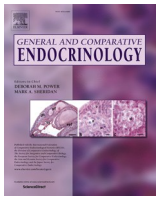
General and Comparative Endocrinology 347 (2024) 114426

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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



FGFs function in regulating myoblasts differentiation in spotted sea bass (*Lateolabrax maculatus*)

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ARTICLE INFO

Keywords:
FGFs
Myoblasts
Differentiation
Spotted sea bass

ABSTRACT

Fibroblast growth factors (FGFs) are a family of structurally related peptides that regulate processes such as cell proliferation, differentiation, and damage repair. In our previous study, fibroblast growth factor receptor 4 (*fgfr4*) was detected in the most significant quantitative trait loci (QTL), when identified of QTLs and genetic markers for growth-related traits in spotted sea bass. However, knowledge of the function of *fgfr4* is lacking, even the legends to activate the receptor is unknown in fish. To remedy this problem, in the present study, a total of 33 *fgfs* were identified from the genomic and transcriptomic databases of spotted sea bass, of which 10 were expressed in the myoblasts. According to the expression pattern during myoblasts proliferation and differentiation, *fgf6a*, *fgf6b* and *fgf18* were selected for further prokaryotic expression and purification. The recombinant proteins FGF6a, FGF6b and FGF18 were found to inhibit myoblast differentiation. Overall, our results provide a theoretical basis for the molecular mechanisms of growth regulation in economic fish such as spotted sea bass.

1. Introduction

As one of the most important traits in aquaculture, the growth performance of fish is always considered to be the primary trait in selective breeding. The development of skeletal muscle, the largest tissue in fish, is considered as the decisive factor for the growth of the fish (Zimmerman and Lowery, 1999). In mammal, skeletal muscle, consists of multinucleated myofibrils (Abe et al., 2004), begin to proliferate from muscle precursor cells, at the stage of myoblasts, and subsequently differentiate and fuse to form myotubes (Baj et al., 2005; Demonbreun et al., 2011). In other words, myoblast proliferation and differentiation are the two main components of myogenesis and skeletal muscle development (Chargé and Rudnicki, 2004). Myoblasts are mononuclear cells with a strong capacity to proliferate and differentiate (Bornemann et al., 1999), and play important roles in the growth and development of skeletal muscle, as well as regeneration and repair of damage (Adams, 2006).

Teleost muscle originates in the early embryo, with some myogenic precursor cells (MPCs) present in the somatic and external cell layers of the embryo (Johnston et al., 2011). In zebrafish, *myod* and *myf5* expression is evident prior to the completion of gastrulation (Coutelle et al., 2001). Myogenesis involves proliferation, migration, fusion and terminal differentiation of myoblasts (Griffin et al., 2010). A continuous skeletal muscle cell lineage was established in the muscle of juvenile rockfish, and a certain number of multinucleated myotubes were observed after induced differentiation (Kong et al., 2021). Meanwhile, the development and regeneration of skeletal muscle is controlled by a complex regulatory process involving a variety of growth factors (Rescan, 2008). Spotted sea bass, an important commercial fish in China, with around 200,000 tons production each year. However, the lack of selective breeding of spotted sea bass leads to the degeneration of genetic characteristics, such as the decline in the growth rate and decreased disease-resistant ability (Zhang et al., 2023a; Zhang et al., 2023b). To provide a useful framework for determining the genetic basis of growth traits in spotted sea bass, in our previous study we constructed a high-density linkage map with 6883 SNP markers spanning 2189.96 cM. For body weight and body length QTL mapping, 24 significant QTLs, including 318 SNPs and 30 candidate genes were identified (Liu et al., 2020). Among these candidate genes, fibroblast growth factor receptor 4 (*fgfr4*) was detected in the most significant quantitative trait loci (QTL).

Fibroblast growth factors (FGFs) were discovered in 1974 (Gospodarowicz, 1974) and can be classified as paracrine, endocrine or

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https://doi.org/10.1016/j.ygcen.2023.114426
Received 19 October 2023; Received in revised form 11 December 2023; Accepted 11 December 2023
Available online 14 December 2023

autocrine according to their mode of action (O'Neill, 2008). Except for the autocrine type, FGFs exert their biological effects by binding to the specific receptors FGFRs (Luo et al., 2006). In skeleton muscle, the main receptor expressed is *fgfr4* (Cool et al., n.d.; Kästner et al., 2000; Olwin et al., 1994), while the main ligand is *fgf6* (Armand et al., 2006, 2003; Coulier et al., 1994). Irene Marics et al. have shown that *fgfr4* is the only receptor that is highly expressed during chicken muscle differentiation, and inhibition of *fgfr4* signaling can lead to the stagnation of muscle progenitor cell differentiation (Marics et al., 2002). Transcriptomic analysis of mouse satellite cells at different ages showed that *fgfr4* was a key gene in satellite cell differentiation, and functional analysis showed that *fgfr4* promoted satellite cell differentiation, while *tgfb2*, *wnt9a* and *fgfr4* synergistic effect weakened senescence satellite cell differentiation during skeletal muscle development (Zhang et al., 2018). These results suggest that FGFR4 plays an important role in myogenic differentiation. In mice, *fgf6* is effective for muscle regeneration by activating and stimulating the proliferation of satellite cells (Floss et al., 1997). By knocking out *fgf6*, satellite cells in mice no longer proliferate and their ability to regenerate after injury is diminished (Zammit et al., 2006). In contrast, the differentiation of myogenic cells was inhibited in C2C12 cells overexpressing *fgf6*, and the expression of *myog*, a marker gene for myogenic differentiation, was decreased (Israeli et al., 2004). It has also been shown that using the exogenous protein FGF6 delivered to growing skeletal muscle, an increase in the number of satellite cells in the injected muscle was found, suggesting that FGF6 is essential for satellite cell growth (Zofkie et al., 2021). Studies on different developmental stages of grass carp showed that the expressions of *fgf6a* and *fgf6b* were positively correlated with muscle fiber diameter and negatively correlated with muscle fiber density, indicating that they play an important role in the regulation of muscle growth of grass carp (Xu et al., 2019). *fgf18* was identified as an important growth-related regulator in genome-wide association studies of growth and body-related traits in large yellow croaker (Zhou et al., 2019). In addition, *fgf18* is required for early chondrocyte proliferation and has been shown to regulate chondrogenesis for bone growth and development (Liu et al., 2007).

In the present study, to identify the *fgfr4* locus, we used bioinformatics to identify families of FGF genes and analyzed the expression of FGFs during differentiation of myoblasts. Based on the results, three recombinant FGFs proteins were purified to further elucidate their role in the differentiation of skeletal muscle myocytes. Our findings link growth traits to QTL results for *fgfr4*, which may be involved in the regulation of skeletal muscle formation, and provide a theoretical basis for molecular mechanisms of growth regulation in the spotted sea bass and other cnidarians.

