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Methylation status and expression patterns of *myomaker* gene play important roles in postnatal development in the Japanese flounder (*Paralichthys olivaceus*)☆

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ABSTRACT

Myomaker is a membrane protein that plays a crucial role in the fusion of myoblasts during muscle growth. DNA methylation, a significant factor, regulates gene expression. The aim of this study was to examine the methylation and mRNA expression patterns of the *myomaker* gene during 8 different postnatal developmental stages in the Japanese flounder (L: 7 days post hatch (dph); M1: 21 dph; M2: 28 dph; M3: 35 dph; J1: 90 dph; J2: 180 dph; A1: 24 months; A2: 36 months). Muscle tissue samples were taken from Japanese flounder at different postnatal development stages to measure the extent of DNA methylation and gene expression. Methylation level in the promoter and exon 1 of *myomaker* was measured using bisulfite sequencing, and the relative expression of *myomaker* during each developmental stage was measured by quantitative PCR. The relative expression levels of *myomaker* were up-regulated from stages L to M2, M3 to J2, and methylation of *myomaker* was negatively correlated with mRNA expression. Furthermore, the CpG site located at −26 bp in the promoter was the lowest methylated region in all developmental stages. These results offer a basis for understanding the mechanism by which *myomaker* regulates muscle formation during postnatal development.

1. Introduction

The Japanese flounder (*Paralichthy olivaceus*) is an economically fish species in Asian mariculture because of its fast growth rate and high market value. Although Japanese flounder has been as an economically important fish species for many years, the genes that govern skeletal muscle growth in the Japanese flounder remain poorly studied. Skeletal muscle is an important tissue that is not only involved in regulating the metabolism, locomotion, and strength of the animal body (Pavlath and Horsley, 2003) but also is an important protein source for humans. The formation of skeletal muscle requires the fusion of mononucleated myoblasts to form nascent multinucleated myotubes. These myotubes then fuse further and produce contractile proteins, forming mature myotubes. Myoblast fusion is an important step during muscle growth and involves a complex series of cellular and molecular events, including cellular migration, cell–cell recognition, cellular adhesion, cytoskeletal reorganization, and membrane coalescence, all leading up to the final membrane fusion event (Abmayr et al., 2003; Abmayr and Pavlath, 2012).

Myoblast fusion is essential in the formation of skeletal muscle fibers and is a complex and tightly controlled process (Chen and Olson, 2005). This process must be highly cell-specific to guarantee that myoblast fusion does not form syncytia with non-muscle cells. Although the transcriptional mechanisms that regulate skeletal muscle growth have been studied in detail (Bentzinger et al., 2012; Berkes and Tapscott, 2005; Buckingham, 2006; Karasik et al., 2005), the mechanisms that coordinate myoblast fusion remain poorly understood, and few muscle-specific proteins that directly regulate myoblast fusion have been identified (Abmayr and Pavlath, 2012; Landemaine et al., 2014; Millay et al., 2013, 2016; Rochlin et al., 2010; Shi et al., 2017; Zhang and Roy, 2017). Recently, some studies have revealed the molecules and signaling pathways that are involved in this process (Abmayr and Pavlath, 2012; Hindi et al., 2013; Rochlin et al., 2010). Among these are important transmembrane proteins that span the entirety of the biological membrane and play vital roles during myoblast fusion, being involved in cellular migration, recognition, and adhesion (Luo et al., 2015). These transmembrane proteins include the mannose receptor, which directs myoblast migration during the fusion of myoblasts (Jansen and Pavlath, 2006), and myoferlin, which is also required for myoblast fusion and is expressed at membrane sites during fusion

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(Doherty, 2005). Transmembrane proteins of the immunoglobulin superfamily, such as Roughest/Irregular-optic chiasma C, Kin of IrreC/Dumbfounded, and Hibris/Sticks-and-stones, are required for myoblast fusion (Artero et al., 2001; Bour et al., 2000; Ruiz-Gómez et al., 2002; Strünkelnberg et al., 2001). However, these proteins are not muscle-specific, and some of them perform functions that are not required for myoblast fusion. Therefore, identifying muscle-specific transmembrane proteins that have direct and essential roles in myoblast fusion is key.

In recent years, Millay et al. (2013) identified a muscle-specific transmembrane protein, transmembrane protein 8c (Tmem8c, also called *myomaker*), which is essential for the fusion of myoblasts. The *myomaker* protein sequence is highly conserved among vertebrates (Millay et al., 2016), and the function of *myomaker* in myogenesis is conserved in both mice and zebrafish (Landemaine et al., 2014; Millay et al., 2013). During myogenesis and muscle regeneration, *myomaker* is expressed transiently and promotes the fusion availability of myoblasts (Millay et al., 2013; Millay et al., 2014). However, the expression pattern of the corresponding gene, *myomaker*, during postnatal muscle development in the Japanese flounder, and the degree to which this gene is methylated during development, remains to be determined. To understand muscle growth and the function of factors that regulate muscle growth in the Japanese flounder, we analyzed the mechanism regulating *myomaker* expression based on methylation patterning.

