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Novel insights into the immune regulatory effects of ferritins from blunt snout bream, *Megalobrama amblycephala*

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ABSTRACT

Ferritins play vital roles in maintenance of iron homeostasis as iron storage proteins. Recently, the immune function of ferritins have attracted increasing attention, especially their roles in defense against pathogenic infections. However, the immune regulatory mechanism of fish ferritins are not well known. In the present study, comparative digital gene expression (DGE) profiling was performed to explore the regulatory effects of the *Megalobrama amblycephala* ferritins (*MamFers*) using *MamFers* overexpressed and control L8824 cells (*Ctenopharyngodon idella* hepatic cell line). Clean reads were aligned to the *C. idella* genome and differential expression analysis was conducted with representative differentially expressed genes pointed out. On that basis, further studies were performed to verify two pivotal regulated pathways in L8824 and EPC (Epithelioma Papulosum Cyprini cell line) cells, respectively. The results showed that *NLRC5* (NOD-like Receptor Family CARD Domain Containing 5) mediated the regulation of *MamFers* on expression of *MHC I* (Major Histocompatibility Complex Class I) and its chaperone *β2M* (Beta-2-Microglobulin) in L8824 cells. Then, *β2M* further mediated the regulation of *MamFers* on hepcidin expression, indicating that *MamFers* regulated the expression of hepcidin via *NLRC5/MHC I/β2M* axis. In addition, *MamFers* regulated the adhesion of *Aeromonas hydrophila* to EPC cells by regulating the expression of two extracellular matrix proteins *Intgβ1* (integrin β1) and *FN* (fibronectin). In a word, the present study provided novel insights into the immune regulatory functions of fish ferritins.

1. Introduction

Iron is an essential microelement for living organisms, while an excess of iron is toxic, thus maintenance of iron homeostasis is vital. Ferritins play pivotal roles as iron storage proteins by storing redundant iron in a nontoxic and biologically available form in this regard. Mammalian ferritins consist of heavy (H) and light (L) subunits, which are responsible for different roles during iron storage. The H subunit is considered to possess ferroxidase activity, whereas L subunit functions in iron nucleation [1]. The ferritin middle chain (M) has been identified in several lower vertebrates, such as fish and amphibians [2], which is deemed to own the characteristic features of both H and L subunits.

Recently, the cross-talk between iron metabolism and immune response has attracted increasing attention, especially the immunomodulatory effects of iron metabolism related proteins. Thereinto, ferritins have been proved to participate in host innate immunity via its iron withholding ability, sequestering excess iron within host cells (e.g. macrophage and hepatocyte) to create a low iron environment and inhibit the proliferation of bacteria [3,4]. Additionally, the regulation of cytokines (e.g. tumor necrosis factor alpha and interleukin 1 alpha) on the expression of ferritins has been reported previously [5], while only few studies focus on exploring the regulatory effects of ferritins on immune response and iron metabolism.

A previous study has defined the regulation of ferritin on the expression of cytokines in the rat hepatic stellate cells through the NF-κB (Nuclear factor kappa B) signaling pathway, providing strong evidence that ferritin acts as an iron-independent pro-inflammatory mediator [6]. In regard to the regulatory role in iron metabolism, exogenous

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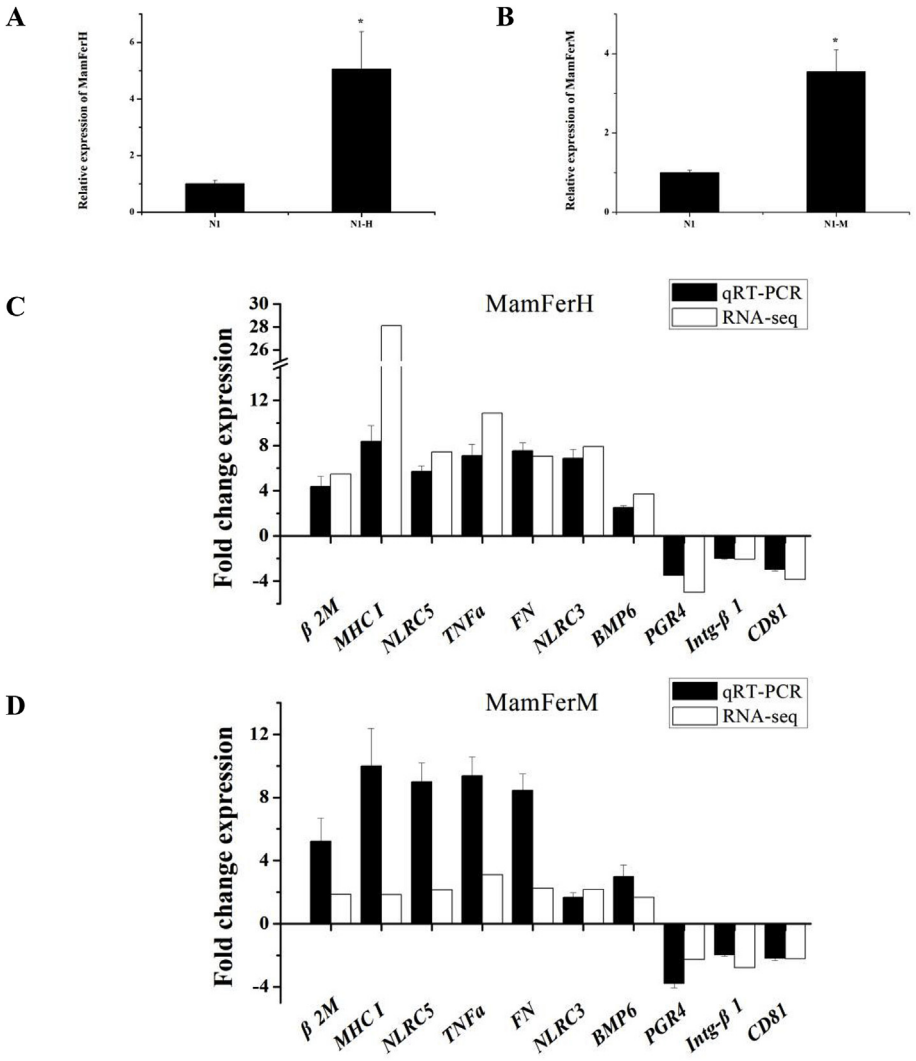