2. Materials and methods

2.1. Ethics statement

All experimental animals were purchased from Lijin Shuangying Aquatic Seed Co., LTD., (Dongying, China). All animal procedures used in this study were approved by the Ethics Committee for Animal Experiments of Ocean University of China and were performed in accordance with the Guidelines for Animal Experimentation of Ocean University of China. After anesthetized with 100 ng/mL 3-aminobenzoate methanesulfonic acid (MS-222) to minimize misery, experimental individuals were dissected and processed. The field studies did not concern endangered or protected species.

2.2. Cell culture and induced differentiation

52 spotted sea bass (wet weighing 28.46–31.35 g, about 200 days old) were used in the present study. The experiment individuals were disinfected with 75 % ethanol after anesthetization. Skeletal muscle tissues were sampled with adipose tissues removed and cut into small pieces (approximately 1 mm^3). The muscle tissues were cultured into a cell culture dish (175 cm^2) with L-15 growth medium (GM, G-CLONE, Beijing, China) which mixed 20 % fetal bovine serum (FBS, ABSIN, Shanghai, China), 1 % 100 U/mL penicillin and 100 μg/mL streptomycin (ABSIN, Shanghai, China) under a 25℃, CO_2-free incubator (Jinghong, Shanghai, China). Every 3–4 days, the whole medium was replaced with fresh medium until cell emigration and passaging. When grew to approximately 70 % abundance, the primary cells were isolated with 0.25 % trypsin (Servicebio, Wuhan, China). The isolated cell suspension was precultured in the incubator for 2 h to remove the fibroblasts.

To induce cell differentiation, culture medium was replaced with differentiation medium (DM) with 2 % horse serum (G-CLONE, China) and 1 % penicillin–streptomycin-gentamicin solution when the myoblasts reached 70 %-80 % confluence. To quantify the efficiency of myofibroblast fusion, myofibroblasts were fixed in 4 % paraformaldehyde for 10 min on day 4 of induced differentiation, stained with DAPI (Biosharp, China) for 3–5 min and then the DAPI staining solution was aspirated, and washed with PBS 2–3 times for 3–5 min each time, and the nuclei of the cells were observed under the fluorescence microscope.

Table 1
Primers sequences used for QRT-PCR.

Primers	Sequence (5′-3′)	Size (bp)	Amplification efficiency
α-tubulin-F	AGGTCTCCACAGCAGTAGTAGAGC	89 bp	99 %
α-tubulin-R	GTCCACCATGAAGGCACAGTCG		
ki67-F	CAGTGAGGCAGTCCAACGCTTC	136 bp	96 %
ki67-R	GGGAGTTGTTACAGTGGTCGTCTTC		
myog-F	TCCATCCAGCCTGTCACCTCAC	144 bp	101 %
myog-R	ACCTTCTTCAGACGCCTCTTCTCC		

2.3. RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from all cultured cell samples descripted before via TRIzol reagent (Vazyme, China) according to the reagent manual. RNA quantity and purity were evaluated by a Biodropsis BD-1000 nucleic acid analyser (OSTC, China) and electrophoresis using a 1 % agarose gel. The total RNA was reverse transcribed into cDNA using 1 μg of DNA per 20 μL reaction via the Prime ScriptTM RT reagent kit (TaKaRa, Japan) according to the reagent manual.

For quantitative real-time PCR (qPCR) analysis, cyclic amplification was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, China) and genes specific primers (Table 1). qPCR was performed using the Applied Biosystems StepOneplus real-time quantitative fluorescent PCR system with three biological replicates and three independent technical replicates per sample. Spotted sea bass alpha-Tubulin was used as reference gene and the $2^{-\Delta\Delta CT}$ algorithm was used to estimate the relative expression level of each gene.

2.4. Gene family identification and phylogenetic analysis

The *fgf* gene sequences were obtained from the Ensembl and NCBI databases for several representative vertebrates, including: human (*Homo sapiens*), mouse (*Mus musculus*), nile tilapia (*Oreochromis niloticus*), large yellow croaker (*Larimichthys crocea*) and zebrafish (*Danio rerio*). Using their amino acid sequences as a reference, the genome (PRJNA408177) and RNA-Seq (SRR4409341, SRR4409397) databases of spotted sea bass were searched by TBLASTN (E = 1e-5) to obtain preliminary candidate *fgf* genes of spotted sea bass. ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) was used to predict open reading frames and translate amino acid sequences. The relative molecular weights (MW, kDa) and isoelectric points (pI) of FGFs proteins were predicted by ExPASyPort-Param. Copy numbers of the corresponding *fgf* genes were determined from the genomic databases of

Table 2
Primers sequences used for orf cloning and plasmid construction.

Primers	Sequence (5′-3′)
Primer for ORF cloing	
fgf6a-F	ATGGCCGTTGCGCAAAGG
fgf6a-R	TCATATTCGGGGTAGGAAGTGCG
fgf6b-F	ATGGCCATTGCGCAAAGG
fgf6b-R	TCATAGTCGTGGGAGGAAATGTGT
fgf18- F	ACGCTGACCGTCTTATGT
fgf18-R	AGGGAGGTGTTCAGTCGTGT
Primer for plasmid construction	
PE-*fgf6a*-FPE-*fgf6a*-F	tcacagagaacagattggtggaTACCCGCTGCCGAGCGGC
PE-*fgf6a*-R	agtggtggtggtggtggtcttaTATTCGGGGTAGGAAGTGCGTTAC
PE-*fgf6b*-F	tcacagagaacagattggtggaTATCCGATTCCGAGCAGGAC
PE-*fgf6b*-R	agtggtggtggtggtggtcttaTAGTCGTGGGAGGAAATGTG
PE-*fgf18*-F	tcacagagaacagattggtggaGTCAACTTCAGCGTGCATGTGG
PE-*fgf18*-R	agtggtggtggtggtggtcttaCTAGCGGGGCCCAGTAGC

spotted sea bass and several other representative vertebrates and compared.

According to the FGF amino acid sequences of several species, ClustalX 2.1 and ESPript 3.0 (https://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) were used to perform multiple alignments of the amino acid sequences. A phylogenetic tree was reconstructed from the multiple alignments of the amino acid sequences with Neighbor-joining (NJ) and Jones-taylor-thornton (JTT) methods using MEGA-X. The values on the trees represent bootstrap scores of 1,000 iterations, indicating the credibility of each branch.

2.5. Gene cloning and sequence analysis

The gene cloning primers for *fgfs* were designed according to the gene sequence identified from the genome and transcriptomic databases of spotted sea bass (Table 2). ORF sequence was amplified by 2 × Phanta Max Master Mix (Dye Plus) (Vazyme, China) using muscle tissue cDNA as template. PCR products were extracted and purified by Gel DNA extraction kit (Vazyme, China). The products were constructed into pCE2 TA/Blunt Zero vector (Vazyme, China) by TOPO method, and the sequence accuracy was verified by sequencing.

