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The transcriptional regulation of an antimicrobial peptide hepcidin1 in *Oryzias melastigma* upon EE2 exposure involved in a new pathway with a novel transcriptional regulatory element HepERE

Min Zhang[a], Meng-Fei Zhang[a,b,c], Fang-Yi Chen[a,b,c], Qian Cui[a], Bing-Zhen Yang[a], Yong Liu[a], Hui Peng[a,b,c], Ke-Jian Wang[a,b,c,*]

[a] State Key Laboratory of Marine Environmental Science, College of Ocean & Earth Science, Xiamen University, Xiamen, Fujian, China
[b] State-Province Joint Engineering Laboratory of Marine Bioproducts and Technology, Xiamen University, Xiamen, Fujian, China
[c] Fujian Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources, Xiamen University, Xiamen, Fujian, China

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ABSTRACT

17α-ethinylestradiol (EE2) exerts endocrine disrupting effect and immunotoxic effect on marine animals, including modulation of hepcidin expression. The antimicrobial peptide hepcidin displays a crucial role in innate immunity in fish against invading pathogens. It is known that the transcription of hepcidin in mammals is individually regulated by many stimuli, including inflammation, iron overload, anemia or hypoxia, through several distinct molecular pathways. The canonical mechanism for endocrine disrupting effects is mediated by an estrogen receptor (ER) and estrogen responsive element (ERE), whereas the underlying mechanism for immunotoxic effect is still unclear. In this study, a hepcidin from *Oryzias melastigma* (OM-hep1) was found to be down-regulated upon EE2 exposure and was associated with ERα. Unlike the revealed signaling pathways for hepcidin regulation in mammals, it was revealed by promoter activity analysis that the OM-hep1 transcription was not associated with canonical immune-associated and hormone-associated regulatory elements, known as the nuclear factor κB (NF-κB), signal transducer and activator of transcription 3 (STAT3), ERE and estrogen-related receptor responsive element (ERRE). Further analysis through a series of base mutations revealed a short fragment from −315 to −289 bp on the OM-hep1 promoter with high activity. This fragment was composed of a putative ERE-like element (23 bases) plus an adjacent down-streamed four bases motif GTGT. Replacement of either of the core bases (GGTCA) of ERE-like or GTGT motif showed non-activity and non-response to EE2 exposure, thus a new hepcidin-associated element named as HepERE was revealed. Evidences from electrophoretic mobility shift assay (EMSA) and surface plasmon resonance (SPR) assay demonstrated that the EE2-mediated down-regulation of OM-hep1 expression was associated with ERα binding to HepERE but not classical ERE. Taken together, a novel signaling pathway was revealed and the regulatory mechanism associated with the ERα and HepERE element on immunomodulation of OM-hep1 expression upon EE2 exposure was first reported here.

1. Introduction

The immune system of aquatic animals is particularly sensitive to pollutants [1], which probably enhances the susceptibility of fish to disease by interfering with their immune homeostasis. Even though no specific pathogen responsible for the disease has been determined, a general suppression in immune function and resistance against infections due to the presence of aquatic pollutants is therefore thought to be an important inducement to a higher infectivity of the disease in aquaculture [2,3]. However, little convincing evidence has been provided so far for the interaction between disease occurrence in marine animals and contamination of the culture environment. Given that, studies on the interaction between the toxic effects caused by typical environmental pollutant exposure, such as to 17β-estradiol (E2), 17α-ethinylestradiol (EE2), benzo [a]pyrene or bisphenol A (BPA), and the alterations of the immune status in marine animals have attracted much attention [4–9], a reasonable conclusion could have been expected based on the data accumulated from such studies.

* Corresponding author. State Key Laboratory of Marine Environmental Science, College of Ocean & Earth Science, Xiamen University, Xiamen, Fujian, 361102, China.
E-mail address: wkjian@xmu.edu.cn (K.-J. Wang).

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Among various efficient components of fish immune system, the antimicrobial peptides (AMPs), which are well known to play important roles in innate immunity through effective defense against various bacterial, fungal and viral invasion, are attracting much more attention. Hepcidin is a small, cationic, cysteine-rich antimicrobial peptide. It was originally identified from human plasma and urine [10,11] and has since been widely isolated and investigated in various species such as mammals, amphibians and fishes [12,13]. In human, hepcidin gene is reported dominantly expressed in liver and functions as an iron-regulatory hormone in the normal homeostatic iron regulation. Compared to human, fish hepcidin is abundant in liver as well as kidney [14,15]. In its role as an antimicrobial peptide, fish hepcidin are primarily associated with pathogens infection or inflammation. During inflammation, the hepcidin expression is usually mediated through the Janus kinase (JAK)/STAT pathway [16–18]. The transcriptional regulation of hepcidin by iron is involved in the bone morphogenetic protein/Sma- and Mad-related protein (BMP/SMAD) pathway [19,20]. Anemia or hypoxia negatively regulate hepcidin expression respectively through inhibiting the binding of C/EBPα and STAT3 to hepcidin promoter, or the inhibition of BMP/SMAD4-dependent signaling [21,22].

Interestingly, fish hepcidin can be modulated by environmental estrogenic pollutants like EE2 and BPA [4,7,23], suggesting that the expression of fish hepcidin might be regulated through different signal pathways. Previous study demonstrates that estrogen can regulate hepcidin expression through classical ERα/ERE pathway [24]. As reported in mammals, ERE binding sites are found existent in the 5′-flanking region of the hepcidin gene [25,26] and ERα binding to the ERE half-site participates in the negative regulation of E2-mediated human hepcidin transcription [25]. However, inconsistent observation is obtained using different experimental conditions like in hepatocytes where E2 induced hepcidin expression via the G-protein-coupled receptor 30 (GPR30)/BMP6-dependent signaling [27]. Those reports indicate the complexity of hepcidin regulation upon exposure to estrogen. However, less studies have been involved in elucidating the regulatory mechanism of fish hepcidin transcription upon environmental estrogenic pollutants exposure.

Marine medaka (*Oryzias melastigma*) is considered as a promising marine fish model for ecotoxicological studies due to its small size, short generation cycle, high spawning rate, and a wide range of salinity adaptation [28–31]. Thus the present study used *O. melastigma* and a typical environmental pollutant EE2 for the immunotoxic mechanism study. To investigate the immunotoxic effects of EE2 on marine medaka, the antimicrobial peptide hepcidin, which is a very important component in the fish innate immune system, was chosen for elucidating the transcriptional regulatory mechanism *in vivo* and *in vitro*. The hepcidin gene OM-hep1 has been identified in our previous study, exhibiting potent antibacterial, antitumor and antivirus activity [28,32].

2. Materials and methods

2.1. Animals, chemicals waterborne exposure and sample preparation

All animal procedures were carried out in strict compliance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the animal welfare and ethics committee of Xiamen University. The marine medaka (*O. melastigma*) fertilized eggs were kindly provided by the State Key Laboratory in Marine Pollution, City University of Hong Kong. Our laboratory has already established a self-propagating population of *O. melastigma* for more than six years. The fish were maintained in the breeding room in aerated 30 ± 1‰ artificial seawater at 6.5 ± 0.2 mg L^{-1} O_2, 25 ± 2 °C with a 14:10 light/dark cycle. Males were separated from females by visual morphological determination, and the healthy male medaka (5 months old) were chosen for the toxicological experiment.

After acclimated in 10-L glass tanks for three days, seventy-two male fish were exposed to EE2 (EE2-exposed group, 5, 500 ng/L) and DMSO (solvent control group, $V_{DMSO}/V_{seawater}$ = 1/20000) for 12 and 48 h, respectively. Ninety percent of the water in each tank was replaced daily with new aerated artificial seawater dosed with the appropriate amount of EE2 or DMSO. Each group of 24 fish were used. Twelve fish were collected at each time-point post EE2 and DMSO exposure, individually. The liver tissue randomly collected from three fish were pooled as one sample and thus there were four replicates (n = 4) at each dose of EE2. Then, the collected samples were frozen immediately in liquid nitrogen and stored at −80 °C before use. The fish were fasted during the exposure duration.

