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The mechanism of immune related signal pathway Egr2-FasL-Fas in transcription regulation and methylated modification of *Paralichthys olivaceus* under acute hypoxia stress

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ABSTRACT

Apoptosis genes *Egr2*, *Fas* and *FasL* are related to immune responses. However, the mechanism of these genes inducing apoptosis in fish are still not very clear. An acute hypoxia treatment (1.73 ± 0.06 mg/L) for 24 h was carried out on Japanese flounder (*Paralichthys olivaceus*). The increasingly dense apoptotic signals at 3 h, 6 h, 12 h by TUNEL in skeletal muscle indicated that hypoxia could quickly affect muscle growth and development. Furthermore, we concluded that the Egr2-FasL-Fas signal pathway, which was located at the upstream of apoptotic executor protein caspases, was related to the apoptosis by quantitative real-time PCR, protein concentration detection in ELISA and double gene in situ hybridization methods. The mechanism of the pathway was researched in transcription regulation and epigenetic modification by dual-luciferase reporter assay and bisulfite modified method, respectively. Egr2, as a transcription factor, could up-regulate the expression of *FasL* gene. And its binding site was mainly between −479 to −1 of *FasL* gene promoter. The 5th CpG dinucleotides (−514) methylation levels in *FasL* gene were significantly affected by hypoxia, and they were negatively correlated with its expressions. These suggested that the −514 site may be a very important site to regulate the *FasL* gene expression. Above results, we concluded that hypoxia activated the immune related signal pathway Egr2-FasL-Fas to induced skeletal muscle apoptosis to affect growth and development of Japanese flounder. The study revealed the mechanism of hypoxia induced apoptosis, which could provide a reference for fish immunity and aquaculture management.

1. Introduction

Apoptosis plays a vital role in immune responses [1–3], and it can avoid the damage caused by inflammation [4]. Hypoxia, caused by natural factors or human factors, is a global environmental problem affecting the behavior, growth, survival and reproduction of many organisms. It could induce apoptosis in many species and cells, such as mesenchymal stem cells of rat (*Rattus norvegicus*), central nervous system of sturgeon (*Acipenser shrenckii*), heart cells of zebrafish (*Danio rerio*), umbilical vein endothelial cells of human (*Homo sapiens*), embryoid body cells of mouse (*Mus musculus*), intestinal cell of *Pelteobagrus vachelli*, hypothalamus cell of gibel carp (*Carassius gibelio*) [5–10]. More deeply, the expressions of related genes (such as *bcl-2*, *bcl-xl*, and *krox24*) were changing when hypoxia was occurring [11].

Cysteine-dependent aspartyl-specific proteases (caspases) are widely recognized factors related to apoptosis, including caspase-3, caspase-6, caspase-7, caspase-8, caspase-9 [12–14]. The apoptosis was divided into intrinsic pattern of mitochondrial dependent pathway, endoplasmic reticulum dependent pathway and extrinsic pattern of death receptor mediated signal transduction pathway [15–17]. Augstein et al. [18] found that the activity of caspase-3, a major effector of apoptosis, was enhanced by both Fas (factor associated suicide) and its ligand (factor associated suicide ligand, FasL). Furthermore, caspase 8 was activated by DISC (death-inducing signaling complex), which included Fas [19]. In other words, Fas receptor could rapidly mobilize caspase proteins to perform apoptosis after being bound by its ligand FasL [20]. Fas (alternate name CD95, APO1, ALPS1A, APT1, FAS1, FASTM, TNFRSF6), a membrane receptor protein, could be activated by binding to FasL

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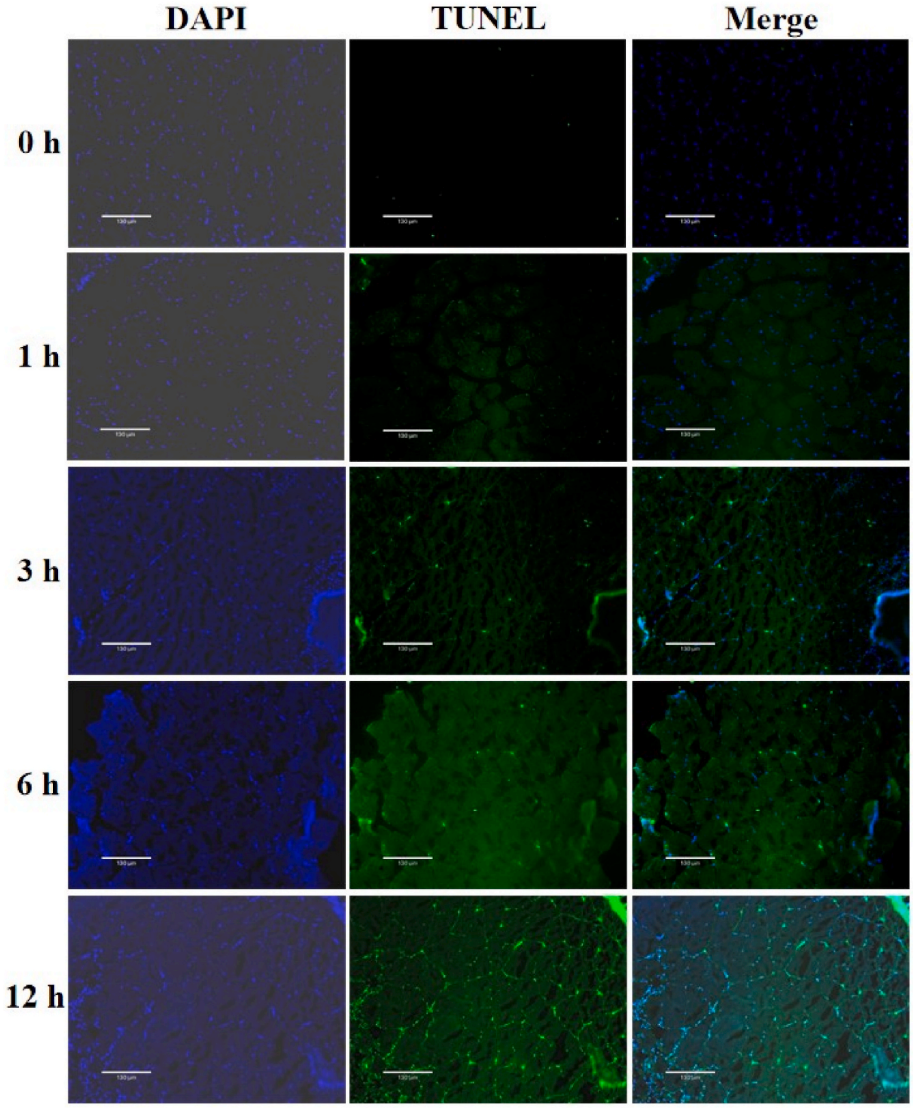

Fig. 1. Histochemical observation of apoptosis signal by TUNEL. Times on the left indicated hypoxia stress times. Scale bar: The white line in the lower left corner of each photograph represents the length of 130 μm.

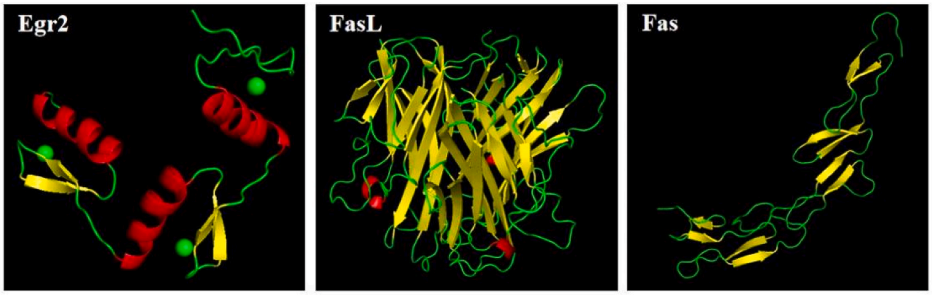

Fig. 2.1. The three-dimensional structures of Egr2, FasL ans Fas protein in Japanese flounder. The α helix, β sheet and loop are colored in red, yellow and green, respectively.

