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Transcriptomic analysis revealing hepcidin expression in *Oryzias melastigma* regulated through the JAK-STAT signaling pathway upon exposure to BaP

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ABSTRACT

Our previous study revealed that an antimicrobial peptide hepcidin, can be significantly up-regulated either with LPS challenge or upon exposure to Benzo[a]pyrene (BaP) in red sea bream, but the molecular mechanism involved in whether the transcriptional expression of hepcidin induced by LPS or BaP is regulated through a similar signaling pathway is not yet known. To elucidate the underlying molecular mechanism, the marine model fish *Oryzias melastigma* was exposed to 1 μg/L BaP as well as challenged with 5 μg of LPS per fish. Samples at 3 h post-LPS challenge, and 2 d and 3 d post-BaP exposure were separately collected for transcriptome analysis. General analysis of the predicted immune-associated unigenes based on the transcriptomic data showed that the percentages of modulated immune-associated genes were 7% with LPS challenge, and 3% and 7% with BaP exposure at 2 and 3 days, respectively. Genes involved in functions like antimicrobial activity, neutrophil activation, and leukocyte chemotaxis were up-regulated with LPS challenge, whereas more than half of the immune associated genes including the KLF family were down-regulated upon BaP exposure, indicating a difference in the modulated immune genes between LPS challenge and BaP exposure. Specific comparative analyses of the immune-associated signal pathways NOD, TOLL, NF-κB and JAK-STAT with LPS challenge or upon exposure to BaP, indicated that most of the modulated genes in association with the NOD, TOLL and NF-κB pathways were induced with LPS challenge but only a few after exposure to BaP, suggesting that BaP exposure was generally not associated with any of the three signal pathways. Interestingly, further transcriptomic analysis revealed that 5 of the 8 modulated genes associated with the JAK-STAT pathway were down-regulated, while 2 inhibiting genes were up-regulated after BaP exposure for 2 days whereas LPS challenge resulted in only less than half modulated, suggesting the possibility of down-regulation caused by BaP exposure through JAK-STAT pathway. Further testing using an EPC cell culture demonstrated that expression of the hepcidin1 gene was less involved in the known signal pathways, such as c/EBP, BMP, and NF-κB, but instead mostly in association with the JAK-STAT pathway upon BaP exposure.

1. Introduction

Marine pollution, caused by persistent organic pollutants, environmental hormones and heavy metals, has been a great concern in recent years. Among other consequences associated with this environmental contamination, it poses a potential threat to the fishery industry, as many pollutants have toxic effects on living animals including fish. In particular, they can alter the immune status of fish, a phenomenon known as “immunotoxicity”. The term “immunotoxicology” was first used in a scientific publication by Moore JA (Moore, 1979) in 1979, with many relevant manuscripts published afterwards (Caspritz and Hadden, 1987; Koller, 1980; Loose, 1986). Immunotoxicological studies in aquatic animals came much later than in the field of human medicine, and at first many studies were mainly focused on the alteration of the overall immune status of aquatic animals, which is often used as an indicator of environmental safety (Hussain, 1996; Wester et al., 1994). In recent years, many researchers are gradually starting to explore underlying immunotoxic mechanisms upon exposure to aquatic contaminants (Ge et al., 2017; Xu et al., 2018).

Polycyclic aromatic hydrocarbons (PAHs) are aquatic pollutants that can easily penetrate biological membranes and accumulate in organisms, showing carcinogenic, mutagenic and toxic effects (Li et al., 2016). BaP is one of the most widely distributed and well-studied PAHs. With concentrations up to 0.1–4.2 μg/L in contaminated areas (Hose

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et al., 1982; Huang et al., 2012), BaP in natural waters can be prevalent enough to modulate the immune system of marine creatures. It has been proven to suppress the progenitor of pre-B cell formation as well as drive a polarized T cell response (Hong et al., 2016; Near et al., 1999), showing a toxic effect on adaptive immunity. Khaniyan, M. has reported that BaP injection can inhibit the production of immunoglobulin M (IgM) and antimicrobial activity in orange spotted grouper (Khaniyan et al., 2016). In addition, BaP exposure can inhibit serum antibacterial activity and delay the significant expression of hepcidin to show an immune response against lipopolysaccharide (LPS) challenge in red sea bream (Bo et al., 2014). The immunotoxic effect upon BaP exposure was first reported in 1983, where the authors found that exposure to BaP with a dosage of 5 μg/g body weight (BW) could lead to a reduced number of IgM and immunoglobulin G (IgG) antibody plaque forming cells (Dean et al., 1983). Subsequently, there have been many immunotoxic studies published over the last 20 years, and the immunotoxicity of hormones, PAHs and heavy metals has recently been reported in aquatic animals (Bruneau et al., 2015; Thilagam et al., 2009; Ye et al., 2011). However, studies on the toxic effects induced by BaP sometimes have ambiguous results. BaP has been reported to depress innate and adaptive immune responses in mice at high concentration exposures (Dean et al., 1983; Urso and Gengozian, 1980), which can also induce an immunotoxic response in fish (Carlson et al., 2004; Khaniyan et al., 2016). However, the efficiency of BaP exposure is partially concentration related. For example, low-dose BaP exposure activates mediastinal lymph node cells in mice (Yanagisawa et al., 2016). Additionally, some studies show that lower concentrations of certain pollutants actually enhance the immune response of fish against bacterial infection (Yang et al., 2015). These conflicting observations have resulted in an incomplete understanding of the underlying immunotoxic mechanisms involved in pollutant exposure. Recently, focusing on the aquatic pollutant bisphenol A (BPA), Yang et al. showed that the toxic effect caused by BPA was involved in a synergistic interaction of ERα and NF-κB transcription factors (Yang et al., 2015). Additional studies have reported that immunotoxicological mechanisms are involved in different signaling pathways (for example the NF-κB and Janus kinase(JAK)-STAT signaling pathways) through which the modulation of immune systems is thought to be activated upon pollutant exposure (Guo et al., 2017; Hermann and Kim, 2005); however, the underlying mechanisms have been less studied.

