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The putative mature peptide of piscidin-1 modulates global transcriptional profile and proliferation of splenic lymphocytes in orange-spotted grouper (*Epinephelus coioides*)

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ABSTRACT

Piscidins are important components in protecting microbial infections in teleost. The present study purified and identified a truncated peptide, whose sequence was very close to that of putative mature peptide of epinecidin-1 (piscidin-1) in orange-spotted grouper (*Epinephelus coioides*), Epi-1 (also named as short form of ecPis-1, ecPis-1S). The immunomodulatory effects of ecPis-1S on splenic lymphocytes of orange-spotted grouper were explored *in vitro*. The transcriptome study was carried out by *De novo* transcriptome sequencing (RNA-Seq) in splenic lymphocytes of orange-spotted grouper. Regarding the profiles of gene expressions, 2994 genes were up-regulated and 2679 genes were down-regulated in the splenic lymphocytes stimulated by ecPis-1S. In the case of differential expression genes, 330 genes were involved in immune related pathways. Among them, 34 genes were involved in T cell receptor signaling pathway, 31 genes in natural killer cell mediated cytotoxicity and 23 genes in leukocyte transendothelial migration, respectively. Immune-related genes selected for qRT-PCR verification, such as interleukin-1β (*il-1b*), tumor necrosis factor α (*tnfa*), T cell antigen receptor (*tcr*), major histocompatibility complex class I (*mhc I*), and *mhc II* were significantly up-regulated by ecPis-1S ($p < 0.05$). ecPis-1S could significantly enhance the proliferation of splenic lymphocytes of orange-spotted grouper *in vitro* ($p < 0.05$). In addition, the result of qRT-PCR revealed that ecPis-1S also significantly up-regulated cell cycle-related genes, including cyclin A (*cyca*), cyclin-dependent kinase 2 (*cdk2*), *cdk4*, cell division cycle protein 6 (*cdc6*), and transforming growth factor β (*tgfb*) ($p < 0.05$), which suggested that ecPis-1S promoted the proliferation of lymphocytes by activating cell division cycle. In conclusion, the results indicated that the mature peptide of piscidin-1 in orange-spotted grouper could act as immune modulator and play an important role in regulation of the immune response in fish.

1. Introduction

Antimicrobial peptides (AMPs), also called host-defense peptides (HDPs), are widely distributed from prokaryotes to eukaryotes [1]. AMPs present several common features, such as low molecular weight (less than 10 kDa), positive charged, and with an amphipathic structure [2]. AMPs are considered to play an essential role in defeating pathogens including viruses, bacteria, fungi and parasites [3]. In recent years, they have attracted considerable attention as a possible alternative of antibiotics. Furthermore, accumulated evidences have shown that AMPs are multifunctional peptides that could also act as immune modulators and signaling molecules. In human, an amphipathic α-helical peptide [4], cathelicidin LL-37 could increase the production of cytokines and chemokines, and participate in pro- and anti-inflammatory signaling [5–7]. Similar immunomodulatory effects have been discovered in human defensins [3], which are another group of cationic peptides with disulfide bond linkages that could modulate the immune and inflammatory responses [8].

In order to prevent microbial infections, fish have also developed multiple AMPs as the first line of defense, including pleurocidin, cathelicidin, β-defensin, piscidin and hepcidin [1,9,10]. As an important member of AMPs in fish, piscidins comprise a family of cationic amphipathic polypeptides, and have multiple functions in microorganism elimination [7,10–18] and tumor cells dissociation *in vitro* [19–21]. Their genes can be induced by various stimuli, including bacteria [22], components of bacteria [23], parasites [24], viruses [25] and poly I:C [26]. Interestingly, increasing amount of studies has been reported to show the immunomodulatory activities of piscidin family members in

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recent years. For examples, tilapia piscidin 3 (TP3) and 4 (TP4) could increase immune-related genes expression when electro transferred into skeletal muscle in Nile tilapia (*Oreochromis niloticus*) [27]. Epinecidin-1, a member of grouper piscidins [10,19], was first cloned from *Epinephelus coioides* [28]. The putative mature peptide (known as Epi-1) which was synthetized has been reported to modulate fish immune responses [29–33]. In an oral administration study, immune-related genes including *tnf1*, *il1b* and *nfkb* were up-regulated in *E. coioides* and zebrafish (*Danio rerio)* when fed with the recombinant epinecidin-1 protein [34]. In another study, electroporation of the epinecidin-1 gene into *E. coioides* and subsequent infection with *Vibrio vulnificus* affected the expression of immune-related genes, in which Myeloid differentiation factor 88 (*myd88*), *il-8*, *tnfa*, *tnfb* and interferon regulation factor 2 (*irf2*) were up-regulated [35]. However, the exact immunomodulatory mechanism of piscidins in fish immune system is still unclarified.

Up to now, no report has addressed the processing of the pro-piscidins by local protease. No evidence has showed whether the putative mature form of epinecidin-1 (Epi-1) is present in *E. coioides*. Furthermore, more evidences are needed to reveal the immunomodulatory functions of piscidin in teleost. Our previous study cloned and characterized three novel piscidins from *E. coioides*, and named them as *ecpis-2*, *ecpis-3* and *ecpis-4*, respectively [10]. Since *epinecidin-1* is a member of piscidin family reported earlier in *E. coioides*, we changed the name of *epinecidin-1* into *ecPis-1* in that paper [10]. In present study, we purified and identified a truncated peptide of ecPis-1, which showed 100% identity and 85.7% coverage with putative mature peptide Epi-1 or the short form of ecPis-1 (ecPis-1S), implying that prepro-peptide of ecPis-1 underwent cleavage processing to remove signal peptide and prodomain of pro-peptide. To reveal further details of the immunomodulatory functions of piscidin in grouper, the effects of ecPis-1S on proliferation of lymphocytes and gene expressions were explored *in vitro* with primary culture of splenic lymphocytes of orange-spotted grouper.

