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Iron-metabolic function and potential antibacterial role of Hepcidin and its correlated genes (Ferroportin 1 and Transferrin Receptor) in Turbot (*Scophthalmus maximus*)

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ABSTRACT

Antimicrobial peptide plays an important role in fish immunity. The small molecular antimicrobial peptide Hepcidin in turbot was studied and reported in this paper. The Ferroportin 1 (*FPN1*) and Transferrin Receptor (*TFR*) genes, which are related to Hepcidin, were cloned in turbot. The characteristics of Hepcidin and its related genes were studied, including an analysis of the expression patterns and cloning of the Hepcidin promoter, the relationship between Hepcidin and NF-κB and the regulation of iron-metabolism. The results showed that the promoter of *SmHepcidin* contains the binding sites of NF-κB, and NF-κB may directly or indirectly receive feedback signals from *SmHepcidin*. In the liver, spleen and kidney, in which there was an increased *SmHepcidin* expression level, *SmFPN1* dramatically decreased and *SmTFR* was also either decreased or exhibited no obvious change after bacterial/viral infection and an injection of exogenous Hepcidin protein. RNAi experiments in turbot kidney cells confirmed the expression changes of these gene patterns. Furthermore, the administration of exogenous Hepcidin protein, which regulates the level of chelatable iron in cells, further confirmed the function of Hepcidin in iron metabolism. It is speculated that the rapidly increased expression of *SmHepcidin* may induce changes in the expression of related genes, and that the *in vivo* chelatable iron concentration which participates in the antibacterial process was also changed when exogenous pathogens are present in turbot. It is suggested that SmHepcidin plays a defensive role against pathogenic infection.

1. Introduction

Antimicrobial peptides (AMPs), also known as host-protective peptides, are small, positively charged peptides that play an important role in the innate immune system by the formation of a hole in membranes or disordering pathogen cells by dissolving the cell wall so as to protect the body from bacteria, fungi, viruses and other pathogenic infections [1]. In general, AMPs are secreted in the saliva, mucus, circulatory system and other pathogen target areas [2].

Hepcidin is a small molecule AMP that also regulates the concentration of iron in the cell and is expressed mainly in the liver [3]. Previous studies reported that human Hepcidin, a 25 amino acid AMP expressed in the liver named LEAP-1, was originally isolated from the urine and plasma [3,4]. To date, there are three different isforms of human Hepcidin having antibacterial activity, and these are comprised of 20, 22 and 25 amino acid residues, respectively [4]. Although Hepcidin genes have been successfully cloned from a variety of different fish [5,6], further investigation is needed. The existing reports are mainly focused on the mammalian forms, while the function of Hepcidin in fish is as yet unclear. In fish, most reports have suggested that Hepcidin has no direct antimicrobial activity; nevertheless it may be involved in the resistance to pathogenic infection. In the course of infection by bacteria leading to inflammation, Hepcidin can regulate the concentration of serum iron and it also controls the level of iron in cancer cells [7,8]. The probably reason is Hepcidin limits the growth of harmful cells by inducing a lack of iron [9]. Membrane iron transport protein Ferroportin 1 (Fpn1), also known as iron regulatory transporter 1 (Fe-regulated transporter 1, IREG1) or metal transporter protein (metal transport protein 1, MTP1), is a transmembrane iron export protein that has an important physiological role in cellular iron transport. Hepcidin-Fpn1 regulates the

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amount of iron which cells absorb that has been released into the blood from macrophages, liver cells and the intestine. This finding has been supported by a number of studies which also have shown that the impact of Hepcidin on Fpn1 caused by a direct effect [10]. Currently, the cDNAs of Fpn1 have been cloned from the zebrafish, frog, mouse, rat and human. Transferrin Receptor (TFR), a cell membrane receptor, is a double-stranded trans-membrane glycoprotein connected by a disulfide bond has an MW of 18 kDa [11–13]. In human, the TFR gene located on the long arm of chromosome No. 3. TfR plays a critical role in the regulation of cellular uptake of iron [14–17]. The relationship of Hepcidin with the iron-metabolism genes, as well as how they are involved in the resistance to infection, required further investigation.

Turbot (*Scophthalmus maximus*) is a widely cultured marine fish of considerable economic importance in Europe and China. However, with the deterioration of the environment in recent years, fish diseases continue to occur at a high rate, resulting not only in serious economic losses, but impairing the development of the industry. In order to explore the molecular mechanisms for disease resistance and host–pathogen interactions in this species, certain immune-related genes, including Hepcidin, the CXC chemokine, Nramp (natural resistance associated macrophage protein) and Akirin have been cloned from turbot [18–21]. Meanwhile, turbot kidney cell lines have been established [22]. These studies have laid the foundation for further study of the function of Hepcidin in the antimicrobial pathways.

In this investigation, the promoter sequence of *SmHepcidin* was cloned. *Fpn1*, a Hepcidin-related gene, and the *TFR* have been cloned in turbot. The results of infection assays reveal the expression patterns of Hepcidin and the related genes *Fpn1* and *TFR*. Hepcidin promoter cloning and the regulation of iron of Hepcidin were also investigated.

2. Materials and methods

Ethics Statement:

All the experimental animal programs involved in this study were approved by the Yellow Sea Fisheries Research Institute's animal care and use committee, and followed the experimental basic principles.

2.1. Experimental animals and cell lines

Turbot having an average weight of 100 g obtained from the Haiyang Fisheries Company in Yantai were raised in a tank with seawater (16 °C). For the cloning and tissue expression analysis of SmFPN1 cDNA, 11 tissues consisting of the brain, gill, skin, muscle, fin, heart, liver, spleen, kidney, head kidney and intestine were collected from three individuals for the purpose of RNA extraction. To investigate the response to bacterial and viral challenge, tissues from liver, spleen, and kidney were collected from three individuals for RNA extraction.

The *S. maximus* kidney cell line (SMKC) [22] developed by Wang Na in our lab was used in the present study. The cells were cultured in modified eagle's medium containing 10% fetal calf serum (JIBC) and antibiotics, as described. For infection, SMKC cells were grown on a 6-well culture plate (8×10^5 cell/well) with serum-free medium.

2.2. Bacterial/viral challenge and function tests of the synthesized Hepcidin protein in vivo

The bacterium *Vibrio anguillarum*, which has been shown to be pathogenic in turbot [23], was cultured at 28 °C to mid-logarithmic growth on 2216E medium, centrifuged to collect the bacteria and suspended in 0.9% saline [24,25]. The number of bacteria in the suspension was measured with a cell counter. A final concentration of 7×10^6 cfu of *V. anguillarum* was used, and 0.9% saline was used as the negative control [26]. After 6 h, 12 h, 24 h, 48 h, 72 h and 96 h, tissues (three individuals) were used for RNA extraction. For the negative control, tissues were taken 12 h after the saline challenge.

For viral infection, Lymphocystis disease virus (LCDV) was used for virus susceptibility analysis. The virus was isolated and used to detect viral susceptibility in a previous study [27]. The culture and titration determinations were performed as described [27,28]. Lymphocystis disease virus (LCDV 1×10^8 TCID50/mL, 100 μL per fish) challenge methods liked bacterial challenge; tissues were isolated for RNA extraction at 6 h, 12 h, 24 h, 48 h, 72 h and 96 h. And the negative control was medium MEM challenged. For SMKC infection, after 2 days of culture, cells were treated for 1 h with 1 μL LCDV (1×10^8 TCID50/mL) or Poly I:C (500 μg/mL, Sigma), followed by a supplementation with medium, and immediately frozen at −70 °C until RNA extraction.

For function tests of the synthesized Hepcidin protein *in vivo*, the methods also liked bacterial challenge at a final concentration of 1 μg/g of body weight. At 24 h, 48 h and 72 h, three of the experimental turbot were selected to isolate the organs. For RNA extraction, the isolated organs were stored at −80 °C. The organs of untreated fish were isolated after injection for 48 h, 72 h, and 96 h, with the organs stored at −80 °C for RNA extraction.

2.3. Cloning of the Ferroportin 1 and Transferrin Receptors

For the cloning of Fpn1 and TFR, liver cDNA of turbot was used as the template. The primers FPN-s1, FPN-a1, TFR-S3 and TFR-A3 were designed based on homology with human, mouse and zebrafish Fpn1 and TFR. Then the 5′ and 3′ fragments were amplified according to the BD SMART™ RACE cDNA amplification kit, using the designed primers FPN-5-A3, FPN-5-A5, FPN-3-S4, FPN-5-S5, TFR-5-A1, TFR-5-A2, TFR-3-S2 and TFR-3-S3 (Table 1).