DNA methylation is an important epigenetic process that is used to modulate gene expression without the alteration of DNA sequences (Egger et al., 2004; Jablonka and Lamb, 1998), and it is the first discovered epigenetic modification (Holliday and Pugh, 2009; Riggs, 1975). In general, the methylation of CpG islands suppresses the transcription of mammalian and vertebrate somatic cell genes (Bird, 2002; Plass and Soloway, 2002). For example, Ding et al. (2013) reported that the methylation pattern of *cyp17-II* limits its expression in flounders. It is thought that DNA methylation patterns may affect gene expression differently during different stages of postnatal muscle development. However, the relationship between gene expression and DNA methylation in *myomaker* during different postnatal developmental stages has not yet been studied in the Japanese flounder. Hence, we analyzed the relative expression and DNA methylation of *myomaker* during 8 different postnatal development stages in the Japanese flounder. Muscle samples were taken during each developmental stage, and the expression of *myomaker* in the muscle tissue was measured using quantitative PCR. A methylation map of CpG sites was then developed using bisulfate sequencing. Collectively, our results elucidate the roles of *myomaker* in muscle growth and the function of *myomaker* DNA methylation in the regulation of gene expression in the Japanese flounder.

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. We collected 8 different developmental stages (According to the classification of the developmental stage of Minami (1982)) of Japanese flounder: L (7 dph, larva), M1 (21 dph, pre-metamorphic larva), M2 (28 dph, metamorphic climax larva), M3 (35 dph, post-metamorphic juvenile), J1 (about 90 dph, juvenile), J2 (about 180 dph, juvenile), A1 (about 24 months, adult), A2 (about 36 months, adult). In our experiment and data analysis, 3 fish (The fish of stage L, M1, M2, M3 were so small, so we used more than one individual as one sample) were used in each stage. All fish were euthanized using tricaine methanesulfonate (MS-222). 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 L, M1, M2 and M3, we cut off redundant tissue and only retain muscle tissue, and the muscle tissue of stage J1, J2, A1 and A2 was obtained from the dorsal part near the head.), and then stored at −80 °C for genomic DNA and total RNA isolation.

2.3. RNA isolation

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. Quantitative real Time-PCR

The relative expression levels of *myomaker* mRNA were determined by total RNA extracted from muscle of Japanese flounder. Quantitative real time 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 quantitative PCR 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 *myomaker* quantitative PCR conditions were as follows: denaturation at 95 °C 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, 2001). A probability level of $P < 0.05$ was considered statistically significant.

Table 1
Nucleotide sequences of primers used for real time PCR in the experiment.

Primer name	Sequence (5′–3′)	Product size	Tm (°C)	Accession no.
Myomaker	F: TCACTGCGTCTTCACAGC	217 bp	56.00 °C	XM_020109955.1
Myomaker	R: GTAGACCATGGCCTCCAT			
18S	F: ATTGACGGAAGGGCACCAC	134 bp	60.00 °C	EF126037.1
18S	R: ATGCACCACCACCCACAGA			

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

Primer name	Sequence (5′–3′)	Product size	Tm (°C)	Accession no.
Myomaker-P1	F: GAGGATTGAGGGATTTGAGGATATTT	249 bp	57.59 °C	XM_020109955.1
Myomaker-P1	R: ACCTCCATATAAAAACCCCTCTTAAC			
Myomaker-E1	F: ATATGGAGGTTATGGTTTATTTTTTT	224 bp	51.28 °C	
Myomaker-E1	R: TTAAATAATCTAAATTTCCACCAACT			

