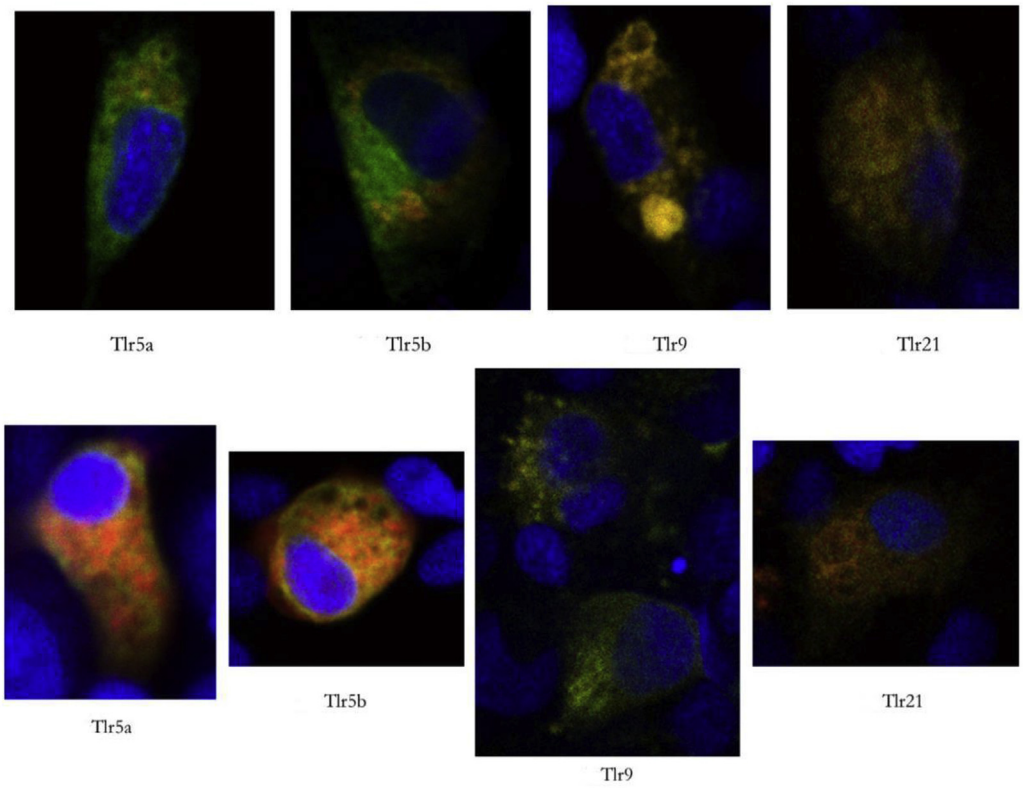

Fig. 5. Subcellular localization of four Tlrs of blunt snout bream. Above: co-localization map with early endosomal marker Rab5; Below: co-localization with late endosomal marker Rab7.

Matlr5b, pcDNA 3.1(+)-Matlr9 and pcDNA 3.1(+)-Matlr21, were experimental treatments. Triplicate cell samples were collected at 0, 6, 12, 18, 24, 36 48, and 72 h post-transfection.

The EPC cells that were maintained on coverslips in 6-well plates were transfected with plasmid pEGFP-N1-Tlrs by Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions. One day (24 h) post the transfection, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained by Hoechst. The cells were mounted with 50% glycerol and observed by fluorescence microscope (Leica, Germany).

2.5. Determination of irf3, irf7, isg15, mx1, pkr, and viperin gene expression

The qPCR primers (Table 2) for *β-actin* (KF844250), *irf3* (KF844251), *irf7* (KJ027520), *isg15* (KM099174), *mx1* (KM099175), *pkr* (KM099176) and *viperin* (KM099177) gene were synthesized by Tianyi Huiyuan Biological Engineering (Wuhan) Co., Ltd.

Total RNA from EPC cells that was collected at each time point after transfection as described in 2.4, was isolated using an RNA rapid extraction kit. Reverse transcription was performed using PrimeScript™ RT reagent Kit according to the manufacturer's instructions. qPCR was performed on the RocheLightCycler480 (Switzerland) system using cDNA templates from EPC cells collected at all time-points and SYBR Green dye. The reaction solution contained 10 μL SYBR Premix Ex Taq TM II, 1 μL (10 μmol/L) of forward and reverse primers each, 2 μL cDNA template, and 6 μL ddH_2O. The qPCR protocol was 95 °C for 30 s, 95 °C for 5 s, and 60 °C for 45 s for 40 cycles. Triplicate reactions were run for each sample and results analyzed quantitatively by the $2^{-\Delta\Delta CT}$ method. Data were calculated as mean ± SD using Microsoft Excel and IBM SPSS 19.0 (SPSS Inc., USA). Comparison between the two groups was performed using independent sample *t*-test. We applied two significance thresholds: $P < 0.05$ was considered as a statistically significant difference, and $P < 0.01$ as a highly significantly difference.

2.6. Cloning of blunt snout bream irf1, irf2, irf6, irf7, irf8 and irf9 gene promoters and luciferase activity assays

Primers (Table 3) for the conventional PCR were designed according to the genomic sequence of blunt snout bream [23], and synthesized by Tianyi Huiyuan Biological Engineering (Wuhan) Co., Ltd. PCR was performed on Eppendorf Mastercycler PCR machine with the cDNA