(alternate name CD95L, APO1L, ALPS1B, APT1LG1, APTL, CD178, TNFSF6, TNLG1A, FASLG, GLD) [19]. Fas and FasL belong to tumor necrosis factor (TNF) family and TNF receptor (TNFR) family, respectively. Hypoxic stimulation could induce the overproduction of FasL or Fas and then caused apoptosis in microglia and heart cells of rats [21,22]. The Egr (early growth response) family members, including Egr1 (NGFI-A, krox24, zif268, G0S30, TIS8, ZENK), Egr2 (krox20, NGF1-B), Egr3 (PILOT), and Egr4 (NGFI-C), are associated with growth, development, differentiation and apoptosis, and exquisitely sensitive to environmental changes, such as hormone, hypoxia, ultraviolet irradiation [23–28]. They, as transcription factors, mainly transmit signals in cells by share three-zinc finger motifs composing highly conserved DNA-binding domain, whose transcriptional activation consensus sequence is GCGGGGGCG [29]. Specifically, Egr2 could regulate *FasL*

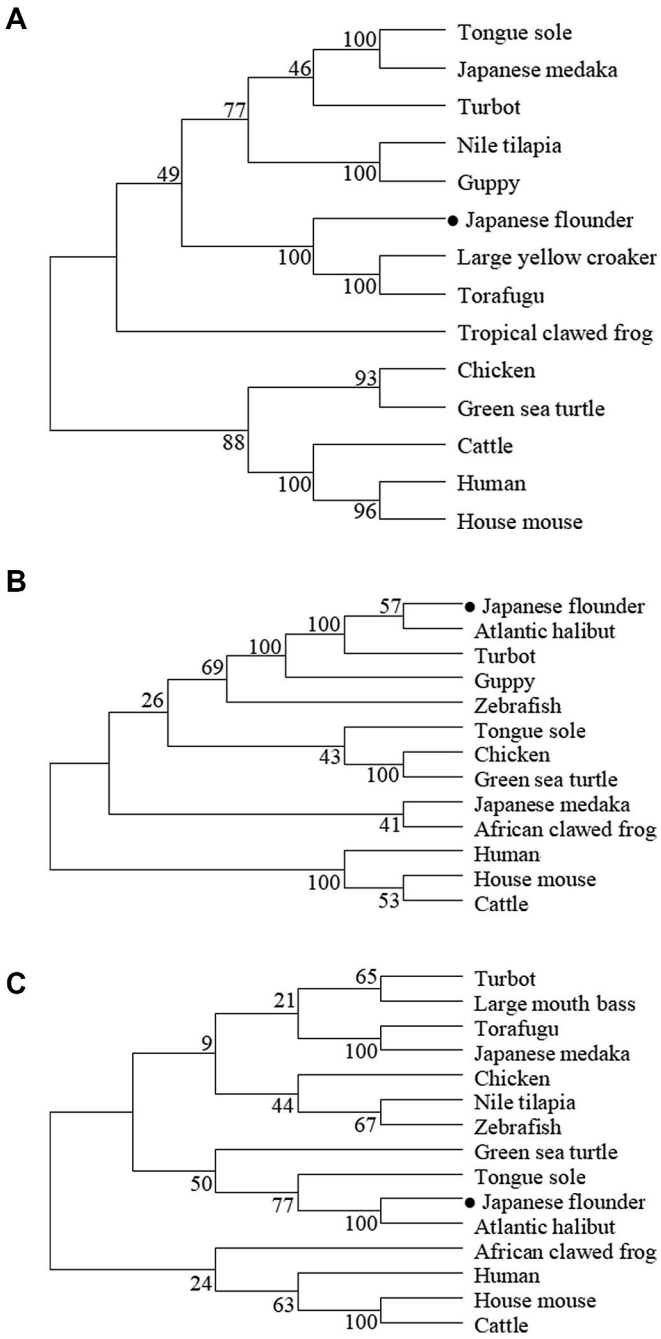

Fig. 2.2. Phylogenetic trees of Egr2 (**A**), FasL (**B**) and Fas (**C**). The location of Japanese flounder was marked with solid black dot. The accession numbers of Egr2 protein are: Japanese flounder (*Paralichthys olivaceus*): XP_019938344.1; Nile tilapia (*Oreochromis niloticus*): XP_003454109.1; Large yellow croaker (*Larimichthys crocea*): XP_010735936.1; Tongue sole (*Cynoglossus semilaevis*): XP_008312384.1; Guppy (*Poecilia reticulata*): XP_008436442.1; Japanese medaka (*Oryzias latipes*): XP_004080832.1; Torafugu (*Takifugu rubripes*): XP_003977992.1; Human (*Homo sapiens*): XP_011537729.1; Turbot (*Scophthalmus maximus*): AWP17168.1 putative; House mouse (*Mus musculus*): XP_030100730.1; Tropical clawed frog (*Xenopus tropicalis*): NP_001093725.1; Chicken (*Gallus gallus*): XP_040530696.1; Green sea turtle (*Chelonia mydas*): XP_037761498.1; Cattle (*Bos taurus*): XP_002698898.1. The accession numbers of FasL protein are: Japanese flounder (*Paralichthys olivaceus*): XP_019936396.1; Atlantic halibut (*Hippoglossus hippoglossus*): XP_034438821.1; Tongue sole (*Cynoglossus semilaevis*): XP_024908296.1; Turbot (*Scophthalmus maximus*): XP_035503040.1; Guppy (*Poecilia reticulata*): XP_008405554.1; Japanese medaka (*Oryzias latipes*): XP_004068323.1; Human (*Homo sapiens*): NP_000630.1; Chicken (*Gallus gallus*): NP_001026730.2; House mouse (*Mus musculus*): NP_034307.1; African clawed frog (*Xenopus laevis)*: XP_018115349.1; Zebrafish (*Danio rerio*): NP_001036166.1; Green sea turtle (*Chelonia mydas*): XP_007061261.2; Cattle (*Bos taurus*): NP_001092329.1. The accession numbers of Fas protein are: Japanese flounder (*Paralichthys olivaceus*): XP_019945341.1; Atlantic halibut: (*Hippoglossus hippoglossus*): XP_034464318.1; Turbot (*Scophthalmus maximus*): XP_035461411.1; Tongue sole (*Cynoglossus semilaevis*): XP_008320651.1; Largemouth bass (*Micropterus salmoides*): XP_038577398.1; Torafugu (*Takifugu rubripes*): XP_011601556.2; Nile tilapia (*Oreochromis niloticus*): XP_013126838.1; Zebrafish (*Danio rerio*): XP_021323082.1; Human (*Homo sapiens*): AKB11528.1; House mouse (*Mus musculus*): ABI24113.1; African clawed frog (*Xenopus laevis*): XP_018080527.1; Chicken (*Gallus gallus*): XP_015143837.3; Green sea turtle (*Chelonia mydas*): XP_037755582.1; Japanese medaka (*Oryzias latipes*): AAS91707.1; Cattle (*Bos taurus*): DAA14967.1.

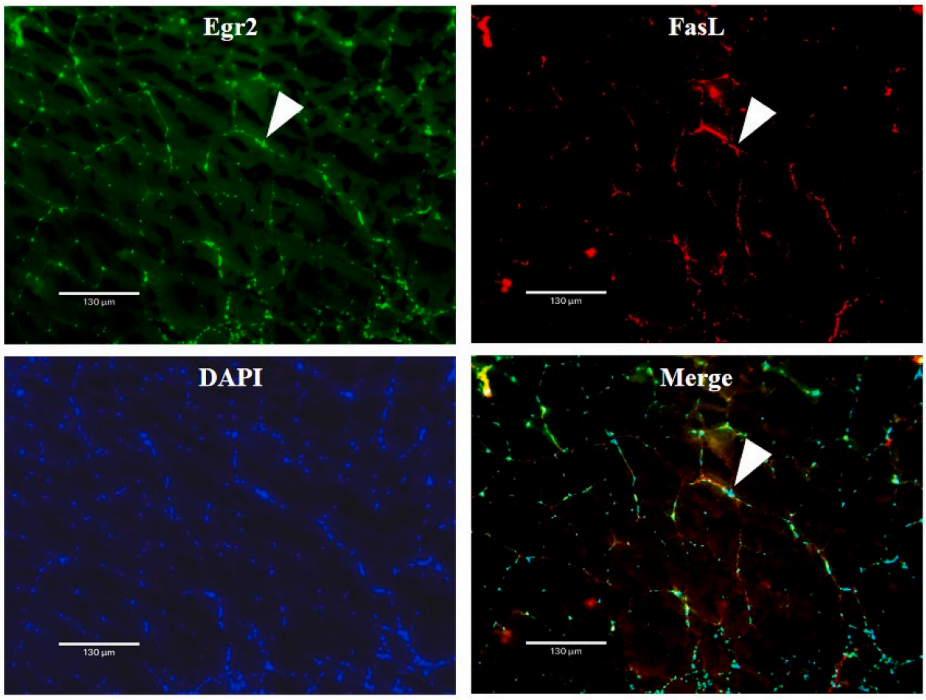

Fig. 3.1. The double in situ hybridization (D-ISH) result of *Egr2* and *FasL* RNAs in skeletal muscle.

expression by binding to the FasL regulatory element (FLRE) in mice and human cells [29–31]. As a result, Egr2 no other members in Egr family was selected as the target factor to study its relationship with apoptosis in Japanese flounder (*Paralichthys olivaceus*).

