Fish and Shellfish Immunology 86 (2019) 335–346

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Cloning and characterization of the LEF/TCF gene family in grass carp (*Ctenopharyngodon idella*) and their expression profiles in response to grass carp reovirus infection

Denghui Zhu[a,b], Rong Huang[a], Liangming Chen[a,b], Peipei Fu[a,b], Lifei Luo[a,b], Libo He[a], Yongming Li[a], Lanjie Liao[a], Zuoyan Zhu[a], Yaping Wang[a,*]

[a] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, 430072, China
[b] University of Chinese Academy of Sciences, Beijing, 100049, China

ARTICLE INFO

Keywords:
Grass carp
Reovirus infection
LEF/TCF
HMG
Subcellular localization
mRNA expression
Immune

ABSTRACT

T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) proteins from the High Mobility Group (HMG) box family act as the main downstream effectors of the Wnt signaling pathway. HMGB proteins play multifaceted roles in the immune system of mammals. To clarify the immunological characteristics of LEF/TCF genes in grass carp (*Ctenopharyngodon idella*), five LEF/TCF genes (TCF7, LEF1, TCF7L1A, TCF7L1B, and TCF7L2) were identified and characterized. All five LEF/TCF proteins contained two characteristic domains: a HMG-BOX domain and a CTNNB1_binding region. Phylogenetic tree analysis revealed that the LEF/TCF proteins were represented different lineages. These results of subcellular localization showed that four of the LEF/TCF genes were localized exclusively within the nucleus, while TCF7L2 was localized in the cytoplasm and nucleus. The mRNA expression profiles of these LEF/TCF family genes differed across different tissues. The mRNA expression levels of TCF7, TCF7L1A, and TCF7L2 changed significantly in liver after grass carp reovirus (GCRV) challenge; TCF7 and TCF7L1A responded early while TCF7L2 responded late. This suggests that these genes may participate in GCRV-related immune responses. Moreover, TCF7 promoted Bcl6 transcription in response to the GCRV challenge. These findings further our understanding of the function of LEF/TCF genes in teleosts.

1. Introduction

The Wnt signaling pathway is one of the major signaling mechanisms regulating cell-fate during embryogenesis and in adult tissues [1]. Most studies of immunity and blood cells have focused on this pathway, in which β-catenin plays a central role [2–4]. T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) proteins from the High Mobility Group (HMG) box family act as the main downstream effectors of the Wnt signaling pathway [5]. HMGBs function in a number of foundational cellular processes, such as transcriptional regulation, DNA repair, recombination, differentiation, and extracellular signaling, as well as in the nucleosome [6,7]. HMGB proteins function as a ligand that can evoke inflammatory responses, and as sensors for nucleic acid-mediated immune responses in mammals [8,9]. A previous study demonstrated that HMGB2a and HMGB2b in grass carp not only mediated antiviral immune responses, but were also involved in responding to viral/bacterial PAMPs challenges [10].

Nuclear DNA-binding TCF/LEF proteins and their transcriptional cofactor β-catenin represent the key components of the canonical branch of the Wnt signaling pathway. In the nucleus, β-catenin associates with LEF/TCF to activate transcription of Wnt signaling target genes [11]. The mammalian TCF/LEF family comprises four nuclear factors TCF7, LEF1, TCF7L1, and TCF7L2 (also known as TCF1, LEF1, TCF3, and TCF4, respectively) [12]. However, five factors have been identified in zebrafish: TCF1, LEF1, TCF3a, TCF3b, and TCF4 [13–16].

The LEF/TCF family exhibits extensive patterns of alternative splicing, alternative promoter usage, and activities of repression, as well as activation [5]. Many studies have highlighted the important biological consequences that this complexity has in development and disease. Runx3 can interact with all four TCF/LEF family genes, and this interaction attenuates TCF4-β-catenin signaling during intestinal tumorigenesis [17]. In mature $CD8^+$ T cells, TCF-1 and LEF-1 regulate the generation, maturation, and longevity of memory $CD8^+$ T cells in response to viral or bacterial infection [18–20]. TCF-1 restrains the expression of interleukin 17 (IL-17A) in developing thymocytes and activates $CD4^+$T cells [21]. In addition, TCF-1 can interact with Foxp3

* Corresponding author. Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China.
E-mail address: wangyp.ihb@gmail.com (Y. Wang).

https://doi.org/10.1016/j.fsi.2018.11.057
Received 11 May 2018; Received in revised form 5 September 2018; Accepted 26 November 2018
Available online 27 November 2018

[22]. TCF7 and LEF1 are considered to play an essential role in the development of Natural killer T (NKT) cells [23]. TCF1 is required for the T follicular helper (TFH) cell response to viral infection [24]. TFH cells express high levels of B cell lymphomas 6 (Bcl6), a transcriptional repressor, which is essential for TFH cell differentiation [25–27]. TCF-1 can bind to the Bcl6 promoter and enhance transcription of Bcl6 [28]. Therefore, the LEF/TCF family has an important role in the immune system.

Innate immunity is crucial for teleosts as it protects them against viral infection and bacterial invasion [29,30]. Grass carp (*Ctenopharyngodon idella*), an economically important freshwater aquaculture species in China, accounts for more than 18% of total freshwater aquaculture production [31]. However, each year grass carp production is reduced by 30% or more by disease, which causes tremendous economic losses to grass carp farmers and restricts the further development of grass carp aquaculture. Of all fish diseases, grass carp hemorrhagic disease caused by grass carp reovirus (GCRV) is one of the most damaging and pathogenic [32]. Currently, there are limited drugs and vaccines available to target infection by this pathogen. Thus, a better understanding of the innate immune response in grass carp could accelerate disease-resistant breeding.

The main purposes of this study were to clone the full-length cDNA of the LEF/TCF gene from *C. idella* and examine the phylogenesis of LEF/TCF, and to study tissue-specific expressions of the gene and its response to GCRV challenge to better understand the antiviral immune mechanism in *C. idella*. Such functional experiments will aid future research into fish immune systems.

2. Materials and methods

2.1. Ethics statement

All animal experiments were complied with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). All protocols were approved by the committee of the Institute of Hydrobiology, Chinese Academy of Sciences (CAS). The reference number obtained was Y11201-1-301 (Approval date: 30 May 2016). All surgeries were performed under eugenol anesthesia (final concentration: 100 mg/L) and all efforts were made to minimize suffering.

2.2. Experimental animals, GCRV exposure, and sample collection

A total of 100 tails of healthy grass carp at 3-months-old were used in the study. The grass carp, weighing approximately 10 g with an average length of 7 cm, were obtained from the Guanqiao Experimental Station, Institute of Hydrobiology, Chinese Academy of Sciences (CAS), and acclimatized in aerated freshwater at 28 °C for 1 week before processing. The grass carp were fed twice daily, at 9:00 a.m. and 5:00 p.m., during the experimental period. The virus was identified as GCRV-GD108.

After grass carp were confirmed as healthy, they were exposed to the virus by feeding as follows. Dead fish with apparent symptoms of GCRV infection were collected and homogenized together with an equal volume of 0.75% saline. Low-speed centrifugation was conducted at $8000 \times g$ for 20 min at 4 °C to remove impurities. The supernatant was filtered using a 0.45-mm Millex filter (Millipore, USA), and used as the source virus. The titer of virus in the supernatant was determined using RT-qPCR (3.12×10^3 copy/ul) using specific primers (Table 1) for the S6 segments of the GCRV. The supernatant of virus was mixed with an equal amount of commercial feed. The experimental group of fish was fed the feed mixture on the first day, then with commercial feed on the subsequent days. The temperature was maintained at 26–28 °C throughout the experiment.

Three healthy grass carp were sacrificed, and tissue samples of skin, gill, intestine, liver, spleen, head kidney, middle kidney, muscle, and brain tissues were isolated and homogenized in TRIzol reagent (Invitrogen, USA). All samples were prepared for full-length cDNA cloning and tissue expression pattern analysis of the LEF/TCF genes.

To observe the effect of viral infection on the mRNA expression of the LEF/TCF family from the immune system, three fish were randomly sampled as a group, and their gills, liver, spleen, and intestine were harvested on days 1, 2, 3, 4, 5, and 6 post-GCRV infection.

All the samples of RNA were extracted according to the manufacturer instructions for TRIzol reagent. RNA samples were incubated in RNase-free DNase I (Promega, USA) to eliminate any contaminating genomic DNA. Random primers and a ReverTra Ace kit (Toyobo, Japan) were used to reverse transcribe the RNA into cDNA.

