Comparative Biochemistry and Physiology, Part B 223 (2018) 16–22

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

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

DNA methylation levels and expression patterns of *Smyd1a* and *Smyd1b* genes during Metamorphosis of the Japanese Flounder (*Paralichthys olivaceus*)

Shuxian Wu[a,b], Yajuan Huang[a,b], Siping Li[a,b], Haishen Wen[a,b], Meizhao Zhang[a,b], Jifang Li[a,b], Yun Li[a,b], Changwei Shao[c,d], Feng He[a,b,*]

[a] The Key Laboratory of Mariculture, Ministry of Education,Ocean University of China, 266003 Qingdao, China
[b] Fisheries College, Ocean University of China, 266003 Qingdao, China
[c] Key Laboratory for Sustainable Development of Marine Fisheries, Ministry of Agriculture, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, China
[d] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China

ARTICLE INFO

Keywords:
DNA methylation
Gene expression
Japanese flounder
Smyd1a
Smyd1b

ABSTRACT

Japanese flounder (*Paralichthys olivaceus*) undergoes metamorphosis by changing its body from the larval to the juvenile form, and this process involves muscle development. Smyd1, a histone methyltransferase, plays a role in the skeletal muscle. In the present study, the *Smyd1a* and *Smyd1b* expression patterns and their 5′ UTR and exon 1 DNA methylation levels were analyzed during metamorphosis of the Japanese flounder. Sample were analyzed 21 days post-hatching (dph) (with no migration of right eye; M1stage), 28 dph (during migration of right eye; M2 stage), and 35 dph (after migration of right eye; M3 stage). The results show that *Smyd1a* expression was highest in the M2 stage and then decreased, whereas *Smyd1b* expression continued to rise during the three stages. Methylation levels of CpG sites at positions −2318 and −2217 of the *Smyd1a* P region (−2462 to −2181 region of the 5′ UTR), and the CpG sites at positions −351, −330, −284, −190, and − 92 of the *Smyd1b* promoter, with both regions containing putative transcription factor binding sites, showed significant differences in the three stages ($p < 0.05$). Interestingly, the methylation levels of these CpG sites were negatively correlated with mRNA expression. We inferred that binding of the predicted transcription factors might be affected by methylation of the CpG sites and thus modulate gene expression. Taken together, our results suggest that DNA methylation in the *Smyd1a* and *Smyd1b* genes participates in the regulation of metamorphosis, and epigenetics may provide clues for further studies of the mechanisms of metamorphosis in the Japanese flounder.

1. Introduction

Smyd1, a histone methyltransferase containing the conserved SET-MYND functional domain, is specifically expressed in the heart and skeletal muscle (Hwang and Gottlieb, 1997; Hwang and Gottlieb, 1995). Previous research has shown that *Smyd1* plays an important role in myofiber maturation and contraction in zebrafish and is essential for cardiac differentiation and morphogenesis in mice (Gottlieb et al., 2002; Tan et al., 2006). Sequence analysis showed that *Smyd1* genes are present in two copies (*Smyd1a* and *Smyd1b*) in zebrafish (Sun, 2008). In mice, targeted deletion of the *Smyd1* gene resulted in embryonic lethality (Gottlieb et al., 2002). Interestingly, knockdown of *Smyd1a* expression resulted in a normal myofibril alignment, with little effect on muscle development (Gao et al., 2014). In contrast, knockdown of *Smyd1b* expression affected sarcomere organization (Li et al., 2013). Previous studies have shown that *Smyd1* plays an important role in the early stages of muscle development. However, it is still unclear whether this gene plays a role in the metamorphosis of marine fish.

Epigenetic modifications such as DNA methylation, RNA interference, and histone modification play an important role in the regulation of gene expression (Paszkowski and Whitham, 2001; Stefanska and Macewan, 2015; Portela and Esteller, 2010). DNA methylation can also affect the expression of genes by interacting with transcription factors or altering chromatin structure (Razin and Kantor, 2005). The 11 zinc finger-containing DNA binding protein CTCF, which is highly conserved in vertebrates, can block the interaction between the enhancer and promoter but binds only to non-methylated sites (Bell et al., 1999). In addition, MeCP2 leads to the formation of an inactive

* Corresponding author at: The Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, 266003 Qingdao, China.
E-mail address: hefengouc@ouc.edu.cn (F. He).

https://doi.org/10.1016/j.cbpb.2018.05.002
Received 12 December 2017; Received in revised form 22 May 2018; Accepted 22 May 2018
Available online 01 June 2018
1096-4959/

chromatin structure by binding to methylated DNA and the corepressor Sin3A (Jones et al., 1998; Nan et al., 1998). However, some methylation events associated with transcription factors do not have obvious effects on gene expression. The binding site of the transcription factor Sp1 contains a central CpG, and Sp1 is able to bind to its methylated site and promote transcription (Höller et al., 1988). At present, research on Smyd1 mostly focuses on its role as a histone methyltransferase, as Smyd1 inhibits the transcription of genes in a histone deacetylase-dependent manner (Gottlieb et al., 2002). No reports on the methylation levels of the *Smyd1a* and *Smyd1b* genes were found in the literature.

Japanese flounder is a valuable marine fish, but also an important marine aquaculture fish. The Japanese flounder undergoes metamorphosis during development, and in this process it changes from a symmetrical pelagic larva to an asymmetrical benthic juvenile, accompanied by obvious external characteristics (eye migration and craniofacial remodeling) (Klaren et al., 2008). These changes are associated with methylation and expression patterns of some genes. Assessment of global DNA methylation showed significant differences in methylation pattern during lamprey metamorphosis (Covelo-Soto et al., 2015). During metamorphosis of the Japanese flounder, IGF-IR-1 and IGF-IR-2 mRNA levels vary markedly (Zhang et al., 2011). However, genes methylation patterns during metamorphosis of the Japanese flounder have not been reported. Because of the economic importance of Japanese flounder, study of the methylation pattern and mRNA expression levels of muscle-related genes during metamorphosis is required for a better understanding of the growth and development of this marine fish.

In this study, we investigated the DNA methylation and relative gene expression patterns of *Smyd1a* and *Smyd1b* during metamorphosis of the Japanese flounder. The results will help elucidate the functional relationship between *Smyd1a* and *Smyd1b* and their role in metamorphosis during development of the Japanese flounder from an epigenetic perspective.

