Comparative Biochemistry and Physiology, Part B 219–220 (2018) 33–43

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

The DNA methylation status of *MyoD* and *IGF-I* genes are correlated with muscle growth during different developmental stages of Japanese flounder (*Paralichthys olivaceus*)

Yajuan Huang, Haishen Wen, Meizhao Zhang, Nan Hu, Yufeng Si, Siping Li, Feng He*

The Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao 266003, China

ARTICLE INFO

Keywords:
Japanese flounder
MyoD and *IGF-I*
DNA methylation
Gene expression
Muscle growth

ABSTRACT

Many genes related to muscle growth modulate myoblast proliferation and differentiation and promote muscle hypertrophy. MyoD is a myogenic determinant that contributes to myoblast determination, and insulin-like growth factor 1 (IGF-I) interacts with MyoD to regulate muscle hypertrophy and muscle mass. In this study, we aimed to assess DNA methylation and mRNA expression patterns of *MyoD* and *IGF-I* during different developmental stages of Japanese flounder, and to examine the relationship between *MyoD* and *IGF-I* gene. DNA and RNA were extracted from muscles, and DNA methylation of *MyoD* and *IGF-I* promoter and exons was detected by bisulfite sequencing. The relative expression of *MyoD* and *IGF-I* was measured by quantitative polymerase chain reaction. IGF-I was measured by radioimmunoassay. Interestingly, the lowest expression of *MyoD* and *IGF-I* emerged at larva stage, and the mRNA expression was negatively associated with methylation. We hypothesized that many skeletal muscle were required to complete metamorphosis; thus, the expression levels of *MyoD* and *IGF-I* genes increased from larva stage and then decreased. The relative expression levels of *MyoD* and *IGF-I* exhibited similar patterns, suggesting that *MyoD* and *IGF-I* regulated muscle growth through combined effects. Changes in the concentrations of IGF-I hormone were similar to those of *IGF-I* gene expression. Our results the mechanism through which *MyoD* and *IGF-I* regulate muscle development and demonstrated that *MyoD* interacted with *IGF-I* to regulate muscle growth during different developmental stages.

1. Introduction

Myogenesis, defined as the development and growth of vertebrate skeletal muscle, is the result of a set of well-characterized events. Specification is the initial event which occurs when precursor cells, arising deep within the somites, become myoblasts. Myoblasts then undergo proliferation, and differentiation to fuse into myotubes. These myotubes then mature into myofibers, which are the core unit of muscle, and grow by recruiting other myoblasts (Johansen and Overturf, 2005).

The development of vertebrate skeletal muscle is molecularly regulated by a family named myogenic regulatory factors (MRFs), including myogenic determining factor (*MyoD*), Myf6 (*MRF4*), myogenin (*MyoG*) and genes myogenic factor 5 (*Myf5*) (Buckingham et al., 2003; Molkentin and Olson, 1996; Pownall et al., 2002; Puri and Sartorelli, 2000). Moreover, the members of MRFs are basic helix-loop-helix (bHLH) transcription factors, which together play a vital role in myoblast determination and differentiation and in the development of pre-myoblasts to forming muscle fiber (Buckingham, 1992). *MyoD* was the first myogenic regulatory gene identified (Davis et al., 1987) and is one of the earliest MRFs involved in determination and terminal differentiation of skeletal muscle (Berkes and Tapscott, 2005; Buckingham, 2006). Furthermore, the MyoD protein contributes to myoblast determination, which is activated in proliferating myoblasts before overt differentiation, and converts undifferentiated myoblasts into myotubes (Rudnicki et al., 1992). *MyoD* can also initiate and maintain the differentiation and development of skeletal muscle during myogenesis (Weintraub, 1993a, 1993b). However, few studies have examined the methylation level and expression level of MyoD gene during different developmental stages. Hence, we analyzed the methylation and expression levels of the *MyoD* gene during different developmental stages.

The development of vertebrate skeletal muscle is also modulated by insulin-like growth factors (IGFs) (Castillo et al., 2002; Castillo et al., 2004; Howard et al., 1985; Le Bail et al., 1998; Peterson et al., 2004; Shunsuke et al., 2000; Velloso, 2008). For example, the size of the skeletal muscle in transgenic mice's skeletal muscle is increased when the level of *IGF-I* is increased (Coleman et al., 1995). Li et al. (2009) found that *IGF-I* functions as a polypeptide growth factor and that *IGF-I*

* Corresponding author at: 5 Yushan Road, Ocean University of China, Qingdao 266003, China.
E-mail address: hefengouc@ouc.edu.cn (F. He).

https://doi.org/10.1016/j.cbpb.2018.02.005
Received 23 October 2017; Received in revised form 25 January 2018; Accepted 22 February 2018
Available online 25 February 2018
1096-4959/

can regulate growth during all stages of development in vertebrates. *IGF-I* promotes the proliferation and differentiation of skeletal muscle cells and stimulates myotube hypertrophy *in vitro* (Hawke and Garry, 2001). Additional studies have also shown that *IGF-I* positively regulates the proliferation and differentiation of skeletal muscle cells. Furthermore, some studies have demonstrated that *MyoD* gene interacts with *IGF-I* to regulates muscle hypertrophy and muscle mass (Aguiar et al., 2013; Marcio and Maeli, 2012). *IGF-I* also affects muscle growth and myoblast proliferation in fish (Castillo et al., 2004; Cleveland et al., 2010; Díaz et al., 2009). However, few studies have examined these roles of these genes in muscle development in the Japanese flounder.

Epigenetics refers to modifications that induce changes in genetic activity or associated proteins without changing the fundamental DNA sequence (Egger et al., 2004; Jablonka and Lamb, 1998). DNA methylation is an important epigenetic process involved in modulation of gene expression and was the first type of epigenetic modification to be discovered (Holliday and Pugh, 1975; Riggs, 1975). In general, methylation of CpG islands suppresses gene transcription in mammalian somatic cells and other vertebrates (Bird, 2002; Plass and Soloway, 2002). For example, DNA methylation serves to silence gene expression by hampering the binding of certain transcription factors and through the recruitment of repressive chromatin machinery in zebrafish (Wu et al., 2011). Many previous studies have examined the gene silencing effects of methylation of CpG islands and decentralized sites in promoter and exon 1 regions (Antequera and Bird, 1999; Maruyama et al., 2002; Waki et al., 2003). However, DNA methylation in the coding regions also causes gene silencing; for example, a recent study showed that the silencing of the *PDHA2* gene was regulated by DNA methylation in the coding regions (Pinheiro et al., 2010). Additionally, Ding et al. (2013) reported that the methylation patterns of specific CpG sites in coding regions block the expression of *cyp17-II* gene. Thus, the DNA methylation patterns of the promoter and exons may play important roles in modulating gene expression at different stages of post-embryonic muscle development.

The Japanese flounder is an important economic fish species for mariculture in Asia due to its fast growth rate and high market value. However, few studies have evaluated the roles of specific genes in skeletal muscle growth in this species. Zhang et al. (2006) analyzed the expression patterns of the *MyoD* gene during embryogenesis and the *IGF-I* gene during metamorphosis in the Japanese flounder (Zhang et al., 2011a, 2011b). Additionally, some previous studies have examined the relationships between the *IGF-I* and *MyoD* expression (Miyake et al., 2007; CNKI, 2014). However, no studies have evaluated *MyoD* gene expression and DNA methylation levels of *MyoD*, and the relationships between *IGF-I* gene expression and DNA methylation levels of *IGF-I*, and the relationship between *MyoD* and *IGF-I* gene during postembryonic development have not been explored in the Japanese flounder. Hence, in this study, we aimed to elucidate the factors affecting muscle growth and differentiation in the Japanese flounder. To this end, we analyzed the methylation patterns and relative expression levels of the *MyoD* and *IGF-I* genes during five different stages of development in this important fish species.

2. Materials and methods

2.1. Ethics statement

All animal experiments were conducted in accordance with the guidelines and approval of the respective Animal Research and Ethics Committees of Ocean University of China. The field studies did not involve endangered or protected species. The fish were all euthanized by tricaine methanesulfonate (MS-222).

2.2. Experimental fish and data collection

Healthy Japanese flounder were collected from Donggang District Institute of marine treasures in Rizhao of Shandong province, and brought them to Ocean University of China, where they were temporary reared in a 500 L bucket in seawater. About 1000 individuals of larval 7dph (about 50 individuals as one sample) (stage A), 40 individuals of juvenile about 90dph (stage B), 40 individuals of juvenile about 180dph (stage C) and 80 individuals of adult about 24 months (stage D) and 36 months (stage E) were collected. In our experiment and data analysis, 3 fishes (The fish of stage A were so small, so we used about 50 individuals as one sample) were used in each stage. All fish were euthanized using tricaine methanesulfonate (MS-222), and then blood was obtained from the caudal vein by using a heparinized 1 ml syringe (The fish of stage A and stage B were too small to obtain blood). Body weight, body height, body length and total length were measured at each growth stage. Tissue samples were collected and immediately frozen in liquid nitrogen (In stage A, we cut off redundant tissue and only retain muscle tissue under the microscope), and then stored at −80 °C for genomic DNA and total RNA isolation.