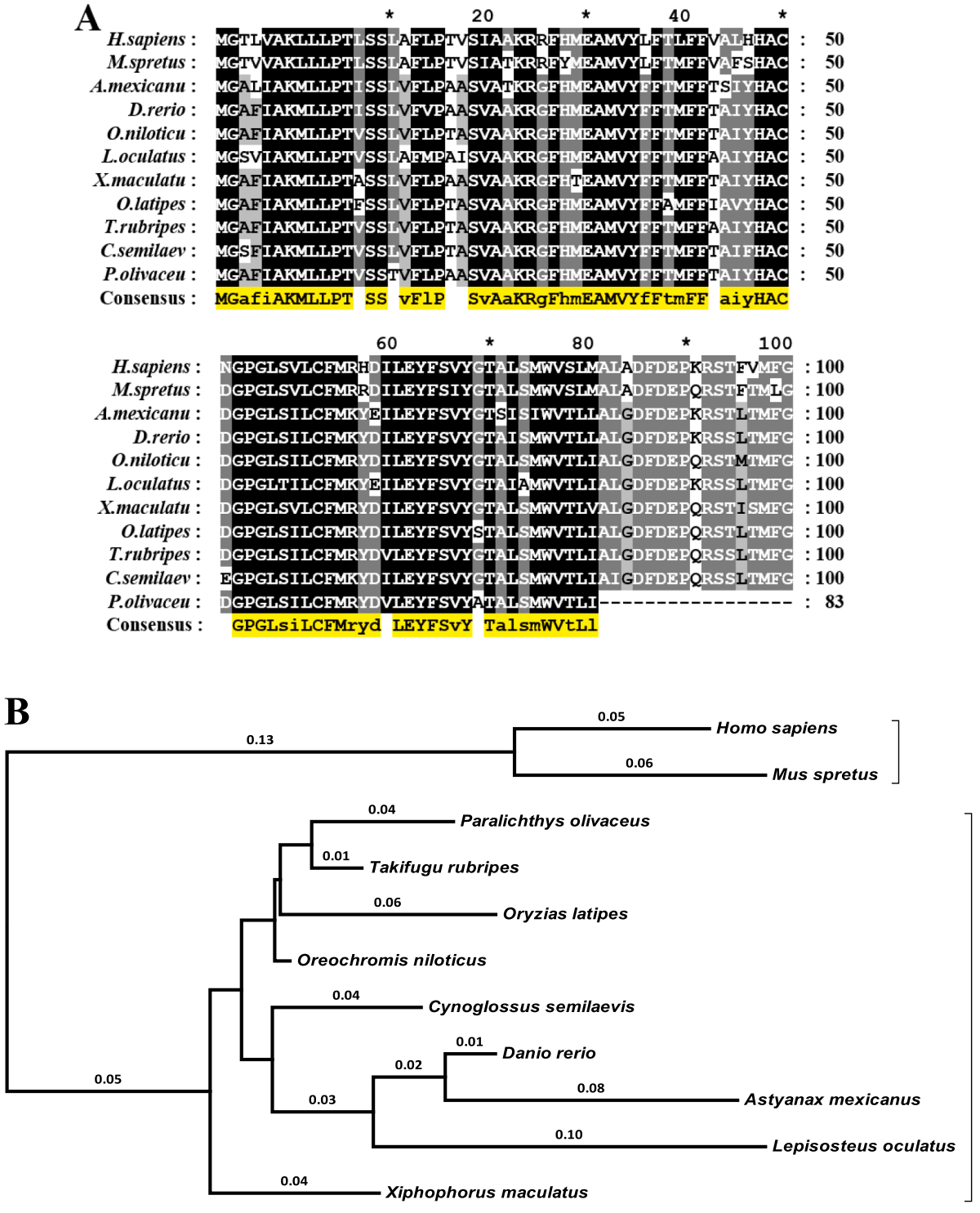

Fig. 1. Fig. 1A shows cross-species alignment of Myomaker proteins. Identical residues in all orthologs are shown at the bottom in yellow. Fig. 1B shows phylogenetic trees of Myomaker. The numbers above branch display branch length.

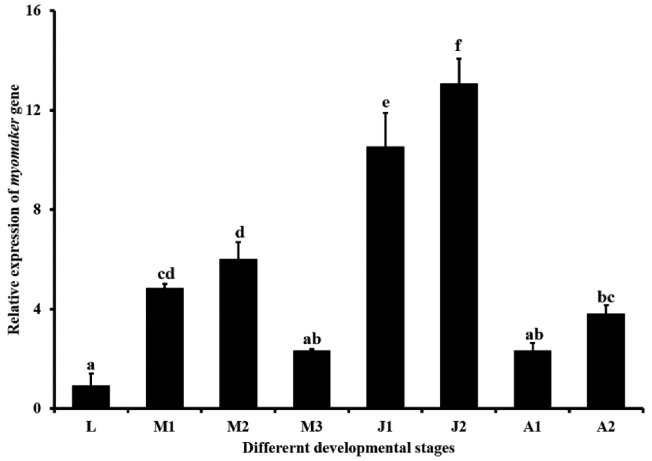

Fig. 2. Muscle mRNA expression of *myomaker* from 8 different developmental stages in Japanese flounder. Each histogram represents the mean of three determinations. Values are expressed as mean ± standard error of mean. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

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, amino acid sequence and phylogenetic of myomaker

The presumptive transcription factor was predicted using online Jaspar software (http://jaspar.genereg.net/). The *myomaker* amino acid sequences of the various species were obtained from Ensembl (https://asia.ensembl.org/index.html) and NCBI (https://www.ncbi.nlm.nih.gov/). The conserved gene sequence of *myomaker* was identified with the other species including *Homo sapiens* (ENSG00000187616), *Mus musculus* (ENSMUSG00000009214), *Astyanax mexicanus* (ENSAMXG00000014260), *Danio rerio* (ENSDARG00000103988), *Oreochromis niloticus* (ENSONIG00000013892), *Lepisosteus oculatus* (ENSLOCG00000002633), *Xiphophorus maculatus* (ENSXMAG00000000047), Takifugu rubripes (ENSTRUG00000009850) and *Cynoglossus semilaevis* (GenBank accession no. XM_008336442.1) by multiple sequence alignment. The phylogenetic tree was constructed using the Neighbor-Joining (NJ) method within the Molecular Evolutionary Genetics Analysis (MEGA 6.0) software. Furthermore, the topological stability of the trees was evaluated under the 1000 bootstrap replications.

2.7. DNA bisulfite modification and analysis

In each developmental stage, 3 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 exon1 of *myomaker* (GenBank accession no. XM_020109955.1) were identified by online MethPrimer design software (http://www.urogene.org/methprimer/). 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. Statistical analysis

The data 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 correlation between gene expression and methylation extent was 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. Analysis of amino acid sequences and phylogenetic analyses

To examine the evolutionary relationships between versions of *myomaker* in the Japanese flounder and those in other species, amino acid sequence analysis was conducted, and a phylogenetic tree was constructed. We analyzed 11 amino acid sequences from 11 species, the results of which are shown in Fig. 1. The cross-species alignment of *myomaker* sequences revealed that the gene is highly conserved among the tested species (Fig. 1A). The teleost and mammal versions of *myomaker* were grouped into two diverse lineages, with the version found in the Japanese flounder being grouped into the teleost lineage (Fig. 1B).