2. Materials and methods

2.1. Ethics statement

The study was approved by the respective Animal Research and Ethics Committees of Ocean University of China. The field studies did not involve endangered or protected species.

2.2. Experimental animal collection

Japanese flounder larvae [21, 28 and 35 days post hatching (dph)] were provided by the aquatic farm, Jimo, China. According to the classification of the developmental stage of Minami (1982) and combined with the right eye migration, we divided the metamorphic stages of Japanese flounder into three stages, M1 (21 dph, no migration of right eye), M2 (28 dph, during migration of right eye), M3 (35 dph, finished migration of right eye). The larvaes were stored at −80 °C for islation of genomic DNA and total RNA.

2.3. Genomic DNA isolation

Genomic DNA was extracted from the larvaes (we cut off redundant tissue and only retain muscle tissue under the microscope) using Marine Animal Genomic DNA Kit (TransGen, China). About 4–5 individuals were needed in the stages of M1 and M2 for the extraction of each DNA sample and 2–3 individuals for M3 stage. The DNA concentration was measured using the nucleic acid analyzer Biodropsis BD-1000 (OSTC, China), and the integrity by agarose gel electrophoresis examination. The DNA was stored at −20 °C.

2.4. DNA bisulfite modification and sequencing

Genomic DNA was modified using the BisulFlash DNA Modification Kit (EpiGentek, USA) following the manufacturer's instructions. Primers were designed on the Oligo 6.0 according to the bisulfate-modified DNA sequences. After that the methylation specific PCR was performed, the PCR products were separated by agarose gel electrophoresis. The Quick Gel Extraction Kit (TransGen, China) was used to purify the products of separation. And then purified products were cloned into the pEASY-T1 vector (TransGen, China), and transferred into *Trans1*-T1 Phage Resistant Chemically Competent Cell (TransGen, China). For three biological repetitions of each stage, 7–10 individual clones were sequenced to evaluate the methylation level. The efficiency of bisulfite modification was calculated by a formula that the percentage of the number of converted cytosines on the total number of cytosines (except the cytosines of CpG dinucleotides).

2.5. RNA isolation and reverse transcription

Total RNA was extract by RNAiso Reagent Kit (TaKaRa, Japan). The concentration of RNA was quantified by the nucleic acid analyzer Biodropsis BD-1000 (OSTC, China), and the integrity by agarose gel electrophoresis examination. The PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Japan) was used to obtain the cDNA. The cDNA was stored at −20 °C.

2.6. Quantitative real-time PCR and expression analysis

Quantitative real-time PCR was conducted by the Roche LightCycler480 (German) and SYBR Premix Ex Taq™ (TliRNaseH Plus) Kit (Takara, Japan). It was used to determinate the relative expressions of *Smyd1a* (GenBank accession no. MH_349346) and *Smyd1b* (GenBank accession no. MH_349347). Primers are listed in Table 1, the Japanese flounder 18 s (GenBank accession no. EF126037.1) was used as the endogenous reference gene. The amplified system was formed from 10 μl SYBR®Premix Ex Taq (TliRNaseH Plus), 0.4 μl ROX Reference Dye, 0.4 μl PCR Forward Primer, 0.4 μl PCR Reverse Primer, 2 μl cDNA template, add RNase-free water to 20 μl. The reaction completed according to the procedure of 95 °C for 30 s, 40 cycles of 95 °C for 5 s, Tm for 30 s. All samples were run in triplicate. And then we calculated the relative expression by the method of comparative threshold ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001).

2.7. Genetic structure and phylogenetic analysis of Smyd1a and Smyd1b

The online MethPrimer design software (http://www.urogene.org/methprimer/) was used to achieve CpG-rich regions. The presumptive transcription factor binding site was predicted using PATCH™ public 1.0 online software (http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi). The Open Reading Frame (ORF) Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) was used for predicting the coding sequence of *Smyd1a* and *Smyd1b*. The *Smyd1* amino acid sequences of the various species were obtained from Ensembl and GenBank. The phylogenetic tree was constructed using the Neighbor-Joining (NJ) method within the Molecular Evolutionary Genetics Analysis (MEGA 4.0) software. Furthermore, the topological stability of the trees was evaluated under the 1000 bootstrap replications.

2.8. Statistical analysis

The data were presented as means ± SE. The statistical differences were analyzed by one-way ANOVA and Duncan's multiple range tests using SPSS 19.0. The Pearson correlation coefficient (r) between the methylation level and the mRNA expression level was determined by independent samples t-test. p-Value < 0.05 was considered statistical

Table 1
Primers used for bisulphate PCR and quantitative real-time PCR.

Primer names	Sequence (5′–3′)	Product size (bp)	Tm (°C)	Accession no.
Smyd1a P region* (BS-PCR)	F:TTTTTAGGTTTTTTGGGTGAGATTTT R:AAAAACCTTAACATTTAATTCCTCCT	282	55.4	
Smyd1a exon1 (BS-PCR)	F:TTGGAAAAGGGAGGGGTTTGAAGG R:AATCCCTCTTATCCAAACCCCCAC	306	60.5	MH_349346
Smyd1a (qRT-PCR)	F:CAACTCACCTGGAAAAGG R:GCACTCTTGCTTGTGTTC	235	53.6	
Smyd1b promoter (BS-PCR)	F:TTGTGGATTAAAATATTTAAGGAGGA R:CCAAACAAAACACAAAAATCAAAAAC	356	53.2	
Smyd1b exon1 (BS-PCR)	F:AGTGTTGTATTGTTTGATGTAGGG R:CTCACTCCTAAAATCTAAACCCAA	337	60.2	MH_349347
Smyd1b (qRT-PCR)	F:AAGTGGTCTTCACCGAGC R:ACTCCTGCTTGTGCTCGT	177	53.6	
18s (qRT-PCR)	F:ATTGACGGAAGGGCACCAC R:ATGCACCACCACCCACAGA	134	65.0	EF126037.1

Note: *: The −2462 to −2181 region of 5′ UTR.

significance.

3. Results

3.1. Structure analysis of Smyd1a and Smyd1b gene

As shown in Fig. 1A, the *Smyd1a* P region contains a CpG-rich region (located at −2368 bp to −2240 bp) with 12 CpG sites. Some putative transcription factor binding sites are located near these CpG sites and some at a CpG site. Putative binding sites for the transcription factors SRF, GATA-1, LVc, SP1, c-Ets-1, RAR-beta2, and c-Ets-2 were found. These transcription factors have various regulatory functions (Fig. 1B). The *Smyd1b* promoter contains 15 CpG sites (Fig. 1C), and similar to *Smyd1a*, the predicted transcription factor binding sites are at or near a CpG site. Putative binding sites for the transcription factors c-Ets-2, SP1, E2F-1, RAR-alpha1, and GATA-1 were found.

