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Myostatin-deficient medaka exhibit a double-muscling phenotype with hyperplasia and hypertrophy, which occur sequentially during post-hatch development

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ABSTRACT

Myostatin (MSTN) functions as a negative regulator of skeletal muscle mass. In mammals, MSTN-deficient animals result in an increase of skeletal muscle mass with both hyperplasia and hypertrophy. A MSTN gene is highly conserved within the fish species, allowing speculation that MSTN-deficient fish could exhibit a double-muscled phenotype. Some strategies for blocking or knocking down MSTN in adult fish have been already performed; however, these fish show either only hyperplastic or hypertrophic growth in muscle fiber. Therefore, the role of MSTN in fish myogenesis during post-hatch growth remains unclear. To address this question, we have made MSTN-deficient medaka (*mstnC315Y*) by using the targeting induced local lesions in a genome method. *mstnC315Y* can reproduce and have the same survival period as WT medaka. Growth rates of WT and *mstnC315Y* were measured at juvenile (1–2 wk post-hatching), post-juvenile (3–7 wk post-hatching) and adult (8–16 wk post-hatching) stages. In addition, effects of MSTN on skeletal muscle differentiation were investigated at histological and molecular levels at each developmental stage. As a result, *mstnC315Y* show a significant increase in body weight from the post-juvenile to adult stage. Hyper-morphogenesis of skeletal muscle in *mstnC315Y* was accomplished due to hyperplastic growth from post-juvenile to early adult stage, followed by hypertrophic growth in the adult stage. Myf-5 and MyoD were up-regulated in *mstnC315Y* at the hyperplastic growth phase, while myogenin was highly expressed in *mstnC315Y* at the hypertrophic growth phase. These indicated that MSTN in medaka plays a dual role for muscle fiber development. In conclusion, MSTN in medaka regulates the number and size of muscle fiber in a temporally-controlled manner during posthatch growth.

Introduction

Myostatin (MSTN) or growth and differentiation factor-8 (GDF-8) is a member of the transforming growth factor-β (TGF-β) superfamily, and functions as a negative regulator of skeletal muscle mass (McPherron et al., 1997). MSTN-null mice generated by gene targeting show a drastic and widespread increase in skeletal muscle with a combination of hyperplasia and hypertrophy. Each muscle of mutant mice weighs 2–3 times compared to those of wild-type mice. Naturally occurring mutations in the MSTN gene have been identified in double-muscling cattle breeds (Kambadur et al., 1997; McPherron and Lee, 1997), dogs (Mosher et al., 2007) and even humans (Schuelke et al., 2004). MSTN protein is synthesized in skeletal muscle as a propeptide, which is processed by proteolytic cleavage to produce the mature form at the C-terminus. The C-terminal homodimer is known to be the biologically active molecule. The C-terminal amino acid sequence is highly conserved across mammalian and avian species (McPherron and Lee, 1997). Their identities are 95–100% among mouse, rat, human, porcine, chicken, turkey, baboon, bovine and ovine. Furthermore, in fish (for example, zebrafish, Atlantic salmon, sea perch, European seabass, bastard halibut and Chilean flounder), the C-terminal domain reaches identities higher than 88% comparing with mammalian MSTN (Delgado et al., 2008). According to these studies, there is a high possibility that the MSTN gene and its biological function should be conserved in vertebrates.

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Previous studies about the effects of MSTN in fish have demonstrated inconsistent phenotypes in body weight. Lee et al. (2009) reported that MSTN knock-down zebrafish using vector-based RNA interference exhibited a 1.4-fold increased body weight at 4 months. In contrast, Xu et al. (2003) showed that over-expression of MSTN prodomain as a dominant-negative form in zebrafish resulted in an only slightly increased body weight at 2.5 months. Sawatari et al. (2010) also revealed that over-expression of a dominant-negative form of MSTN in medaka did not change the body weight at 6 months. Therefore, it remains unclear whether blocking the MSTN function in fish leads to body weight gain. These reports also show conflicting phenotypes in muscle fibers. MSTN knock-down zebrafish resulted in only hypertrophy, while the over-expression of dominant-negative forms in zebrafish and medaka affected only hyperplasia. It is quite possible that these seemingly contradictory phenotypes in fibers are due to the incomplete inhibition of the MSTN signaling pathway. Thus, previous morphological evidence could not have demonstrated whether MSTN plays an inhibitory role in hyperplastic or hypertrophic muscle growth in fish. To accurately understand the MSTN function in fish, the generation of a completely MSTN-suppressed fish is required. Moreover, there is an important consideration in the evaluation of muscle growth in fish. Hyperplasia and hypertrophy in fish can continue during posthatch growth (Johnston, 1999; Rowe and Goldspink, 1969); however, in the studies reported previously, the effects of MSTN on myogenesis have been analyzed at single or a few points, but not throughout life. Therefore, there is some possibility that change of phenotypes is overlooked in transgenic fish for vector-based RNA interference and MSTN prodomain over-expression. To address these problems, further studies has been required using other techniques, which is able to completely inhibit MSTN function during posthatch growth.

In the current study, we made medaka with a MSTN deficient signaling pathway (called MSTN deficient medaka or *mstnC315Y* in the present report), corresponding to the naturally occurring mutation in double-muscled Piedmontese cattle (Kambadur et al., 1997), by targeting induced local lesions in the genome (TILLING) method (Taniguchi et al., 2006). *mstnC315Y* have the ability to reproduce and have the same survival period as WT medaka. These fish have allowed analysis of the effects of MSTN on myogenesis throughout life. As a result, the *mstnC315Y* shows a double-muscling phenotype with hyperplasia and hypertrophy. Moreover, we elucidated that MSTN in medaka regulated the number of muscle fibers at the post-juvenile stage and the size of those at the adult stage. Therefore, *mstnC315Y* mutant medaka provides an opportunity to elucidate the MSTN function in fish.