3.2. Expression of myomaker mRNA in muscle tissue

The expression patterns of *myomaker* are shown in Fig. 2. The relative expression of *myomaker* increased from stages L to M2, declined from stages M2 to M3, increased from stages M3 to J2, and finally increased from stages A1 to A2, with the highest expression being observed in stage J2 ($P < 0.05$) and the lowest in stage L.

3.3. Structure analysis of myomaker gene

The *myomaker* (GenBank Accession no. XM_020109955.1) contains one CpG rich region, a CpG island that included 9 CpG sites located in the region from −81 bp to + 61 bp (Fig. 3A). The promoter of *myomaker* has 249 bp and included 13 CpG sites, 11 of which were located at or near putative transcription factor binding sites such as MEF2C, Myog, Myod1, USF1, and SP1 (Fig. 3B). The *myomaker* exon 1 has 224 bp. The exon 1 of myomaker had 7 CpG sites, all of which were located at or near the presumed binding sites of transcription factors such as Myod1, MEF2C, Myog, USF1, SP1, PAX3, and PAX7 (Fig. 3C).

3.4. Methylation of myomaker and CpG sites during different developmental stages

To quantify the methylation of *myomaker* in *P. olivaceus*, we chose to analyze the promoter and exon 1 regions of *myomaker*. The muscle tissues of Japanese flounders were sampled at different stages of development and used in this experiment to extract DNA. The PCR products of bisulfite modification were evaluated by agarose gel electrophoresis, and the results revealed that all products were consistent with the anticipated objective strap size (Fig. 4A). Part of the sequencing diagram is shown in Fig. 4B. Evaluation of the efficiency of bisulfite treatment showed that all unmethylated cytosine was converted to thymine in all copies of the three different samples of the CpG dinucleotide sequences analyzed. This suggests that the DNA modification

Fig. 3. The gene structure analysis of *myomaker*. The long red line indicates the *myomaker* gene structure and the two empty boxes indicate the regions of measuring the methylation level (from −148 bp to +101 bp and from +92 bp to +315 bp) in promoter and exon 1 are marked with blue line and green line, covered the CpG rich region. The start codon was showed in pentagram. GC Percentage in y-axis shows GC content in sequence (It usually contains at least 200 bp), and the CpG in x-axis indicates where the CpG is located (Fig. 3A). Fig. 3B and 3C show a partial sequence of promoter and exon 1, respectively. The yellow boxes indicate CpG sites on coding regions of *myomaker* gene and the green box shows the start codon of *myomaker* gene. 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.

procedure was highly efficient.

The CpG dinucleotide methylation statuses of the promoter and exon 1 of *myomaker* were measured by bisulfite conversion and subsequent DNA sequencing. The DNA methylation patterns are shown in Fig. 5. The promoter and exon 1 showed similar levels of methylation in all 8 developmental stages. Methylation in the promoter and in exon 1 gradually declined from stages L to M2, increased from stages M2 to M3, declined from stages M3 to J2, increased from stages J2 to A1, and finally declined from stages A1 to A2, with the methylation being highest in stage A1 and the lowest in stage J2.

We found 13 CpG sites in the *myomaker* promoter, and the methylation levels of each of the CpG sites at each developmental stage are shown in Fig. 6A. The DNA methylation level of each CpG site had similar trend. Fig. 6B shows the average methylation of each CpG site

