

cDNA clone and expression analysis of α -Tropomyosin during Japanese flounder (*Paralichthys olivaceus*) metamorphosis

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Abstract: Tropomyosin (TM) plays a critical role in skeletal and cardiac muscle development and function. To assess the functional significance of α -TM in Japanese flounder (*Paralichthys olivaceus*) development and metamorphosis, cDNA from Japanese flounder was cloned and α -TM mRNA measured during development and metamorphosis. The full-length cDNA is 1 191 bp, including a 5'-untranslated region of 114 bp, a 3'-UTR of 222 bp, and an open reading frame of 855 bp encoding a polypeptide of 284 amino acids. Real-time quantitative PCR revealed that α -TM mRNA is initially expressed in unfertilized ovum, indicating the α -TM gene is maternal. Relatively low mRNA levels were observed in different embryonic stages. A higher level of α -TM mRNA was detected 3 days post hatching (dph), while the highest level was measured at 29 dph (metamorphic climax) after which it declined towards the end of metamorphosis. The expression of α -TM mRNA was up-regulated in thyroid hormone-treated larvae at 36 dph, but there was no marked difference at other stages when compared to control animals. After thiourea treatment, the expression of α -TM mRNA declined slightly. These data provide basic information that can be utilized in further studies into the role of α -TM in *P. olivaceus* development and metamorphosis.

Keywords: *Paralichthys olivaceus*; Cloning; Expression; α -Tropomyosin; Thyroid hormone

Tropomyosins (TMs) are a family of actin binding proteins that are in all tissues and always association with polymerized actin. Each tropomyosin molecule is bound to one troponin complex which is made up three components: the calcium-binding protein (troponin-C), the inhibitory protein (troponin-I) and troponin-binding protein (troponin-T). α -Tropomyosin (α -TM) plays a critical role in skeletal and cardiac muscle development and function (Marston et al, 2013) and gene mutations lead to myopathy. α -TM regulates muscle contraction by combining with the actin protein filaments and controlling myosin-head-actin interaction on thin filaments under the influence of troponin and Ca^{2+} . As a consequence, tropomyosin controls the actin-myosin cross-bridge cycle and contraction (Gordon et al, 2000). Tropomyosin is present in muscle and non-muscle cells and is an α -helical protein that forms a coiled-coil dimer. In striated muscle, tropomyosin mediates the interactions between the troponin complex and actin so as to regulate muscle contraction.

The Japanese flounder (*Paralichthys olivaceus*) is an important commercial fish and undergoes a special post-embryonic metamorphosis, involving transformation from a bilateral pelagic larva to an asymmetrical benthic juvenile with both eyes on the same side of the body. Metamorphosis in fish takes place in certain species that experience an abrupt ontogenetic transformation from larval to juvenile form associated with a transition to new habitat (Wilbur, 1980; Youson, 1988).

Its asymmetrical shape is unique in vertebrates. The process is largely controlled by thyroid hormone (TH) and exogenous thyroid hormone can induce early metamorphosis of flounder larvae (Schreiber, 1998). During

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metamorphosis skeletal muscle cells change morphology, involving troponin and myosin light chains prior to translating body morphology from planktonic to benthic life (Yamano *et al.*, 1991; Yamano *et al.*, 1994). Thiourea (TU) can inhibit flounder metamorphosis (Inui & Miwa, 1985). Li *et al.* (2012) previously suggested that the expression of tropomyosin is related to amphioxus sarcomere, muscle and nerve cord formation, and participate in body model building (Li *et al.*, 2012). Muscle is one of the obvious organizational changes during flounder metamorphosis but the role of tropomyosin in flounder metamorphosis remains unclear.

In the present study, we used RT-PCR methods to measure α -TM mRNA expression to understand (1) the expression of α -TM during flounder development and metamorphosis, and (2) whether α -TM expression is modulated by TH. We also cloned the complementary DNA (cDNA) sequence, including the complete open reading frame (ORF) of the tropomyosin gene in Japanese flounder. Finally, nucleotide composition, molecular evolution, protein structure and potential functional region were analyzed using bioinformatics methods.

