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Identification of a second *follistatin* gene in grass carp (*Ctenopharyngodon idellus*) and its regulatory function in myogenesis during embryogenesis

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ABSTRACT

Follistatin can antagonize the function of myostatin as a competitive binding protein and promote muscle growth *in vivo*. Here, we report the isolation and characterization of a second follistatin gene *fst2* in grass carp (*Ctenopharyngodon idellus*). The grass carp *fst2* cDNA was 1,376 bp in length, with an open reading frame (ORF) encoding 350 amino acid residues. A relatively low sequence identity of 78% was found between grass carp Fst2 and its paralog Fst1. Sequence and phylogenetic analyses suggest that the grass carp *fst2* originated from fish-specific gene duplication. In adult fish, *fst2* mRNA expression was observed in most tissues but was strongly expressed in the eyes, muscles, skin and ovary. Grass carp *fst2* mRNA could be detected as early as 16 h post-fertilization (hpf), while *fst1* mRNA was detected throughout embryogenesis. Using *in situ* hybridization, *fst2* transcripts were detected in the anterior somites at 24 hpf and in the brain and posterior somites at 36 hpf. Meanwhile, *fst1* mRNA was transcribed mainly in the optic vesicle and at the cephalic mesoderm at 12 hpf, in the eyes, cephalic mesoderm and at the lateral edge of most somites at 24 hpf, and mainly in the brain at 36 hpf. Furthermore, overexpression of *fst2* mRNA markedly affected the formation of the embryonic midline and somite structures. Based on comparisons with *fst1*, our findings suggest that *fst2* retained the ancestral functions of regulating muscle development and growth during embryogenesis in grass carp.

1. Introduction

Myostatin (MSTN), a secreted protein belonging to the transforming growth factor beta (TGF-β) family, inhibits muscle differentiation and growth in mammals (McCroskery et al., 2005; McPherron et al., 1997). Follistatin (FST), also known as activin-binding protein, is an autocrine glycoprotein that is expressed in nearly all tissues of higher animals (Link and Nishi, 1997; Nakamura et al., 1990). FST can antagonize the function of MSTN as a competitive binding protein and promote muscle growth *in vivo* (Amthor et al., 2004). FST in mammals has been demonstrated to markedly increase muscle mass by hyperplasia, hypertrophy or a combination of both (Lee and McPherron, 2001). Transgenic expression of FST in mice results in dramatic increases in muscle mass, both hypertrophy and hyperplasia, and even greater effects are detected in *Mstn* knockout mice (Lee and McPherron, 2001). *Fst* gene knockout mice have been shown to die immediately after birth, having a reduced amount of muscle tissue, and this finding is consistent with a role for FST in regulating myogenesis (Lee et al., 2010; Matzuk et al., 1995).

Mammals and birds have single copies of the *Fst* gene, while teleost fish may harbor duplicated *fst* genes (Jaillon et al., 2004). The *fst1* gene is universal among teleosts, the sequence of which is closely related to its ortholog in higher vertebrates (Macqueen and Johnston, 2008). As teleost fish experienced an additional genome-wide duplication event (Crow et al., 2006; Taylor et al., 2003), it is possible that many teleost genomes have two *fst* genes. The second *fst* gene, *fst2*, was recently found in several fish species within the superorder Ostariophysi, such as zebrafish, fathead minnow and the catfish (Dal-Pra et al., 2006; Macqueen and Johnston, 2008).

In zebrafish, *fst1* transcripts are first detected at the 70% epiboly stage in cephalic mesoderm and subsequently in the ventral region of rostral somites (Bauer et al., 1998; Thisse, 2001). Analysis of *fst2* mRNA expression shows that it begins at the 14-somite stage in the proximal region of ventral and dorsal parts of the somites during zebrafish embryogenesis. Recent studies have indicated that overexpression of the *fst1* transgene can promote zebrafish muscle growth by enhancing myofiber hyperplasia (Li et al., 2011). Compared to the ample information available on *Fst* structure and function in mammals, relatively little is known about the conserved and divergent molecular structures, expression and biological function of duplicated *fst* genes in fish, especially in aquacultural important species. Determining which *fst* copy has retained the regulatory function of the ancestral gene on muscle development and growth is important for future genetic improvement in aquaculture fish.