2.4. Cloning of the promoter region

Using the Genome Walking Kit (TAKARA), the Hepcidin promoter region was cloned. According as the manual of the Genome Walking Kit, the HP A2, and HP A3 and HP A4 primers were designed (Table 1).

2.5. Sequence analysis

In order to obtain full-length cDNA, the 5′ and 3′ cDNA sequences as well as the conserved sequences were obtained with DNAMAN software. Translation was performed using DNASTAR software and homologous sequences were searched by BLAST using http://www.ncbi.nlm.nih.gov/ sites containing blastn, blastp and tblastp. The alignment the amino acid sequences of FPN1 and TFR from the different species was performed using the ClustalW alignment program, and the phylogenetic tree was constructed on the basis of the proportion of the amino acid differences (p-distances) determined by the Neighbor-joining (NJ) method [29] using MEGA 3 software [30]. The amino acid sequences of the following proteins were used in the alignment with SmFPN1: XP_002191941 [*Taeniopygia guttat*] XP_002703601 [*Bos taurus*] XP_003207470 [*Meleagris gallopavo*] NP_058613 [*Mus musculus*] NP_579849 [*Rattus norvegicus*] NP_001012931 [*Gallus gallus*] AAI63874 [*Danio rerio*] XP_002808055 [*Macaca mulatta*] NP_001125633 [*Pongo abelii*] ABL75285 [*Xenopus (Silurana) tropicalis*] EAX10901[*Homo sapiens*] XP_515981 [*Pan troglodytes*].

Table 1
Oligonucleotide primers used in the experiments.

Name	Sequence 5′–3′	Purpose used
FPN-s1	ATGGGGGGATCGGATGTGG	Amplified
FPN-a1	CCTCTCTCGGACTCAATCACAT	FPN1 fragments
TFR-S3	GCGTGAGCTTCGCTGAGAAGGT	Amplified
TFR-A3	CAGGTAACCCTCCAGCCACTC	TFR fragments
FPN-5-A3	GCGAGACACTGAGGTCCAGGGGG	FPN15′RACE
FPN-5-A5	GGTCAATAATCCTCACTGTGGCGTT	FPN15′RACE
FPN-3-S4	CATTGGCTGCGGCTTCATCTCTG	FPN13′RACE
FPN-5-S5	CCTCAGTGTCTCGCCCTTCG	FPN13′RACE
TFR-5-A1	GTGATGGTCTGAGCCACGATTT	TFR2 5′RACE
TFR-5-A2	TGTGGTTGAAAGAAGGGAAGCC	TFR2 5′RACE
TFR-3-S2	ATTCAAGCCAAGGAGGAGCAT	TFR2 3′RACE
TFR-3-S3	AAGGAGGAGCATTGTGTTTGCGAGC	TFR2 3′RACE
HP A2	AGAAGCCACAGCCCTTGTAGGC	Promoter cloning
HP A3	CAAAGGGAGATGTGGCTCTGTCG	
HP A4	AGTGTCATTGCTCCCTGCCTCTTCC	
HP-M-S1	TGCCGCTGGTGCTGCAACTGCTGCAAGCGCAA	Mutation
HP-M-A1	GAACTTGCAGCACATGCC	
HP-M-S2	gcGAATTCATGTGCCGCTGGTGCTGCAAC	Plasmid construction
HP-M-A2	gcCTCGAGTTAGAACTTGCAGCACAT	
hp-f2	gcGAATTCATGCAGAGCCACATCTCCCTT	
hp-r2	gcCTCGAGGAACTTGCAGCAGAAGC	
β-actin-s1	GCTGTGCTGTCCCTGTA	RT-PCR for β-actin
β-actin-a1	GAGTAGCCACGCTCTGTC	
hp-r-f2	CTCCCTTTGCCGCTGGTG	RT-PCR
hp-r-r2	AGCCCTTGTTGGCCTTGC	for SmHepcidin
FPN-rt-51	GGCAGACATGAACGCCACA	RT-PCR for SmFPN1
FPN-rt-31	AAGCCGCAGCCAATGAAAT	
TFR-R-S1	CTTCCCTTCTTTCAACCACACC	RT-PCR for SmTFR
TFR-R-A1	CACCAATGACCACATACCGATC	

The amino acid sequences of the following proteins were used in the alignment with SmTFR: CAM13354 [*D. rerio*] CAF97624 [*T. nigroviridis*] CBN82172 [*Dicentrarchus labrax*] AAI67309 [*X. tropicalis*] ADP20546 [*Heterocephalus glaber*] ABY87967 [*Zygodontomys brevicauda*] EDM11407 [*R. norvegicus*] ABX89905 [*Calomys musculinus*] XP_001101412 [*M. mulatta*] BAD92491 [*H. sapiens*] ABY87967 [*Z. brevicauda*] ABD65249 [*Tursiops truncatus*] NP_001009312 [*Felis catus*] XP_003229578 [*Anolis carolinensis*] NP_990587 [*Gallus gallus*].

The promoter region was analyzed with Promoter Scan (http://www-bimas.cit.nih.gov/molbio/proscan/). The conserved domains of the protein were analyzed at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The SWISS-MODEL program (http://www.expasy.ch/) was used to predict the tertiary structure. The molecular weight and isoelectric point of the protein were predicted using ProtParam. The transmembrane domain of the protein was predicted on http://www.sbc.su.se/%7Emiklos/DAS/.

2.6. Protein synthesis

The Hepcidin protein was synthesized by the Invitrogen Company based on the Hepcidin amino acid sequences (QSHISLCRWC CNCCKANKGC GFCCKF), for which the synthesized purity was greater than 95%.

2.7. RNAi

siRNAs targeting the Hepcidin gene were designed and synthesized by GenePharma, and the siRNA oligos were purified by HPLC. According to the mature Hepcidin peptide sequence, the three beginning, middle and end gene targets of the siRNA were designed in the forepart, the middle and the rear (Table 2), respectively. The siRNA transfected into SMKC by lipo2000 (Invitrogen), subsequently the level of Hepcidin, Fpn1 and TFR expression were detected by realtime PCR.

Table 2
Oligonucleotide primers used in RNAi.

ID	Sequence 5′–3′
siRNA1	GCACAUCAAGAGACGUCAATT
	UUGACGUCUCUUGAUGUGCTT
siRNA2	GCUCGCCUUUGUUUGCAUUTT
	AAUGCAAACAAAGGCGAGCTT
siRNA3	CAACAAGGGCUGUGGCUUCTT
	GAAGCCACAGCCCUUGUUGTT

2.8. RNA extraction and cDNA synthesis

Total RNA was isolated from 500 mg powdered frozen SMKC cells or fish tissues by homogenization in 5 mL TRIzol (Invitrogen), held at room temperature for 5 min. An aliquot of chloroform (1 mL) was added to each extract, and the resulting mixture was centrifuged (10 min, 13,000 *g*). The aqueous layer was transferred to a clean tube, and the RNA firstly precipitated by the addition of 3 mL isopropanol, and then pelleted by centrifugation (15 min, 13,000 *g*). The RNA pellet was washed twice with 75% ethanol and re-suspended in diethylpyrocarbonate-treated water. After DNA removal (Turbo DNA-free kit, Ambion), RNA integrity was detected using agarose gel electrophoresis, and the concentration of RNA was quantified spectrophotometrically. The first strand cDNA was synthesized from 500 ng total RNA using a PrimeScript® RT reagent kit (Takara), following the recommendation of the manufacturer Quantitative Real-Time PCR (RT-qPCR).

2.9. Quantitative real-time PCR

The RT-qPCR protocol adhered to the 'Minimum Information for Publication of Quantitative Real-Time PCR experiments' guidelines [31]. The relative mRNA steady-state level was measured by RT-qPCR. The quantitative RT-PCR (RT-qPCR) was conducted on an Applied Biosystems 7500 Real-Time PCR System with SYBR® Premix Ex Taq™ (Takara). For normalization, the β-actin gene was used as reference gene [32–34]. The RT-qPCR primer pairs for *SmHepcidin*, *SmFPN1*, *SmTFR* and β-actin were designed with the guidelines of a product size 150–250 bp and a Tm of 60 ± 1 °C. The cDNA of 11 normal and infected tissues was chosen for the detection of *SmHepcidin*, *SmFPN1* and *SmTFR* expression using the gene-specific primer primers hp-r-f2, hp-r-r2 (for SmHepcidin); FPN-rt-

Table 3
The motif scans of the SmHepcidin promoter.