The antimicrobial peptide hepcidin was first discovered in humans in 2000 and named in 2001 (Krause et al., 2000; Park et al., 2001). It is predominantly expressed in the liver and functions as an iron-regulatory hormone during normal homeostatic iron regulation. In fish, hepcidin is highly expressed not only in the liver but also in the kidneys (Wang et al., 2009b; Yang et al., 2007) and has multiple variants in different fish species, suggesting that hepcidin and its variants are important components of innate immune systems (Yang et al., 2011). Interestingly, we found in a previous study that BaP can induce hepcidin mRNA expression in red sea bream, and the expression pattern of hepcidin is similar to that induced with LPS challenge (Bo et al., 2014; Wang et al., 2009a). The results make us speculate about whether the regulation of hepcidin mRNA expression is through a similar signal pathway; however, no clear answer is yet reported. As it is known now, hepcidin synthesis can be transcriptionally regulated by several stimuli, such as being induced by inflammation and iron overload, or suppressed by anemia and hypoxia (Kong et al., 2014; O'Shea and Plenge, 2012; Viatte and Vaulont, 2009). The immune response of hepcidin is usually mediated by cytokine interleukin (IL-6) through the JAK-STAT signaling pathway (Wrighting and Andrews, 2006). Additionally, physiological concentrations of reactive oxygen species (ROS) could induce hepcidin expression directly through phosphorylated STAT3 (Millonig et al., 2012). The transcriptional regulation of hepcidin by iron is involved in the BMP/SMAD pathway, whereas anemia and hypoxia negatively regulate hepcidin expression through inhibiting the binding of C/EBPα and STAT3 to the hepcidin promoter and inhibiting BMP/SMAD4-dependent signaling, respectively (Ganz, 2011). Comprehensively, theses work suggest that the expression of fish hepcidin upon exposure to BaP might be regulated through different signal pathways. However, unlike the immune-associated mechanisms of hepcidin regulation, the related mechanisms underlying exposure to environmental pollutants are less known.

Based on our previous observations of hepcidin expression, which was either induced by LPS challenge or BaP exposure, we hereby designed a transcriptomic study in which we aim to investigate the differences in the modulated genes and signal pathways with LPS challenge and BaP exposure, and furthermore to elucidate the potential signaling pathway involved in regulation of hepcidin expression during fish exposure to BaP.

2. Materials and methods

2.1. Chemicals

BaP, DMSO, MS-222 (Ethyl 3-aminobenzoate methanesulfonate salt) and LPS (from *E. coli* 055:B5) were purchased from Sigma-Aldrich (China). Acetone was purchased from Sinopharm (China). TRIzol was purchased from Invitrogen (USA). NSC 74859 was purchased from Tocris (Britain). A BaP stock solution was prepared by dissolving the chemical in acetone to a concentration of 200 mg/L, or dissolved in DMSO to a concentration of 1 mmol/L. A LPS stock solution was prepared by dissolution in MilliQ to a final concentration of 5 g/L.

2.2. Fish maintenance and exposure

The medaka (*Oryzias melastigma*) used in our experiment were a kind gift from the City University of Hong Kong and have been reared in our laboratory for over 10 generations. The fish were reared in artificial seawater at a salinity of 30‰, a temperature of 28°C, and a photoperiod of 14 h-light/10 h-dark. Sexually mature 4-month old male medaka were chosen and randomly placed into 20 L glass tanks. At least three replicate tanks were set for each treatment group for preparing biological replicates. Fish were anesthetized by MS-222 (0.25 g/L) before the operation. LPS was injected intraperitoneally at a dosage of 5 μg per fish, and the STAT3 inhibitor NSC 74859, was used at a dose of 1 μg per fish. The control group received identical volume of sterile saline. The experiment was carried out for 5 days, and the sampling times included 3, 6, 12, 24, 48, 72, 96, and 120 h. The initial concentration of BaP was 1 μg/L when the control group received the same volume of acetone, and the exposure media was renewed every day. The same sampling times were used for the LPS challenge. At least 3 fish were anesthetized and sampled at each time point from each treatment group. Their liver was transferred to liquid nitrogen immediately and kept at −80 ℃ until RNA extraction.

2.3. Transcriptomic library construction and annotation

RNA was extracted by TRIzol and quantified using Nano Drop 2000. To obtain as many gene transcripts as possible, the liver RNA for sequencing was equally mixed from various exposure experiments as follows: LPS and saline at 3 h, PDTC and saline at 6 h, and BaP and acetone at 2 or 3 days. A total amount of 3 μg RNA was used as input material for the RNA sample preparations. The sequencing library was generated using a NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) and then sequenced on an Illumina HiSeq™ 2500 platform, generating 125 bp paired-end reads. After removing Illumina adapter sequences and filtering out sequences that did not meet the quality thresholds, all sequences were assembled *de novo* using Trinity (r20140413p1). The resulting transcripts were annotated using NCBI blast (2.2.28+, e-value $< 1 \times 10^{-5}$), KAAS (r140224, e-value $< 1 \times 10^{-10}$), hmmscan (HMMER 3, e-value < 0.01) and blast2go (b2g4pipe_v2.5, e-value $< 1 \times 10^{-6}$).

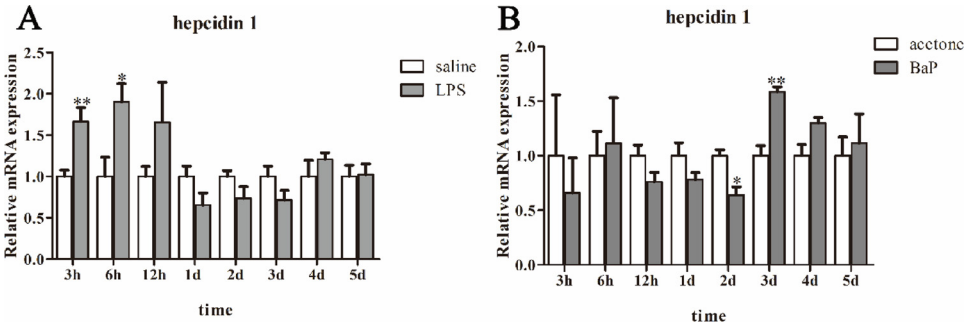

Fig. 1. *Hepcidin1* mRNA expression modulated with LPS challenge or upon BaP exposure. A) The expression pattern of *hepcidin1* mRNA in medaka with LPS treatment. B) The expression pattern of *hepcidin1* mRNA in medaka upon BaP exposure. Results are normalized to 16 s and represented as fold increase ± SEM (*error bars*) in comparison with the untreated control group. Statistical significant data were calculated by student's *t*-test. n ≥ 3, * $p < 0.05$, ** $p < 0.01$.