Fig. 1. Expression patterns of *MamFers* and differentially expressed genes post pEGFP-N1-*MamFers* plasmids transfection in L8824 cells. Relative expression of *MamFerH* (**A**) and *MamFerM* (**B**) genes post pEGFP-N1-*MamFers* plasmids transfection (pEGFP-N1 as control) in L8824 cells have been detected using qRT-PCR assay. The expression patterns of differentially expressed genes was comparative analyzed via DGE and qRT-PCR assays post *MamFerH* (**C**) and *MamFerM* (**D**) overexpression. Values of qRT-PCR were described as mean ± SE (n = 3) and the asterisks indicate statistically significant differences (*, $P < 0.05$), Fold change of DGE represent the expression level ratios of treatment group to Mock (N1) group. Full name of gene abbreviations, *β2M*: Beta-2 microglobulin; *MHC I*: major histocompatibility complex, class I molecules; *NLRC5/3*: NOD-like receptor family CARD domain containing 5/3; *TNFα*: tumor necrosis factor alpha; *BMP6*: Bone morphogenetic protein 6; *FN*: fibeonectin; *PRG4*: Proteoglycan 4;*Intgβ1*: integein beta 1.

ferritin has been discovered to up-regulate hepatic expression of bone morphogenetic protein 6 and hepcidin in mice [7]. In addition, the ferritin H-deleted mice showed induced liver hepcidin mRNA levels and reduced duodenal expression of *DMT1* (Divalent Metal Transporter 1) mRNA, indicating that intestinal ferritin H subunit is required for the accurate control of iron absorption [8]. Similarly, knockdown of β2M (Beta-2-Microglobulin), the chaperone of MHC I proteins (Major Histocompatibility Complex Class I) [9], would perturb the subcellular distribution of hepcidin [10]. Additionally, *in vitro* and *in vivo* studies have revealed that *MHC I* and *β2M* genes could be transcriptionally regulated by *NLRC5* (NOD-like Receptor Family CARD Domain Containing 5) [11,12], whether *β2M* and its upstream pathways participated in the regulation of ferritins to hepcidin is not clear. Thus, the regulatory effect of fish ferritins warrant further investigation.

Megalobrama amblycephala, belonging to *Megalobrama*, Cyprinidae, closely related with *Danio rerio*, *Ctenopharyngodon idella* and *Cyprinus carpio*, is one of the major species in Chinese freshwater polyculture system, which is under the threat of bacterial septicemia caused by *Aeromonas hydrophila* infection [13]. Our previous study has characterized *M. amblycephala* ferritin H and M subunits (*MamFerH* and *MamFerM*), their expression was significantly up-regulated upon *A. hydrophila* infection, and the recombinant proteins possess effective iron depriving and bacteriostatic activity [14]. In order to better understand the transcriptional regulatory roles of the two *M. amblycephala* ferritin genes (*MamFers*), digital gene expression (DGE) profiling of the *MamFers* overexpressed L8824 cells (*C. idella* hepatic cell line) was performed in the present study. The differentially expressed genes were identified, and representative regulatory pathways were verified. These results would assist in understanding the immune functions of fish ferritins, especially their roles in defense against bacterial infection.

2. Materials and methods

2.1. Plasmids construction and siRNAs preparation

Primers containing *Xho* I and *BamH* I restriction enzyme splicing site were used to amplify the coding regions of *MamFers* genes (Supplemental Table 1). The PCR products were cloned into vector pEGFP-N1 (Clontech, USA) and sequenced to confirm the specificity. All siRNAs (Supplemental Table 2) used in the present study were synthesized in GenaPharma Corporation (Shanghai, China), including a negative scrambled siRNA selected as control.

2.2. Cell culture and transfection assay

The identities of ferritin genes between *M. amblycephala* and *C. idella*, *C. carpio* or *Pimephales promelas* were higher than 90%, and sequences alignment have been provided in the Supplemental Fig. 1 with the regions targeted by siRNAs or qRT-PCR primers labeled. Thus, L8824 (*C. idella* hepatic cell line) and EPC (Epithelioma Papulosum Cyprini cell line) stored in the Cell Collection Centre for Freshwater Organisms of Huazhong Agricultural University were used in the

Table 1
Representative differentially expressed genes.