3.2. Phylogenetic analysis of Smyd1a and Smyd1b

To examine the evolutionary relationship of Japanese flounder *Smyd1a* and *Smyd1b* with other species, a phylogenetic tree was constructed with the MEGA4.0 software using the Neighbor-Joining method. We analyzed 17 amino acid sequences from 12 species and the clustering results are shown in the Fig. 2. Japanese flounder *Smyd1a* and *Smyd1b* were grouped into the two diverse lineages. All teleost *Smyd1a* and *Smyd1b* are placed into two different clades.

3.3. The DNA methylation patterns and expression levels of Smyd1a

Analysis using agarose gel electrophoresis showed that the PCR products after bisulfite modification were of the same size as the target amplified product (Fig. S1A, SI Appendix). We randomly selected 12 sequencing results of the *Smyd1a* exon (containing 38 cytosines outside CpG dinucleotides) showing that only 10 cytosines were not converted to thymine, and the bisulfite modification efficiency achieved was 97.8%. Part of the sequencing diagram was shown in Fig. S1B (SI Appendix). This proved that the conversion efficiency of bisulfite modification was very efficient.

The DNA methylation levels of the *Smyd1a* P region and exon 1 are shown in Fig. 3A. There are 12 CpG sites in the P region of *Smyd1a*, and methylation levels were high in all stages. DNA methylation levels decreased from M1 to M2 and increased slowly from M2 to M3. There are 4 CpG sites in the exon 1 of *Smyd1a*, and methylation levels decreased from M1 to M2 and increased from M2 to M3. Methylation levels of the P region were higher in each stage than those of exon 1. Methylation levels of the *Smyd1a* P region and exon 1 were not significantly different in the three stages of metamorphosis. Methylation levels of a single CpG site are shown in Fig. S2 (SI Appendix). The methylation pattern of the single CpG site in the *Smyd1a* P region was the same as that of the P region. In contrast, the methylation levels at the −2318 and −2217 sites showed significant difference ($p < 0.05$) (Fig. 3B).

Smyd1a mRNA expression levels are shown in Fig. 3C. Expression levels were high in the M2 stage, and lowest in the M1 stage. Expression levels from M1 to M3 first increased and then decreased. In addition, methylation levels of the CpG sites located at the *Smyd1a* P region −2318 ($r = -0.865$) and − 2217 ($r = -0.932$) positions were negatively correlated with the expression levels of *Smyd1a* (Fig. 3D).

3.4. The DNA methylation patterns and expression levels of Smyd1b

As shown in Fig. 4A, 15 and 11 CpG sites are located in the promoter and exon 1 of *Smyd1b*, respectively. Methylation levels at the promoter were relatively high in the M1 stage, decreased in the M2 stage, and increased again in the M3 stage. The methylation pattern of exon 1 in the three stages was relatively flat, with a slow upward trend. Methylation levels in the *Smyd1b* promoter and exon 1 were also not significantly different in the three stages of metamorphosis. The methylation levels of the single CpG site in the *Smyd1b* promoter were the same as those of the promoter (Fig. S3, SI Appendix). In contrast, the methylation levels at the −351, −330, −284, −190, and −92 sites were significantly different ($p < 0.05$) in the three stages of metamorphosis, and the methylation pattern was the same as that of the promoter (Fig. 4B).

Expression levels of *Smyd1b* showed a continuous increase in the three stages of metamorphosis, and the levels in the M3 stage were much higher than in the M2 stage (Fig. 4C). Unexpectedly, its expression levels were not negatively correlated with methylation levels of CpG sites of the *Smyd1b* promoter at the −351, −330, −284, −190, and −92 positions (Fig. 4D). From the M2 to the M3 stage, both methylation and expression levels tended to increase.

3.5. The relationship of methylation and expression levels between Smyd1a and Smyd1b

The methylation levels of the P region of *Smyd1a* were higher than those of the *Smyd1b* promoter at every stage, but the change pattern is the same. *Smyd1a* showed the highest methylation levels in the M1 stage, whereas the highest methylation levels of *Smyd1b* was observed at the M3 stage (Fig. S4A, SI Appendix). A comparison of exon 1 methylation levels between *Smyd1a* and *Smyd1b* is shown in Fig. S4B (SI Appendix). Methylation levels of *Smyd1a* are highest in the M1 stage, whereas those of *Smyd1b* are highest in the M3 stage. The expression levels of *Smyd1a* were lower than those of *Smyd1b* in every stage. The