2.3. Cloning the full-length cDNA of TCF/LEF1 family genes

The sequences of the *Danio rerio* LEF/TCF family genes (*D. rerio TCF7*, Accession no. NM_001012389.2; *D. rerio LEF1*, Accession no. NM_131426.1; *D. rerio TCF7L1A*, Accession no. NM_131269.2; *D. rerio TCF7L1B*, Accession no. NM_131296.2; and *D. rerio TCF7L2*, Accession no. NM_131259.1) were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/nuccore/). Then, specific fragments of the grass carp LEF/TCF were obtained by blast searching the sequences of zebrafish with the draft genome of the grass carp [33]. Specific primers (Table 1) for amplification of LEF/TCF genes were designed based on the sequences obtained above. The first strand cDNA described above was used as the template for PCR. The 5′ and 3′ ends of the LEF/TCF genes were obtained using the SMARTer™ RACE cDNA Amplification Kit (Invitrogen, USA). The PCR conditions were as follows: 94 °C for 2 min, 35 cycles of 94 °C for 10 s, annealing at 62 °C for 30 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. The PCR products were purified using a Gel Extraction Kit (Omega, USA), and the purification products were ligated into pMD18-T vectors (Takara, Japan). The ligation products were transferred into *E. coli* DH5a cells (TransGen, China) and cultured at 37 °C for 12 h. Positive colonies were selected for sequencing by a commercial company (Tsing ke, China).

2.4. Sequence analysis

ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) was used to predict the location of the open reading frame (ORF). The nucleotide and predicted amino acid sequences of the LEF/TCF family in grass carp were analyzed using The Sequence Manipulation Suite (STS) (http://www.bio-soft.net/sms/). The online CDD (Conserved Domain Data base) tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and the Simple Modular Architecture Research Tool (SMART) program (http://smart.emblheidelberg.de/) were used to analyze the protein domain features. The online SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide. NLS and NES were predicted using putative patterns and NetNES 1.1 Server (http://www.cbs.dtu.dk/services/NetNES/). Multiple sequence alignments were performed using the ClustalW 2.1 program (http://www.ebi.ac.uk/tools/clustalw2.1). The phylogenetic tree was constructed based on the complete amino acid sequences using MEGA 5.0 software (http://www.megasoftware.net/index.html). In addition, *Bos taurus*, *Homo sapiens*, and *Mus musculus* were introduced as outgroups.

2.5. Expression analysis of the LEF/TCF genes in different tissues

For tissue distribution analysis of the LEF/TCF family, RT-qPCR was performed on 10 tissues samples form healthy grass carp (as mentioned in 2.2). The housekeeping gene β-actin was used as the reference gene and the expression level in the liver was used as the reference line (1.0) for qRT- PCR. Specific primers (Table 1) of β-actin and the LEF/TCF family were designed for RT-qPCR. Relative expression levels of LEF/TCF family mRNA in different tissues were examined using RT-qPCR in

Table 1
Primers for full-length cDNA cloning and RT-qPCR.

Gene	Primer name	Sequence(5'→3')	Application
TCF7	TCF7-F	GGCGACGACAACAACAATCAAA	partial sequence obtaining
	TCF7-R	GCTCTCTGTTTTAATTCTGCTGAAGTC	
	TCF7-5′Rout	ATCAGGAACGGGTATCCAGAATACG	5′RACE
	TCF7-5′Rin	CACGTCGTCCAGATGTTCTCG	
	TCF7-3′Rout	AAGCAGCAGGACTCCAGCTCAG	3′RACE
	TCF7-3′Rin	CAGCAGGACTCCAGCTCAGATAA	
	TCF7-RTF	GCGTTCACGGAGAGCGACCT	RT-qPCR
	TCF7-RTR	TGGGCACGTCGTCCAGATGT	
LEF1	LEF1-F	GCCGCAGTTGTCAGGTGGA	partial sequence obtaining
	LEF1-R	GAACTCAAGGTTTGCCTTTAGGATT	
	LEF1-5′Rout	CTTGTCGTGGTAAGAGTCTGGTGTT	5′RACE
	LEF1-5′Rin	TGCGTCGTGATTGTTACTGTTGG	
	LEF1-3′Rout	ATCTCGGGAAGAGCAAGCTAAGTAT	3′RACE
	LEF1-3′Rin	CAATTATGGAAAGAAAAAGAAGCGG	
	LEF1-RTF	GCCCAAAAGACCTCACATCAAGA	RT-qPCR
	LEF1-RTR	TGGCCTGTACCTGAGGCAGG	
TCF7L1A	TCF7L1A-F	TACAGGTCCTGCTAAGAGAAGAAAGTT	partial sequence obtaining
	TCF7L1A-R	TTTTACTCCACTGATTTGGTGACCA	
	TCF7L1A-5′Rout	GCCTGATAAATCTCCCTAAATCCAC	5′RACE
	TCF7L1A-5′Rin	TGGGAGATAGCGATCCGTTGG	
	TCF7L1A-3′Rout	TCACCACCAAACCCGAGGGAAGAGC	3′RACE
	TCF7L1A-3′Rin	CCAGTCGGCGACCCTCCTTT	
	TCF7L1A-RTF	ACGCCTCCATGTCTAGTCTGGTATC	RT-qPCR
	TCF7L1A-RTR	CACTACTTTGGCCCTCATTTCCTT	
TCF7L1B	TCF7L1B-F	GTTTGGTAAGTTTGTCAGGACGACA	partial sequence obtaining
	TCF7L1B-R	AAGCAGATGATCAAATATTGACCAATAA	
	TCF7L1B-5′Rout	GTGACCGTGAGTCTTTGAGTGCTG	5′RACE
	TCF7L1B-5′Rin	TAGAAATGGGTAGCCTGGATAATGG	
	TCF7L1B-3′Rout	GACCAGCTCCTCAGGCAGTCAG	3′RACE
	TCF7L1B-3′Rin	CTCTTCACTCCCATCATGCTTTGTT	
	TCF7L1B-RTF	CCCAGCGGCTCACACGAAGT	RT-qPCR
	TCF7L1B-RTR	TGCGGCCGAGGATCTGGTTG	
TCF7L2	TCF7L2-F	TCGGGATTCGGGGATCACA	partial sequence obtaining
	TCF7L2-R	AACACAGAATGAAAAAAGACAGAAGACA	
	TCF7L2-5′Rout	CCTGGCATCTTTAAGTGCTTGTCTA	5′RACE
	TCF7L2-5′Rin	TAGATATGTGCGAGCGGTCGGT	
	TCF7L2-3′Rout	CTTAGCTCTGTTGGACAACTCCG	3′RACE
	TCF7L2-3′Rin	CCCACATCGGCGTGTCATTC	
	TCF7L2-RTF	ATCCCTCAACAGCTCGAAACATC	RT-qPCR
	TCF7L2-RTR	TCGTTCTTTCCTGGCTAACTCGTA	
CTNNB1	β-catenin-F	GGCCCACAGGCTCTGGTCAA	RT-qPCR
	β-catenin-R	GCATGCCACCAGCCTCAACG	
Bcl6	Bcl6-F	CAGTGGGCTCTTCTACTCAATCTTTACC	RT-qPCR
	Bcl6-R	TCAGTGTGAGACGTGAAGTGTACATAAAC	
β-actin	β-actin-F	TCGGTATGGGACAGAAGGAC	RT-qPCR
	β-actin-R	GACCAGAGGCATACAGGGAC	
S6 segments	GCRV-F	AGCGCAGCAGGCAATTACTATCT	RT-qPCR
	GCRV-R	ATCTGCTGGTAATGCGGAACG	

a CFX96 real-time PCR detection system (Bio-Rad, USA). The RT-qPCR cycling conditions were as follows: 95 °C for 2 min, 40 cycles of 95 °C for 10 s, annealing at 62 °C for 20 s, and 72 °C for 30 s, followed by a the Melt Curve was constructed. The expression level of LEF/TCF family mRNA was calculated using the $2^{-\Delta\Delta CT}$ method [34].

2.6. Temporal expression analyses of the LEF/TCF genes after GCRV infection

To examine the effects of viral infection on the mRNA expression of the LEF/TCF family in the immune system, RT-qPCR was performed on samples as described in 2.2. The special primers and RT-qPCR cycling conditions were the same as in 2.5. The housekeeping gene β-actin was used as the reference gene and the expression level of the untreated groups (0 days) was set as the baseline (1.0) for qRT- PCR. The relative expression was calculated as the ratio of gene expression in the treated groups at each time point (from 1 to 6 days after the GCRV challenge) relative to that in the untreated groups (0 days).

2.7. The mRNA expression of β-catenin, TCF7 and Bcl6 after GCRV infection

To understand the mechanism that the TCF7 gene was down-regulated throughout the test period in the gill, the mRNA of β-catenin was observed in the gill. To observe the regulation pattern of Bcl6 by TCF7 after GCRV infection, RT-qPCR was performed on liver tissues (as described in 2.2). The housekeeping gene β-actin was used as the reference gene and the expression level of the untreated groups (0 days) was set as the baseline (1.0) for RT-qPCR. The specific primers for RT-qPCR are listed in Table 1. The relative expression was calculated as the ratio of gene expression in the treated groups at each time point (from 1 to 6 days after GCRV challenge) relative to that in the untreated groups (0 days).

2.8. Subcellular localization of LEF/TCF

A CIK cell line, provided by China Center for Type Culture Collection, was cultured in Medium 199 (Sigma, USA) supplemented with 10% fetal bovine serum (FBS; Biosource, USA), 100 IU/ml of

Table 2
Primers in the plasmid vector construction. The nucleotides in lowercase mark the restriction enzyme site. 'CCG' and 'CGC' in the 5′ terminal represent protective bases.

