

Molecular structure of the largemouth bass (*Micropterus salmoides*) *Myf5* gene and its effect on skeletal muscle growth

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Received: 9 June 2008 / Accepted: 12 August 2008 / Published online: 28 August 2008
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Abstract Myogenic Regulatory Factors (MRFs), a family of basic helix-loop-helix (bHLH) transcription factors, play important roles in regulating skeletal muscle development and growth. *Myf5*, the primary factor of MRFs, initiates myogenesis. Its expression pattern during somitomyogenesis in some fish has been revealed. To further study its effect on fish muscle during postembryonic growth, characterization and function analysis of *myf5* cDNA were carried out in largemouth bass. The 1,093 bp cDNA sequence was identified by RT-PCR and 3'RACE, then the ORF of *Myf5* cDNA was cloned into the expression vector pcDNA3.1(-)/mycHisB. The recombinant plasmid pcDNA3.1(-)/mycHisB-Myf5 was injected into the dorsal muscle of tilapias. RT-PCR and histochemical results showed that the exogenous gene was transcribed and translated in vivo. Its effect on muscle growth focused on myofiber hypertrophy in white muscle 60 days post injection. This indicated that overexpression of *Myf5* can promote myogenesis during the fish muscle postembryonic growth period.

Keywords *Myf5* · Largemouth bass · Expression vector · Muscle growth

Introduction

The cell fate of skeletal muscle precursor is determined by the expression of myogenic regulatory factors (MRFs) that include *Myf5*, *MyoD*, *myogenin* and *MRF4*. These nuclear proteins all contain a basic helix-loop-helix (bHLH) domain. By the bHLH domain, they can dimerize with E-proteins to form protein dimmers [1–4] and bind to the consensus DNA sequence (CANNTG) as an E-box to activate the transcription of muscle-specific genes, such as the muscle creatine kinase gene and myosin light chain gene [5–7]. MRFs are expressed specifically in muscle cells, and forced expression in other cell types could induce the muscle differentiation programs in these cells [8, 9].

Myf5, as the first expressed MRF, plays an essential role in the specification and proliferation of myoblasts [10–12]. Human *Myf5* was discovered in 1989, and found to be capable of converting mouse 10T1/2C3H fibroblasts into myosin expressing myotubes [13]. *Myf5* gene-knockout mice exhibited delayed development of the epaxial musculature and died from severe rib abnormalities, suggesting that *Myf5* primarily regulates the epaxial musculature formation [14–17]. In fish, several MRFs have been cloned and their molecular mechanisms on muscle development and growth have been partially revealed. For example, *Myf5* expression patterns during somitomyogenesis have been uncovered in zebrafish [18], striped bass [19], flounder [20] and sea perch [21]. In morpholino-injected zebrafish embryos, abnormal muscle development and defective somite patterning have been observed, suggesting that *Myf5* plays a similar role in zebrafish and mammals [22]. Its function on postembryonic muscle growth was only reported in rainbow trout. During rainbow trout postnatal development, the expression of MRFs were revealed to be correlated with the muscle growth pattern [23], but the concrete mechanism of the promotion of

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muscle fiber postembryonic growth is still unclear. Unlike mammals, most of fish skeletal muscles grow dramatically during the post-larval life, involving continuous myofiber hyperplasia and hypertrophy processes [24]. The proliferation of a population of myogenic progenitor cells (MPCs) showing varying degrees of commitment to terminal differentiation to myoblasts contribute to these two processes [25]. Thus, MRFs may also have an important role in regulating muscle formation and growth during the postnatal period in fish. Investigating the effect of *Myf5* on postembryonic fish muscle growth will be an important part of the molecular basis of muscle development and growth.

Largemouth bass has become an important cultured commercial freshwater species in China, and is a good subject for the study of fish growth as it has rapid postnatal growth. Here we report the isolation and characterization of the largemouth bass *Myf5* cDNA, and the effect of its overexpression on postembryonic muscle growth in fish.