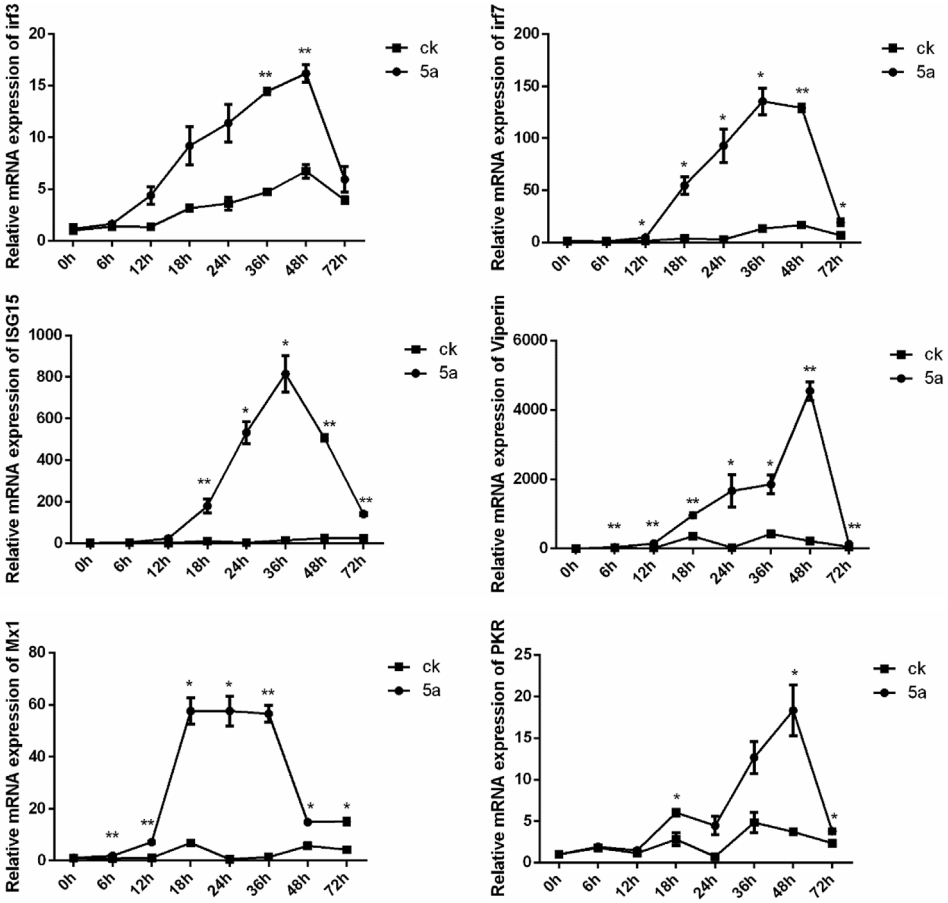

Fig. 6. Effects of overexpression of *tlr5a* on the expression of downstream interferon associated immune factors in EPC cells. Data are shown as average ± standard deviation derived from three independent experiments. $*P < 0.05$, $**P < 0.01$, when compared to control group at the same time point post-transfection.

from EPC cells as template. The PCR reaction solution contained 12.5 μL high-fidelity PrimerSTAR Max DNA polymerase Mix, 1 μL (10 μmol/L) of forward and reverse primers each, 1 μL cDNA template, and ddH_2O up to the total of 25 μL. The PCR reaction protocol was 94 °C for 5 min, 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 90 s, 40 cycles, hold at 16 °C. Amplified DNA was analyzed by agarose gel electrophoresis, cloned using a pMD18-T vector, and then sequenced by Tianyi Huiyuan Biological Engineering (Wuhan) Co., Ltd. EPC cells were seeded in 24-well plates at a density of 1×10^5 cells/ml for 24 h. Co-transfection was performed with the overexpression plasmid, target promoter luciferase plasmid, and pRL-TK (internal control reporter vector). After 24 h of transfection, cells were washed with PBS, lysed with passive lysis buffer (Promega), and assayed for luciferase activities in a luminometer by the Dual-Luciferase Reporter Assay System (Promega). The luciferase reading of each sample was first normalized against the pRL-TK levels, and the relative light unit intensity was presented as the ratio of firefly luciferase to renilla. Comparison between the two groups was performed using independent sample *t*-test.

3. Results

3.1. Characterization of the blunt snout bream tlr5a, tlr5b, tlr9 and tlr21

Full length ORFs of four blunt snout bream *tlr* genes, *tlr5a*, *tlr5b*, *tlr9*, and *tlr21* are 2646, 2637, 3177 and 2961 bp in length respectively. Comparison of these sequences, obtained from cDNA, to the corresponding genomic data showed that each gene possessed one exon. Moreover, *tlr5a* and *tlr5b* are located on the same chromosome.

The amino acid sequences of *tlr5a* and *tlr5b* were analyzed, and the results revealed that both of them are a membrane form of TLR5. *tlr5a* contained 12 LRR motifs, a LRR-CT motif, two low complexity regions, a transmembrane region and a TIR domain. *tlr5b* included 12 LRR motifs, a LRR-CT motif, a transmembrane region and a TIR domain. *tlr9* had 15 LRR motifs, a LRR-CT motif, a low complexity region, a transmembrane region and a TIR domain. *tlr21* included 12 LRR motifs, a transmembrane region and a TIR domain. The schematic diagrams of Tlr5a, Tlr5b, Tlr9 and Tlr21 are shown in Fig. 1.

3.2. Expression of TLRs in different tissues in blunt snout bream and in response to A. hydrophila infection

qPCR was carried out to study the tissue distribution TLRs in different tissues of blunt snout bream. As shown in Fig. 2, *tlr5a*, *tlr5b*, *tlr9* and *tlr21* were constitutively expressed in all nine examined tissues. *tlr5a* was highly expressed in liver, but the expression was low in intestines and blood. The expression of *tlr5b* was highest in spleen, kidney and heart, and lowest in blood. *tlr9* expression was high in kidney, and low in gill and blood. Expression of *tlr21* was high in gill, but low in heart and brain.

In order to study the responses of *tlr5a* and *tlr5b* to the bacterial challenge with *A. hydrophila*, tissues were collected at different time points was, and total RNA extracted for qPCR analysis. As shown in Fig. 3, and compared with the control (0 h), expression level of *tlrs* was significantly up-regulated in liver 6 h after the stimulation ($P < 0.05$). Expression patterns of *tlrs* genes were similar, but the absolute gene expression level and duration of upregulation were differed ($P < 0.05$). *tlr5b* was most highly upregulated. In spleen, the highest expression level of *tlr5b* and *tlr9* was observed 6 h after the bacterial stimulation. From 0 h to 12 h, the expression of *tlr5a* first increased and then decreased. The expression of *tlr21*was relatively low in spleen. In