Transcriptional regulation widely exists in organisms, including three elements: transcription factors, target genes and regulatory binding sites [32]. Wu et al. [33] found that transcription factor Egr1 could activate the *WNT5A* gene expression by binding to the GCGGGGGTG sequence from −52 to −44 site in promoter. Wu et al. [34] found that transcription factor Nrf2 could activate the *GST* gene expression by binding to the GCTGAGTCA sequence near −852 site in promoter. Epigenetic modification, including DNA modification, RNA modification and histone modification, is also an effective way to regulate gene expression in many species. The 5-methylcytosine of cytosine-phosphate-guanine (CpG) in genome DNA is one of it, which is widely existed in eukaryote, and it is related to important biological processes such as gene expression regulation, transposon silencing, gene imprinting, X chromosome silencing [35]. Especially, methylation levels of CpG dinucleotides in CpG islands, CpG enrichment areas, are related to gene expression [36,37]. In CpG islands, hypermethylation and hypomethylation are mostly related to gene silencing and expression, respectively [38,39]. Morán et al. [40] found that methylation levels affected the survival of hatchery-reared brown trout (*Salmo trutta*). The 5-methyl in CpG dinucleotides may affect the activation function of transcription factor by blocking they binding to specific sequences, for example, Navarro-Martín et al. [41] found that SF-1 and Foxl2 transcriptional activation functions were suppressed after increasing the *cyp19a* gene methylation level.

Hypoxia in natural water and aquaculture water is widespread in all over the world. Japanese flounder, an important marine economic fish, is widely cultivated in East Asia (such as China, Japan and Korea) [42]. However, we are still not fully understanding the mechanism of functional immune related signal pathway Egr2-FasL-Fas under hypoxia in this species. On the other hand, apoptosis related factors have been widely concerned in cancer research, and the executive factors caspases of apoptosis were widely concerned, but their upstream regulatory factors (such as Egr2, FasL, Fas) were rarely noticed in Japanese flounder.

In our study, we aimed to understand the functional mechanism of immune related signal pathway Egr2-FasL-Fas under acute hypoxia stress in Japanese flounder. The study will provide a theoretical basis for immune functional research activated by environmental factors in fish and a reference for the aquaculture industry.

2. Materials and methods

2.1. Ethics statement

The guidelines of Animal Research and Ethics Committees of Ocean University of China were strictly followed to treat to all experimental Japanese flounder and perform all experimental operations.

2.2. Hypoxic treatment and sampling

Japanese flounder were collected from Qingdao HaoRuiYuan aquaculture Co., Ltd, China. All individuals were temporarily reared one week to acclimatize environment before hypoxic treatment (about 30 ind/m^2). Specifically, the seawater was filtered and disinfected in one aquaria tank (1.5*1.5*0.6 m^3 with about 1000 L seawater) before raising fish and changed once a day in the acclimatization period. The environmental parameters were as follows, water temperature maintained at 18.51 ± 0.07 °C (range: 17.7–19.3 °C), pH at 7.56 ± 0.02 (range: 7.3–7.7), salinity at 29.0 ± 0.1 ppt (range: 28–30 ppt), dissolved oxygen (DO) (YSI EcoSense DO200A dissolved oxygen meter, USA) at 7.47 ± 0.03 mg/L (range: 7.06–7.91), photoperiod at 14 h light: 10 h dark. Two feedings (at 9:00 a.m. and 3:00 p.m.) were carried out with Surgreen® commercial compound feed for marine fish (Surgreen, China) daily, in which residual feed and feces were removed in time. But all fish were treated with fasted for 24 h before hypoxic treatment. Subsequently, all individuals were randomly distributed to two DO treatment groups with triplicate in 6 rectangular tanks (1.0*0.6*0.6 m^3 with about 300 L seawater, about 30 ind/m^2), in which the half were treated by normoxia [7.43 ± 0.03 mg/L (range: 7.06–7.91)] and the remaining half by hypoxia [1.73 ± 0.06 mg/L (range: 1.10–2.08). Except for no water changing and DO concentration (maintained by

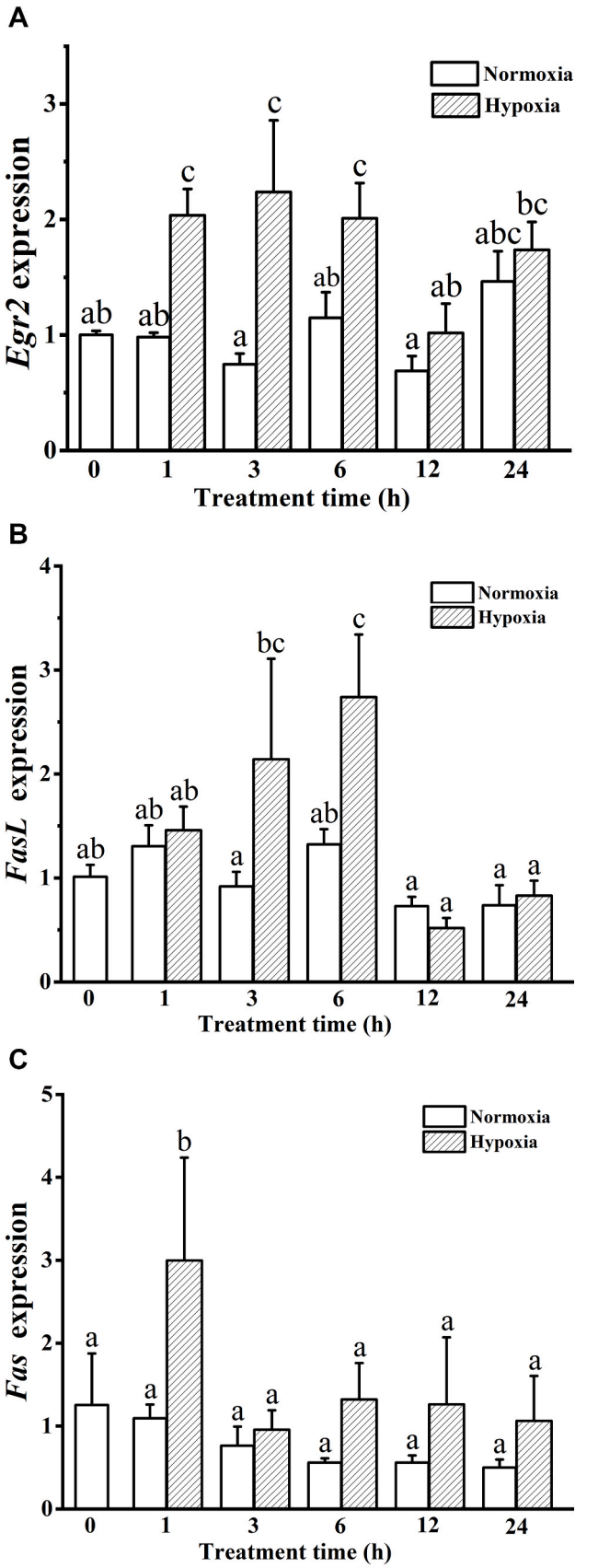

Fig. 3.2. The mRNA relative expression of *Egr2* (**A**), *FasL* (**B**) and *Fas* (**C**) in skeletal muscle at different normoxia and hypoxia times. Different letters indicate significant differences ($P < 0.05$).