Gene	Primer name	Sequence(5'→3′)	Application
TCF7	TCF7-SubF	CCGctcgagGTATGCCGCAGCTGAACGGT	pEGFP-TCF7
	TCF7-SubR	CGCggatccGGAACAGTCAAGAGAGTTATCTGAGCT	
LEF1	LEF1-SubF	CCGctcgagGTATGCCGCAGTTGTCAGGTGG	pEGFP-LEF1
	LEF1-SubR	CGCggatccGATGTACGCCGTTTTCATTCTCTG	
TCF7L1A	TCF7L1A-SubF	CCGctcgagGTATGCCTCAATTAAACGGAGGAGG	pEGFP-TCF7L1A
	TCF7L1A-SubR	CGCggatccCTCCACTGATTTGGTGACCAGG	
TCF7L1B	TCF7L1B-SubF	CCGctcgagGTATGCCACAGCTTAACGGAGGT	pEGFP-TCF7L1B
	TCF7L1B-SubR	CGCggatccGTCAGCTGATTTGGTTACCAACG	
TCF7L2	TCF7L2-SubF	CCGctcgagGTATGCCGCAGCTGAACGGC	pEGFP-TCF7L2
	TCF7L2-SubR	CGCggatccGTCTATCGACTTAGTTACGAGTGAAAGG	

penicillin (Sigma), and 100 mg/ml of streptomycin (Sigma). Cells were incubated at 28 °C in a 5% CO_2 humidified atmosphere, and the medium was changed every 3 days.

pEGFP-N3 (Clontech, USA) was used as the original plasmid. The vectors that were used to analyze the subcellular localization of the LEF/TCF family were constructed by ligating the fragment that contained a complete ORF to pEGFP-N3. Sense primers with the *XhoI* site and antisense primers with *BamHI* (Table 2) were designed to amplify the complete ORF of the LEF/TCF family. Then, the fragments were double-cleaved using *XhoI* and *BamHI* and the pEGFP-N3 was digested with the same enzymes. The target fragments were purified, ligated with T4 ligase, and used to transform bacteria to select positive clones. Finally, we verified that the vectors were successfully constructed using sequencing and restriction enzymes analysis. The recombinant plasmids were named pEGFP-TCF7, pEGFP-LEF1, pEGFP-TCF7L1A, pEGFP-TCF7L1B, and pEGFP-TCF7L2.

For transfection, CIK cells were plated into 6-well plates (NEST China) at a density of 5×10^5 cells/ml. After approximately 24 h, transfection was performed with Hieff TransTM Liposomal Transfection Reagent (YEASEN, China) according to the manufacturer instructions. At 24 h post-transfection, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. For nuclear staining, cells were incubated in 0.1 mg/ml Hoechst 33342 for 10 min in the dark. The cells were then mounted with 50% glycerol and observed under a fluorescence microscope (Olympus, Germany).

2.9. Statistical analysis

Data were expressed as the mean ± standard deviation (S.D.). All the data were analyzed using SPSS 18.0 software, and significant differences between means were determined using a one-way ANOVA. Differences were considered significant at $P < 0.05$, denoted by *.

3. Results

3.1. Sequence analysis of the LEF/TCF family

Five LEF/TCF family genes in grass carp, TCF7, LEF1, TCF7L1A, TCF7L1B, and TCF7L2 (GenBank accession numbers: MH161193, MH161194, MH161195, MH161196, and MH161197, respectively), also known as TCF1, LEF1, TCF3A, TCF73B, and TCF4, respectively, were successfully cloned. The sequence details of the cDNAs and proteins are listed in Table 3 and Fig. S1. All five LEF/TCF proteins contained two characteristic domains: a HMG-BOX domain of DNA-binding and a CTNNB1_binding region (Fig. 1). The five LEF/TCF proteins were non-secreted proteins without a signal peptide in the SignalP 4.0 Server.

The sequence similarity of the LEF/TCF family in *C. idella* ranged from 49% to 82%. TCF7L1A and TCF7L1B showed the highest similarity (Table 4). Moreover, CiTCF7, CiLEF1, CiTCF7L1B, and CiTCF7L2 showed the highest similarities with *D. rerio* TCF7 (84%), *D. rerio* LEF1 (98%), *D. rerio* TCF7L1B (88%), and *D. rerio* TCF7L2 (99%) respectively. However, CiTCF7L1A was highly similar to *Notothenia coriiceps* TCF7L1A (84%) (Table 5 and Fig. S2).

3.2. Phylogenetic analysis

To gain a deeper understanding of the molecular evolutionary relationships between the LEF/TCF family in other species, a phylogenetic tree was constructed based on the homologous amino acid sequences from other teleost fish (*Danio rerio, Xenopus laevis, Larimichthys crocea, Clupea harengus, Ictalurus punctatus, Salmo salar, Oryzias latipes,* and *Cynoglossus semilaevis)* and non-fish (*Homo sapiens, Bos taurus, Mus musculus,* and *Gallus gallus*). The accession numbers of the sequences used to construct the tree are given after the species names in the tree. As shown in Fig. 2, the LEF/TCF genes were separated into four groups containing TCF7, LEF1, TCF7L1, and TCF7L2. The dendrogram also showed that all LEF/TCF in grass carp were closely related to those in zebrafish.

3.3. Tissue distribution of the LEF/TCF mRNA

As shown in Fig. 3, all five LEF/TCF mRNA were expressed in all 10 tissues sampled. However, the relative expression levels of LEF/TCF mRNA were different. The expression of TCF7 was high in the spleen, moderate in the gill, brain, and head kidney, and low in the muscle, liver, and heart (Fig. 3A). LEF1 and TCF7L2 had higher levels in the brain (Figs. 3B and 4E), and LEF1 was less expressed in other tissues. However, TCF7L2 was moderately expressed in the spleen, muscle, and skin, and low levels were observed in the liver, head kidney, and middle kidney. Figs. 3C and 4D shows that the expression patterns of TCF7L1A and TCF7L1B were similar, which showed high expression levels in the spleen, moderate levels in the skin, gill, muscle, brain, and heart, and

Table 3
The sequences details of the cDNA and the proteins in *C. idellus*.

Gene	Full lengths	ORF lengths	Lengths of 5′UTR	Lengths of 3′UTR	Protein lengths	Molecular mass	Theoretical pI
TCF7	1583bp	1095bp	140bp	348bp	364aa	41.32 kDa	8.24
LEF1	1146bp	1104bp	146bp	195bp	367aa	41.36 kDa	6.82
TCF7L1A	2679bp	1905bp	481bp	293bp	634aa	69.78 kDa	9.66
TCF7L1B	2209bp	1722bp	300bp	187bp	573aa	62.88 kDa	8.47
TCF7L2	2858bp	1833bp	330bp	695bp	610aa	66.93 kDa	8.99

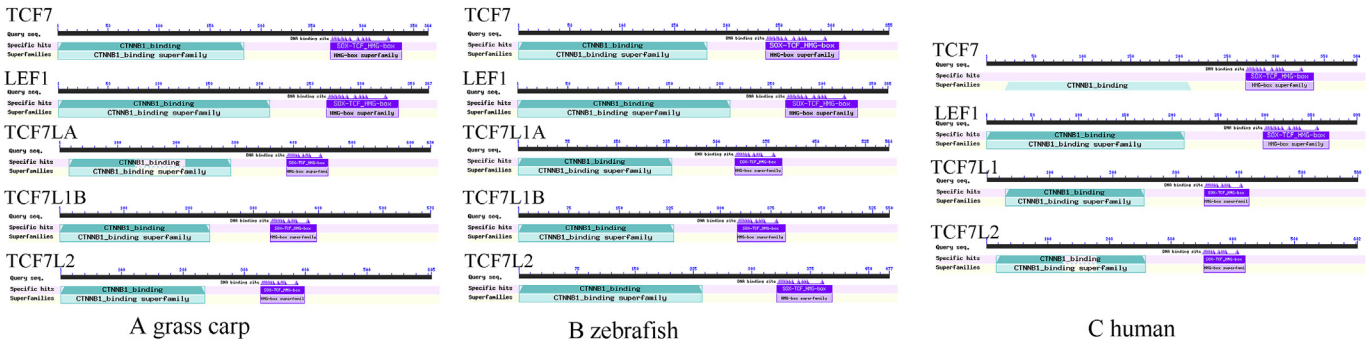

Fig. 1. The protein domain analyze of grass carp (A), zebrafish (B) and human (C) LFF/TCF proteins. The deduced LEF/TCF protein structures of grass carp were analyzed by the online tool CDD (Conserved Domain Database) at NCBI.

Table 4
Comparison of sequence similarity of TCF Family genes in *C. idellus*.

LEF/TCF proteins	length (aa)	C. idella LEF1	C. idella TCF7L1A	C. idella TCF7L1B	C. idella TCF7L2
C. idella TCF7	364	53%	54%	51%	53%
C. idella LEF1	367		61%	49%	51%
C. idella TCF7L1A	634			82%	53%
C. idella TCF7L1B	573				58%
C. idella TCF7L2	610				

Table 5
Sequence identities of the deduced amino acid of LEF/TCF genes between *C. idellus* and other species.