Based on the observation of OM-hep1 transcription upon EE2 exposure, the fish were first intraperitoneally injected with ER inhibitor ICI 182780 (1 mM) followed by 500 ng/L EE2 exposure for 12 h. Before injection, the fish were anesthetized using 0.025% MS-222. We used 27 fish in this experiment. The injection volume was 8.8 μL/fish (m = 1 mM × 8.8 μL × 606.77 g/mol ≈ 5.34 × 10^{-3} mg, weight = 0.53 ± 0.04 g/fish, final dose = m/weight = 10.075 mg/kg ≈ 10 mg/kg). The dosage of ICI 82780 used in fish was based on the dosage/body weight which is used in mice [33]. The control groups were injected with the same volume of DMSO diluted in sterile MilliQ. We strictly treated chemical reagents followed standard biosecurity. The used water with chemical exposure during our experiments was finally centrally collected and treated by the special department in Xiamen University.

EE2 (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mg L^{-1} stored at 4 °C; ICI 182780 (Tocris Bioscience) was dissolved in DMSO at 100 mM, and diluted into 1 mM using MilliQ before injection. Tricaine methanesulfonate (MS-222) anesthetic (Sigma-Aldrich) was dissolved in seawater and used at 0.025 g/L.

2.2. RNA isolation, reverse transcription and qPCR

Total RNA of the liver of adult fish was extracted using TRIzol Reagent (Invitrogen, USA) following the manufacturer's instructions. After being treated with RNase-free DNase I (Promega, USA to remove DNA contamination), total RNA quantification was measured using a Nanodrop 2000 spectrophotometer (Thermo), and RNA quality was verified by electrophoresis on ethidium bromide-stained 1.5% agarose gels. Then, 1 μg was reverse-transcribed into cDNA using a PrimeScript™ RT Reagent Kit (Takara). Several pairs of oligonucleotide PCR primers (seen in Supplemental Table 1) were designed according to conserved regions of myeloid differentiation marker 88 (MyD88), NF-κB (p65), JAK2 and STAT3 mRNA sequences from other fish, available in the National Center for Biotechnology Information. The qPCR analysis was performed using the fluorescent dye Power SYBR Green PCR Master Mix and ABI 7500 System as previously described [15]. Previous studies have provided evidence to prove the stability of ribosomal protein L7 (RPL7) gene expression in different treatment conditions, specifically in endocrine disrupting chemicals (EDCs)-exposed experiments [34,35]. Therefore, RPL7 was used as endogenous control in our EE2-exposed experiments. The specific primers used were listed in Supplementary Table 1. The relative expression levels (fold change) of the tested genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.3. OM-hep1 promoter cloning and recombinant plasmid constructions

The full-length genomic DNA and cDNA sequence were obtained by our previous work [28]. Genomic DNA was isolated from the liver of *O. melastigma* using a DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's instructions. The Genome Walking Kit (TaKaRa, Japan) was used to isolate the 5′-flanking region of the OM-hep1 gene. Based on the published sequences, several designed gene-specific primers as well as four shorter arbitrary degenertes (AP1, AP2, AP3, AP4) were used to amplify the upstream region of OM-hep1 gene via nested PCR. Specific PCR products were purified and sequenced (Supplementary Table 1). Transcription factor binding sites (TFBS) in the obtained 5′-

flanking region of OM-hep1 gene were predicted using the Genomatix/MatInspector web site (https://www.genomatix.de/).

The 5′-flanking region sequence of OM-hep1 was amplified from genomic DNA using the specific primers with *Xho* I and *Pst* I restriction sites, and was then inserted into the *Xho* I and *Pst* I digested promoterless vector (pEGFP-1), resulting a recombinant plasmid pEGFP-Hep1. Several immune-associated TFBS, such as STAT3 and NF-κB, and hormone-associated TFBS, such as ERE and ERRE, which were respectively involved in the inflammation-mediated and hormone-mediated hepcidin regulation, were screened. Based on the position of the above putative binding sites, ten serial deletion sequence fragments were obtained using PCR and respectively constructed into *Kpn* I/*Xho* I sites of PGL3-basic vector (Clontech), containing a firefly luciferase gene (Supplementary Table 2). Then, the mutants of the novel HepERE element designed based on the OM-hep1 promoter sequence were constructed through overlap extension PCR to confirm the regulating roles of the HepERE element. All the constructed plasmids were verified by sequencing. Finally, their promoter activities were determined using dual-luciferase assays.

2.4. Cell culture, transfection and luciferase assay

Endothelial progenitor cells (EPC, *Cyprinus carpio*) and Human Embryonic Kidney 293 T cells (HEK293T) were cultured following the protocol. EPC (stored in our laboratory) were maintained in phenol red free L-15 medium (Gibco), supplemented with 10% charcoal/dextran stripped fetal bovine serum (FBS, Gibco), penicillin (100 μg/mL) (Gibco), and streptomycin (100 μg/mL) (Gibco). They were cultured at 25 °C without any CO_2. The HEK293T cells (stored in our laboratory) were cultured in DMEM medium containing 10% FBS (Gibco), penicillin (100 U/mL) (Gibco) and streptomycin (100 μg/mL) (Gibco). HEK293T cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

EPC cells were seeded at a density of 1×10^5 cells/mL in a 48-well plate overnight. The recombinant pEGFP-Hep1 plasmid was transfected into EPC cells with Lipofectamine 2000 (Invitrogen) at the optimal ratio of 0.4 μg: 1 μL (plasmid: transfection reagent), while pEGFP-1 was used as a negative control. To determine the EGFP expression, images of the transfected EPC cells were collected with fluorescence microscopy (Nikon AZ100) at 24 h post-transfection, For the luciferase activity assay of recombinant constructs, EPC cells were co-transfected with 0.4 μg series plasmids and 2 ng of internal control renilla luciferase plasmid pRT-TK per well using Lipofectamine 2000, the empty vehicle PGL3-basic was used as negative control. The internal control plasmid pRT-TK was used for normalization of transfection efficiency. After being transfected for 24 h, cells were starved in serum-free L-15 medium for 24 h and then washed with 1 × HBSS twice before collection in lysis buffer. The cellular extracts were analyzed for luciferase activity using the Dual-Luciferase® Reporter Assay System (Promega) on a GloMax™20/20 luminometer (Promega).

To investigate the effect of EE2 on OM-hep1 promoter, EE2 (or other estrogenic compounds E2, BPA) at a final concentration of 5×10^4 ng/L was added to EPC medium after the cells starved for 24 h, an equal amount of DMSO (less than 0.005%) was used as control. Cells were subsequently harvested as described above. Each experiment was performed in triplicate and repeated at least three times.

2.5. Electrophoretic mobility shift assay

The ORF (Open Reading Frame) sequence of OM-ERα was constructed into pCMV-HA vector. HEK293T cells were transfected with pCMV-HA-ERα for 48 h. Nuclear extracts were prepared using nuclear extraction kits (Viagene Biotech Inc.). An oligonucleotides probe containing the full sequence of HepERE element within the hepcidin promoter was biotin labeled by Viagene Biotech Inc. Equal amounts of labeled and complementary oligonucleotides were gradually allowed to cool to room temperature to allow the annealing of double-stranded oligonucleotides.

