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Transgenic overexpression of *follistatin 2* in blunt snout bream results in increased muscle mass caused by hypertrophy

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ABSTRACT

As a competitive binding protein, follistatin (FST) antagonizes myostatin (MSTN) function and promotes muscle growth. Overexpression of *FST* in transgenic mice results in dramatically increased muscle mass caused by hyperplasia and/or hypertrophy. Previously, we identified two functional divergent *fst* genes (g*cfst1* and g*cfst2*) in grass carp (*Ctenopharyngodon idella*). The objectives of this study were to determine if g*cfst* genes have similar roles on promoting muscle growth in fish. We generated a heterozygous g*cfst1*- and g*cfst2*-transgenic F0 generation in blunt snout bream (*Megalobrama amblycephala*). We then produced a homozygous gynogenetic F1 generation, and eventually a homozygous transgenic F2 generation. Driven by the zebrafish skeletal muscle-specific gene *Mylz2* promoter, high levels of g*cfst1* or g*cfst2* mRNAs were expressed in embryonic somites and adult muscles in homozygous g*cfst1*- or g*cfst2*-transgenic F2 blunt snout bream, respectively. In contrast to g*cfst1*-transgenic fish or wild-type controls, adult g*cfst2*-transgenic F2 blunt snout bream exhibited a double-muscling phenotype and marked increases in body height and thickness. Overexpression of *gcfst2* caused significant fiber size enlargement in skeletal muscle in g*cfst2*-transgenic F2 blunt snout bream. Our findings suggest that enhanced muscle growth induced by *gcfst2* overexpression in transgenic blunt snout bream is caused by hypertrophic muscle growth. This approach may be used to promote directional breeding in important aquaculture species such as blunt snout bream in the future.

Statement of relevance: To promote directional breeding in important aquaculture species blunt snout bream, our research papers presents novel data on fish breeding. Our article complies with the Policy Statement for submission of manuscripts to the Genetics Section.

1. Introduction

Myostatin (MSTN), also known as growth differentiation factor-8 (GDF-8), is a secreted protein belonging to the transforming growth factor beta (TGF-β) superfamily (McPherron et al., 1997; McPherron and Lee, 1997; Joulia et al., 2003; McCroskery et al., 2005). Expressed almost exclusively in mammalian skeletal muscle, MSTN plays a strong inhibitory role in the regulation of skeletal muscle (McCroskery et al., 2003). Knockout mutations of the *mstn* gene give rise to the double-muscling phenotype in several mammalian species, including cattle, mice, humans, sheep and dogs (Stinckens et al., 2011). The high conservation of mature MSTN in fish compared with mammals indicates a similar function in inhibiting muscle growth (Gabillard et al., 2013; Zheng et al., 2015). A double-muscling effect has recently been generated in adult transgenic zebrafish by suppression of *mstn-2* using antisense RNA (Lee et al., 2009). Furthermore, a significant double-muscle phenotype was generated by the *mstn-1 C315Y* ENU-mutant in medaka (Chisada et al., 2011). More recently, depletion of *myostatin b* (*mstn-2*) promotes somatic growth and lipid metabolism in zebrafish (Gao et al., 2016).

The autocrine glycoprotein follistatin (FST) antagonizes the function of MSTN as a competitive binding protein and promotes muscle growth *in vivo* (Nakamura et al., 1990; Link and Nishi, 1997; Amthor et al., 2004). FST overexpression in transgenic mice results in dramatic increases in muscle mass caused by hyperplasia and/or hypertrophy, with even greater effects detected in *Mstn*-knockout mice (Lee and McPherron, 2001). *Fst1* overexpression promotes muscle growth in zebrafish by enhancing myofiber hyperplasia (Li et al., 2011). In contrast, *Fst* gene knockout mice have a reduced amount of muscle tissue (Matzuk et al., 1995; Lee et al., 2010). These findings are consistent with a role for FST in regulating myogenesis. Many teleost fish, including grass carp (termed as g*cfst1* and g*cfst2*), have two functional divergent *fst* genes as a result of a genome-wide duplication event (Crow et al., 2006; Zhong et al., 2013). The g*cfst2* transcripts are detected mainly in the somites, while g*cfst1* mRNA is transcribed mainly in the cephalic mesoderm during grass carp embryogenesis (Zhong et al., 2013). Based on this pattern of expression, we hypothesized that *gcfst2* plays a critical role in enhancing muscle growth by antagonizing the function of MSTN. To test this hypothesis we performed experiments using the *Tgf2* transposon system to produce homozygous transgenic F2 blunt

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snout bream lines expressing high levels of gc*fst1* and gc*fst2* mRNAs in embryonic somites and adult muscles driven by the strong zebrafish skeletal muscle-specific *mylz2* promoter. Compared to gc*fst1*-transgenic fish, gc*fst2*-transgenic F2 adult fish exhibited higher body mass caused by muscle hypertrophy. Our findings suggest that the negative regulatory functions of gc*fst2* in myostatin-related inhibition of muscle growth are retained and may be used to promote directional breeding in important aquaculture species such as blunt snout bream in the future.

2. Materials and methods

2.1. Whole-mount in situ hybridization

A 790-bp PCR fragment of gc*fst1* amplified by primers 5′-GCCCTGGGAAGAGATGTAAAATGA-3′ and 5′-CTTCCAATCATAAGTCCTTTCTGTAGC-3′ and a 796-bp PCR fragment of gc*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 then rehydrate embryos were stored at −20 °C for a minimum of 24 h. Whole-mount embryo *in situ* hybridization analysis using digoxigenin (DIG)-labeled RNA riboprobes was conducted at 24 or 36 hours post-fertilization (hpf) as described (Zhong et al., 2013). Embryos from grass carp or blunt snout bream at 12, 24 or 36 hpf were permealized by proteinase K treatment. Then, 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 to produce purple, insoluble precipitates. Photographs were taken with a Nikon SMZ1500 microscope (Tokyo, Japan).