Materials and methods

Experimental fish

Largemouth bass and Nile tilapia were obtained from Pearl River Fisheries Research Institute. Nile Tilapia, which also belong to Perciformes, were used as the experimental animals to evaluate the effect of largemouth bass *Myf5* on muscle growth for its close relationship to largemouth bass, easier raising and handling, and fewer diseases usually occur when kept in captivity than largemouth bass itself. The total length of the Nile tilapia used in this study ranged from 10 to 14 cm. The Nile tilapia during this period showed the high growth rate. These Nile tilapia which all came from the full-sibs family, were injected with plasmids and further cultured for two months under controlled conditions (temperature $24 \pm 1^\circ\text{C}$; photoperiod 14:10 light:dark). All the fish were anaesthetized before handling.

Isolation of largemouth bass *Myf5* cDNA

Total RNA was extracted from the trunk muscle of the largemouth bass weighing 400 g, using the SV Total RNA Isolation System (Promega). First-strand cDNA was synthesized using the TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa). Three primers were designed with reference to the known nucleotide sequences of *Myf5* from fish such as zebrafish, carp, striped bass, flounder, and rainbow trout. The sense primer F1 used was located at the initiation codon and the nested sense primer F2 used was located at the downstream of F1; F1: ATGGA(T/C)GTCTTCTC(G/A/C)(A/C)CATCCC and F2: CGCCATCCAGTACATCGAGAG. The antisense primer used was R1: TCACAG(G/T)ACGTGG

TAGACGGG. The PCR was performed using F1 and oligo dT adaptor primer (in kit, including dT and M13 Primer M4): GTTTTCCCAGTCACGAC. The parameters were 28 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 1 min, with an additional initial 3-min denaturation at 94°C and a 5-min final extension at 72°C . Then the nested PCR was performed using F1 and R1. 3'RACE was conducted using F2 and the M13 Primer M4 (obtained from the kit) according to the parameters above. The PCR products were subsequently cloned into the pMD19 T-vector (TaKaRa) and sequenced. The two fragments were then spliced to obtain the ORF and the 3'UTR.

Construction of the recombinant plasmid pcDNA3.1(-)/mycHisB-Myf5

Two primers were designed to modify the *Myf5* open reading frame (ORF) of largemouth bass, including an extra *ApaI* and *XbaI* adapter respectively. The sense primer F3: ATAGGGCCACCATGACGTCTTCTCACCATC, and antisense primer R2: AGATCTAGAAACAGGACGTGGTAGAC, were synthesized (Invitrogen). After PCR amplification with PfuDNA polymerase, the product was digested with *ApaI* and *XbaI*, and cloned into the same sites of the pcDNA3.1(-)/mycHisB plasmid (Invitrogen) to generate the recombinant pcDNA3.1(-)/mycHisB-Myf5 expression plasmid which was driven by the cytomegalovirus promoter (CMV).

Route of plasmid intramuscular injection

Tilapia were anaesthetized using 100 mg/l MS-222 (Sigma) to avoid the fish struggling and the resulting muscle contractions which lead to a decrease of DNA-expression in mice [26]. About 40 μg pcDNA3.1(-)/mycHisB-Myf5 and pcDNA3.1(-)/mycHisB plasmid were dissolved in 100 μl 0.75% NaCl respectively. The recombinant plasmid was injected into the tilapia dorsal muscle at the fifth scale in the lateral line of the right side to a depth of 5–7 mm at an angle of 45° , and the same position of the opposite side with the same depth was injected using the non-recombinant plasmid as a control. 48 h post-injection, total RNA was extracted from the muscle at the injected place using Trizol (Invitrogen), then was digested by RNase-Free DNase (Promega) to confirm no plasmid in RNA sample. RT-PCR was then conducted to examine the locally transcription of the exogenous gene using the sense primer F4: GCCATCCATTACATGAGAGTC located at nucleotide position 331 of the largemouth bass *Myf5* ORF and the antisense primer R3: CCTCTTCTGAGATGAGTTTTTTG located in the myc tag of the recombinant plasmids. On the eighth day post-injection, immunohistochemistry was used to examine the translation of the exogenous gene. The recombinant plasmid expresses the *Myf5:mycHis* fused protein which can be detected with the Anti-His Tag mouse monoclonal antibody.