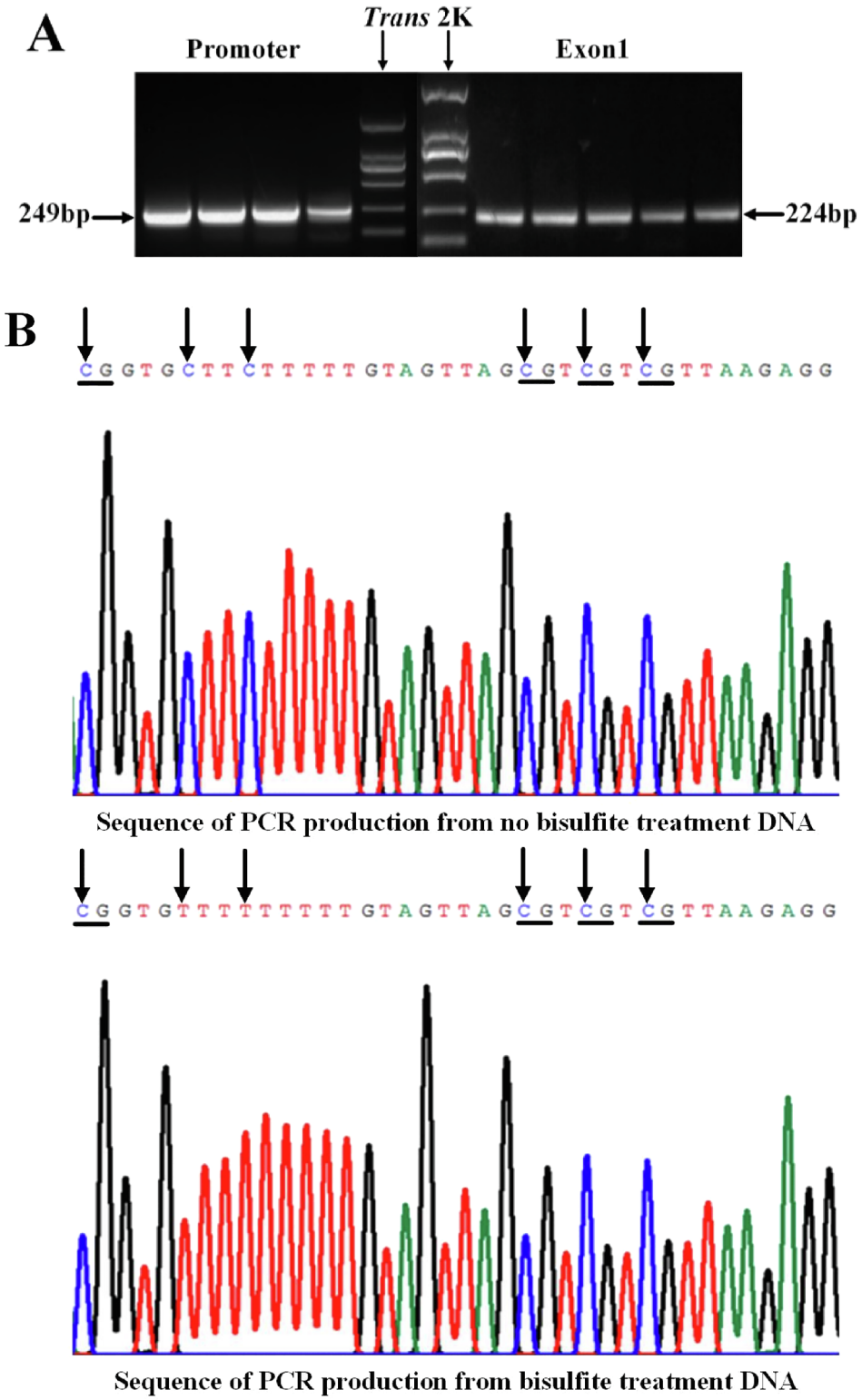

Fig. 4. The result of PCR production electrophoresis of bisulfite treatment DNA in promoter and exon 1 (A) and its partial sequence (B). The arrow indicates the position of Cytosine site, and underlines show the position of CG site (B).

and the methylation of a site at position −26 bp, which was methylated to a markedly lower degree than that in the other sites.

The methylation of 7 CpG sites in *myomaker* exon 1 during the 8 developmental stages shown in Fig. 6C. The DNA methylation of every CpG site in exon 1 had similar trend. Fig. 6D shows the average methylation of each CpG site and the methylation of a site at position +147 bp, which was methylated to a markedly lower degree than that in the other sites.

3.5. Relationship between myomaker methylation and expression during each developmental stage

DNA methylation has a significant effect on the regulation of gene expression and is critical in the control of gene expression during