2.3. RNA isolation and reverse transcriptase-PCR

The muscle tissue of Japanese flounder was used to extract RNA. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The concentration of extracted total RNA was measured by the nucleic acid analyzer Biodropsis BD-1000 (OSTC, China), and 1% agarose gel was used to check the integrity of RNA. Reverse transcription was carried through a two-step method with Prime Script™ RT reagent Kit (TaKaRa, Dalian, China). The synthesized cDNA was stored at −20 °C until used.

2.4. qRT-PCR

The relative expression levels of *MyoD* and *IGF-I* mRNA were determined by total RNA extracted from muscle of Japanese flounder. qRT-PCR was performed using SYBR Premix Ex Taq™ (TliRNaseH Plus) Kit (Takara, Japan, Codeno. RR420A) on Applied Biosystems 7300 machine (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. The primers used for qPCR are given in Table 1. The amplification efficiency of these primer pairs was over 99%. Reactions were executed in a 20 μl volume containing 10 μl SYBR®Premix Ex Taq (TliRNaseH Plus), 0.4 μl PCR Forward Primer, 0.4 μl PCR Reverse Primer, 2 μl cDNA template, add RNase-free water to 20 μl. The *MyoD* qPCR conditions were as follows: denaturation at 95 °C

Table 1
Nucleotide sequences of primers used for Real Time PCR in the experiment.

Primer name	Sequence (5′ to 3′)	Product size	Tm	Accession no.
MyoD	F: GTCAGTCCACCAGGAGAGGA	117 bp	57.45 °C	DQ184914.1
MyoD	R: TGCTGGAGAGACAGTCGAGA			
IGF-I	F: GTTTGTGTGTGGAGAGAGAG	229 bp	55.40 °C	NW_017859759.1
IGF-I	R: TTTGTGCCCTGCGGTACTAA			
18S	F: ATTGACGGAAGGGCACCAC	134 bp	60.00 °C	EF126037.1
18S	R: ATGCACCACCACCCACAGA			

for 30 s, 40 cycles of denaturation at 95 °C for 5 s, annealing at Tm for 30 s, and extension at 72 °C for 30 s. 18S ribosomal RNA, as reference gene, was amplified under the same conditions. Each sample was run in triplicates. Negative control was also run by using all the qPCR reagents without the cDNA template, and the relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2012). A probability level of $P < 0.05$ was considered statistically significant.

2.5. Genomic DNA isolation

Genomic DNA was extracted from muscle samples at different developmental stages using Marine Animal DNA Kit (TransGen, Beijing, China) following the manufacturer's instructions. The concentration and purity of DNA were measured by the nucleic acid analyzer Biodropsis BD-1000 (OSTC, China), and the integrity of DNA was evaluated by agarose gel electrophoresis. The Genomic DNA was stored at −20 °C for later use.

2.6. Analysis of genetic structure and amino acid sequence of MyoD and IGF-I

Online software ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) was used to find *MyoD* and *IGF-I* gene open reading frame (ORF). Transcription factor was predicted using Jaspar software (http://jaspar.genereg.net/). The conserved gene sequence of *MyoD* was identified with the other species including *Sparus aurata* (GenBank accession no. AF478568.1), *Oreochromis niloticus* (GenBank accession no. FJ907953.1), *D. rerio* (GenBank accession no. AF318503.2), *Oreochromis aureus* (GenBank accession no. GU246721.1), *O. mykiss* (GenBank accession no. X75798.1), *Epinephelus coioides* (GenBank accession no. HM190250.1) and *Solea senegalensis* (GenBank accession no. FJ009109.1) by multiple sequence alignment. The conserved gene sequence of *IGF-I* was identified with the other species including *Paralichthys lethostigma* (GenBank accession no. DQ221741.1), *Platichthys stellatus* (GenBank accession no. KC709503.1), *Siniperca knerii* (GenBank accession no. JQ794831.1), *Siniperca scherzeri* (GenBank accession no. JQ794830.1), *Epinephelus coioides* (GenBank accession no. KR269818.1), *Lates calcarifer* (GenBank accession no. XM_018697285.1). The transcription factor binding site of *MyoD* coding region was analyzed according to Zhang et al. (2006).

2.7. DNA bisulfite modification and analysis

In each developmental stage three fish were used to process the bisulfite modification. DNA samples (200 ng) were sodium bisulfite-modified using the Methylamp™ DNA Modification Kit (QIAGEN) according to the manufacturer's instructions. The promoter and exons CpG rich regions of *MyoD* (GenBank accession no. DQ184914.1) and *IGF-I* (GenBank accession no. NW_017859759.1) were identified by NCBI (https://www.ncbi.nlm.nih.gov/). Primers were designed according to the known sequences by Oligo 6.0 (Table 2). The PCR products were cloned into a pEASY-T1 vector (TransGen, Beijing, China). For each fish typically 10 clones were sequenced to determine the methylation level. To evaluate the efficiency of bisulfite modification, we calculated the percentage of the number of converted cytosines on the total number of cytosines (excluding cytosines of CpG dinucleotides). The formula is as follows: The conversion percentage = [the number of converted cytosines (excluding cytosines of CpG dinucleotides)] / [the total number of cytosines (excluding cytosines of CpG dinucleotides)] × 100%.

2.8. IGF-I radioimmunoassay (RIA)

Stage B used liquid supernatant of muscle homogenate to measure the concentration of IGF−I. Blood of stage C, D and E were centrifuged for 10 min at 4 °C at 10,000 rpm. Plasma was removed and stored at −80 °C for later use. Plasma and muscle homogenate IGF-I levels were determined by I^{125} radioimmunoassay (The fish in stage A were too small to determine the concentration of IGF-I), using diagnostic kits from Diagnostic Products Corporation (Tianjin Nine Tripods Medical and Bioengineering Co. Ltd., Sino–US joint venture enterprise).

2.9. Statistical analysis

Data reported were expressed as means ± standard error. All qRT-PCR expression data were log-transformed to ensure normality. Data was analyzed by one-way ANOVA followed by Duncan's multiple range tests to determine significant differences between samples using SPSS 19.0. The correlations between gene expression, methylation extent, and hormone changes were initially examined by spearman tests using SPSS19.0 (SPSS Co. Ltd., Chicago). The correlation coefficient was calculated by Excel. Statistical significance was determined at *P*-value < 0.05.

3. Results

3.1. Expression of MyoD and IGF-I mRNA in muscle tissue

The expression levels of *MyoD* and *IGF-I* mRNAs in the muscles during the five different developmental stages are showed in Fig. 1. From Fig. 1, *MyoD* and *IGF-I* expression levels increased from stage A to stage C and then decreased from state C to state E, with the highest expression observed in at stage C ($P < 0.05$) and lowest expression observed in stage A ($P < 0.05$).

3.2. Structural analysis of the MyoD and IGF-I genes

Next, we performed structural analysis of the *MyoD* (GenBank Accession no. DQ184914.1) gene and found that this gene had three CpG rich regions, i.e., CpG island 1 (including 10 CpG sites located at −22 bp to +134 bp), CpG island 2 (including 37 CpG sites located at +283 bp to +609 bp) and CpG island 3 (including 11 CpG sites located

Table 2
Primers used for bisulphate PCR (BS-PCR).

Primer name	Sequence (5′ to 3′)	Product size	Tm	Accession no.
MyoD-P1	F: GGTTGTTGATTGGTTAGATTTTAGTG	264 bp	56.02 °C	DQ184914.1
MyoD-P1	R: ACTCCATAATTCAATCCTTAACCCAA			
MyoD-E1	F: TTTGGGTTTGTAAGGTTTGTAAGTGG	327 bp	57.59 °C	
MyoD-E1	R: ACCTATCAATCCCTAAACTCACCATA			
MyoD-E2	F: AAGTATTAATTAGTGTTTGGTTTTAT	240 bp	49.71 °C	
MyoD-E2	R: TAAATCAAATTAAAATCCTAACTAAC			
IGF-I-P1	F: TTTGTGTAATGTAGATAAATGTGAGG	261 bp	54.44 °C	NW_017859759.1
IGF-I-P1	R: CCTTAAAAACATCACATAAATACCAC			
IGF-I-E1	F: TTTGGAAATTTTGGTATGTATTGTGT	127 bp	52.86 °C	
IGF-I-E1	R: TTACAAAACTACCTACATTAAAACTT			