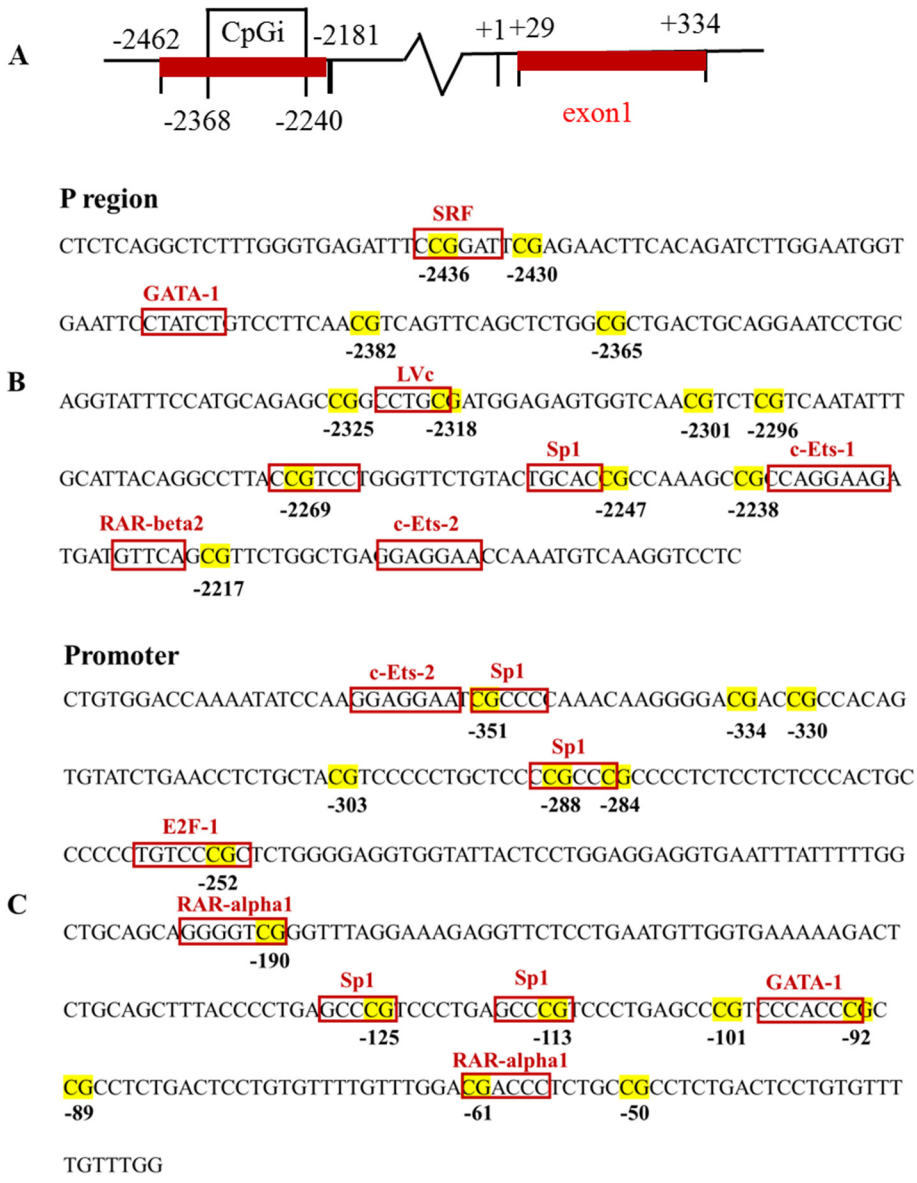

Fig. 1. Structure analysis of *Smyd1a* and *Smyd1b* gene. The long black line indicates the *Smyd1a* gene structure and the empty black box indicate the CpGi region (CpG island). The regions of measuring the methylation level (from −2462 bp to −2181 bp and from +29 bp to +334 bp) in 5′ UTR and exon1 are marked with red line (Fig. 1A.) The predictive transcription factors and putative binding sites on the *Smyd1a* (B) P region and *Smyd1b* (C) promoter. The CpG dinucleotide sites are represented by the yellow shade. The red frames represent the putative binding sequences of the transcription factors and the predictive transcription factors are indicated above them.

expression levels of both *Smyd1a* and *Smyd1b* increased significantly ($p < 0.05$) from M1 to M2, whereas *Smyd1a* levels decreased and *Smyd1b* levels continued to increase significantly ($p < 0.05$) from M2 to M3. The expression levels of *Smyd1a* were highest in the M2 stage, whereas *Smyd1b* levels were highest in the M3 stage (Fig. S4C, SI Appendix). Fig. 5 shows a comparison between average methylation and expression levels of *Smyd1a* and *Smyd1b*.

4. Discussions

Japanese flounder metamorphosis is an important period in its growth and development, and is accompanied by an asymmetric remodeling of some tissues and organs (Schreiber, 2006). These changes in shape and function are related to methylation of genes and transcriptional or post-transcriptional regulation of expression. Previous studies in other species have shown that differences in methylation and gene expression underlie metamorphosis (Covelo-Soto et al., 2015; Zhang et al., 2011). Therefore, the study of *Smyd1* gene expression during metamorphosis of the Japanese flounder is particularly informative. In this study, we examined the methylation and expression levels of *Smyd1a* and *Smyd1b* genes during metamorphosis.

Methylation-sensitive amplified polymorphism analysis showed that the dramatic morphological changes may be related to changes in DNA methylation during metamorphosis of European eel (*Anguilla anguilla*, L.) (Trautner et al., 2017). In addition, DNA methylation is involved in the epigenetic regulation of post-natal development of sea lamprey (*Petromyzon marinus*) (Covelo-Soto et al., 2015). To investigate the relation between metamorphosis and epigenetics in the Japanese flounder, we determined the methylation levels of two genes involved in muscle development, *Smyd1a* and *Smyd1b*. The results show that the changes in methylation levels of *Smyd1a* and *Smyd1b* showed the same pattern during metamorphosis. Changes in promoter and exon 1 methylation levels during the three stages of metamorphosis were not significant. It is noteworthy that significant differences were observed in the methylation levels of some CpG sites in the *Smyd1a* P region and *Smyd1b* promoter. At position −2318 and −2217 of the *Smyd1a* P region and at position −351, −330, −284, −190, and −92 of the *Smyd1b* promoter, methylation levels decreased from stages M1 to M2 and increased from stages M2 to M3. These sites may play an important role in metamorphosis.

During metamorphosis of the Japanese flounder, the expression level of related genes would presumably change (Bao et al., 2005; Gong et al., 2005). In this study, we examined the expression levels of the *Smyd1a* and *Smyd1b* genes during metamorphosis. The expression