	D.rerio	*C.harengus*	*G.gallus*	*H.sapiens*	*B.taurus*	*C.semilaevis*	*I.punctatus*	*O. mykiss*	*M.musculus*
TCF7	84%	74%	67%	52%	52%	69%	78%	81%	
LEF1	98%	87%	83%	78%	78%				77%
TCF7L1A	77%	70%	64%	61%	57%				64%
TCF7L1B	88%	85%	68%	69%	67%				67%
TCF7L2	99%	85%	95%	78%	83%	86%	90%		95%

low levels in the liver, intestine, head kidney, and middle kidney.

3.4. The temporal expressions of LEF/TCF genes after GCRV infection

Fig. 4 shows the expression levels of grass carp LEF/TCF family genes at different time points compared with the control group (0 days). After GCRV infection, the mRNA expression levels of TCF7 in the spleen, intestine, and liver tended to increase then decrease. In the spleen, the TCF7 expression level peaked (2.1-fold, $P < 0.05$) on day 3, and rapidly recovered to the initial level and remained stable until the end of the experiment. On day 2, the expression of TCF7 reached its highest level in liver and intestine (3.6-fold, $P < 0.05$ and 1.6-fold, $P < 0.05$, respectively), then quickly declined to the lowest level (0.42-fold, $P < 0.05$ and 0.42-fold, $P < 0.05$, respectively) on day 4. In the gill, the TCF7 was down-regulated throughout the test period (Fig. 4A). The expression of LEF1 after GCRV infection is shown in Fig. 4B. In gill and spleen, the expression of LEF1 reached its highest level on day 5 (2.5-fold, $P < 0.05$ and 1.9-fold, $P < 0.05$, respectively), and then was significantly down-regulated. In the liver, the expression level of LEF1 was the highest on day 1, then decreased and reached its lowest level on day 4. In the intestine, LEF1 expression level did not change significantly in the first 2 days, then reached its highest level on day 3, and then decreased on day 4.

TCF7L1A responded strongly to GCRV challenge (Fig. 4C). In the liver, the expression of TCF7L1A was up-regulated at all time points compared to day 0 and reached a peak (19.9-fold, $P < 0.05$) at day 1. In the gill, there was no significant change in the expression of TCF7L1A, which was slightly up-regulated on day 4.

TCF7L1A was moderately up-regulated in the spleen and intestine on days 2 and 4, and the highest expression levels (3.7-fold, $P < 0.05$ and 3.5-fold, $P < 0.05$, respectively) were detected on day 2, which then slightly declined and dropped to the initial levels.

TCF7L1B expression after GCRV challenge is shown in Fig. 4D. The mRNA of TCF7L1B was always down-regulated in the gill and spleen and reached its lowest level on day 6. After GCRV infection, the expression of TCF7L1B was slightly up-regulated in the liver and intestine and reached a peak (1.6-fold, $P < 0.05$ and 1.9-fold, $P < 0.05$, respectively) on day 2, and then slowly became down-regulated in the liver. However, in intestine, TCF7L1B expression remained below the initial level after day 2.

There were sight changes in TCF7L2 expression (Fig. 4E) in spleen. In the gill, the mRNA level was down-regulated after GCRV exposure and sharply reached a peak after 5 days (2.5-fold, $P < 0.05$). In the liver, the mRNA expression was up-regulated after GCRV infection and peaked on day 5 (11.2-fold, $P < 0.05$), following a down-regulation and remained higher than the original level. In the intestine, the expression of TCF7L2 showed a dynamic change.

3.5. The mRNA expression of β-catenin,TCF7 and Bcl6 after GCRV infection

After GCRV infection, the mRNA levels of β-catenin was down-regulated throughout the test period in gill (Fig. 5).

Since TCF7 significantly changed in the liver after GCRV infection, the mRNA expression of Bcl6 was examined in liver tissue. As shown in Fig. 6, the mRNA levels of TCF7 were up-regulated on days 1, 2, and 5 in liver and Bcl6 was also up-regulated on days 1, 2, and 5. The mRNA levels of TCF7 and Bcl6 reached a peak on day 2. From Fig. 6, it is evident that the expression patterns of TCF7 and BCL6 were the same. Taken together, these results suggest that TCF7 can enhance the

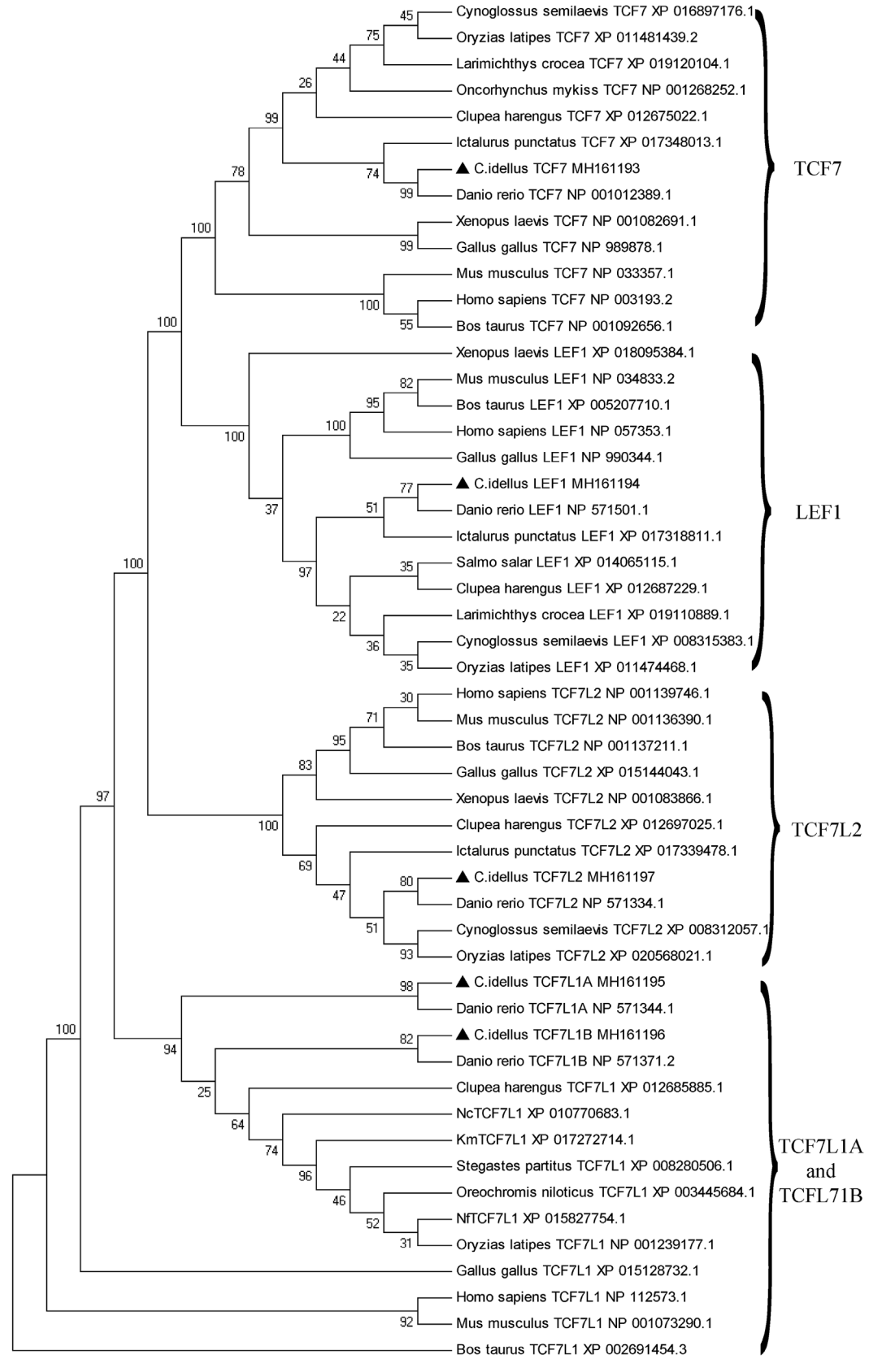

Fig. 2. Neighbor-joining phylogenetic tree analysis. The phylogenetic tree of LEF/TCF amino acid were constructed by MEGA5 software using neighbor-joining (N–J) method. LEF/TCF from grass carp *(Ctenopharyngodon idella)* were marked by triangle. The accession numbers of the sequences used to construct the tree are given after the species names in the tree.