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The grass carp, *Ctenopharyngodon idellus*, belonging to the superorder Ostariophysi, has been accepted as a principal species in Chinese freshwater fish polyculture systems, with a total production of over 4 million tons in 2007 (FBMA, 2008). Feeding expenses during the breeding period are significantly reduced with this herbivorous fish species, and its rapid growth provides a useful model for studying muscle growth (Liu et al., 2009; Xia et al., 2010; Yuan et al., 2011). In the present study, a second *fst* gene (*fst2*) was cloned and identified from grass carp. The phylogenetic relationships and structures of grass carp Fst proteins were determined, and their expression patterns were identified in various adult organs and during embryogenesis. Further gain-of-function analysis provided strong evidence to support that *fst2* may be involved in the embryonic formation of the midline and somite structures. Our molecular and functional analyses suggest that *fst2* may play important roles in muscle development and growth during embryogenesis in grass carp.

2. Materials and methods

2.1. Animals

All experiments were conducted following guidelines approved by the Shanghai Ocean University Committee on the Use and Care of Animals. Embryos were generated by grass carp (*C. idellus*) with natural crosses. Adult grass carp were sacrificed by immersion in MS-222 (tricaine methanesulfonate, Sigma, St. Louis, MO). Tissues including brain, eyes, gill, skin, heart, liver, spleen, kidney, intestine, slow-twitch muscle, fast-twitch muscle, ovary and testis, were rapidly dissected, frozen in liquid nitrogen and stored at −80 °C until use. These grass carp were obtained from the Qingpu Fish Breeding Experiment Station, Shanghai, China.

2.2. Molecular cloning of grass carp fst2 cDNA

Total RNA was isolated from grass carp embryos at 36 h post-fertilization (hpf) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and subsequently treated with DNase (Promega, Madison, WI, USA) to remove contaminating genomic DNA. First-strand cDNA was reverse-transcribed from total RNA using Reverse Transcriptase M-MLV (TaKaRa, Tokyo, Japan) with oligo-dT primers according to the manufacturer's instructions.

Based on the zebrafish *fst2* mRNA sequences (GenBank Accession No. NM_001109831.1, 2182 bp), reverse transcription (RT)-PCR was performed to amplify partial cDNA fragments of grass carp *fst2* using the primers 5′-TGATCGCGCTTCTCATATGGT-3′ (sense) and 5′-CTTCGGAGATGGCAGGTGTTG-3′ (antisense). A 633-bp partial PCR fragment of grass carp *fst2* was cloned, sequenced and used to design nested gene-specific primers for 5′ RACE analysis (5′-ATGGAACAGCAGAAAGTACAAGCC-3′, 5′-CACCACCATTGAAGATCAGCCA-3′) and 3′ RACE analysis (5′-GCACACACGCAGCGAGGTTTATTCC-3′, 5′-CGATGGGAAAACGTACCGGAATGA-3′). The 5′ and 3′ ends of the *fst2* mRNA were amplified using the SMART RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) following the manufacturer's protocol. PCR products were gel-purified, ligated into the T/A cloning vector pMD-19T (Takara, Dalian, China) and transformed into *Escherichia coli DH5α* competent cells. Positive clones were examined by PCR and direct sequencing.

2.3. Sequences and phylogenetic analyses

The nucleotide sequence of *fst* cDNA was analyzed using BioEdit 7.0.0.1 (Hall, 1999). The sequences of Fst proteins from different species were compared by the NCBI BLASP search program. Alignment of the putative amino acid sequences of the Fst proteins was performed with the Clustal X 1.81 program (Thompson et al., 1997). Positions with gaps were eliminated, and a phylogenetic tree of Fst putative proteins was constructed using MEGA 5.05 (Tamura et al., 2011) with *p*-distance and 1000 bootstraps replicates.