Name	TFD #	Strand	Location	Weight
INF.1	S01152	+	116	1.044
ATF	S01940	+	148	3.721
ATF	S01059	+	148	1.157
CREB	S00969	+	148	3.442
CREB	S00489	–	152	1.147
ATF/CREB	S00534	–	153	1.138
CREB	S00144	–	154	2.549
NF-κB	S01498	+	178	1.08
AP-2	S00346	+	241	1.355
AP-2	S01936	+	242	1.108
JCV_repeated_sequenc	S01193	–	247	1.658
T-Ag	S00974	+	277	1.086
UCE.2	S00437	+	279	1.278
AP-2	S00180	–	292	1.064
(Sp1)	S01027	–	296	2.233
TFIID	S01540	+	322	1.971
TFIID	S00087	+	324	2.618

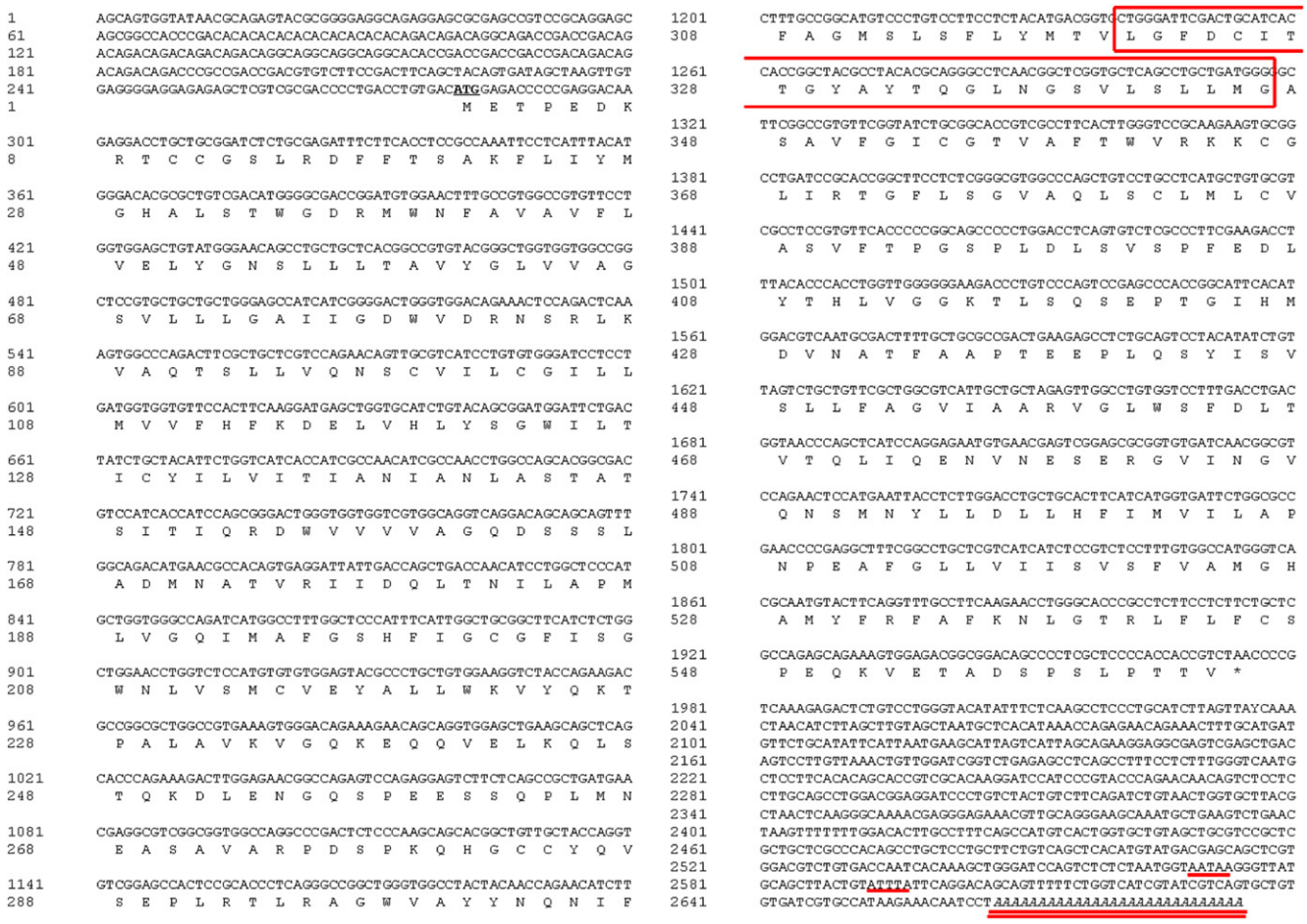


Fig. 1. Nucleotide sequence (above) and deduced amino acid sequence (below) of *SmFpn1*. Nucleotides were numbered from the first base at the 5′ end. Amino acids, shown in one letter abbreviations, were numbered from the initiating methionine. The box indicates the HBD domain, AATAA box were underlined, and poly(A) were double lined. The stop codon was marked by an asterisk.

51, FPN-rt-31 (for SmFPN1); TFR-R-S1, TFR-R-A1 (for SmTFR) (Table 1). The primers, β-actin-s1 and β-actin-a1 (Table 1), were used to amplify the β-actin fragment (Table 1).

All RT-qPCRs were performed in a 20 μL volume containing 1 μL first strand cDNA, 10 μL SYBR® Premix Ex Taq™, 0.4 μL ROX Reference Dye II, 0.4 μL PCR forward/reverse primers (10 μM) and 7.8 μL nuclease-free water. The thermo-cycling conditions for the reaction were as follows: 95 °C for 30 s, followed by 40 cycles consisting of 95 °C for 5 s and 59 °C for 34 s. The reaction was carried out with 3 duplicates of each sample. The negative control for RT-qPCR was also in 20 μL volume without template, and the positive control were performed in a 20 μL volume containing 1 μL diluted (1:10 v/v, 1:50 v/v, 1:100 v/v, 1:1000 v/v, 1:5000 v/v) plasmid (500 ng/μL), which recombined with *SmHepcidin*, *SmFPN1* or *SmTFR*. Data (normalized Ct values) from the treated and control tissues templates were compared and the 2-ΔΔCT method was selected as the relative quantification calculation method. The efficiency of the primer designed assays was calculated as described [35] using the following equation: $E = (10(-1/\text{slope})) - 1$. The amplification efficiencies of the *SmActin*, *SmHepcidin*, *SmFPN1* and *SmTFR* designed primer assays and the three real-time PCR were ≥92%.

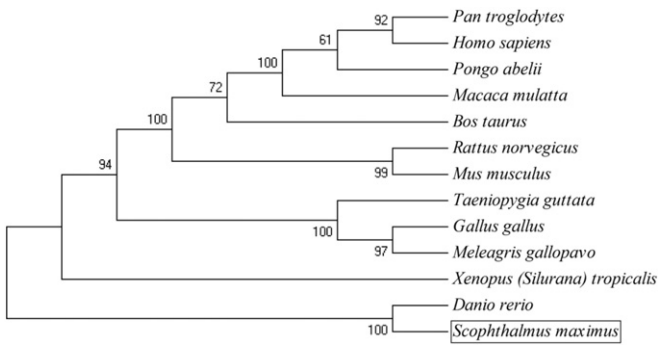


Fig. 2. Phylogenetic tree, constructed by the neighbor-joining algorithm using ClustalW Multiple Alignment, Showing the relationship between Fpn1 proteins. The SmFpn1 protein was boxed.

2.10. Plasmid construction

According to the Hepcidin ORF, the primers HP-F2, HP-R2 (restriction sites: EcoR I and Xho I) were designed for making the PcDNA3.1-Hepcidin vector. The primers FPN-s1, FPN-a1, TFR-S3 and TFR-A3 were designed based on homology with the *FPN1 and TFR* of the human, mouse and zebrafish. Based on the sequences of 20 mouse and human Hepcidiin genes, a 7–26 amino acid sequence of *SmHepcidin* was captured, and we mutated A in the 16th position into R, and F in the 22nd position into M. Using the primers HP-M-S2 and HP-M-A2, the Hepcidin mutation was inserted into pcDNA3.1^{+} (Table 1).