2. Material and methods

2.1. Isolation of piscidin-1 from orange-spotted grouper

Healthy orange-spotted groupers (weight 602 ± 19.39 g, length 32 ± 2.53 cm) were obtained from the Fish Market of Nanshan District, Shenzhen, Guangdong Province, China. The groupers were challenged by intraperitoneal injection of *V. parahaemolyticus* (1×10^8 colony-forming units, CFU) and 12 h later the mucus was obtained by scratching the skin. The crude mucus was homogenized with 30 mL ethanol/0.7 mol/L HCl (v/v, 3:1), and then preserved at 4 °C overnight. The sample was centrifuged at 4000 g for 30 min, and the supernatant was collected. The supernatant containing crude proteins and peptides was separated by gel filtration chromatography. Samples were then purified by reversed phase high performance liquid chromatography (HPLC) on a Shimadzu system (Japan) using a C18 reverse phase column (Inertsil ODS3, 5 μm, 4.6 × 250 mm) running a gradient of two solvents, A: H_2O, 0.1% trifluoroacetic acid, and B: H_2O, 70% acetonitrile, 0.1% trifluoroacetic acid. A linear gradient of 30%–70% solvent B over 35 min was used at a flow rate of 1 mL/min.

Eight fractions were collected for LC-MS/MS analysis. In brief, the freeze-dried sample (collected fraction) was suspended in water containing 0.1% formic acid and 2% acetonitrile, then subjected to TripleTOF 5600 (Sciex, USA) mass spectrometry (MS) coupled with nano LC-Ultra 1D plus system (Eksigent) for online nano ESI-LC-MS analysis. MS/MS spectra were acquired on high sensitivity mode with accumulation time of 80 ms per spectra; the threshold intensity of the precursor ions was180cps with a charge state of from +2 to +5. The ionization parameters were set as follows: ion spray voltage of 2400 V, curtain gas of 35psi, nebulizer gas of 5 psi, an interface heat temperature of150 °C.The MS raw data was submitted to the protein pilot v.4.5 (Sciex, USA) for performing the database search analysis with the following search parameters: (i) the peptide sequence, ecpis-1L0 (the deduced amino acid sequence of *ecpis-1*: MRCIALFLVLSLVVLMAEPGEGFIFHIIKGLFHAGKMIHGLVTRRRHGVEELQDLDQRAFEREKAFA) was appended to a protein sequence database; (ii) Neither enzyme digestion nor cysteine alkylation; (iii) instrument type: TripleTOF 5600; (iv) ID focus: biological and amino acid modifications; (v) search pattern: thorough ID.

2.2. Synthesis of custom peptide

The peptide ecPis-1S (GFIFHIIKGLFHAGKMIHGLV) with a purity of > 95% was generated at GL Biochem (Shanghai) Ltd. (China) using the solid reaction method. The purity of the peptide was determined by high-performance liquid chromatography and mass spectrometry. The peptides were reconstituted in sterile deionized water and stored at −80 °C.

2.3. Preparation of primary cultured splenic lymphocytes

For analysis of proliferation and gene expression upon *in-vitro* stimulation, splenic lymphocytes were collected as followed. Fish were anesthetized with an overdose of MS222 (Sigma-Aldrich, USA). Spleens were removed aseptically and immersed in RPMI-1640 medium immediately. After dismissed, tissues were gently mashed by pressing with the flat surface of a syringe plunger against a sterile sieve. Splenocytes were collected and washed twice, diluted to 2×10^7 cells/mL and laid on the Lymphocyte Separation Medium (Corning, USA). Cells were harvested after centrifuging at 1000 g for 30 min at room temperature. After the blood red cells were disrupted, lymphocytes were suspended in medium. Cell concentration and viability were determined by trypanblue dye exclusion with a haemocytometer. Viability of cells was approximately 90%. Cells were cultured at 27 °C and 5% CO_2 overnight. After centrifuging at 800 g for 10 min, supernatant was discarded, while the cells were suspended in RPMI-1640 medium and diluted into 2×10^7 cells/mL.

2.4. Transcriptome analysis

2.4.1. In-vitro stimulation of splenic lymphocytes by esPis-1S

For RNA-Seq, cells were diluted into 1×10^7 cells/mL each well and divided into two groups with five replicates. The experimental group (ecPie-1S) was added with esPie-1S at 4.0 μmol/L, while control group (ecPis-free, no esPie-1S added) with RPMI-1640 Medium. Twelve hours later, cells of five replicates in each group were collected and pooled, centrifuged at 400 g for 10 min and the precipitates were preserved at −80 °C.

2.4.2. Total RNA extraction and cDNA library construction

Total RNA was extracted from splenic lymphocytes using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. The integrity of RNA was assessed with Agilent 2100 Bioanalyzer (Agilent, USA). Only RNA with high quality (OD_{260}/OD_{280} = 1.9–2.1) was used for further cDNA library construction. Equal amount of RNA from each group (esPis-1S and ecPis-free) was pooled to provide templates for RNA-Seq library construction. Total RNA was digested by DNase I (NEB, USA), purified by Dynabeads mRNA purification kit (Invitrogen, USA), and then the poly (A)-containing mRNA was fragmented into 200–250 bp with Fragmentation buffer (Ambion, USA). The fragmented RNA was transcribed into the first strand cDNA using random hexamer primer N6 and SuperScript II reverse transcriptase (Invitrogen, USA), followed by synthesis of the second strand cDNA with second strand master mix (Invitrogen, USA). Then, the double-stranded cDNA was purified with QIAquick PCR purification kit (QIAGEN, Germany); end repaired, and followed by addition of A-tail and ligated adapters. The adapter-ligated fragments were enriched by PCR amplification with PCR primer cocktail and master mix (Thermo Fisher Scientific, USA).

PCR products were purified with AMPure XP beads (Beckman, USA) to get the final cDNA library. Two libraries were prepared and sequenced independently using an Illumina HiSeq2000 platform (Illumina, USA), with 2 × 90 bp read length (BGi tech, Shenzhen, China).

2.4.3. Sequencing and assembly

RNA-Seq raw reads were filtered to discard any dirty reads, including reads with adapters, reads with unknown nucleotides (bases N) higher than 5%, and reads with more than 20% of low quality bases (base quality ≤ 10). All clean reads were assembled by Trinity short reads assembling program (http://trinityrnaseq.sf.net) with default parameters to get unique transcript fragments (unigenes). Unigenes were further processed for sequence splicing and redundancy removal with TIGR Gene Indices Clustering Tools (TGICL) to acquire non-redundant unigenes.