MATERIALS AND METHODS

Animal experiments

Flounder larvae and juveniles were reared in seawater kept at 16 ± 1 °C at the Beidaihe Center Experiment Station (Chinese Academy of Fishery Sciences, Hebei). Larvae from 3–13 days post hatching (dph) were in a 2 000 L tank at 16–17 °C. At 14 dph, larvae were divided into three groups and cultured from 15–42 dph. The control group (control) was exposed to natural seawater. The TH group (thyroid hormone, T4) was exposed to seawater containing a 0.1 mg/L concentration of TH over the course of the experiment (Inui & Miwa, 1985). The TU group was exposed to seawater containing a 30 mg/L concentration of TU over the whole experiment (Inui & Miwa, 1985). All protocols and procedures were approved by the Shanghai Ocean University Review Committee for the Use of Animal Subjects.

Sample collection

Larvae were carefully separated into developmental stages as described by Minami (1982). We collected samples at 0 h (unfertilized egg), 26 hour post fertilization (hpf, gastrula stage), 71 hpf (heart-beating stage), 3 dph, 9 dph, 14 dph, 17 dph (pre-metamorphosis, the

stage prior to the start of eye migration, Stage D), 23 dph (pro-metamorphosis, the right eye moved toward the dorsal margin but still could not be seen from the left/ocular side, Stage F), 29 dph (climax metamorphosis, the right eye has become visible from the ocular side while not reached the dorsal midline, Stage G), and 36 dph (post-metamorphosis, the right eye has just located on the dorsal margin, Stage H). Whole larvae ($n=3$ pools, 5 specimens/pool), including the control (9, 14, 17, 20, 23, 29 and 36 dph), TH treatment (17, 20, 23, 29 and 36 dph), and TU treatment (17, 20, 23, 29 and 36 dph), were collected for RNA isolation. Adult tissues/organs samples ($n=3$ specimens) were separately dissected, immediately frozen in trizol and maintained at -80 °C for RNA extraction.

RNA isolation

Total RNA was isolated from whole larvae using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RQ1 RNase-free DNase (Promega, Madison, WI, USA) was used to remove genomic DNA contamination. RNA concentration was quantified using an NANODRO 2000 (ThermoFisher Scientific) and RNA integrity was detected by agarose-gel electrophoresis, and $1.8 < A260 / A280$ ratios < 2.0 .

Tropomyosin gene cloning and sequence analysis Design of primer sequences

A few species of tropomyosin mRNA were used as query sequences through the basic local alignment search tool (BLAST) program to retrieve high homological domain from the GenBank database. Primers (Table 1) were designed according to these domains using Primer Premier 5.0. Gene-specific primers (Table 1) were designed in the cloned PCR fragments for 5'-rapid amplification of cDNA ends (5'-RACE) and 3'-RACE, respectively.

Table 1 Designed primers used in the experiment

Primer name	Primer sequence (5'–3')
α -Tm Primer F	TTAGCGTTCAGTTAGGCACA
α -Tm Primer R	AGCTTGGTCAGGGCAGTT
α -Tm3' RACE Outer Primer	GCAACTGCCCTGACCAAGC
α -Tm3' RACE Inner Primer	GAAGGCTGCTGATGAGAGCGAGA
α -Tm5' RACE Outer Primer	CTTGTCGGTGAGGACCTTGATC
α -Tm 5' RACE inner Primer	GACGAGCTTACGGGCCACCT
α -Tm qPCR-F	ACCACGCCCTCAACGACA
α -Tm qPCR-R	GGACAGGAAAGCGAAGACA

Synthesis of cDNA

The first-strand cDNA of each sample was synthesized from 500 ng total RNA GoScript Reverse Transcription System (Promega). PCR was performed using the primers (α -TM F+ α -TM R) with cDNA as a template. PCR products were purified from 1% agarose gel, and cloned into PMD-19T vectors. The plasmid was transformed into DH5 α competent cells. Positive clone were selected on lysogeny broth-amp plates supplemented with X-gal and IPTG. The cloned α -TM cDNAs of *P. olivaceus* were sequenced.

