



Generation of fast growth Nile tilapia (*Oreochromis niloticus*) by myostatin gene mutation

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ABSTRACT

Tilapia is the second most prolific species grown in aquaculture after carp, and is widely grown in >100 countries. Myostatin (MSTN) has been proved to be a negative regulator of skeletal muscle growth. Mutation of *MSTN* gene resulted in significant increase in both body size and muscle mass in vertebrates, mostly with a species-specific effect. To generate a new strain in Nile tilapia (*Oreochromis niloticus*) with fast growth traits, the molecular characterization, gene expression and functional studies of *mstn* gene were extensively investigated. Phylogenetic and synteny analyses indicated that *mstnb*, but not *mstna*, might be the orthologous gene of mammalian *MSTN* gene. Expression pattern analyzed by qRT-PCR demonstrated that *mstnb* is abundant in muscle, while *mstna* is dominantly expressed in the brain. Fluorescence in situ hybridization (*FISH*) analysis also demonstrated that *mstnb* is exclusively expressed in the basement membrane of white skeletal muscle fibers, further suggesting the possible role of *mstnb* gene in tilapia muscle growth. To verify the roles of *mstnb* in skeletal muscle growth, we obtain the *mstnb*^{-/-} mutant using CRISPR/Cas9 gene editing technology. Compared with the wild type (WT) fish, the *mstnb*^{-/-} mutants showed a typical double-muscle phenotype with increased muscle mass, sticking out between head and dorsal fin, from 5 months after hatching (mah). Morphological observation revealed an excessive proliferation of white muscle fibers at 7 mah. The average body weights (21.67 ± 8.26 g), body heights (4.46 ± 0.65 cm) and body widths (2.36 ± 0.53 cm) of the *mstnb*^{-/-} fish were 49.45%, 32.74% and 37.21% higher than those of the *mstnb*^{+/+} fish (body weight, 14.50 ± 8.60 g; body height, 3.36 ± 0.45 cm; body width, 1.72 ± 0.18 cm) at 5 mah. The results showed that the growth performance parameters of the *mstnb*^{-/-} fish, including weight gain rate (1.99 times), condition factor (1.77 times) and specific growth rate (1.23 times), were significantly higher than the *mstnb*^{+/+} fish under laboratory conditions at 5 mah. In addition, the parameters of WGR, CF, SGR and FE were significantly increased in *mstnb*^{-/-} Nile tilapia than those of *mstnb*^{+/+} Nile tilapia and GIFT tilapia for a 90-day feeding trial under the wild natural environment. Interestingly, sexually dimorphic effects on muscle growth have been found with an increase of growth performance of skeletal muscle in *mstn*^{-/-} XY fish, but not *mstn*^{-/-} XX fish at 7 mah. Taken together, this study, for the first time, proved that *mstn* gene mutation might promote the generation of new fast-growing male strain of Nile tilapia.

Abbreviations: MSTN/mstn, myostatin; WT, wild type; WGR, weight gain rate; CF, condition factor; SGR, specific growth rate; FE, feed efficiency; *FISH*, fluorescence in situ hybridization; mah, months after hatching; GMT, genetically male tilapia; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9; TGF-β, transforming growth factor-β; MRFs, myogenic regulatory factors; *myod1*, myogenic differentiation 1; *myf5*, myogenic factor 5; *panx3*, pannexin 3; *vegfa*, vascular endothelial growth factor Ba; *fgf11b*, fibroblast growth factor 11b.

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1. Introduction

Aquaculture sustainability is the key to future human nutritional security and united nations also adopted “Zero hunger” as one of the sustainable development goals (Naylor et al., 2000; Naylor et al., 2021). However, achievements of these goals are very difficult due to unavailability of good quality strain, and proper and universal management practices. Notably, tilapias are the most popular aquaculture species cultured over 127 countries (2017) due to their high nutritional values, and better economic returns (Yue and Lin, 2016; Wang and Lu, 2016; Barroso et al., 2019). Extensive research efforts by traditional breeding techniques, including improved breeding strategies, hybridization, monosex production, genetically male tilapia (GMT) progeny, have been made to promote the production efficiency of tilapia (Eknath and Hulata, 2009; Ghosal and Chakraborty, 2017). Development of new strain of fish with enhanced economic traits (e.g. higher growth, more muscle mass) by genome editing and similar advanced biotechnology approaches will be an enchanted feather in tilapia farming. Particularly, clustered regularly interspaced short palindromic repeats associated with Cas9 (CRISPR/Cas9)-based approaches have developed rapidly in recent years to be an efficient, time-saving technology (Cong et al., 2013; Doudna and Charpentier, 2014; Li et al., 2019).