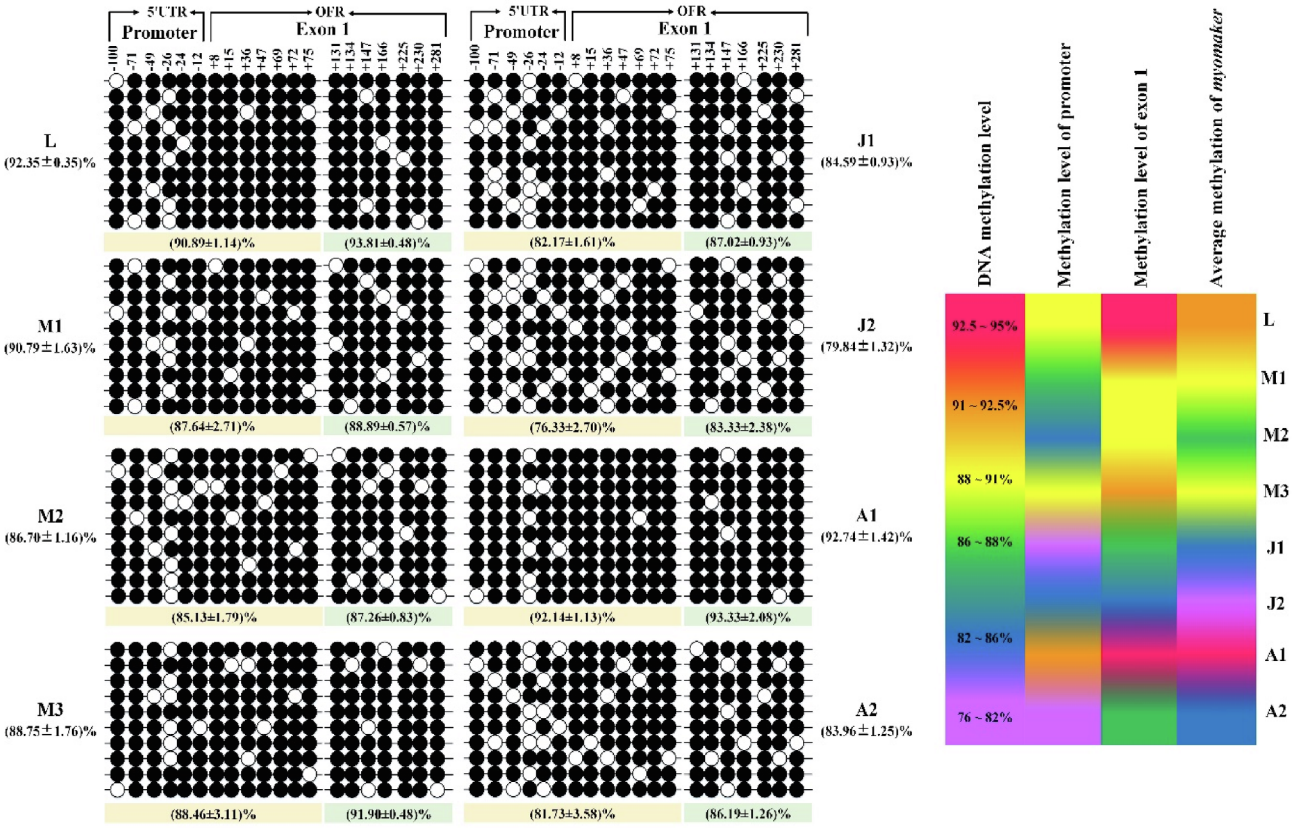

Fig. 5. DNA methylation patterns of promoter and exon 1 in *myomaker*. An open circle represents an unmethylated CpG, and a solid circle represents a methylated CpG. Each line represents one sequenced clone. The first line indicates the localizations of studied CpG sites related to the sequence of *myomaker*. The percentage indicates the methylation level (the percentage on the left and right indicates the average methylation level of *myomaker* at different developmental 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 exon 1.

development. Thus, we analyzed the relationship between the methylation and gene expression of *myomaker* in muscle tissue. As shown in Fig. 7, the methylation level of promoter and exon 1 had similar tendency. The relationship between the methylation of the promoter and exon 1 and overall gene expression is also provided in Fig. 7. The average methylation of *myomaker* was strongly negatively correlated with gene expression in all developmental stages ($r = -0.828$, $P < 0.05$). The amount of methylation at the promoter and exon 1 was also completely opposite to the relative gene expression of each region ($r = -0.864$, $P < 0.01$; $r = -0.843$, $P < 0.01$respectively).

4. Discussion

Myomaker is involved in boosting myoblast fusion in mice, zebrafish, and chickens (Landemaine et al., 2014; Luo et al., 2015; Millay et al., 2013). However, the roles of *myomaker* in the postnatal developmental stages of fish has not been fully elucidated. The results of this study demonstrate that DNA methylation is essential for regulating *myomaker* mRNA expression. Importantly, this study is the first to demonstrate the important roles of *myomaker* in modulating Japanese flounder skeletal muscle growth during postnatal stages. These results not only provide insight regarding the function of *myomaker*, which is important for muscle growth during postnatal stages, but also provide testimony for some regulatory factors of gene expression during different developmental stages.

Previous studies have focused on the function of *myomaker* during embryonic development and have shown that *myomaker* is essential for the promotion of myoblast fusion in zebrafish, mice, chickens, and so on (He et al., 2017; Landemaine et al., 2014; Lu Meng, 2016; Luo et al., 2015; Millay et al., 2013; Zhang and Roy, 2017). In this study, we examined the expression of *myomaker* during postnatal development in the Japanese flounder. We found that the relative expression of *myomaker* is up-regulated from stages L (7 days post hatch) to stage M2 (28 days post hatch). The Japanese flounder undergoes a typical metamorphosis over this period, during which its muscle tissues undergo drastic morphological changes (Yamano et al., 1991; Yamano et al., 1994). Zhang and Shi (2003) have reported that the skeletal muscles of the flounder are composed of thin layers of muscle fibers and that the myofibril content of these muscles is lower prior to metamorphosis, with the muscle fibers becoming thicker and having higher myofibrillar content by the metamorphic climax. The increased expression of *myomaker* may be conducive to the accelerated fusion of myoblasts and the formation of skeletal muscle fibers during metamorphosis. We found that the expression of *myomaker* increases from stages M3 to J2, which makes sense considering the faster growth rate of juvenile fish relative to adults. We speculate that the up-regulated expression of *myomaker* during growth from stages M3 to J2 is needed for myoblast fusion and the formation of mature muscle fibers. The slightly raised expression of *myomaker* from stages A1 to A2 may be due to muscle regeneration taking place in adult fish. Previous studies have demonstrated that *myomaker* is essential for muscle regeneration during postnatal development (Millay et al., 2014). Our results showed that a large amount of muscle fibers formed during postnatal development, and so we can conclude that *myomaker* is essential for the regulation of myoblast fusion and for the generation of muscle fibers during postnatal development. The *myomaker* gene can thus be considered a candidate gene for