In order to obtain the 3' and 5' end sequences of α -Tm, nested 3' and 5'-RACE PCR were performed with 5'-Full RACE Core Set and 3'-Full RACE Core Set (TAKARA) using the DNase treated total RNA. The reaction system and conditions were designed according to the technical manual. The RACE products were sequenced and contiged with PCR fragments above, then the whole sequence was submitted to GenBank.

Bioinformatics analysis

The TM gene coding regions and structural domains were predicted using ORF finder and Pfam. Molecular masses and isoelectric point (pI) were predicted using the web server ProtParam (<http://www.expasy.org/tools/protparam.html>). For the phylogenetic analysis of α -TM genes, 12 α -TM protein sequences were selected. MEGA 5.2 was used to build a phylogenetic tree via the neighbor-joining (NJ) method, with 1 000 bootstraps. The α -TM protein sequences were aligned using ClustalX 2.0 to identify homologous regions.

mRNA expression (quantitative real-time PCR)

The first-strand cDNA of each sample was synthesized from 500 ng total RNA GoScript Reverse Transcription System (Promega). The reaction system and conditions were designed according to the technical manual. The first-strand cDNAs were kept at -20°C . A standard curve was generated for each pair of primers based on known quantities of cDNA (10-fold serial dilutions corresponding to cDNA transcribed from 500–0.05 ng of total RNA) to estimate amplification efficiencies. All calibration curves exhibited correlation coefficients >0.995 , and the corresponding real-time PCR efficiencies (E) were 0.96–0.99.

Real-time PCR was performed in Bio-Rad CF96 (Bio-Rad). The 20 μL reaction mixture consisted of 10 μL SYBR Premix Taq ($\times 2$), 0.4 μL forward primer (10 $\mu\text{mol/L}$), 0.4 μL reverse primer (10 $\mu\text{mol/L}$) and 1 μL Kunming Institute of Zoology (CAS), China Zoological Society

PCR template. All experiments were performed triplicate. The relative expression levels of TM were normalized to GAPDH and β -actin. PCR was as follows: initial denaturation and enzyme activation for 3 min at 95°C , followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Fluorescence was collected at the end of each round of amplification.

The relative mRNA expression for each gene was determined using the ($2^{-\Delta\Delta\text{Ct}}$) method (Scheffe et al, 2006). The results of real-time PCR analysis were presented as mean \pm SE. The relative expression level of the α -TM gene in the E1 (unfertilized ovum) was used as the calibrator. Groups were compared using one-way ANOVA, followed by a Tukey test to identify statistically distinct groups. Significant differences were accepted for $P < 0.05$.

RESULTS

cDNA cloning and sequence analyses of α -Tm

RT-PCR was conducted using two pairs of primers (Table 1). The sizes of the PCR products were 438 bp. Further research was conducted by 5'-RACE and 3'-RACE, and PCR products were 727 bp and 624 bp, respectively. The full length α -TM cDNA was 1 191 bp including a 5'-untranslated region (UTR) of 114 bp, a 3'-UTR of 222 bp, and an open reading frame (ORF) of 855 bp encoding a polypeptide of 284 amino acids (GenBank accession number: KF996501).

α -TM is a two-chained α -helical coiled-coil protein that binds along the length of the actin filament and regulates muscle contraction in cooperation with Tn (troponin) and myosin. The predicted molecular size and theoretical PI of α -TM is 27×10^3 and 4.69, respectively. There are nine amino acids (LKEAET-RAE) on the C-end, which is a conservative area and characteristic of the tropomyosin gene region. The sequence (EELDRAQE) in 97–104 and sequence (KLEEAEEK) in 112–118 were stability control regions of α -TM. The amino acids 258–284 and 175–190 were two distinct regions that bind to TnT (troponin T) (Figure 1).