2.4.4. Functional unigenes annotation and classification

All unigenes were aligned by BLASTx using cut-off E-value of $1E^{-5}$ to identify the coding sequence (CDS) in known protein databases, including NCBI non-redundant protein database (NR: http://www.ncbi.nlm.nih.gov), Swiss-Prot database (http://www.uniprot.org), Kyoto Encyclopedia of Genes and Genomes (KEGG: http://www.genome.jp/kegg), and Clusters of Orthologous Groups of Proteins database (COG: http://www.ncbi.nlm.nih.gov/COG/). For unigenes which did not align to any protein database, they would be scanned using ESTScan (http://www.ch.embnet.org/software/ESTScan2.html) to produce predicted coding region and direction. The Blast2GO program (http://www.blast2go.com/b2ghome) was used to obtain gene ontology (GO) (http://www.geneontology.org/) annotation of the unigenes based on that BLASTx against Nr annotation. The WEGO software was used to perform GO functional classification [36]. The KEGG pathway and KEGG Orthology (KO) of unigenes were analyzed based on that BLASTx against the KEGG database.

2.4.5. Identification of differentially expressed genes

Differential expression analysis was performed with comparison between two libraries of splenic lymphocytes to identify the differentially expressed genes (DEGs). All DEGs were then tested by GO functional enrichment analysis and KEGG pathway analysis with GOatools (http://github.com/tanghaibao/GOatools) and KOBAS (http://kobas.cbi.pku.edu.cn/home.do).

2.5. Quantitative reverse transcription real-time PCR (qRT-PCR)

For qRT-PCR, splenic lymphocytes were prepared following steps of the Part 2.3. Lymphocytes were treated with esPie-1S at 0.5 μmol/L and 4.0 μmol/L respectively. Four replicates were performed for each treatment. After 12 h and 24 h, cells were collected and preserved at −80 °C. Total RNA extraction was performed as described above. Total RNA of the tissues was extracted using RNAiso Plus (TaKaRa, Dalian, China) following the manufacturer's protocol. One micrograms of total RNA was reverse transcribed using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) to obtain first strand cDNA. Gene-specific primers were designed based on the *E. coioides* transcriptome sequences using Primer Premier 5.0. The primer sequences are listed in Table 1. *Beta-actin* of *E. coioides* was used as endogenous control. qRT-PCR was performed in a 20 μL solution containing 10 ng of template cDNA and SYBR Premix Ex Taq II (TaKaRa) using LightCycler 480 (Roche, Switzerland) at 95 °Cfor 1-min pre-incubation, followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. Finally, the melting curve was analyzed to detect single amplification. Fluorescent signal accumulation was recorded at the 60 °C 1 min phase during each cycle under the control of LightCycler 480 Software 1.5. The relative quantities of the target genes expressed as fold variation over β-actin were calculated using the $2^{-\Delta\Delta Ct}$ comparative Ct method [10].

Table 1
Sequences of primers used in this study.

Primer name	Squences(5'→3')
IL-1F	TTGAGGGCAGAACTGTGGCT
IL-1R	CAGGCTGTCTTTGGTAATCGTC
IL-8F	TGGCCGTCAGTGAAGGGAGTCTA
IL-8R	CAGTGGGAGTTTGCAGGTATCAGC
TCRβF	CTCGTCCTGCGTGTTTGA
TCRβR	ACAGTGAGTTTTGTCCCC
MHCIF	ACAGCTACGACGGAAACGAC
MHCIR	TCCCAACCAATCCACACACT
MHCIIF	CTTCATCAGCCTCTACACAGC
MHCIIR	CTCAGCGTTCTTCACACCCA
TNFαF	AGCGGAAAGCCAGACATCACGAC
TNFαR	CTTGGCTTTGCTGCTGATTCGACTC
TGFβF	ACAACAACCCGCTACCTCG
TGFβR	TCCGCTCATCCTCATTTCC
SmadF	CGTGGATTTCAGCAGCATATT
SmadR	TCAAAATAGGCAATGGAGCAC
cdc6F	TGCTGCTGGTTCTGGATG
cdc6R	ACGAGACAGAGGCGAGACTTT
cycAF	TCTCCCCTCACAATCAATGC
cycAR	CGTGGATTTCAGCAGCATATT
cdk2F	GGGTGACTGAGTTCTTTGAGG
cdk2R	AGGGGACGTATGGAGTGGTT
cdk4F	TGGACCAAGACCTCAAGACG
cdk4R	AATGCAAGACCGCACAGTAAC
NFATF	GCTGCGGTGTGGGGTTGT
NFATR	ATGAGGCGGGGCGTTGGT
β-actinF	ATCGCCGCACTGGTTGTTGACA
β-actinR	GGCCCATACCCACCATCACTCC

2.6. Lymphocyte proliferation assay

The lymphocyte proliferation assay consists of three treatments: lymphocytes were stimulated with only ecPis-1S,co-stimulated with ecPis-1S and Concanavalin A (ConA), and co-stimulated with ecPis-1S and lipopolysaccharide (LPS). A control was performed with no ecPis-1S for each treatment. Four replicates were performed for each treatment. Splenic lymphocytes were prepared following steps of the Part 2.3. Lymphocytes (2×10^7 cells/well) were incubated into 96-well culture plates, and ecPis-1S, ConA, and LPS were added at a series of concentrations. The final concentrations of ecPis-1S were 0.25, 0.5, 1.0, 2.0, and 4.0 μmol/L for each treatment. The final concentration of ConA was 0.25 μg/mL, and that of LPS was 4 μg/mL. The plates were incubated in a humid atmosphere with 5% CO_2 (Thermo) at 28 °C for 24 h. In the last 4 h of incubation, 20 μL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 5 mg/mL) was added into each well. The supernatants were discarded and then dimethyl sulfoxide (DMSO, 100μL/well) was added into each well. Finally, A_{490} was tested as the index of ecPis-1S stimulating the proliferation of lymphocytes proliferation [37].

2.7. Statistical analysis

All data was analyzed using SPSS (version 16.0) software and expressed as Mean ± SE. Significant differences between infected group and control group at each time point were determined using the Duncan test ($p < 0.05$).