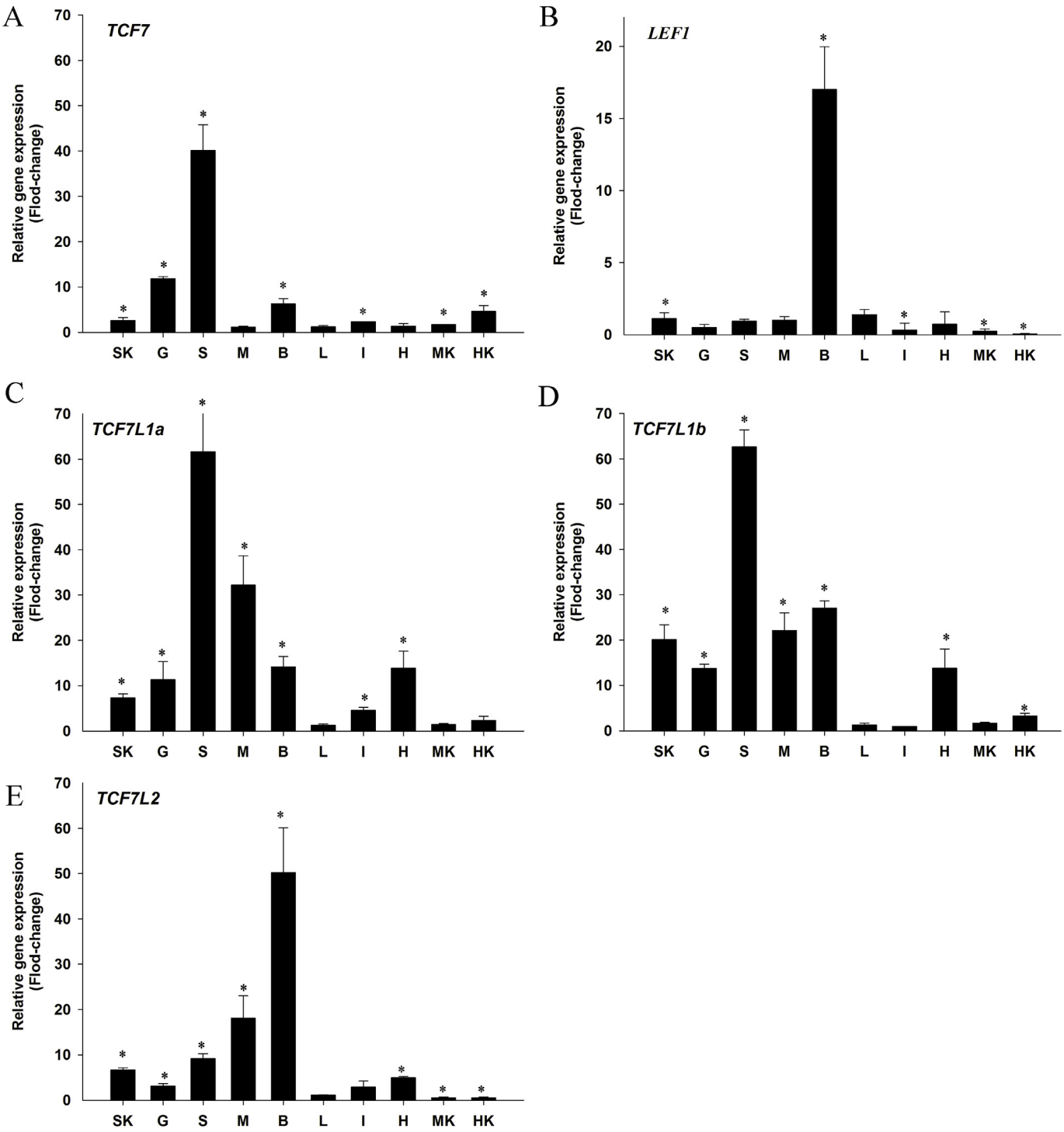

Fig. 3. Tissue distribution of LEF/TCF genes mRNA expression in healthy grass carp. The 10 examined tissues are indicated in abbreviations. SK: skin; G: gill; S: spleen; B: brain; L: liver; HK: head kidney; MK: middle kidney; H: heart; M: muscle; I: intestine; Asterisks (*) indicates significant differences of mRNA expressions between the tissues and liver ($P < 0.05$).

expression of Bcl6.

3.6. Subcellular localization of LEF/TCF genes

To observe the localization of LEF/TCF in cells and assess their localization differences, the constructed vectors were transferred into CIK cells. The empty plasmid pEGFP-N3 was transfected at the same time as the positive control. The results of subcellular localization experiments showed that the four LEF/TCF genes were exclusively concentrated in the nucleus of CIK cells, except for TCF7L2 that was expressed in the cytoplasm and nucleus. The control EGFP was distributed throughout the entire cell (Fig. 7).

4. Discussion

Wnt signaling drives cell fate decisions, proliferation, survival activities, and changes in cell shape [5]. LEF/TCF that are sequence-specific DNA-binding transcription factors play an important role in

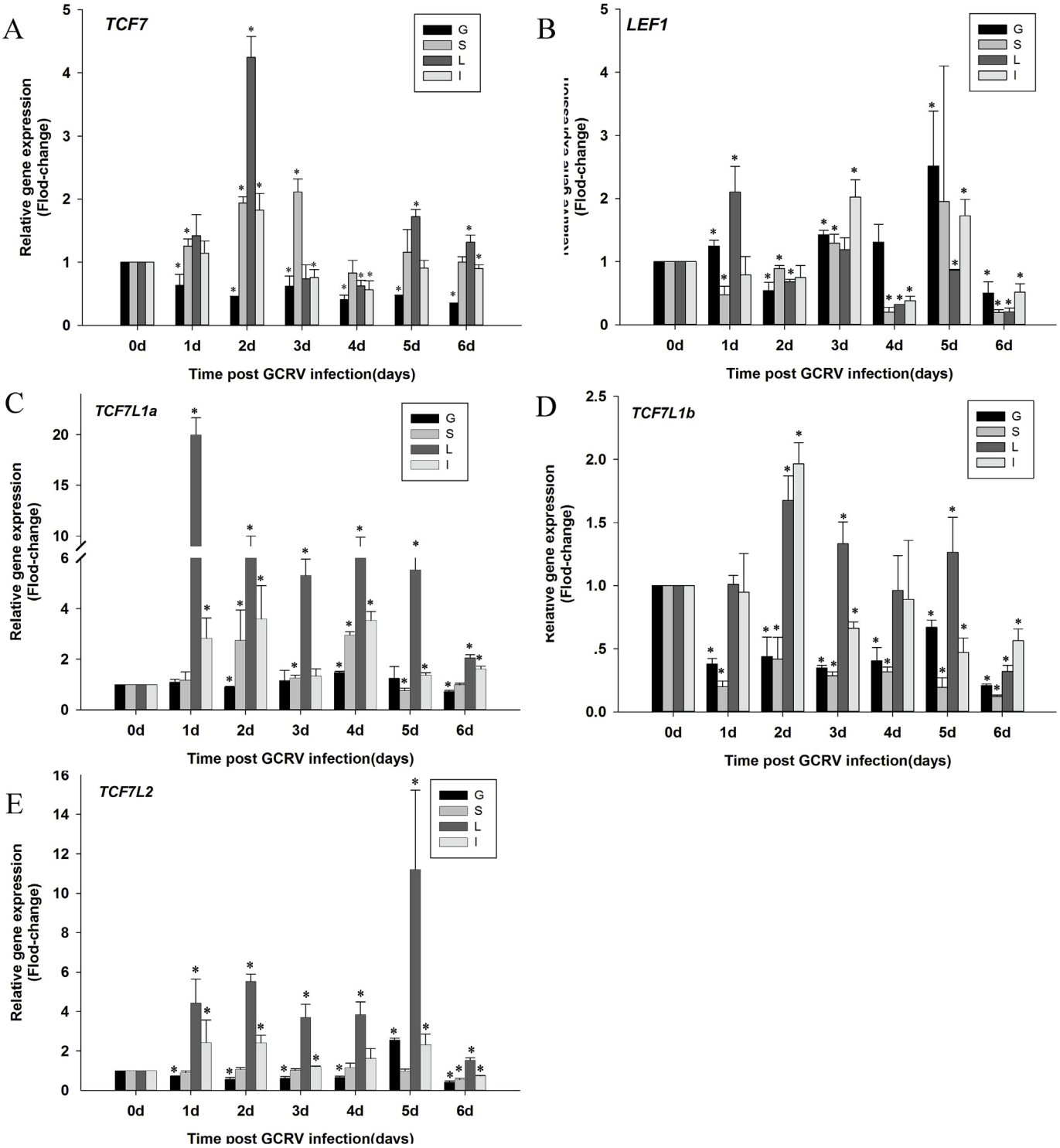

Fig. 4. Temporal expression patterns of the LEF/TCF genes in the immune tissues of *C. idella* following a GCRV infection. (A) TCF7, (B) LEF1, (C) TCF7L1A, (D) TCF7L1B, and (E) TCF7L2. RNA was isolated from the gills (G), liver (L), spleen (S), and intestine (I) and subjected to a RT-qPCR analysis. The expression of the LEF/TCF genes in the untreated groups (day 0) was set at 1.0. β-actin was used as the internal control to normalize the relative expression level of the target gene. Asterisks (*) indicates significant differences of mRNA expressions between the treated groups (1–6 days) and the untreated groups (day 0) ($P < 0.05$). The results were based on three independent experiments and expressed as mean values ± SD.

mediating canonical Wnt signaling by recruiting β-catenin to Wnt target genes [35]. Previous studies of LEF/TCF have focused on human and mouse models. However, little is known about the roles and transcriptional regulatory mechanism of LEF/TCF in teleosts. The purpose of this study was to analyze the characteristics the LEF/TCF genes in grass carp.