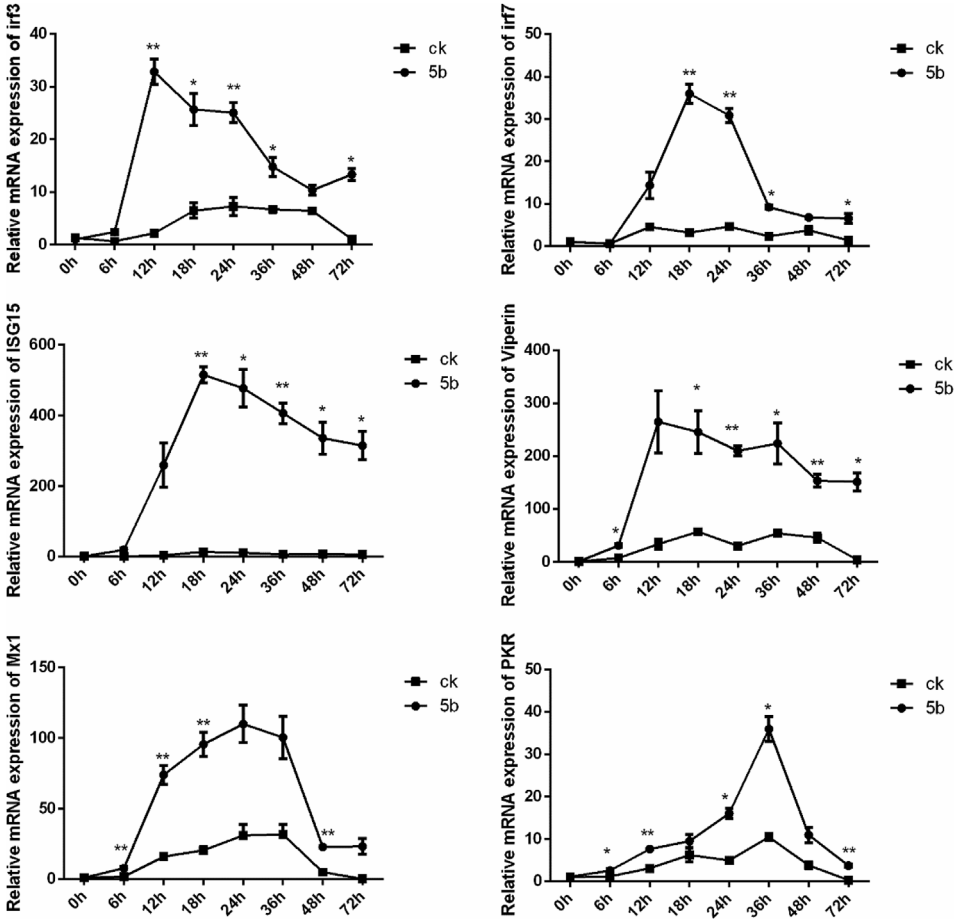

Fig. 7. Effects of overexpression of *tlr5b* on the expression of downstream interferon associated immune factors in EPC cells.

kidney, the response pattern of *tlrs* was similar to spleen. In intestine, the highest expression level of *tlr5b*, *tlr9* and *tlr21* was achieved 12 h after the bacterial stimulation, while the highest expression of *tlr5a* occurred at 24 h. In gills, the expression of *tlr5a* was decreased throughout the bacterial infection; the expression of *tlr5b* peaked at 12 h, whereas the expression at other time points. The expression of *tlr9* increased from 0 h to 24 h and then decreased. The response pattern of *tlr21* was similar to *tlr9*.

3.3. Construction of pEGFP-N1-tlrs and pcDNA3.1(+)-tlrs overexpression vectors and determination of cell efficiency

Transfection of EPC cells with empty pEGFP-N1 and pEGFP-N1-Tlrs vectors resulted in the highest GFP expression 48 h post-transfection. Transfection efficiency was above 50%. No fluorescence was detected in non-transfected EPC cells under fluorescent light (Fig. 4).

3.4. Subcellular localization of Tlr from blunt snout bream proteins

Green fluorescence of Tlr5a-EGFP was distributed in the cytoplasm of transfected cells, in a filiform or reticulated pattern (Fig. 5), suggesting that Tlr5a may be located in the plasma membrane of transfected cells. Tlr5b-EGFP showed similar localization pattern to Tlr5a-EGFP. Tlr9-EGFP was distributed in the cytoplasm of transfected cells and aggregated in some regions of the endosome. Tlr21-EGFP distributed in the endoplasmic reticulum. As a negative control, the empty pEGFP was distributed uniformity in transfected cells.

3.5. Gene expression of downstream interferon-associated factors in EPC cells overexpressing tlrs from blunt snout bream

qPCR was used to detect the mRNA transcription levels of *irf3, irf7, isg15, mx1, pkr* and *viperin* genes after the EPC were transfected with empty pcDNA3.1(+) vector and overexpression pcDNA3.1(+)-Tlrs vector for 0 h, 6 h, 12 h, 18 h, 24 h, 36 h, 48 h and 72 h. The expression of *irf3*, *irf7*, *isg15*, *mx1*, *pkr*, and *viperin* genes was significantly up-regulated in EPC cells transfected with pcDNA3.1(+)-Matlrs plasmid (Figs. 6–9). In response to the *tlr5a* overexpression in carp EPC cells, the highest relative expression of *irf3 viperin* and *pkr* at 48 h post-transfection was 16.2, 4549, and 18.3-fold, respectively. As for *irf7* and *isg15*, the expression peaked at 36 h, and for *mx1* it peaked at 24 h post-transfection. In response to the *tlr5b* overexpression in carp EPC cells, the expression of all genes except *mx1* peaked earlier than in response to the *tlr5a* overexpression. In response to *tlr9* overexpression, the expression of other genes peaked at 48 h and 72 h post-transfection: *irf3* - 48 h (85.2-fold), *isg15* - 48 h (2410.1-fold), *mx1* - 48 h (283.9-fold), *irf7* - 72 h (58.1-fold), *pkr* - 72 h (29.5-fold) and *viperin* - 72 h (15216.4-fold). In response to *tlr21* overexpression, the highest expression of *irf3*, *irf7* and *pkr* occurred at 72 h post-transfection (120.2-fold, 68.1-fold and 36.4-fold respectively), and the highest expression of *isg15* and *mx1* occurred at 48 h post-transfection (645.1-fold and 1563.1-fold respectively). The expression of *viperin* exhibited a marked increase at 36 h, a slight decrease at 48 h, and it peaked at 72 h post-transfection (15216.4-fold). These four overexpression vectors upregulated the expression of *isg15* and *viperin* more than the expression of other genes. Compared with EPC cells transfected with empty pcDNA3.1 (+) vector, the gene expression of *irf3, irf7, isg15, mx1, pkr* and *viperin* genes in the cells transfected with pcDNA3.1(+)-Matlrs was up-regulated. This