Gene ID	Gene name	KEGG pathway sub-categories	N1	N1−H		N1-M	
			FPKM	FPKM	FC	FPKM	FC
Adhesion/junctional modification							
CI01000016_09317824_09332379.EXON	Extracellular matrix protein 1	(1) ECM-receptor interaction	187.75	38.86	0.21	111.62	0.59
CI01000001_06071846_06084571.EXON	Biglycan		36.45	18.41	0.50	19.14	0.53
CI01000004_05936410_05942402.EXON	Proteoglycan 4		967.57	194.14	0.20	425.98	0.44
CI01000037_01538390_01570296.EXON	Fibronectin	(2) ECM-receptor interaction/Focal adhesion	118.41	834.22	7.05	266.79	2.25
CI01000060_00281612_00291567.EXON	Thrombospondin-1		35.24	6.73	0.19	70.17	1.99
CI01000080_00681850_00695789.EXON	Thrombospondin-4b		252.82	12.70	0.05	201.81	0.80
CI01000032_00846052_00850360.EXON	Collagen alpha-1(X) chain		809.70	30.79	0.04	300.94	0.37
CI01000053_02071290_02083892.EXON	Integrin alpha-E	(3) ECM-receptor interaction/Focal adhesion/	18.75	2.68	0.14	8.02	0.43
CI01000036_01428324_01454913.EXON	Integrin alpha-V	Regulation of actin cytoskeleton	1396.43	688.01	0.49	1716.18	1.23
CI01000304_06123328_06162242.EXON	Integrin alpha-1		85.96	14.97	0.17	56.22	0.65
CI01000070_04679660_04692608.EXON	Integrin beta-1		258.94	127.17	0.49	93.58	0.36
CI01000053_01124489_01129327.EXON	Cell adhesion molecule 1	(4) Cell adhesion molecules (CAMs)	144.11	39.09	0.27	97.00	0.67
CI01000350_02241412_02243039.EXON	Connective tissue growth factor		216.84	40.93	0.19	220.90	1.02
Immune system process/defense response							
CI01046163_00002590_00013429.EXON	Major histocompatibility complex class I-related gene	(5) Antigen processing and presentation	4.04	18.80	4.65	5.94	1.47
CI01000319_05704764_05712826.EXON	Major histocompatibility complex class I-related gene		1.19	33.39	28.11	2.18	1.83
CI01000020_04807118_04808453.EXON	Beta-2-microglobulin		756.40	4143.89	5.48	1417.31	1.87
CI01000013_05483212_05505616.EXON	NLRC5	(6) NOD-like receptor signaling pathway	1.20	8.89	7.44	2.57	2.15
CI01000099_00003573_00007424.EXON	NLRC3		5.93	46.83	7.90	12.89	2.17
CI01000016_05381573_05383796.EXON	Interleukin-11	(7) Cytokine-cytokine receptor interaction	6.16	18.83	3.06	5.39	0.87
CI01000350_00650752_00674046.EXON	Interleukin-10 receptor subunit beta		24.46	98.01	4.01	32.64	1.33
CI01000095_02409472_02411189.EXON	Tumor necrosis factor		2.27	24.63	10.86	7.05	3.10
CI01000009_09031859_09038439.EXON	Tumor necrosis factor-inducible gene 6		8.41	10.45	1.24	19.29	2.29
Gene ID	Gene name		N1	N1−H		N1-M	
			FPKM	FPKM	FC	FPKM	FC
CI01000071_04430037_04431564.EXON	C−C motif chemokine 7	(7) Cytokine-cytokine receptor interaction	4.26	427.32	100.31	19.60	4.60
CI01000016_05229439_05231984.EXON	C-type lectin domain family 1	(8) C-type lectin receptor signaling pathway	53.04	7.93	0.15	22.51	0.42
CI01000034_04019323_04045212.EXON	C-type mannose receptor 2	(9) Phagosome	188.44	76.83	0.41	184.30	0.98
CI01000010_03204475_03207149.EXON	Macrophage-expressed gene 1		22.79	144.06	6.32	40.38	1.77
CI01000344_01208746_01214936.EXON	Lysosome-associated membrane glycoprotein 3	(10) Lysosome	159.33	1027.73	6.45	332.93	2.09
CI01000110_03027125_03028515.EXON	Lysozyme		153.60	540.44	3.52	220.62	1.44
CI01000001_13329841_13334948.EXON	CD63		412.78	1674.71	4.06	412.05	1.00
CI01072362_00000096_00000637.EXON	CD9 antigen	(11) Hematopoietic cell lineage	53.29	623.54	11.70	187.71	3.52
CI01000180_01423287_01433910.EXON	CD81	(12) B cell receptor signaling pathway	51.13	13.38	0.26	22.84	0.45
CI01000047_03501347_03512538.EXON	Interferon regulatory factor 3	(13) RIG-I-like receptor signaling pathway/	57.57	199.11	3.46	113.23	1.97
CI01000330_01209985_01218019.EXON	Interferon regulatory factor 7	Toll-like receptor signaling pathway	36.66	302.78	8.26	79.02	2.16
Iron metabolism							
CI01000327_01644946_01696545.EXON	BMP6	(14) Cytokine-cytokine receptor interaction	2.47	9.13	3.70	4.10	1.66
CI01000023_00364216_00367202.EXON	Heme oxygenase	(15) Iron metabolism signaling pathway	40.09	7.57	0.19	51.32	1.28
CI01000027_07022724_07029894.EXON	Metalloreductase STEAP4		57.79	26.37	0.46	78.96	1.37
CI01000091_01265220_01283983.EXON	Hephaestin-like protein 1		7.17	27.96	3.90	12.48	1.74
CI01000009_11803818_11808988.EXON	Solute carrier family 40 member 1		170.94	414.70	2.43	310.06	1.81
Transcription factor							
CI01000211_00090092_00102914.EXON	JAK2	(16) JAK-STAT signaling pathway	70.60	141.71	0.53	85.00	1.20
CI01000009_11835682_11873220.EXON	STAT4		76.28	595.27	7.80	214.79	2.82
CI01000071_00720193_00720785.EXON	Polyubiquitin	(17) Proteasome	19.68	483.56	24.57	64.15	3.26
CI01000108_00875430_00878425.EXON	TRAF family member-associated NF-kappa-B activator	(18) NF-kappa B signaling pathway	14.04	40.57	2.89	16.59	1.18
CI01000000_02516813_02517287.EXON	Nuclear factor NF-kappa-B p105 subunit		11.72	31.06	2.65	18.48	1.58
CI01000012_13593620_13605408.EXON	Nuclear factor NF-kappa-B p100 subunit		44.26	111.99	2.53	55.38	1.25

present study for functional study of *M. amblycephala* ferritin genes. These cells were cultured in Medium 199 containing 10% fetal bovine serum (Gibco, CA, USA) with penicillin and streptomycin (100 U/mL). Plasmids or siRNAs transfection assay were conducted in L8824 or EPC cells using Lipofectamine2000 transfection reagent according to the manufacturer's protocol. Cells were collected for RNA extraction or prepared for bacterial adhesion assay at 48 h post transfection.

2.3. RNA isolation and illumina sequencing

Total RNA of L8824 or EPC cells was extracted using TRIzon reagent (CoWin Biosciences, Beijing, China) according to the manufacturer's instructions. The quality and concentration of total RNA were determined using NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) and Agilent Bioanalyser 2100 (Agilent Technologies, Palo Alto, CA, USA), respectively. The first strand cDNA was synthesized from 1 μg total RNA using the PrimeScript® RT reagent Kit With gDNA Eraser (TaKaRa) following the manufacturer's protocol and stored at −20 °C for qRT-PCR assay. In addition, sequencing libraries were constructed using total RNA of L8824 cells transfected with pEGFP-N1-MamFers or pEGFP-N1 (control) plasmids, and Illumina sequencing was carried out by Novogene Company (Beijing, China) on a Hiseq4000