EMSA was performed using Non-Radioactive EMSA Kits with Biotin-Probes (Viagene Biotech Inc.). (1) For the binding reaction, 3.0 μL of the nuclear protein extracts, 1.5 μL 10 × binding buffer, 1.0 μL Poly (dI:dC) and 9.0 μL dH_2O were mixed well and incubated for 20 min at room temperature, then 0.5 μL biotin-labeled probe biotin-HepERE (6.6 nM) was added into the mixture and reacted for at least 20 min; Negative reaction was performed under identical condition on the binding reaction by adding 3.0 μL dH_2O to replace nuclear protein extracts; (2) For the competition reaction, 3.0 μL of the nuclear protein extracts, 1.5 μL 10 × binding buffer, 1.0 μL Poly (dI:dC), 1.5 μL unlabeled cold probe (265 nM, 40-fold over that of biotin-probe) and 7.5 μL dH_2O were mixed well and incubated for 20 min at room temperature, then 0.5 μL biotin-HepERE (6.6 nM) was added into the mixture and reacted for at least 20 min; (3) For the supershift EMSA reaction, 3.0 μL of the nuclear protein extracts, 1.5 μL 10 × binding buffer, 1.0 μL Poly (dI:dC) and 8.0 μL dH_2O were mixed well and incubated for 20 min at room temperature, followed by adding 0.5 μL biotin-HepERE (6.6 nM) into the mixture and reacting for at least 20 min; then 1.0 μL anti-OM-ERα antibody (prepared by Abmart Inc. with OM-ERα amino acid sequence provided by our laboratory) was added into the mixture and reacted for at least 30 min.

After that, samples were separated using nondenaturing 6.5% polyacrylamide gel electrophoresis and transferred to a Hybond-N nylon membrane (GE Healthcare, Piscataway, NJ). After transfer, the nylon-membrane was removed and rinsed in 0.5 × TBE. Next, the membrane was placed in a UV linker to crosslink DNA at 600 mJ/cm^2 for 4 min. The membrane with immobilized and bound DNA was blocked and applied with streptavidin-horseradish peroxidase conjugate for 20 min, respectively. After thorough washing with 1 × washing solution, the membrane was equilibrated with 1 × equilibrate buffer for 5 min, and then placed into a Chemiluminescence Imager, with 2 mL chemiluminescent substrate solution added onto the surface of the membrane, and the EMSA result detected.

2.6. Surface plasmon resonance measurements

Surface plasmon resonance (SPR) was performed using a Biacore T200 system (GE Healthcare UK Ltd.) to analyze nucleic acid-to-protein interactions. To monitor the interactions of HepERE with human ERα (hERα), the biotin-HepERE was immobilized on a Sensor Chip SA until the calculated amount of DNA that gave a maximum HepERE binding capacity of 300 RU was bound. Various concentrations (2.34375, 4.6875, 9.375, 18.75, 37.5, 75, 150 nM) of purified hERα were then applied to the HepERE immobilized Sensor Chips for 120 s in 10 mM HEPES, pH 7.4, containing 150 mM NaCl, 3 mM EDTA and 0.05% (v/v) surfactant P20 at a flow rate of 30 μL/min. The chip surface was then regenerated with 5 mM NaOH at a flow rate of 10 μL/min for 45 s. This assay was carried out at room temperature. The apparent equilibrium constants (K_D) for HepERE with hERα was calculated from the association and dissociation curves using BIA evaluation software (GE Healthcare UK Ltd.).

2.7. Statistic analysis

All results were presented as mean ± S.E. Statistical analysis was performed using SPSS software (version 13). One-way analysis of variance (ANOVA) and independent-samples *t*-test and were used to determine the expression difference between or within groups. Difference was accepted at $p < 0.05$.

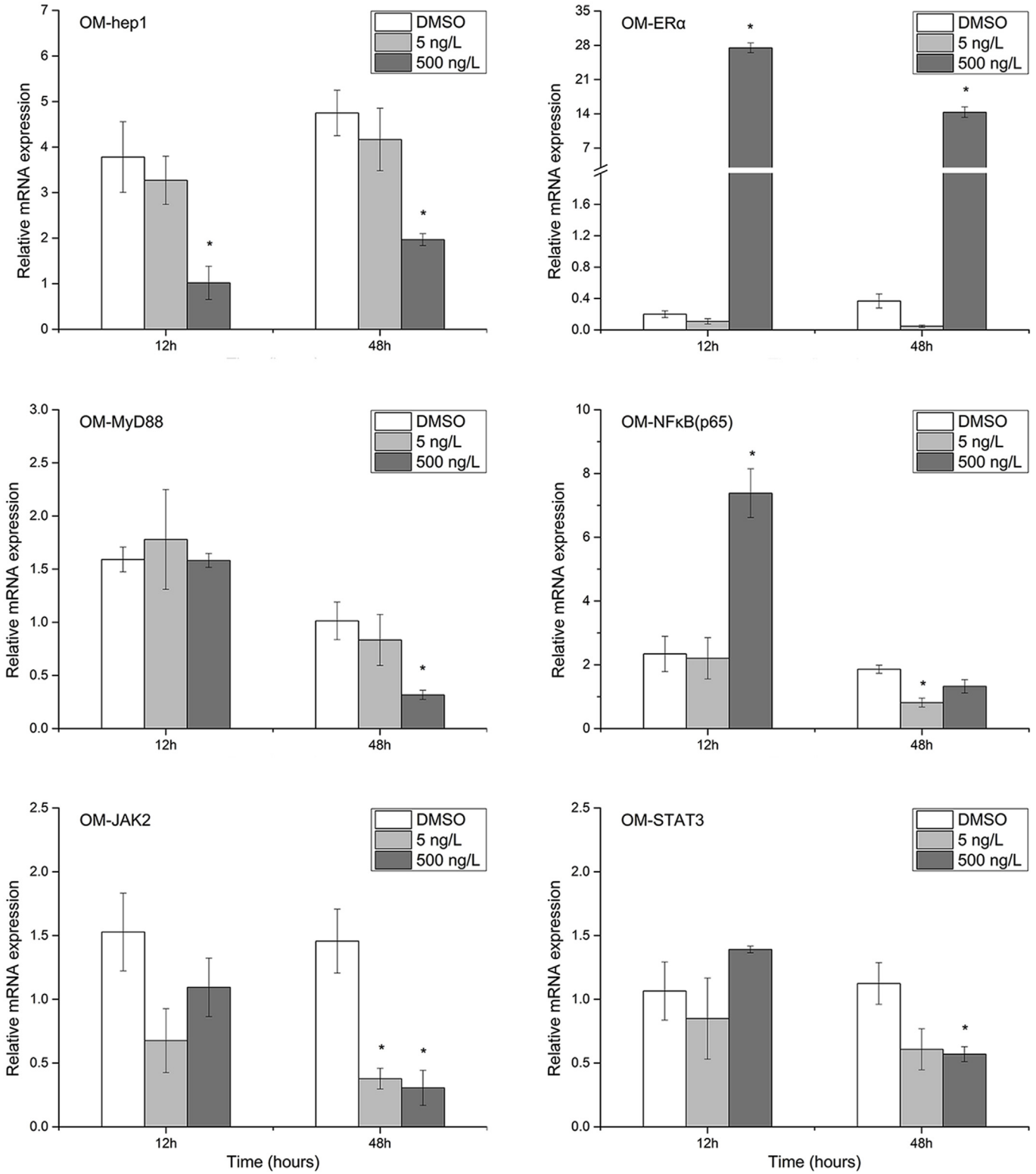

Fig. 1. Effects of EE2 exposure on gene expression of immune-associated genes (OM-hep1, OM-JAK2, OM-STAT3, OM-MyD88, OM-NF-κB(p65)) and estrogen receptor α OM-ERα in the liver of male *O. melastigma* (5 months old). Male medaka liver were sampled at 12 and 48 h upon EE2 exposure, each liver sample was pooled from 3 fish, n = 4. The gene expression fold change was normalized to *RPL7* and denoted relative to that of the solvent control group (0.005% DMSO, v/v). Data were analyzed using one-way ANOVA statistical analysis and expressed as mean ± S.E. (n = 4, 12 fish). The asterisk indicates statistically significant differences ($p < 0.05$).