2.4. Identification of differentially expressed genes (DGEs) using Illumina

3 μg RNA extracted from Medaka liver was used for DGE analysis on each sample. cDNA libraries were sequenced on an Illumina Hiseq 2500 platform and 50 bp single-end reads were generated. After removing disqualified reads, clean data were mapped onto the assembled transcriptome above. DESeq (1.10.1) was used for determining the differential expression between treatment and its own control group. Genes with a *p-value* < *0.01* found by DESeq were assigned as differentially expressed.

2.5. Quantitative RT-PCR (q-PCR)

The RNA (1 μg) from individual liver samples was reverse-transcribed using a PrimeScript TM RT-PCR Kit (TaKaRa, China PR). Next, q-PCR was performed on a Qiagen Rotor-Gene Q using Power SYBR Green PCR Master Mix (Roche, USA). The primer sequences used in the q-PCR study, with amplification efficiencies calculated within 90–110%, are presented in Table A1. 16 s was quantified to normalize the relative expression levels for selected genes. A gene's expression level was calculated based on the standard curve for each plate. Statistically significant difference were tested by a student's *t*-test with interaction analysis using IBM SPSS Statistics 20. Statistical significance was accepted at $p < 0.05$.

2.6. Western blot

Medaka livers were harvested and lysed by RIPA with PMSF on ice. Protein concentrations were determined by G250. Equal amounts of protein were loaded and separated on 10% SDS-polyacrylamide gels and blotted onto PVDF membranes. Membranes were blocked using 5% skim milk with 0.05% PBS-Tween (T-PBS) for 2 h at 37°Cand then incubated with primary antibodies (see Table A2) diluted in skim milk/T-PBS overnight at 4°C. 5% BSA, T-PBS was used for blocking nonspecific binding and for dilution of the anti-p-RelA antibody. After a wash in T-PBS for 20 min, the secondary antibodies were diluted in 5% BSA and incubated at 37°C for 1 h followed by another washing step in T-PBS. The blot was developed using chemiluminescence and exposed to CCD for 1–3 min.

2.7. Hepcidin1 promoter constructs and luciferase assays

In our previous study, the sequence of OM-hep1 promoter was cloned and sequenced (GenBank no. MF774223) (Zhang et al., 2018). Based on that, STAT-binding site deletion mutants of the recombinant pGL3-basic plasmid were constructed by PCR and all of the constructs were verified by sequencing. Endothelial progenitor cells (EPC, Cyprinus carpio, stored in our laboratory) were maintained in L-15 medium (Gibco), supplemented with 10% charcoal/dextran stripped fetal bovine serum (FBS, Gibco) and 5 mmol/L HEPES with a pH of 7.2. They were cultured at 26°C in the absence of CO_2. For luciferase assays, EPC cells were seeded at a density of 10^5 cells/mL in 48-well plates overnight, and then co-transfected with 0.4 μg of the targeted luciferase constructs and 10 ng of pRL-TK plasmid with Fugen 6 (Promega) following the manufacturer's instructions with BaP or an equal amount of DMSO treatment for 48 h. Then, cells were washed with 1 × HBSS twice before collection in a lysis buffer. The cellular extracts were analyzed for luciferase activity using the dual-luciferase reporter assay system (Promega).

3. Results

3.1. Different expression patterns of the hepcidin1 gene induced by LPS or BaP

Hepcidin is known as an important antimicrobial peptide in the liver, which can be induced by LPS or BaP in many species (Wang et al., 2009a). The gene expression changes after LPS challenge or BaP exposure are shown in Fig. 1. Hepcidin1 mRNA showed a 1.7-fold change at 3 h post-LPS injection. The largest change in its level was at 6 h, reaching a 1.9-fold change over the control. However, hepcidin1 mRNA transcription was significantly inhibited at 48 h (2 d), but significantly up-regulated at 72 h (3 d) upon BaP exposure.

3.2. Assembly of the medaka transcriptome

Sequencing generated 91.2 M 125 bp reads from the library. Following raw data processing, a total of 11 G clean bases were obtained and input into the transcriptome assemblies. A total of 58,183 transcripts were assembled with an average length of 919 bp and an *N*50 of 1681 bp. 60.57% of the 46,792 unigenes, which is the longest among the homologous transcripts, were annotated using Nr, Nt, KO, SwissProt, PFAM, GO and KOG databases.

3.3. General analysis using the transcriptomic data of the immune-associated unigenes either with challenge by LPS or upon exposure to Bap at 2 and 3 days

The adult male fish (20 fish for each sample, 3 samples for each group) were either injected with LPS for 3 h or exposed to BaP for 2 or 3 days. Judging by a *p-value* < *0.01*, it was found that there were 276 unigenes up-regulated and 180 unigenes down-regulated upon LPS challenge. Interestingly, there were also 228 unigenes up-regulated and 558 unigenes down-regulated upon exposure to BaP at 2 days. After 3 days of BaP exposure, there were 175 unigenes up-regulated and 172 unigenes down-regulated (Fig. 2A).