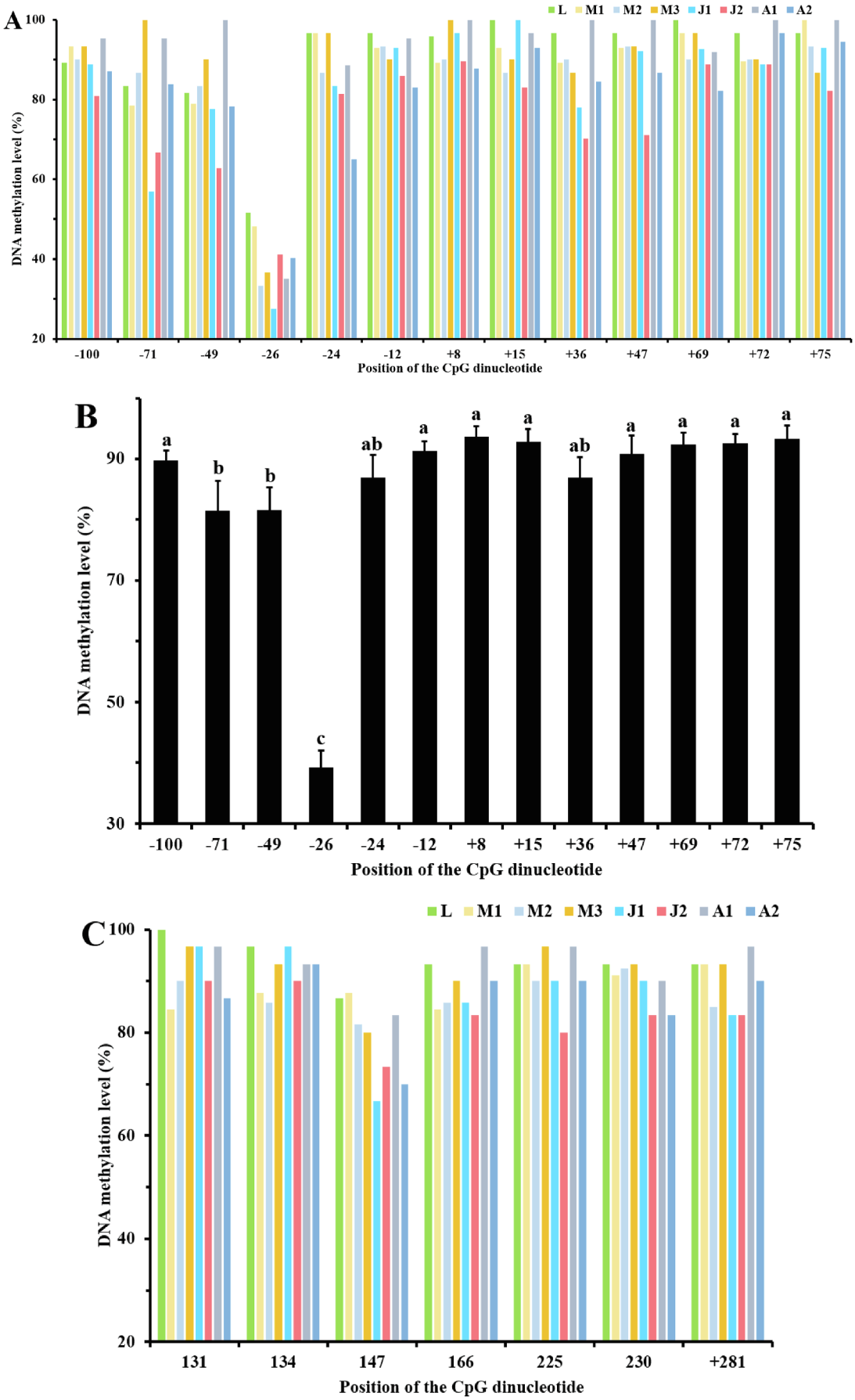

Fig. 6. The DNA methylation level of promoter and exon 1 of *myomaker* at different developmental stages. Fig. 6A and B indicate methylation level of each CpG site in promoter. Fig. 6C and D indicate methylation level of each CpG site in promoter.

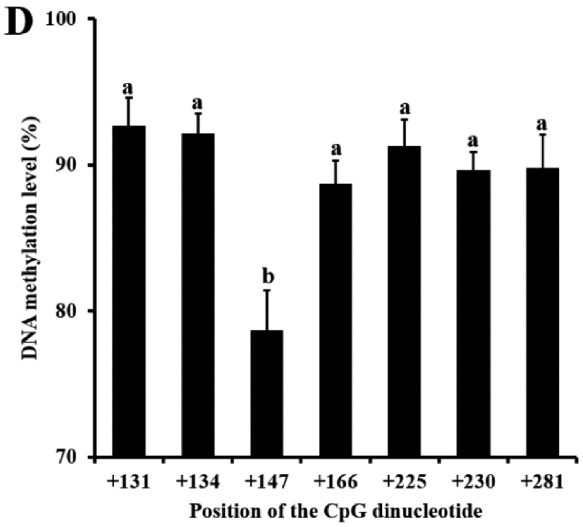

Fig. 6. (*continued*)

molecular breeding in fish. It should be noted that in this study, we examined *myomaker* expression only during postnatal development, and the specific function of *myomaker* in the embryonic stages of the Japanese flounder should be determined.

DNA methylation is an important epigenetic modification in the regulations of gene expression. Ordinarily, DNA methylation inhibits transcription, and the reversion of methylation is often associated with gene activation (Cedar, 1988). In some cases, DNA methylation can directly interfere with transcription factor binding (Ehrlich and Ehrlich, 1993). The methylation of coding regions can also repress gene expression significantly (Hsieh 1997; Irvine et al., 2002); thus, we chose the promoter and exon 1 as key regions at which to measure methylation. The methylation of the promoter and exon 1 of *myomaker* was negatively correlated with their expression. Our results are similar to those of other studies that have shown that methylation can regulate gene expression during the development of the skeleton and ovaries in animals (Attwood et al., 2014; Bird, 2002; Day et al., 2013; Ding et al., 2013; Jin et al., 2016; Laker and Ryall, 2016; Miyata et al., 2015; Ng and Bird, 1999). The decreased methylation of *myomaker* may be caused by the large demand for mature skeletal muscle during metamorphosis and the fast growth rate of juveniles.