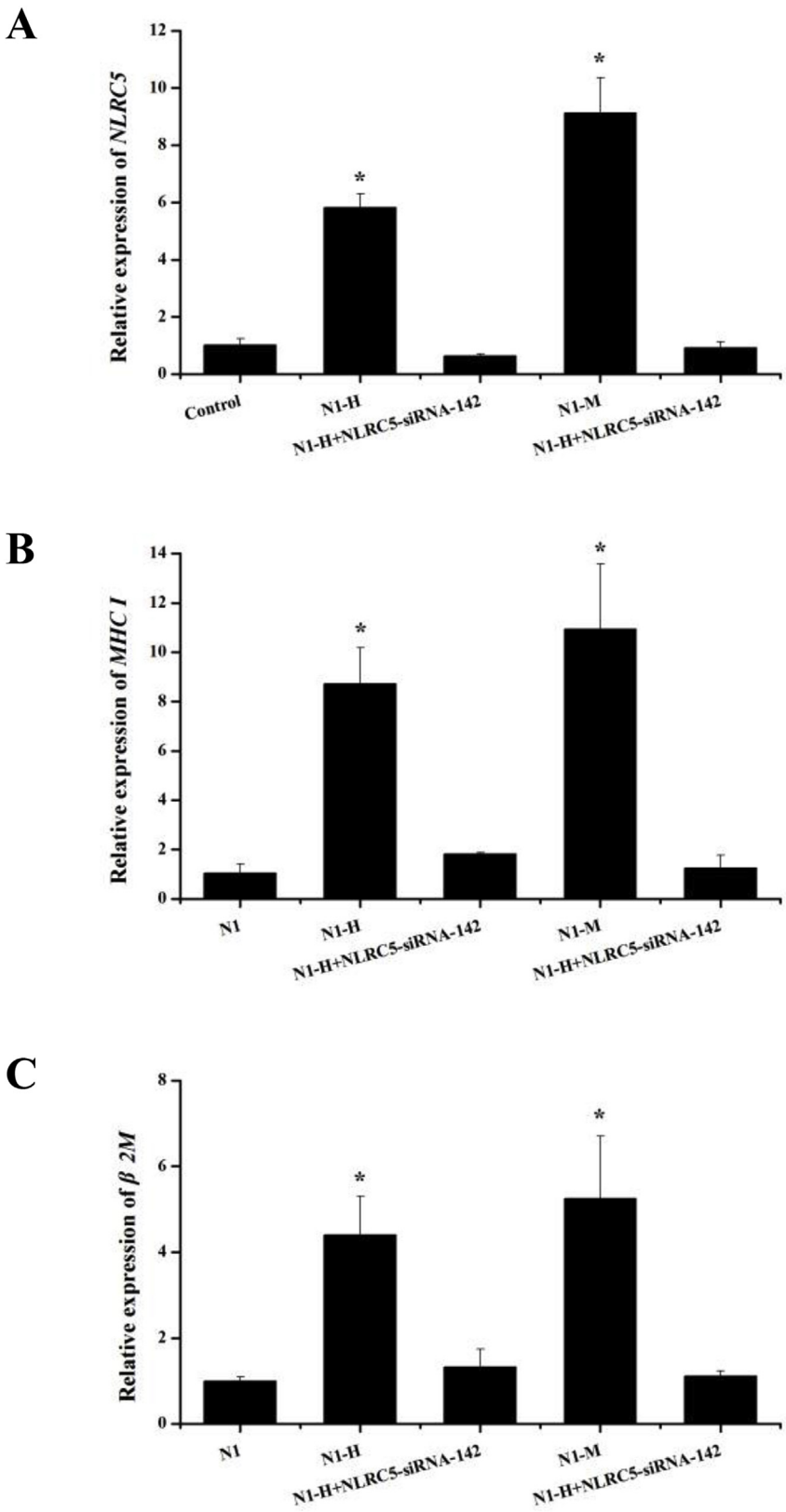

Fig. 2. Mediation of *NLRC5* to the regulation of *MHC I* and *β2M* expression by *MamFers*. The interference effect of siRNA-*NLRC5* on the induced expression of *NLRC5* gene post *MamFers* overexpression in L8824 cells was detected using qRT-PCR assay (**A**). Mediation of *NLRC5* to the regulation of *MamFers* on the expression of *MHC I* (**B**) and *β2M* (**C**) genes was detected via co-transfection of siRNA-*NLRC5* and pEGFP-N1-*MamFers* plasmids. The values of qRT-PCR were described as mean ± SE (n = 3) and the asterisks indicate statistically significant differences (*, $P < 0.05$).