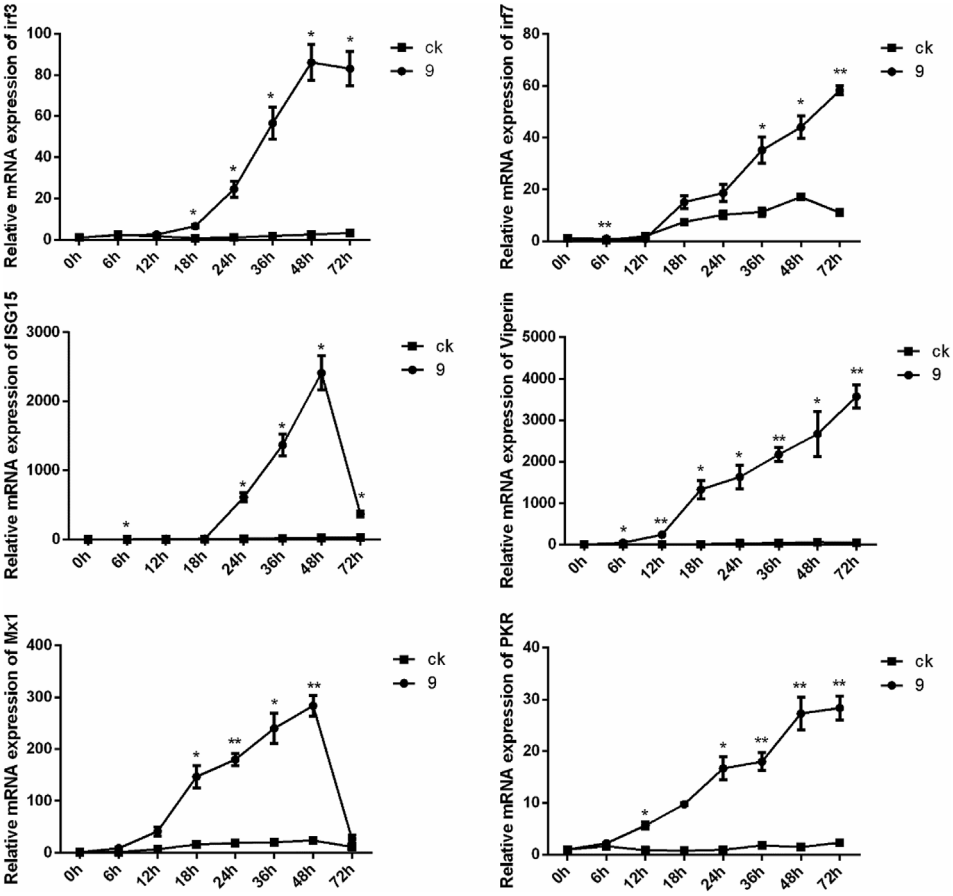

Fig. 8. Effects of overexpression of *tlr9*on the expression of downstream interferon associated immune factors in EPC cells.

indicates that overexpression of *tlrs* in carp EPC cells can up-regulate the expression of *irf3, irf7, isg15, mx1, pkr, and viperin* genes (Figs. 6–9). Different expression patterns of those genes also indicate different functions of *tlr5a* and *tlr5b*, and *tlr9* and *tlr21*.

3.6. tlrs from blunt snout bream facilitate the promoter activities of irf7

The full-length promoters of six blunt snout bream *irf* genes, named *irf1-p*, *irf2-p*, *irf6-p*, *irf7-p*, *irf8-p* and *irf9-p* were obtained by PCR. We used JASPAR database (http://jaspar.genereg.net/) to predict their elements, such as TATA box, CAAT box, GAGA box, etc. (Fig. 10). To identify the role of blunt snout bream *tlrs* in the induction of *irfs*, luciferase reporter assays were performed to examine the promoter activities of *irfs* upon the overexpression of *tlrs* from blunt snout bream. As shown in Fig. 11 and Fig. 12, in cells with overexpressed *tlrs*, the activity of *irf7* promoter was significantly increased, but promoters of other *irfs* weren't increased. However, the overexpression of *tlr5b* increased the activity of *irf1-p* and *irf7-p*. The same results were found in HK293T cells and carp EPC cells (Figs. 11 and 12).

4. Discussion

TLRs play a key role in the innate immunity via the recognition of conserved microbial molecular patterns and activation of immune inflammatory responses. Here, we studied the role of TLRs in blunt snout bream and the effects of overexpression of *tlrs* on the expression of downstream interferon-associated immune factors in EPC cells. Investigating changes in host cells by introducing cloned genes via eukaryotic expression vectors is an effective approach for exploring gene functions [24]. Sequencing analysis showed that the insertion of genes at multiple cloning sites of the pEGFP-N1 (*TLRs* and *GFP*) and pcDNA3.1(+) (*TLRs*) plasmids did not cause any frameshifts in the reading frames of these genes. This suggests that the construction of pEGFP-N1-Matlrs and pcDNA3.1(+)-Matlrs expression vectors was successful. Both these vectors carry the promoter for cytomegalovirus (CMV), and pEGFP-N1 vector also carries the GFP protein, so they can be used to determine the efficiency of transfection using eukaryotic vectors and show the location of target genes.

Two types of Tlr5 exist in teleost fish: the membrane form - Tlr5M, and the soluble form - Tlr5S [25]. The Tlr5M of teleost fish is orthologous to the mammalian TLR5, which contains LRR motifs, transmembrane region, and TIR domain. These are the common three domains of TLRs, among which the LRR domain is in charge of recognizing different PAMPs. Tlr5S is a soluble form, without the transmembrane and TIR domains, that exists in some teleost fish [26], but it has not been found in mammalian genomes yet. In this study, two *tlr5* genes were cloned from blunt snout bream and both of them included an LRR domain, a transmembrane region, and a TIR domain, so they correspond to the membrane form of TLR5. Overall, the protein sequences of Tlr5a and Tlr5b from blunt snout bream had 81% similarity, with the highest similarity in the TM and TIR domains, and the lowest similarity in the LRR domain. This suggests that Tlr5a and Tlr5b may be interacting with bacteria in different ways. The same phenomenon was reported in zebrafish, common carp [27], Indian major carp [28] and grass carp [29], all of which belong to the *Cyprinidae* family. However, *tlr5* has been lost in some fish species, such as Atlantic cod [30], and probably functionally replaced by other PRR families with overlapping ligand profiles [31].