3. Results

3.1. The regulation of OM-hep1 upon EE2 exposure involved in OM-ERα

The hepcidin gene OM-hep1 was obtained from marine medaka (*O. melastigma*) in our laboratory previously. In this study, we isolated the partial cDNA sequence of four immune-related genes (OM-MyD88, OM-NF-κB(p65), OM-JAK2 and OM-STAT3). It was noted that the expression of all of the tested immune-related genes including OM-hep1, OM-MyD88, OM-NF-κB(p65), OM-JAK2, OM-STAT3 were affected upon EE2 exposure (Fig. 1). Interestingly, we found that the OM-hep1 expression significantly deceased upon 500 ng/L EE2 exposure at 12 and 48 h, whereas the OM-ERα expression was markedly up-regulated, showing possible involvement of OM-ERα pathway in EE2-exposed OM-hep1 expression. Thus, OM-hep1 was chosen for investigating the molecular mechanism of immunotoxicity upon EE2 exposure.

3.2. Inhibition of OM-ERα blocks the EE2-mediated down-regulation of OM-hep1 expression

Based on the alterations of OM-hep1 and OM-ERα, we speculated that OM-ERα might be involved in the EE2-mediated regulation of OM-hep1 transcriptional expression. To assess this assumption, pre-injection of the ER inhibitor ICI 182780 was carried out followed with EE2 exposure, resulting in a notable observation that the inhibition of OM-hep1 was effectively blocked by ICI 182780 (Fig. 2A). In addition, the significant up-regulation of both OM-ERα and biomarker gene OM-*vtg*1 were efficiently abolished, implying a successful application of ICI 182780 (Fig. 2B and C).

3.3. Bioinformatic analysis of the promoter region of OM-hep1

To investigate the transcriptional regulatory mechanism of OM-hep1 upon EE2 exposure, a 2558 bp length of the 5′-flanking region of the OM-hep1 gene (GenBank no. MF774223) was successfully isolated using genomic walker technology. The 5′-flanking sequence of the OM-hep1 gene was then examined using MatInspector, and several putative TFBS were identified. The initiation codon ATG was defined as position +1. There were several hormone-associated elements such as ERE, ERRE and androgen responsive element (ARE) and immune-associated elements such as STAT3 and NF-κB binding sites (Fig. 3A) screened with the OM-hep1 promoter. We defined two regions in OM-hep1 promoter, the proximal region (from −716 to −137 bp, located near the transcription start site) and the distal region (from −2558 to −2202 bp). In the proximal region, one putative imperfect ERE-like element (AGGA GGTCAaaaTTTCCCCAGTG) was flanked with four NF-κB binding sites (NF-κB_{1-4}) and two STAT3 binding sites ($STAT3_{1-2}$) (Fig. 3B). In the distal region, there was a hormone-related ERRE element, and another ERRE existed between the proximal and distal regions (Fig. 3B).

3.4. Identification of functional HepERE in the promoter region of OM-hep1

The recombinant plasmid pEGFP-Hep1 was successfully constructed with the EGFP gene under the control of the OM-hep1 promoter. After the pEGFP-Hep1 was transiently transfected into EPC cells, it was observed that the OM-hep1 promoter region could drive EGFP expression, exhibiting promoter activity (Fig. 4A). To further investigate which element was involved in the transcriptional activity of OM-hep1, ten recombinant plasmids were generated by continuous deletion of the OM-hep1 promoter sequence based on the position of putative immune-associated and hormone-associated TFBS. The results showed that the luciferase activity of recombinant plasmid pGL3-(-315/+27) was significantly higher than all other recombinants after being transfected into EPC cells (Fig. 4B), indicating that the immune-associated NF-κB and STAT3 elements were not involved in OM-hep1 regulation and the region between −315 and −284 bp was functional in the OM-hep1 promoter.

In view of the fact that both the predicted immune-associated elements NF-κB and STAT3 and hormone-associated ERRE and ARE did not show any significant activities (Fig. 4B), we further carried out

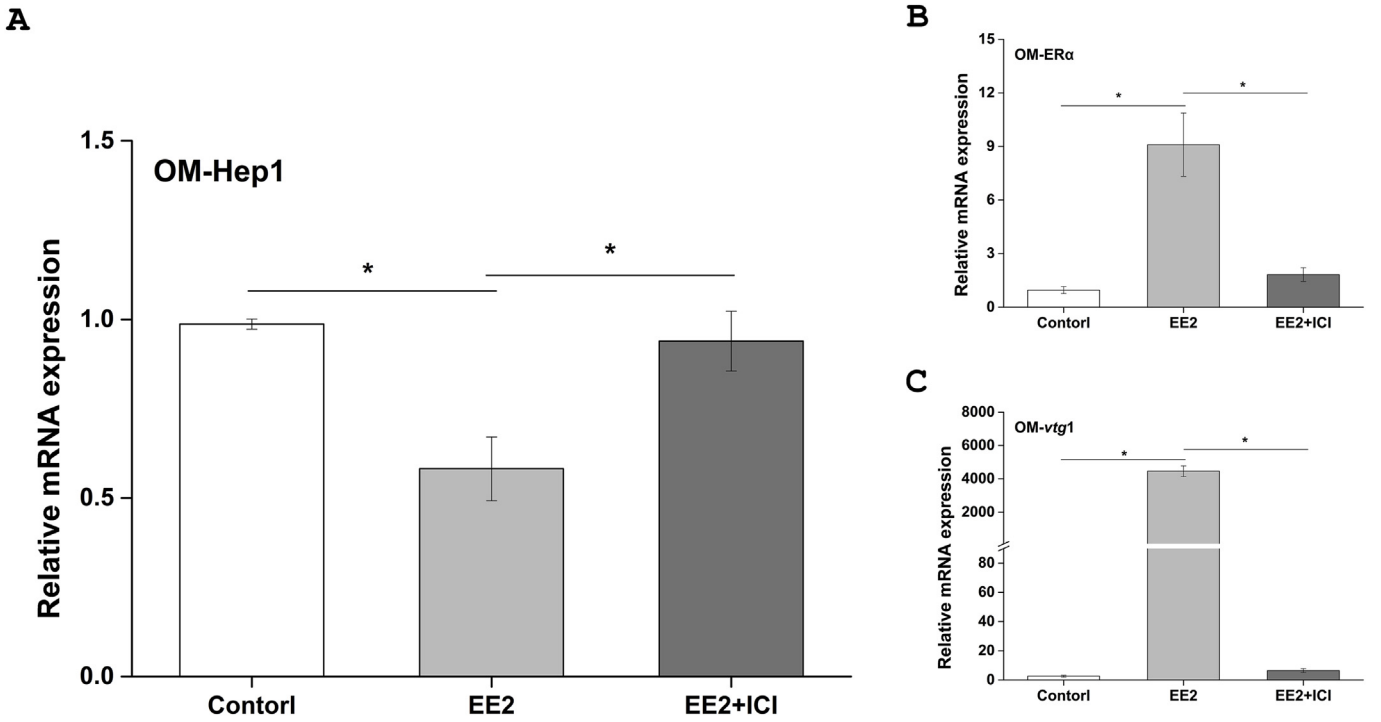

Fig. 2. Inhibition of OM-ERα blocked the EE2-mediated down-regulation of OM-hep1 expression. Pre-injection of ICI 182780 abolished EE2-mediated down-regulation of OM-hep1 **(A)** as well as up-regulation of OM-ERα **(B)** and OM-*vtg*1 **(C)** in liver at 12 h (n = 3). Data were analyzed using the independent T-test and expressed as mean ± S.E. (n = 3). The asterisk indicates significant difference ($p < 0.05$).