2.6. Expression and purification of recombinant proteins

To amplify the coding sequences of FGF6a, FGF6b, and FGF18, homologous primers (Table 2) were designed and the amplified fragments were inserted into the pET-28a-SUMO expression vector. The constructed plasmids were then transformed into *E. coli* BL21 (Ang yu, China). Positive clones of E. coli were incubated in Luria Broth (LB) medium containing glucose (2 g/L, Sigma-Aldrich), ampicillin (100 mg/L, Sigma-Aldrich, St. Louis, MO, USA) and chloramphenicol (34 mg/L, Sangon Biotech, Shanghai, China) at 37 °C.

When the OD600 value reached 0.4 to 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma-Aldrich) was added to a final concentration of 0.4 mM and the temperature was lowered to 16 °C. After 16 h of induction, bacterial cells were collected by centrifugation at 3,500 g for 10 min at 4 °C. Lysis buffer (500 mM NaCl, 20 mM phosphate buffer, 10 mM imidazole, pH 7.4) was used to resuspend the cells and ultrasonic lysis was performed on ice. After lysis, the supernatant was collected by centrifugation at 12,000 g for 10 min at 4 °C and filtered through 0.22 μm filter. The recombinant proteins in the supernatant were purified on the TED-Ni column (Beyotime Biotechnology, Shanghai, China), and their concentrations were determined by the BCA kit (Beyotime Biotechnology, Shanghai, China) after dialysis in ice bath, and the correct protein expression was verified by SDS-PAGE electrophoresis. The purified FGF6a, FGF6b, and FGF18 were snap-frozen in liquid nitrogen and stored in batches at −80 °C.

Table 3
Characteristics of *fgf* genes in spotted sea bass.

Subfamily classification	Gene name	Chromosome number	ORF length (bp)	Amino acid length (aa)	Molecular weight (kDa)	Isoelectric point (pI)
FGF1	*fgf1a*	chr14	450	149	17.04	8.52
	fgf1b	chr12	609	202	22.32	8.57
	fgf2	chr14	468	155	17.19	9.73
FGF4	*fgf4a*	chr1	618	205	22.94	9.22
	fgf4b	chr14	567	188	20.97	10.42
	fgf5	chr8	687	228	25.87	10.52
	fgf6a	chr6	627	208	23.12	9.28
	fgf6b	chr22	624	207	23.50	9.85
FGF7	*fgf3*	chr1	726	241	27.37	10.77
	fgf7	chr1	609	202	23.66	9.27
	fgf10a	chr17	975	324	35.57	10.05
	fgf10b	chr8	756	251	27.54	10.96
	fgf22	chr2	672	223	24.46	10.12
FGF8	*fgf8a*	chr13	642	213	25.10	10.55
	fgf8b	chr3	609	202	23.35	10.92
	fgf17	chr8	645	214	25.02	10.65
	fgf18	chr14	591	196	22.51	10.69
	fgf24	chr14	576	191	22.35	10.06
FGF9	*fgf16*	chr14	654	217	24.64	9.04
	fgf20a	chr3	684	227	25.11	8.69
	fgf20b	chr14	633	210	23.29	7.86
FGF11	*fgf11a*	chr23	750	249	28.21	9.97
	fgf11b	chr10	483	160	27.37	9.98
	fgf12a	chr12	780	259	29.01	9.75
	fgf12b	chr5	552	183	20.23	9.65
	fgf13a	chr14	768	255	28.46	8.85
	fgf13b	chr12	696	231	26.08	10.18
	fgf14a	chr11	630	209	23.23	9.36
	fgf14b	chr24	603	200	22.05	10.28
FGF19	*fgf19*	chr1	636	211	23.17	7.09
	fgf21	chr21	621	206	23.18	6.78
	fgf23a	chr22	792	263	29.42	5.73
	fgf23b	chr6	747	248	27.96	7.78

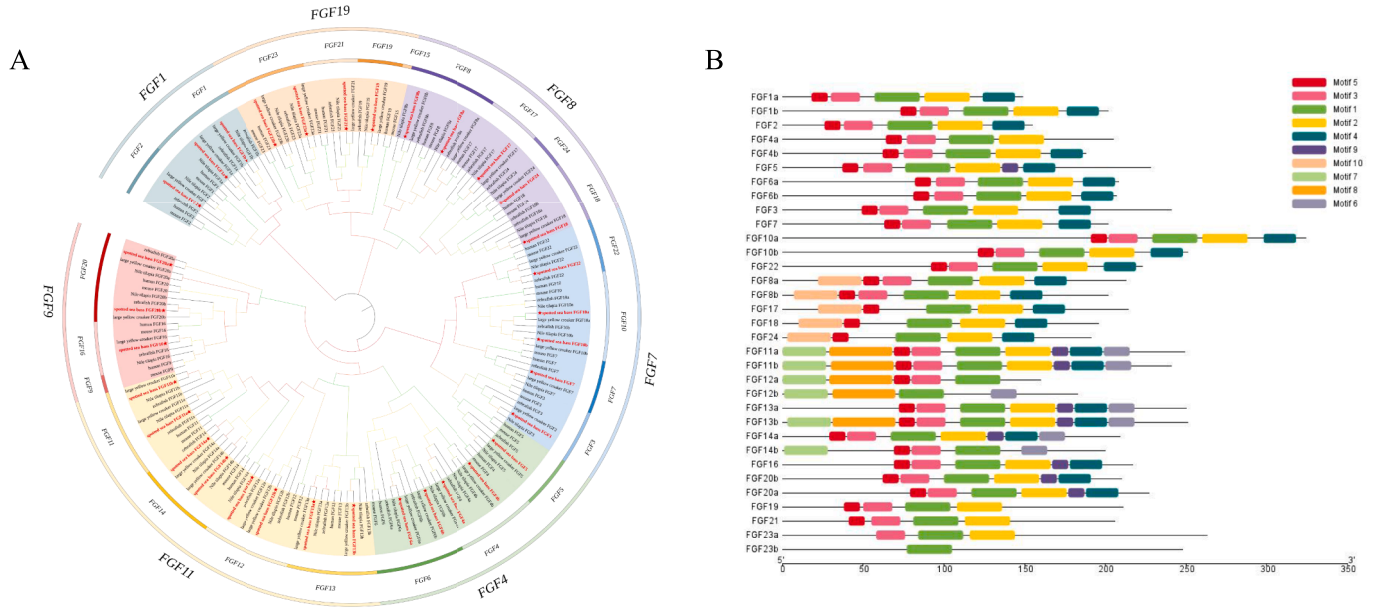

Fig. 1. (A) Phylogenetic relationships of *fgf* genes in spotted sea bass and selected species, including human (*Homo sapiens*), mouse (*Mus musculus*), Nile tilapia (*Oreochromis niloticus*), large yellow croaker (*Larimichthys crocea*) and zebrafish (*Danio rerio*). The phylogenetic tree constructed using MEGA X software based on the Neighbor-Joining method and Jones-Taylor-Thornton (JTT) model with 1000 replicates The *fgf* gene of spotted sea bass was marked with bold red font and "★".The *fgf* genes identified were labelled with different colors in the outer circle; The different colors of the outer circle mark seven different subfamilies; Branches of different colors in the circle are used to represent the size of self-development value. (B) Motif analysis of FGF family in spotted sea bass. Different motifs are represented by different box colors. The bits indicate amino acid conservation in each position. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.7. Western blot

Western blotting was used to recognize the recombinant proteins containing 6 × His epitope tag and ensure the successful expression and purification. Each protein sample was separated on 15 % acrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Germany). The membranes were blocked in TBS/0.5 % Tween (TBST) containing 5 % skim milk and then incubated overnight at 4 °C with monoclonal rabbit His-tagged antibody (1:1000, Cell signaling technology, USA). After washing 3 times for 10 min in TBST (Tris Buffered Saline with Tween 20) buffer, the PVDF membrane was further incubated for 2 h with diluted in polyclonal goat anti-rabbit IgG (1:5000, Beyotime, China) as secondary antibody. Target proteins were visualized using the BeyoECL Plus (Beyotime, China).