Interestingly, we also found that a CpG site at the −26 bp position in the promoter of *myomaker* was methylated significantly less than that in other CpG sites. This CpG site was located near the putative transcription factors MyoG and MyoD. *Myog* and *MyoD* are myogenic regulatory factors (MRFs) and could be responsible for the activation of numerous downstream genes that initiate muscle cell differentiation (Berkes and Tapscott, 2005; Braun and Gautel, 2011). *Myog* is important in the terminal differentiation of myoblasts, whereas *MyoD* controls myogenic determination genes (Berkes and Tapscott, 2005; Braun et al., 1989; Edmondson and Olson, 1989). As transcriptional factors, MyoG and MyoD play essential roles in muscle-specific gene transcription (Blais et al., 2005; Cao et al., 2006). Luo et al. (2015) confirmed that both MyoG and MyoD transcription factors can directly bind to *myomaker* and initiate its expression. However, when a single base in the binding site changes, MyoG and MyoD transcription factors cannot bind to the sequence efficiently, and thus, cannot initiate gene expression. In our study, the low methylation of the −26 bp CpG site in both the promoter and exon 1 may have resulted in the increased expression of *myomaker*. We also suggest that the sequence near the −26 bp CpG site may be involved in the activation of *myomaker* transcription. Our previous study showed that the expression of *MyoD* was up-regulated from stages L to J2, then decreased from stages J2 to A2 (without stage M1, M2 and M3) (Huang et al., 2018), a pattern different from that seen in *myomaker*. Variation in the expression patterns of *MyoD* and *myomaker* has also been reported in chickens (Luo et al., 2015). These results suggest that *MyoD* and *myomaker* play different roles in muscle growth. The regulatory mechanisms underlying MyoG and MyoD during myoblast differentiation remain unclear. The specific functions and the mechanisms of MyoG and MyoD in myoblast fusion during postnatal development remain unknown and require further study. The function of this sequence using gene knockout experiments should also be elucidated.

5. Conclusion

We researched the expression patterns of the *myomaker* gene during

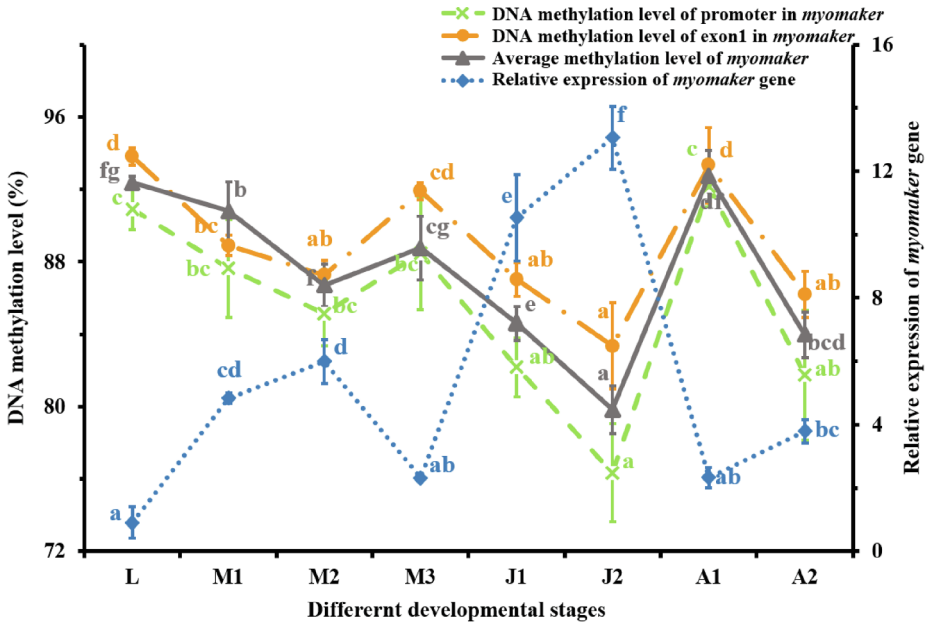

Fig. 7. The correlation between gene expression and CpG methylation level of *myomaker* gene at different developmental stages. Values represent mean ± SEM. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

8 different postnatal developmental stages in the Japanese flounder. The results showed that the expression of *myomaker* increased from 7 to 28 dph over the course of postnatal development, indicating that the *myomaker* gene is an important gene involved in the regulation of muscle growth during metamorphosis. The expression of the *myomaker* gene increased from 35 to 180 dph, indicating that the *myomaker* gene is an important regulatory gene during the juvenile stage of the Japanese flounder. Methylation was the lowest at a CpG site located at −26 bp at the putative Myog and MyoD recognition sequences, suggesting that the effect of these transcription factors on binding efficiency could affect gene expression. The average methylation level of CpG islands was negatively correlated with gene expression during postnatal development, suggesting that DNA methylation patterns in the *myomaker* promoter and exon 1 were involved in controlling gene expression. Our results may help to elucidate the molecular mechanisms behind fish muscle growth from an epigenetic perspective during different developmental stages. This study also reinforces the idea that *myomaker* plays an important role in postnatal development, especially in metamorphosis period and juveniles.

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Conflict of interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.04.017.

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