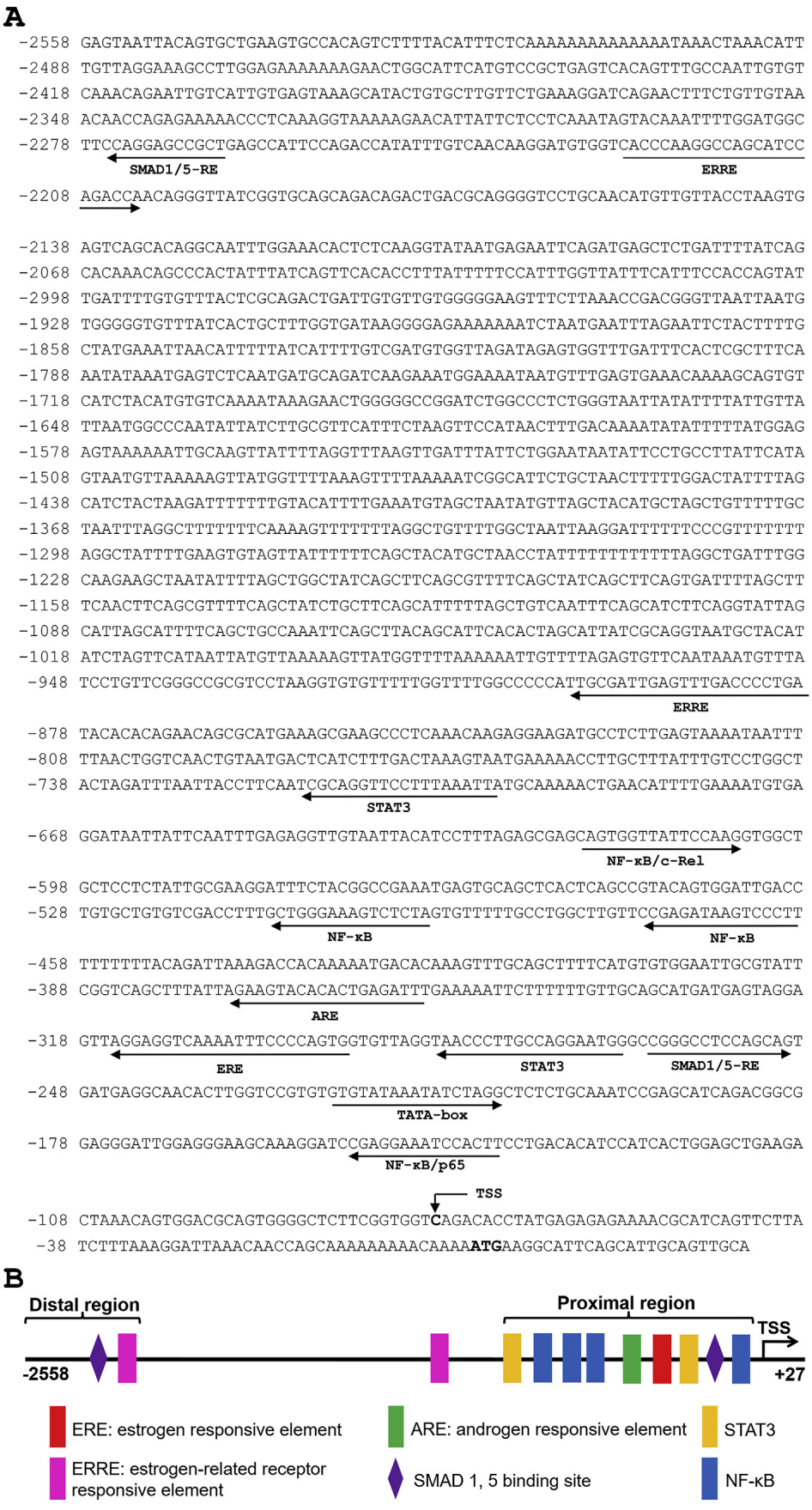

Fig. 3. Sequence analysis of the 5′-flanking region of the OM-hep1 gene. **(A)** The locations of potential transcription factor binding sites (TFBS) of ERE, ERRE, ARE, NF-κB, STAT3, TATA-box, and SMAD1/5 binding site are underlined by arrows. The base A of OM-hep1 start codon (ATG) is at the +1 position. **(B)** Diagram of the 5′-flanking region of the OM-hep1 gene, with multiple predicted immune-related and hormone-related TFBS located in the proximal region and distal region.