2.8. Statistical analysis

All statistical procedures were performed using SPSS 21.0 software

Table 4
Copy numbers of *fgf* genes in several representative vertebrates.

Subfamily classification	*fgf* Genes	Human	Mouse	Zebrafish	Large yellow croaker	Tilapia	Spotted sea bass
FGF1	*fgf1*	1	1	2	2	2	2
	fgf2	1	1	1	1	1	1
FGF4	*fgf4*	1	1	1	2	2	2
	fgf5	1	1	1	1	1	1
	fgf6	1	1	2	2	2	2
FGF7	*fgf3*	1	1	1	1	1	1
	fgf7	1	1	1	1	1	1
	fgf10	1	1	2	2	2	2
	fgf22	1	1	1	1	1	1
FGF8	*fgf8*	1	1	2	2	2	2
	fgf17	1	1	1	1	1	1
	fgf18	1	1	2	1	1	1
	fgf24	0	0	1	1	1	1
FGF9	*fgf9*	1	1	0	0	0	0
	fgf16	1	1	1	1	1	1
	fgf20	1	1	2	2	2	2
FGF11	*fgf11*	1	1	2	2	2	2
	fgf12	1	1	2	2	2	2
	fgf13	1	1	2	2	2	2
	fgf14	1	1	1	2	2	2
FGF19	*fgf15*	0	1	0	0	0	0
	fgf19	1	0	1	1	1	1
	fgf21	1	1	1	1	1	1
	fgf23	1	1	1	2	2	2
	Total	22	22	31	33	33	33

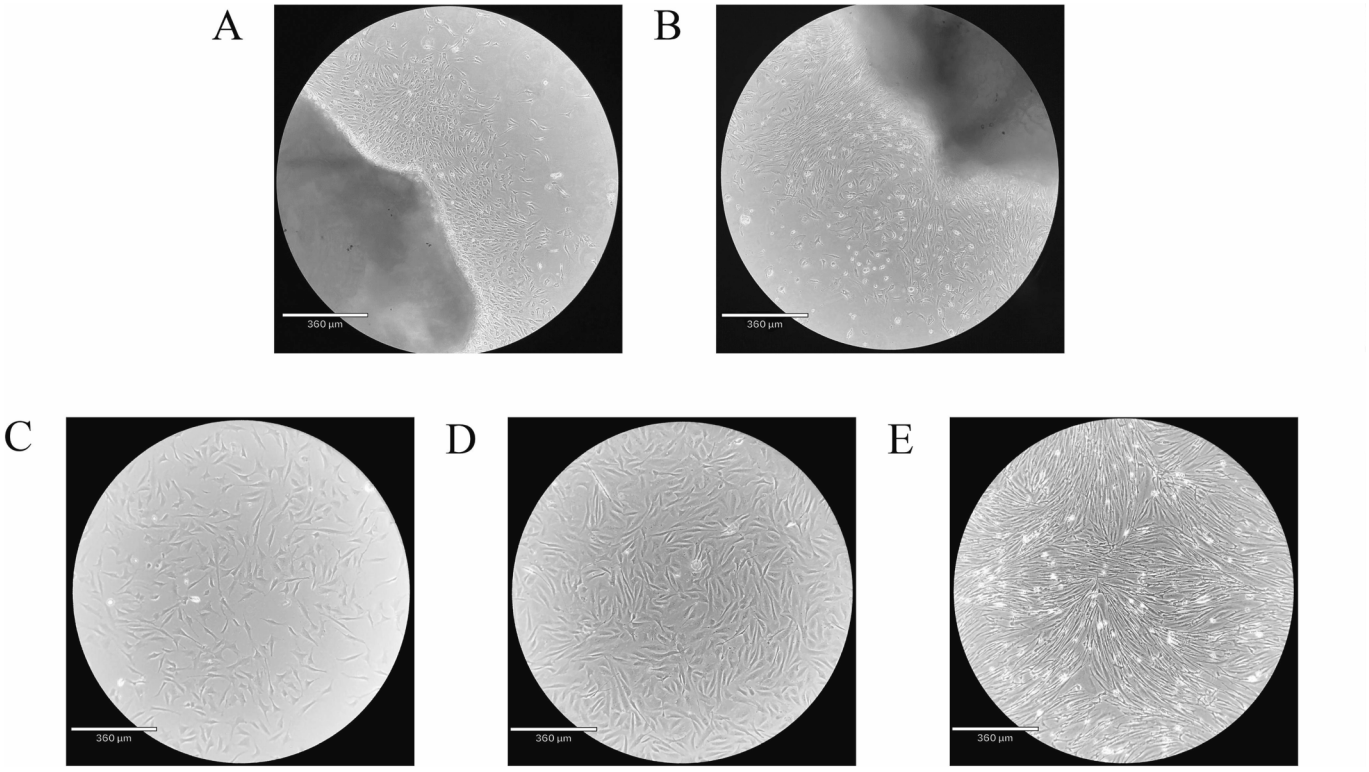

Fig. 2. Isolation, culture and induced differentiation of myoblasts from spotted sea bass. (A) Myoblasts transplanted on day 9. (B) Myoblasts transplanted on day 12. (C) Morphology of P1 generation myoblasts cultured in vitro for 0 day. (D) Morphology of P1 generation myoblasts cultured in vitro for 2 days. (E) Morphology of P1 generation myoblasts after induced differentiation for 4 days in vitro.

and data were expressed as mean ± standard error of the mean (SEM). Data were analysed using one-way ANOVA followed by LSD and Dunnett T3 multiple range tests, with differences considered significant when $P < 0.05$. Statistical analyses were performed with reference to previous reports (Davis et al., 2010; Du Toit et al., 2018).