According to the sequence alignment result, the *P. olivaceus* α -TM protein sequence shares similarities above 90% with homologs from other vertebrates. Phylogenetic analysis of α -TM from *P. olivaceus* and other vertebrates using protein sequences is shown in Figure 2. *P. Olivaceus* is closest to *E. coioides*, sharing a high level of similarity of 98%, and farthest from lancelets (*G. gallus*) at a low level of 93%. Similarities within all the other species are between 94%–97%. This

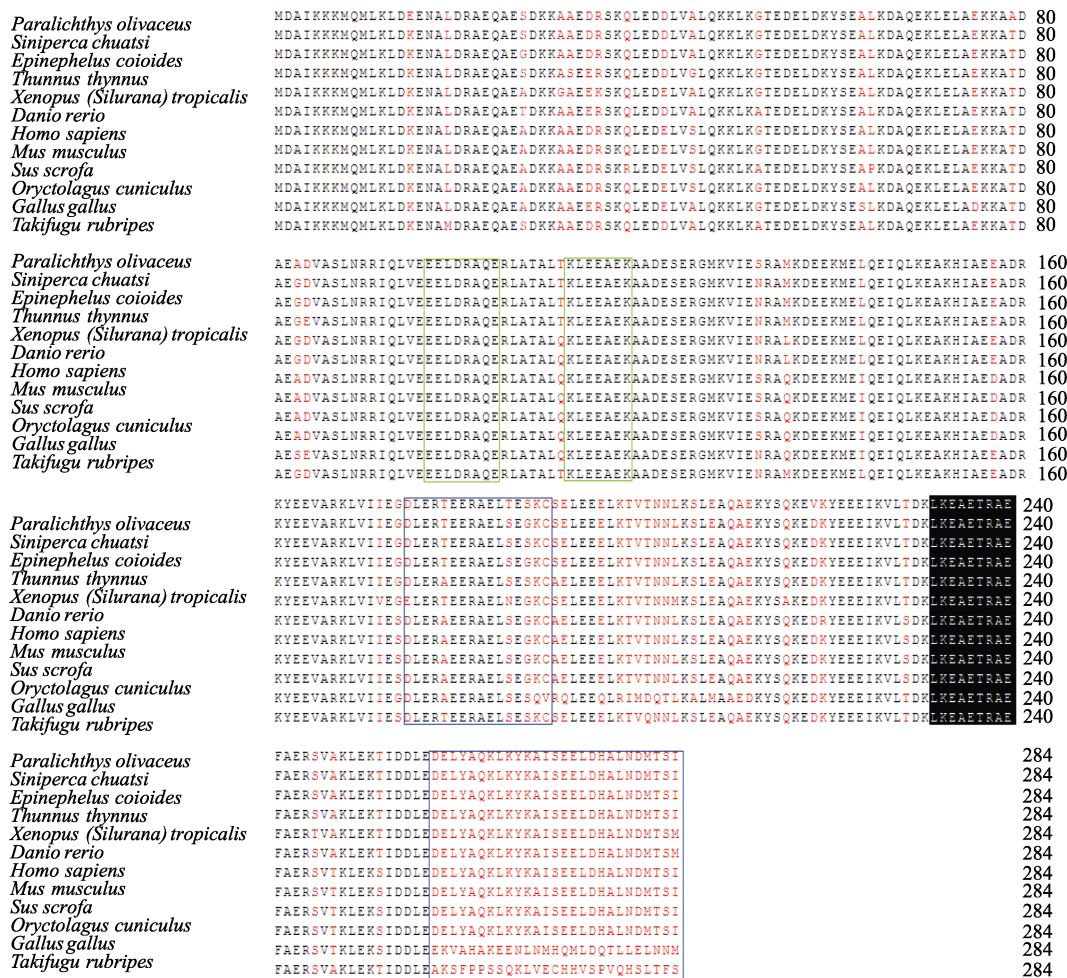


Figure 1 Amino acid sequence alignment for *Paralichthys olivaceus* tropomyosin

Black font represent same amino acid residues, red font represents similar amino acid residues. Green box contains stability control region of α -TM (97-104:EELDRAQE;112-118 KLEEA EK). Blue box contains binds to TnT region. Black blocks represent characteristic amino acid residues of tropomyosin.