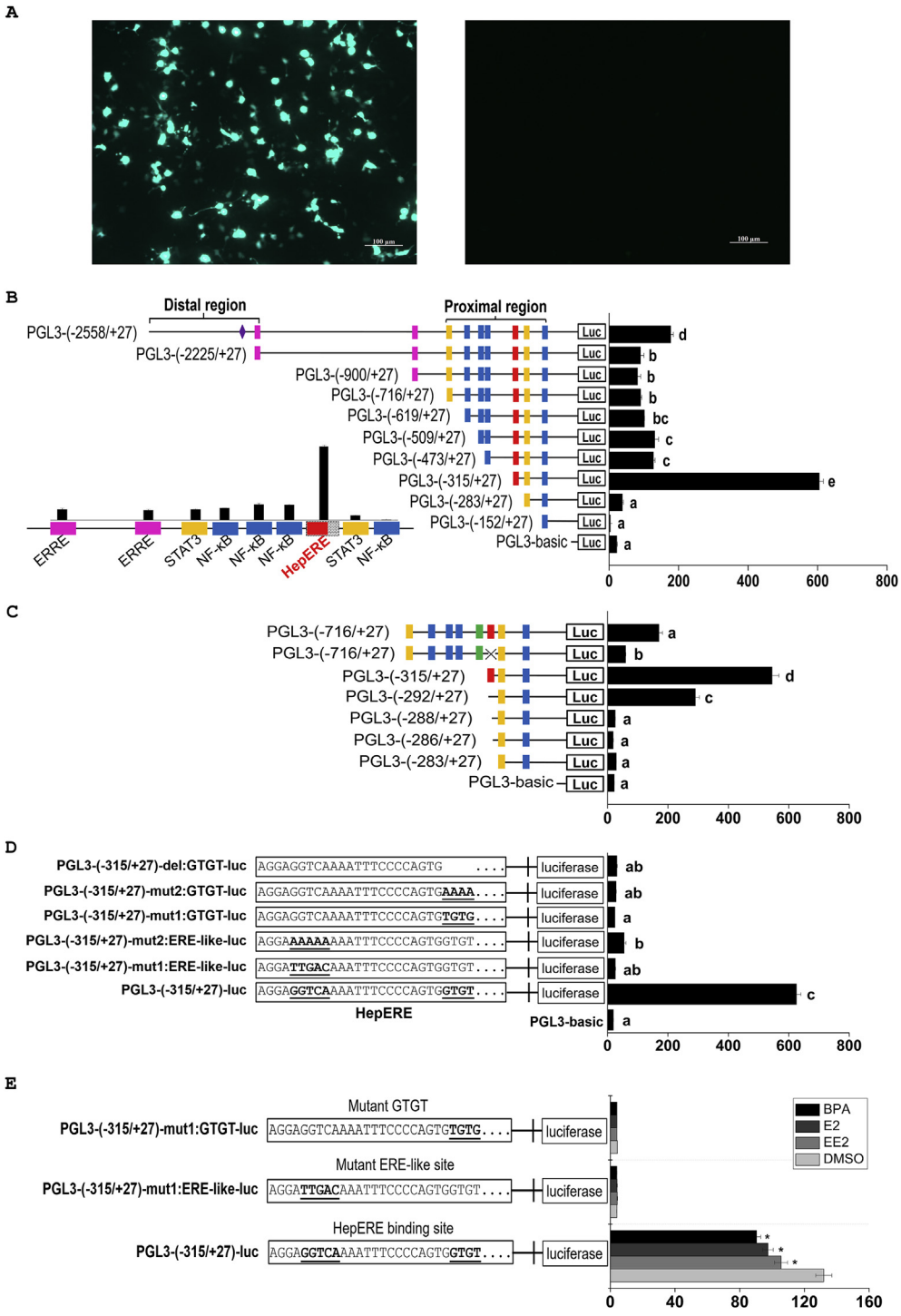

Fig. 4. Identification of the functional HepERE element in the promoter region of OM-hep1. **(A) left**, Fluorescence image of EPC cells with transfected pEGFP-Hep1; **right**, Control with transfected pEGFP-1, scale bar = 100 μm. EPC cells were transfected with 0.4 μg recombinant pEGFP-Hep1 plasmid or promoterless pEGFP-1 plasmid, and were imaged by fluorescence microscopy at 24 h post transfection. **(B)** Serial 5′-deletion and luciferase activity analysis of OM-hep1 promoter. **(C)** Functional analysis of the putative key region ranging from −315 to −284 bp by serial deletion. Variable promoter activities ($p < 0.05$) were calculated using one-way ANOVA and denoted by latters (a, b and c). The dotted rectangular box on the left is a visual representation deduced from **(B)** and **(C)**. **(D)** Functional analysis of the mutants of the novel HepERE element (−315 to −289 bp). The sequences of the HepERE element and designed mutant sequences based on PGL3-(-315/+27)-luc are shown on the left. The results were calculated using one-way ANOVA method. * indicate significant difference ($p < 0.05$) compared with the control plasmid PGL3-(-315/+27)-luc. Each bar represents the mean ± S.E. (n = 4). EPC cells were co-transfected with 0.4 μg series plasmids or PGL3-basic (negative control) and 2 ng of internal control plasmid pRT-TK per well using Lipofectamine 2000. After being transfected for 24 h, cells were starved in serum-free L-15 medium for 24 h. Then, they were detected using dual-luciferase reporter assay system. **(E)** Effect of the mutation of the HepERE element in EE2, E2, BPA-challenge promoter activity of OM-hep1. The mutations were based on the recombinant plasmid PGL3-(-315/+27)-luc. The transfection progress was the same as described above, however, EE2 (E2, or BPA) at final concentration 5×10^4 ng/L or equal amount of DMSO (less than 0.005%, used as control) was individually added to the medium after EPC cells starved for 24 h. The relative luciferase activity was detected at 24 h post-challenge of EE2. The asterisk indicates significant difference compared with DMSO-treated control ($p < 0.05$). All the results were from at least three independent experiments.

studies on exploring new elements located at the region ranging from −315 to −284 bp, because of the highest activity in this region. The results of sequential deletions assay in Fig. 4C showed that the luciferase activity of recombinant pGL3-(-288/+27) was similar to pGL3-(-283/+27), suggesting that −315 to −289 bp was crucial for the high promoter activity of OM-hep1. Therefore, this region (−315 to −289 bp) was defined as a new key transcriptional regulatory element of the OM-hep1 promoter, named as HepERE. HepERE consists of a putative ERE-like element (23 bases) and an adjacent down-streamed four bases motif GTGT. Interestingly, if the ERE-like element was deleted maintaining intact the GTGT motif, the recombinant pGL3-(-292/+27) still possessed a relatively high luciferase activity with almost half of the pGL3-(-315/+27) (Fig. 4C), suggesting that ERE-like together with the GTGT motif were equally important to HepERE. To further assess the importance of the putative ERE-like and GTGT motif, mutations of the bases of substitution assays were applied in our study. It was demonstrated that replacement with the core bases of either ERE-like (GGTCA) or GTGT motif (GTGT) could inactivate the transcriptional activity of the OM-hep1 promoter (Fig. 4D). The overall results suggested that the determined HepERE element contributed to the transcription of the OM-hep1 gene in marine medaka liver.

To further confirm whether the HepERE was necessary for the response to the treatment of EE2 or other estrogenic compounds (such as E2, BPA) in the OM-hep1 promoter activity, wild type plasmid pGL3-

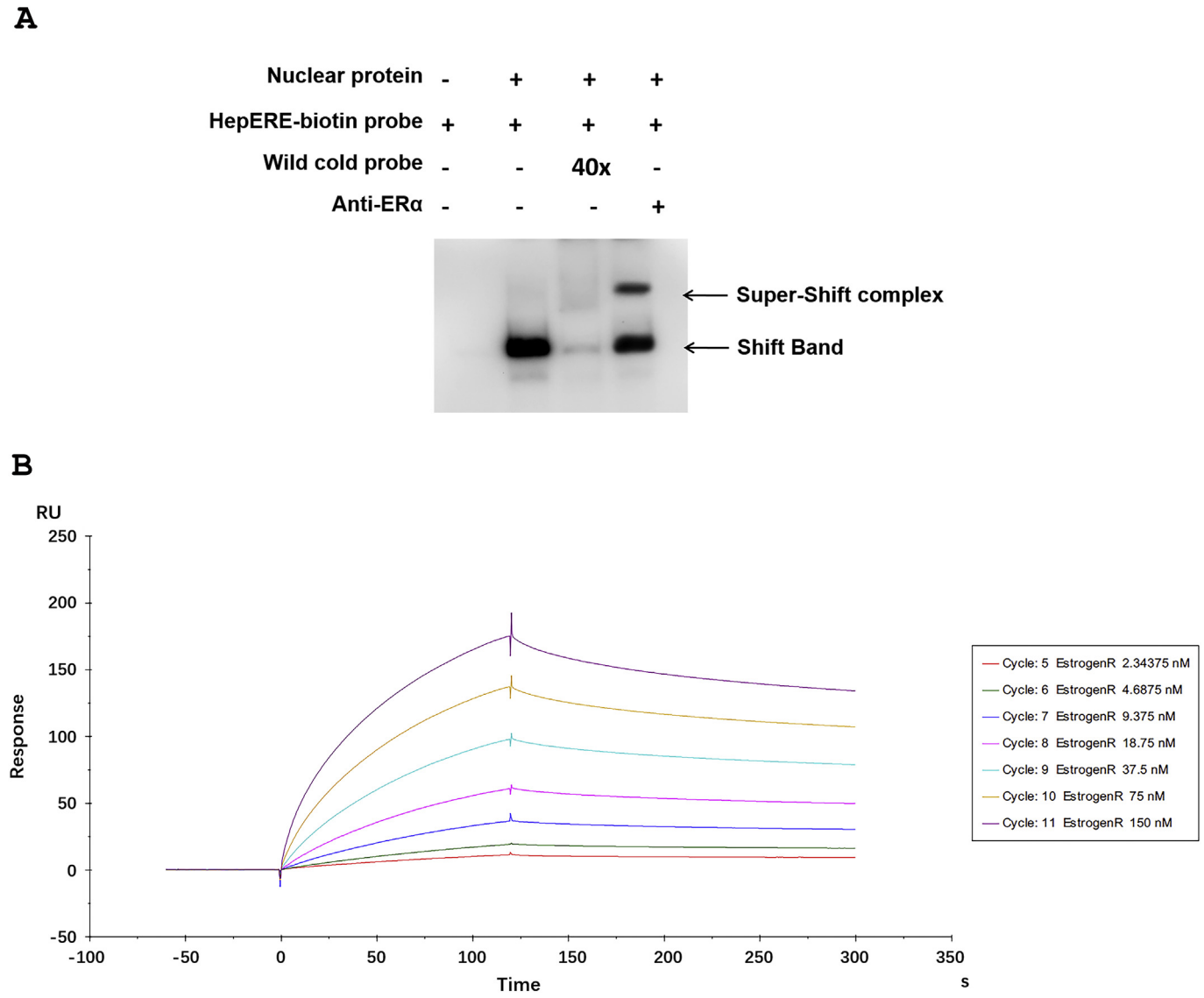

Fig. 5. Analysis of ERα protein binding to the HepERE element. **(A)** EMSA analysis of the binding of OM-ERα to the HepERE element in OM-hep1 promoter. Nuclear proteins were isolated from the OM-ERα-over-expressed HEK293T cells that transfected with pCMV-HA-ERα for 24 h and incubated with biotin-labeled oligonucleotides (HepERE). For competition EMSA, a 40-fold excess of unlabeled wild cold probes was added during the pre-incubation period. For supershift EMSA, anti-ERα antibody was added. The arrows indicate supershift, HepERE binding complex. **(B)** SPR analysis of the *in vitro* binding of hERα protein to HepERE element. The SPR sensorgrams for hERα association and dissociation are shown from sensor-immobilized 27-base pair sequence (HepERE).