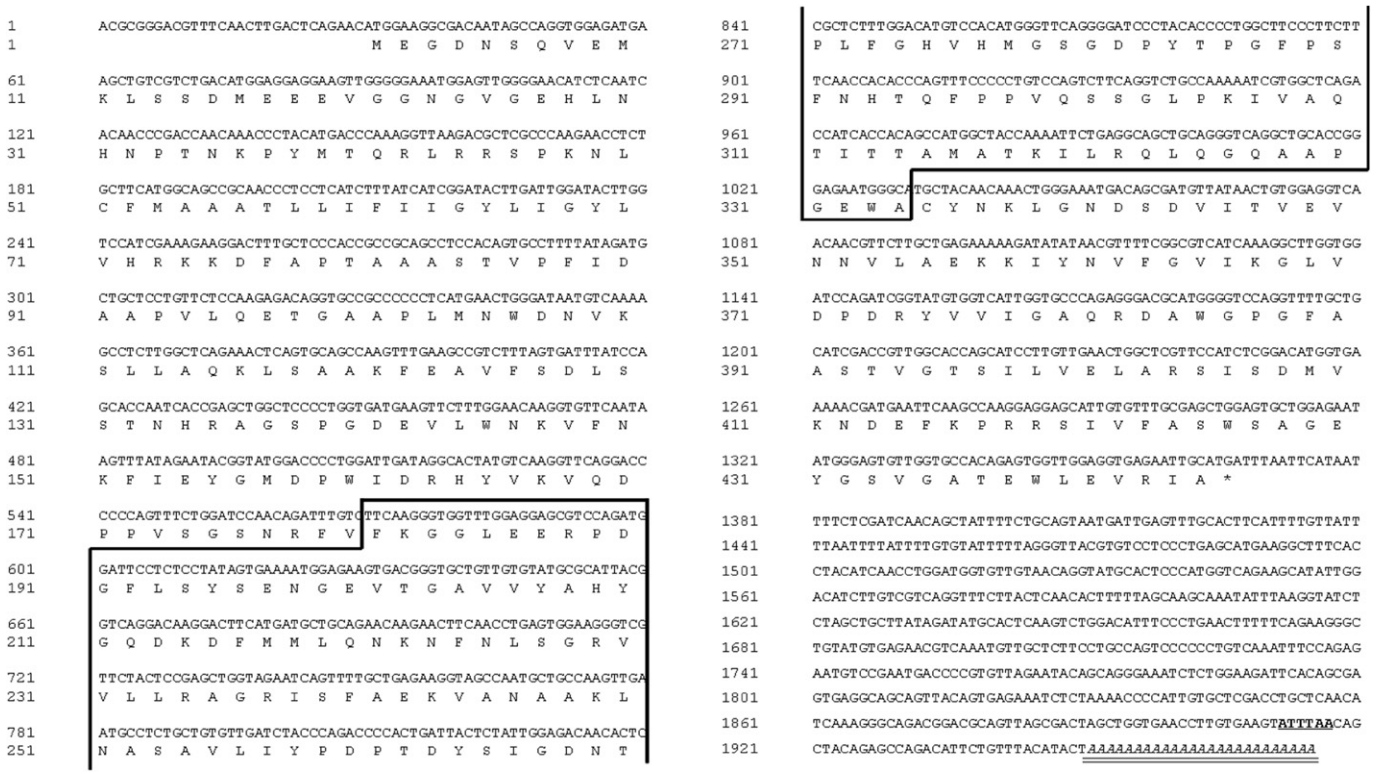


Fig. 3. Nucleotide sequence (above) and deduced amino acid sequence (below) of *SmTfR*. Nucleotides were numbered from the first base at the 5′ end. Amino acids, shown in one letter abbreviations, were numbered from the initiating methionine. The box indicates the PA_TfR conserved domain, ATTTA box were underlined, and poly(A) were double lined. The stop codon was marked by an asterisk.

2.11. *S. maximus kidney cell line (SMKC) cells transfection*

According to the Lipofectamine 2000 transfection kit instructions, THE SMKC was developed and used for the transfection experiments.

2.12. *Detection of intracellular chelatable iron in SMKC using Phen Green FL staining*

The medium (MEM) was removed, washed twice with PBS and Phen Green FL (Invitrogen, 20 μmol/L, a DMSO preparation) was added. After incubation at 25 °C for 30 min in the dark, the Phen Green FL was removed, washed twice with PBS, and an appropriate amount of medium was added and incubated for 15 min. Under the (CLSM), a specified amount of cells (usually 8–9 cells) was selected. The control group was comprised of a certain volume of distilled water (100 μL) and the experimental group was the same volume of synthetic SmHepcidin protein (10 μg/mL) added to cells. The wavelengths of 492 nm and 517 nm were utilized to observe the fluorescence intensity of Phen Green FL. The detection time was 1233 s and the fluorescence values were scanned every 18 s and this was repeated three times [36].

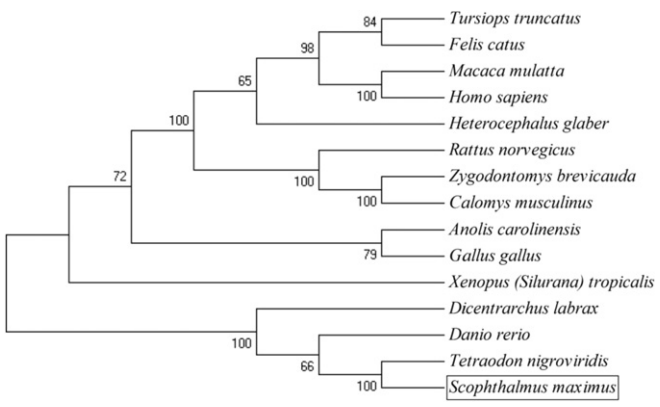


Fig. 4. Phylogenetic tree, constructed by the neighbor-joining algorithm using ClustalW Multiple Alignment, showing the relationship between TfR proteins. The SmTfR protein was boxed.

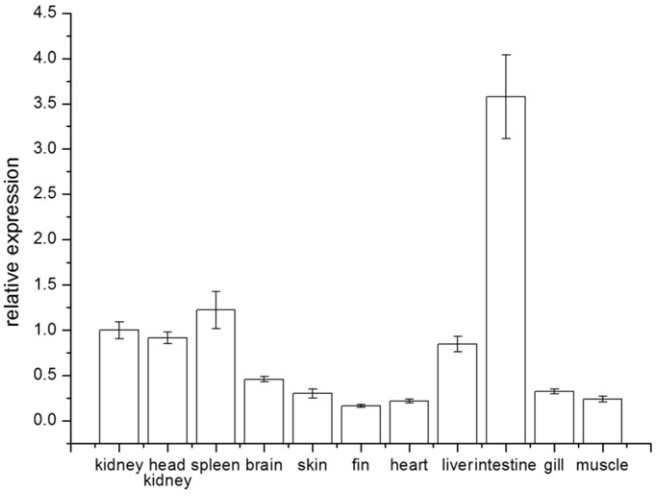


Fig. 5. The expression pattern of *SmFpn1* in turbot different tissues was detected by the quantitative RT-PCR. *SmFpn1* mRNA was expressed in all tissues detected with higher expression levels in the intestine. The β-actin gene was used as an internal control to calibrate the cDNA template for all the samples.

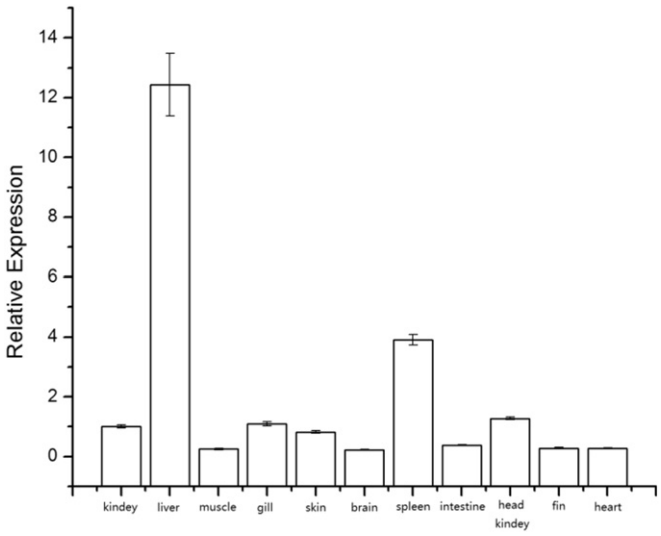


Fig. 6. The expression pattern of *SmTfR* in turbot different tissues was detected by the quantitative RT-PCR. *SmTfR* mRNA was expressed in all tissues detected with higher expression levels in the liver. The β-actin gene was used as an internal control to calibrate the cDNA template for all the samples.