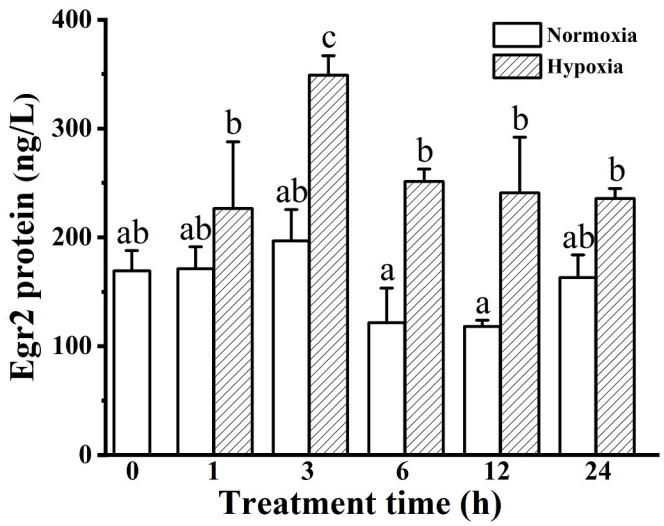

Fig. 3.3. The Egr2 protein concentration in muscle homogenate by ELISA. Different letters indicate significant differences ($P < 0.05$).

pumping air or nitrogen from a steel cylinder), other water parameters (temperature, pH, and salinity) were kept same as the acclimation period.

After hypoxic or normoxic treatment for 0 h (control), 1 h, 3 h, 6 h, 12 h, 24 h, samples were collected, respectively. In detail, tricaine methane sulfonate (MS-222, 200 mg/L) was used to anesthetize fish, whose weight (45.64 ± 1.80 g) and length (15.3 ± 0.5 cm) were measured immediately. Skeletal muscles were obtained after dissection. Some were promptly frozen in liquid nitrogen, afterwards stored at −80 °C for DNA extraction, RNA extraction and tissue homogenate preparation. Others were fixed into 4% paraformaldehyde for morphological observation and double in situ hybridization (D-ISH).

2.3. Histochemical observation

Frozen slices of muscle tissue cross section were obtained according Kanda et al. [43] with minor modifications. Specifically, skeletal muscle tissue was dehydrated in 30% sucrose solution after fixed in 4% paraformaldehyde solution for 24 h; subsequently, they were embedded in OCT embedding agent (SAKURA, Japan). Afterwards, muscle tissue slices (thickness = 7 μm) were obtained by cutting the embedding tissues by a tissue slicer (LEICA TP-1020, Germany). TransDetect® In Situ Fluorescein TUNEL Cell Apoptosis Detection Kit (Trans, China) was used to detect skeletal muscle cell apoptosis. It should be noted that we stained nucleus with DAPI by using the antifading mounting medium with DAPI (G-GLONE, China). Photos were taken by fluorescence microscope (ECHO RVL-100-G, USA).

2.4. Bioinformatics analysis

The online software of Gene Structure Display Server (GSDS 2.0) (http://gsds.gao-lab.org/index.php) was used to analyze gene (*Egr2*, *FasL*, *Fas*) sequence. The physical and chemical characteristics of these three proteins (Egr2, FasL and Fas) were analyzed. Concretely, the online software of ProtParam tool (https://web.expasy.org/protparam/) was used to predict their molecular weight and theoretical isoelectric point (pI), the Simple Modular Architecture Research Tool (SMART) (http://smart.embl.de/) the domains, the TMHMM - 2.0 (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0) the transmembrane helices number. Furthermore, preliminarily proteins three-dimensional structures were predicted by the online software of SWISS-MODEL (https://swissmodel.expasy.org/interactive) and depicted deeply by PyMOL 2.5.2 software.

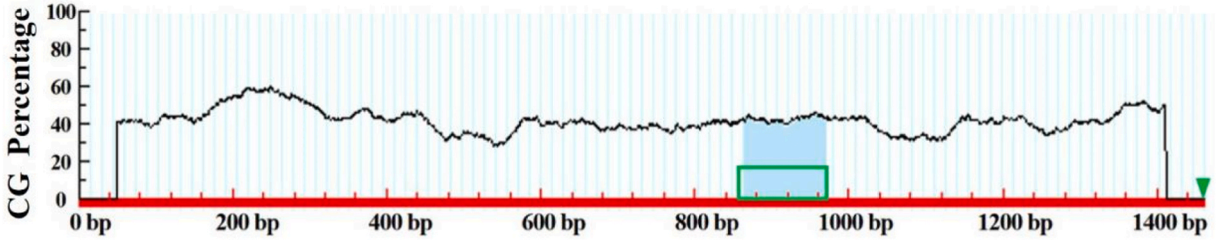

Fig. 4.1A. The methylation status measuring region of *FasL* gene. The abscissa indicated *FasL* gene promoter; ordinate denoted CG percentage; light blue shaded part showed the CpG island [106 bp (from −596 to −491); green box represented the methylation status measuring region [115 bp (from −603 to −489), with 5 CpG dinucleotides]; and the green inverted triangle represented the position of start codon (ATG).

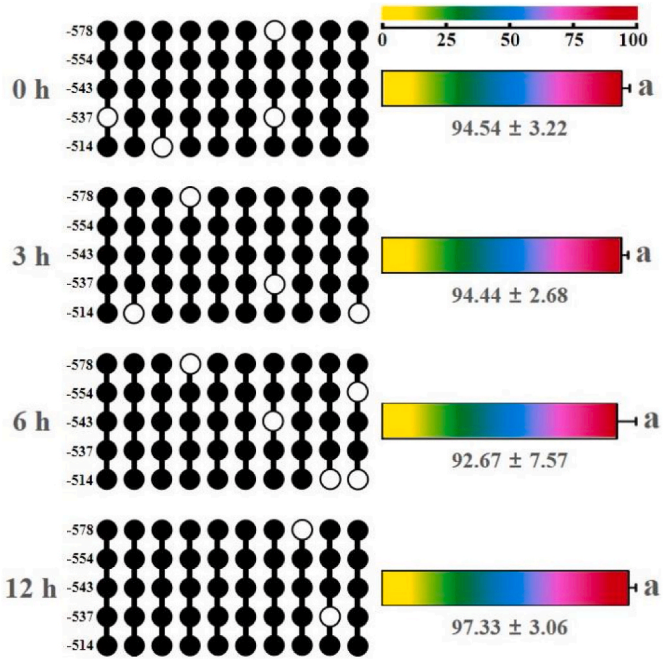

Fig. 4.1B. The methylation status and levels of *FasL* gene. Methylated or unmethylated C (cytosine) in CpG dinucleotides was denoted by filled or open circles, respectively. The left numbers of the circles (−578, −554, −543, −537, −514) indicated the CpG dinucleotides location relative to the start codon. The columns on the right represented methylated levels (M ± SE). Same letters indicated non-significant differences ($P > 0.05$).

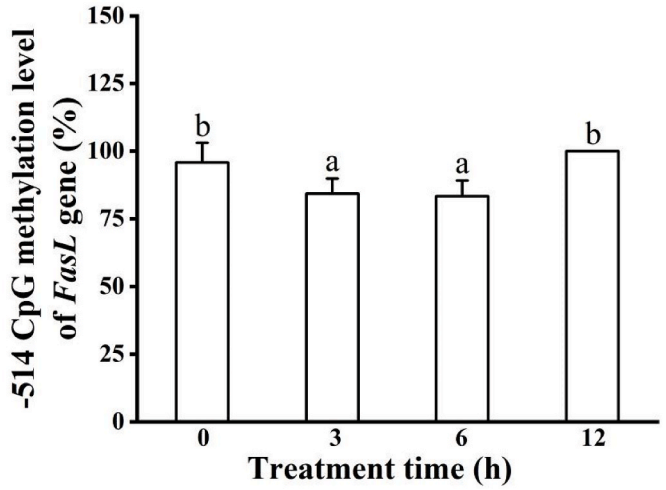

Fig. 4.1C. The methylated levels of the 5th CpG dinucleotides (−514).