Based on the transcriptome data, approximately 7% (31) of the unigenes were associated with immunity among the significantly modulated unigenes upon challenge by LPS, whereas only approximately 3% (28) of the unigenes were associated with immunity upon exposure to BaP at 2 d and approximately 7% (25) at 3 d (Fig. 2B). Venn analysis of immune and signal transduction genes showed that only a few genes could be shared with the three treatment groups (Fig. 2C). Besides, almost all of the modulated unigenes associated with immune

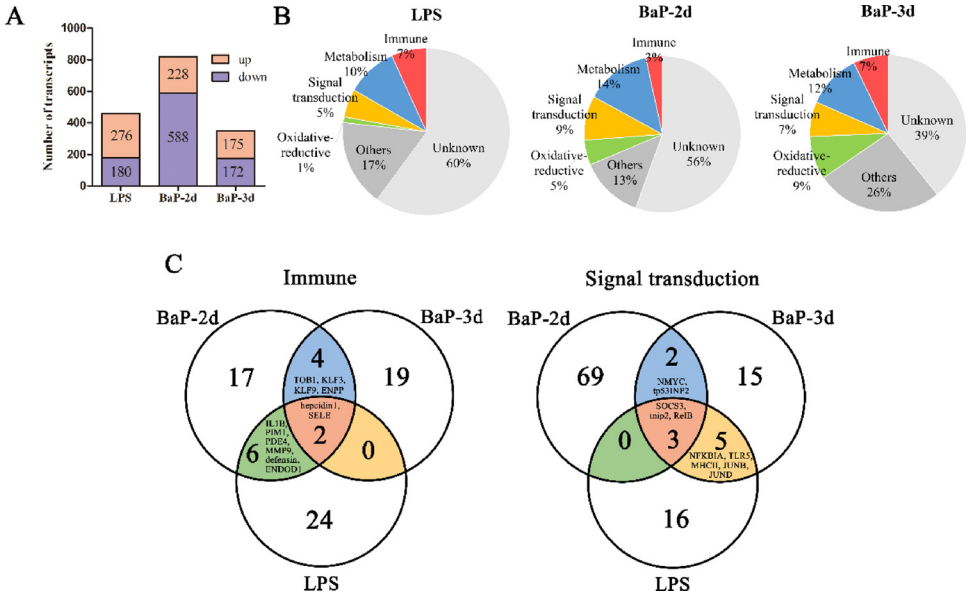

Fig. 2. Gene ontology analysis of differentially expressed genes by LPS challenge or upon BaP exposure. A) the modulated gene numbers with challenge of LPS, or upon exposure to BaP for 2 days or 3 days; B) Pie Chart showed the percentage of genes in immunity, metabolism, signal transduction and other functions. C) Venn analysis of immune and signal transduction associated genes. Among the modulated immune-associated genes there were only a few genes shared with three treatments. Otherwise, only a few signal pathways were shared with LPS and BaP treatments.

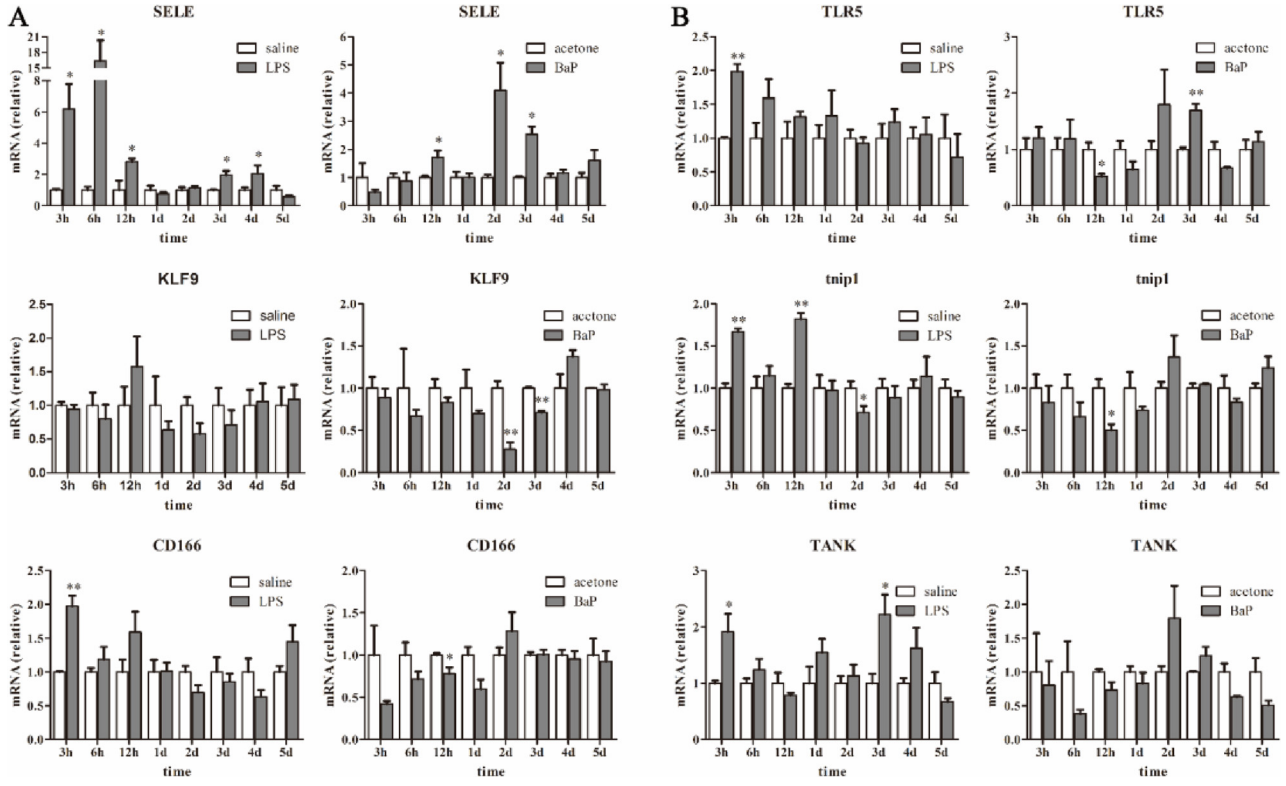

Fig. 3. Verification of the transcriptome data by q-PCR. A) Relative expression of selected immune genes by q-PCR. B) Relative expression of selected signal transduction associated genes by q-PCR. Fish were injected by 5 μg/L LPS or exposure to 1 μg/L BaP in water for a time span of five days. Results are normalized to 16 s and represented as fold increase ± SEM (*error bars*) compared with untreated control group. Statistical significant was calculated by student's *t*-test. n ≥ 3, * $p < 0.05$, ** $p < 0.01$.

response were up-regulated by LPS challenge, but more than half of immune genes were down-regulated upon BaP exposure.