Monomeric flagellin, a structural component of the bacterial flagella, is an agonist of mammalian TLR5 [32]. *A. hydrophila* is a single-flagellum bacterium. Previous studies have shown that bacterial flagellins secreted by both pathogenic and commensal bacteria have the

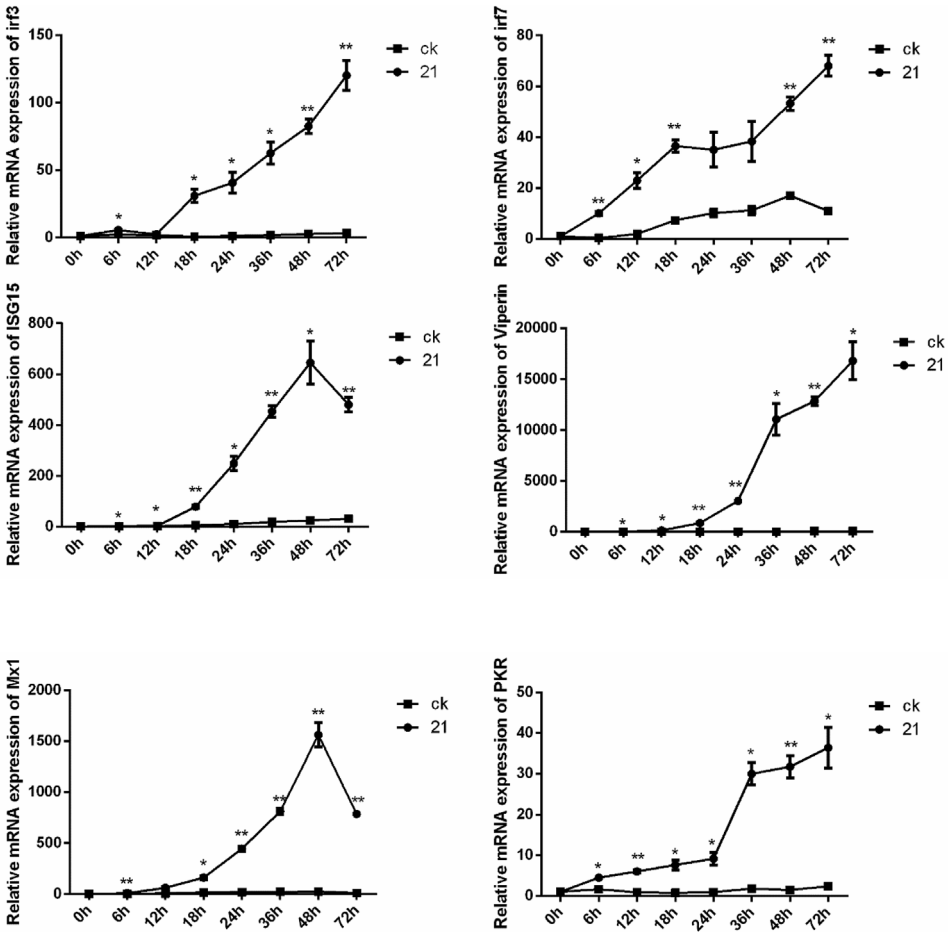

Fig. 9. Effects of overexpression of *tlr21* on the expression of downstream interferon associated immune factors in EPC cells.

potential to activate the epithelial chemokine secretion and activate the production of proinflammatory cytokines [33]. Flagellin recognition system is conserved across fish and mammals, but slightly more complex in some fish species due to the existence of two forms of Tlr5 [34]. For example, in rainbow trout (*Oncorhynchus mykiss*) both forms play an active part in the ligand recognition: flagellin first stimulates the Tlr5M, which induces the downstream immune response genes, like *NF-κB*. Induced Tlr5S recognizes flagellin in the circulation and then binds to Tlr5M to amplify the NF-κB signaling cascade through a positive-loop synergetic mechanism [34]. In addition to its expression in colonic epithelium, *tlr5* is expressed in internal tissues such as heart, brain, spleen, kidney, and testis, suggesting a wide role for TLR5 in the host defense [9]. Whereas in the blunt snout bream, the expression of *tlr5a* was highest in liver, expression of *tlr5b* was highest in spleen. The expression patterns of *tlr5a* and *tlr5b* from blunt snout bream were similar to zebrafish, but different from grass carp [29]. After blunt snout bream infection with *A. hydrophila,* expression of *tlr5a* and *tlr5b* was increased in most tissues. The expression level of *tlr5b* was few times higher than that of *tlr5a* in liver, intestine and gill tissues, whereas the relationship was inversed in spleen and kidney. These results indicate that *tlr5a* and *tlr5b* from blunt snout bream respond to bacterial stimulation and work together against the bacterial invasion. In comparison to terrestrial animals, the environment that the fish inhabit is more complex. Thus, the two forms of TLR5 may be a beneficial strategy for teleost fish to adapt and survive in a complex environment.