3. Results

3.1. Identification of piscidin-1 from mucus of grouper after challenged by V. parahaemolyticus

According to the result of gel filtration chromatography (Fig. S1), fractions were collected and further purified by HPLC. Peak fractions separated by HPLC were concentrated by freeze drying (Fig. 1A and B), and then sent to LC-MS for identification. As the result of LC-MS, a

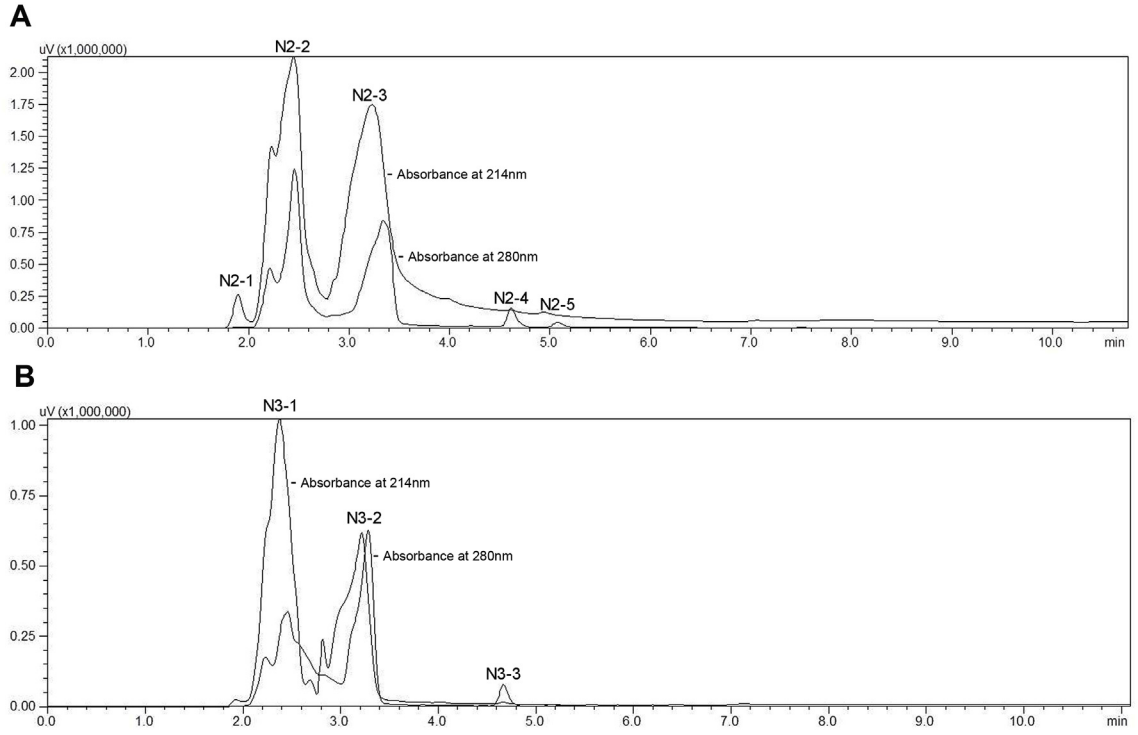
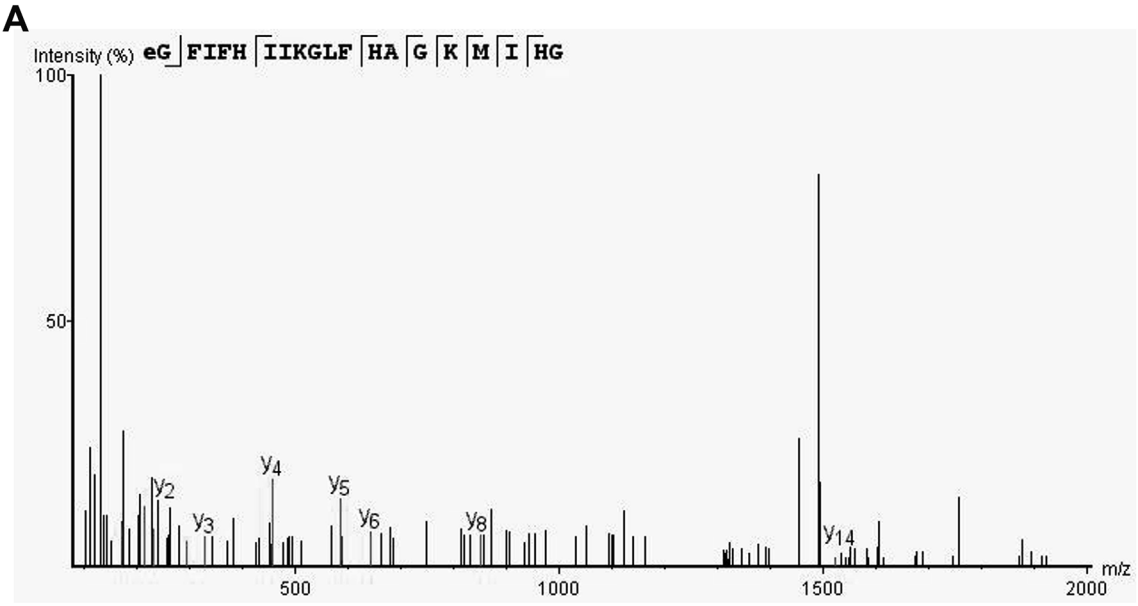


Fig. 1. A chromatogram of gel filtration peak samples from mucous of orange-spotted grouper (*E. coioides*) obtained by reversed phase HPLC. A. HPLC chromatogram of gel filtration peak 2 sample (N2), five fractions were collected, indicated by the numbers (N2-1, 2, 3, 4, and 5) over peaks respectively. B. HPLC chromatogram of gel filtration peak 3 sample (N3), three fractions were collected, indicated by the numbers (N3-1, 2, and 3) over peaks respectively.



B

Peptide	Uniq	-10lgP	Mass	Length	ppm	m/z	z	RT	Fraction	Scan	Source File	Area Sample 2	#Spec	#Spec Sample 2	Start	End	PTM
G.E(-18.01) GFIFHIIKGLFHAGKMIHG.L	Y	15.39	2233.1982	20	4.4	745.4100	3	12.48	2	20222	DL N-2-2 2017120233.wiff	2.5E3	1	1	21	40	Pyro-glu from E

Fig. 2. The result of LC-MS analysis of HPLC fraction No. N2-2. (A) The MS/MS spectrum of N2-2. (B) The result of database search analysis for N2-2.

peptide was identified, EGFIFHIIKGLFHAGKMIHG (Fig. 2), which was identical to Epi-1 (GFIFHIIKGLFHAGKMIHGLV) with a coverage of 85.7%.