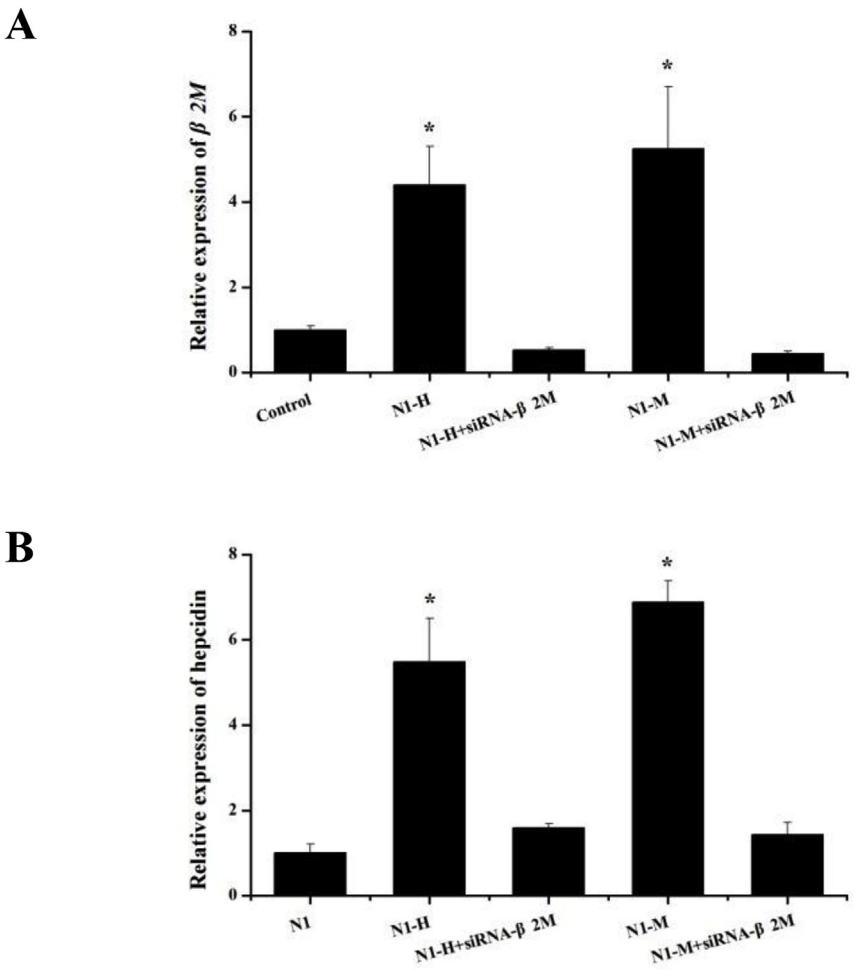

Fig. 3. Mediation of *β2M* to the regulation of hepcidin expression by *MamFers*. The interference effect of siRNA-*β2M* on the induced expression of *β2M* gene post *MamFers* overexpression in L8824 cells was detected using qRT-PCR assay (**A**), and co-transfection of siRNA-*β2M* and pEGFP-N1-*MamFers* plasmids could mediate the induced effect of *MamFers* on the expression of hepcidin gene (**B**). The values of qRT-PCR were described as mean ± SE (n = 3) and the asterisks indicate statistically significant differences (*, $P < 0.05$).

platform.

2.4. Sequencing quality control and reads mapping

Clean reads were obtained by removing reads with adaptors and poly-N, ambiguous nucleotides and low quality reads from raw data. Then, index of the reference genome (*C. idella* genome) was built using Bowtie 2 [15] and paired-end clean reads were aligned to the reference genome using TopHat v2.0.12 [16]. HTSeq was used to count the reads numbers mapped to each gene [17]. FPKM (expected number of fragments per kilobase of transcript sequence per millions base pairs sequenced) of each gene was calculated based on the length of the gene and reads count mapped to this gene.

2.5. Differential expression analysis

Differential expression analysis between pEGFP-N1-*MamFers* and pEGFP-N1 transfected L8824 groups was performed using the DEGSeq package 1.12.0 [18]. The resulting *P*-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Corrected *P*-value of 0.05 and log2 (Fold change) of 1 were set as the threshold for differential expression. GO (Gene Ontology) enrichment and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis of differentially expressed genes was implemented by the GOseq R 2.12 package [19] and KEGG database, respectively. The expression patterns of randomly selected differentially expressed genes were verified using qRT-PCR assay as described below. In addition, co-transfection of pEGFP-N1-*MamFers* plasmids and *siRNAs* of downstream genes were conducted in L8824 or EPC cells to assess the immune regulatory roles of *MamFers*. The L8824 cells was used to study the regulation of *MamFers* on the expression of hepcidin via *NLRC5/MHC I/β2M* axis, and the EPC cells was used to study the effect of *MamFers* on the adhesion of *A. hydrophila* and its related pathways.

2.6. Quantitative real-time PCR analysis

Expression patterns of mRNA were analyzed using quantitative real-time PCR (qRT-PCR) as previously reported [20]. Briefly, qRT-PCR was performed on a LightCycler® 480 II real-time PCR detection system (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) using LightCycler® 480 SYBR Green I Master Mix (Roche Diagnostics) according to the manufacturer's protocol. Relative expression levels of target genes were measured in terms of threshold cycle value (Ct) by the $2^{-\Delta\Delta Ct}$ method [21] using *18S rRNA* as internal reference [22–24]. All reactions were performed in triplicate and primers were listed in Supplemental Table 1. The expression levels of genes in the Mock (N1) library were set to 1, and the relative expression levels determined by gene expression profiling and qRT-PCR were presented as fold change.