Myostatin (MSTN) is a member of the transforming growth factor- β (TGF- β) superfamily and it is a well-known negative regulator of myogenesis in skeletal muscle development. Mutation of *Mstn* gene induces skeletal muscle hypertrophy in mice (McPherron et al., 1997) and also known promote the development of “double-muscle phenotype” in various vertebrates (Grobet et al., 1997; Li et al., 2020; Mosher et al., 2007). Natural mutant carrying 11 bp deletion in *Mstn* gene was identified in a group of cattle and promoted as breed named “Belgian Blue cattle” also possesses double-muscle phenotype (Kambadur et al., 1997). So far, the “double-muscle model” has been successively obtained through *Mstn* gene editing in goats (*Capra hircus*) (Ni et al., 2014), sheep (*Ovis aries*) (Crispo et al., 2015), pigs (*Sus*) (Cai et al., 2017; Li et al., 2020; Wang et al., 2017), chickens (*Gallus gallus domesticus*) (Kim et al., 2020) and quail (*Coturnix japonica*) (Lee et al., 2020). These reports highlight the prospects of gene editing in efficient, enhanced and profitable livestock production.

Fast-growing strains with higher production of edible muscle are usually preferred in aquaculture. Two or four copies of *mstn* genes, which were identified as *mstna* (*mstn1*) and *mstnb* (*mstn2*), had been identified in most of diploid and tetraploid fish species (Pie and Alvares, 2006). In zebrafish (*Danio rerio*) and sea bream (*Pagrus major*), expression analysis revealed that *mstnb* is mainly expressed in brain, muscle, and intestine, whereas *mstna* seems to be restricted to brain (Xu et al., 2003). Studies on *mstn* gene editing are mainly focused on model fish and only a few studies in farmed fish. Among these, *mstn* gene deficiency by gene editing resulted in obvious “double-muscle phenotype” in small model fish of zebrafish (Gao et al., 2016) and medaka (*Oryzias latipes*) (Chisada et al., 2011). Moreover, *mstn* gene mutation (mosaic, at F0/F1 generation) led to increased numbers of muscle fibers in some aquaculture fish, such as common carp (*Cyprinus carpio*) (Shahi et al., 2022; Zhong et al., 2016), channel catfish (*Ictalurus punctatus*) (Khalil et al., 2017), blunt snout bream (*Megalobrama amblycephala*) (Sun et al., 2020) and loach (*Misgurnus anguillicaudatus*) (Tao et al., 2021). However, due to long life cycle and maturation time, homozygous *mstn* mutant lines have been constructed only in two aquaculture species, i.e. yellow catfish (*Tachysurus fulvidraco*) (Zhang et al., 2020) and red sea bream (Kishimoto et al., 2018).

In this study, using tilapia, characterization and expression analysis of two *mstn* genes were investigated. Subsequently, a *mstnb*^{-/-} mutant line was successfully generated in tilapia, and the effects of *mstn* deficiency on muscle growth were comprehensively examined. To our best knowledge, this is the first report showcasing the fact the fast-growing tilapia can be produced using genome editing tools.

2. Materials and methods

2.1. Animals

Nile tilapias were acclimatized in recirculating freshwater tanks at 26 ± 2 °C under a natural photoperiod before use. All mono-females (XX) and mono-males (XY) were obtained by artificial fertilization of eggs from normal females (XX) with sperm from either sex-reversed males (XX) or super males (YY), respectively. In this study, we wanted to investigate the sex-biased growth difference of *mstn* gene editing. Therefore, monosex fertilized eggs were used for microinjection and gene editing experiments. Notably, the XX males were generated by treating fry with the aromatase inhibitor Fadrozole (200 μ g/g diet) (Novartis Company, AG, Switzerland) according to previous report (Sun et al., 2014). And production of YY fish was carried out by crossing XY male fish and XY sex reversed female fish, which was obtained by 17 β -estradiol treatment (50 μ g/g diet) (Sigma, USA) from 3 to 35 days after hatching (dah). Animal experiments were conducted in accordance with the regulations of the Guide for Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animal Experimentation at Southwest University.

2.2. Phylogenetic analyses, syntenic and amino acid alignment analyses

Sequences were analyzed using the EditSeq and Megalign program with the Laser gene sequence analysis software package (DNASTar, Madison, WI, USA). Phylogenetic tree was generated by the neighbor-joining (NJ) method using the program MEGA5.0 software with a bootstrap of 1000 replicates to assess the confidence in the phylogeny (Huey et al., 2019; Tamura et al., 2013). For syntenic analysis, position and orientation of *Mstn* genes and their adjacent genes on the chromosome were determined using Genomicus (<http://www.genomicus.biologie.ens.fr/genomicus-89.01/cgi-bin/search.pl>) (Eckhardt et al., 2007). Sequences were downloaded from NCBI and Ensembl, and amino acid comparison analysis of *Mstn* gene of human, sheep, cow and tilapia by Clustal X was carried out for sequence alignment (Larkin et al., 2007).