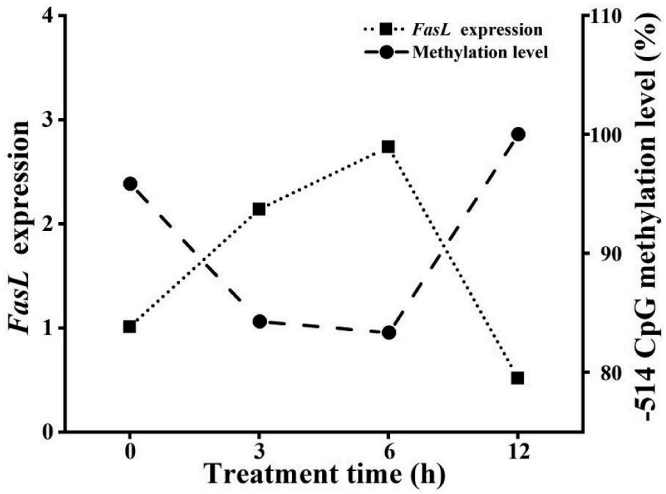

Fig. 4.2A. The changing trends of DNA methylation levels (−514 CpG dinucleotides) and mRNA relative expression of *FasL* gene.

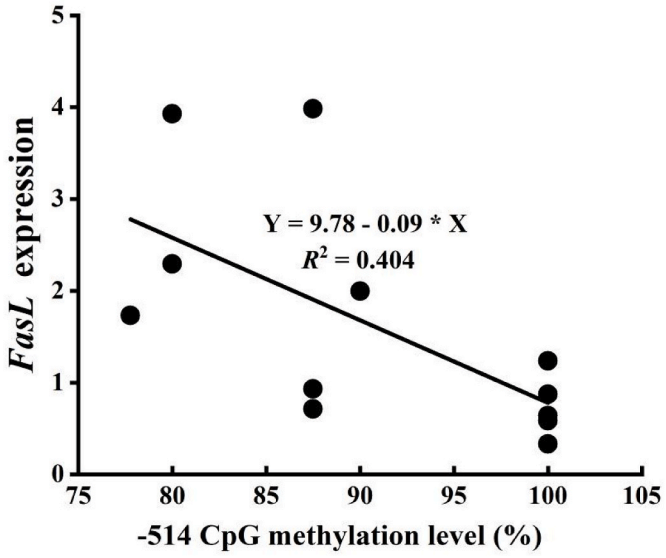

Fig. 4.2B. The correlation of mRNA relative expression and DNA methylation (−514 CpG dinucleotides) level of *FasL* gene.

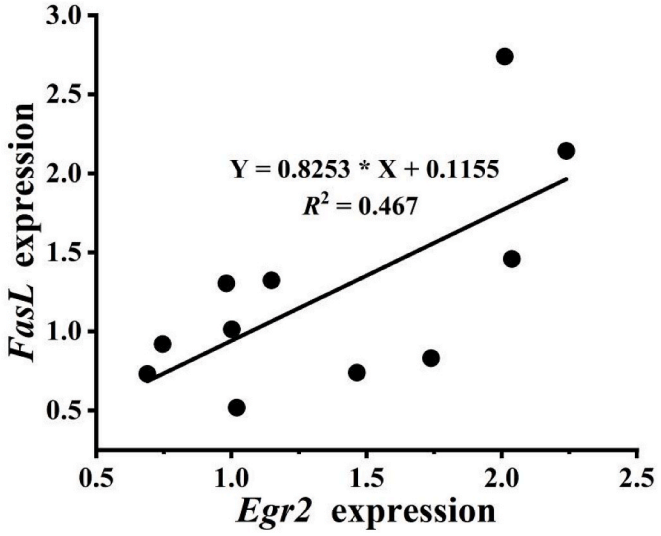

Fig. 5.1. The correlation of *FasL* and *Egr2* mRNA relative expressions.

Phylogenetic trees were constructed by Molecular Evolutionary Genetics Analysis software (MEGA 7.0) with Neighbor-Joining (NJ) method (under 1000 bootstrap replications) to analyze the protein (Egr2, FasL and Fas) evolutionary relationships of Japanese flounder with other species.

In addition, in order to know the other members of the Egr family in Japanese flounder, we analyzed and compared their protein (Egr1, Egr2 and Egr3) related information as above.

2.5. Double in situ hybridization of Egr2 and FasL RNAs

Three steps were divided in this experiment, including obtaining DNA template by high fidelity PCR, RNA probe by transcription, and hybridization.

Firstly, DNA templates of *Egr2* (479 bp) and *FasL* (509 bp) were amplified from cDNA by high fidelity PCR (Phanta® Max Super-Fidelity DNA Polymerase Kit, Vazyme, China). cDNA used here was obtained as described in 2.6 (relative expressions of *Egr2*, *FasL*, and *Fas* genes). The primers (Table A.1C in Appendix) were added three protective bases-SP6 promoter sequence (cgc-atttaggtgacactatagaagcg) or T7 promoter sequence (ccg-taatacgactcactatagggagaca) in the 5' end of forward primer or reverse primer, respectively, after they were designed by Primer Premier 5 software (Premier, Canada). Subsequently, FastPure® Gel DNA Extraction Mini Kit (Vazyme, China) was used to obtain the target DNA templates, which were sequenced by Sangon Biotech (China) to ensure the purified products being pure and their sequences being correct.

Secondly, transcription system was as follows, 1 μg DNA template, 2 μL 10*RNA Polymerase Reaction Buffer (NEB, USA), 1 μL Ribonuclease Inhibitor (TRANS, China), 2 μL T7 RNA Polymerase (Roche, Switzerland), 2 μL 10*NTP and RNase free H_2O were mixed to 20 μL. Here, *Egr2* probes and *FasL* probes were labeled by digoxigenin (DIG) labeled NTP (DIG RNA Labeling Mix 10* conc, Roche, Switzerland) and Biotin labeled NTP (Biotin RNA Labeling Mix 10* conc, Roche, Switzerland), respectively.

Thirdly, exogenous RNA probes hybridized endogenous RNA according to the methods in Kanda et al. [43] and Li et al. [44] papers. Photos were obtained using fluorescence microscope (ECHO RVL-100-G, USA).

2.6. Relative expressions of Egr2, FasL, and Fas genes

RNA isolater Total RNA Extraction Reagent (Vazyme, China) was used to obtain total RNA in skeletal muscle, whose concentration and integrity were detected by nucleic acid analyzer Biodropsis BD-1000 (OSTC, China) and agarose gel electrophoresis, respectively. Subsequently, cDNA was synthetized using HiScript® III RT SuperMix for q-PCR (+gDNA wiper) (Vazyme, China). Afterwards, mRNAs (*Egr2*, *FasL*, *Fas*, *18S*) relative expressions were measured by using ChamQ™ SYBR® Color q-PCR Master Mix (High ROX Premixed) (Vazyme, China) and an Applied Biosystems StepOne Plus Real-Time PCR System (Applied Biosystems, USA) in quantitative real-time PCR (q-PCR), in which all primers (Table A.1A) [except for *18S* primers, which was referred to Huang et al. [45] were designed by the online software of Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). In the q-PCR, the system was 10 μL, including 2 × ChamQ SYBR Color q-PCR Master Mix (High ROX Premixed) 5 μL, Forward Primer (F) 0.2 μL, Reverse Primer (R) 0.2 μL, cDNA template (4 × diluted) 1 μL, and ddH_2O 3.6 μL; the procedure was 95 °C for 30 s, 40 cycles of 95 °C for 10 s and annealing temperature which was showed in Table A.1A for 30 s. Primers have been tested for eligible in their melt curve and amplification efficiency. Triplicate in all samples was conducted as technical replicates. We used comparative threshold ($2^{-\Delta\Delta Ct}$) method to calculate the relative expressions.

In addition, considering the potential regulatory relationship of Egr2 on *FasL* gene, we performed linear regression analysis between their expressions.

2.7. Egr2 protein concentration in muscle homogenate

Ground fully skeletal muscle (1-time mass, g) in a Tissue Grinder (DHS, China) was mixed with normal saline (9-times volume, mL) under the condition of ice-water bath. The supernatant of the mixture after centrifugation (2500 rpm, 10 min, 4 °C) was 10% muscle tissue homogenate. Subsequently, the instruction of Fish Egr2 ELISA Kit (Wuhan Saipei Biotechnology Co., Ltd, China) was strictly followed to detect the Egr2 protein concentration in muscle homogenate.