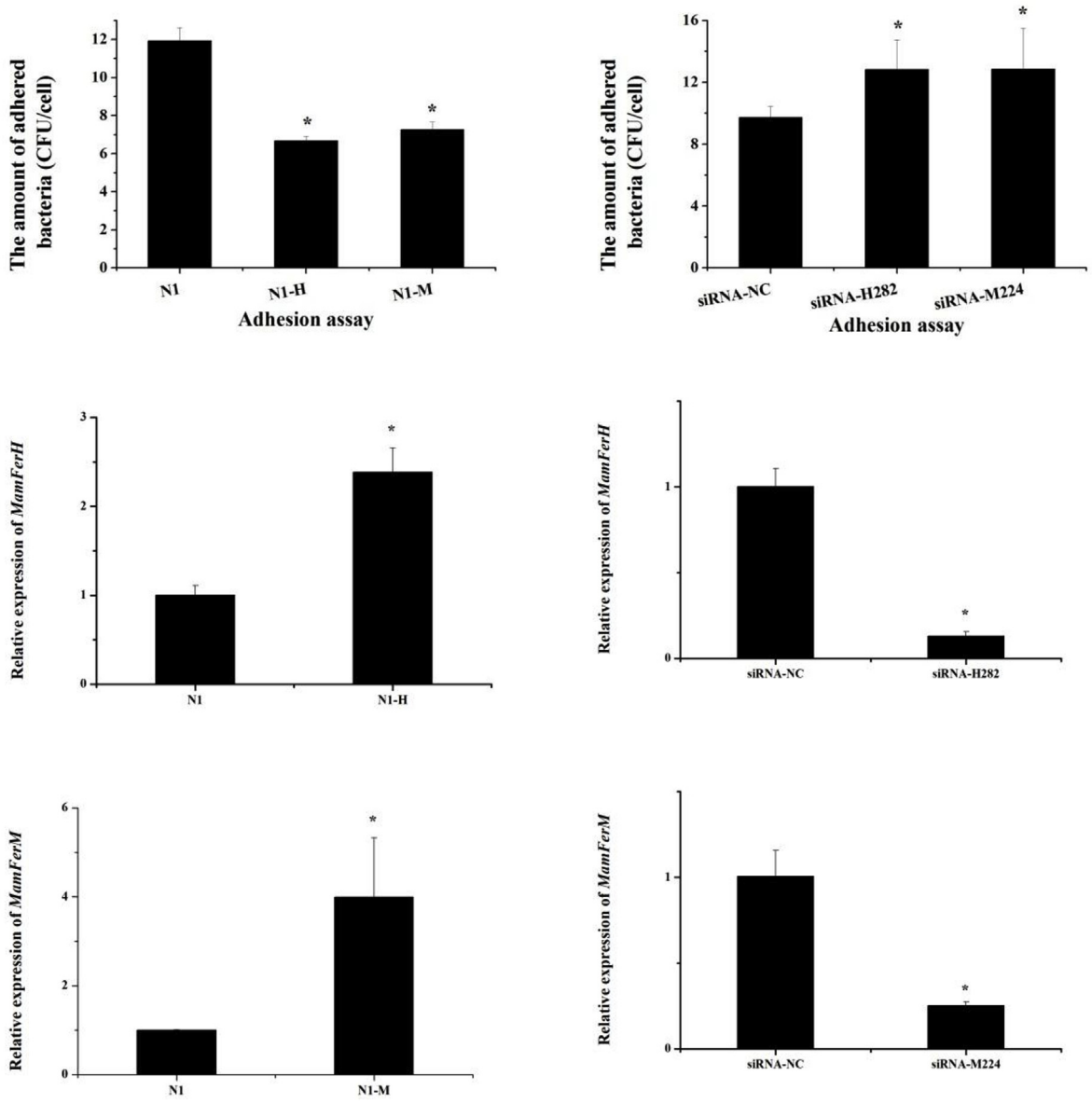

Fig. 4. Regulation of *MamFers* on the adhesion of *A. hydrophila* to EPC cells. Relative expression of *MamFers* genes and the adhesion of *A. hydrophila* post pEGFP-N1-*MamFers* plasmids (pEGFP-N1 as control) or siRNA-*MamFers* (siRNA-NC as control) transfection were detected in EPC cells. Values were described as Mean ± SE (n = 3), differences were determined by one-way analysis of variance (ANOVA). The asterisks indicated statistically significant differences ($P < 0.05$).

2.7. Bacterial adhesion assay

It was reasonable to use EPC, a kind of fish epithelial cell line, for the study of bacterial adhesion. EPC cells cultured in 24-well plates was transfected with pEGFP-N1-*MamFers* plasmids or siRNA-*MamFers* according to the method described in section 2.2, and the overexpression or knockdown effects were detected by qRT-PCR. Similarly, co-transfection of pEGFP-N1-*MamFers* plasmids and siRNA-fibronectin (*FN*) or addition of anti-integrin β1 (Intgβ1) antibody post siRNA-*MamFers* transfection was performed to assess the function of *FN* or *Intgβ1* in the regulation of *MamFers* to bacterial adhesion. The EPC cells were infected with *A. hydrophila* at the Multiplicity of Infection (MOI) of 10:1 for 45 min at 28 °C 48 h post transfection. Then, cells were gently washed with PBS for 3 times. After 15 min incubation with 1% Triton X-100, the cell lysates were plated to analyze the number of bacteria adhered to EPC cells. The experiment was performed in triplicate, and statistical difference was calculated using Student's t-test with significant difference level set at $P < 0.05$.

2.8. Statistical analysis

In the present study, data were presented as mean ± SE. The statistical significance was assessed by one-way analysis of variance (ANOVA) using SPSS 17.0, and $P < 0.05$ was considered as statistically significant difference.

3. Results

3.1. Sequencing quality and reads mapping

Upon transfection of pEGFP-N1-*MamFers* plasmids in L8824 cells, the expression of *MamFerH* and *MamFerM* genes up-regulated about 5.1-fold and 3.5-fold, respectively (Fig. 1A and 1B). Then, total RNA of pEGFP-N1-*MamFers* and pEGFP-N1 transfected L8824 cells was isolated for RNA-seq, and all raw sequence data were deposited in NCBI Sequence Read Archive (SRA) with accession number listed in Supplemental Table 3. The proportion of clean reads to raw reads was

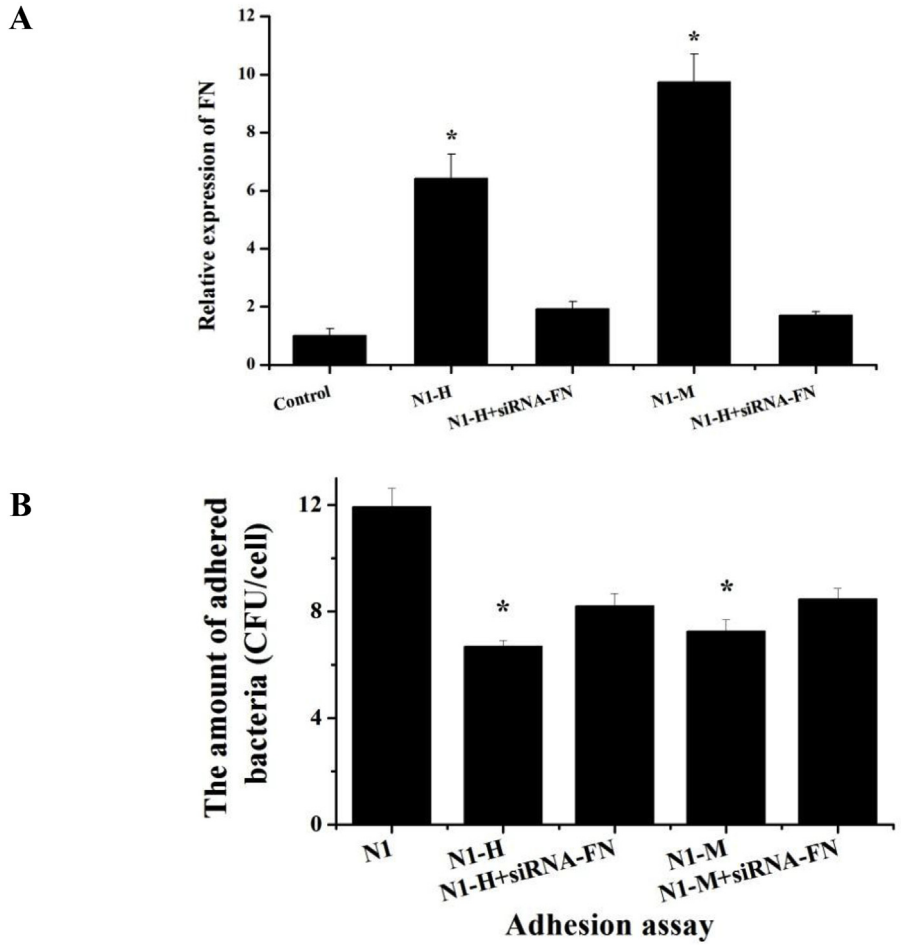

Fig. 5. Mediation of *FN* to the regulation of *A. hydrophila* adhesion by *MamFers*. The interference effect of siRNA-*FN* on the induced expression of *FN* gene post *MamFers* overexpression in EPC cells was detected using qRT-PCR assay (**A**), and co-transfection of siRNA-*FN* and pEGFP-N1-*MamFers* plasmids could relieve the inhibition effect of *MamFers* on the adhesion of *A. hydrophila* to EPC cells (**B**). Values were described as Mean ± SE (n = 3), differences were determined by one-way analysis of variance (ANOVA). The asterisks indicated statistically significant differences ($P < 0.05$).