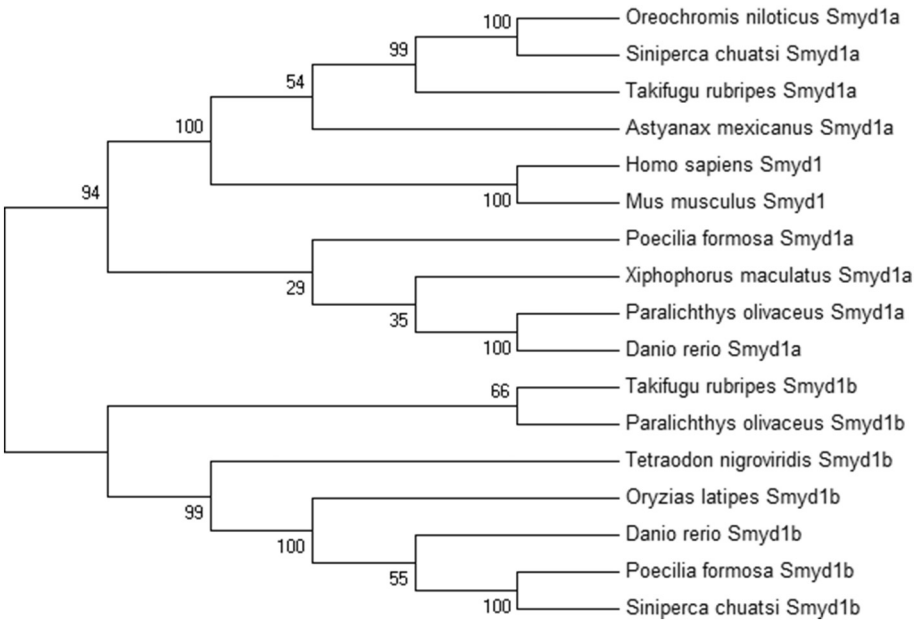

Fig. 2. Phylogenetic trees of *Smyd1a* and *Smyd1b*. The accession numbers are: Ensembl: *Oreochromis niloticus Smyd1a*: ENSONIG00000012570; *Takifugu rubripes Smyd1a*: ENSTRUG00000013032; *Takifugu rubripes Smyd1b*: ENSTRUG00000015565; *Astyanax mexicanus Smyd1a*: ENSAMXG00000009168; *Homo sapiens Smyd1*: ENSG00000115593; *Mus musculus Smyd1*: ENSMUSG00000055027; *Poecilia Formosa Smyd1a*: ENSPFOG00000006798; *Poecilia Formosa Smyd1b*: ENSPFOG00000007466; *Xiphophorus maculatus Smyd1a*: ENSXMAG00000010713; *Paralichthys olivaceus Smyd1a*: MH_349346; *Paralichthys olivaceus Smyd1b*: MH_349347; *Danio rerio Smyd1a*: ENSDARG00000009280; *Danio rerio Smyd1b*: ENSDARG00000091253; *Tetraodon nigroviridis Smyd1b*: ENSTNIG00000016679; *Oryzias latipes Smyd1b*: ENSORLG00000003877; GenBank: *Siniperca chuatsi Smyd1a*: AHG32674.1; *Siniperca chuatsi Smyd1b*: AHG32675.1.

levels of the *Smyd1a* and *Smys1b* were different during metamorphosis. *Smyd1a* showed the highest and lowest expression levels in the M2 and M1 stages, respectively. In contrast, the expression levels of *Smyd1b* increased in the three stages. A previous study has shown that Japanese flounder skeletal muscle is composed of thin layers of muscle fibers with a low content of myofibrils before metamorphosis. At the peak of metamorphosis, muscle fibers become thick and myofibrils are abundant (Zhang and Shi, 2003). *Smyd1b* is essential for myofibrillogenesis in zebrafish skeletal muscle (Li et al., 2013). In zebrafish embryos, *Smyd1a* and *Smyd1b* play a role in myofibril assembly, although *Smyd1b* is more important than *Smyd1a* (Gao et al., 2014). We hypothesize that both *Smyd1a* and *Smyd1b* play an important role in the muscle development of Japanese flounder during metamorphosis, but higher expression levels of *Smyd1b* are required for muscle fibers to become thick during metamorphosis. On the other hand, *Smyd1a* has a relatively large supporting role in the early stages of metamorphosis, and its expression levels decrease in the later stages. Further research is needed to uncover the biological significance of different expression patterns of *Smyd1a* and *Smyd1b* during metamorphosis of the Japanese flounder.

DNA methylation can affect the availability of proteins by altering the chromatin structure of the corresponding genes (Bird, 2007). CpG methylation inhibits the viral genome during mammalian development (Nan et al., 1998). Many studies have described that methylation of gene promoter regions results in its transcriptional suppression (Soria et al., 2002; Zhu et al., 2017; Lazo-De-La-Vega-Monroy et al., 2017). Recent studies showed that DNA methylation in the coding region also strongly inhibits gene expression (Hsieh, 1997; Irvine et al., 2002; Graessmann et al., 1994). To elucidate the relationship between DNA methylation and *Smyd1a* and *Smyd1b* genes expression during metamorphosis, we studied the relationship between methylation levels and *Smyd1a* and *Smyd1b* expression levels. In every stage, the methylation levels of the P region of *Smyd1a* were higher than those of the *Smyd1b* promoter and the expression levels of *Smyd1a* were lower than those of *Smyd1b*. The methylation levels of the *Smyd1a* P region and exon 1 were negatively correlated with the corresponding expression levels. These results suggest that the methylation levels of *Smyd1a* inhibit its expression. However, no negative correlation between methylation levels in the *Smyd1b* promoter and exon 1 and the corresponding expression levels was observed, indicating that methylation may not play a dominant role in affecting *Smyd1b* gene expression. These results may also be related to the special period of metamorphosis, in which the level of hormones, gene expression and other factors would change (Zhang et al., 2011; Yoshikawa et al., 2013; Manzon et al., 2014). Further research is needed to clarify this issue.

A previous study has shown that methylation of CpG sites inhibits the binding of transcription factors, leading to gene silencing (Deng et al., 2001). Methylation of CpG sites can also activate the binding of specific transcription factors (Chatterjee and Vinson, 2012). However, some transcription factors are not affected by CpG site methylation (Harrington et al., 1988). In our study, the putative transcription factor binding sites of the P region of the *Smyd1a* and *Smyd1b* promoters were identified. Most of the predicted transcription factor binding sites are located near or at CpG sites, and binding of transcription factors at these sites also affected gene expression. For example, the transcription factor Sp1, which binds to the CpG sites at positions −351 and −284 of the *Smyd1b* promoter, has been reported to have an effect on the expression of many genes. In addition, Sp1 enhances human insulin receptor gene promoter activity (Chen et al., 1994). Studies have shown that SP1 could inhibit the transcriptional activity of the MEPE gene in mouse pre-osteoblasts (Liu et al., 2017). The transcription factor GATA-1, which binds to the CpG site at position −92, has been shown to activate the EpoR promoter in fibroblast transfection assays (Zon et al., 1991). The transcription factors serum response factor (SRF) and EBs, binding at other CpG sites, also affect *Smyd1* gene expression. It has been reported that *Smyd1* is associated with SRF, and that *Smyd1* forms a complex with SRF and enhances SRF DNA binding activity (Ye et al., 2016). In mouse embryonic bodies (EBs), the expression of *Smyd1* is reduced owing to deletion of the transcription factor Ets2 (Islas et al., 2012). Therefore, we inferred that binding of the predicted transcription factors may be affected by methylation of CpG sites, thus affecting gene expression, but this issue remains unclear.

As a histone methyltransferase, Smyd1 specifically methylates histone H3 at lysine 4 (Tan et al., 2006), and thus histone methylation is related to gene expression. In the future, histone methylation levels will be determined to further verify gene expression level.