Further analysis found that LPS challenge up-regulated immune genes were involved in functions like antimicrobial activities, neutrophil activation and leukocyte chemotaxis, but most of the immune functional unigenes were inhibited after BaP exposure for 2 or 3 days, which were associated with the KLF family, T cell activation, neutrophil activation at 2 d of exposure and antimicrobial activity at 3 d of exposure (Table A3).

To verify the transcriptome data, immune associated genes and signal pathway associated genes were chosen for detection using q-PCR from 1 h to 5 d with LPS challenge or 3 h to 5 d of BaP exposure (Fig. 3). The transcriptome verification rate was above 70%, and the expression pattern of those genes showed some similarity with hepcidin1.

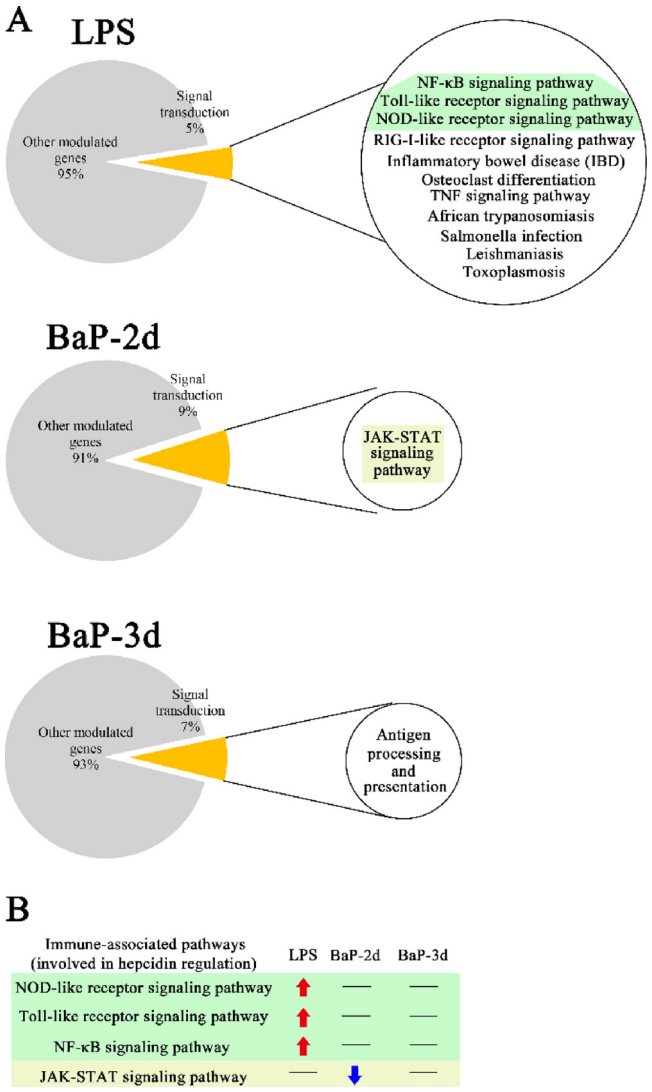

Fig. 4. Immune associated pathway analysis based on transcriptome data. A) Transcriptomic analysis indicated that more immune-associated signal pathways was significantly modulated by LPS challenge ($p\text{-}val < 0.05$), whereas only one immune-associated signal pathway JAK-STAT was modulated upon exposure to BaP for 2 days, and only one signal pathway antigen processing and presentation was modulated upon exposure to BaP for 3 days. B) Four signal pathways NOD, TOLL, NF-κB and JAK-STAT which is known to regulate hepcidin expression were modulated with LPS challenge or BaP exposure, and JAK-STAT alone was modulated upon BaP exposure for 2 days.

3.4. Specific analysis of the potential immune signal pathways using transcriptomic data - NOD, TOLL, NF-κB, and JAK-STAT – after LPS challenge or exposure to BaP for 2 or 3 days

Immune associated signaling pathways were regulated with LPS challenge or exposure to BaP. With the standard of $p\text{-}val < 0.05$, KEGG enrichment indicated the difference on immune pathway modulation. Based on transcriptome data, only one immune associated pathways could be significantly enriched in BaP-2d or BaP-3d group, in contrary, 11 immune associated pathways were enriched in LPS challenge group (Fig. 4A). Multiple pathways which are associated with hepcidin regulation, including the NOD-like receptor signaling pathway, the Toll-like receptor signaling pathway, the NF-κB signaling pathway, and the JAK-STAT signaling pathway could be modulated with LPS challenge or upon BaP exposure at 2 or 3 days (Fig.4B, Table A4).

For the NOD-like receptor signaling pathway, the 14 modulated genes involved in pathway activation were compared in this study. 12 genes were up-regulated with LPS challenge, whereas only 2 were up-regulated after 2 days of BaP exposure and only 1 after 3 days of exposure.

For the Toll-like receptor signaling pathway, 11 genes were modulated. 9 genes were up-regulated with LPS challenge, whereas only 2 were up-regulated after exposure to BaP for 2 days and 1 after 3 days of exposure.

For the NF-κB signaling pathway, 17 genes were modulated. 15 genes were up-regulated with LPS challenge (including 3 inhibiting protein-coding genes), whereas only 5 were up-regulated after exposure to BaP for 2 days and 1 after 3 days of exposure. To confirm the regulation of the NF-κB signaling pathway with LPS challenge, immunoblotting was used to detect the protein expression level of the canonical NF-κB signaling pathway (Fig. A1). Western blotting showed that although NFKBIA was transitionally up-regulated, it decreased with LPS stimulation as our results also showed that IκBα was degraded at the protein level. In addition, the phosphorylation level of RelA was up-regulated. The transcriptome data as well as western blotting demonstrated that NF-κB pathway was up-regulated by LPS challenge (Fig. 5A). Throughout the comparison, it could be determined that LPS stimulation was closely related to the three signaling pathways - NOD, Toll and NF-κB – but not BaP exposure.