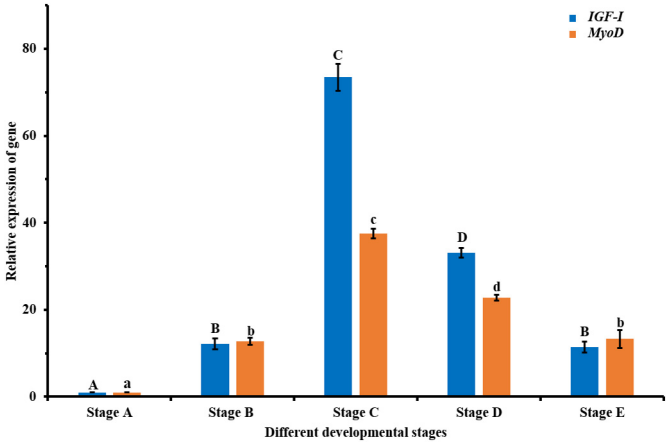

Fig. 1. Muscle mRNA expression of *MyoD* and *IGF-I* from five different developmental stages in Japanese flounder. Each histogram represents the mean of three determinations. Values are expressed as mean ± standard error of mean. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

at +2113 bp to +2343 bp) (Fig. 2A). As shown in Fig. 2B, the promoter of *MyoD* was 264 bp in length and included nine CpG sites. All the CpG sites in the promoter were located at or near the putative transcription factors binding sites, such as that for MEF2C, USF1, SP1, MyoG, MyoD1. Additionally, the predicted CpG-rich region in *MyoD* exon 1 was 327 bp in length (Fig. 2C) and included 30 CpG sites. We found that six of the CpG sites were located at or near the presumptive binding sites for transcription factors such as MyoD1, MyoG, AP-1. As shown in Fig. 2D, the forecasted CpG-rich regions in *MyoD* exon 3 had 13 CpG sites, six of which were located at or near the constructive binding sites for transcription factors, such as USF1, MyoD1, MyoG. As shown in Fig. 5A, the promoter in *IGF-I* had 12 CpG sites, six of which were located at transcription factors binding sites, such as USF1, MEF2C, MyoG, SP1, MyoD1.Six CpG sites were located at *IGF-I* exon 1, and all CpG sites were located at the putative binding sites of transcription factors, such as USF1, Myog, SP1, Myod1(Fig. 5B).

3.3. Relationship between MyoD DNA methylation and expression during different developmental stages

The polymerasechain reaction (PCR) products of bisulfite modification were evaluated by agarose gel electrophoresis, and the result revealed that all products were consistent with the expected band sizes (Fig. S1A, SI Appendix). Part of the sequencing diagram is shown in Fig. S1B (SI Appendix). Evaluation of the efficiency of bisulfite treatment showed that Cs were all converted to Ts in all copies of three different samples of CpG dinucleotide sequences. This findings implied that the DNA modification procedure was very efficient.

The CpG dinucleotides methylation status of promoter and two exons in *MyoD* was detected by bisulfite conversion and subsequent DNA sequencing in muscle tissues. The DNA methylation patterns are shown in Fig. 3. The promoter and exons showed different methylation levels in the *MyoD* gene; the methylation level of exon 3 was much higher than those of the promoter and exon 1. Additionally, the methylation level in promoter sequence was decreased from stage A to stage C and then gradually increased from stage C to stage E, with the maximum and minimum levels of methylation observed during stage A and stage C, respectively. The methylation levels in exon 1 and exon 3 were decreased from stage A to stage B and then gradually increased from stage B to stage E; the minimum and maximum methylation levels were observed in stage B and stage A, respectively.

There were 9 CpG sites in *MyoD* promoter; the methylation level of each CpG sites is shown in Fig. S2A (SI Appendix). DNA methylation levels decreased from stage A to stage C and then increased from stage

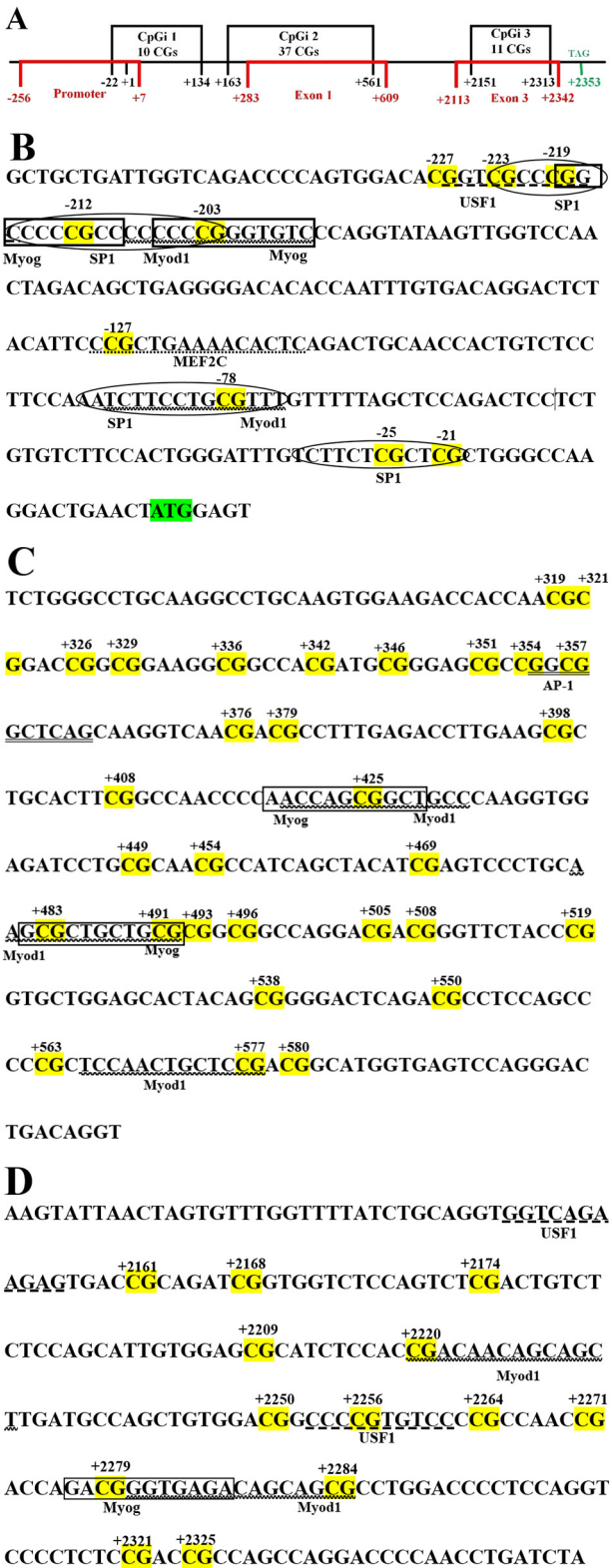

Fig. 2. The gene structure analysis of *MyoD*. The long black line indicates the *MyoD* gene structure and the three empty black boxes indicate the CpGi1, CpGi2 and CpGi3 CpG rich region (*i.e.* CpG island). The regions of measuring the methylation level (from -256 bp to +7 bp, from +283 bp to +609 bp and from +2113 bp to +2342 bp) in promoter and two exons are marked with red line, almost covered the CpG rich regions. The stop codon was located at +2353 bp site, showed in green color (Panel A). The yellow boxes indicate CpG site on coding regions of *MyoD* gene (Panels B and C). The binding sequences of different transcription factors are marked with different sign and the name of the transcription factors were marked under the corresponding transcription factor. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