3.2. Transcriptome analysis

3.2.1. Sequencing results and De novo assembly

After the RNA-seq was performed, 64.37 Mb and 53.52 Mb raw reads were obtained from esPis-free and ecPie-1S, respectively (Table S1). Raw reads were filtered, and dirty reads were removed. Two clean reads, 52.76 Mb and 48.06 Mb, with Q20% of 97.77% and 98.38%, were obtained from two groups respectively. The clean reads from two libraries were further assembled into 64 508 unigenes. The total length of all unigenes was 54 938 110 nt and average length was 852 nt. N50 was 1445 nt. In the control group (ecPis-free), the total consensus sequences, total length and mean length were 740bp, 67 690 bp and 33 851 111 bp, respectively. In the esPie-1S treatment group (ecPie-1S), the total consensus sequences, total length and mean length were 1036bp, 61 760 bp and 37 859 538 bp, respectively (Table S2).

3.2.2. Annotation of assembled unigenes

A total of 64 508 unigenes were compared against the protein databases including the NCBI non-redundant (NR) database and Swiss-Prot database by BLASTx, and nucleotide (NT) database by BLASTn. BLAST results showed that 31 043 (48.12%), 39 581 (61.36%) and 26 582 (41.21%) unigenes significantly matched with the annotated sequences in NR, NT and Swiss-Prot databases, respectively (Table S3). Among these genes, 13.9% of unigenes possessed an E-value between $1e^{-5}$ and $1e^{-15}$ (Fig. S2–A) and over 92.6% of unigenes shared more than 40% similarities with the annotated sequences in the NCBI database (Fig. S2–B). The species distribution according to these unigenes showed that *E. coioides* unigenes had the largest number of hits to *Nile tilapia* (52.3%), followed by *Japanese medaka* (11.9%), *Fugu rubripes* (11.4%), *Tetraodon nigroviridis* (4.8%), *Dicentrarchuslabrax* (4.0%), *Brachidaniorerio* (2.3%) and *Anoplopoma fimbria* (1.6%) (Fig. S2–C).

3.2.3. GO and COG classification of transcriptome sequences

Based on the database, a total of 41 978 assembled unigenes were aligned respectively to the COG (9847 unigenes, 15.26%), KEGG (21 861 unigenes, 33.89%), and GO (18 123 unigenes, 28.09%) (Table S3). GO is a standardized system for categorizing genes and classifying gene functions across species [38]. Based on the GO analysis, 18 123 unigenes were classified into three categories: biological process (97 295 unigenes), cellular component (62 765unigenes) and molecular function (24 222 unigenes) (Fig. S3). These unigenes were further divided into 57 subcategories. Within biological process category, the most common subcategories included cellular process, metabolic process, single organism process, biological regulation, regulation of biological process and response to stimulus. Within the cellular component, the seven most abundant subcategories were cell, cell part, organelle, organelle part, membrane, macromolecular complex and membrane part. In terms of molecular function, the most common subcategories included binding, catalytic activity, transporter activity, enzyme regulator activity, nucleic acid binding transcription factor activity, molecular transducer activity and protein biding transcription factor activity.

The assembled unigenes were further aligned to the COG database to phylogenetically classify the proteins encoded by the unigenes. Totally, 24 415 unigenes were classified into 25 functional categories (Fig. S4). The predominant category was the general function prediction only (18.41%), followed by translation, ribosomal structure and biogenesis (14.4%), transcription (9.02%), replication, recombination and repair (8.54%), cell cycle control, cell division, chromosome partitioning (4.71%), posttranslational modification, protein turnover, chaperones (6.03%), signal transduction mechanisms (5.80%), and cell wall/membrane/envelope biogenesis (5.35%).

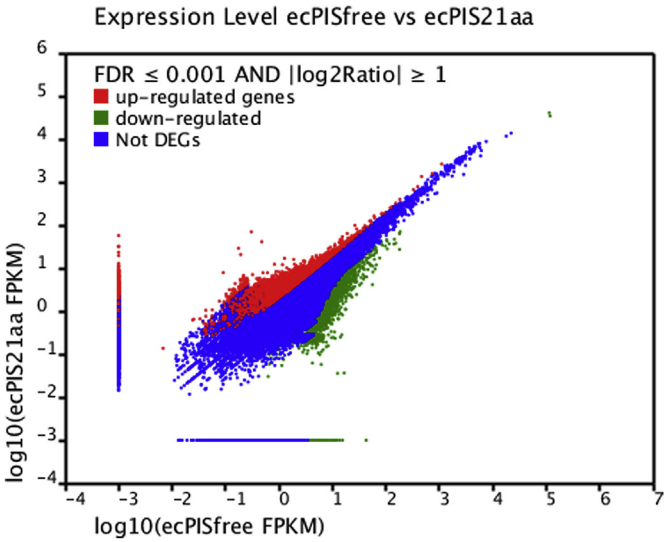


Fig. 3. Differential gene expression profile of ecPis-1S relative to ecPis-free. The horizontal axis is relative expression of ecPis-free group and vertical axis is relative expression of ecPis-1S group. All Unigenes were separated into three levels. Dots in red indicated upregulation (when log2(ecPIS21aa_FPKM/ecPISfree_FPKM) > 0), green indicates down-regulation (when log2(ecPIS21aa_FPKM/ecPISfree_FPKM) < 0), and blue indicates similar expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2.4. Differentially expressed genes(DEGs)analysis

To quickly screen the significant DEGs from the transcriptome, the ecPis-1S group was compared with the control group. A fold-change abs value of more than 2 and FDR p-value of less than 0.001 were considered significant. The results yielded 2994 up-regulated genes and 2679 down-regulated genes (Fig. 3).

3.2.5. Analysis of key genes related to immune responses

Among differential expression genes stimulated by ecPis-1S, 330 genes were involved in immune related pathways (Fig. 4), including complement and coagulation cascades, intestinal immune network for IgA production, antigen processing and presentation, T cell receptor signaling pathway, Toll-like receptor signaling pathway, leukocyte transendothelial migration, Fc gamma R-mediated phagocytosis and chemokine signaling pathway. Thirty four genes were involved in T cell receptor signaling pathway, 31 genes in natural killer cell mediated cytotoxicity and 23 genes in leukocyte transendothelial migration, respectively.