In this study, five family genes of LEF/TCF were identified and characterized from grass carp: TCF7, LEF1, TCF7L1A, TCF7L1B, and TCF7L2. We predicted that all five LEF/TCF proteins contained two characteristic domains: a HMG-BOX domain and a CTNNB1_binding region. The region of highest homology between LEF-1/TCF family genes was the HMG DNA-binding domain and the amino-terminal

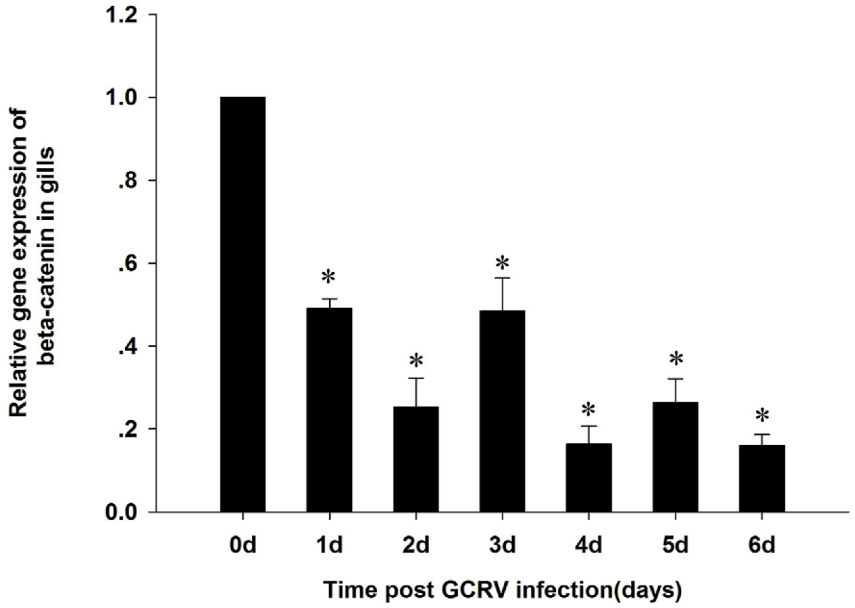

Fig. 5. The mRNA expression of β-catenin in gill. The expression of the genes in the untreated groups (day 0) was set at 1.0. β-actin was used as the internal control to normalize the relative expression level of the target gene. Asterisks (*) indicates a significant level ($P < 0.05$). The results were based on three independent experiments and expressed as mean values ± SD.

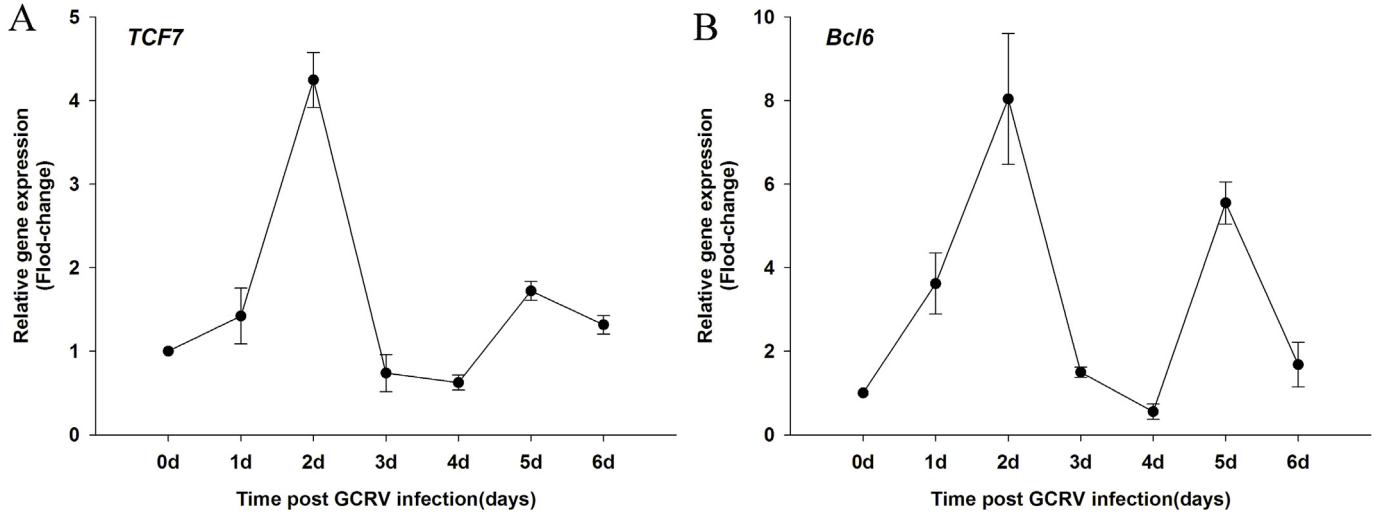

Fig. 6. The mRNA expression of TCF7and Bcl6 in liver after GCRV infection. (A) TCF7, (B) Bcl6. The expression of the genes in the untreated groups (day 0) was set at 1.0. β-actin was used as the internal control to normalize the relative expression level of the target gene.

β-catenin-binding domain [36], which was also highly conserved across fish and mammals. The LEF/TCF proteins exhibited remarkable amino-acid sequence conservation in the high-mobility group DNA-binding domain (HMG; ~95–99% sequence identity) and nuclear localization signal (NLS). The NLS is recognized directly by importin alpha subunits for nuclear import [37]. Comparison of amino acid sequences and phylogenetic trees across different species showed that the LEF/TCF proteins in grass carp and zebrafish were highly related. Therefore, it is possible that, like zebrafish, LEF/TCF proteins in grass carp also have a similar structure–function relationship as mammalian LEF/TCF, and that these proteins conform to represent a conserved trait.

HMGBs occur in a wide variety of eukaryotes and are generally considered to be nuclear proteins [38,39]. All five LEF/TCF proteins were non-secreted proteins without a signal peptide. Under basal conditions without GCRV, poly (I: C) and LPS challenge, all HMGBs are exclusively localized in the nucleus [40]. In this study, four LEF/TCF genes of grass carp were localized in the nuclear, except for TCF7L2 that showed protein expression in the cytoplasm and nucleus. The expression pattern of TCF7L2 was different from that of other family genes. The nuclear localization may be attributed to the NLSs found in the HMGBs [41,42], as well as the DNA-binding activity that prevents the shuttling of HMGBs from the nucleus to the cytoplasm. However, once in the nucleus, all LEF/TCF genes bind to the consensus sequence CCTTTGWW. The HMG box recognizes its site in the minor groove, and the nuclear localization signal contacts the phosphate backbone to elevate DNA binding 100-fold. This cooperation results in nanomolar affinity for the DNA sequence, a value that is merely 20–40-fold above its affinity for any double-stranded DNA [43–45]. Therefore, LEF/TCF can play a regulatory role in the nucleus by associating with β-catenin and binding DNA.

All tested genes of the LEF/TCF family were detected in all tissues sampled, but the mRNA expression profiles of LEF/TCF differed between tissues (Fig. 3). The expression levels of TCF7, TCF7L1A, and TCF7L1B was high in spleen. It is possible that they are related to the

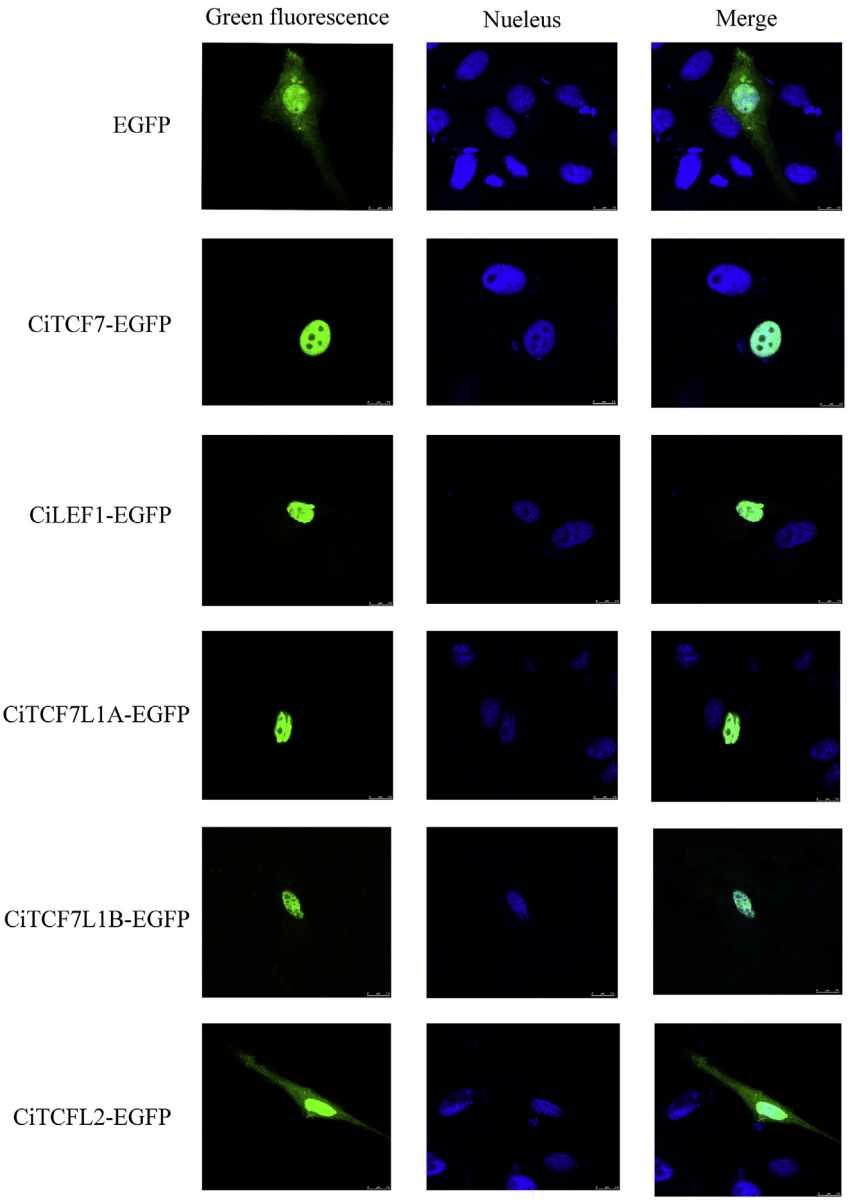

Fig. 7. Subcellular localization of LEF/TCF proteins in CIK cells. CIK cells were plated in 6-well plates. At 24 h post-transfection, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100. Green fluorescence showed the distribution of EGFP or EGFP-tagged proteins, and blue fluorescence showed the nuclei stained with Hoechst 33342 under a 63 × oil immersion objective lens (scale bar, 7.5 μm). All samples were visualized using a confocal microscope.