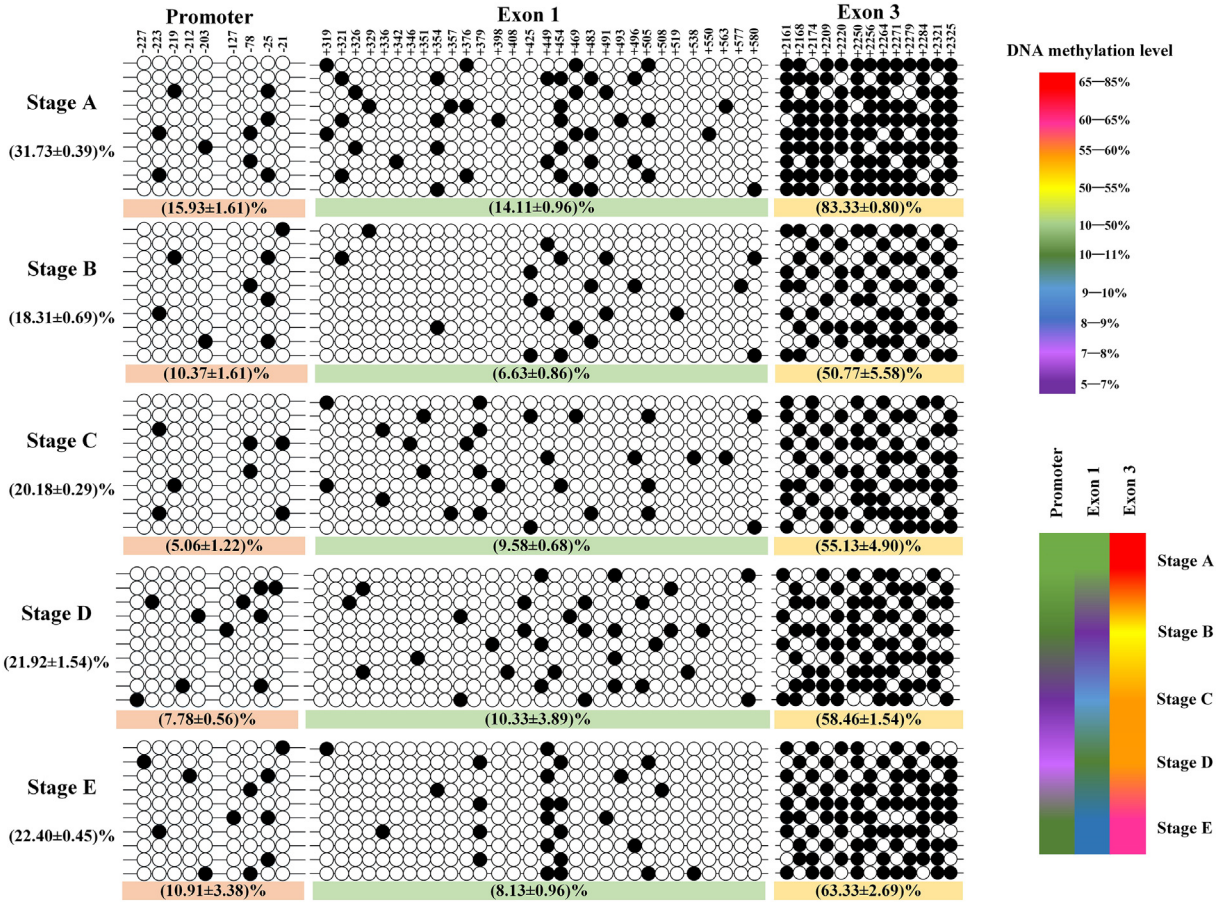

Fig. 3. DNA methylation patterns of promoter and two exons in *MyoD*. An open circle represents an unmethylated CpG, and a solid circle represents a methylated CpG. Each line represents one sequenced clone. The first line indicates the localizations of studied CpG sites related to the sequence of *MyoD*. The percentage indicates the methylation level (the percentage on the left indicates the average methylation level of *MyoD* at different development stages), calculated as the number of methylated CpG sites per total number of CpG sites in each stage, data as mean ± SEM. Average methylation was calculated for all CpG sites in each stage. 3 fish samples were used, and for each fish typically 10 clones were used to determine DNA methylation levels. Different colors in the right show different methylation level of promoter and exons.

C to stage E; the peak level was observed in stage A. Moreover, the methylation level of CpG sites at −203 bp was the lowest, and that at −25 bp was the highest. The changes in methylation levels for the 30 CpG sites in *MyoD* exon 1 are detailed in Fig. S2B (SI Appendix). The DNA methylation level of each CpG site in exon 1 tended to decreased from stage A to stage B and then increased from stage B to stage E, with peak methylation observed in stage A. In addition, the CpG site at +354 bp, +357 bp, +425 bp and so on were also in transcription factor binding sequences. The methylation levels of 13 CpG sites in exon 3 are shown in Fig. S2C (SI Appendix). At these sites, the DNA methylation levels first decreased and then increased, with peak methylation levels observed in stage A.

As shown in Fig. 4, the methylation levels of the promoter and exon 1 is relatively low as compared with those of exon 3; however, the average methylation levels of the promoter and two exons in *MyoD* gene showed similar trends. The results demonstrated that the average methylation level in stage A was higher than those in stage B, stage C, stage D and stage E. The relationships between DNA methylation levels of in the promoter and two exons of the *MyoD* gene and expression of the *MyoD* gene are also given in Fig. 4. The average methylation level of *MyoD* gene was strongly negatively correlated with gene expression during different developmental stages. The methylation level of the promoter was also negatively correlated with gene expression ($r = -0.643$); methylation levels were high in stage A and low in stage C, but then increased from stage C to stage E.

3.4. Changes in methylation and expression levels of the IGF-I gene during different developmental stages

There were 12 CpG sites in the *IGF-I* promoter; the methylation level of each CpG sites is shown in Fig. S3A (SI Appendix). DNA methylation levels decreased from stage A to stage C and then increased from stage C to stage E, with peak methylation observed in stage A. Moreover, the methylation level was the highest in the CpG site at –64 bp. Changes in the methylation trends of six CpG sites in *IGF-I* exon 1 are shown in Fig. S3B (SI Appendix). The DNA methylation of each CpG site in exon 1 tended to decreased from stage A to stage C and then increased from stage C to stage E, with peak methylation observed in stage A. In addition, the methylation level was the highest in the CpG sites at +355 bp.

Next, we analyzed the methylation levels of the promoter and exon 1 in the *IGF-I* gene. As shown in Fig. 6, the methylation level of *IGF-I* gene was relatively low compared with that in the *MyoD* gene, although similar trends were observed between the two genes. The methylation levels of the promoter and exon 1 sequences in *IGF-I* exhibited trends similar to those of the average methylation level; the average methylation level of *IGF-I* promoter decreased from stages A to stage C and then increased from stage C to stage E, with the highest methylation level observed in stage A (Fig. 7). The relationship between the DNA methylation level *IGF-I* and the expression of the *IGF-I* gene is shown in Fig. 7. The average methylation level of *IGF-I* was correlated with *IGF-I* gene expression during the different developmental stages ($r = -0.884$). The methylation level of *IGF-I* exon 1 was also negatively

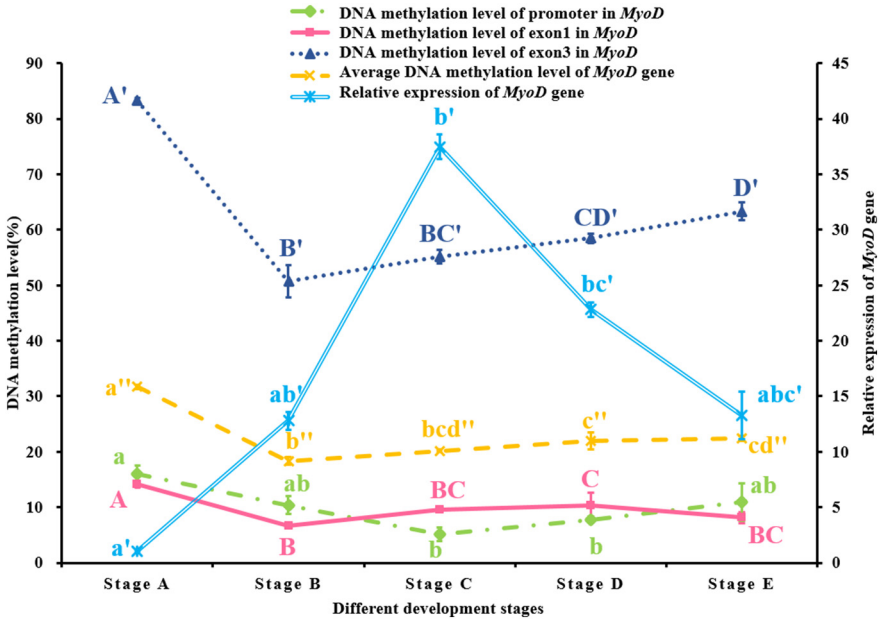

Fig. 4. The correlation between gene expression and CpG methylation level of *MyoD* gene at different development stages. Values represent mean ± SEM. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

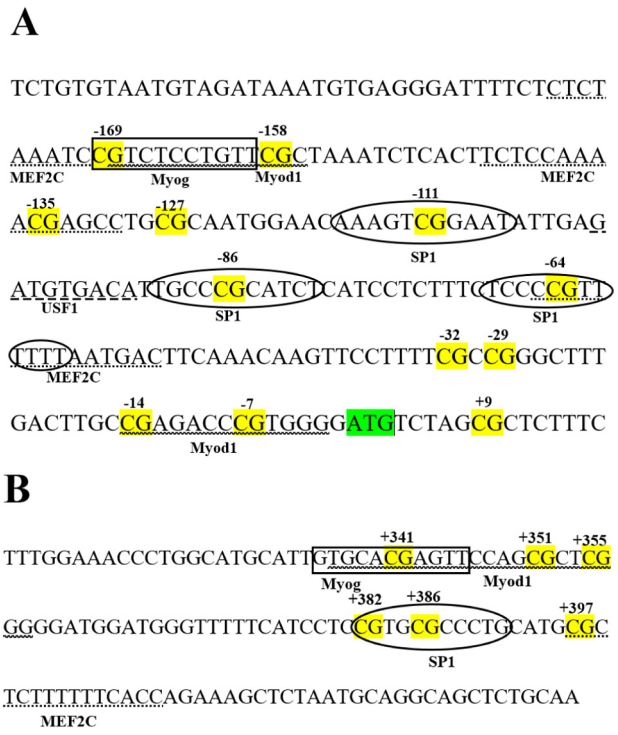

Fig. 5. The gene structure analysis of *IGF-I*. The yellow boxes indicate CpG site on promoter and exon1 of *IGF-I* gene (Fig. 7A and B). The binding sequences of different transcription factors are marked with different sign and the name of the transcription factors were marked under the corresponding transcription factor. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

correlated with gene expression.

3.5. Changes in MyoD and IGF-I gene expression during different developmental stages

As shown in Fig. 8, the methylation level of *MyoD* was strongly negatively correlated with its gene expression (r = −0.643); similar results were observed for the *IGF-I* gene (r = −0.884) during different developmental stages. The transcription level of *IGF-I* gene increased initially and then descended, similar to the observed changes in the expression of the *MyoD* gene ($r = 0.972$).

3.6. Relationships between growth traits and expression and methylation levels during different developmental stages

Next, we evaluated the relationships among growth traits, expression levels, and methylation levels during different developmental stages of the Japanese flounder. As shown in Fig. S4A (SI Appendix), as body weight increased, the relative expression levels of the *MyoD* and *IGF-I* genes first increased and then decreased; in contrast, the average methylation levels of the *MyoD* and *IGF-I* genes initially decreased and then increased gradually. As shown in Fig. S4B (SI Appendix), as body length increased, the relative expression levels of *MyoD* and *IGF-I* genes first increased and then decreased; in contrast, the average methylation levels initially decreased and then increased gradually.