(-315/+27), and the ERE-like mutant plasmid pGL3-(-315/+27)-mut:ERE-like or GTGT mutant plasmid pGL3-(-315/+27)-mut:GTGT, were transiently transfected into EPC cells. The luciferase activity of pGL3-(-315/+27) was significantly decreased with each treatment of three estrogenic compounds (Fig. 4E). More importantly, mutation of the ERE-like or GTGT motif significantly nullified the pGL3-(-315/+27) promoter activity, which led to the absence of the response of this fragment of promoter activity to EE2, E2, and BPA treatment.

3.5. ERα protein binds to HepERE with high affinity

To determine whether the HepERE on the OM-hep1 promoter could be recognized with OM-ERα, EMSA was performed using biotin-labeled oligonucleotides corresponding to HepERE of the OM-hep1 gene. Recombinant plasmid pCMV-OM-ERα was constructed in our study. The OM-ERα protein was successfully over-expressed in HEK293T cells after they were transfected with pCMV-OM-ERα for 24–72 h. The nuclear protein extracts were obtained at 48 h after transfection with a concentration of 1.86 μg/μL using the bicinchoninic acid (BCA) method. The results in Fig. 5A show that the nuclear protein (implying the OM-ERα protein) could efficiently bind to biotin-labeled DNA probe (lane 2, from left to right) and the binding was competed by adding a 40-fold excess of unlabeled wild cold HepERE probe (lane 3). More importantly, an anti-OM-ERα antibody caused a supershift of the protein-DNA binding complex (lane 4). Collectively, these EMSA data demonstrated that the HepERE could bind to OM-ERα protein. To further verify these results of EMSA and explore the binding affinity between HepERE and OM-ERα protein, DNA/protein-binding assay using SPR was carried out in the present study. Since there was failure of prokaryotic expression concerning the OM-ERα protein *in vitro*, hERα was applied in SPR performance because of the high homology and conservative evolution of the DNA binding domain in ERα. Biotin-labeled HepERE was first immobilized on a Sensor Chip SA until the binding capacity reached 300 RU. The values of association rate constant (Ka) and dissociation rate constant (Kd) were 1.377×10^5 $(Ms)^{-1}$ and 0.001182 s^{-1}, respectively. The dissociation constant (K_D) was calculated as Kd/Ka ≈ 8.6 nM, which indicated high-affinity recognition of the HepERE by the hERα protein (Fig. 5B).

4. Discussion

It was clear from our study that an environmental pollutant EE2 had a toxic effect not only on the endocrine system but also on the immune system of a fish. The exposure of EDCs is known to cause toxic effects on the immune system of marine animals but no report until now has revealed the underlying molecular mechanism related to EDCs immunotoxic effects. As observed in China, many coastal areas are more or less contaminated with environmental pollutants, and some researchers think that this kind of aquatic environment could not be suitable to raise fish or other marine economic animals. The simple reason is that a decrease of resistance or survival rates in marine animals may result from this contamination, which generates toxic effects on the marine animals by inducing physiological changes or altering immune homeostasis. Previous studies attribute a general suppression of immune function in marine fish to the presence of aquatic EDCs [2,3]; and EDCs can regulate the immune response in fish [4,6–8,36]. However, the underlying molecular mechanism has not yet been clarified, owing to the difficulty in choosing applicable model animals or lack of suitable immunologic factors to be evaluated. We realized that elucidation of the signaling pathway would much facilitate our understanding of the molecular mechanism involved upon exposure to EDCs. In the present study, the antimicrobial peptide hepcidin OM-hep1 was chosen as an immunologic factor to investigate the molecular mechanism associated with the EE2-exposed immunotoxic effect, mainly because many studies on hepcidin and its variants in different fish species have been carried out by our group for more than 15 years, as well as the fact that some of the knowledge acquired is concerned not only with the antimicrobial activities of hepcidin, but also with the immunotoxicity associated with hepcidin in fish upon exposure to E2, EE2, or BPA [4,5,14,15,28,32,37–39].

EE2 is known to regulate endogenous hormone-related gene transcription via ERs, such as vitellogenin (*vtg*) gene, which is thought to be a usable biomarker of endocrine disruption by xenoestrogen [40,41]. In addition, it has a similar property as reported that the homeostasis of the fish immune system can be modified by xenoestrogen [4–7,42–45]. For instance, EE2 exposure compromises both the cellular and humoral immune systems of rainbow trout in a tissue-dependent manner [7], and modulates the immune response of gilthead seabream involving in increasing interleukin-1β, interleukin-6, etc. [6]. The modulation of hepcidin transcription expression by xenogenous estrogen was also screened among the EDCs-affected immune-related genes. A previous study on largemouth bass reveals that E2 exposure leads to down-regulation of hep1 transcription but not hep2, whereas the up-regulation of hep-2 expression induced by bacterial challenge can be blocked by E2 [46]. In our previous study, two hepcidin variants (OM-hep1 and OM-hep2) are identified in marine medaka, both of which show a rapid and remarkable up-regulation in liver and spleen with the challenge of *Vibrio parahaemolyticus* [28]. Similar to the observation on largemouth bass, liver OM-hep1 alone was down-regulated upon EE2 exposure at 12 and 48 h, but OM-hep2 was not significantly affected by EE2 exposure in our study (data not shown). Thus, OM-hep1 was very available to be used as a candidate immune factor for investigating the EE2-mediated immunotoxic mechanism on marine medaka.

OM-ERα was significantly up-regulated upon 500 ng/L EE2 exposure in the present study. Meanwhile, the OM-hep1 transcription was down-regulated. Coincidentally, the down-regulation of OM-hep1 expression was effectively restrained by ER inhibitor ICI 182780, implying a possible direct involvement of OM-ERα in the transcriptional expression of OM-hep1 upon EE2 exposure. However, in order to elucidate the EE2-mediated regulatory mechanism of the OM-hep1 gene, another key factor of the transcriptional regulatory element on the promoter of OM-hep1 must be revealed further.

In our study, several immune-associated transcriptional factor binding sites, such as STAT3 and NF-κB elements, were screened in the promoter of OM-hep1 (Fig. 3), and are in the promoter of human and mice hepcidins [10,11,47] for their conservation among different species. Many previous studies on hepcidins have revealed that the hepcidin mRNA expression is individually regulated through several distinct signaling pathways, as it can be transcriptionally induced by infectious/inflammatory stimuli or iron overload, and suppressed by anemia, iron deficiency or hypoxia [16,19,21,48]. For infectious/inflammatory stimuli, the immune responses are normally mediated through the JAK2/STAT3 pathway [16–18]. For iron metabolism, the reverse effect on hepcidin transcriptional expression is mainly mediated through the BMP/SMAD pathway [19,20]. Under conditions of either anemia or hypoxia, hepcidin expression is down-regulated through the ROS-mediated prevention of C/EBPα and STAT3 binding to hepcidin promoter, or the inhibition of BMP/SMAD4-dependent signaling [21,22]. Thus, we first tried to investigate whether OM-hep1 transcriptional expression was regulated through the immune-associated signaling pathways such as the NF-κB and JAK/STAT3 pathways. Although several immune-associated genes are involved in the NF-κB or JAK/STAT3 signaling pathways upon EDCs exposure [4,49,50], our study on hepcidin transcription failed to obtain a similar conclusion, suggesting that there was no obvious involvement of immune-related signaling pathways in the transcriptional regulation of OM-hep1 upon EE2 exposure. Dual-luciferase assay showed that several NF-κB and STAT3 binding sites predicted on the OM-hep1 promoter were inactivated in OM-hep1 promoter activity, implying these immune-associated signaling pathways were not directly involved in EE2-mediated OM-hep1 regulation.