[*Paralichthys olivaceus*] gb|KF996501; [*Siniperca chuatsi*] gi|339896195; [*Epinephelus coioides*] gi|295792268; [*Thunnus thynnus*] gi|38175083; [*Xenopus (Silurana) tropicalis*] gi|165971165; [*Danio rerio*] gi|55962544; [*Homo sapiens*] gi|63252898; [*Mus musculus*] gi|509182; [*Sus scrofa*] gi|148222268; [*Oryctolagus cuniculus*] gi|157787199; [*Gallus gallus*] gi|45382323; [*Takifugu rubripes*] gi|74136093.

molecular phylogeny agrees with the evolutionary relationships for these species.

Expression of α -TM mRNA during embryonic and larval development

Markedly low levels of α -TM transcripts were detected in the unfertilized egg and gastrula stages, but reached a certain level at the heart beating stage (Figure 3). A relatively high level of α -TM mRNA was present until 3 dph, but from 9 dph to 14 dph it sharply decreased and remained low. From 17 dph, α -TM mRNA gradually increased and reached its highest level at 29 dph, when the larvae were at metamorphic climax; levels declined visibly at the end of metamorphosis.

Effect of TH on α -TM mRNA expression during metamorphosis

Larvae at 14 dph were exposed to exogenous TH (0.1 mg/L) and TU (30 mg/L), and levels of α -TM mRNA were determined in larvae sampled at 3, 9, 15 and 22 days after TH and TU treatment (Figure 4). In the treatment, the expression of GAPDH and β -actin were smooth. No significant differences in α -TM mRNA levels were observed in TH- and TU-administrated larvae at 23 dph. Importantly, TH-treated larvae exhibited significantly higher α -TM mRNA levels than untreated and TU-treated larvae at 36 dph ($P<0.05$). The relatively lower levels of α -TM mRNA were detected in TU-treated

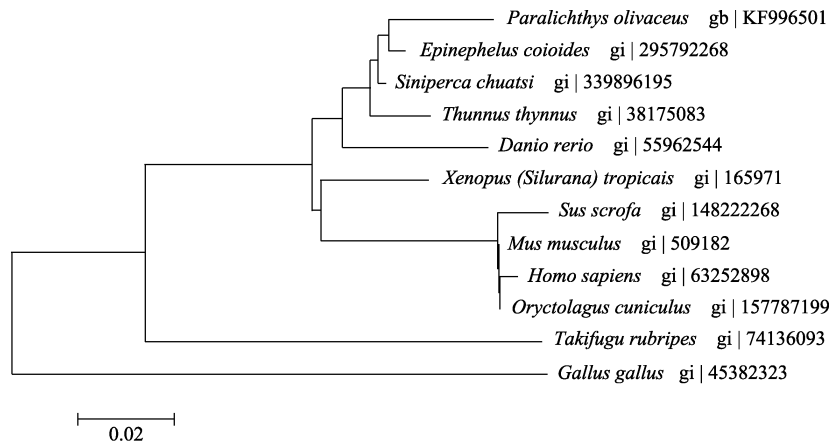


Figure 2 Phylogenetic tree of tropomyosin

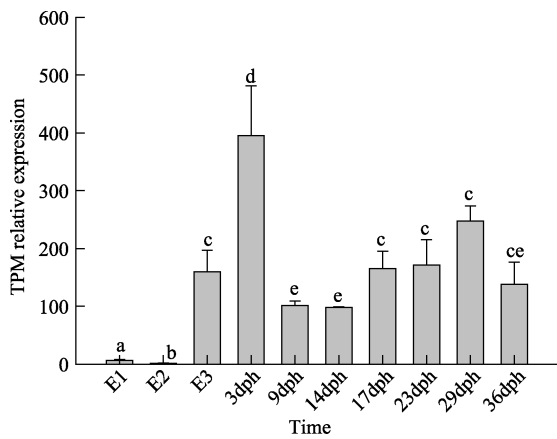


Figure 3 The relative expression of tropomyosin in different development periods

E1: unfertilized ovum; E2: gastrula; E3: Heart beating stage; 3 dph: 3 days post hatching; 9 dph: nine days post hatching; 14 dph: 14 days post hatching; 17 dph: 17 days post hatching; 23 dph: 23 post hatching; 29 dph: 29 days post hatching; and 36 dph: 36 days post hatching.