2.8. DNA methylated status detection

According to the changing trend of *FasL* gene relative expression, four groups (0 h, 3 h, 6 h, 12 h) were chosen to detect the promoter methylation levels of *FasL* gene. Genome DNA was extracted from skeletal muscle by using FastPure® Cell/Tissue DNA Isolation Mini Kit (Vazyme, China), and subsequently modified it by BisulFlash™ DNA Modification Kit (EpiGentek, USA). The TaKaRa EpiTaq™ HS (for bisulfite-treated DNA) Kit (Takara, Japan) was used to conduct methylation-specific PCR (MS-PCR), in which primers (Table A.1B) were designed by the online MethPrimer design software (http://www.urogene.org/methprimer/). In addition, the software was also used to predict CpG island and determine the measuring sequence (Fig. 4.1A).

After obtained the target sequence by purifying MS-PCR product using FastPure® Gel DNA Extraction Mini Kit (Vazyme, China), we connected the sequence with pEASY-T1 vector (TransGen, China) to construct promoter sequence contained plasmid. The plasmid was transferred into *Trans*1-T1 phage resistant chemically competent cell (TransGen, China), subsequently. Three biological repetitions per treatment group and about 10 (8–10) clones per individual were followed. For ensure the credibility of experimental results, the percentage of converted cytosines (excluding cytosines of CpG dinucleotides) was calculated to evaluate the bisulfite modification efficiency. In detail, the formula was the number of converted cytosines (excluding cytosines of CpG dinucleotides)/total number of cytosines (excluding cytosines of CpG dinucleotides) × 100.

In addition, due to the possible effect of methylation on expression referred in introduction, we also performed linear regression analysis between methylation levels and expressions.

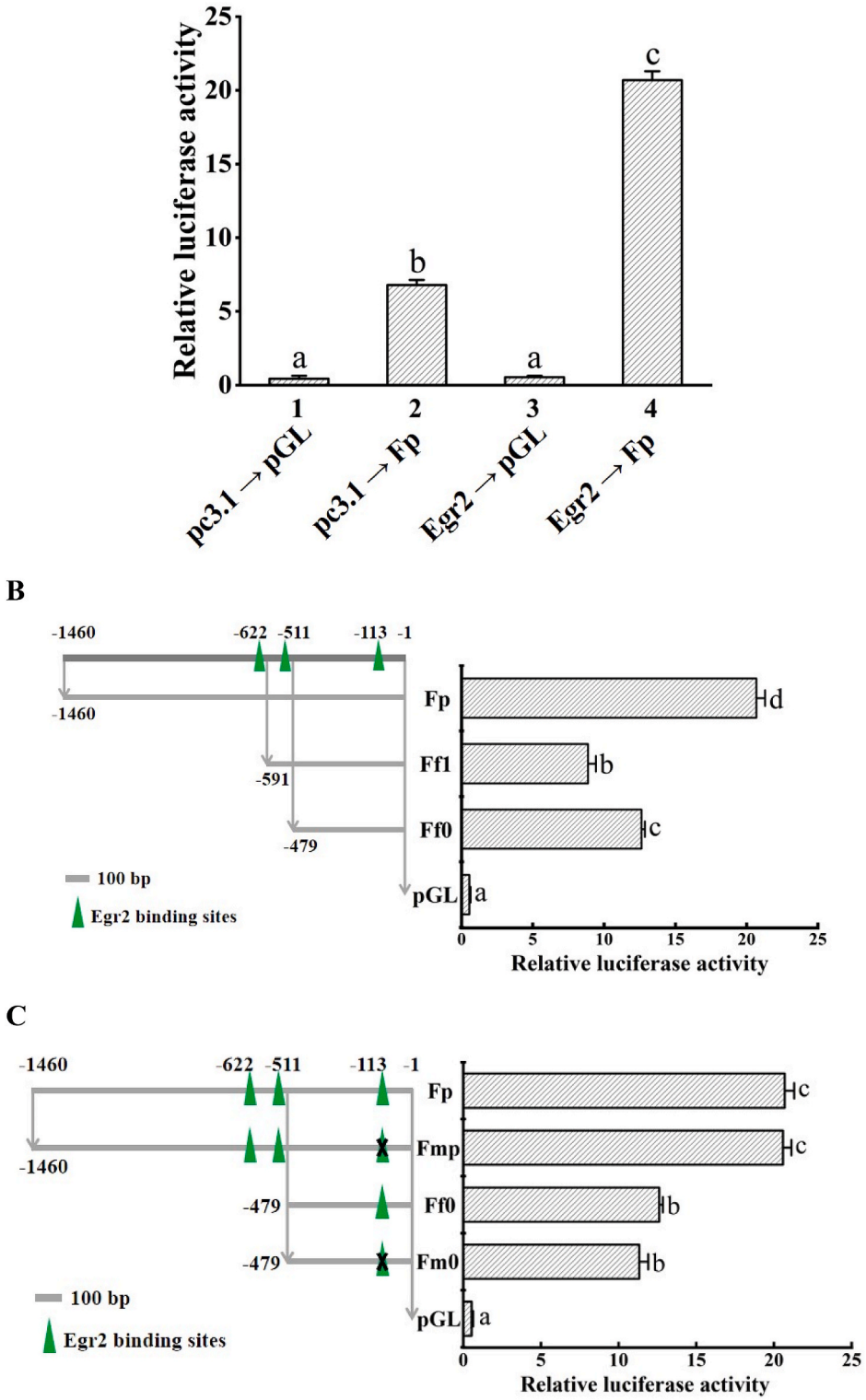

Fig. 5.2. The results of dual-luciferase reporter assay. (**A**) The transcriptionally regulation of transcription factor Egr2 on *FasL* gene promoter. The pGL3 and pc3.1 represented circular pGL 3-Basic plasmid and circular pcDNA3.1 (+) plasmid, respectively, which were not linked to exogenous sequence. Reporter plasmid pGL~FasL was constructed by *FasL* gene promoter sequence and fragmented pGL3-Basic plasmid digested by double endonuclease (SacI and HindIII). Expression plasmid pc3.1~Egr2 was constructed by Egr2 coding sequence and double endonuclease (HindIII and BamHI). Transfection efficiency was characterized by the PRL-TK control plasmid, which expressed Renilla luciferase (Rluc). The arabic numerals near abscissa indicated different treatment groups. (**B**) The fragmentation deletion result. The green high triangle icons (△) represented the putative Egr2 binding sites which were predicted by JASPAR online software. The three light gray horizontal lines (Fp, Ff1, Ff0) represented reporter plasmids (pGL~Fp, pGL~Ff1, pGL~Ff0), which were connected by different length *FasL* gene promoter sequence and fragmented pGL 3-Basic digested by double endonuclease (SacI and HindIII), respectively. Plasmids of Fp and pGL were same as pGL~FasL and pGL3 in Fig. 5.2A, respectively. (**C**) The bases mutation result. Fmp and Fm0 indicated the two mutant plasmids (pGL~Fmp, pGL~Fm0), whose sequences near −113 (CTCTATGGGTGGTTA) were deleted by fusion high fidelity PCR. The Fp, Ff0 and pGL plasmids were same as that in Fig. 5.2B. Different letters indicated significant differences ($P < 0.05$).

2.9. Dual-luciferase reporter assay

Dual-luciferase reporter assay was used to verify the transcription factor Egr2 regulating *FasL* gene expression.

The Egr2 coding sequence (CDS) and *FasL* gene promoter sequence were obtained by high fidelity PCR, in which primers (Table A.1D) was designed by CE Design V1.04 (Vazyme, China). Expression plasmid (pc3.1~Egr2) and reporter plasmid (pGL~FasL) were constructed by strictly following the instruction of ClonExpress® Ultra One Step Cloning Kit (Vazyme, China). Here, the expression plasmid of pc3.1~Egr2 was constructed by pcDNA 3.1(+) plasmid connecting Egr2 CDS after digested by double restriction endonuclease [HindIII and BamHI (NEB, USA)]. Similarly, the reporter plasmid of pGL~FasL was constructed by pGL3-Basic plasmid connecting *FasL* gene promoter sequence after digested by double endonuclease [SacI and HindIII (NEB, USA)]. These plasmids were transferred into *Trans*1-T1 phage resistant chemically competent cell (TransGen, China), respectively. After target sequences were sequenced by Sangon Biotech (China), plasmids were

extracted by using EndoFree Mini Plasmid Kit II (TIANGEN, China).