3.3. Proliferation of lymphocytes stimulated by ecPis-1S

Since transcriptome results showed that cell cycle related genes were up-regulated, it was postulated that ecPis-1S might play an important role in proliferating cells. Accordingly, lymphocytes were treated with different concentrations of ecPis-1S *in vitro*. Results showed that ecPis-1S could significantly promote lymphocytes proliferation with no dose-dependent effect (Table 2–A). In addition, there were no significant differences in proliferation between groups treated with ConA alone and in combination with ecPis-1S (Table 2–B). Similar results were observed in LPS co-treated group (Table 2–C).

3.4. qRT-PCR validation

Based on the result of transcriptome data and proliferation induced by ecPis-1S, 13 genes related to immune response and cell division cycle were selected for qRT-PCR performance (Fig. 5). All genes were significantly up-regulated only at 4.0 μmol/L ecPis-1S, except cdc6

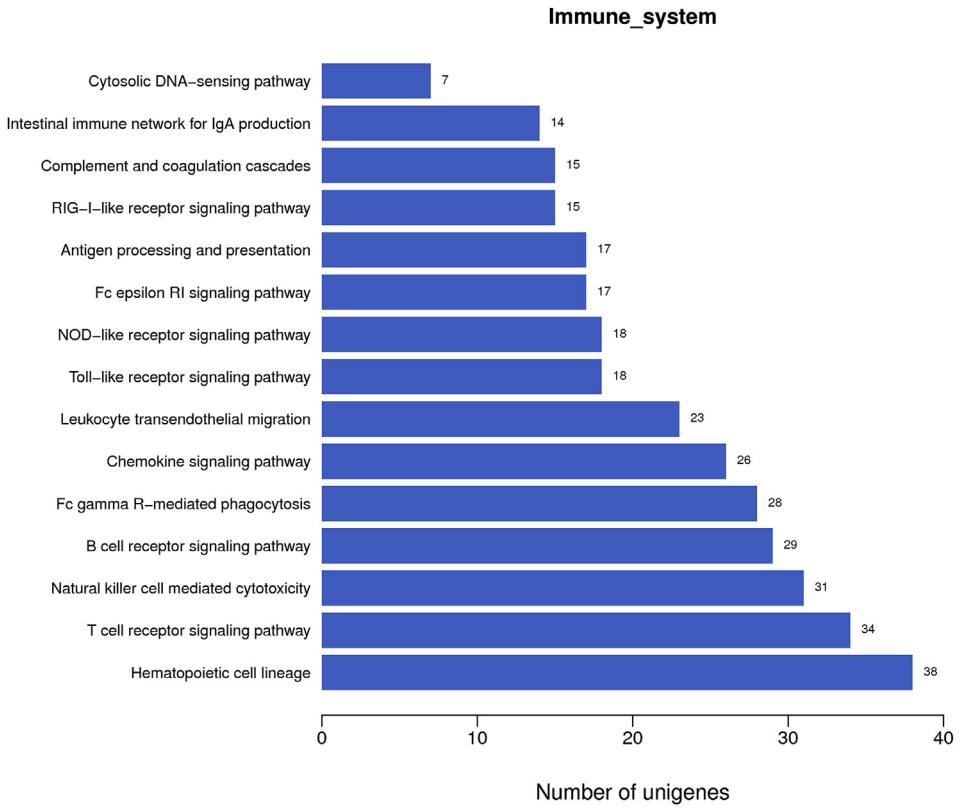


Fig. 4. Distribution of differential genes involved in immune related pathway stimulated by ecPis-1S comparing to ecPis-free.

Table 2
Effect of ecPis-1S on splenic lymphocytes proliferation (A_{490}).

Concentration of ecPis-1S (μmol/L)	A	B	C
0	0.222 ± 0.004^{a}	0.247 ± 0.015^{ab}	0.258 ± 0.020^{ab}
0.25	0.264 ± 0.011^{b}	0.244 ± 0.010^{a}	0.260 ± 0.019^{a}
0.5	0.252 ± 0.004^{b}	0.250 ± 0.012^{ab}	0.250 ± 0.019^{ab}
1	0.236 ± 0.006^{a}	0.257 ± 0.008^{ab}	0.233 ± 0.028^{ab}
2	0.263 ± 0.011^{b}	0.269 ± 0.005^{b}	0.256 ± 0.027^{ab}
4	0.255 ± 0.013^{b}	0.260 ± 0.018^{ab}	0.218 ± 0.025^{b}

A. Single stimulation of ecPis-1S; B. Co-stimulation of ecPis-1S with ConA; C. Co-stimulation of ecPis-1S with LPS. The results were expressed as mean ± standard error (SE) from four determinations (n = 4).a-bBars in the figure without the same superscripts differ significantly ($P < 0.05$).

which was up-regulated at both 0.5 and 4.0 μmol/L post 24 h stimulation. Expressions of *tcr* and *mhc II* were significantly decreased in 12 h, while increased in 24 h. *Il-1b*, *mhc I*, *tnfa*, nuclear factor of activated T cell (*nfat*), and *tgfb* were significantly up-regulated in 24 h. *Il-8* was down-regulated in 12 h while with no differential change in 24 h. *Cyca*, *cdk2* and *cdk4* were up-regulated with 4.0 μmol/L ecPis-1S in both 12 h and 24 h.

4. Discussion

AMPs are widely expressed at epithelial surfaces and first known as their ability to kill a broad range of pathogens. Most of them are cationic peptides and possess hydrophobic surfaces that can interact with negative charged bacterial film and insert into the lipid bilayer, forming transmembrane channel or leading membrane disruption [39]. Further study of antimicrobial mechanism also uncovered the immunological properties of AMPs, including modulating pro- and anti-inflammatory responses, enhancing chemo attraction, enhancing extracellular and intracellular bacterial killing, direct effect on adaptive immunity, regulation of apoptosis and even wound healing [40–43]. In human, defensin and LL-37 in modulating immune system are most documented that they have been used as immunostimulant and/or immunoadjuvant [44–46]. However, immunomodulatory mechanisms of them are complicated and not well elucidated. For example, LL-37 can mediate immune responses via activation of putative cell receptors, including Toll-like receptors (TLRs), receptor tyrosine kinases (RTKs), G protein-coupled receptors (GPCRs), and ligand-gated ion channel (LGIC) [47]. Consequently, NF-κB, IRF and NFAT would be activated and regulate the activation of immune cells and cytokine release, which brings up chemokine-like activity and accelerates the phagocytosis and proliferation of immune cells [46–49]. Moreover, LL-37 can regulate MAPK p38 signaling and affect cytokines releasing by interacting with intracellular protein GAPDH [50].