higher than 96% with the Q20 values higher than 96% in all samples, and the GC contents ranged from 46.38% to 47.25% (Supplemental Table 4). Clean reads mapped to *C. idella* genome ranged from 87.26% to 88.29% and most clean reads mapped to unique sites (83.86%–85.53%) (Supplemental Table 5).

3.2. Identification and verification of differentially expressed genes

Differentially expressed genes was identified according to the criterion of fold change ≥2 (*P*-value ≤ 0.05). A total of 1398 (782 up-regulation and 616 down-regulation) and 55 (32 up-regulation and 23 down-regulation) unigenes showed significantly differential expression post *MamFerH* and *MamFerM* overexpression, respectively. Representative differentially expressed genes were grouped into 4 categories: junction/adhesion, immune system process/defense response, iron metabolism and transcriptional regulation (Table 1), based on GO enrichment analysis, KEGG pathway analysis and literature search. Nine genes, including *β2M*, *MHC I*, *NLRC5*, *NLRC3*, *TNF α*, *FN*, *BMP6* (Bone morphogenetic protein 6), *PRG4* (Proteoglycan 4) and *Intg-β1*, were randomly selected for qRT-PCR (Fig. 1C and 1D) to validate the differentially expressed genes identified by comparative DGE analysis. Further studies were performed to verify *MamFers* regulated signaling pathways that predicted via DGE sequencing.

3.3. NLRC5 mediated the regulation of MamFers on β2M

As shown in Fig. 2A, overexpression of *MamFers* up-regulated the expression of *NLRC5* gene, while the induced expression could be suppressed post co-transfection of siRNA-*NLRC5* and pEGFP-N1-*MamFers*. Furthermore, the regulatory effect of *NLRC5* on the *MHC I* gene was also verified in the present study. As shown in Fig. 2B and 2C, overexpression of *MamFers* could increase the expression of *MHC I* and its molecular chaperone *β2M*, while the induction effect could be suppressed by co-transfection of siRNA-*NLRC5* and pEGFP-N1-*MamFers*, indicating that *NLRC5* mediated the regulation of *MamFers* on *MHC I* and *β2M* genes. Specifically, *MamFers* could induce the expression of *NLRC5*, which could further promote the expression of *MHC I* and *β2M* axis.

3.4. β2M mediated the regulation of MamFers on hepcidin

As shown in Fig. 3A, the induced expression of *β2M* post *MamFers* overexpression could be inhibited by the co-transfection of siRNA-*β2M* and pEGFP-N1-*MamFers* plasmids. In addition, the up-regulated expression of hepcidin gene was detected post *MamFers* overexpression, while co-transfection of siRNA-*β2M* with pEGFP-N1-*MamFers* plasmids could down-regulate its enhanced expression, indicating *β2M* mediated the regulation of *MamFers* on hepcidin expression (Fig. 3B). Overall, *MamFers* could regulate the expression of hepcidin gene via *NLRC5/MHC I/β2M* axis.

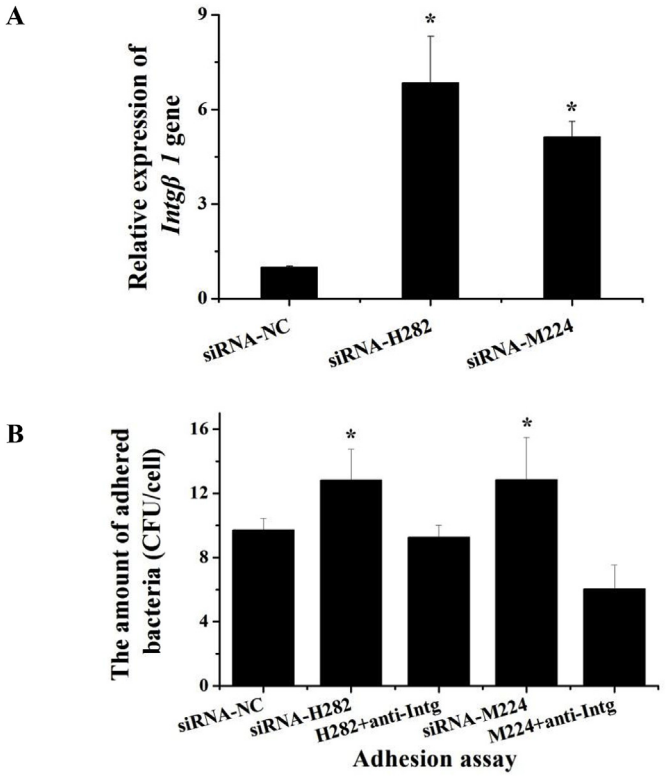

Fig. 6. Mediation of *Intgβ1* to the regulation of *A. hydrophila* adhesion by *MamFers*. Regulation of *MamFers* on the expression of *Intgβ1* gene was detected using qRT-PCR assay post knockdown of *MamFers* in EPC cells (**A**), and the addition of anti-Intgβ1 antibody post siRNA-*MamFers* transfection could suppress the up-regulated adhesion of *A. hydrophila* to EPC cells (**B**). Values were described as Mean ± SE (n = 3), differences were determined by one-way analysis of variance (ANOVA). The asterisks indicated statistically significant differences ($P < 0.05$).