2.13. Luciferase reporter assay

SMKC cells were transfected with various plasmids: pcDNA3.1-Hepcidin, pcDNA3.1-Hepcidin-m (its protein sequence same as the protein sequence of human), pcDNA3.1, pNFκB-luc and pRL-TK. After 36 h transfection, cells were harvested and the luciferase activities were measured with a luminometer (Berthold Technologies) using a Luciferase Reporter Gene Assay Kit (Beyotime, China). All of the luciferase assay experiments were performed with the co-transfection of Renilla (20 ng) as the internal control. Each experiment was performed in triplicate, and the data represents the means ± SD of three independent experiments after being normalized to the Renilla activity.

3. Results

3.1. Cloning and sequence analysis of the SmHepcidin promoter sequence

Based on the published sequence of *SmHepcidin* (AY994075) [19], we acquired the 273 bp ORF of *SmHepcidin* encoding 89 aa. The protein molecular weight was determined to be 9.7 kDa and the isoelectric point (pI) 7.65.

Using a multiplex PCR method, the promoter sequence was cloned, with a length 591 bp. Analysis of the promoter sequence revealed that the main motifs of the sequence, i.e. CREB, NF-κB, AP2, etc., that were all important binding sites of the transcription factor (Table 3).

3.2. Cloning and sequence analysis of SmFPN1 and SmTFR

The *SmFPN1* ORF in turbot had a length of 1695 bp, and the 5′ and 3′ end fragments were acquired by RACE with the primers FPN-5-A3, FPN-5-A5, FPN-3-S4 and FPN-5-S5, and the adaptor primers UPM and NUP. Finally, the three fragments were combined into a 2542 bp form, containing a 5′UTR of 280 bp, an ORF of 1695 bp encoding a polypeptide of 564 amino acids and a 719 bp 3′UTR with

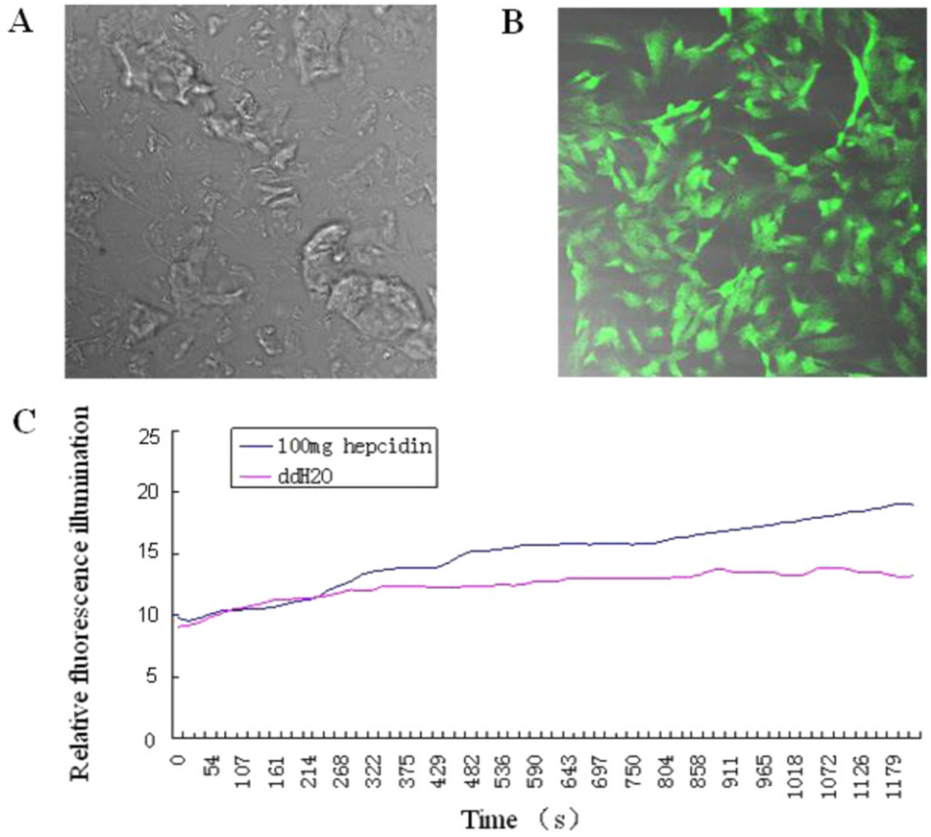
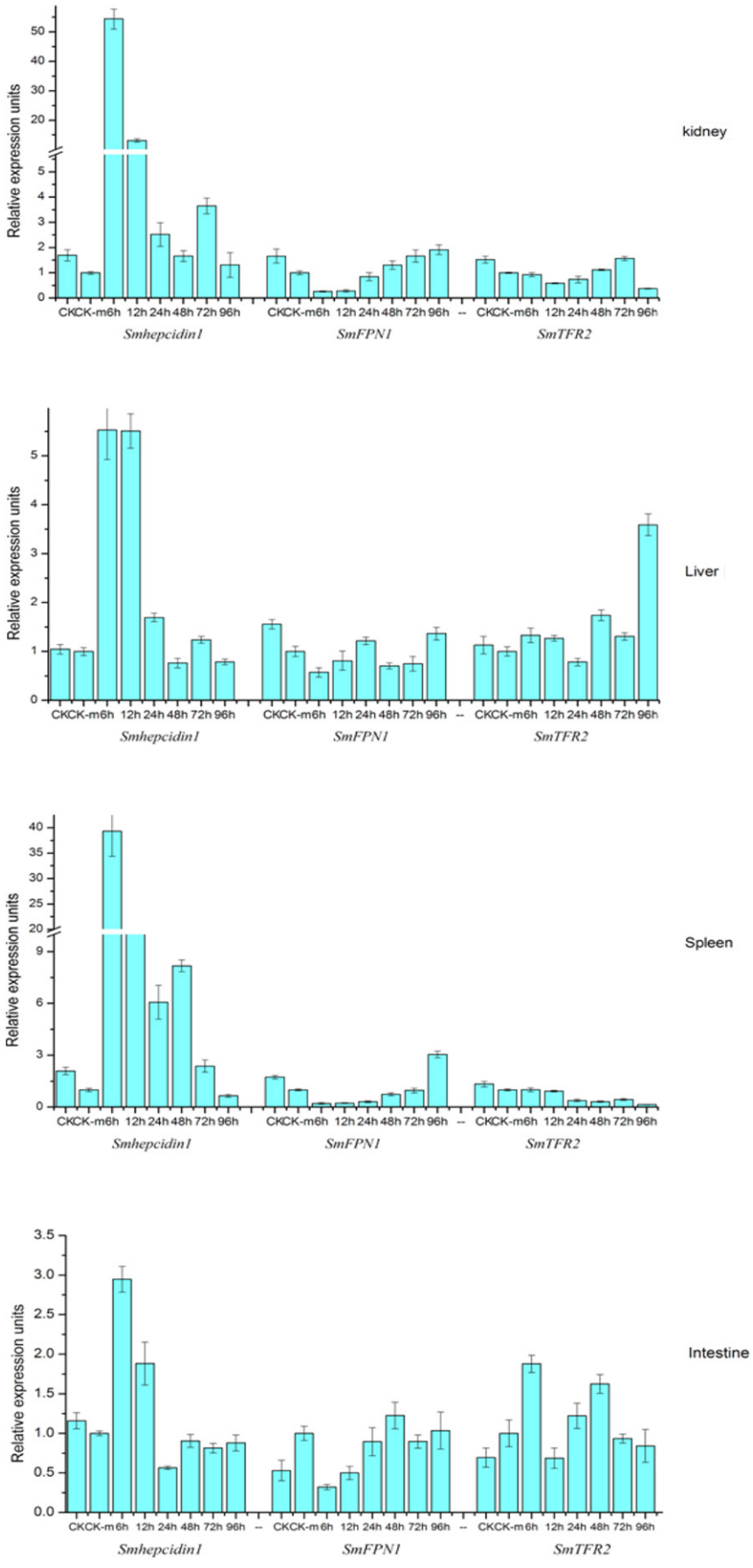


Fig. 7. The fluorescence intensity of iron by Phen Green FL in different time. (A) The cell before luciferase staining, (B) the cell after luciferase staining was observed by confocal laser scanning microscope(CLSM), (C) the fluorescence intensity of iron after ScHepcidin added in different time.


Relative expression units
kidney
Liver
Spleen
Intestine
CKCK-m6h 12h 24h 48h 72h 96h
Smhepcidin1
SmFPN1
SmTFR2

a poly (A) tail (Fig. 1). By phylogenetic analysis, the genes were clustered together with the fish *FPN1* (Fig. 2).