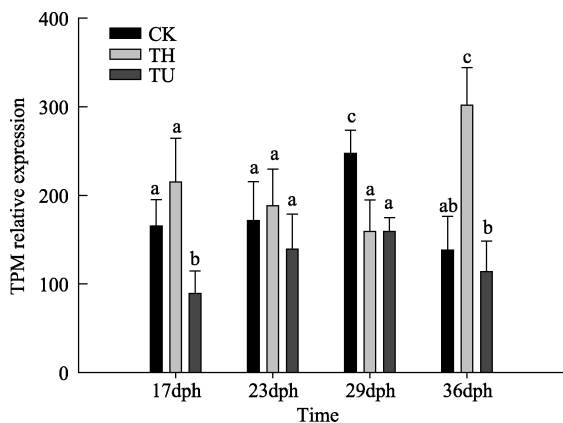


Figure 4 Expression of Tropomyosin during metamorphosis of NC, TH- and TU-treated larvae

17 dph: 17 days post hatching; 23 dph: 23 days post hatching; 29 dph: 29 days post hatching; and 36 dph: 36 days post hatching.

larvae compared with controls at 17, 23, 29 and 36 dph.

DISCUSSION

There are four TM genes: TPM1 (α -TM), TPM2 (β -TM), TPM3 (γ -TM) and TPM4 (δ -TM). (Jagatheesan et al, 2010) formed by a family of four alternatively spliced genes that display highly conserved nucleotide and amino acid sequences. We isolated the full length cDNA of the α -TM gene from *P. olivaceus*. The deduced protein sequence is 284 amino acids, the same as other species in striated muscle. It is 93%–98% homologous to α -TM of other teleosts. High sequence homology illustrates that the function of this gene is highly conserved. An 8-residue sequence (EELDRAQE) with five charged residues in 97–104 and a 7-residue sequence (KLEEA EK) with five charged residues in 112–118 are regarded as likely sources of the contributions that result in the stability observed in full-length tropomyosin (Kirwan & Hodges, 2010). Previous studies determined that TnT binds to α -TM in two distinct regions: at the carboxyl end of the molecule (amino acids 258–284), and internally in the region of amino acids 175–190 (Pearlstone & Smillie, 1983; Sumida et al, 2008). We found that these sites in Japanese flounder, mammals and other teleosts were conserved. Phylogenetic analysis revealed that the *P. olivaceus* α -TM locates on the same group as all fish α -TM.

α -TM is expressed early during flounder development and remains transcribed throughout its lifetime, which is the same for Mariappan in mouse (Muthuchamy et al, 1993). Tropomyosin plays an important role in controlling calcium regulated sarcomeric contraction through its interactions with actin and the troponin complex. We found high levels of α -TM mRNA

expression at 3 dph, which may be due to transition from a quiescent state to sport state for flounder. α -TM mRNA remained at lower levels until 29 dph because a great deal of energy is required to meet the needs of metamorphosis during metamorphosis, and thus the growth rate decreased and α -TM mRNA expression declined. TM is an essential protein, if knocked out the

developing mouse dies between 10–14 embryonic days (Rethinasamy *et al.*, 1998). The Glu180Gly and Asp175Asn mutations can cause hypertrophic cardiomyopathy (HCM) (Bing *et al.*, 2000). TH and TU may promote or inhibit flounder metamorphosis, respectively (Fu *et al.*, 2012), but here the expression of α -TM was relatively unaffected.