3.5. Fibronectin mediated the regulation of MamFers on A. hydrophila adhesion

As shown in Fig. 4, *MamFers* overexpressed EPC cells inhibited the adhesion of *A. hydrophila*, while knockdown of *MamFers* could promote its adhesion, indicating the negative regulation of *MamFers* on *A. hydrophila* adhesion. DGE profiling of *MamFers* overexpressed L8824 cells identified numerous differentially expressed adhesion/junctional modification pathway related genes, of which *FN* and *Intgβ1* attracted great attentions. As shown in Fig. 5, overexpression of *MamFers* could induce the expression of *FN* in EPC cells, but suppress the adhesion of *A. hydrophila*, while co-transfection of siRNA-*FN* with pEGFP-N1-*MamFers* plasmids could relieve the inhibition effect on *A. hydrophila* adhesion, indicating that *FN* participated in the negative regulation of *MamFers* on bacterial adhesion.

3.6. Integrinβ1 mediated the regulation of MamFers on A. hydrophila adhesion

As shown in Fig. 6, knockdown of *MamFers* in EPC cells could increase the expression of *Intgβ1* gene as well as the adhesion of *A. hydrophila* (Fig. 4), while addition of anti-Intgβ1 antibody post siRNA-*MamFers* transfection could suppress the up-regulated adhesion of *A. hydrophila*, indicating that *Intgβ1* also participated in the regulation of *MamFers* on bacterial adhesion. That is, *MamFers* suppressed the adhesion of *A. hydrophila* to EPC cells by inducing the expression of *FN* and suppressing the expression of *Intgβ1* gene.

4. Discussion

Comparative DGE analysis via high throughput sequencing technologies has been accepted as a effective approach to assess transcriptional response upon different challenging conditions [25,26]. In the present study, comparative DGE sequencing and differential expression analysis was performed to analyze the regulatory roles of *MamFerH* and *MamFerM* genes. Representative differentially expressed genes was categorized according to GO enrichment, KEGG analysis and literature search, of which two representatively regulatory signaling pathways were further verified.

Attaching to the host cell surface should be the first stage of bacterial infection. That is, adhesion to host cells or modification of host cellular junction is the prerequisite and foundation of bacterial invasion [27]. Thus, cellular junction may function as the basic barrier in protecting host against bacterial invasion. A bunch of host cellular junction related genes were differently expressed in the present study, including *FN*, *Intgβ1*, thrombospondin, collagen, Biglycan, PRG 4, Extracellular matrix protein 1, Cell adhesion molecule 1, and Connective tissue growth factor, etc. Plenty of these genes involved in the Extracellular matrix (ECM)-receptor pathway, such as *FN*, PRG 4, Extracellular matrix protein 1 and *Intgβ1*, of which Intgβ1 is a kind of transmembrane protein that connecting ECM and cytomembrane. Additionally, ECM-Intg pathway is pretty important during bacterial invasion, as many bacteria firstly need to adhere to Intgβ1 directly or indirectly.

The present study revealed that *MamFers* can regulate the adhesion of *A. hydrophila* to EPC cells, and the regulation was mediated by *FN* and *Intgβ1*. As a kind of transmembrane glycoproteins, Intg might be the primary target during bacterial infection [28]. Bacteria can bind to Intg proteins in two ways, directly bind to Intg proteins by secreting adhesin (e.g. *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Shigella flexneri* and *Helicobacter pylori*) [29–31], or indirectly bind to Intg proteins via ECM proteins (e.g. *Staphylococcus aureus*, *Streptococcus pyogenes*, *Porphyromonas gingivalis*, *Neisseria gonorrhoeae*, *N. meningiditis*, *Bartonella henselae* and *Mycobacterium leprae*) [32]. Certainly, the two types are not always mutually exclusive, since some bacteria can bind to Intg proteins both directly and indirectly (such as *Y. enterocolitica*) [33,34]. The present study indicated that *MamFers* could regulate *A. hydrophila* adhesion via regulating *Intgβ1* and *FN* expression.

The adaptive immune system of fish has not yet completely evolved, thus the innate immune system is extremely important in defense against bacterial infection. Fish innate immune system include physical barrier, and cellular or humoral immunity, which can be regulated by chemokine, interferon, complement and the like molecules. In the present study, expression of several immune genes are affected post *MamFers* overexpression, such as *MHC I*, *β2M*, *NLRC3/5*, interleukin, tumor necrosis factor, interferon regulatory factors and so on, indicating the regulation of *MamFers* on host innate immune system. Thereinto, MHC I is a kind of antigen presenting protein that widely expressed in multiple cell surface, which can be regulated by *NLRC5* both *in vitro* and *in vivo* [11,12]. *NLRC5* is one of the most important members of the NOD like receptor family, playing vital roles in the host immune system as pattern recognition receptor and immune regulatory factor [35,36]. The present study is the first to reveal the regulation of *MamFers* on the expression of *NLRC5/MHC I/β2M* axis, and provides a new perspective of the immune regulatory functions of fish ferritins.

Iron is an essential microelement for almost all living organisms and participates in various physiological activities. However, an excess of iron not only stimulates the generation of toxic free radical, but also facilitates the proliferation of pathogenic microorganism. Hence, maintenance of iron homeostasis is extremely important, of which ferritins play pivotal roles as iron storage proteins. Meanwhile, iron is the centre of nutrient source strife between host and pathogens, thus many iron metabolism related genes participated in host immune response via iron segregation. In the present study, overexpression of *MamFers* would induce the expression of several iron metabolism

related genes, such as hephaestin, metalloreductase STEAP4, solute carrier family 40 member 1, *BMP6*, etc. Previous study revealed that knockdown of *β2M* would perturb the subcellular distribution of two important iron metabolism related proteins: HFE (hereditary hemochromatosis protein) and hepcidin [10]. Similarly, the present study also revealed that *β2M* could mediate the regulation of *MamFers* to hepcidin expression. However, the precise regulatory mechanism has not been fully understood and still need further study.

In summary, comparative DGE sequencing and differential expression analysis was performed in the present study to analyze the regulatory functions of *MamFerH* and *MamFerM* genes, of which two representatively regulatory pathways were further verified. The present study indicated that *MamFers* regulated the expression of hepcidin in L8824 cells via *NLRC5/MHC I/β2M* axis. In addition, *MamFers* could regulate *A. hydrophila* adhesion to EPC cells by regulating the expression of *Intgβ1* and *FN*. What is more, these findings would be helpful in understanding the immune defense system of *M. amblycephala* and developing of targeted immune agents.

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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.2019.01.045.

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