Materials and methods

Cloning of medaka MSTN gene

RNA was extracted from wild-type medaka larvae (Kyoto-Cab, a substrain of Cab) by RNeasy® Mini Kit (QIAGEN Sciences, MD, USA) according to the manufacturer's instructions. cDNA was synthesized using PowerScript (BD Biosciences Clontech, Palo Alto, CA, USA). To identify medaka MSTN gene orthologs, we used the basic local alignment search tool (BLAST) to search the medaka genome database (http://www.ensembl.org/Oryzias_latipes/blastview). Medaka MSTN cDNA sequences were then determined using a combination of RT-PCR and rapid amplification of cDNA ends (RACE). RACE products were generated using SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech). The 5′ RACE (1st: 5′-AGATCTTCACCTCAATGAACGGTTGCAG-3′, nest: 5′-TCTGCTGAGGTGACGGCTAAGTCATTTC-3′) and 3′ RACE primers (1st: 5′-GACGAGCATGCATCTACGGAGACAATTA-3′, nest: 5′-ATGGAGAGCCAAAGTGTTGCCTTTTCTC-3′) were used. The cDNA sequence was used to retrieve the genomic sequence from the draft medaka genome assembly.

Generation of MSTN mutant medaka

Generation of MSTN mutant medaka was carried out as described previously (Taniguchi et al., 2006). To find the mutations in the region of interest, the third exon of the medaka MSTN gene was screened using the forward primer #1 (5′-GGACAGCTGCCAAATAGTGC-3′) and reverse primer #1 (5′-AGGCTGACTGCTGCCTCTAC-3′) by PCR (92 °C for 60 s; 12 cycles of 92 °C for 20 s, 65 °C for 20 s with a decrement of 0.6 °C per cycle, 72 °C for 30 s; 20 cycles of 92 °C for 20 s, 58 °C for 20 s and 72 °C for 30 s; 72 °C for 180 s [ABI 9700 dual 384 well GeneAmp PCR system]). The PCR products were treated with an ExoSAP-IT kit (GE Healthcare). Sequencing reaction was then carried out using a BigDye terminator ver. 3 kit (Applied Biosystems) and the forward primer #1. Sequencing products cleaned up by ethanol precipitation were run on automated ABI 3730xl DNA analyzers (Applied Biosystems). *In vitro* fertilization was carried out using sperm with the desired mutation and the progeny were genotyped by sequencing. Heterozygous fish carrying the same mutation were back-crossed with wild-type medaka, at least 4 times. To verify the genotype from this incross, the DNA was amplified by PCR with forward primer #2 (5′-TCAGGAGAAGTAGGCAGCTGTG-3′) and reverse primer #2 (5′-CATGGAGGGGATCTTACCGTAG-3′). The 546-bp PCR product obtained was sequenced using the forward primer #3 (5′-ATGAGACCCTGACAGCCATC-3′). The resulting sequence chromatograms were analyzed for the expected single nucleotide polymorphism (guanine-to-adenine replacement) at one or both alleles (Fig. 2A). Wild-type fish (WT) and homozygous mutant line (*mstnC315Y*) obtained from the incross of heterozygous mutant parents were used for all the following experiments.

Nuclear protein preparation

Nuclear extract was prepared using the modified protocol previously described by (Lizotte et al., 2005). Skeletal muscle was frozen with liquid nitrogen and crushed into a fine powder. The obtained powder (200 mg) was mixed in 3 ml of the lysis buffer containing 50 mM Tris (pH 7.5), 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, 1 mM NaF and 1:20 protease inhibitor cocktail (Complete Mini EDTA free, Roche Diagnostics K. K., Basel, Switzerland), and was homogenized at low speed for 1 min using a mechanical homogenizer (Physcotron NS-310E, Microtec Co., Ltd., Japan). The homogenate was centrifuged at 450×*g* and 4 °C for 15 min to remove tissue debris. The supernatant was mixed in an equal volume (3 ml) of the isotonic solution consisting of 10 mM Hepes (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 1 mM NaF and 1:20 protease inhibitor. Nuclei were pelleted by centrifugation at 2000×*g* and 4 °C for 15 min. The pellet was resuspended in the hypertonic solution consisting of 300 mM Hepes (pH 7.5), 30 mM $MgCl_2$, 1.4 M KCl, 0.5 mM DTT, 1 mM NaF and 1:20 protease inhibitor. To allow the nuclei to shrink, the resuspension was incubated on ice for 20 min. These were then centrifuged at 2000×*g* and 4 °C for 15 min. The final pellet was resuspended in the storage solution containing 20 mM Hepes (pH 7.5), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 25% (v/v) glycerol, 1 mM NaF and 1:20 protease inhibitor. The protein concentration was determined with the BCA Protein Assay (Pierce, IL, USA).

SDS-PAGE and western blotting

Nuclear proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This technique was performed using 4% (w/v) acrylamide stacking gel followed by a 5–20% gradient separation gel. Proteins were transferred to a PVDF membrane (0.2 μm Pall Fluoro Trans® W membranes, Pall Corp., MI, USA). Firstly, the membrane was washed with TBS (20 mM Tris–HCl, pH 7.5, 150 mM NaCl) for 5 min and then blocked with blocking