Further analysis of the JAK-STAT signaling pathway interestingly showed that among 12 modulated genes, BaP exposure resulted in 3 genes up-regulated (including 2 inhibiting protein-coding genes) and 5 down-regulated at 2 d and 2 genes up-regulated and 2 down-regulated at 3 d, whereas LPS resulted in 3 genes up-regulated and 1 down-regulated. In consideration of IL-6 (Fig. A2), which could not be annotated in the transcriptome data, its expression was tested by q-PCR, which showed it was down-regulated after BaP exposure at 2 d but showed no significant change with LPS challenge (Fig. 5B). The result suggests that gene expression modulated during BaP exposure might be related to the JAK-STAT signaling pathway.

3.5. Detailed analysis of the association between hepcidin1 expression modulated at 2 and 3 days of BaP exposure with the JAK-STAT signaling pathway

As it is reported that there are four signal pathways involved in hepcidin regulation, we performed a detailed analysis of the involved genes, which are usually related to each signaling pathway: such as c/EBP, BMP-SMAD, NF-κB and JAK-STAT. We found that the key genes like *Samd1/4/5/8* of the BMP-SMAD signaling pathway and *Gdf15* of the c/EBP signaling pathway did not show any response. The NF-κB signaling pathway showed no significant response while the JAK-STAT signaling pathway was inhibited at 2 d during BaP exposure.

In addition, regulation of the oxidation-reduction system was analyzed by transcriptomics after BaP exposure for 3 days (Fig. A3). A majority of the up-regulated genes analyzed in the study were ROS-generation associated, which were classified to be dehydrogenase, oxygenase or in the P450 family. The CYP1/2 families, which belong to Phase I enzymes, were about 1.5-fold higher in expression versus the control, while *Cyp3c* was only approximately 1.2-fold higher. Three retinal-associated genes showed a significant expression, among which *Rdh11* and *Rdh8* were up-regulated. Meanwhile, *Oat1*, the terminal oxidase in mitochondrial electron transport which can eliminate endogenous and exogenous organic anions, and *Cox7a2*, were down-regulated.

The regulation of hepcidin1 expression in Medaka by the JAK-STAT signaling pathway was confirmed by NSC 74859, a specific STAT3 inhibitor, which successfully inhibited hepcidin1 mRNA expression in this study (Fig. 6A). To confirm this result, luciferase reporter constructs containing wild-type hepcidin1 promoters (WT 716 bp, and WT 315 bp) or with STAT-binding sites were transfected into an EPC cell line. Results showed that the STAT-binding sites not only inactivate the transcriptional activity of the hepcidin1 promoters, but also nullified the

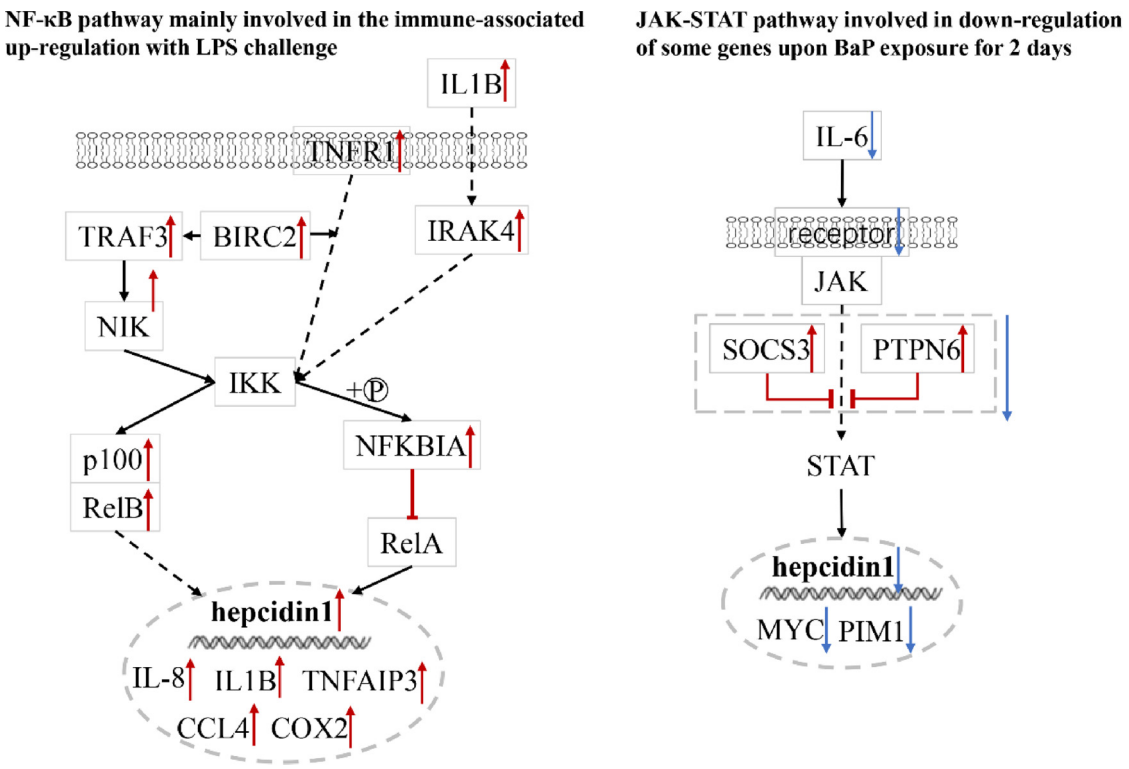

Fig. 5. Transcriptomic analysis further showed that hepcidin gene was up-regulated through NF-κB with LPS challenge, whereas it was down-regulated through JAK-STAT upon exposure to BaP. A) The regulation of NF-κB signaling pathway by LPS challenge. B) The regulation of JAK-STAT signaling pathway upon BaP exposure.

induction of the hepcidin1 promoter activity with BaP treatment (Fig. 6 (B, C)).