3. Results

3.1. Identification of the FGF gene family in spotted sea bass

A total of 33 *fgf* genes, identified from the genomic and transcriptomic database of spotted sea bass, were divided into seven subfamilies, named FGF1, FGF4, FGF7, FGF8, FGF9, FGF11 and FGF19. The members in each subfamily were shown in Table 3, with *fgf9* and *fgf15* missing in spotted sea bass. T weight and isoelectric points of the predicted amino acid range from 17.19 kDa to 35.57 kDa and 5.73 to 10.96, respectively. To further determine the phylogenetic relationships of the FGF family in spotted sea bass, a phylogenetic tree was also constructed (accession number shown in **Table. S1**). The phylogenetic analyses results showed that the *fgf* of spotted sea bass clustered with their corresponding homologues, and all *fgf* genes were divided into seven subfamilies, which was consistent with the identification results (Fig. 1**A**). The phylogenetic tree confirmed the identification of the *fgf* and provided a basis for the identification and annotation of the *fgf* of spotted sea bass.

Fgf copy numbers in representative higher vertebrates were presented in Table 4. Results showed that the copy numbers ranged from 22 to 33 with double copies of *fgf* in teleost. Notably, *fgf24* was only present in teleost compared with *fgf9* which was only existed in mammals. In addition, *fgf19* and *fgf15* were missing in mice and humans, respectively.

Structural domain analysis of the FGF family was also performed (Fig. 1**B**). Results showed that similar motifs were found for the same subfamily of FGFs. Motif 1 was present in all 33 FGFs. Whereas motif 2, motif 3, and motif 5 were present in all FGFs except FGF23 which may have lost function due to the deletion of some motifs. In addition, motif6, motif 7, and motif 8 were specific to the FGF11 subfamily and motif 10 was specific to the FGF8 subfamily. These different motifs divided the 33 FGFs into different subfamilies.

3.2. In vitro culture of spotted sea bass myoblasts showed proliferation and differentiation capacity

The isolated cultured spotted sea bass myoblasts and the induced differentiation stages was shown in Fig. 2. To identify the different developmental stage of myoblasts, cells samples after 72 h of proliferation and 96 h of differentiation were collected (Fig. 3**A**). mRNA level of *ki67*, a marker gene for cell proliferation, showed downregulated after differentiation ($P < 0.05$). mRNA level of *myog*, a marker gene for cell differentiation, upregulated significantly after differentiation ($P < 0.05$, Fig. 3**B**). The differentiation ability of myoblasts was demonstrated by differentiation medium with 2 % horse serum. Results showed a large amount of multinucleated myotubes stained by DAPI were observed after 96 h differentiation (Fig. 3**C**), indicated the isolated myoblasts have a good capacity for myogenic differentiation in spotted sea bass.

3.3. Expression patterns of the FGF family in myoblast proliferation and differentiation in spotted sea bass

Based on transcriptomic data (RNA-seq data, accession number: PRJNA859992) (Zhang et al., 2022), 15 *fgf* genes and certain paralogues were found expressed during myoblasts proliferation and differentiation in spotted sea bass. The expression levels of *fgf1*, *fgf2*, *fgf6a*, *fgf6b*, *fgf7*, and *fgf18* were differentially expressed between these two stages (Fig. 4). Among them, *fgf6a*, *fgf6b* and *fgf18* are significantly increased in the differentiation phase. Therefore, *fgf6a*, *fgf6b* and *fgf18* will be selected for the study below.

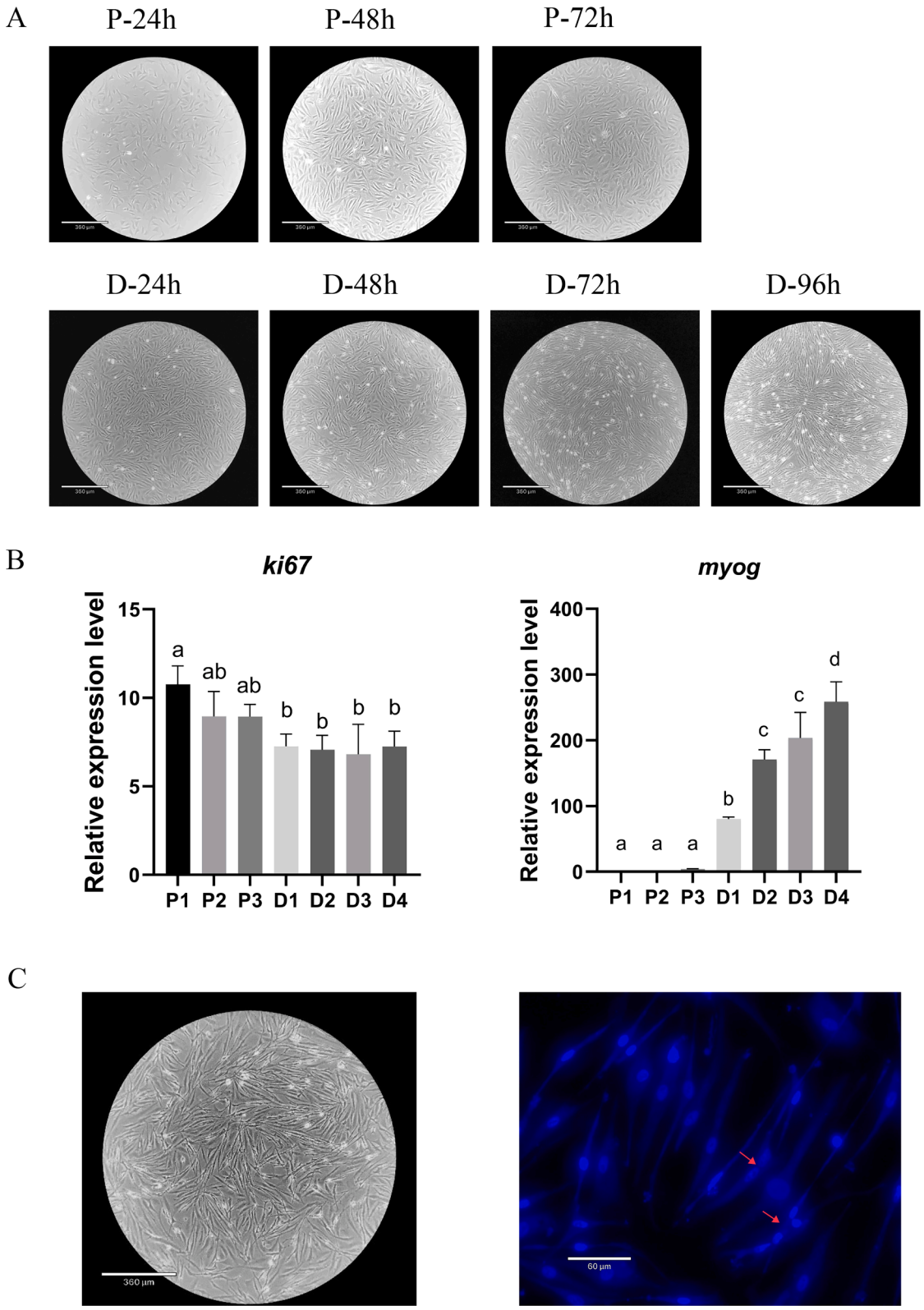

Fig. 3. Identification of myoblast development stages in spotted sea bass. (A) The state of cultured myoblasts of spotted sea bass at different stages, including "P": cell proliferation stage: 24 h、48 h、72 h; "D": cell differentiation stage: 24 h、48 h、72 h、96 h. (B) Expression of marker genes at different developmental stages in spotted sea bass myoblasts, including *ki67*, cell proliferation stage marker genes and *myog*, cell differentiation stage marker genes. The data are shown as the mean ± S.E.M. (n = 3), and different letters indicate significant differences ($P < 0.05$). (C) Identification of differentiation ability of myoblasts in spotted sea bass. Light microscopic picture of myoblasts of spotted sea bass after induced differentiation for 72 h; DAPI nuclear staining picture of myoblasts of spotted sea bass after induced differentiation for 72 h.