The muscle of the injection position was cut transversely into $1 \times 1 \times 1 \text{ cm}^3$ and sectioned using routine paraffin sectioning. The Anti-His Tag mouse Monoclonal Antibody (BOSTER, China) was used as the first antibody (diluted 1:100 in PBS). All steps were carried out according to the SABC (Strept-Avidin Biotin Complex) kit protocol (BOSTER, China); the diaminobenzidine (DAB) substrate kit (BOSTER, China) was used for visualization. Sections were then rinsed and lightly stained with hematoxylin.

Evaluation of myofiber diameter and density

Sixty days post-injection, three individual tilapias were analyzed. The muscles of the same injection places in two sides was transversely cut into $0.5 \times 0.5 \times 1 \text{ cm}^3$ tissue small blocks and fixed with formalin for 24 h, and then embedded in paraffin, sectioned and stained with hematoxylin/eosin. The same hemi-myotomes in experiment and control sections from the same position of the two sides were selected for the measurement of diameters and densities of the muscle fibers (fibers were assumed to be roughly cylindrical). In one section, consecutive five different microscope fields (10×10) from the outer myofiber layers to inner were photographed, and these five fields almost covered the

hemi-myotome in the section. The five pictures were measured respectively, and added up to obtain the mean value of the myofiber diameter and density. More than 500 white muscle fibers were observed per fish. The red muscle fibers are a thin layer on the body surface, and the cell numbers are limited. Thus more than 150 red muscle fibers were analyzed. A Student's *t*-test was used to determine whether significant differences existed between the recombinant plasmid injection sites and the non-recombinant plasmid injection sites.

Results

Sequences of nucleotide and deduced amino acid of largemouth bass *Myf5*

The cDNA length of largemouth bass *Myf5* was 1,093 bp and contained a 723 bp ORF with a 370 bp flanking region at the 3'-end (GenBank accession no. EU555403). The deduced amino acid sequence of largemouth bass *Myf5* revealed a 240 amino acid polypeptide that contained a bHLH domain located at amino acid positions 56–124 (Fig. 1). No signal peptide was found using the SignalP3.0 analysis software on line (<http://www.cbs.dtu.dk/service/SignalP>).

Fig. 1 Nucleotide and deduced amino acid sequences of largemouth bass *Myf5* cDNA. The bHLH domain is underlined; THA polyadenylation signal ATTTAAA is shown in dark