2.2. Transgenic construction

Total RNA was isolated from grass carp (*Ctenopharyngodon idella*) embryos at 24 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. Reverse transcription (RT)-PCR was performed to amplify 969 bp of the open reading frame (ORF) of gc*fst1* using the primers 5′- CGCGGATCCATGCTAAGGATGCTAAAGCG-3′ (sense) and 5′-CCATCGATGTATTACTTACAGTTGCAAGATCC-3′ (antisense) or 1053 bp of the ORF of gc*fst2* using the primers 5′-CGCGGATCCTGAGGATGCTACAGTTACCG-3′ (sense) and 5′-CCATCGATGGTTTATCCAGTCAGTAACAGAG-3′ (antisense). Restriction endonuclease recognition sites (underlined) were incorporated into the primers for directional cloning.

The gc*fst1* and gc*fst2* ORFs were then purified and cloned into the pMD-19T vector (Takara, Dalian, China). The resulting plasmids, pMD-19T-gc*fst1* and pMD-19T-gc*fst2*, and pTgf2-Mylz2-RFP (Guo et al., 2015) were digested with *Bam*HI and *Cla*I (Takara, Shanghai, China). Linear fragments of gc*fst1* or gc*fst2* and pTgf2-Mylz2 were gel-purified and ligated using T_4 DNA ligase (Takara, Shanghai, China) to generate pTgf2-Mylz2-gc*fst1* and pTgf2-Mylz2-gc*fst2* (Fig. 2a, b). All plasmids were verified by DNA sequencing.

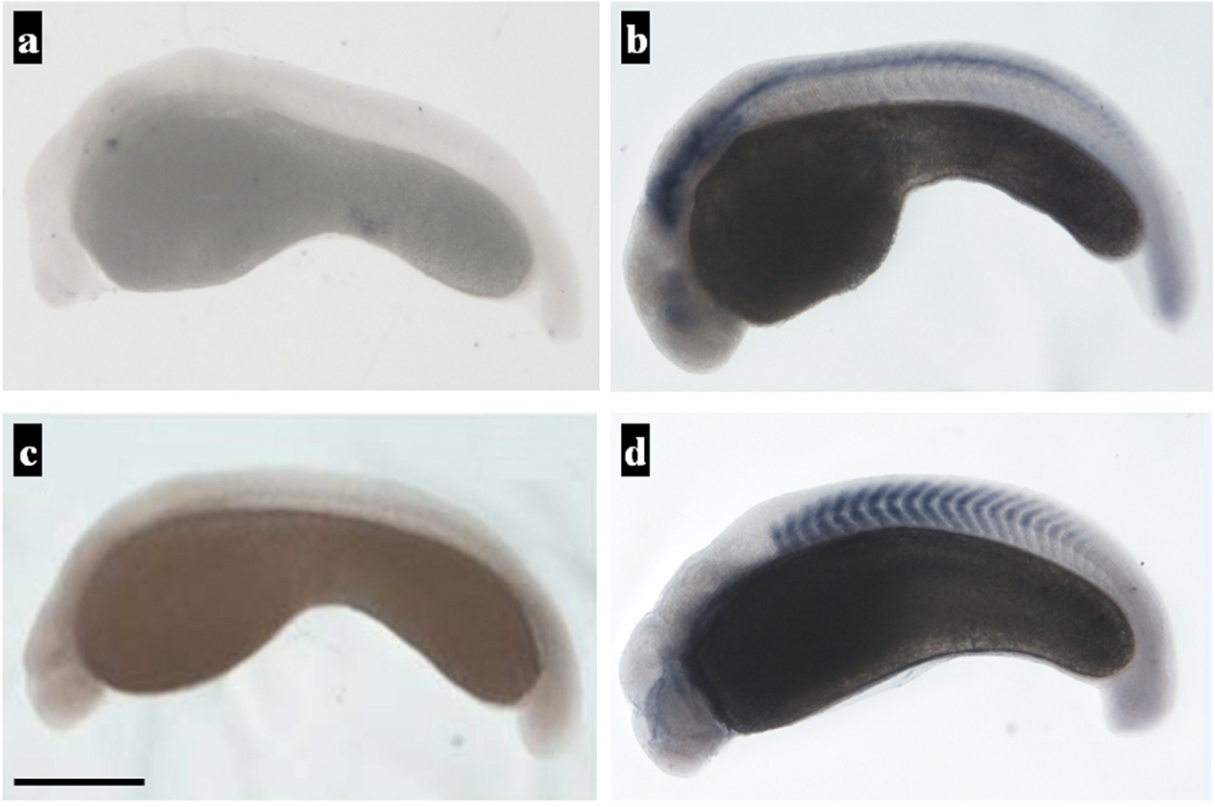

Fig. 1. Embryonic expression of gc*fst1* and gc*fst2* mRNAs in the wild-type grass carp. (a–d) Whole-mount embryo *in situ* hybridization analysis was conducted at 24 h post-fertilization using the sense probes for gc*fst1* (a) or gc*fst2* (c) and the antisense probes for gc*fst1* (b) or gc*fst2* probe (d). All embryos are viewed laterally with the head to the left. Scale bar = 600 μm.