5. Conclusions

In this study, we determined the pattern of methylation and expression of *Smyd1a* and *Smyd1b* genes during metamorphosis of the Japanese flounder. The results show that the gene expression patterns of *Smyd1a* and *Smyd1b* were different, which may result from differences in the function of both genes during metamorphosis or from the time point chosen for the M3 stage being earlier than that of the down-

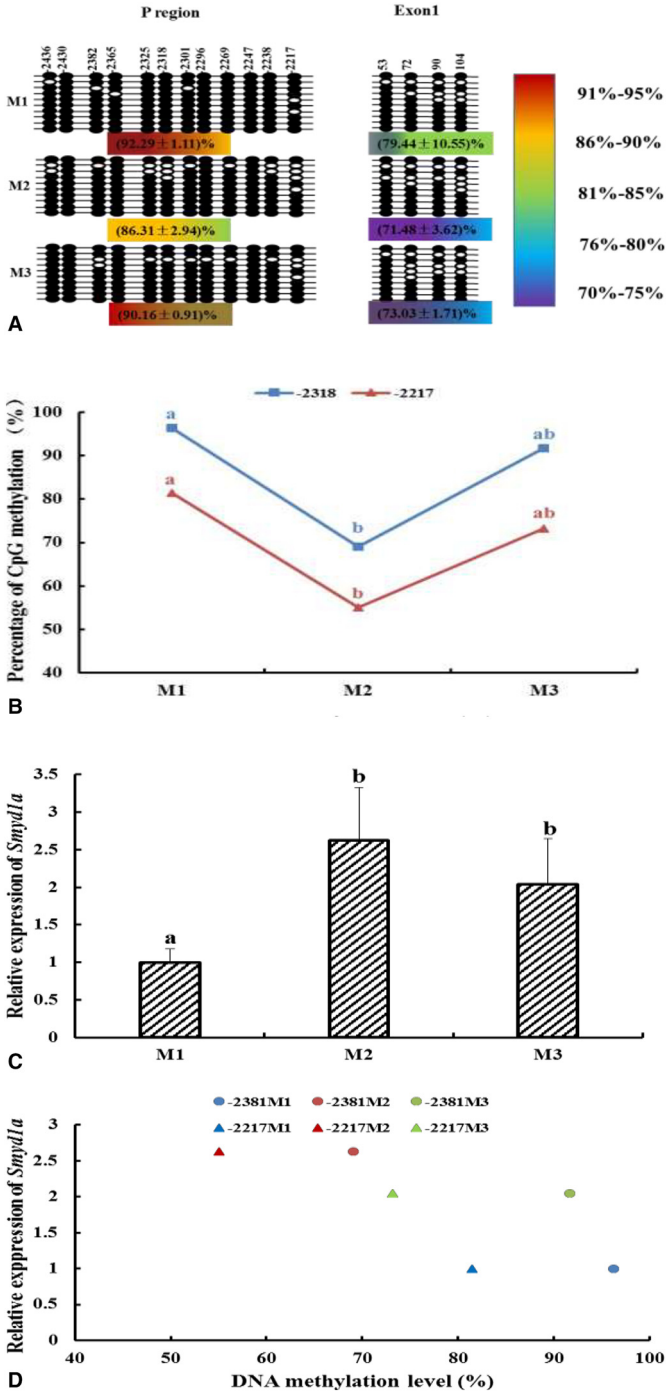

Fig. 3. DNA methylation patterns and expression levels of *Smyd1a*. DNA methylation patterns of P region and exon 1 in *Smyd1a* (A). The methylation state was represented by a circle, open circles were unmethylation and filled circles were methylation. The numbers above represent the location of the CpG relative to the starting codon. The percentage was average methylation level, data is expressed by mean ± SEM. The methylation levels of two CpG sites with significant difference of the *Smyd1a* P region (B). The mRNA expression levels of *Smyd1a* gene from metamorphic stages M1 to M3 (C). Values are shown as mean with SEM. Significant differences between stages are indicated by different letters. Fig. 3D. The correlation between DNA methylation levels (−2318 and −2217 sites of P region) and mRNA expression levels of *Smyd1a* gene at three metamorphic stages of Japanese flounder.