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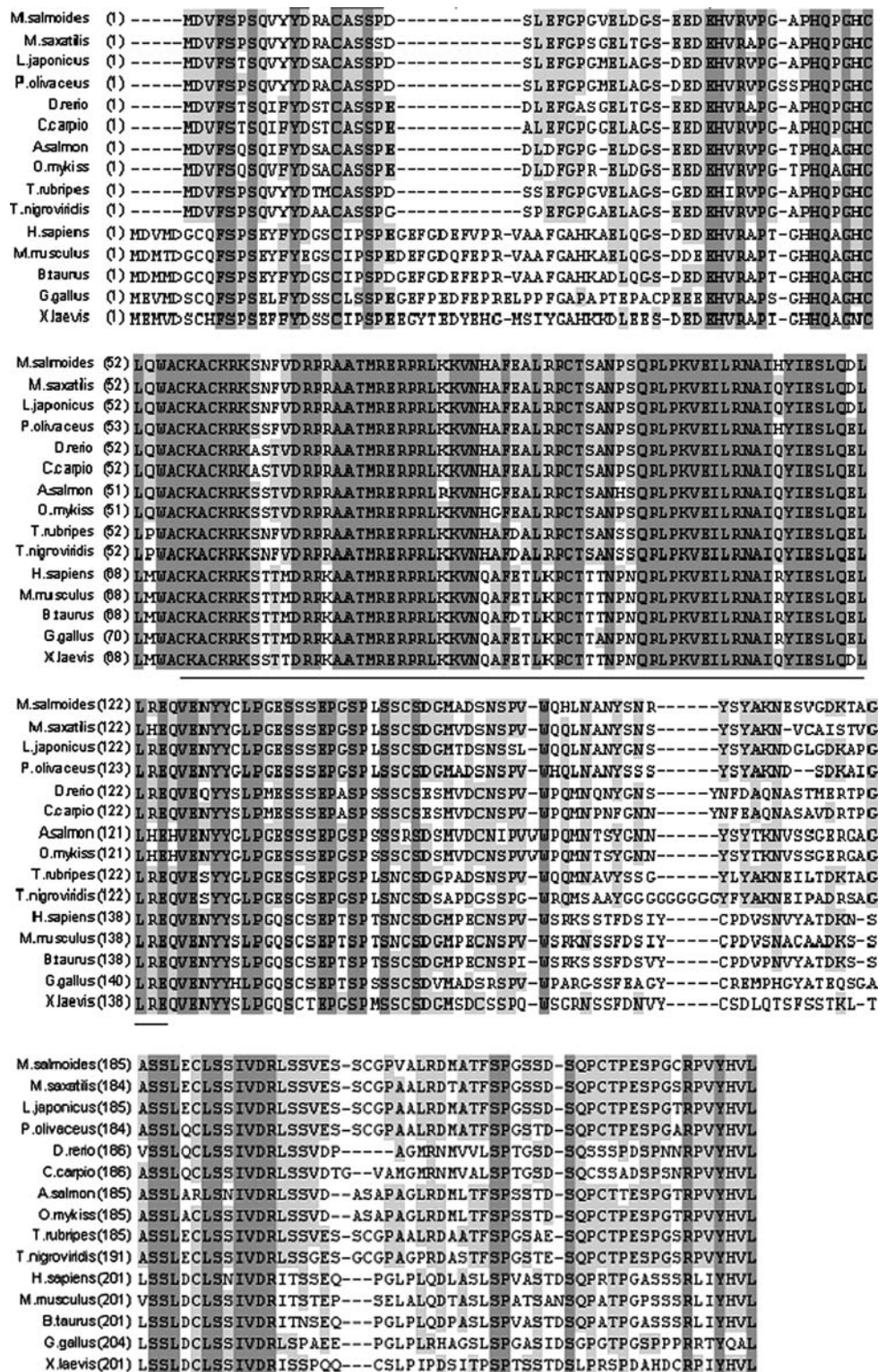
1  ATG GAC GTC TTC TCA CCA TCC CAG GTC TAC TAC GAC AGA GCG TGT GCT TCG TCT CCA GAT
1  M D V F S P S Q V Y Y D R A C A S S P D
61  AGC CTG GAG TTT GGA CCC GGT GTG GAG CTT GAT GGT TCT GAG GAG GAC GAG CAT GTC AGG
21  S L E F G P G V E L D G S E E D E H V R
121 GTT OCC GGG GCA OCT CAC CAG CCG GGA CAC TGT CTC CAG TGG GOC TGC AAG GOC TGC AAG
41  V P G A P H Q P G H C L Q W A C K A C K
181  CGC AAG TCC AAC TTT GTG GAC CGC AGA CCG GCT GOC ACC ATG CGT GAG CGC CCG CCG CTG
61  R K S N F V D R R R A A T M R E R R R L
241  AAG AAG GTC AAC CAC GCG TTC GAG GCT TTG AGA CGT TGC ACC TOG GCC AAC OCC AGC CAA
81  K K V N H A F E A L R R C T S A N P S Q
301  CGT CTG CCA AAG GTG GAG ATC CTG CGC AAC GOC ATC CAT TAC ATT GAG AGT CTG CAG GAC
101  R L P K V E I L R N A I H Y I E S L Q D
361  CTG CTA CGA GAG CAG GTG GAA AAC TAC TAC TGC CTA OCT GGA GAG AGC AGC TCT GAG OCT
121  L L R E Q V E N Y Y C L P G E S S S E P
421  GGT AGC CCA CTG TCC AGC TGC TCT GAC GGC ATG GCT GAC AGC AAC AGT CCA GTG TGG CAA
141  G S P L S S C S D G M A D S N S P V W Q
481  CAT CTG AAT GCA AAC TAC AGC AAC AGA TAT TCA TAT GCG AAA AAT GAG AGT GTG GGC GAT
161  H L N A N Y S N R Y S Y A K N E S V G D
541  AAG ACA GCT GGA GOC TCT AGT CTG GAG TGT CTC TCC AGC ATC GTT GAT CGC TTG TCC TCG
181  K T A G A S S L E C L S S I V D R L S S
601  GTG GAG TCC AGC TGC GGA CCG GTG GCT CTG AGA GAC ATG GCC ACC TTC TCC OCT GGG AGC
201  V E S S C G P V A L R D M A T F S P G S
661  TCC GAC TCG CAG OCC TGC ACG CCG GAG AGC COC GGA TGC AGG OCC GTC TAC CAC GTC CTG TGA
221  S D S Q P C T P E S P G C R P V Y H V L *
AGGA AACCTTGCCTTTA ACGTGGCTATA TTGOCACAGTCAGGCGCCAGCTTTCACCA AACACCAGCTGCA TTTGCAACAAGAAGAGAT AAGA
ACTAGTT TTGTGCAAT TTT AAAAGAGTCTGAAT TTGAGGACCTGTGGCAAGTAGCTTT TTTGTACATGAGATTGTAAATA TGTGA TGT AATT
GCCCATT TAT TCTATACATGCT AT TAT ACTCAA TGAGACATA TTTAA TTA TGAGAGT ACA TG TAA TGT TGCATA ATTCCA ACATGAAA TGGT
ATTT AAGCAGTTT TCA TTC TTA CT TTCCATGTTGAA TTT CATGA CATTAAATCTTTT CACTGTT TTTGTGTAA AAAAAGAA AGA AAA AAA AA
AA