References

- Bing W, Knott A, Redwood C, Esposito G, Purcell I, Watkins H, Marston S. 2000. Effect of hypertrophic cardiomyopathy mutations in human cardiac muscle α -tropomyosin (Asp175Asn and Glu180Gly) on the regulatory properties of human cardiac troponin determined by *in vitro* motility assay. *Journal of Molecular and Cellular Cardiology*, **32**(8): 1489-1498.
- Fu YS, Shi ZY, Wang GY, Li WJ, Zhang JL, Jia L. 2012. Expression and regulation of miR-1, -133a, -206a, and MRFs by thyroid hormone during larval development in *Paralichthys olivaceus*. *Comparative Biochemistry and Physiology. Part B: Biochemistry & Molecular Biology*, **161**(3): 226-232.
- Gordon AM, Homsher E, Regnier M. 2000. Regulation of contraction in striated muscle. *Physiological Reviews*, **80**(2): 853-924.
- Inui Y, Miwa S. 1985. Thyroid hormone induces metamorphosis of flounder larvae. *General and Comparative Endocrinology*, **60**(3): 450-454.
- Jagatheesan G, Rajan S, Wieczorek DF. 2010. Investigations into tropomyosin function using mouse models. *Journal of Molecular and Cellular Cardiology*, **48**(5): 893-898.
- Kirwan JP, Hodges RS. 2010. Critical interactions in the stability control region of tropomyosin. *Journal of Structural Biology*, **170**(2): 294-306.
- Li XY, Lin YS, Zhang HW. 2012. Phylogenetic analysis and expression patterns of tropomyosin in amphioxus. *Zoological Research*, **33**(4): 389-394. (in Chinese)
- Marston S, Memo M, Messer A, Papadaki M, Nowak K, McNamara E, Ong R, El-Mezgueldi M, Li XC, Lehman W. 2013. Mutations in repeating structural motifs of tropomyosin cause gain of function in skeletal muscle myopathy patients. *Human Molecular Genetics*, **22**(24): 4978-4987.
- Minami T. 1982. The early life history of a flounder *Paralichthys olivaceus* [in Wakasa Bay, Japan Sea, Japan]. *Bulletin of the Japanese Society of Scientific Fisheries*, **48**(11):1581-1588.
- Muthuchamy M, Pajsk L, Howles P, Doetschman T, Wieczorek DF. 1993. Developmental analysis of tropomyosin gene expression in embryonic stem cells and mouse embryos. *Molecular and Cellular Biology*, **13**(6): 3311-3323.
- Pearlstone JR, Smillie LB. 1983. Effects of troponin-I plus-C on the binding of troponin-T and its fragments to alpha-tropomyosin. Ca^{2+} sensitivity and cooperativity. *Journal of Biological Chemistry*, **258**(4): 2534-2542.
- Rethinasamy P, Muthuchamy M, Hewett T, Boivin G, Wolska BM, Evans C, Solaro RJ, Wieczorek DF. 1998. Molecular and physiological effects of α -tropomyosin ablation in the mouse. *Circulation Research*, **82**(1): 116-123.
- Scheffe JH, Lehmann KE, Buschmann IR, Unger T, Funke-Kaiser H. 2006. Quantitative real-time RT-PCR data analysis: current concepts and the novel "gene expression's C_T difference" formula. *Journal of Molecular Medicine*, **84**(11): 901-910.
- Schreiber AM & Specker JL. 1998. Metamorphosis in the Summer Flounder (*Paralichthys dentatus*): Stage-Specific Developmental Response to Altered Thyroid Status. *General and comparative endocrinology*, **111**(2): 156-166.
- Sumida JP, Wu E, Lehrer SS. 2008. Conserved Asp-137 imparts flexibility to tropomyosin and affects function. *Journal of Biological Chemistry*, **283**(11): 6728-6734.
- Wilbur HM. 1980. Complex life cycles. *Annual review of Ecology and Systematics*, **11**: 67-93.
- Yamano K, Miwa S, Obinata T, Inui Y. 1991. Thyroid hormone regulates developmental changes in muscle during flounder metamorphosis. *General and Comparative Endocrinology*, **81**(3): 464-472.
- Yamano K, Takano-Ohmuro H, Obinata T, Inui Y. 1994. Effect of thyroid hormone on developmental transition of myosin light chains during flounder metamorphosis. *General and Comparative Endocrinology*, **93**(3): 321-326.
- Youson JH. 1988. First metamorphosis. *Fish Physiology*, **11**(Part B): 135-196.