3.7. Changes in IGF-I level during different developmental stages of the Japanese flounder

Next, we examined changes in IGF-I level corresponding with muscle development during the four different developmental stages. As shown in Fig. 9, IGF-I levels increased from stage B to stage C and then decreased, with peak levels observed in stage C. The results showed that IGF-I levels showed changes similar to those of *MyoD*. As shown in Fig. 9, changes in the concentration of IGF-I were consistent with changes in the relative expression levels of the *IGF-I* gene.

4. Discussion

In recent years, the role of epigenetics in fish muscle growth has not been well studied. Although, some studies have assessed quantitative and molecular genetics (Akolkar et al., 2016; Tan et al., 2002; Zhang et al., 2006; Salem et al., 2005) as well as the epigenetics of fish reproduction (Si et al., 2016; Ding et al., 2013), no studies have examined the epigenetics of Japanese flounder muscle growth. DNA methylation has been extensively studied owing to its involvement in most cellular processes. Moreover, DNA methylation regulates gene expression and is

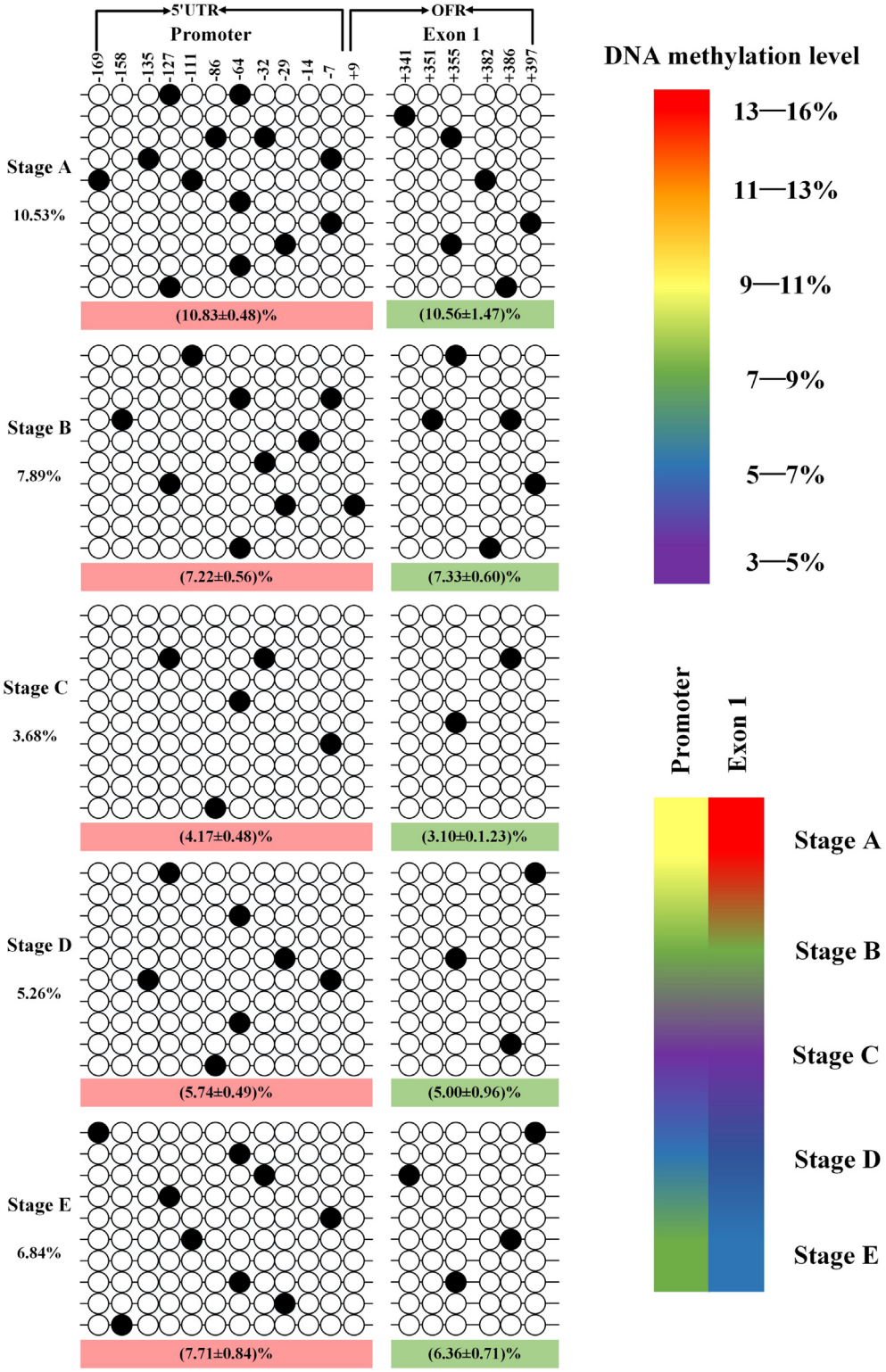

Fig. 6. DNA methylation patterns of promoter and exon1 in *IGF-I*. An open circle represents an unmethylated CpG, and a solid circle represents a methylated CpG. Each line represents one sequenced clone. Numbers in the first line indicates the localizations of studied CpG sites related to the sequence of *IGF-I*. The percentage indicates the methylation level (the percentage on the left indicates the average methylation level of each stage), calculated as the number of methylated CpG sites per total number of CpG sites in each stage, data as mean ± SEM. Average methylation was calculated for all CpG sites in each stage. 3 fish samples were used, and for each fish typically 10 clones were used to determine DNA methylation levels. Different colors in the right show different methylation level of promoter and exons.

critical for establishing patterns of gene repression during development (Cedar and Bergman, 2009). The Japanese flounder is an important economic fish and exhibits a rapid growth rate due to fast-skeletal muscle growth. Skeletal muscle growth is regulated by many genes, including *MyoD* gene, which is necessary for the determination of skeletal muscle specification and differentiation. Additionally, the *IGF-I* gene is responsible for the proliferation and differentiation of skeletal muscle cells.

Fu et al. (2011) reported that microRNAs play an important role in regulating gene expression during Japanese flounder metamorphosis. In present study, we analyzed the DNA methylation levels of *MyoD* and *IGF-I* gene in five different stages from myogenesis to muscle maturation in order to determine whether epigenetic modification of *MyoD* and *IGF-I* was responsible for larvae and adult skeletal muscle growth and development. We found that the highest and lowest average DNA methylation level of *MyoD* gene appeared in stage A and stage C, respectively; in contrast, the lowest average DNA methylation of *IGF-I* gene was observed in stage B. We hypothesize that this phenomenon

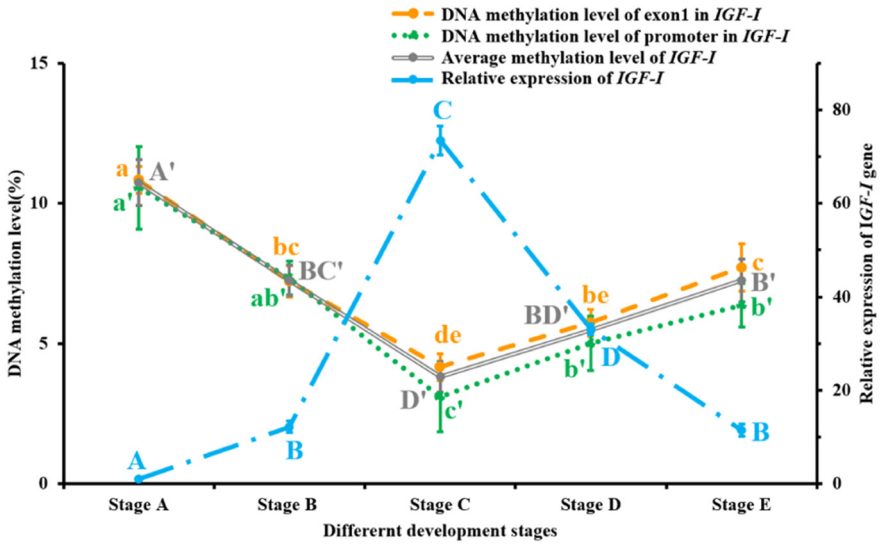

Fig. 7. The correlation between gene expression and CpG methylation level of *IGF-I* gene at different development stages. Values represent mean ± SEM. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

may be caused by metamorphosis and demethylating. Indeed, the progression of metamorphosis from stage A to stage B requires a significant amount of skeletal muscle. Moreover, demethylation is known to occur after fertilization. Both of these processes can result in dramatic epigenetic changes.