Transfection of cells with GFP fusion protein showed that Tlr5a and Tlr5b from blunt snout bream exhibited similar localization patterns. After the transfection of cells with overexpression vectors, pcDNA3.1(+)–Matlr5a and pcDNA3.1(+)–Matlr5b, both vectors quickly induced downstream immune responses in EPC. These results suggest that Tlr5a and Tlr5b of blunt snout bream may have similar functions in signal transmission and ligand recognition as their mammalian homologue TLR5. A highly similar result has been reported in grass carp: CiTLR5a and CiTLR5b exhibited a similar localization (cytoplasm of CIK cells) and also induced downstream immune responses after the cells were stimulated by flagellin from *Salmonella typhimurium* (expression levels of CiTLR5a, CiTLR5b, and downstream genes such as MyD88, NF-κB, IRF7, IL-1b and TNF-a, were up-regulated) [29]. However, the localization of Tlr5a and Tlr5b from blunt snout bream was different from zebrafish orthologues zftlr5a and zftlr5b in Hela cells, which were evenly distributed in the cytoplasm, but not present in the cell membrane or organelles [35]. Purified flagellin from *Helicobacter pylori* could induce the NF-κB activation in HEK cells transfected with TLR5 [36,37]. In human HT-29 cells, enteropathogenic *Escherichia coli* infection could recruit TLR5 to the cell surface. In non-stimulated cells, most TLR5 proteins were located in intracellular compartments and few were observed on the cell surface [38]. The localization of TLR5 in mammals is similar among different cell types, and flagellin infection can induce the localization of TLR5 to the cell surface. However, the localization of Tlr5 in teleost fish is not identical. These differences may be attributed to the usage of different cell lines, or to the existence of different paralogues of Tlr5 gene in fish, as these may also have distinct functional profiles. However, expression patterns of downstream genes induced by these two paralogues in EPC cells were different, which suggests that they may play different regulatory roles in the immune system of blunt snout bream. In zebrafish, Tlr5b does not signal as a conventional TLR5 homodimer, but instead cooperates with its paralogue Tlr5a to form a unique heterodimeric TLR5 that responds

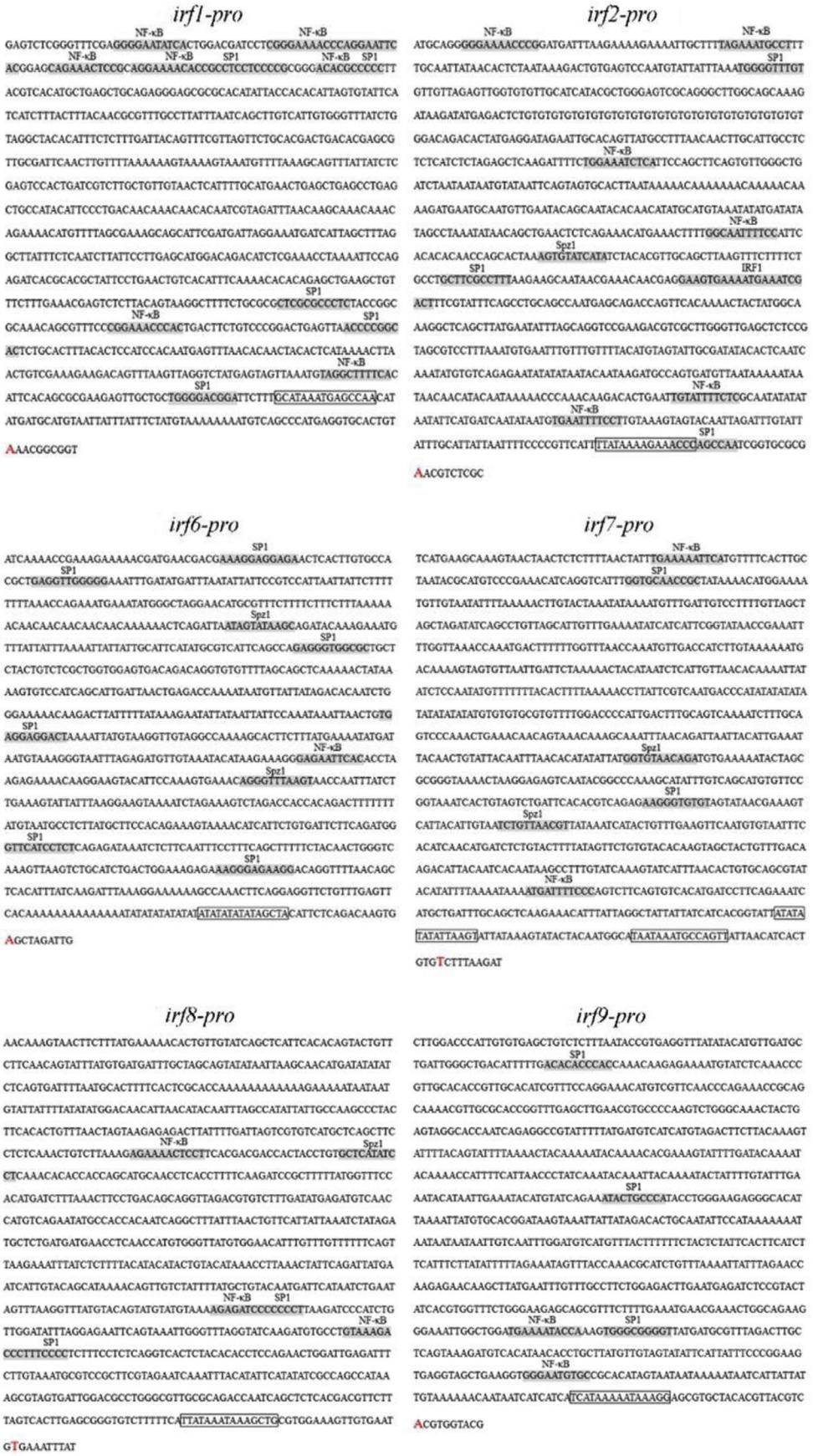

Fig. 10. Promoter functional area prediction map. The gray areas represent transcription factor binding sites, red letters indicate transcription initiation sites, and boxed nucleotides indicate the TATA boxes.