4. Discussion

In this study, a total of 46,792 unigenes were screened with transcriptomic analysis using the standard of *p-val* < *0.01*. As shown in Table S1 and S2, many significantly differentially expressed genes, including immune-associated genes and those involved in signal pathways associated with immune responses, have been revealed. General analysis of the immune-associated unigenes either with LPS challenge or after exposure to Bap for 2 to 3 days, based on transcriptomic data, revealed that LPS challenge and BaP exposure could both significantly modulate the mRNA expression of these genes. The number of significantly modulated genes were 456, 786 and 347, respectively, by LPS challenge and BaP exposure at 2 d and 3 d. These modulated genes were involved in various biological functions including immunity, metabolism and transcription. Based on the data obtained, it could be determined that environmental contaminants like BaP have real effects on biological processes at the transcriptional level. General analysis of the predicted immune-associated unigenes based on transcriptomic data showed that the percentages of immune-associated genes were 7%, 3% and 7% for LPS challenge, BaP exposure at 2 d, and BaP exposure at 3 d, respectively. The results obtained from LPS challenge were expected, as LPS is known as an immune stimulant that induces proinflammatory expression. Unexpectedly, we found that BaP exposure could result in the modulation of 7% immune-associated genes after 3 days, markedly different from that induced after 2 days of exposure with only 3% modulated immune-associated genes. Through general analysis of the transcriptomic data, we can infer that BaP exposure is an immune stimulant that alters the transcriptional expression of various genes, including immune-associated genes. This result guided us to further investigate whether the regulation mechanisms associated with LPS challenge or BaP exposure are similar or different. Our analysis found that almost all modulated unigenes associated with an immune response were up-regulated with LPS challenge, while mostly down-regulated after BaP exposure. Additionally, the modulated immune genes were also different from each other among the three treatments; for example, the KLF family was only modulated after BaP exposure while neutrophil activation associated genes showed no significant change after BaP exposure. The results demonstrated that the modulated genes with LPS challenge were different from those modulated after exposure to BaP, and that the expression patterns were almost conversely related to each other. This further indicates that their regulation mechanisms either with LPS challenge or BaP exposure were not similar: that is, they proceeded through different signal pathways.

Subsequently, we used transcriptomic data to compare the immune-associated signal pathways of NOD, TOLL, NF-κB, and JAK-STAT with the altered immune responses with LPS challenge or after exposure to BaP. 14 genes involved in the NOD-like signaling pathway and 11 genes involved in the Toll-like signaling pathway were modulated with LPS challenge, whereas less than 3 genes were up-regulated after BaP exposure for 2 or 3 days. Based on this result, most of the modulated genes in association with either the NOD-like or Toll-like receptor signaling pathway were mainly activated with LPS challenge but not BaP exposure. Additionally, LPS may activate NF-κB as well as the MAPK pathway through the NOD and Toll-like receptor signaling pathways, of which BaP showed no effect, as evidenced by our finding that more genes were modulated with LPS challenge than after exposure to BaP. Compared with the 17 genes modulated with LPS challenge, only 5 were up-regulated after exposure to Bap for 2 days and 1 after BaP exposure for 3 days, which indicated that the regulation of the NF-κB signaling pathway was similar to the NOD and Toll-like signaling pathways. The immunoblotting assay further demonstrated that LPS challenge activated the NF-κB signaling pathway. Through this detailed comparison, it is understood that LPS stimulation was highly related to the three signal pathways (NOD, TOLL and NF-κB), but BaP exposure was generally not associated with them.

Furthermore, as there are four signal pathways involved in hepcidin regulation, we performed a detailed analysis of the involved genes

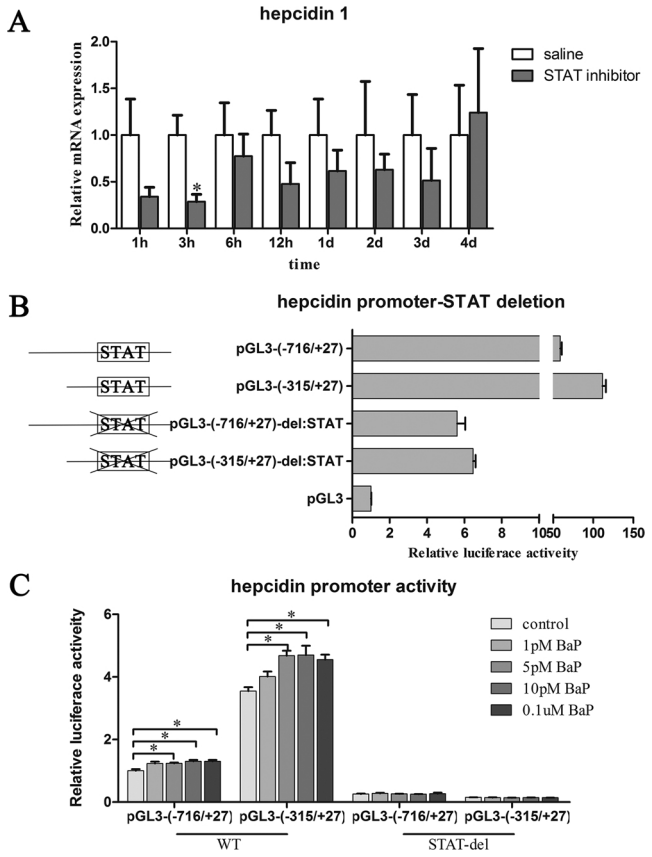

Fig. 6. Confirmation of the association between the hepcidin1 gene expression and the activity of JAK-STAT pathway upon BaP exposure *in vivo* and *in vitro*. A) STAT inhibitor NSC 74859 inhibited *hepcidin1* expression in Medaka. Results are normalized to 16 s and represented as fold increase ± SEM (*error bars*) in comparison with the untreated control group. Statistical significant data were calculated by student's *t*-test. B) Deletion analysis of *hepcidin1* promoter. C) Up-regulation of *hepcidin1* upon BaP exposure is STAT-dependent. (B). (C) Wide type (WT) and STAT-del mutants were transfected into EPC cells. The luciferase activity was determined and normalized to Renilla luciferase activity. (* $p < 0.05$; n ≥ 4).