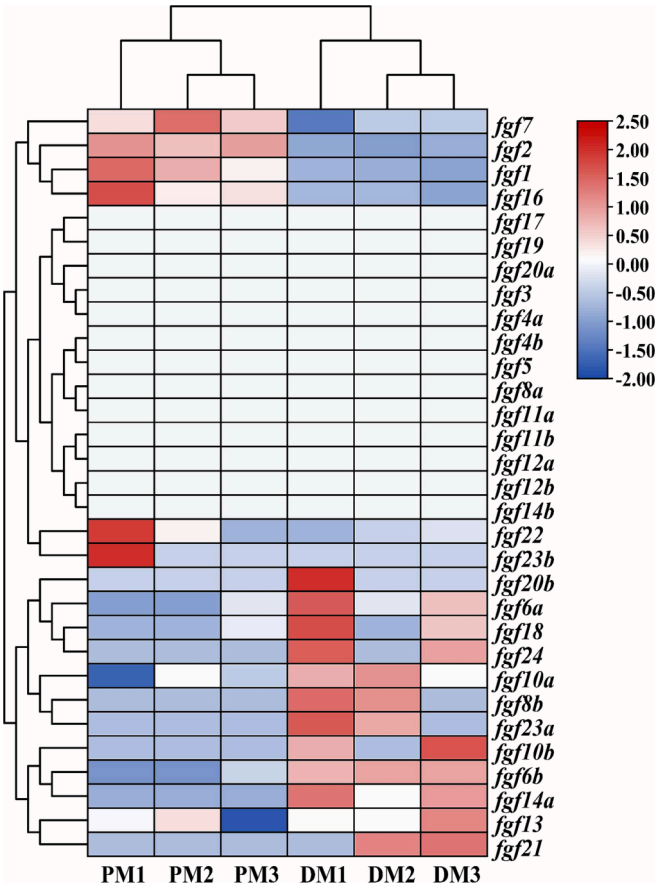

Fig. 4. The expression of FGF family members of spotted sea bass at two time points of skeletal muscle cell proliferation and differentiation. The three samples at proliferation stage were represented as PM1, PM2 and PM3, the three samples at the differentiation stage were represented as DM1, DM2 and DM3. Data for the relative expression levels of genes are obtained by RNA-seq after taking $\log_2$ (FPKM + 1). The color scale represents expression levels, and red indicate higher expression while blue indicate lower expression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Chronological expression of fgf6a, fgf6b and fgf18 during proliferation and differentiation of myoblasts in spotted sea bass

To confirm that *fgf6a*, *fgf6b* and *fgf18* were involved in the process of myoblasts proliferation and differentiation, mRNA levels of these genes were tested from the previous in vitro cultured myoblasts (Fig. 5). As shown in the results, the relative expression of *fgf6a*, *fgf6b* and *fgf18* showed no significant different in the proliferation phase, whereas increased significantly in the differentiation phase ($P < 0.05$).

3.5. FGF6a, FGF6b and FGF18 inhibited the differentiation process of myoblasts

The ORF sequences of *fgf6a*, *fgf6b* and *fgf18* (Accession number: OQ989161、OQ989162 and OQ989171) cloned from spotted sea bass were 627 bp, 624 bp and 591 bp in length, encoding 208, 207 and 196 amino acids, respectively. The FGF6A, FGF6B, and FGF18 proteins have similar tertiary structures (Fig. 6**A**), with N-terminal signal peptide and internal pseudo-threefold symmetry (beta-clover topology). FGF6a, FGF6b and FGF18 recombinant proteins (rFGF6a, rFGF6b and rFGF18) with 33.01 kDa, 33.18 kDa and 34.39 kDa were obtained by induced expression (Fig. 6**B, C, D, E**). In addition, the SDS-PAGE results were further validated by western blot (Fig. 6**F**).

Induced-differentiated myoblasts was treated with different concentrations of rFGF6a, rFGF6b and rFGF18 for 48 h. Expression levels *myog*, a marker gene for myogenic differentiation, was tested to determine the effect of the rFGFs on myoblasts differentiation. Results showed 2, 4, and 8 nmol/mL of rFGF6a and rFGF6b caused a significant decrease in *myog* level compared to the control group ($P < 0.05$). Treatment with rFGF6a and rFGF6b in 4 nmol/mL had the strongest inhibitory effect which *myog* expression reduced to 60 % and 71 % of the control group, respectively (Fig. 7**A, 7B**). However, rFGF18 could significantly decreased *myog* expression at 8 nmol/mL, where *myog* expression was reduced to 76 % of the control group (Fig. 7**C**). These results indicated that FGF6a, FGF6b and FGF18 could inhibit myoblasts differentiation, with differences in the starting concentration, the degree of inhibition and the most potent doses.

4. Discussion

Skeletal muscle development is a multi-step and dynamic process that involves the generation of myogenic precursors into myoblasts, proliferation of myoblasts, differentiation of myoblasts into multinucleated myotubes as they exit the growth cycle, and subsequent maturation into myofibers (Zheng et al., 2021). The myoblasts of spotted sea

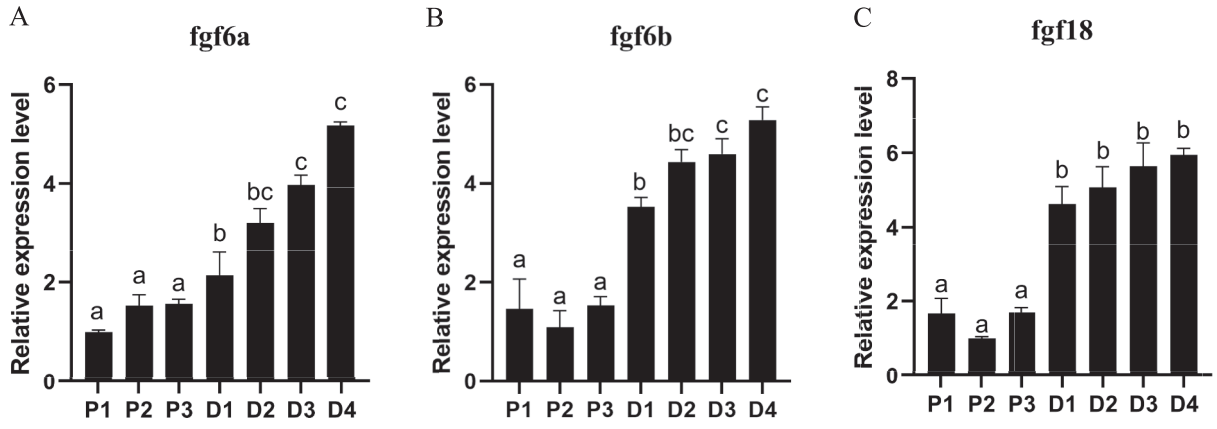

Fig. 5. Temporal expression of *fgf6a*, *fgf6b* and *fgf18* at different stages of myoblast proliferation and differentiation. (P) Stages of cell proliferation (24 h、48 h、72 h); (D) Stages of cell differentiation (24 h、48 h、72 h、96 h). The data are shown as the mean ± S.E.M. (n = 3), and different letters indicate significant differences ($P < 0.05$).