The *SmFPN1* gene encodes a putative protein of 564 amino acids (Fig. 1) with a predicted molecular mass of 61.5 kDa and an isoelectric point (pI) of 5.61. The protein is a transmembrane protein, and the transmembrane region contains a total of 10 transmembrane domains by transmembrane domain prediction analysis. Analysis of the conserved domains showed that the protein has a 24 aa of Hepcidin binding domain (HBD) (Fig. 1).

The Turbot *SmTFR* ORF had a length of 1338 bp, and the 5′ and 3′ end fragments were acquired by RACE using the BD SMART™ RACE cDNA amplification kit with the primers TFR-5-A1, TFR-5-A2, TFR-3-S2 and TFR-3-S3, and the adaptor primers UPM and NUP. Finally, the three fragments were combined into a 1975 bp form, containing a 5′UTR of 29 bp, an ORF of 1338 bp encoding a polypeptide of amino acids and a 608 bp 3′UTR with a poly (A) tail (Fig. 3).

The *SmTFR* gene encodes a putative protein of 445 amino acids (Fig. 3) with a predicted molecular mass of 48.7 kDa and an isoelectric point (pI) of 6.27. The conserved domain analysis showed that the protein has the conserved domain PA_TfR. Phylogenetic analysis showed the SmTFR cluster together with the fish analyzed (Fig. 4).

3.3. Expression profile of SmFPN1 in various tissues

Quantitative real-time RT-PCR was employed to quantify the expression of *SmFPN1* mRNA in different turbot tissues in normal individuals. The mRNA transcripts of *SmFPN1* were detected in all the tissues examined, with the highest expression in the intestine, and a higher expression in the liver, spleen, kidney and head kidney (Fig. 5).

In different tissues in normal individuals, the expression of *SmTFR* mRNA was also detected by Quantitative real-time RT-PCR, with the highest expression in the liver, and a higher expression in the spleen, kidney and head kidney (Fig. 6).

3.4. Detection of intracellular chelatable iron

After adding synthetic Hepcidin to cells, the fluorescence intensity of Phen Green FL was increased over time, and obviously different from the control ($p < 0.01$) because chelatable iron reducing its quenching to Phen Green FL. In the control, although the fluorescence intensity of Phen Green FL was slightly increased, it was not significantly different (Fig. 7).

3.5. Expression profile of SmHepcidin, SmFPN1 and SmTFR after bacterial/viral challenge and function tests of synthesized Hepcidin in vivo as well as RNAi

The real-time PCR results showed that after the bacterial challenge, *SmHepcidin* expression rapidly increased in the course of sampling in the liver, spleen, kidney and intestine. The level of *SmHepcidin* expression peaked at 6 h, and the level of *SmHepcidin* expression increased several times more than the control. Then *SmHepcidin* expression continued to decline until it reached the normal level. With the *SmHepcidin* expression, the *SmFPN1* expression reached a minimum value at 6–12 h, and *SmTFR* expression declined at 12 h, and then began to rise slowly. The *SmFPN1* response was more sensitive than *SmTFR* to *SmHepcidin* (Fig. 8).

LCDV is an important pathogen in many fish species which results in lymphocystis disease [37]. After the viral challenge, the real-time PCR results showed that the gene expression pattern was similar to the bacterial challenge, but the *SmFPN1* and *SmTFR* response to *SmHepcidin* was of lower sensitivity, as the expression only began to increase after 12–24 h (Fig. 9).

After function tests of synthesized Hepcidin *in vivo*, *SmHepcidin*, *SmFPN1* and *SmTFR* exhibited a different expression pattern in different tissues (Fig. 10). In the liver, the expression in *SmHepcidin*, *SmFPN1* and *SmTFR* was identical to the expression level after infection. Meanwhile, the response of *SmFPN1* and *SmTFR* to *SmHepcidin* was very obvious. However, in the spleen and kidney, the expression of *SmHepcidin* exhibited almost no change.

At the 5′ end, the middle and 3′ end of the *SmHepcidin* gene, three siRNA were designed and named siRNA1, 2 and 3. After the transfection of SMKC and extraction of RNA, the real-time PCR analysis results showed that siRNA1, 2 and 3 were able to effectively silence the *SmHepcidin* expression of the transduced cells and the expression was reduced by 2–7 folds, while the expression levels of *SmFPN1* and *SmTFR* were increased (Fig. 11).

3.6. Luciferase reporter assay

We cotransfected the eukaryotic expression vector pcDNA3.1-Hepcidin mutation or pcDNA3.1-Hepcidin with pNFκB-luc and pRL-TK into SMKC cells, and pcDNA3, pNFκB-luc and pRL-TK cotransfected into SMKC cells were used as the negative control. The results showed by the analysis of the changing fluorescence intensity, the overexpression of SmHepcidin in SMKC cells of cotransfected pcDNA3.1-Hepcidin mutation or pcDNA3.1-Hepcidin with pNFκB-luc and pRL-TK, resulted in NF-κB promoting the expression of the downstream reporter gene, causing increased fluorescence intensity, which the SmHepcidin mutant protein (Hepcidin-m) induced. In summary, SmHepcidin is able to increase the level of activation of NF-κB through an activation of certain signaling pathways (Fig. 12).

4. Discussion

4.1. SmHepcidin, SmFPN1 and SmTFR in iron homeostasis

4.1.1. SmHepcidin plays an important role in iron homeostasis

In this paper, the cloned *SmHepcidin* sequence is the same as that of the published Hepcidin of turbot (AM113708.1). The *SmHepcidin* gene encodes a putative protein of 26 amino acids. The fragment also has eight conserved cysteine sites, the same as the human form. The prediction results of the protein tertiary structure indicate that the 8 cysteine residues form four disulfide bonds and constitute the skeleton-like structure, which plays a role in stabilization.

Hepcidin, like other antimicrobial peptides, has two main functions, regulating iron-metabolism [38,39] and having broad-spectrum antimicrobial activity [40]. As an important element in the fish immune system, the expression of Hepcidin is regulated by external factors exert control through specific transcription factors acting on the Hepcidin promoter in different signaling pathways so as to change the Hepcidin transcription level. Therefore, the cloning of the Hepcidin promoter of turbot and the analysis of the main

Fig. 8. The quantitative RT-PCR analysis of the expression level of *SmHepcidin*, *SmFpn1* and *SmTfR* mRNA, relative to β-actin mRNA, of spleen(A), liver(B), kidney(C) after infection with *Vibrio anguillarum*. Figures show *SmHepcidin*, *SmFpn1* and *SmTfR* relative expression levels at 6 h, 12 h, 24 h, 48 h, 72 h and 96 h post bacterial injection, CK-m means the tissues at 12 h after saline solution injection, and CK means the fish live in normal condition. All data are expressed as mean ± S.D. ($n = 3$). Significant differenced across Saline were indicated with two asterisk at $P < 0.01$.