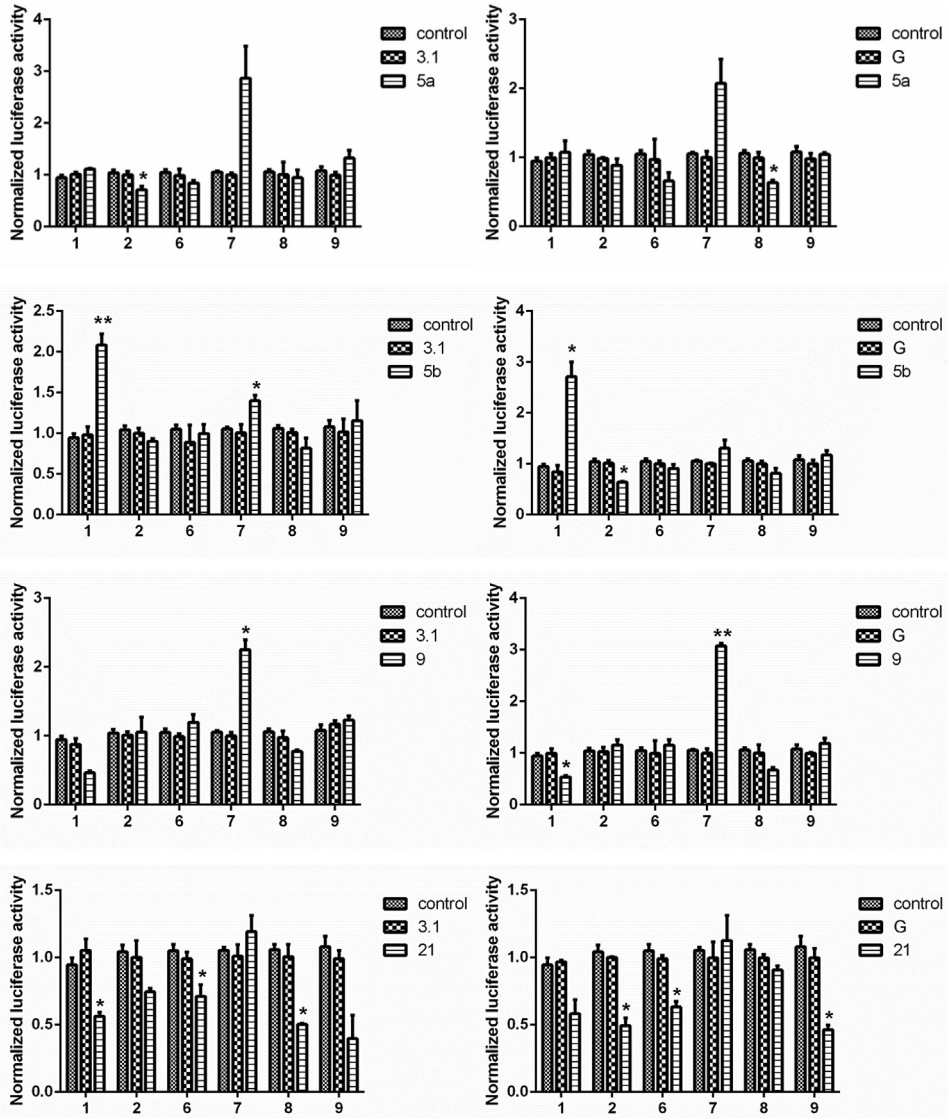

Fig. 11. Overexpression of pcDNA3.1 recombinant plasmid and pEGFP recombinant plasmid activated *irfs* promoter in 293T cells. The numbers 1, 2, 6, 7, 8 and 9 on the x-axis represent *irfs*: *irf1, irf2, irf6, irf 7, irf8* and *irf9*. *$P < 0.05$, **$P < 0.01$, when compared to control group at the same time point post-transfection.

to the flagellin [39]. Here, we found that both paralogues could independently regulate the transcription of interferon-associated genes. As fish Tlr5s generally show more complex and diverse regulation in signal transmission than in mammals, this suggests that functions of these two genes in teleosts may be species-specific.

TLR9 has been identified in mammals and several fish species, such as zebrafish [40], carp [41], grass carp [42], Japanese flounder [10] and puffer fish [43]. The predicted Tlr9 from blunt snout bream TIR domain had high sequence similarity to the TIR domains of other known TLR9 molecules. TLR9 recognizes, and is essential for the inflammatory response to bacterial DNA and unmethylated CpG sequences [44,45]. Tlr9 LRR8 contains two CXXC motifs, which are important for CpG binding. In this research we found high expression of *tlr9* from blunt snout bream in kidney and spleen. In comparison, this gene was highly expressed in kidney and gills of common carp [41]; in Japanese flounder, *tlr9* was highly expressed in peripheral blood, gills, intestines, kidney and spleen [10], but *Danio rerio tlr9* (*Daretlr9*) was highly expressed in blood, and at lower levels in muscle, liver and spleen [40]. These differences in tissue expression patterns among different studies may be species-specific. In mammals, TLR9 is essential for the recognition of CpG ODN in mice and confers responsiveness to CpG ODN (CpG oligodeoxynucleotides) in human cell lines [46,47]. In our study, after the bacterial infection, copy numbers of *tlr9* from blunt snout bream mRNA increased in studied tissues. This is similar to a report in Japanese flounder challenged with *Edwardsiella tarda*; after the challenge, immunohistochemical detection showed that the number of TLR9-expressing cells markedly increased in tissues of infected fish. We found that the expression of *tlr9* from blunt snout bream was increased at 6 h in all studied tissues (except the intestine), especially in liver and kidney. In contrast, in the early stage of *E. tarda* infection in Japanese flounder, *tlr9* expression did not clearly increase in any of the studied tissues.

Tlr21 from blunt snout bream has 12 LRR motifs, which broadly fits the range reported in other teleosts: TLR21 from large yellow croaker also had 12 LRRs, olive flounder - 18 LRRs, orange-spotted grouper - 16 LRRs, and grass carp - 17 LRRs [11,12,48,49]. The TIR domain, which has an important role in the TLR signal transduction, is highly conserved in comparison to Tlr21 in other fish species, all of which have three conserved BOX motifs. This suggests that Tlr21 from blunt snout bream might play a similar role in the intracellular signal transduction. Tissue expression patterns of *tlr21* from blunt snout bream showed that the gene was broadly expressed in most examined tissues, with the most prominent expression in gills, followed by kidney and liver. Similar expression patterns were also reported in zebrafish, large yellow