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An alignment of the amino acid sequence of several Myf5 homologs reveals that the largemouth bass Myf5 shared higher identity with fish Myf5 than other vertebrate species (Fig. 2). For example, largemouth bass Myf5 exhibits 93%, 92%, 92%, 82%, 76% and 75% identity with sea perch [21],

striped bass [19], flounder [20], rainbow trout [27], carp [28] and zebrafish [18]; and 56%, 56%, 57%, 57% and 57% identity with chicken [29], bovine [30], mice [31], humans [13], and the African clawed frog [32], respectively. The alignment results showed that Myf5 polypeptide sequences

Fig. 2 Comparison of the amino acid sequences and domains of the Myf5 protein among vertebrates. The sequence of the bHLH domain is underlined. The putative amino acid sequence of largemouth Myf5 (GenBank accession no. EU555403) is compared to the amino acid sequences of Stripped bass (*Morone saxatilis*, AF463525); Sea perch (*Lateolabrax japonicus*, DQ407725); Flounder (*Paralichthys olivaceus*, DQ872515); Zebrafish (*Danio rerio*, AF270789); Carp (*Cyprinus carpio*, AB012883); Atlantic salmon (*Salmo salar*, DQ452070); Rainbow trout (*Oncorhynchus mykiss*, AY751283); Fugu rubripes (*Takifugu rubripes*, AY445319); Pufferfish (*Tetraodon nigroviridis*, DQ453127); Human (*Homo sapiens*, X14894); Mouse (*Mus musculus*, NM_008656); Cow (*Bos taurus*, M95684); Chicken (*Gallus gallus*, X73250) and African clawed frog (*Xenopus laevis*, X56738). The alignment was generated using vector NTI 7.0 software. Identical and similar residues are shaded black and gray, respectively



of largemouth bass, sea perch and striped bass, which all belong to Perciformes, are highly conserved.

Identification of the recombinant plasmid pcDNA3.1(-)/mycHisB-Myf5 for the intramuscular injection

When the 6,156-bp constructed recombinant plasmid was double-digested with *Apa*I and *Xba*I, a 729 bp DNA fragment was obtained as expected. Sequencing results revealed that the ORF was complete and correct.

RT-PCR result to confirm transcription of the exogenous gene

The recombinant plasmid pcDNA3.1(-)/mycHisB-Myf5 and non-recombinant plasmid pcDNA3.1(-)/mycHisB were injected into trunk muscle of Nile Tilapia. RT-PCR was used to detect the transcription of the exogenous gene. The right side of the tilapia dorsal muscle injected with the pcDNA3.1(-)/mycHisB-Myf5 plasmid was selected as the experimental group, and the same position of the opposite side of the fish that was injected with the pcDNA3.1(-)/mycHisB plasmid was used as the control. On the third day post-injection, RT-PCR was used to examine the exogenous gene transcription with using the sense primer located in the largemouth bass *Myf5* ORF and the antisense primer located in the myc tag of the recombinant plasmid. The results demonstrated that the experimental side of the fish where the pcDNA3.1(-)/mycHisB-Myf5 plasmid had been injected showed *myf5mycHis* expression, while no transcription was detected in the control side (Fig. 3).