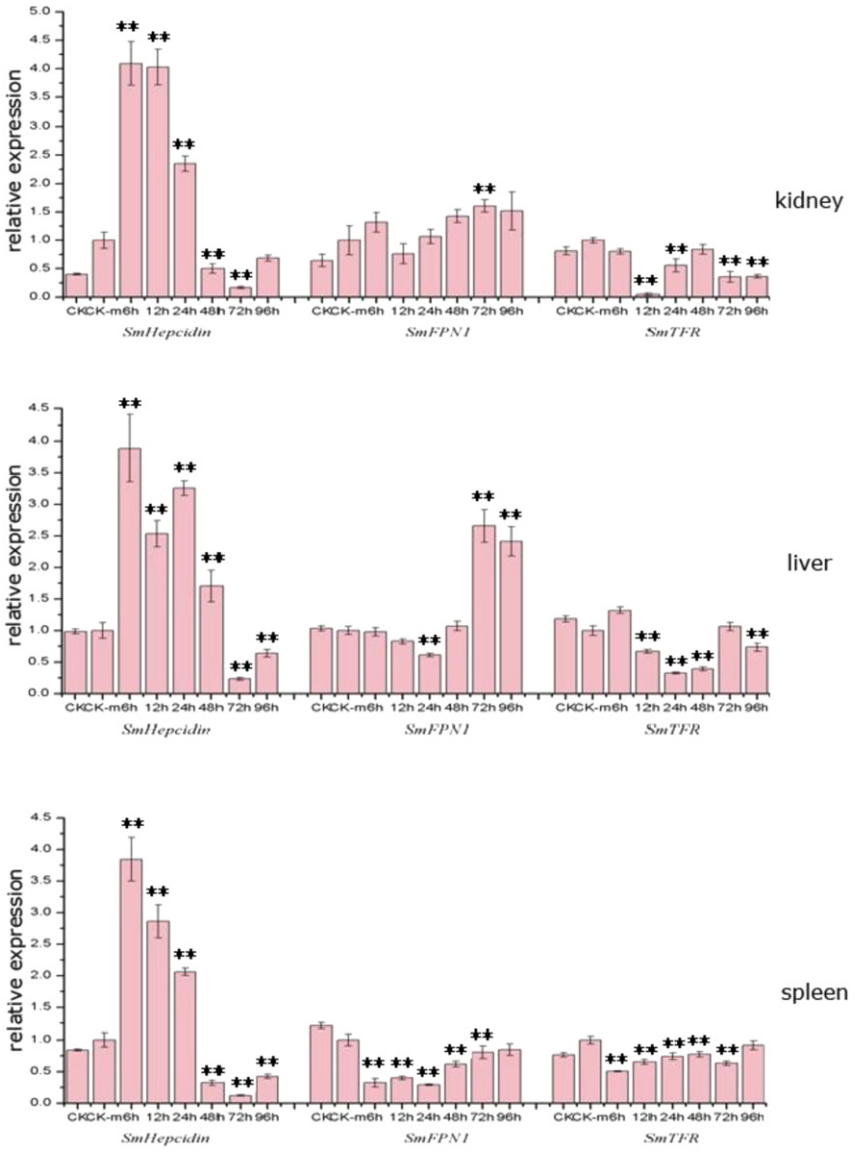


Fig. 9. The quantitative RT-PCR analysis of the expression level of *SmHepcidin*, *SmFpn1* and *SmTfR* mRNA, relative to β-actin mRNA, of spleen(A), liver(B), kidney(C) after infection with LCDV. Figures show *SmHepcidin*, *SmFpn1* and *SmTfR* relative expression levels at 6 h, 12 h, 24 h, 48 h, 72 h and 96 h post virus injection, CK-m means the tissues at 12 h after medium injection, and CK means the fish live in normal condition. All data are expressed as mean ± S.D. ($n = 3$). Significant differenced across Saline were indicated with two asterisk at $P < 0.01$.

motif components enables an investigation of the changing pattern of Hepcidin in turbot, the results of which showed that the promoter region of SmHepcidin includes two important transcription factors CCAAT enhancer binding sites for CREB and NF-κB, respectively. These two sites correspond to the two different signaling pathways involved in Hepcidin [41,42]. The former mainly acts through the BMP-SMAD1 ~4 signaling pathway which regulates Hepcidin, while the latter acts mainly through the NF-κB signaling pathway, which regulates the expression of Hepcidin.

In order to verify the function of the Hepcidin protein *in vitro*, the function of synthetic Hepcidin and the importance of Hepcidin in iron-metabolism were verified using iron fluorescence staining. The results showed that, when adding the synthetic Hepcidin to SMKC cells, the fluorescence increased, indicated that chelatable iron can be reduced because it is likely to be used or occupied. May be it was that Hepcidin increased the ability of cells fixed iron, which could reduce the ability of pathogenic bacteria using iron. The results also demonstrate that the synthetic Hepcidin has biological activity.

4.1.2. Tranmembrane protein Ferrorportin 1 distribution and the role in iron metabolism

The transmembrane protein Ferrorportin 1 interacts with Hepcidin in the control of iron homeostasis using Hepcidin-FPN1 as the core [10]. Through gene and protein sequencing analysis, a fragment of 24 aa was found in SmFPN1 which has conserved HBD sites that be relevant to the activity of Hepcidin. Alignment analysis of the HBD with human FPN1 showed that the region is highly conserved, and may be closely related to the broad-spectrum antimicrobial capability of Hepcidin [8]. We compared the SmFPN1 transmembrane domain with other species' published Ferroportin, the result show they all have ten transmembrane domains. *SmFPN1* is mainly expressed in the intestine, and liver, perhaps because intestinal epithelial cells and liver cells are the main site of iron storage and activity. Under normal conditions, FPN1 is highly expressed in these areas, and is used to transport the iron in the intestinal epithelial cells into the bloodstream and then into other tissues and organs of the body.

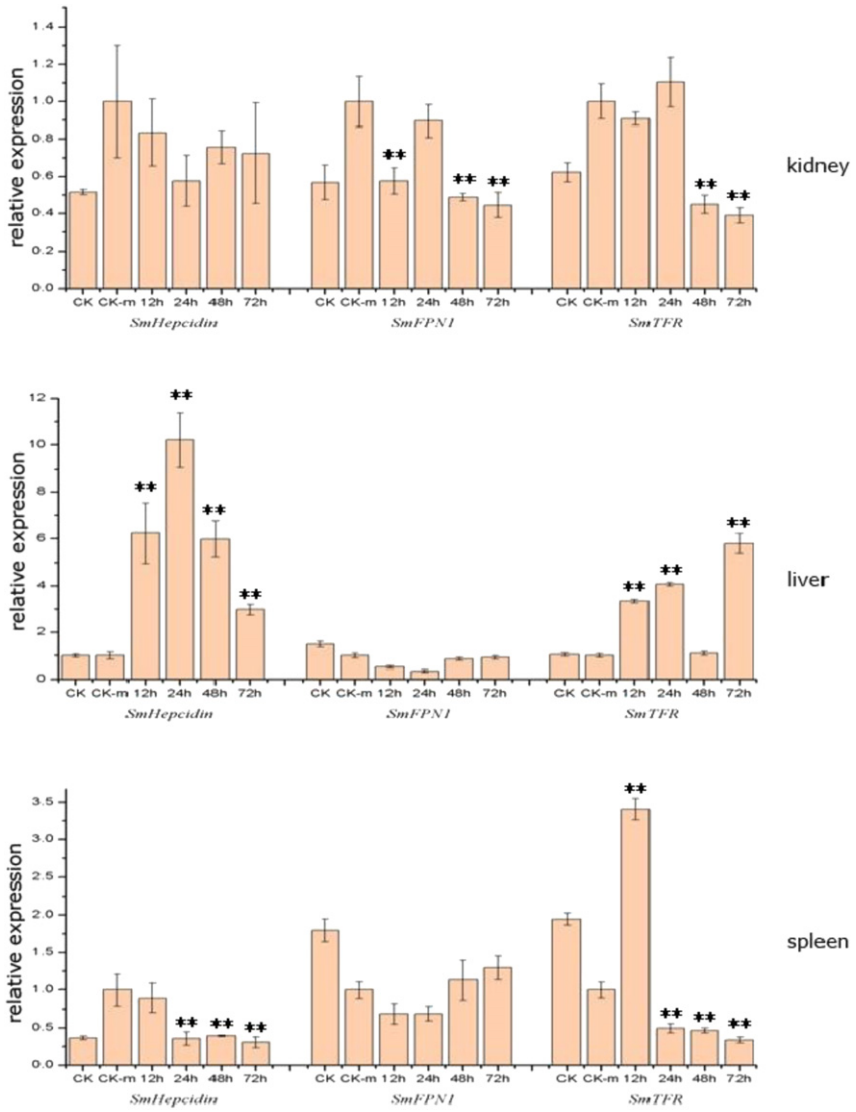


Fig. 10. The quantitative RT-PCR analysis of the expression level of *SmHepcidin*, *SmFpn1* and *SmTfR* mRNA, relative to β-actin mRNA, of spleen(A), liver(B), kidney(C) after injection with synthesized Hepcidin. Figures show *SmHepcidin*, *SmFpn1* and *SmTfR* relative expression levels at 6 h, 12 h, 24 h, 48 h and 72 h post protein injection, CK-m means the tissues at 12 h after saline solution injection, and CK means the fish live in normal condition. All data are expressed as mean ± S.D. ($n = 3$). Significant differenced across Saline were indicated with two asterisk at $P < 0.01$.

4.1.3. The TFR has an important role in iron-metabolism

The Transferrin Receptor has an important role in the process of transferring iron into cells. In this process, transferrin (TF) first forms the iron complex, and then the two TF complexes HEF and TFR, which together constitute the iron transport complexes, transfer extracellular iron into the cells through endocytosis [43]. Next, IL-6 acts on SMAD in order to activate the transcription which induces the production of Hepcidin. The concerted effect of TFR and Hepcidin is indirect, but other studies have shown that Hepcidin is beneficial for the formation of the TF-HEF-TFR complex [44]. The reciprocal promotion by TFR and Hepcidin enables a rapidly increased expression of Hepcidin and a rapid regulation of iron homeostasis.