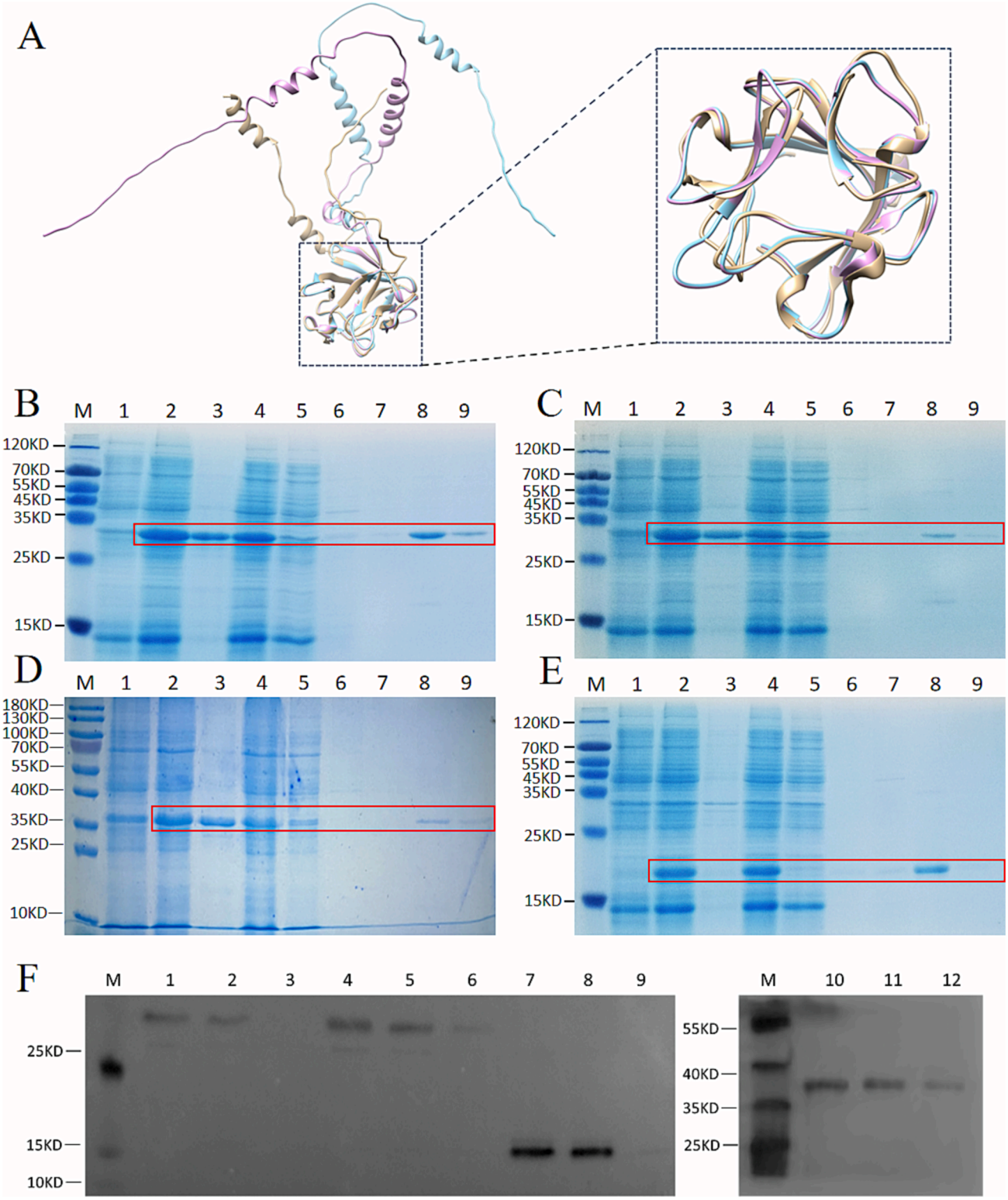

Fig. 6. Protein tertiary structure prediction and SDS-PAGE electrophoresis of purified FGF6a, FGF6b and FGF18 recombinant proteins. (A) Superimposed diagram of FGF6a, FGF6b and FGF18 tertiary structures. Tan represents FGF18, plum represents FGF6a, and sky blue represents FGF6b (B) FGF6a combinant protein (33.01 kDa); (C) FGF6b recombinant protein (33.18 kDa); (D) FGF18 recombinant protein (34.39 kDa); (E) PET-28a-SUMO empty carrier protein (15.96 kDa); M: Protein molecular weight standard; 1: Total protein of E. coli before IPTG induction; 2: Total protein of E. coli after IPTG induction; 3: Sediment of E. coli after IPTG induction; 4: Supernatant of E. coli after IPTG induction; 5: Flow-through fractions; 6–7: Washing fractions; 8–9: Elution fractions with target protein. (F) WB detection of pET-28a-SUMO empty body protein and FGF6a, FGF6b, FGF18 recombinant proteins. 1/2/3: FGF6a recombinant protein; 4/5/6: FGF6b recombinant protein; 7/8/9: PET-28a-SUMO empty carrier protein; 10/11/12: FGF18 recombinant protein; M: Protein molecular weight standard; 1/4/7/10: Total protein of E. coli after IPTG induction; 2/5/8/11: Supernatant of E. coli after IPTG induction; 3/6/9/12: Sediment of E. coli after IPTG induction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