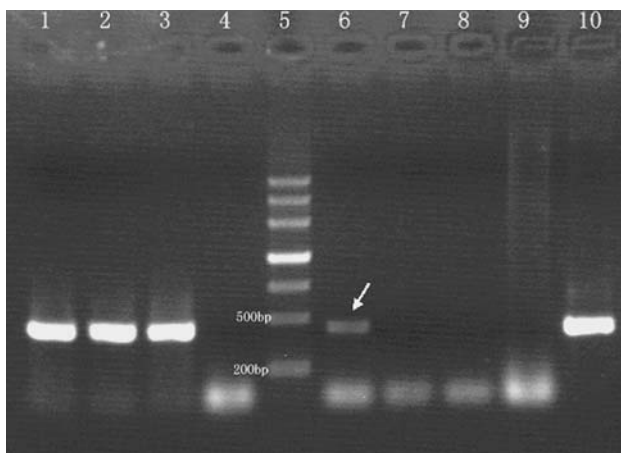


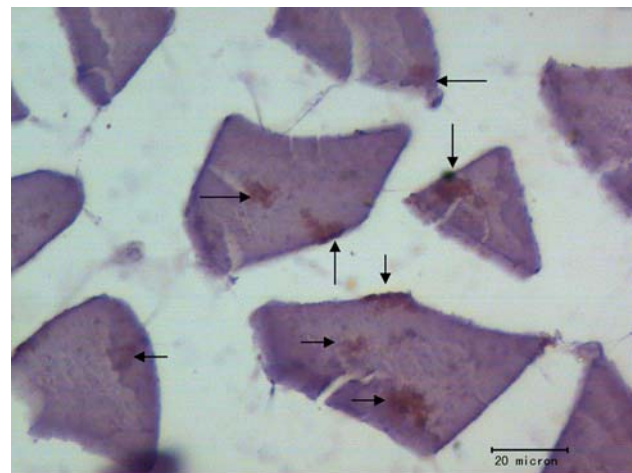
Fig. 3 RT-PCR to examine *myf5mycHis* transcription in vivo. Lane 1: beta-actin from the experimental side of the injected fish. Lane 2: beta-actin from the control side of the injected fish. Lane 3: beta-actin from non-injected fish. Lane 4: blank control. Lane 5: MarkerIII. Lane 6: *myf5-mycHis* from the experimental side of the injected fish. Lane 7: *myf5-mycHis* from the control side of the injected fish. Lane 8: *myf5-mycHis* from non-injected fish. Lane 9: blank control. Lane 10: the positive *myf5-mycHis* control (421 bp)

Immunohistochemistry results to confirm translation of the exogenous gene

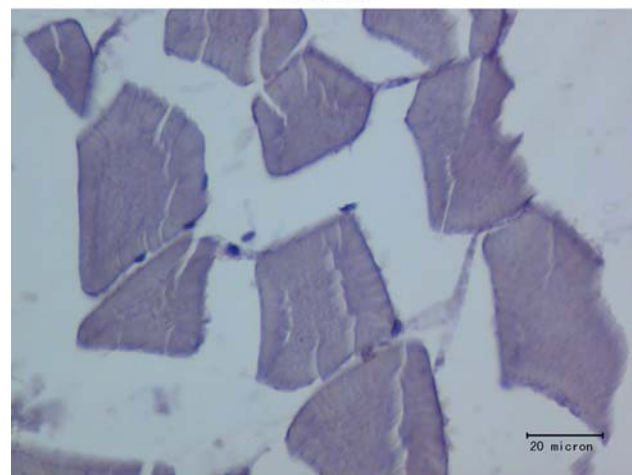
Expression of *myf5mycHis* was confirmed by immunohistochemical staining of the transfected muscle 7 days after injection. The Anti-His Tag mouse Monoclonal Antibody (BOSTER, China) was used as the first antibody and the Biotin-Goat anti-mouse IgG (BOSTER, China) was used as the second antibody. Immunohistochemical sections of tilapia muscle are shown in Fig. 4. The transfected muscle cells were strongly stained around the injection site in the experimental side, but not stained on the control side.