We also verified the expression levels of *MyoD* and *IGF-I* gene during five different developmental stages. Carani et al. (2014) found that there was no differences in *MyoD* gene expression from the early juvenile stage to the post-juvenile stages in *Arapaima gigas*. In *Oncorhynchus mykiss*, the results showed significant differences in different developmental stages; the expression level of *MyoD* was low at 14 days post-fertilization (dpf), peaked at 37dpf, then gradually decreased and remained low until the 3-year-old stage, and increased after the 3-year-old stage, reaching its highest levels during this stage (Johansen and Overturf, 2005). Our analysis of *MyoD* gene expression showed results different from those in previous reports of teleosts, whereas relative expression of *IGF-I* gene was consistent with those of previous studies. Barbara et al. (2010) demonstrated that expression of *IGF-I* gene decreased in Large White fish from 60 to 210 days of age. Moreover, the *IGF-I* gene has been shown to stimulate proliferation and differentiation in different species, including chickens and zebrafish (Duclos, 2005; Fuentes et al., 2013; Li et al., 2014; Tollefsens et al., 1989). Metamorphosis appeared during 7 to 90 days post-hatching (dph), and we assumed that a large amount of muscle was needed during this phase to complete metamorphosis. Consistent with this, the relative expression of *MyoD* and *IGF-I* genes increased to meet the requirements for the forming skeletal muscle during metamorphosis. Previous studies have also reported that the expression of *IGF-I* (Zhang et al., 2011a, 2011b) and IGF-IR (Hildahl et al., 2007) expression levels increased during metamorphosis. The results implied that *MyoD* and *IGF-I* gene had positive effect on skeletal muscle formation during metamorphosis. Furthermore, the relative expression of the *IGF-I* gene showed variations similar to that of the *MyoD* gene. This indicated that the *MyoD* and *IGF-I* genes were involved in muscle growth during different developmental stages.

Our findings also demonstrated that *MyoD* interacted with *IGF-I* to

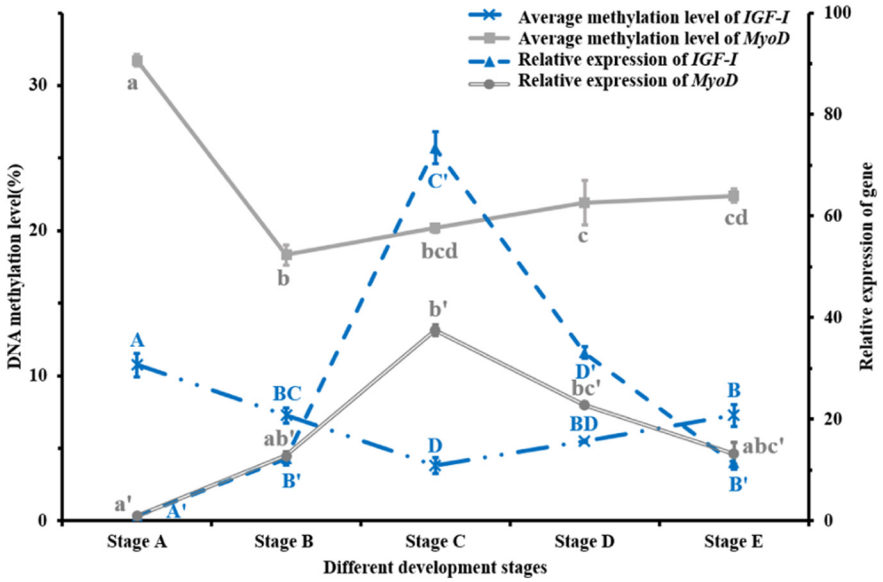

Fig. 8. The change trends of *MyoD* and *IGF-I* at different development stages. Values as mean ± SEM. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

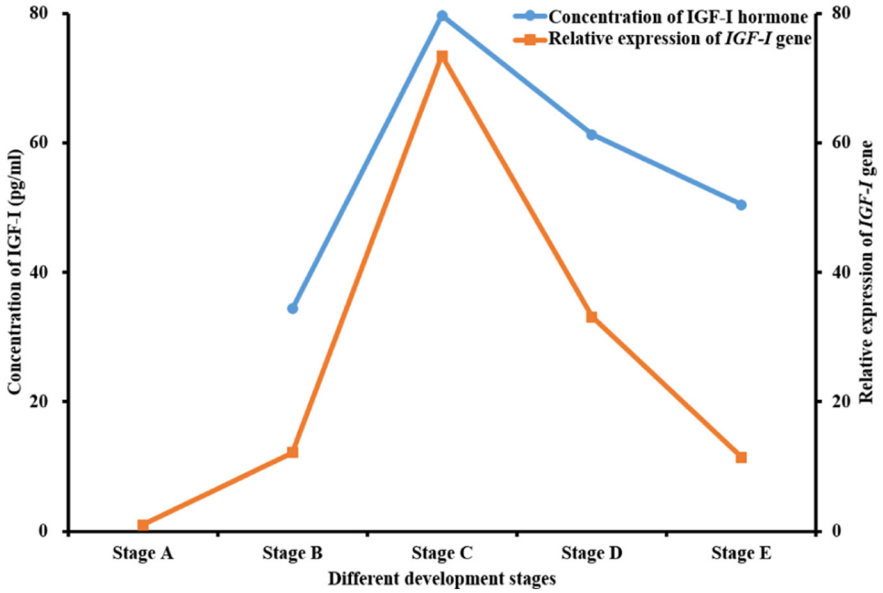

Fig. 9. The concentration of IGF-I of the plasma or muscle homogenate in RIA.

regulate Japanese flounder muscle hypertrophy and muscle mass. Some studies have obtained similar resultsin other species, including rats (Aguiar et al., 2013) and transgenic zebrafish (*Danio rerio*) (Marcio and Maeli, 2012). Interestingly, our study showed that *MyoD* methylation was not completelynegatively correlated with gene expression throughout the entire developmental process. Specifically, from stage A to stage C, the methylation level first decreased and then increased, whereas the expression level was increased throughout all developmental stages. From stage A to stage B, the Japanese flounder underwent metamorphosis. We hypothesized that variations in DNA methylation patterns after birth were affected by the metamorphosis stage. Because gene expression continued to increase, we assumed that the skeletal muscles of fish needed to continue growing to maintain regular growth after metamorphosis.

DNA methylation plays a vital role in regulation of gene expression, and an appropriate level of DNA methylation is necessary for gene function (Jones and Takai, 2001; Morgan et al., 2005). However, DNA methylation is not the only factor affecting gene expression. Some studies have reported that environmental factors, histones and transcription factors can also influence epigenetic status (Down et al., 2008; Jaenisch and Bird, 2003; Su et al., 2011). Interestingly, the CpG site of *MyoD* at +354 bp and +357 bp CpG were located at the AP-1 transcription factor binding site. Li et al. (2008) reported that the AP-1 regulates a wide range of cellular processes, including cell proliferation, death, survival and differentiation. Reducing AP-1 binding at the *MyoD* promoter resultes in increased *MyoD* expression in mice (Pedraza-Alva et al., 2009). Moreover, the CpG sites at −212 bp, +425 bp, +483 bp, +491 bp CpG site and so on of *MyoD* gene and −169 bp, −158 bp, −14 bp, −7 bp, +341 bp, +351 bp, +355 bp of *IGF-I* gene were located at binding sites for the Myog or Myod1 transcription factors, respectively. Myog and Myod1 are bHLH factors, which have been shown to regulate myogenesis in mammals, birds, sea urchins, nematodes, frogs and insects (Emerson, 1993; Olson and Klein, 1994; Olson, 1990; Sassoon, 1992; Weintraub et al., 1991). Furthermore, MyoG acted synergistically with MyoD to modulate a set of genes normally expressed late in the program of myogenic differentiation (Cao et al., 2006). DNA methylation can cause chromatin to condense, thereby blocking transcription (Boerboom et al., 1999; Fü et al., 2001). Additionally, the CpG site at −227 bp, −223 bp and +2256 bp CpG site of *MyoD* gene were located at the binding site for the USF1 transcription factor, which is involved in decreasing the activity of *MyoDa* and inhibiting MyoD-dependent autoactivation in *Xenopus* (Gao et al., 1997; Lun et al., 1997) by binding to the promoter. We speculate that high CpG methylation at positions +354 bp and so on decreased the combination rate of the MyoG, MyoD1, AP-1, MEF2C, SP1 and USF1 corresponding with their recognition sequences and then reduced *MyoD* and *IGF-I* transcriptional activity during stage A to stage E; DNA methylation levels of *MyoD* and *IGF-I* were the lowest during stage B and stage C, respectively, and the binding rates of transcription factors with their recognition sequences increased, resulting in upregulation of gene expression. However, further studies are needed to determine the combination rate of the MyoG, MyoD1, AP-1 and USF1. Thus, DNA methylation together with other factors may regulate gene expression. Additional studies should evaluate whole-genome methylation levels to identify other genes that regulate muscle growth. Moreover, lower methylation may occur between stage A and stage B due to metamorphosis, and low DNA methylation tended to elevate gene expression; however, additional work is required to determine the exact nature of these trends and to identify the factors affecting gene expression and muscle growth.

Muscle growth in teleost fish is also regulated by the growth hormone (GH)/IGFs axis (Castillo et al., 2004; Castillo et al., 2002; Green et al., 1985; Le Bail et al., 1998; Moriyama et al., 2000; Peterson et al., 2004). The *IGF-I* gene belonges to this axis. Our findings demonstrated that changes in the concentration of IGF-I hormone were similar to those in the *IGF-I* gene. Similarly, Stratikopoulos et al. (2008) reported that liver-specific knockout of the *Igf1* gene in mice significantly reduces serum IGF-1 levels, suggesting that the *IGF-I* gene plays a role in regulating IGF-I hormone levels. In addition, the levels of IGF-Ihormone, *IGF-I*, and *MyoD* may alter muscle growth together during different developmental stages.