Plasmids were transfected into human embryonic kidney 293T (HEK293T) cells, which were resuscitated (37 °C) from cryopreserved in liquid nitrogen. They were used to be transfected when were cultured to 3rd or 4th generation, whose growth was normal and steady with DMEM/High Glucose culture medium (Servicebio, China) with 10% fetal bovine serum (FBS) (absin, China). Concretely, about 10^5 293T cells per well were placed into 24 well plates (Corning, USA). When they confluence reached to 50%–70%, plasmids were transfected into them by Lipofectamine™ 3000 Reagent (Invitrogen, USA). In order to quantify the transfection efficiency as control, PRL-TK plasmid (Promega, USA), which could express Ranilla luciferase, was transfected at the same time. Triplicate was conducted here.

After transfection 48 h, we detected double-fluorescence values using SYNERGY HTX multi-mode reader (BioTek, USA) and Dual-Luciferase kit (Promega, USA).

To determine the specific binding sites of transcription factor Egr2, fragment deletion and bases mutation experiments were performed according to the predicted binding sites of Egr2 transcription factor on *FasL* promoter sequence by JASPAR online software (http://jaspar.genereg.net/). Similar to above, fragment deleted plasmids (pGL~Ff1, pGL~Ff0) were constructed using the fragmented promoter sequence of *FasL* gene and the fragment plasmid digested by double endonuclease [SacI and HindIII (NEB, USA)]. The fragmented sequences were obtained by high fidelity PCR, whose primers sequences were shown in Table A.1D. The bases mutation plasmids (pGL~Fmp, pGL~Fm0) were obtained by fusion PCR, whose primers sequences were also shown in Table A.1D.

2.10. Statistical analysis

Data were showed as mean ± standard error (M ± SE). One-way ANOVA with Duncan's post hoc test was conducted to determine statistical differences under the premises of normal distribution and variance homogeneity. Statistical analyses were performed in SPSS 22.0, graphs making in OriginPro 9.0. The *P* value was set at 0.05.

3. Results

3.1. Apoptotic signal in skeletal muscle tissue

With the extension of hypoxia time, apoptosis signals became denser. In detail, apoptotic signals sporadically appeared at 3 h, and they were very dense when hypoxia stress for 12 h (Fig. 1). However, apoptosis signal did almost not appear in 0 h and 1 h groups.

3.2. Genetic structure and phylogenetic analysis

Egr2, *FasL* and *Fas* gene have 2, 4 and 9 exons, respectively (Table A.2, Fig. A.1). Three zinc finger (ZnF_C2H2) structures related to transcriptional activation function were predicted in the secondary structure of Egr2 protein C-terminal, and their amino acid position were 322–346, 352–374, 380–402, respectively. Furthermore, we found three ligand binding sites (tumor necrosis factor receptor, TNFR) and one receptor binding site (tumor necrosis factor, TNF) in the receptor Fas and the ligand FasL, whose number of trans membrane helices were two and one, respectively (Table A.3, Fig. A.2). In the three-dimensional structures, α helix, β sheet and loop were all existed in Egr2 and FasL proteins, however, Fas did not contain α helix (Fig. 2.1). In the Egr family, only three members (Egr1, Egr2, Egr3) were found in Japanese flounder. They all contained low complexity domains and ZnF_C2H2 domains (Fig. A.3).

The phylogenetic tree of Egr2 was mainly divided into two branches (fish and other higher vertebrates) except for tropical clawed frog (*Xenopus tropicalis*), which was clustered in the fish branch (Fig. 2.2A). Similar to Egr2, the phylogenetic trees of FasL and Fas were also basically divided into two branches (Fig. 2B, C). Special species were chicken (*Gallus gallus*), green sea turtle (*Chelonia mydas*), African clawed frog (*Xenopus laevis*) in FasL tree, and chicken (*Gallus gallus*), green sea turtle (*Chelonia mydas*) in Fas tree, which were all clustered in fish branch. Interestingly, the Atlantic halibut (*Hippoglossus hippoglossus*) and tongue sole (*Cynoglossus semilaevis*), which were same as Japanese flounder belonging to Pleuronectiformes in taxonomy, were indeed closely clustered with Japanese flounder in the Fas phylogenetic tree; but turbot (*Scophthalmus maximus*) which was more adjacent to Japanese flounder in taxonomy (belonging to Bothidae) was far away from it in the tree.

3.3. Genes expressions and protein concentration

Egr2 and *FasL* mRNAs were co-located near the nucleus of muscle cells (Fig. 3.1) by D-ISH.

The relative expression of *Egr2*, *FasL* and *Fas* increased first and then decreased (Fig. 3.2A, B, C). More specifically, *Fas* expression was significantly different at 1 h hypoxia stress compared with its control; differences of *FasL* expression were significant at 3 h and 6 h; and significant differences of *Egr2* expression were appeared at all these three time points. However, the time points of significant differences in Egr2 protein concentration were 3 h, 6 h and 12 h (Fig. 3.3).

3.4. DNA methylation levels

Twelve sequencing results were randomly selected to calculate the bisulfite modification efficiency. Only 4 cytosines (18 cytosines outside 5 CpG dinucleotides per 115 bp) were not converted to thymines, indicating that bisulfite modification efficiency was 98.15%, which was very efficient (Fig. A.4 in Appendix showed part of a sequencing result). The overall methylation levels of these five CpG dinucleotides were high (95.64 ± 0.87%) and not significant difference (Fig. 4.1B). However, significant difference was found in 5th CpG dinucleotides (−514), whose methylated levels showed a downward trend first and then an upward trend (Fig. 4.1C). Due to the significant difference in the 5th CpG dinucleotides, we conducted linear regression analysis between its methylated levels and mRNA relative expression levels, whose result showed that they were negatively correlated ($R = -0.636$, $R^2 = 0.404$; Fig. 4.2A, B).

3.5. Egr2 transcriptionally regulated FasL

Considering the possible regulation of Egr2 on *FasL*, we conducted linear regression analysis between their expressions, whose result showed that they had a positive correlation ($R = 0.684$, $R^2 = 0.467$, Fig. 5.1). In the dual-luciferase reporter assay, the relative luciferase activities were significantly higher when expression plasmid pc3.1~Egr2 and reporter plasmid pGL~FasL were simultaneously added in 4th than that in 1st, 2nd, 3rd (c Vs. a, b, a; Fig. 5.2A). In its fragmentation deletion experiment, the first significant difference appeared between pGL and Ff0 (a Vs. c), which indicated that the Egr2 binding sites were located between −479 ~ −1 (Fig. 5.2B). As a result, the predicted Egr2 binding sequence was mutated. Unexpectedly, significant difference was not found between Fm0 and Ff0 (b Vs. b), Fmp and Fp (c Vs. c) (Fig. 5.2C).

4. Discussion

Apoptosis induced by hypoxia was widespread, such as umbilical vein endothelial cells in human, embryonic stem cells in mouse, heart cells in zebrafish, cardiomyocytes in chicken [5,46,7]. In this study, the apoptotic signal in skeletal muscle tissue indicated that such DO level (1.73 ± 0.06 mg/L) did cause immune response of cells apoptosis in Japanese flounder muscle. Furthermore, apoptosis signals at 3 h meant that fish responded quickly to hypoxia, and the dense signals at 12 h

reflected the continuous damage of hypoxia. These implied that environmental stress should be avoided in time in aquaculture of Japanese flounder.

Protein domain analyses showed that the C-terminal of Egr and FasL proteins was more important than N-terminal. This importance was also confirmed in human FasL protein, whose dysfunction was discovered when only three amino acids were deleted in C-terminal [20,47]. The conservation and the results of phylogenetic trees indicated that these factors (Egr2, FasL and Fas) may perform similar functions in Japanese flounder. After confirmed the co-expression of *Egr2* and *FasL* mRNAs by D-ISH, the significant differences of gene (*Egr2*, *FasL* and *Fas*) expressions and Egr2 protein concentrations demonstrated that these three factors were indeed affected by hypoxia. Therefore, their regulatory mechanism was studied subsequently. It was worth mentioning that the time points of Egr2 expression differences and protein concentration differences were not completely consistent. We speculated that there may be two reasons. On the one hand, it took time to translate the mRNAs to protein sequences, and fold to active proteins. On the other hand, their units were different, in which mRNA expression was the relative expression to *18S*, but protein was the absolute concentration (ng/L).