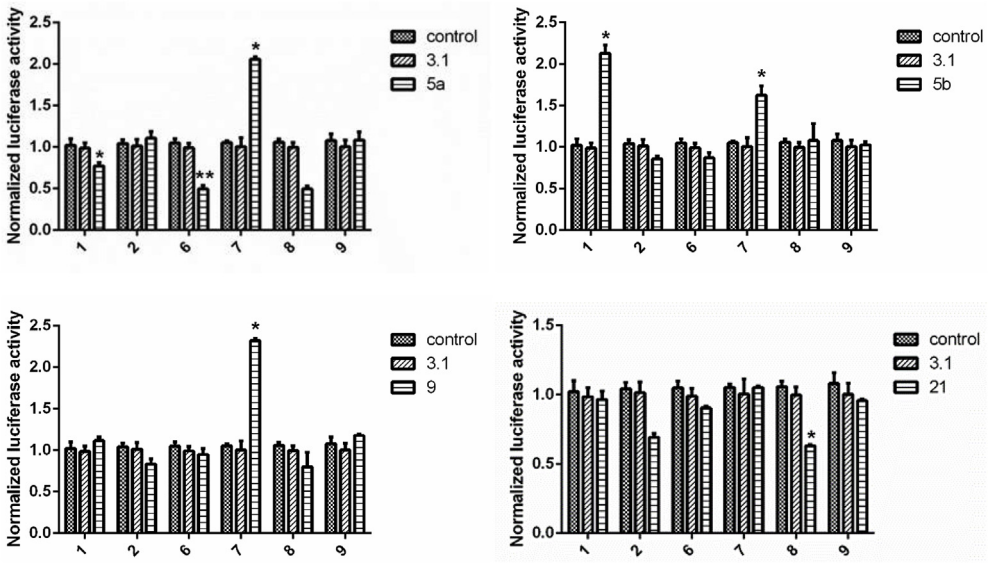

Fig. 12. Overexpression of pcDNA3.1 recombinant plasmid activated *irfs* promoter in EPC cells. The numbers 1, 2, 6, 7, 8 and 9 on the x-axis represent *irfs*: *irf1, irf2, irf6, irf 7, irf8* and *irf9*.

croaker, orange spotted grouper and grass carp [11,12,48–50]. After the bacterial challenge, the expression of *tlr21* was significantly up-regulated in liver, from which we deduce that indicating that in blunt snout bream *tlr21* is induced after bacterial infection in immune system-related tissues. This is similar to the large yellow croaker, where *LcTLR21* gene transcripts increased significantly in all examined tissues after challenges with LPS, *Vibrio parahaemolyticus* and PolyI:C [48]. Similar findings were reported in other fish species, such as *Epinephelus coioides*, *Paralichthys olivaceus* and *Ctenopharyngodon idella* [11,12,48,49].

In this research, the subcellular localization of Tlr9 and Tlr21 in blunt snout bream was identical: mainly in the cytoplasm of EPC cells. In human gastric tissues, subcellular localization of TLR9 showed that it was not only expressed in the cytoplasm, but also on the cell surface. The same phenomenon was observed in the human B cell line TK6 with the same TLR9 antibody. In a chronic active *Helicobacter pylori* gastritis, TLR9 expression in the gastric epithelium was exclusively localized at the basolateral pole of the epithelial cells without detectable expression at the apical pole [36]. Those results suggest that the surface localization of TLR9 may play an important role in the detection of damaged cells and the exogenous DNA introduced by infection [51]. The function of this gene can be preliminarily determined by its localization [52]. In order to interact with its ligand and to initiate signaling, TLR9 needs to be translocated from endoplasmic reticulum to the endosomes [53]. In humans, TLR9 is located in the endoplasmic reticulum of non-immune cells. When immune cells are stimulated by CpG DNA, TLR9 is transferred from the endoplasmic reticulum to the endosome, and interacts with it [53,54]. In fish cells, the situation is more complicated. CpG ODNs colocalize with endogenous SsTLR9 in primary salmon mononuclear phagocytes. However, salmon CHSE cells accumulate significant amounts of CpGs in endolysosomes but fail to relocate transgenic SsTLR9 into these compartments. Similarly, TO cells (a salmon head kidney-derived cell line) do not translocate CpGs into the CpG-containing compartments [55]. As mammals do not have *TLR21*, the functions of this protein are not particularly well understood. While teleosts possess both Tlr9 and Tlr21, birds and amphibians do not have *Tlr9*. Preliminary results indicate that TLR9 and TLR21 are functionally very similar. In chicken, the function of chTLR21 was similar to the mammalian TLR9, as both genes can recognize the same type of ligands. However, there are some functional differences: chTLR21 acts as a receptor for synthetic ODNs, as well as for the DNA of bacterial origin [13,56]. Previous studies demonstrated that Tlr21 was preferentially localized in the endoplasmic reticulum in zebrafish and chicken [13,50]. In large yellow croaker, in HEK-293T cells, *LcTLR21* was mainly localized in the cytoplasm. These results indicate that Tlr9 and Tlr21 from blunt snout bream might have similar functions in cell signaling transduction. After transfection of cells with overexpression vector, both *tlr9* and *tlr21* from blunt snout bream induced downstream immune response in the EPC. These results suggest that Tlr9 and Tlr21 of blunt snout bream may have similar functions to TLRs of mammals in signal transmission and ligand recognition. The induction by *tlr9* occurred earlier than that of *tlr21*, but *tlr21* was more highly upregulated. It is possible that both genes can induce downstream immune factors and that they play complementary roles. However, in zebrafish, zebTLR9 and zebTLR21 recognized CpG-ODN with different CpG motifs: GACGTT or AACGTT were preferentially recognized by TLR9, while TLR21 responded preferentially to the GTCGTT motif [50]. As in zebrafish, different expression levels of *tlr9* and *tlr21* inducing interferon-associated immune factors may also be caused by different ligands recognized by the two genes. It indicates that they are not functionally redundant, and may play a synergistic role in the regulation of signal transduction.

Results of the luciferase reporter assays showed that in blunt snout bream the four *tlrs* can directly affect the activity of *irf* promoters. *tlr5a*, *tlr5b* and *tlr9* promoted the activity of *irf7* promoter, but *tlr5b* also promoted the activity of *irf1* promoter, which suggests that *tlr5b* has more diverse functions in the signal transduction. Previous studies of the IRF family in mammals revealed two distinct mechanisms for the activation of IRF7 promoter: through IFN and virus infection [57]. This is similar to our results, which indicate that IRF7 promoter is activated by TLRs and independently by the IFNs, and that, just as in mammals, IRF7 plays an important role in the interferon pathways in fish.

These results suggest that *tlrs* from blunt snout bream might play important roles in immune responses in fish, specifically in regulating the Irf and inflammatory pathways. These findings provide a strong basis for future studies of the fish innate immune system and its evolution, as well as new directions for the studies of congenital immune regulation in fish.

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