Furthermore, we focused on the ERα/ERE signaling pathway since this pathway is normally involved in E2/EE2-mediated gene regulation and some related information shows that the transcription of hepcidin in mammals [26] and fish [46] can be down-regulated upon exposure to the xenogenous E2 or EE2. A previous study using HepG2 and HuH7 cell lines treated with an xenogenous E2 reports that the hepcidin transcription is regulated through the interaction of the ERα and ERE element (GGTCAnnnTGACC), which exists in the human hepcidin promoter [26]. Following this report, a conclusion seems to be derived that the immunotoxic effect caused by xenoestrogen can be mediated through the ERα/ERE signaling pathway, during which the OM-hep1 transcriptional regulation might be mediated upon EE2 exposure. However, our study did not obtain a similar result. We found that the ERE-like and ERRE elements on the promoter of OM-hep1 were weakly activated in the promoter activity, meaning that the most effective transcriptional regulatory elements might not have been revealed.

Unexpectedly, we discovered that a region between −315 and −284 bp on the promoter of OM-hep1 showed a high luciferase activity. After further sequential deletion analysis, a novel transcriptional regulatory element located ranging from −315 to −289 bp (AGGAGGTCAaaaTTTCCCCAGTGGTGT) on OM-hep1 promoter was determined, and defined as HepERE. The HepERE consisted of a putative ERE-like element (23 bases) plus an adjacent down-streamed four bases motif GTGT. The potential ERE-like element present in HepERE attracted us to make further tests on its possibly lone role in regulating hepcidin expression without GTGT but this failed (Fig. 4D). Furthermore, replacement of either the core bases (GGTCA) of the ERE-like element or the GTGT motif resulted in an inactivation of OM-hep1 transcription expression. The confirmed result was obtained using a HepERE-intact construct with which EE2 treatment down-regulated the luciferase activity, whereas no change was observed with the HepERE-mutant constructs. Furthermore, it was confirmed that the ERE-like element linked with the GTGT motif became the core transcriptional regulatory element to regulate OM-hep1 transcription, showing an inseparable and synergistic action (Fig. 4). Therefore, HepERE was likely to be the pivotal element functional in the signaling pathway by regulating the OM-hep1 transcriptional expression when exposed to EE2. In order to investigate the differences in the activation of the promoter between EE2 and others estrogenic compounds, the promoter experiments upon E2 and BPA exposure was carried out. The result showed

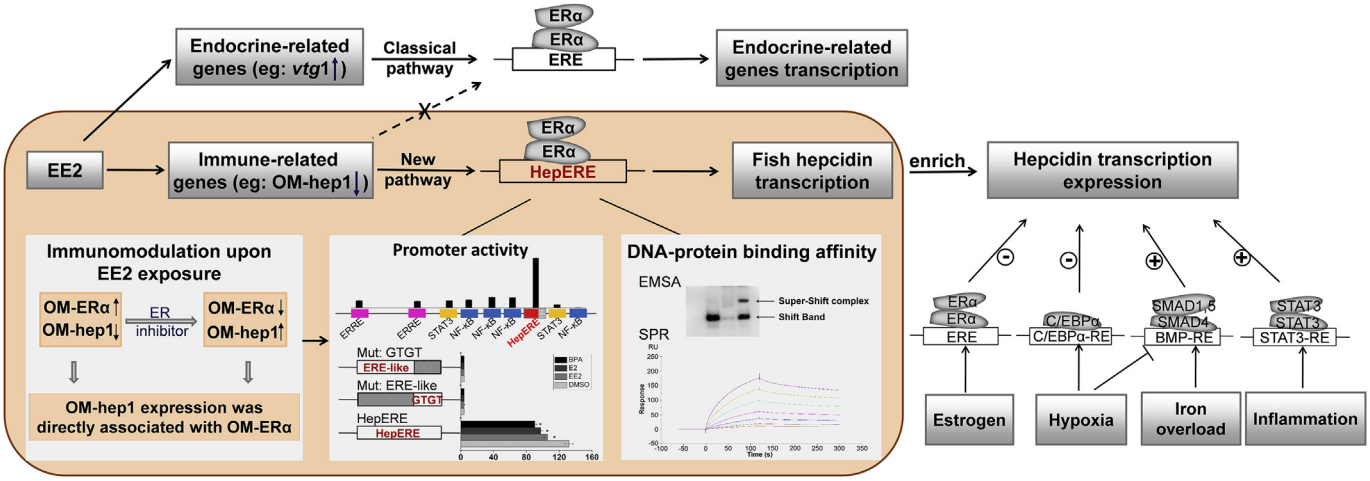

Fig. 6. Schematic representation of the classical and our new revealed pathways of EE2-mediated gene transcription [24].

that the estrogenic activity of either E2 or BPA was somewhat lower than that of EE2. Thus, it was possible that like EE2, the E2 and BPA exposure or possible other estrogenic compounds was involved in the similar pathway with a novel transcriptional regulatory element HepERE as reported in the study, however, this is a preliminary finding and needs much more work to confirm this conclusion.

Finally, the *in vitro* EMSA and SPR assays were carried out in our present study to verify that the new revealed HepERE element was undoubtedly involved in the transcriptional regulation of the OM-hep1 gene. The results indicated that the OM-ERα protein could bind to the HepERE element on the OM-hep1 promoter, exhibiting a high binding affinity (K_D = 8.6 nM) (Fig. 5A and B), which further confirmed our finding. Recent *in vivo* and *in vitro* studies indicate that estrogen deficiency remarkably induces hepatic hepcidin expression in ovariectomized mice [25], and estrogen exposure negatively regulates human hepcidin expression through the binding of ERα with the ERE element in hepcidin promoter [26]. These reports seem to be in contradiction to our finding. However, it is noteworthy in our study that the regulatory element sequence of HepERE was located at −315 to −289 bp on the OM-hep1 promoter, which was distinct from the location of ERE site on the promoter of human hepcidin on which ERE is located, respectively, at −1244 to −1232 bp [25] and −2474 to −2462 bp [26]. More importantly, the objectives of both studies in mammals are to elucidate the role of E2 in iron homeostasis as well as a mechanism to compensate for iron loss during menstruation involved in osteoporosis and breast cancer diseases [25,26], whereas our purpose was to clarify the interaction between the EE2-exposed toxic effects and immunomodulation of fish hepcidin expression at transcriptional level upon EE2 exposure. From this, we can see that which signaling pathway the hepcidin transcriptional expression is involved in is dependent on its functional activation even though exposed to the same pollutant EE2. In addition, both previous reports and our present results are in accordance with the known facts that mammal and fish hepcidin expression is selectively regulated by different signaling pathways corresponding to multiple stimulants, such as the inflammation-mediated JAK/STAT3 pathway, iron metabolism-mediated BMP6/SMAD pathway and hypoxia-mediated C/EBPα pathway, as well as the newly revealed EE2-mediated ERα/HepERE pathway in our study (Fig. 6).

Taken together, an unknown regulatory mechanism associated with ERα and HepERE on immuomodulating OM-hep1 expression upon EE2 exposure was clearly shown in our study. The new signaling pathway was not relevant to any immune-associated pathways, even though via ERα it was also significantly distinct from the canonical estrogen signaling pathway for the endocrine disrupting effects associated with ERα/ERE. Briefly, for regulating hepcidin transcription, EE2 first combined with the receptor OM-ERα, and then OM-ERα recognized and bound with the newly revealed regulatory element HepERE on the OM-hep1 promoter region and thus initiated the activation of down-regulation of OM-hep1. In addition, the newly revealed EE2-mediated ERα/HepERE pathway for hepcidin transcriptional expression significantly enriched our knowledge of the regulatory mechanism of mammal and fish hepcidin expression in correspondance with multiple stimulants (Fig. 6). To our knowledge, this is the first report elucidating the transcriptional regulatory mechanism of fish hepcidin upon EE2 exposure, which is of important scientific significance and which provides guiding significance for exploring immunotoxic mechanisms mediated by different environmental pollutants in the future.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fsi.2018.08.028.

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