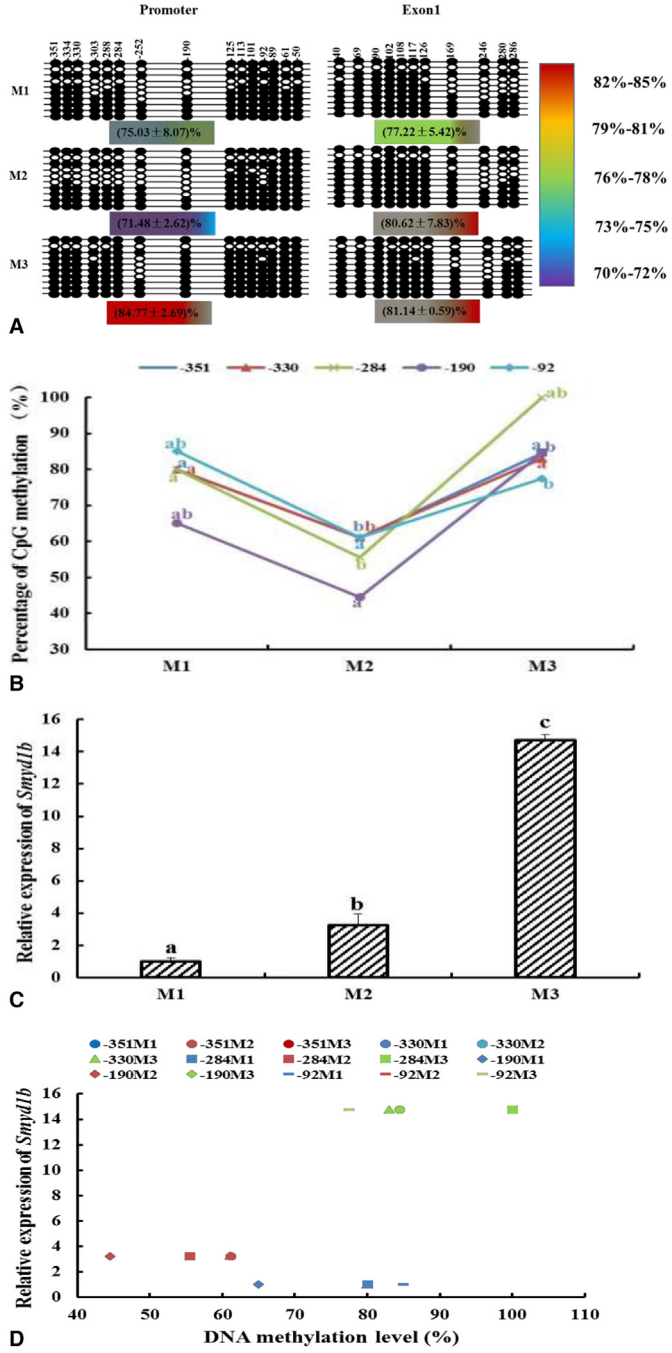

Fig. 4. DNA methylation patterns and expression levels of *Smyd1b*. DNA methylation patterns of promoter and exon 1 in *Smyd1b* (A). The methylation state was represented by a circle, open circles were unmethylation and filled circles were methylation. The numbers above represent the location of the CpG relative to the starting codon. The percentage was average methylation level, data is expressed by mean ± SEM. The methylation levels of five CpG sites with significant difference of the *Smyd1b* promoter (B). The mRNA expression levels of *Smyd1b* gene from metamorphic stages M1 to M3 (C). Values are shown as mean with SEM. Significant differences between stages are indicated by different letters. Fig. 4D. The correlation between DNA methylation levels (−351, −330, −284, −190 and − 92 sites of promoter) and relative expression levels of *Smyd1b* gene at three metamorphic stages of Japanese flounder.

regulation of *Smyd1b*. The methylation patterns of *Smyd1a* and *Smyd1b* were similar. The negative correlation between methylation and expression levels of *Smyd1a* indicated that methylation may be one of the factors affecting gene expression. There was no negative correlation between the methylation status and expression of *Smyd1b*, indicating that methylation of *Smyd1b* had no significant effect on its expression. The combination of transcription factors may also affect expression of

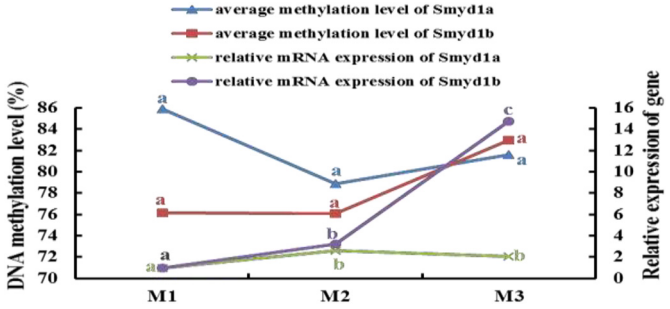

Fig. 5. The correlation between DNA methylation levels and relative expression levels of *Smyd1a* and *Smyd1b* at three metamorphic stages of Japanese flounder. Significant differences between stages are indicated by different letters.

Smyd1a and *Smyd1b*, but further studies are needed to clarify this issue. Our results may help to elucidate the molecular mechanisms of metamorphosis in the Japanese flounder from the perspective of epigenetics.

Acknowledgments

This research was supported by the National Nature Science Foundation of China (31672642) and AoShan Talents 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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpb.2018.05.002.

References

Bao, B., Yang, G., Liu, Z., Li, S., Wang, Z., Ren, D., 2005. Isolation of SFRS3 gene and its differential expression during metamorphosis involving eye migration of Japanese flounder *Paralichthys olivaceus*. Biochim. Biophys. Acta Gen. Subj. 1725, 64–70.

Bell, A.C., West, A.G., Felsenfeld, G., 1999. The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. Cell 98, 387.

Bird, A., 2007. Perceptions of epigenetics. Nature 447, 396–398.

Chatterjee, R., Vinson, C., 2012. CpG methylation recruits sequence specific transcription factors essential for tissue specific gene expression. Biochim. Biophys. Acta 1819, 763.

Chen, H., Walker, G.E., Taylor, S.I., Mckeon, C., 1994. Proximal enhancer of the human insulin receptor gene binds the transcription factor Sp1. Diabetes 43, 884.

Covelo-Soto, L., Saura, M., Morán, P., 2015. Does DNA methylation regulate metamorphosis? The case of the sea lamprey (Petromyzon marinus) as an example. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 185, 42–46.

Deng, G., Chen, A., Pong, E., Kim, Y.S., 2001. Methylation in hMLH1 promoter interferes with its binding to transcription factor CBF and inhibits gene expression. Oncogene 20, 7120.

Gao, J., Li, J., Li, B.J., Yagil, E., Zhang, J., Du, S.J., 2014. Expression and functional characterization of Smyd1a in myofibril organization of skeletal muscles. PLoS One 9, e86808.

Gong, X., Bao, B., Yang, G., Ren, D., 2005. Construction of cDNA library and analysis of parvalbumin gene sequence in metamorphosing flounder *Paralichthys olivaceus*. J. Fish. China 29, 467–472.

Gottlieb, P.D., Pierce, S.A., Sims, R.J., Yamagishi, H., Weihe, E.K., Harriss, J.V., Maika, S.D., Kuziel, W.A., King, H.L., Olson, E.N., 2002. Bop encodes a muscle-restricted protein containing MYND and SET domains and is essential for cardiac differentiation and morphogenesis. Nat. Genet. 31, 25.

Graessmann, A., Sandberg, G., Guhl, E., Graessmann, M., 1994. Methylation of single sites within the herpes simplex virus tk coding region and the simian virus 40 T-antigen intron causes gene inactivation. Mol. Cell. Biol. 14, 2004.

Harrington, M.A., Jones, P.A., Imagawa, M., Karin, M., 1988. Cytosine methylation does not affect binding of transcription factor Sp1. Proc. Natl. Acad. Sci. U. S. A. 85, 2066–2070.

Höller, M., Westin, G., Jiricny, J., Schaffner, W., 1988. Sp1 transcription factor binds DNA and activates transcription even when the binding site is CpG methylated. Genes Dev. 2, 1127.

Hsieh, C.L., 1997. Stability of patch methylation and its impact in regions of transcriptional initiation and elongation. Mol. Cell. Biol. 17, 5897.

Hwang, I., Gottlieb, P.D., 1995. Bop: a new T-cell-restricted gene located upstream of and opposite to mouse CD8b. Immunogenetics 42, 353–361.