So far, a certain number of studies have been reported to indicate that teleost AMPs might also have immunomodulatory activity in addition to anti-microbial activity, which was similar to mammalian AMPs. Homologous transcripts of human cathelicidin, defensin and hepcidin were identified in teleost [51], which showed immunomodulatory activity in fish. Expressions of immune-related genes such as *il-8*, *il-1b* and *tnfa* were regulated when treated with these peptides [52–56]. AMPs specialized in teleost such as epinecidin-1, pleurocidin and piscidin could also up-regulate genes involved in immune response [34,57,58]. Epinecidin-1 as an AMP was originally identified in orange-spotted grouper. It was predicted to have the amphipathic α-helical structure with positive charge [28]. The putative mature peptide of Epinecidin-1 (Epi-1) showed immunomodulatory effect against pathogens in teleost and in other models. In studies against the infection of *V. vulnificus*, injection and oral administration of synthetic Epi-1 regulated expression of immune related genes and enhanced survival rate of infected zebrafish [31,59]. Epi-1 also altered the immune related gene expressions of grouper (*E. coioides*) and medaka (*Oryzias latipes*) in nervous necrosis virus (NNV) infection [58,60,61]. However, no study has clarified the immunomodulatory mechanism of

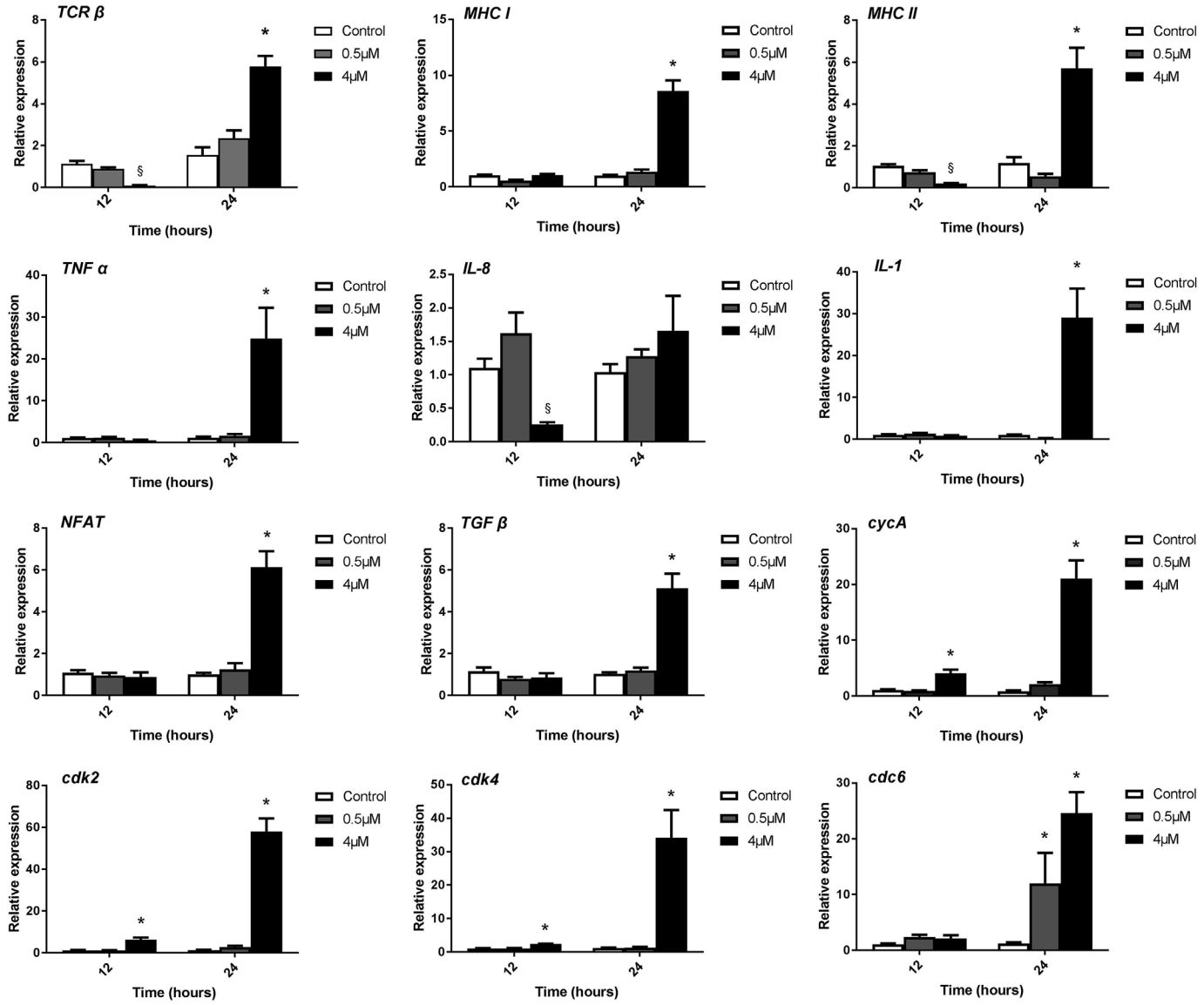


Fig. 5. Quantitative RT-PCR analysis of the expression of immune and cell cycle-related genes 12 h and 24 h after lymphocytes treated with ecPis-1S in Grouper. The results were expressed as mean ± standard error (SE) from four determinations (n = 4). Significant difference ($P < 0.05$) between the ecPis-1S group and the control group was indicated with * (significant increase) or § (significant decrease).

piscidins. Besides, the previous studies on the immunomodulatory effects of piscidins were all done by first stimulating the animal and then taking cells out of the body to analyze, and none of them was done on primary cultured cells.

A truncated peptide of ecPis-1 with 20 aa (EGFIFHIIKGLFHAGKMIHG), which showed 100% identity and 85.7% coverage with Epi-1, was identified in grouper. Piscidins have been found to express highly in skin mucus, spleen, and blood at transcriptional level in fish [51], which were the tissues that we tried to purify the truncated peptides of piscidin-1 from. However, no relevant peptide was obtained from either spleen or blood after repeated attempts. The truncated peptide was identified from mucus after infection of *V. parahaemolyticus*. There might be three reasons that the peptide was identified from mucus rather than spleen and blood. Firstly, mucosal immune system constitutes the first line of defense in fish. After infection, ecPis-1 might be largely secreted and then migrate to mucus, activated by cleavage to exert its multi-functions. Secondly, the degradation rate of ecPis-1S might be slower in mucus than in spleen or blood. Thirdly, gene expression at the level of transcript does not always correspond with that at the level of translation.