immune response. The transcription factor TCF7 initiates the differentiation of TFH cells during acute viral infection [28]. LEF1 and TCF7L2 had a high level of expression in the brain. Recently, studies showed that mutations in components of β-catenin signaling networks were associated with several psychiatric disorders, for example, variants of the TCF7L2 gene have been associated with schizophrenia [46,47]. In adult mouse, Lef1 and TCF7L2 mRNAs are the predominant genes of the LEF/TCF family in the thalamus and midbrain [48]. This indicates that β-catenin and LEF/TCF proteins are involved in the proper functioning of the brain in grass carp. In the present study, TCF7L1A and TCF7L1B in grass carp showed moderate expression levels in heart tissue. In zebrafish, two TCF7L1 homologs, TCF7L1A and TCF7L1B, autonomously restrict cardiomyocytes and promote endothelial specification [49]. Perhaps the TCF7L1A and TCF7L1B in grass carp have a similar function to that in zebrafish in grass carp heart tissue. LEF1 is a regulatory participant in lymphocyte gene expression and differentiation [50]. Therefore, LEF/TCF proteins have different functions, although they are similar in structure.

To clarify the immune characteristics of LEF/TCF genes in fishes, the mRNA expression of LEF/TCF was analyzed in the tissues of the gill, spleen, liver, and intestine after GCRV challenge. The mRNA expression level of TCF7, TCF7L1A, and TCF7L2 changed significantly in liver. In the liver, the mRNA expression of TCF7 and TCF7L1A reached a peak within the first two days. However, TCF7L2 expression was moderately up-regulated and peaked on day 5. Thus, TCF7, TCF7L1A responded early while TCF7L2 responded late. Combined with the results of subcellular localization, TCF7, TCF7L1A, and TCF7L2 showed different expression patterns in response to GCRV infection. We note that TCF7 was down-regulated throughout the test period in gill (Fig. 4A). Since T cell factors (TCF) were activated by signaling through the Wnt/Wingless pathway [51], the results showed that β-catenin was down-regulated in gill after GCRV infection. Thus, TCF7 acting as the main downstream effector of the Wnt signaling pathway was regulated by β-catenin.

In teleosts, spleen and liver play important roles in innate immunity [52]. The liver has one of the largest resident populations of macrophages, natural killer cells, and natural killer T cells, all of which are key components of the innate immune system [53]. To further study the regulatory mechanism of TCF7 after GCRV infection, the mRNA expression of Bcl6 in liver was analyzed (Fig. 6). We found that TCF7 promoted Bcl6 transcription in response to the GCRV challenge. A previous study indicated that TCF7 enhances Bcl6 transcription [28]. Therefore, these results indicated that TCF7, TCF7L1A, and TCF7L2 in grass carp participate in GCRV-related immune responses but their response patterns are different.

In summary, five genes of the LEF/TCF gene family were identified in grass carp. The five showed similar structure, although subcellular localization of TCF7L2 was different from other genes. All LEF/TCF genes were expressed in all organs examined, but their expression levels varied in different tissues. All five were up-regulated at an early stage of infection in vivo. We described the characteristics of LEF/TCF genes and concluded that TCF7, TCF7L1A, and TCF7L2 engaged in antiviral immune responses. TCF7 promoted Bcl6 transcription in response to the GCRV challenge. These findings further our understanding of the function of LEF/TCF in teleosts.

Acknowledgments

Funding: This work was supported by the National Natural Science Foundation of China [grant number 31572614]; and the Strategic Priority Research Program of Chinese Academy of Sciences [grant number XDA08030203].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.057.