Evaluations of muscle fiber change

Sixty days post-injection, the muscle around the injection position from the three individual tilapias were fixed with formalin for 24 h followed by routine paraffin sectioning



a (40×10)



b (40×10)

Fig. 4 Detection of *myf5mycHis* expression in vivo by immunohistochemistry. (a) The positive sample, the fusion protein expressed has been stained as shown by the brown spots, as the arrows indicate and (b) the control sample

and hematoxylin/eosin staining (Fig. 5). The myofiber diameters of white and red muscle were evaluated respectively from the two sides of each tilapia (Table 1). The white muscle fiber diameters of fish 1, 2, and 3 increased 10% (5.2 μm), 9% (4.5 μm) and 7% (3.5 μm) respectively compared with the control side; the average increase was 9%. The size of the red fibers, however, had no significant changes ($P > 0.05$).

Discussions

Polypeptide structure analysis

The bHLH domain is present in some transcriptional regulators and is involved in DNA binding and protein dimerization. This domain of largemouth bass Myf5 is exactly identical to that of other vertebrates and exhibits

Fig. 5 Muscular paraffin sections of experimental and control injection sides in fish 1 (10 \times 10), and the percentage of myofiber cells with different diameters shown in the histogram

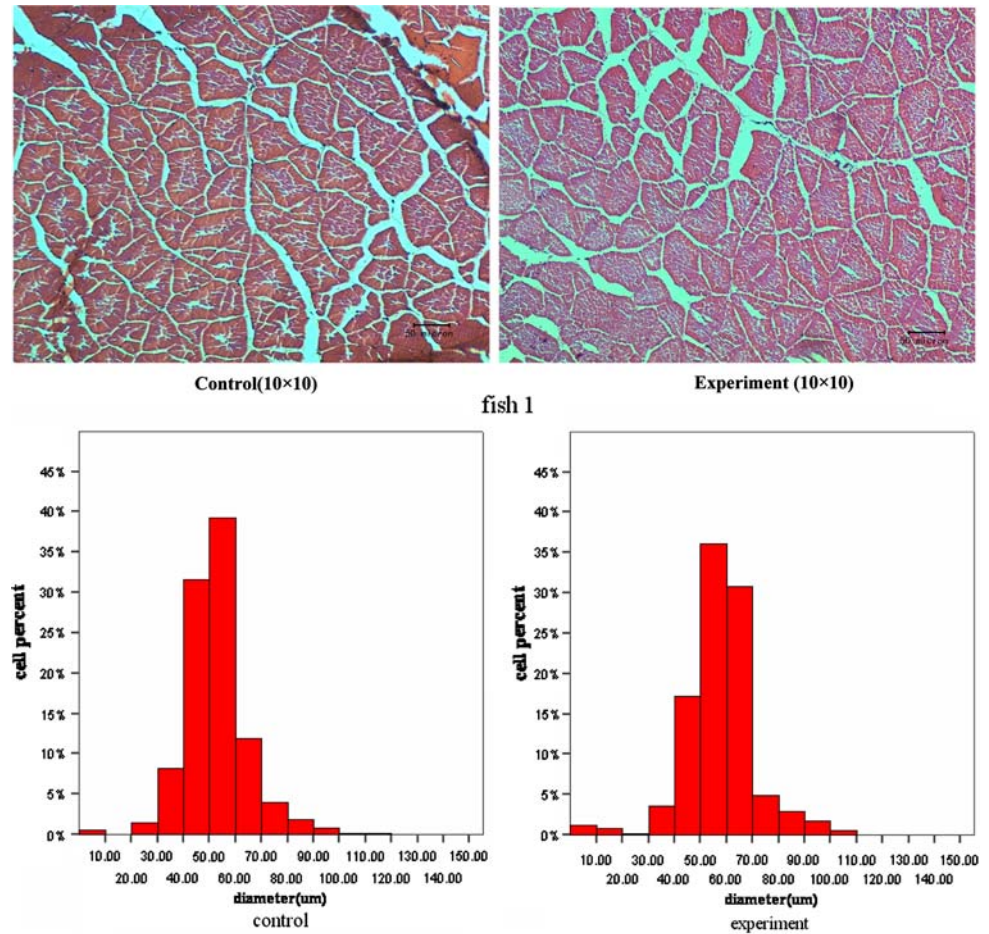


Table 1 Myofiber diameter statistical analysis on day 60 post-injection

	White muscle fiber diameter (μm)		Red muscle fiber diameter (μm)	
	Experiment	Control	Experiment	Control
Fish 1	57.5 \pm 18.6 <i>N</i> = 512	52.7 \pm 12.1 <i>N</i> = 528	39.7 \pm 11.3 <i>N</i> = 264	38.5 \pm 8.0 <i>N</i> = 232
		$P < 0.01$		$P > 0.05$
Fish 2	54.1 \pm 14.8 <i>N</i> = 574	49.6 \pm 12.3 <i>N</i> = 589	29.6 \pm 8.3 <i>N</i> = 236	30.0 \pm 8.4 <i>N</i> = 241
		$P < 0.01$		$P > 0.05$
Fish 3	56.7 \pm 16.4 <i>N</i> = 551	53.2 \pm 17.1 <i>N</i> = 557	35.2 \pm 9.2 <i>N</i> = 157	36.8 \pm 8.7 <i>N</i> = 163
		$P < 0.01$		$P > 0.05$

99%, 97%, 93%, 84% and 84% identity with sea perch, striped bass, zebrafish, mice and humans, suggesting that largemouth bass *Myf5* probably acts as a transcriptional factor to regulate muscle-specific genes, as its homologs do in other species.

The effect of exogenous *Myf5* on fish muscle fiber size and density

Considered with the difficulty in the culture of fish muscle cells and satellite cells, we tried the intramuscular injection of plasmid pcDNA3.1(-)/mycHisB-*Myf5* to study the effect of *Myf5* on fish muscle growth rather than the transient transfection of foreign gene *Myf5* into muscle cells. This strategy was demonstrated available by the RT-PCR and immunohistochemistry results.