Other studies have shown that the two forms of TFR (TFR1 and TFR2), which promotes the expression of Hepcidin, while the TFR2 affinity to iron is lower than that of TFR1 [45]. The *SmTFR* gene encodes a putative protein of 445 amino acids and has the conserved domain PA_TfR. It can be expressed in every tissue, and has the higher expression in immune-related tissues liver and spleen similarly as *SmFPN1*. However, the function of SmTFR is still inadequately understood and required further investigation.

4.2. Expression profile of SmHepcidin, SmFPN1 and SmTFR in turbot

Expression analysis of *SmFPN1* and *SmTFR* in various tissues of adult turbot using Quantitative real-time RT-PCR demonstrated that they could be higher expression in the immune-related organizations (liver, spleen, kindey, head kindey) similar to *SmHepcidin* expression [19].

The results of the changes in the expression levels of *SmHepcidin*, *SmFPN1* and *SmTFR* induced by bacterial challenge of turbot suggest a reason for the opposite expression patterns *SmFPN1* and *SmHepcidin*. *SmFPN1* can directly combine with *SmHepcidin* and this leads to changes in the endogenous *SmFPN1* level due to degradation, and this degradation is not only evident change in the expression of the proteins directly degraded [46], it also is observed in terms of a series of intermediate links which enables SmHepcidin to prevent FPN1

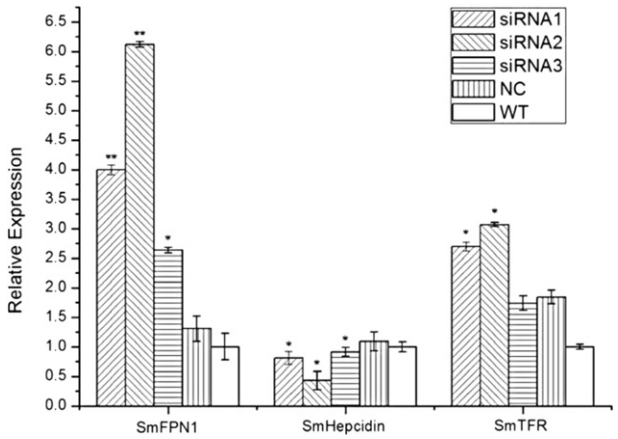


Fig. 11. The quantitative RT-PCR analysis of the expression level of *SmHepcidin*, *SmFpn1* and *SmTfR* mRNA relative to β-actin mRNA of SMKC after transform with *RNAi-Hepcidin*. The figure shows the *SmHepcidin*, *SmFpn1* and *SmTfR* mRNA relative expression levels in siRNA1,2,3 cell line, NC means the cell transformed with randomized siRNA, and WT means the cell cultured under normal condition. All data are expressed as mean ± S.D. ($n = 3$). Significant differenced were indicated with an asterisk at $P < 0.05$, and two asterisk at $P < 0.01$.

transcription. This may be relevant to the fine regulation of cellular protein expression and decreased energy consumption. Similarly, the expression patterns of *SmHepcidin* and *SmTFR* are also opposite to one another, which may be because *SmHepcidin* plays an important role in iron homeostasis. After the bacterial challenge, TFR and TF form a complex which induce the rapid expression of *Hepcidin* through the SMAD pathway [47]. However, an excess of *Hepcidin* also cause an iron deficiency in the blood. This indicates that *SmHepcidin* expression may be considered as a form of feedback regulation, resulting in iron transport complex dissociation and a lower level of related protein transcription that result in a rapid reduction of *Hepcidin* transcription [45,48]. The Similar result also occurred after viral challenge in turbot. After the challenge of synthetic Hepcidin in turbot, a set of very interesting results were obtained. The expression patterns of *SmHepcidin*, *SmFPN1* and *SmTFR* were the same as observed after viral infection in the liver, perhaps because the introduction of exogenous Hepcidin is recognized as an immune stimulus by liver cells, causing the expression levels of *SmHepcidin*, *SmFPN1* and *SmTFR* to subsequently undergo change. However, in the spleen and kidney, the expression level of *SmHepcidin* exhibited almost no change or decreased; meanwhile, the expression levels Changes of *SmFPN1* and *SmTFR* were not similar to bacterial and viral challenge, which suggested the need for further investigation. These

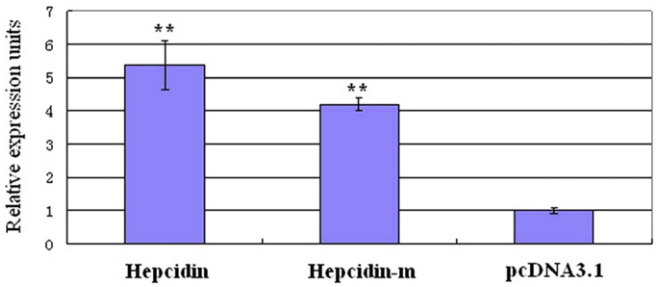


Fig. 12. Hepcidin regulates NF-KB. Hepcidin means SMKC cells cotransfected pcDNA3.1-Hepcidin, pNFκB-luc and pRL-TK, Hepcidin-m means SMKC cells cotransfected pcDNA3.1-Hepcidin-m (Hepcidin mutations), pNFκB-luc and pRL-TK, pcDNA3 said SMKC cells cotransfected pcDNA3.1, pNFκB-luc and pRL-TK. SMKC cells were lysed and luciferase activity was measured with *GloMax20/20 Luminometer*. Error bars represent standard deviations were obtained by measuring each sample three times from three independent experiments. Significant differenced were indicated with two asterisk at $P < 0.01$.

conclusions were supported by gene-silencing experiments of *SmHepcidin*. After silencing of the *SmHepcidin* gene, the expression levels of *SmFPN1* and *SmTFR* were increased, while the expression of *SmTFR* in SMKC transfected by siRNA3 was not significantly changed compared with that of the control, probably because the expression of *SmHepcidin* was not completely inhibited.

Even though intestine is not as a kind of immune organs exists, but as FPN1 has an important function in the intestine, we also examined the expression level of *SmHepcidin*, *SmFPN1*, and *SmTFR* after bacterial infection. From Fig. 8 it can be seen that the expression of the three genes in intestine have the same modes as they in the liver, spleen, kidney after bacterial infection.

4.3. The effect of Hepcidin on the regulation of NF-κB

Previous studies showed that after bacterial infection or an inflammatory response, the body accepts extracellular signals through the Toll-like receptor, degradation of ubiquitinated Iκ-Bs to activate the NF-κB signaling pathway, causing a series of immune-related protein expression events. This is also related to the expression of SmHepcidin regulation by NF-κB [41,49,50]. The SmHepcidin promoter was analyzed in this study, in which an NF-κB binding site was demonstrated. The feedback regulation relationship between SmHepcidin and NF-κB was verified by Luciferase reporter assay. In Luciferase reporter assay, pNFκB-luc, a reporter gene plasmid containing the NF-κB transcription factor binding sites, may be used to detect the NFκB activation level. The over-expression of SmHepcidin improved the level of activation of NF-κB by Luciferase reporter assay. It may because over-expression of SmHepcidin affects the NF-κB complex through some feedback regulation mechanism to increase the level of activation of NF-κB. The upregulated activation of NF-κB as a transcription factor would also change the expression of other immune-related proteins in the NF-κB signaling pathway, which may be a further immunological regulatory mechanism of SmHepcidin, although this requires corroboration. Meanwhile, mutated SmHepcidin induced the expression of the downstream reporter gene, which showed that the tertiary structure of SmHepcidin plays a central role in the process involved in the feedback regulated NF-κB complex change. These signaling pathways SmHepcidin worked through and eventually which transcription factor was led to the activation expression, it can be selected as the future research direction. But whether Hepcidin through reverse control NF-κB expression to regulate the FPN1 and TFR transcription level is not clear, still need further study. In summary, the relationship between NF-κB and Hepcidin, as an important signaling pathway in the fish immune system, is worthy of further investigation.

From the above results, *SmHepcidin* plays a role in the NF-κB signaling pathway, and *SmHepcidin* expression was significantly increased in the course of stimulation with exogenous pathogens. *SmHepcidin* together with *SmFPN1* and *SmTFR* can join in the concerted regulation of intracellular iron. Jiang found that hemoglobin can effectively resist *Staphylococcus aureus*, *Pseudomonas aeruginosa* and other bacteria [51], which suggests that *SmHepcidin* may be affect hemoglobin by regulating the concentration of iron *in vivo*, thus playing a role in the anti-bacterial response.

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