2.4. Rt-PCR

Total RNA was isolated from eight embryos at same developmental stage during embryogenesis or the adult tissues of grass carp using TRIzol reagent (Invitrogen). After DNase treatment, 1 μg RNA was reverse-transcribed to single stranded cDNA using Reverse Transcriptase M-MLV (TaKaRa) and oligo-dT primers. The PCR primers 5′-AGAACGGGAGATGTCAGGTC-3′ and 5′-CTTCGTATGCCACACCAATG-3′ were used to amplify grass carp *fst1*. The primers 5′-CTTCTCATTTGGTTCGCGCA-3′ and 5′-GAACGAGTGTGCACTCCTGAGG-3′ were used to amplify grass carp *fst2*. The PCR product sizes for *fst1* and *-2* were 597 bp and 401 bp, respectively. Both products span 1–2 putative introns. The primers 5′-CCGCTGCCTCTTCTTCCTC-3′ and 5′-CTACCTCCCTTTGCCAGTTTCCGC-3′ were used to amplify *β-actin*. The PCR products were analyzed by electrophoresis and stained with ethidium bromide. The signal intensity of each PCR product was densitometrically semi-quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA) and normalized by the β-actin levels.

2.5. Whole-mount in situ hybridization

A 790-bp PCR fragment of grass carp *fst1* amplified by primers 5′-GCCCTGGGAAGAGATGTAAAATGA-3′ and 5′-CTTCCAATCATAAGTCCTTTCTGTAGC-3′ and a 796-bp PCR fragment of *fst2* amplified by primers 5′-GGACTGTGGTGCTGGAAAGAGGT-3′ and 5′-CTCTGTGCTTAAATGGAGTCTTGCTG-3′ were subcloned into the pGM-T vector (Tiangen, Beijing, China). Each template plasmid was linearized by restriction enzyme digestion, followed by *in vitro* transcription with T7 or SP6 RNA polymerase to generate the antisense or sense RNA riboprobes.

Embryos were collected for *in situ* hybridization, manually dechorionated and fixed overnight at 4 °C in 4% phosphate-buffered paraformaldehyde. Fixed embryos were washed briefly in phosphate-buffered saline (PBS) containing 0.1% Tween-20, transferred to 100% methanol and stored at −20 °C for a minimum of 24 h. Whole-mount *in situ* hybridization using digoxigenin (DIG)-labeled RNA riboprobes was carried out essentially as reported previously, with certain modifications (Thisse and Thisse, 2008; Zou et al., 2009). Briefly, embryos were hybridized with appropriate riboprobes at 60 °C, incubated with anti-DIG antibodies conjugated with alkaline phosphatase (AP), and stained with Roche BM Purple AP substrates (Roche, Basel, Switzerland) to produce purple, insoluble precipitates. Photographs were taken with a Nikon SMZ1500 microscope (Tokyo, Japan).

2.6. In vitro transcription and microinjection

The open reading frames (ORFs) of grass carp *fst1* and *-2* genes were amplified by PCR using *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA). The primer set for *fst1* was 5′-CGCGGATCCGCCACCATGCTAAGGATGCTAAAGCG-3′ and 5′-CCATCGATGTATTACTTACAGTTGCAAGATCC-3′. The primer set for *fst2* was 5′-CGCGGATCCGCCACCTGAGGATGCTACAGTTACCG-3′ and 5′-CCATCGATGGTTTATCCAGTCAGTAACAGAG-3′. The resulting cDNA was cloned into the pMD-19T easy vector (Takara, Dalian, China). To generate the Fst1 and Fst2 expression constructs, the above PCR products were digested with *Hind*III and *Bam*HI and subcloned into the *Hind*III-*Bam*HI sites of the pCS2 + vector. Capped *GFP* mRNA and grass carp