5. Conclusion

In this study, we evaluated the expression patterns of the *MyoD* and *IGF-I* genes during the five different developmental stages. Our results showed that the expression of the *MyoD* and *IGF-I* genes was high during metamorphosis, indicating that the *MyoD* and *IGF-I* genes may be important in regulating muscle growth during metamorphosis. Analysis of DNA methylation levels showed that the CpG sites in the *MyoD* and *IGF-I* promoter and exons were located at binding sites for MyoG, MyoD1, AP-1, MEF2C, SP1 and USF1, suggesting that the binding efficiencies of these transcription factors affected gene expression. The average methylation levels of CpG islands were negatively correlated with gene expression during five different

developmental stages, demonstrating that the patterns of DNA methylation in *MyoD* and *IGF-I* promoter and exons could affect gene expression. The relative expression of *MyoD* gene was similar to that of the *IGF-I* gene. Additionally, changes in serum IGF-I levels were similar to the relative expression of *IGF*-I gene. Our finding suggested *IGF-I* gene and *MyoD* regulated muscle growth together during different development stages in the Japanese flounder. These results provided important insights into the epigenetic mechanisms of fish muscle growth during different development stages.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpb.2018.02.005.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (31672642), Natural Science Foundation of Shandong Province, China (ZR2014CM018) and AoShan Talents Cultivation Program Supported by Qingdao National Laboratory for Marine Science and Technology (2017ASTCP-ES06). It is appreciated that the comments from editors and reviewers have greatly improved our manuscript.

Conflict of interest

The authors declare that they have no competing interests.

References

Aguiar, A.F., Vechetti-Júnior, I.J., Alves De Souza, R.W., Castan, E.P., Milanezi-Aguiar, R.C., Padovani, C.R., Silva, M.D.P., 2013. Myogenin, MyoD and IGF-I regulate muscle mass but not fiber-type conversion during resistance training in rats. Int. J. Sports Med. 34, 293–301.

Akolkar, D.B., Asaduzzaman, M., Kinoshita, S., Asakawa, S., Watabe, S., 2016. Characterization of *Pax3* and *Pax7* genes and their expression patterns during different development and growth stages of Japanese pufferfish *Takifugu rubripes*. Gene 575, 21–28.

Antequera, F., Bird, A., 1999. CpG islands as genomic footprints of promoters that are associated with replication origins. Curr. Biol. 9, 661–667.

Barbara, R., Maria, O., Katarzyna, P., Agata, P.K., Malgorzata, W., Magdalena, R., Marian, R., 2010. Expression of IGF1 and IGF2 genes in muscles during development of pigs representing five different breeds. Ann. Anim. Sci. 10, 231–239.

Berkes, C.A., Tapscott, S.J., 2005. MyoD and the transcriptional control of myogenesis. Semin. Cell Dev Boil. 16, 585–595.

Bird, A., 2002. DNA methylation patterns and epigenetic memory DNA methylation patterns and epigenetic memory. Genes Dev. 16, 6–21.

Boerboom, D., Kerban, A., Sirois, J., 1999. Dual regulation of promoter II and promoter 1f-derived cytochrome P450 aromatase transcripts in equine granulosa cells during human chorionic gonadotropin-induced ovulation: a novel model for the study of aromatase promoter switching. Endocrinology 140, 4133–4141.

Buckingham, M., 1992. Making muscle in mammals. Trends Genet. 8, 144–149.

Buckingham, M., 2006. Myogenic progenitor cells and skeletal myogenesis in vertebrates. Curr. Opin. Genet. Dev. 16, 525–532.

Buckingham, M., Bajard, L., Chang, T., Daubas, P., Hadchouel, J., Meilhac, S., Relaix, F., 2003. The formation of skeletal muscle: from soma to limb. J. Anat. 202, 59–68.

Cao, Y., Kumar, R.M., Penn, B.H., Berkes, C.A., Kooperberg, C., Boyer, L.A., Tapscott, S.J., 2006. Global and gene-specific analyses show distinct roles for Myod and Myog at a common set of promoters. EMBO J. 25, 502–511.

Carani, F.R., Duran, B.O.D.S., Piedade, W.P., Costa, F.A.A.D., Almeidaval, V.M.F.D., 2014. Expression of growth-related factors in skeletal muscle of Pirarucu (*Arapaima Gigas*) during growth. J. Aqua Res. Dev. 5, 1000272.

Castillo, J., Le Bail, P.Y., Paboeuf, G., Navarro, I., Weil, C., Fauconneau, B., Gutiérrez, J., 2002. IGF-I binding in primary culture of muscle cells of rainbow trout: changes during in vitro development. Am. J. Physiol. Regul. Integr. Comp. Physiol. 283, R647–R652.

Castillo, J., Codina, M., Martínez, M.L., Navarro, I., Gutiérrez, J., 2004. Metabolic and mitogenic effects of IGF-I and insulin on muscle cells of rainbow trout. Am. J. Physiol. Regul. Integr. Comp. Physiol. 286, R935–R941.

Cedar, H., Bergman, Y., 2009. Linking DNA methylation and histone modification: patterns and paradigms. Nat. Rev. Genet. 10, 295–304.

Cleveland, B.M., Weber, G.M., Virginia, W., 2010. Effects of insulin-like growth factor-I, insulin, and leucine on protein turnover and ubiquitin ligase expression in rainbow trout primary myocytes. Am. J. Physiol. Regul. Integr. Comp. Physiol. 298, R341–R350.

CNKI, 2014. China clinical medicine. In: Shen, . http://kns.cnki.net/kns/brief/default_result.aspx Accessed 1 May 2014.

Coleman, M.E., Demayo, F., Yin, K.C., Geskell, R., Montgomerylj, C., Schwartz, R.J., 1995. Myogenic vector expression of insulin-like growth factor I stimulates muscle cell differentiation and myofiber hypertrophy transgenic mice. J. Biol. Chem. 270, 12109–12116.

Davis, R.L., Weintraub, H., Lassar, A.B., 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51, 987–1000.

Díaz, M., Vraskou, Y., Gutie, J., Planas, J.V., 2009. Expression of rainbow trout glucose transporters GLUT1 and GLUT4 during in vitro muscle cell differentiation and regulation by insulin and IGF-I. Am. J. Physiol. Regul. Integr. Comp. Physiol. 296, R794–R800.

Ding, Y., He, F., Wen, H., Li, J., Ni, M., Chi, M., Zhao, J., 2013. DNA methylation status of cyp17-II gene correlated with its expression pattern and reproductive endocrinology during ovarian development stages of Japanese flounder (*Paralichthys olivaceus*). Gene 527, 82–88.

Down, T.A., Rakyan, V.K., Turner, D.J., Flicek, P., Li, H., Kulesha, E., Tavare, S., 2008. A Bayesian deconvolution strategy for immunoprecipitation-based DNA methylome analysis. Nat. Biotechnol. 26, 779–785.

Duclos, M.J., 2005. Insulin-like growth factor-i (igf-1) mRNA levels and chicken muscle growth. J. Physiol. Pharmacol. 56, 25–35.

Egger, G., Liang, G., Aparicio, A., Jones, P., 2004. Epigenetics in human disease and prospects for epigenetic therapy. Nature 429, 457–463.

Emerson, C.P., 1993. Skeletal myogenesis: genetics and embryology to the fore. Curr. Opin. Genet. Dev. 3, 265–274.

Fü, R., Said, H.M., Schwerin, M., Vanselow, J., 2001. DNaseI hypersensitive sites and DNA hypomethylation correlate with placental expression. Eur. J. Biochem. 268, 1222–1227.

Fu, Y., Shi, Z., Wu, M., Zhang, J., Jia, L., Chen, X., 2011. Identification and differential expression of MicroRNAs during metamorphosis of the Japanese flounder (*Paralichthys olivaceus*). PLoS One 6, e22957.

Fuentes, E.N., Antonio, J., Molina, A., Thrandur, B., 2013. Regulation of skeletal muscle growth in fish by the growth hormone-insulin-like growth factor system. Gen. Comp. Endocrinol. 192, 136–148.

Gao, E., Wang, Y., Alcorn, J.L., Mendelson, C.R., 1997. The basic helix-loop-helix-zipper transcription factor USF1 regulates expression of the surfactant protein-A gene. J. Biol. Chem. 272, 23398–23406.

Green, H., Morikawa, M., Nixon, T.A., 1985. A dual effector theory of growth hormone action. Differentiation 29, 195–198.

Hawke, T.J., Garry, D.J., 2001. Myogenic satellite cells: physiology to molecular biology. J. Appl. Physiol. 91, 534–551.

Hildahl, J., Galay-Burgos, M., Sweeney, G., Einarsdóttir, I.E., Björnsson, B.T., 2007. Identification of two isoforms of Atlantic halibut insulin-like growth factor-I receptor genes and quantitative gene expression during metamorphosis. Comp. Biochem. Physiol. B 147, 395–401.