which are typically related to each signal pathway: for example, c/EBP, BMP-SMAD, NF-κB and JAK-STAT. We found that the key genes such as *Smad1/4/5/8* of the BMP-SMAD pathway and *Gdf15* of the c/EBP signaling pathway did not show any response based on our transcriptomic analyses. These results indicated that the regulation of hepcidin1 expression upon exposure to BaP was mostly not associated with either the BMP-SMAD or c/EBP signaling pathway. Considering that JAK-STAT is another known pathway for the transcriptional regulation of hepcidin, we also analyzed it in our study. As reported, fish hepcidin exerts its role mainly associated with pathogen infection or inflammation, and its expression is usually regulated through the JAK-STAT pathway during inflammation (Lee et al., 2004; Wrighting and Andrews, 2006; O'Shea and Plenge, 2012; Michels et al., 2015). Unexpectedly, the transcriptomic analyses in this study showed that only 4 genes were modulated with LPS challenge, whereas BaP exposure for 2 days had 12 genes modulated, indicating that BaP exposure at 2 d had more genes down-regulated than with LPS treatment. Thus, we also observed using transcriptomic analysis that many modulated genes were not mediated through the JAK-STAT pathway with LPS challenge, which does not agree with the previous conclusion that hepcidin1 is regulated through JAK-STAT with inflammatory stimulation. In addition, IL-6, which is known as a crucial factor in the regulation of hepcidin1 expression through the JAK-STAT signaling pathway, was significantly down-regulated after BaP exposure for 2 days in this study. Conversely, no significant modulation of IL-6 occurred with LPS challenge and BaP exposure after 3 days. Therefore, it was likely that the modulation of the hepcidin1 gene expression upon BaP exposure was through the JAK-STAT signal pathway. Correspondingly, previous studies have demonstrated that fish hepcidin can be modulated by environmental estrogenic pollutants like EE2 and BPA (Massart et al., 2014; Qiu et al., 2016; Yang et al., 2015), suggesting that the expression of fish hepcidin might be regulated through different signal pathways.

To further confirm this possible conclusion, the association between hepcidin1 expression and the potentially involved JAK-STAT signaling pathway was evaluated in Medaka using an inhibitor of JAK-STAT (NSC 74859) and in the cell culture with a promoter of hepcidin1 (Zhang et al., 2018). As expected, the transcriptional expression of hepcidin1 was significantly downregulated, accompanied with the inhibition of JAK-STAT. In addition, the deletion of the STAT binding site in the hepcidin1 promoter resulted in an inactivation of hepcidin1 transcription, thus both results demonstrated that the JAK-STAT signaling pathway was indeed involved in the regulation of hepcidin1 expression in Medaka. Additional experiments further supported this fact, as we observed that deletion of STAT-RE in the hepcidin1 promoter diminished expression upon exposure to BaP in comparison with the control, suggesting that the transcriptional expression of hepcidin1 was regulated or at least partially regulated by the JAK-STAT signaling pathway upon exposure to BaP.

Although our study revealed through transcriptomic analysis that the modulation of hepcidin1 after BaP exposure for 2 days was associated with the JAK-STAT signaling pathway, it could not be determined whether the modulated genes after 3 days of BaP exposure were also regulated through the same pathway, due to the reduced amount of modulated genes associated with the JAK-STAT pathway at this timepoint. However, our analysis found that BaP exposure for 3 days led to the modulation of the oxidative-reduction system from which ROS are often generated. ROS are known to directly cause the phosphorylation of STAT3 and subsequently modulate hepcidin1 expression. In this study, we observed that the amount of ROS generated with the associated genes, including four P450 family members and three RDH family members as well as others, was up-regulated after BaP exposure for 3 days. We also observed that several genes were down-regulated, which participated in the elimination of ROS like *Oat1*. Further analysis showed that some modulated genes like *Cyp1a1*, *Cyp2aa*, *Cyp2x* as well as *Aox* were all involved in ROS generation, among which *Cyp1a1*, *Cyp2aa*, *Cyp2x* all belong to P450 family members whereas H_2O_2 is a common product of AOX. The P450 family played an important role in BaP phase I metabolism which leads to ROS generation (Anzenbacher and Anzenbacherova, 2001), and ROS is reported to induce hepcidin expression through STAT3 phosphorylation (Millonig et al., 2012). In addition, some studies have indicated that the expression of P450 family members is consistent with hepcidin expression (Hirakawa et al., 2011; Maguire et al., 2017; Wang et al., 2009a). In our study, both the q-PCR data and the transcriptomic analysis data demonstrated that the hepcidin gene was up-regulated after BaP exposure for 3 days, while large amounts of ROS were generated. Therefore, we suggest that the modulated genes after BaP exposure for 3 days might be induced with the generation of ROS, and the regulation of gene expression was likely through STAT, mediated directly by ROS.

5. Conclusion

In conclusion, our study revealed that the transcriptional expression of the antimicrobial peptide hepcidin, which is widely distributed across many fish species, was down-regulated through the JAK-STAT signaling pathway upon exposure to BaP, based on transcriptomic data. This result gives a relatively clear explanation for our previous observations that one hepcidin gene, PM-hepc, identified in *Pagrus major*, was up-regulated either with LPS challenge or upon exposure to BaP

(Bo et al., 2014). The underlying mechanisms involved in the transcriptional regulation of immune-associated genes during LPS challenge and BaP exposure were different, and the modulated genes caused by LPS challenge or exposure to BaP were regulated through different signaling pathways. During this study we also observed that most of the modulated genes with LPS challenge were probably through NF-κB, but relatively less so or only partly in association with JAK-STAT, whereas many immune-associated genes including the hepcidin gene were likely down-regulated through JAK-STAT upon BaP exposure. Similar to other reported environmental pollutants like EE2 and BPA with different regulated signal pathways, here, for the first, we report that the expression of fish hepcidin could be regulated through JAK-STAT during exposure to BaP.

Author contributions

KW designed the study and reviewed and edited the manuscript. QC performed the experiments and wrote the paper. All the authors read and approved the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2018.11.015.

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