References

[1] S. Hoppler, R.T. Moon, Wnt Signaling in Embryonic Development and Adult Tissue Homeostasis, John Wiley & Sons, Inc, 2014.
[2] F.H. Brembeck, M. Rosário, W. Birchmeier, Balancing cell adhesion and Wnt signaling, the key role of β-catenin, Curr. Opin. Genet. Dev. 16 (1) (2006) 51–59.
[3] H. Clevers, Wnt/β-Catenin signaling in development and disease, Cell 127 (3) (2006) 469–480.
[4] Q. Xiao, Z.X. Chen, X.Z. Jin, R.Y. Mao, Z.Q. Chen, The many postures of non-canonical Wnt signaling in development and diseases, Biomed. Pharmacother. 93 (2017) 359–369.
[5] L. Arce, N.N. Yokoyama, M.L. Waterman, Diversity of LEF/TCF action in development and disease, Oncogene 25 (57) (2006) 7492–7504.
[6] M. Štros, HMGB proteins: interactions with DNA and chromatin, Biochim. Biophys. Acta 1799 (1–2) (2010) 101.
[7] T. Ueda, M. Yoshida, HMGB proteins and transcriptional regulation, Biochim. Biophys. Acta 1799 (1–2) (2010) 114.
[8] H. Yanai, T. Ban, T. Taniguchi, High-mobility group box family of proteins: ligand and sensor for innate immunity, Trends Immunol. 33 (12) (2012) 633–640.
[9] H. Yanai, T. Ban, Z.C. Wang, M.K. Choi, T. Kawamura, H. Negishi, M. Nakasato, Y. Lu, S. Hangai, R. Koshiba, HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses, Nature 462 (7269) (2009) 99–103.
[10] Y. Rao, J. Su, C. Yang, L. Peng, X. Feng, Q. Li, Characterizations of two grass carp Ctenopharyngodon idella HMGB2 genes and potential roles in innate immunity, Dev. Comp. Immunol. 41 (2) (2013) 164–177.
[11] M. Rahmani, J.M. Carthy, B.M. Mcmanus, Mapping of the Wnt/β-catenin/TCF response elements in the human versican promoter, Methods Mol. Biol. 836 (2012) 35.
[12] H. Dusan, K. Michal, S. Hynek, K. Vladimir, TCF/LEF transcription factors: an update from the internet resources, Cancers 8 (7) (2016) 70.
[13] E.S. Veien, M.J. Grierson, R.S. Saund, R.I. Dorsky, Expression pattern of zebrafish tcf7 suggests unexplored domains of Wnt/beta-catenin activity, Dev. Dynam. Official Publ. Am. Assoc. Anat. 233 (1) (2005) 233–239.
[14] R.I. Dorsky, A. Snyder, C.J. Cretekos, D.J. Grunwald, R. Geisler, P. Haffter, R.T. Moon, D.W. Raible, Maternal and embryonic expression of zebrafish lef1, Mech. Dev. 86 (1–2) (1999) 147–150.
[15] R.I. Dorsky, M. Itoh, R.T. Moon, A. Chitnis, Two tcf3 genes cooperate to pattern the zebrafish brain, Development 130 (9) (2003) 1937–1947.
[16] C.H. Kim, T. Oda, M. Itoh, D. Jiang, K.B. Artinger, S.C. Chandrasekharappa, W. Driever, A.B. Chitnis, Repressor activity of Headless/Tcf3 is essential for vertebrate head formation, Nature 407 (6806) (2000) 913–916.
[17] K. Ito, A.T. Lim, M.,L. Motoda, M. Osato, L. Chuang, C. Lee, D. Voon, J. Koo, H. Wang, H. Fukamachi, RUNX3 attenuates beta-catenin/T cell factors in intestinal tumorigenesis, Cancer Cell 14 (3) (2008) 226–237.
[18] G. Jeannet, C. Boudousquié, N. Gardiol, J. Kang, J. Huelsken, W. Held, Essential role of the Wnt pathway effector Tcf-1 for the establishment of functional CD8 T cell memory, Proc. Natl. Acad. Sci. U. S. A. 107 (21) (2010) 9777–9782.
[19] X. Zhou, H.H. Xue, Cutting edge: generation of memory precursors and functional memory CD8+ T cells depends on T cell factor-1 and lymphoid enhancer-binding factor-1, J. Immunol. 189 (6) (2012) 2722.
[20] X. Zhou, S. Yu, D.M. Zhao, J.T. Harty, V.P. Badovinac, H.H. Xue, Differentiation and persistence of memory CD8+ T cells depend on T cell factor 1, Immunity 33 (2) (2010) 229.
[21] Q. Yu, A. Sharma, A. Ghosh, J.M. Sen, T cell factor-1 negatively regulates expression of IL-17 family of cytokines and protects mice from experimental autoimmune encephalomyelitis, J. Immunol. 186 (7) (2011) 3946–3952.
[22] J. Vanloosdregt, V. Fleskens, M. Tiemessen, M. Mokry, R. Vanboxtel, J. Meerding, C.G.M. Pals, D. Kurek, M.M. Baert, E. Delemarre, Canonical Wnt signaling negatively modulates regulatory T cell function, Immunity 39 (2) (2013) 298–310.
[23] R. Berga-Bolaños, W.S. Zhu, F.C. Steinke, H.-H. Xue, J.M. Sen, Cell-autonomous requirement for TCF1 and LEF1 in the development of Natural Killer T cells, Mol. Immunol. 68 (2) (2015) 484–489.
[24] T.Q. Wu, H.M. Shin, E.A. Moseman, Y. Ji, B.N. Huang, C. Harly, J.M. Sen, L.J. Berg, L. Gattinoni, D.B. McGavern, P.L. Schwartzberg, TCF1 is required for the T follicular helper cell response to viral infection, Cell Rep. 12 (12) (2015) 2099–2110.
[25] R.J. Johnston, A.C. Poholek, D. Ditoro, I. Yusuf, D. Eto, B. Barnett, A.L. Dent, J. Craft, S. Crotty, Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation, Science 325 (5943) (2009) 1006–1010.
[26] R.I. Nurieva, Y. Chung, G.J. Martinez, X.O. Yang, S. Tanaka, T.D. Matskevitch, Y.H. Wang, C. Dong, Bcl6 mediates the development of T follicular helper cells, Science 325 (5943) (2009) 1001–1005.
[27] Y. Di, S. Rao, L.M. Tsai, S.K. Lee, Y. He, E.L. Sutcliffe, M. Srivastava, M. Linterman, Z. Lei, N. Simpson, The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment, Immunity 31 (3) (2009) 457–468.
[28] L. Xu, Y. Cao, Z. Xie, Q. Huang, Q. Bai, X. Yang, R. He, Y. Hao, H. Wang, T. Zhao, Z. Fan, A. Qin, J. Ye, X. Zhou, L. Ye, Y. Wu, The transcription factor TCF-1 initiates the differentiation of T(FH) cells during acute viral infection, Nat. Immunol. 16 (9) (2015) 991–999.
[29] T. Aoki, J. Hikima, S.D. Hwang, T.S. Jung, Innate immunity of finfish: primordial conservation and function of viral RNA sensors in teleosts, Fish Shellfish Immunol. 35 (6) (2013) 1689–1702.
[30] Z. Liao, Q. Wan, J. Su, Bioinformatics analysis of organizational and expressional characterizations of the IFNs, IRFs and CRFBs in grass carp Ctenopharyngodon idella, Dev. Comp. Immunol. 61 (2016) 97–106.
[31] MOA, China Fishery Statistical Year Book [M], China Agriculture Press, Beijing, 2015, pp. 31–39 PP.
[32] Y. Tian, X. Ye, L. Zhang, G. Deng, Y. Bai, Development of a novel candidate subunit vaccine against Grass carp reovirus Guangdong strain (GCRV-GD108), Fish Shellfish Immunol. 35 (2) (2013) 351–356.
[33] Y. Wang, Y. Lu, Y. Zhang, Z. Ning, Y. Li, Q. Zhao, H. Lu, R. Huang, X. Xia, Q. Feng, X. Liang, K. Liu, L. Zhang, T. Lu, T. Huang, D. Fan, Q. Weng, C. Zhu, Y. Lu, W. Li, Z. Wen, C. Zhou, Q. Tian, X. Kang, M. Shi, W. Zhang, S. Jang, F. Du, S. He, L. Liao, Y. Li, B. Gui, H. He, Z. Ning, C. Yang, L. He, L. Luo, R. Yang, Q. Luo, X. Liu, S. Li, W. Huang, L. Xiao, H. Lin, B. Han, Z. Zhu, Erratum: the draft genome of the grass carp (Ctenopharyngodon idellus) provides insights into its evolution and vegetarian adaptation, Nat. Genet. 47 (8) (2015) 962.
[34] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (4) (2001) 402–408.
[35] E.S. Veien, M.J. Grierson, R.S. Saund, R.I. Dorsky, Expression pattern of zebrafish tcf7 suggests unexplored domains of Wnt/β-catenin activity, Dev. Dynam. 233 (1) (2005) 233.
[36] Q. Eastman, R. Grosschedl, Regulation of LEF-1/TCF transcription factors by Wnt and other signals, Curr. Opin. Cell Biol. 11 (2) (1999) 233–240.
[37] M.G. Prieve, K.L. Guttridge, J. Munguia, M.L. Waterman, Differential importin-α recognition and nuclear transport by nuclear localization signals within the high-mobility-group DNA binding domains of lymphoid enhancer factor 1 and T-cell factor 1, Mol. Cell Biol. 18 (8) (1998) 4819–4832.
[38] L. Sessa, M.E. Bianchi, The evolution of High Mobility Group Box (HMGB) chromatin proteins in multicellular animals, Gene 387 (1–2) (2007) 133–140.
[39] R. Reeves, Nuclear functions of the HMG proteins, Bba-Gene Regul Mech 1799 (1–2) (2010) 3–14.
[40] Y. Rao, J. Su, C. Yang, N. Yan, X. Chen, X. Feng, Dynamic localization and the associated translocation mechanism of HMGBs in response to GCRV challenge in CIK cells, Cell. Mol. Immunol. 12 (3) (2015) 342–353.
[41] M. Stros, HMGB proteins: interactions with DNA and chromatin, Biochim. Biophys. Acta 1799 (1) (2010) 101–113.
[42] T. Bonaldi, F. Talamo, P. Scaffidi, D. Ferrera, A. Porto, A. Bachi, A. Rubartelli, A. Agresti, M.E. Bianchi, Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion, EMBO J. 22 (20) (2003) 5551–5560.
[43] K. Giese, A. Amsterdam, R. Grosschedl, DNA-binding properties of the HMG domain of the lymphoid-specific transcriptional regulator LEF-1, Gene Dev. 5 (12) (1991) 2567.
[44] M. Wetering, Van De, H. Clevers, Sequence-specific interaction of the HMG box proteins TCF-1 and SRY occurs within the minor groove of a Watson-Crick double helix, EMBO J. 11 (8) (1992) 3039–3044.

[45] J.J. Love, X. Li, D.A. Case, K. Giese, R. Grosschedl, P.E. Wright, Structural basis for DNA bending by the architectural transcription factor LEF-1, Nature 376 (6543) (1995) 791–795.
[46] T. Hansen, A. Ingason, S. Djurovic, I. Melle, M. Fenger, O. Gustafsson, K.D. Jakobsen, H.B. Rasmussen, S. Tosato, M. Rietschel, At-risk variant in TCF7L2 for Type II diabetes increases risk of schizophrenia, Biol. Psychiatry 70 (1) (2011) 59–63.
[47] A. Alkelai, L. Greenbaum, S. Lupoli, Y. Kohn, K. Sarner-Kanyas, E. Ben-Asher, D. Lancet, F. Macciardi, B. Lerer, Association of the Type 2 Diabetes Mellitus Susceptibility Gene, TCF7L2, with Schizophrenia in an Arab-Israeli Family Sample, PloS One 7 (1) (2012) e29228.
[48] A. Nagalski, M. Irimia, L. Szewczyk, J.L. Ferran, K. Misztal, J. Kuznicki, M.B. Wisniewska, Postnatal isoform switch and protein localization of LEF1 and TCF7L2 transcription factors in cortical, thalamic, and mesencephalic regions of the adult mouse brain, Brain Struct. Funct. 218 (6) (2013) 1531–1549.
[49] M.R.J. Sorrell, T.E. Dohn, E. D'Aniello, J.S. Waxman, Tcf7l1 proteins cell autonomously restrict cardiomyocyte and promote endothelial specification in zebrafish, Dev. Biol. 380 (2) (2013) 199–210.
[50] A. Travis, A. Amsterdam, C. Belanger, R. Grosschedl, LEF-1, a gene encoding a lymphoid-specific protein with an HMG domain, regulates T-cell receptor alpha enhancer function [corrected], Gene Dev. 5 (5) (1991) 880.
[51] M. Bienz, TCF: transcriptional activator or repressor? Curr. Opin. Cell Biol. 10 (3) (1998) 366–372.
[52] J. Su, T. Huang, J. Dong, J. Heng, R. Zhang, L. Peng, Molecular cloning and immune responsive expression of MDA5 gene, a pivotal member of the RLR gene family from grass carp Ctenopharyngodon idella, Fish Shellfish Immunol. 28 (4) (2010) 712–718.
[53] Z. Li, A.M. Diehl, Innate immunity in the liver, Curr. Opin. Gastroenterol. 19 (6) (2003) 565–571.