Fish postembryonic muscle growth is different from mammals. In mammals, the number of muscle fibers is determined at birth, and muscle fibers only increase in diameter after birth [33]. The muscle fibers absorb additional nuclei derived from the proliferation of so-called satellite cells located between the basal lamina and sarcolemma [34]. In fish, however, postnatal muscle growth is characterized by continuous myofiber hyperplasia and hypertrophy [24]. Fish myogenic progenitor cells (MPCs), responsible for the postembryonic growth in teleosts, are analogous to satellite cells in mammals, but not exactly the same as they are not always located beneath the basal lamina of muscle fibers [35, 36]. The model describing the main events of myogenesis in teleost skeletal muscle is that the pluripotent stem cells are specified to become myogenic progenitor cells (MPCs), and MPC progeny undergo a proliferation phase, and ultimately differentiate into myoblasts which have two cellular fates [25]. First, myoblasts can fuse into short myotubes that can be extended through the absorption of additional myoblasts to form new myofibers. Second, if the myoblasts were absorbed directly by existing fibers, the fiber-myoblast fusion can result in hypertrophy of the fibers [25]. In our experiment, the effect of *Myf5* overexpression mainly focused on muscle fiber hypertrophy and the myofiber diameter was found to increase by 9% on average. Myofiber density, however, did not increase as expected and instead, all decreased (data not shown), suggesting that *Myf5* may be responsible for the hypertrophy of muscle fiber predominantly during the post-larval period of fish. There is a restriction to the methodology used, however, in that an increase in the cross-section area of myofibers can result in a decrease in the number of myofibers fixed in a microscope field. The effect of *myf5* on the process of muscle hyperplasia during the fish postnatal period needs to be further investigated. In our experiment, no significant changes were found for the red fibers. A determination for whether or not MPCs in red

muscle have a different signal regulating pathway during fish postnatal muscle growth is worthy of further study.

In any case, it is likely that overexpression of exogenous *Myf5* activated the differentiation of quiescent MPCs into myoblasts, myoblasts fused into the adjacent fibers and ultimately might lead to the hypertrophy of the existed fiber. In conclusion, this study indicated that the exogenous *Myf5* probably promoted myogenesis during the postembryonic muscle growth period in fish.

Acknowledgments The authors wish to thank Dr. Yongjiang Ma and postgraduate Fengmei Wei from the South China Agriculture University for helping with the evaluation of muscle fiber diameter and density.

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