Hwang, I., Gottlieb, P.D., 1997. The Bop gene adjacent to the mouse CD8b gene encodes distinct zinc-finger proteins expressed in CTLs and in muscle. J. Immunol. 158, 1165–1174.

Irvine, R.A., Lin, I.G., Hsieh, C.L., 2002. DNA methylation has a local effect on transcription and histone acetylation. Mol. Cell. Biol. 22, 6689–6696.

Islas, J.F., Liu, Y., Weng, K.C., Robertson, M.J., Zhang, S., Prejusa, A., Harger, J., Tikhomirova, D., Chopra, M., Iyer, D., 2012. Transcription factors ETS2 and MESP1 transdifferentiate human dermal fibroblasts into cardiac progenitors. Proc. Natl. Acad. Sci. U. S. A. 109, 13016.

Jones, P.L., Veenstra, G.J., Wade, P.A., Vermaak, D., Kass, S.U., Landsberger, N., Strouboulis, J., Wolffe, A.P., 1998. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat. Genet. 19, 187.

Klaren, P.H.M., Wunderink, Y.S., Yúfera, M., Mancera, J.M., Flik, G., 2008. The thyroid gland and thyroid hormones in Senegalese sole (*Solea senegalensis*) during early development and metamorphosis. Gen. Comp. Endocrinol. 155, 686–694.

Lazo-De-La-Vega-Monroy, M.L., Solís-Martínez, M.O., Romero-Gutiérrez, G., Aguirre-Arzola, V.E., Wrobel, K., Wrobel, K., Zaina, S., Barbosa-Sabanero, G., 2017. 11 beta-hydroxysteroid dehydrogenase 2 promoter methylation is associated with placental protein expression in small for gestational age newborns. Steroids 124, 60–66 (Aug).

Li, H., Zhong, Y., Wang, Z., Jie, G., Jin, X., Chu, W., Zhang, J., Fang, S., Shao, J.D., 2013. Smyd1b is required for skeletal and cardiac muscle function in zebrafish. Mol. Biol. Cell 24, 3511–3521.

Liu, Y., Zhang, J., Liu, X., Sun, Y., University, W M, 2017. The study of transcription factor SP1 regulating MEPE gene expression in preosteoblasts of mouse. Genom. Appl. Biol. 36 (7), 2613–2618.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 25, 402–408.

Manzon, L.A., Youson, J.H., Holzer, G., Staiano, L., Laudet, V., Manzon, R.G., 2014. Thyroid hormone and retinoid X receptor function and expression during sea lamprey (*Petromyzon marinus*) metamorphosis. Gen. Comp. Endocrinol. 204, 211.

Minami, T., 1982. The early life history of a flounder *Paralichthys olivaceus* [in Wakasa Bay, Japan Sea, Japan]. Bull. Jpn. Soc. Sci. Fish. 48, 1581–1588.

Nan, X., Ng, H.H., Johnson, C.A., Laherty, C.D., Turner, B.M., Eisenman, R.N., Bird, A., 1998. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature 393, 386.

Paszkowski, J., Whitham, S.A., 2001. Gene silencing and DNA methylation processes. Curr. Opin. Plant Biol. 4, 123.

Portela, A., Esteller, M., 2010. Epigenetic modifications and human disease. Nat. Biotechnol. 28, 1057–1068.

Razin, A., Kantor, B., 2005. DNA methylation in epigenetic control of gene expression. Prog. Mol. Subcell. Biol. 38, 151.

Schreiber, A.M., 2006. Asymmetric craniofacial remodeling and lateralized behavior in larval flatfish. J. Exp. Biol. 209, 610–621.

Soria, J.C., Lee, H.Y., Lee, J.I., Wang, L., Issa, J.P., Kemp, B.L., Liu, D.D., Kurie, J.M., Mao, L., Khuri, F.R., 2002. Lack of PTEN expression in non-small cell lung cancer could be related to promoter methylation. Clin. Cancer Res. 8, 1178.

Stefanska, B., Macewan, D.J., 2015. Epigenetics and pharmacology. Br. J. Pharmacol. 172, 2701–2704.

Sun, X.J., 2008. Genome-wide survey and developmental expression mapping of zebrafish SET domain-containing genes. PLoS One 3, e1499.

Tan, X., Rotllant, J., Li, H., De, D.P., Du, S.J., 2006. SmyD1, a histone methyltransferase, is required for myofibril organization and muscle contraction in zebrafish embryos. Proc. Natl. Acad. Sci. U. S. A. 103, 2713–2718.

Trautner, J.H., Reiser, S., Blancke, T., Unger, K., Wysujack, K., 2017. Metamorphosis and transition between developmental stages in European eel (*Anguilla anguilla*, L.) involve epigenetic changes in DNA methylation patterns. Comp. Biochem. Physiol. Part D Genom. Proteom. 22, 139.

Ye, X., Qian, Y., Wang, Q., Yuan, W., Mo, X., Li, Y., Jiang, Z., Xu, W., Deng, Y., Wan, Y., 2016. SMYD1, an SRF-interacting partner, is involved in angiogenesis. PLoS One 11, e0146468.

Yoshikawa, N., Matsuda, T., Takahashi, A., Tagawa, M., 2013. Developmental changes in melanophores and their asymmetrical responsiveness to melanin-concentrating hormone during metamorphosis in barfin flounder (*Verasper moseri*). Gen. Comp. Endocrinol. 194, 118.

Zhang, J.L., Shi, Z.Y., 2003. Metamorphosis and mechanism during early development of Japanese flounder,*Paralichthys olivaceus*. J. Shanghai Fish. Univ. 12 (4), 348–352.

Zhang, J., Shi, Z., Cheng, Q., Chen, X., 2011. 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.

Zhu, Y.B., Wang, Z.Y., Yin, R.H., Jiao, Q., Zhao, S.J., Cong, Y.Y., Xue, H.L., Guo, D., Wang, S.Q., Zhu, Y.X., 2018. A lncRNA-H19 transcript from secondary hair follicle of Liaoning cashmere goat: identification, regulatory network and expression regulated potentially by its promoter methylation. Gene. http://dx.doi.org/10.1016/j.gene.2017.10.028. (Jan 30, Epub 2017, Oct 17).

Zon, L.I., Youssoufian, H., Mather, C., Lodish, H.F., Orkin, S.H., 1991. Activation of the erythropoietin receptor promoter by transcription factor GATA-1. Proceed. Natl. Acad. Sci. U. S. A. 88, 10638.