In *FasL* gene, the significant difference of methylation level in 5th CpG dinucleotides (−514) indicated that hypoxia may affect gene expression by affecting methylation status of this site, and their negative correlation further proved it, although the overall methylation levels were no significant difference. The change of methylated modification status caused by environment was common. Cytosine methylation was usually associated with chromatin state and gene expression, in which hypermethylation usually meant chromatin condensing and promoter activity inhibition [48]. Navarro-Martín et al. [41] found that high temperature induced hypermethylation of *cyp19a* gene in European sea bass (*Dicentrarchus labrax*). Similar to our study, different CpG dinucleotides had different sensitivity to temperature, and negative correlation between expressions and methylation levels was also found in their research [41]. The overall hypermethylation in *FasL* gene indicated that the constitutive expression was relatively less, which may be related to their vigorous growth stage in the juvenile period. The acute hypoxia stress may increase *FasL* expression by affecting its DNA methylation status to cause apoptosis and disturb the growth and development of Japanese flounder.

On the other hand, both the positive correlations between expressions of *FasL* and *Egr2* genes, and between *FasL* expression and Egr2 protein concentration (Fig. A.5) suggested that Egr2 may transcriptionally regulated *FasL* gene expression liked in mouse and human [29,31]. The results of dual-luciferase reporter assay did illustrate that Egr2 could as transcription factor activate the *FasL* gene transcription. Here, it was worth noting that maybe endogenous transcription factors in 293T cells activated the firefly luciferase expression of reporter plasmid pGL∼FasL in 2nd of Fig. 5.2A. According to the fragment deletion results in Fig. 5.2B, the Egr2 binding site was likely the predicted −113 nearby sequence. However, the results of no significant difference in Fig. 5.2C indicated that the −113 nearby sequence (CTCTATGGGTGGTTA located at −120 to −106 sites) was not the Egr2 binding sequence. Together with these interesting results of significant differences among Ff0, Ff1 and Fp (c Vs. b Vs. d) in Fig. 5.2B, between Fm0 and Fmp (b Vs. c) in Fig. 5.2C, between Ff0 and Fp (b Vs. c) in Fig. 5.2C, we concluded that Egr2 binding sites may located at −105 to −1, −479 to −121, −1460 to −591. Silencer was divided into silencer elements and negative regulatory elements (NREs), which repressed transcription by binding to repressor protein and forming steric hindrance hinders, respectively [49]. The results of fragment deletion in Ff1 and Ff0 implied that silencer may be exist from −591 to −479 of the *FasL* gene promoter sequence. Just as Wang et al. [50] found two silencers (−1080/-801, −500/-201) in the *β-actin* gene of Japanese flounder. These all reflected the potential researching prospect of silencer. However, the predicted transcription factor binding site nearby −511 (AGCGTAGACAT, 11 bp, −516 to −506) was located at the possible silencing region (−591 to −479), so the regulatory function of this sequence on *FasL* gene expression need to be further studied. Mittelstadt and Ashwell [51] found 8 bp sequence (GTGGGTGT) from −214 to −207 was the *FasL cis*-acting regulatory element, which was bound by transcription factor Egr3 in human. These may be due to sites, sequences or species differences of Egr2 and Egr3 transcription factors. As a result, the accuracy of predictions in nonmodel organisms and experimental verifications (e. g. chromatin immunoprecipitation, ChIP; electrophoretic mobility shift assay, EMSA) are needed to strengthen in the future.

Here, we synthesized the results of methylated modification and transcriptional regulation about *FasL* gene. The methylation levels of −514 CpG dinucleotides were significantly affected by hypoxia, but the transcriptionally activated binding sites of Egr2 transcription factor did not include it. Therefore, we speculated that other transcription factors may regulate the *FasL* gene transcription by binding to bases near −514 site. It was supported by the reporter that many other transcription factors could alone or cooperatively activate the *FasL* gene transcription, such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1), specificity protein-1 (Sp-1), interferon regulatory factor-1 (IRF-1), inducible cAmp early repressor (ICER), E26 transformation specific-1 (Ets-1), IFN regulatory factor-1 (IRF-1), FOXO3a (FKHRL1) [47,52]. It was but not limited to them that constituted the network regulating *FasL* expression. However, its species-specificity and tissue-cell specificity is not very clear, which means that their role in Japanese flounder need to be further researched. In addition, the important site (−514) of methylated status was located in the predicted transcription factor binding sites (−516 to −506), so, the interaction of methylated modification and transcription factors should be explored by advanced technology. NAB1 (NGFI-A-binding protein 1) was found to inhibit Egr1 and Egr2 in CV-1 and COS-7 cells of African green monkey (*Cercopithecus aethiops*) [26,27]. Furthermore, it was more interesting of the finding that the repressor NAB2 (NGFI-A-binding protein 2) of Egr1 was transcriptionally regulated by Egr1, which inhibited Egr1 in turn by a negative feedback regulation mechanism [53]. In addition, hepatocellular carcinoma apoptosis could be promoted by nuclear factor of activated T cells 2 (NFAT2) enhancing the expression of *Egr2* in human [54]; HIV Tat protein could be used as a cooperative factor to improve the efficiency of Egr proteins activating *FasL* transcription in human [55]; the dephosphorylated FKHRL1 protein triggered apoptosis by acting on *FasL* gene [56]. Proteins with similar functions in Japanese flounder need to be further found and studied. Many substances (e. g. vitamin D3, nitric oxide, retinoic acid and the transcriptional repressor ICER) that repressed *FasL* expression have also been found [47]. Whether they can be used in aquaculture to resist apoptosis caused by environmental stimuli deserves further research.

Another hypothesis about the results of overall methylation levels and transcriptional regulation was that *FasL* gene may be mainly affected by transcription factor rather than DNA methylation modification status in Japanese flounder.

In summary, as pointed out by Kavurma and Khachigian [47], *FasL* gene expression involved transcription factor regulation, epigenetic modification, protein-protein interaction and phosphorylation. Furthermore, apoptosis induced by hypoxia may associated with multiple signal pathways synthetically. In addition, antiapoptotic factors could also be stimulated by hypoxia [57]. It was a trade-off between adaption to survival or apoptosis to death, which were decided by organisms according to themselves. A homeostasis in normal cells between apoptosis promoting and inhibiting was also existent in a stable environment, and a sudden environmental change could break it and caused a new trade-off decision in organisms [58]. Suhara et al. [59] pointed out that FasL induced apoptosis was a positive feedback loop in cells. FasL likes a point of stress stimulation, which is affected by many environmental factors and substances (such as high temperature, irradiation, progesterone, hydrogen peroxide) and then induces apoptosis [60–63]. However, the complete mechanisms of immune related genes *FasL* and the decision mechanisms, apoptotic metabolic networks need to be deeply explored and elaborated in the future.

5. Conclusion

Acute hypoxia stress caused skeletal muscle cell apoptosis in Japanese flounder. The apoptosis was related to Egr2-FasL-Fas signal pathway. In the pathway, Egr2 could as a transcription factor activate the *FasL* gene transcription. On the other hand, the methylated modification status of −514 CpG dinucleotides may affect *FasL* gene expression. In summary, hypoxia induced apoptosis by acting on immune related signal pathway Egr2-FasL-Fas in transcriptional regulation and epigenetic modification. The study uncovered the mechanism of hypoxia induced apoptosis in Japanese flounder, and could provide a reference for aquaculture.

CRediT authorship contribution statement

Binghua Liu: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. Guangling Li: Validation, Formal analysis, Investigation, Data Curation. Jun Yang: Validation, Investigation. Xiaohui Li: Validation, Investigation. Hao Wang: Validation, Investigation. Jing Yang: Validation, Visualization. Feng He: Conceptualization, Methodology, Resources, Data Curation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. Haishen Wen: Conceptualization, Supervision, Project administration, Funding acquisition.

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Declaration of competing interest

All authors are supporting publication and declaring that no competing financial interest or other conflict exists in this research.

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Appendix A. Supplementary data

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