The peptide identified in this study was a truncated form, which implied that prepro-peptide of ecPis-1 underwent cleavage processing to remove signal peptide and prodomain of pro-peptide. The processing enzymes of the mature peptide and their cleavage sites at the precursor AMPs in fish have not yet been elucidated even though the cleavage site of cod cathelcidin was proposed to be nearby a R-X-R-R motif and that of hepcidins at motif RX(K/R)R site with the same mechanism of mammalian cathelcidin [62] or hepcidin [63]. No report has addressed the processing of the pro-piscidins by local protease. Among fish AMPs, whether the processing of mature peptide of piscidin family exists or not is still inconclusive. In hybrid striped bass (*Morone saxatilis* × *M. chrysops*), lengths of piscidin 1, 2 and 3 were 21–23 aa [64], while piscidin 4 was 44 aa of length [65]. Compared to the sequence of ecPis-1S, the present identified peptide contained an additional glutamic acid (E) in the N-terminal while lacked leucine (L) and valine (V) in the C-terminal. Signal peptide prediction of prepro-peptide of ecPis-1 demonstrated that signal sequence is MRCIALFLVLSLVVLMAEPGEG, which indicated that E and G may not be included in mature peptide [10]. In our previous study (data not shown), it has been verified that the antibacterial activity of Epi-1 remained unchanged whether with G

or not. However, an additional E in the C-terminal might theoretically inhibit its antibacterial activity since glutamic acid is a negative charged amino acid. This result suggested that the sequence of ecPis-1S is very close to the sequence of mature peptide of ecPis-1.

Synthetic Epi-1 was reported to modulate the expression of several pro-inflammatory and immune-relate genes including *il-1b*, *il-10*, *il-22*, *il-26*, *tnfa*, *ifn*γ, *nfkb*, *myd88*, *tlr4a*, *tlr1*, and *tlr3* in fish [27,29,31,34,35]. But no research on systemic regulation of the immune-relate genes by Epi-1 has been reported. In the present study, gene expression profiles of splenic lymphocytes of orange-spotted grouper stimulated by piscidin *in vitro* based on RNA-Seq were obtained. Over 5000 genes were modulated and 330 genes were found involved in immune related pathways, which revealed that ecPis-1S might participate in modulation of innate and adaptive immune systems. Additionally, it was noticed that a certain number of assembled genes were aligned to the cell cycle control and cell division. To investigate whether ecPis-1S could promote the proliferation of lymphocytes or not, a MTT assay was performed. The results showed that the proliferation of lymphocytes was significantly promoted by ecPis-1S without a significant dose-dependent effect from 0.25 μmol/L to 4.0 μmol/L. Furthermore, expressions of genes involved in cell division cycle, such as *cyca*, *cdk2*, *cdk4*, *cdc6*, and *tgfb*, which regulate cell-cycle progression directly or indirectly [66], were all increased under ecPis-1S stimulation. These results demonstrated that ecPis-1S could promote lymphocyte proliferation by activating genes in cell division cycle. When treated by combination of ecPis-1S and LPS or ConA, no significant proliferation was observed in lymphocytes. The reason might be that Epi-1 could disturb LPS binding to cell surface and inhibit the inflammatory [67].

IL-1 and TNF-α are two important pro-inflammatory factors that could induce inflammatory response by regulating the production of cytokines [12]. In the present study, *il-1b* and *tnfa* were up-regulated at 4.0 μmol/L ecPis-1S stimulation post 24 h, demonstrating that piscidin promoted inflammation in grouper. Down-regulation of *il*-8 might have an impact on chemokine secretion since it has been reported that AMPs have chemotactic activity [7,18,68], and this needs to be further confirmed. Adaptive immunity relevant genes selected in this study were also up-regulated, including *tcr*, *mhc I*, *mhc II* and *nfat* in T cell signal pathway, which verified the result of transcriptome analysis that T cell activation, T cell receptor signaling and phagosome pathways could be regulated by piscidin. Although AMPs were first identified as their antimicrobial function, their primary role was now hypothesized as signaling molecular involved in immunomodulation [3]. In human, LL-37 and defensin have been demonstrated to have immunomodulatory effect on immune cells, inducing cytokines and chemokines production [4–8]. In fish, AMPs also display immunomodulatory function. In gilthead seabream (*Sparus aurata)*, β-defensin can chemoattract head kidney leukocytes [69]; In Atlantic salmon (*Salmo salar*), cathelicidins can induce the expression of IL-8 in peripheral blood leukocytes [52]; Fish hepcidins and piscidins are able to modulate the expressions of immune-related genes (which has been discussed above). Recently, epinecidin-1 was reported to modulate MyD88 at protein level through the proteasome degradation pathway [33], which indicated that piscidin was involved in immune regulation. Our result also demonstrated that direct treatment with piscidin would regulate expression of immune related genes of lymphocytes.

In conclusion, the present study purified and identified a truncated peptide of ecPis-1 from *E. coioides*, whose sequence is very close to that of Epi-1 (ecPis-1S), suggesting that prepro-peptide of grouper piscidins undergo cleavage processing to remove signal peptide and prodomain of pro-peptide. To the best of our knowledge, it is the first time that primary cultured cells of teleost fish were used to study for providing insights on the immunomodulatory mechanisms of fish AMPs. By RNA-seq, a total of 64 508 transcripts were assembled and 5673 DEGs were captured in grouper splenic lymphocytes in response to ecPis-1S *in vitro*. A certain number of DEGs relevant to innate immunity were identified, including genes involved in natural killer cell mediated cytotoxicity, leukocyte transendothelial migration, and T cell receptor signaling pathway. Expressions of immune-related genes involved in pro-inflammatory signal pathway and T cell signal pathway were significant up-regulated checked by qRT-PCR. Moreover, the evidences that ecPis-1S get involved in modulation of cell division cycle and stimulate the proliferation of splenic lymphocytes *in vitro* was also reported for the first time.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.12.045.

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