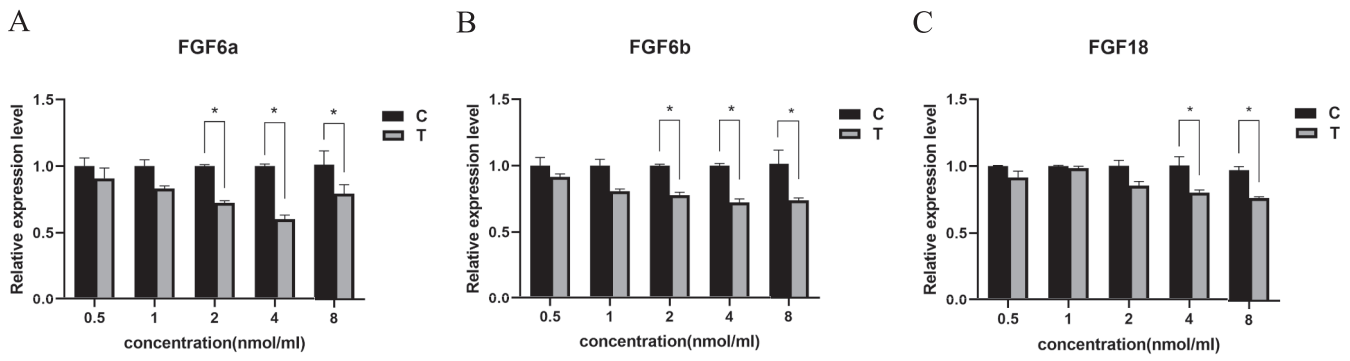

Fig. 7. Relative expression of *myog* in myoblasts of spotted sea bass after treatment by FGF6a, FGF6b and FGF18 proteins. C: Control group, PET-28a-SUMO empty carrier protein treatment; T: treatment group, FGFs protein treatment. The data are shown as the mean ± S.E.M. (n = 3), "*" indicate significant difference ($P < 0.05$).

bass isolated by microexplant culture were spindle-shaped with a few polygonal. The cells showed high competence in proliferation and could reach a density of over 80 % of the culture dish in 2 d. Studies have shown that myoblasts mainly proliferate at higher serum levels (10 % – 20 % serum), while started to differentiate at lower serum levels (1 % – 5 % serum) (Pallafacchina et al., 2010). Our results showed that the relative expression of *ki67*, a marker gene for cell proliferation and plays an important role in cell cycle regulation (Suzuki et al., 2007; Wang et al., 2015), was increased in myoblasts during the proliferation phase than the differentiation phase, while after the induction of differentiation, the expression level of *myog*, a marker gene for myoblast differentiation and regulates the terminal differentiation of mononuclear myoblasts to form multinucleated myotubes (Bustos et al., 2015; Tee et al., 2012), was significantly enhanced than that of the proliferation phase, which is similar to the results to previous studies of other species (Choi et al., 2020; Hayashi et al., 2016; Hayes et al., 2018; Liu et al., 2012).

Fibroblast growth factors (FGFs) are peptide growth factors exist in organisms ranging from nematodes to humans (Ornitz and Itoh, 2001). The FGF family consists of at least 22 members, and due to genome-wide replication events, many genes are multicopied in bony fishes, resulting in more *fgf* family members in teleost than in other vertebrates (Seeger and Paller, 2015). Most of the fibroblast growth factor family share a highly similar internal core region, the FGF domain, with conserved residues involved in binding to receptors. When bound, they cause dimerization of tyrosine kinase receptors, which in turn leads to intracellular conduction. In this study, a total of 33 *fgf* genes were identified from the genome of spotted sea bass, divided into seven subfamilies. Among these *fgf*, homologues of human *fgf* were found in spotted sea bass, except for *fgf9*, which may be lost during the genome of the actinopterygii during evolution (Itoh and Konishi, 2007). In addition, *fgf19* is missing in mice and *fgf15* in humans, and it has been shown that the human *fgf19* and the mouse *fgf15* are direct homologs (Zhuang et al., 2009). In spotted sea bass, *fgf1*, *fgf2*, *fgf6a*, *fgf6b*, *fgf7*, and *fgf18* were expressed in both proliferation and differentiation stages cultured skeletal muscle cells with different expression profiles. Among them, the expression of *fgf6a*, *fgf6b* and *fgf18* were significantly up-regulated, while the rest genes were significantly down-regulated. Accordingly, we further investigated the *fgf6a*, *fgf6b* and *fgf18* function in the subsequent experiments.

In mice, highly expressed in muscle, *fgf6* showed an important function in regeneration by stimulating the proliferation and migration of myoblasts in a dose-dependent manner, thereby promoting muscle differentiation and hypertrophy, while rFGF6 was found to inhibit differentiation of myoblasts in vitro culture system (deLapeyrière et al., 1993; Fon Tacer et al., 2010). In rainbow trout, an extended period of myofibrillar proliferation was accompanied by sustained expression of *fgf6* up to the adult stage (Rescan, 1998). Studies of exogenous FGF6 on mouse C2 myoblasts showed that the expression of myogenic markers and myogenic transcription factors was down-regulated and the differentiation process of C2 myoblasts into myotubes was delayed when added at 25 ng/mL. In addition, it downregulated the expression of *fgfr4* (Pizette et al., 1996). In another study, low concentration of FGF6 (0.8 nmol/mL) promoted myoblasts differentiation through FGFR4-mediated activation of ERK1/2. Immunostaining showed increased expression of MYHC and FGFR4 in C2C12 cells, whereas higher concentration of FGF6 (3.2 nmol/mL) inhibited myoblasts differentiation. These are consistent with our findings that the concentration of FGF6 may influence the differentiation process that occurs in muscle development (Cai et al., 2020). *fgf18* plays a role in the development of a variety of tissues, including the lungs, central nervous system and skeletal muscle (Hagan et al., 2019). *fgf18* supports muscle development in the pharynx in vivo and exogenous *fgf18* can partially rescue the number of myogenic cells in vivo (Feng et al., 2022). In chick embryo limb myoblasts, FGF18 induces premature expression of *myod* and *myf5*, and blocking FGF signaling inhibits endogenous *myod* expression, which is mediated by ERK MAP kinase (Mok et al., 2014). These results all suggest that *fgf6a*, *fgf6b* and *fgf18* have an important role in muscle growth and development.

FGF6a, FGF6b, and FGF18 all belong to secretory extracellular proteins, consisting of 208,207 and 197 amino acids, respectively. The tertiary structure prediction diagram shows that FGF6a, FGF6b and FGF18 all have N-terminal signal peptide and β-clover topological domain. The core domain of the FGF6a and FGF6b were merged together, indicating the same function. While the FGF18 showed slight variation in the beta-turn angle, suggesting the possible variation in the function. However, the N-terminal of these protein containing the signaling peptide and the alpha helix were quite different. The FGF6a and FGF6b were predicted to have a transmembrane structure, which indicating both proteins may act their function in a paracrine pathway. However, the FGF18 was predict to be a secretory protein, without the transmembrane structure. Combined with our invitro experiments, the FGF6a, FGF6b and FGF18 may play the role in inhibiting the differentiation of myoblasts, however, with different pathway.

In summary, our work identified that *fgf6a*, *fgf6b* and *fgf18* down-regulated *myog* expression and inhibited myoblast differentiation in spotted sea bass. Thus, *fgf6a*, *fg f6b* and *fgf18* may be involved in myoblast differentiation as a suppressor. However, the downstream pathways of their activation are unclear and further studies are needed to reveal more about the biological processes.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the National Natural Science Foundation of China [grant number: NSFC, 32072947]、China Agriculture Research System (CARS for Marine Fish Culture Industry) [grant number: CARS-47]、the Shandong Provincial Natural Science Foundation [grant number: ZR2021QC071] and the Key Laboratory of Mariculture of Ministry of Education, Ocean University of China.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2023.114426.

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