Holliday, R., Pugh, J.E., 1975. DNA modification mechanisms and gene activity during development. Science 187 (New Series), 226–232.

Howard, G., Morikawa, M., Mxon, T., 1985. A dual effector theory of growth-hormone action. Differentiation 29, 195–198.

Jablonka, E., Lamb, M.J., 1998. Epigenetic inheritance in evolution. J. Evol. Biol. 11, 159–183.

Jaenisch, R., Bird, A., 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat. Genet. 33, 245–254.

Johansen, K.A., Overturf, K., 2005. Quantitative expression analysis of genes affecting muscle growth during development of rainbow trout (*Oncorhynchus mykiss*). Mar. Biotechnol. 7, 576–587.

Jones, P.A., Takai, D., 2001. The role of DNA methylation in mammalian epigenetics. Science 293, 1068–1070.

Le Bail, P.Y., Gentil, V., Noel, O., Gomez, J.M., Carre, F., Le Goff, P., Weil, C., 1998. Structure, function, and regulation of insulin-like growth factors in fish. Ann. N. Y. Acad. Sci. 839, 157–161.

Li, M., Chiu, J.F., Gagne, J., Fukagawa, N.K., 2008. Age-related differences in insulin-like growth factor-1 receptor signaling regulates Akt/FOXO3a and ERK/Fos pathways in vascular smooth muscle cells. J. Cell. Physiol. 217, 377–387.

Li, X., Bai, J., Ye, X., Hu, Y., Li, S., Yu, L., 2009. Polymorphisms in the 5′flanking region of the insulin-like growth factor I gene are associated with growth traits in largemouth bass *Micropterus salmoides*. Fish. Sci. 75, 351–358.

Li, J., Wu, P., Liu, Y., Wang, D., Cheng, C.H.K., 2014. Gene expression patterns temporal and spatial expression of the four Igf ligands and two Igf type 1 receptors in zebrafish during early embryonic development. Gene Expr. Patterns 15, 104–111.

Livak, K.J., Schmittgen, T.D., 2012. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408.

Lun, Y., Sawadogo, M., Perry, M., 1997. Autoactivation of *Xenopus* MyoD transcription and its inhibition by USF1. Cell Growth Differ. 8, 275–282.

Marcio, E.A., Maeli, L.F., 2012. Muscle-specific growth hormone receptor (GHR) overexpression induces hyperplasia but not hypertrophy in transgenic zebrafish. Transgenic Res. 21, 457–469.

Maruyama, R., Toyooka, S., Toyooka, K.O., Virmani, A.K., Zöchbauer-Müller, S., Farinas, A.J., Minna, J.D., McConnell, J., Frenkel, E.P., Gazdar, A.F., 2002. Aberrant promoter methylation profile of prostate cancers and its relationship to clinicopathological features. Clin. Cancer Res. 8, 514–519.

Miyake, M., Hayashi, S., Sato, T., Taketa, Y., Watanabe, K., 2007. Myostatin and MyoD family expression in skeletal muscle of IGF-1 knockout mice. Cell Biol. Int. 31, 1274–1279.

Molkentin, J.D., Olson, E.N., 1996. Combinatorial control of muscle development by basic helix-loop-helix and MADS-box transcription factors. Proc. Natl. Acad. Sci. USA 93, 9366–9373.

Morgan, H.D., Santos, F., Green, K., Dean, W., Reik, W., 2005. Epigenetic reprogramming in mammals. Hum. Mol. Genet. 14, 47–58.

Moriyama, S., Ayson, F.G., Kawauchi, H., 2000. Growth regulation by insulin-like growth

factor-I in fish. Biosci. Biotechnol. Biochem. 64, 1553–1562.
Olson, E.N., 1990. MyoD family: a paradigm for development? Genes Dev. 4, 1454–1461.
Olson, E.N., Klein, W.H., 1994. bHLH factors in muscle development: deadlines and commitments, what to leave in and what to leave out. Genes Dev. 8, 1–8.
Pedraza-Alva, G., Zingg, J.M., Donda, A., Pérez-Martínez, L., 2009. Estrogen receptor regulates MyoD gene expression by preventing AP-1-mediated repression. Biochem. Biophys. Res. Commun. 389, 360–365.
Peterson, B.C., Waldbieser, G.C., Bilodeau, L., 2004. IGF-I and IGF-II mRNA expression in slow and fast growing families of USDA103 channel catfish (*Ictalurus punctatus*). Comp. Biochem. Physiol. A 139, 317–323.
Pinheiro, A., Faustino, I., Silva, M.J., Silva, J., Sá, R., Sousa, M., Rivera, I., 2010. Human testis-specific PDHA2 gene: methylation status of a CpG island in the open reading frame correlates with transcriptional activity. Mol. Genet. Metab. 99, 425–430.
Plass, C., Soloway, P.D., 2002. DNA methylation, imprinting and cancer. Eur. J. Hum. Genet. 10, 6–16.
Pownall, M.E., Gustafsson, M.K., Emerson, C.P., 2002. Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. Annu. Rev. Cell Dev. Biol. 18, 747–783.
Puri, P.L., Sartorelli, V., 2000. Regulation of muscle regulatory factors by DNA-binding, interacting proteins, and post-transcriptional modifications. J. Cell. Physiol. 185, 155–173.
Riggs, A.D., 1975. X chromosome inactivation, differentiation. Cytogenet. Genome Res. 1975 (14), 9–25.
Rudnicki, M.A., Braun, T., Hinuma, S., Jaenisch, R., 1992. Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. Cell 71, 383–390.
Salem, M., Yao, J., Rexroad, C.E., Kenney, P.B., Semmens, K., Killefer, J., Nath, J., 2005. Characterization of calpastatin gene in fish: Its potential role in muscle growth and fillet quality. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 141, 488–497.
Sassoon, D., 1992. Myogenic regulatory factors: dissecting their role and regulation during vertebrate embryogenesis. Dev. Biol. 156, 11–23.
Shunsuke, M., Felix, G.A., Kawauchi, H., 2000. Growth regulation by insulin-like growth factor-I in fish. Biosci. Biotechnol. Biochem. 64, 1553–1562.
Si, Y., Ding, Y., He, F., Wen, H., Li, J., Zhao, J., Huang, Z., 2016. DNA methylation level of *cyp19a1a* and *Foxl2* gene related to their expression patterns and reproduction traits during ovary development stages of Japanese flounder (*Paralichthys olivaceus*). Gene 575, 321–330.
Stratikopoulos, E., Szabolcs, M., Dragatsis, I., Klinakis, A., Efstratiadis, A., 2008. The hormonal action of IGF1 in postnatal mouse growth. PNAS 105, 19378–19383.
Su, Z., Xia, J., Zhao, Z., 2011. Functional complementation between transcriptional methylation regulation and post-transcriptional microRNA regulation in the human genome. BMC Genomics 12, S15.
Tan, X., Hoang, L., Du, S.J., 2002. Characterization of muscle-regulatory genes, Myf5 and Myogenin, from striped bass and promoter analysis of muscle-specific expression. Marine Biotechnol. 4, 537–545.
Tollefsens, S.E., Lajarasn, R., Mccuskeri, R.H., Clemmons, D.R., Rotwein, P., 1989. Insulin-like growth factors (IGF) in muscle development. J. Biol. Chem. 264, 13810–13817.
Velloso, C.P., 2008. Regulation of muscle mass by growth hormone and IGF-I. Brit. J. Pharmacol. 154, 557–568.
Waki, T., Tamura, G., Sato, M., Motoyama, T., 2003. Age-related methylation of tumor suppressor and tumor-related genes: an analysis of autopsy samples. Oncogene 22, 4128–4133.
Weintraub, H., 1993a. The MyoD family and myogenesis: redundancy, networks, and thresholds minireview. Cell 75, 1241–1244.
Weintraub, H., 1993b. The MyoD family and myogenesis: redundancy, networks, and thresholds. Cell 75, 1241–1244.
Weintraub, H., Davis, R., Tapscort, S., Thayer, M., Krause, M., Benezra, R., Lassar, A., 1991. The myoD gene family: nodal point during specification of the muscle cell lineage. Science 251, 761–766.
Wu, S., Zhang, H., Hammoud, S.S., Potok, M., Nix, D., Jones, D., Cairns, B.R., 2011. DNA methylation profiling in zebrafish. Methods Cell Biol. 104, 327–339.
Zhang, Y., Tan, X., Zhang, P., Xu, Y., 2006. Characterization of muscle-regulatory gene, Myod, from flounder (*Paralichthys olivaceus*) and analysis of its expression patterns during embryogenesis. Mar. Biotechnol. 8, 139–148.
Zhang, J., Shi, Z., Fu, Y., Cheng, Q., 2011a. Gene expression and thyroid hormone regulated transcript of Igf-I during metamorphosis of the flounder, *Paralichthys Olivaceus*. Acta Hydrobiologica Sinica. 35, 355–359.
Zhang, S., Cheng, Q., Chen, X., 2011b. Expression of insulin-like growth factor I receptors at mRNA and protein levels during metamorphosis of Japanese flounder (*Paralichthys olivaceus*). Gen. Comp. Endocrinol. 173, 78–85.