2.3. Expression analyses of *mstn* by qRT-PCR

The transcript levels of *mstna* and *mstnb* in various adult tissues (brain, pituitary, gill, heart, spleen, liver, intestine, ovary, testis, kidney, skeletal muscle and head kidney) was checked by qRT-PCR according to the methods described previously (Yu et al., 2014). Expression profiles of *mstnb* gene in skeletal muscle of XY and XX tilapia were also examined at different developmental stages (2 mah, 3 mah, 4 mah, 5 mah, 7 mah) by qRT-PCR. Moreover, quantitative mRNA expression of three of myogenic regulatory factors (MRFs) myogenic differentiation 1 (*myod1*), myogenic factor 5 (*myf5*), and *myogenin* and genes related to muscle fiber proliferation (pannexin 3, *panx3*; vascular endothelial growth factor Ba, *vegfa*; fibroblast growth factor 11b, *fgf11b*) in skeletal muscles were also investigated by qRT-PCR between *mstnb*^{-/-} and *mstnb*^{+/+} XY fish.

Briefly, total RNA was extracted from the tissues, and total RNA (1.0 μ g) from each sample was reverse transcribed independently using Prime Script RT Master Mix Perfect Real Time Kit according to the manufacturer's instructions (Takara, Dalian, China). All qRT-PCRs were carried out in ABI-7500 fast qRT-PCR machine (Applied Biosystems, USA) in a 20 μ l reactions with a mixture of 10 μ l 2 $\times$ SYBR Premix ExTaq (Takara, Dalian, China), 2 μ l of diluted cDNA or PCR-grade water as negative control, and 1 μ l of each 10 μ M primer, 6 μ l of PCR-grade water. The PCR reactions were initiated from denaturation at 95 °C for 5 min; followed by 40 amplification cycles at 95 °C for 15 s and 60 °C for 30 s. Dissociation protocols were used to measure melting curves. The relative abundance of target genes was evaluated using the formula: $R = 2^{-\Delta\Delta Ct}$ by using *gapdh* as an endogenous control to verify the reaction efficiency (Livak and Schmittgen, 2001). Primer sequences used for PCR

Table 1

Primers used in the present study.

Primer name	Primer sequence (5'-3')	Accession number	Purpose
<i>mstnb</i> -Cas9-F	TAATACGACTCACTATAGCAGCCTTCGTCAGCACCCCTTTTAGAGCTAGAAATAGC		gRNA amplification
<i>mstnb</i> -Cas9-R	TGTAACATTTATGACAGTGGG		
<i>mstnb</i> -T-F	GCTTGCTGATTGCGTTGG		Sequencing and mutant screening
<i>mstnb</i> -T-R	AAGGAGCGGATTCGTATGTG	XM_003458832	
<i>mstnb</i> -P-F	GCGTTGGGTCCAGTAGTTCT		
<i>mstnb</i> -P-R	TAGCGCATTGATCCGTGTCT		
<i>mstnb</i> -Q-F	TGAACCTGATTCCGCTGTCC		
<i>mstnb</i> -Q-R	GTCGATCTTCAAGGAGCGGA		
<i>mstna</i> -Q-F	CGCACAATCCAATCGCCC	XM_003446535	
<i>mstna</i> -Q-R	CCAGCGTCGGTGTCAATCTT		
<i>myod1</i> -Q-F	CGACGGCATGACGGATTTTA		Real-rime PCR
<i>myod1</i> -Q-R	GCTGCTGTTATCGGTGGAGA	XM_005449137	
<i>myogenin</i> -Q-F	CACAGGACAGAAGACTGGGAT		
<i>myogenin</i> -Q-R	TAGCCAGCTTGGTCATACGC	NM_001279526	
<i>myf5</i> -Q-F	CTGCTCTGATGGCATGGCTGA		
<i>myf5</i> -Q-R	CACGATACTGGACAGGCACTC	XM_005456634	
<i>panx3</i> -Q-F	TGAAATCTCCCTGGGGCCT		
<i>panx3</i> -Q-R	CATGAGAGAGTCCCAGCAGT	XM_003449812	
<i>vegfa</i> -Q-F	GTTACCATGGAGCTGATGAGG		Fluorescence in situ hybridization
<i>vegfa</i> -Q-R	ATGTAGGTGTAGGCTGGAGGT	XM_005467503	
<i>fgf11b</i> -Q-F	TGACAAAAGACCAGAGCCACAG		
<i>fgf11b</i> -Q-R	TGCTTTCATCCCTTGTGCTGTC	XM_019363459	
β -actin-Q-F	GGCATCACACCTTCTACAACGA		
β -actin-Q-R	ACGCTCTGTCAGGATCTTCA	XM_003443127	
<i>mstnb</i> -I-F	TAGACACGGATCAATGCGCT		
<i>mstnb</i> -I-R	CTCTGCGAAGGTCACAGCTA	XM_003458832	
<i>mstna</i> -I-F	CCACAGCATCAAGTCGAAA		Fluorescence in situ hybridization
<i>mstna</i> -I-R	GCGTGTCTCCACAGACTCTT	XM_003446535	
<i>laminin</i> -I-F	AATCTGGCCTACTGGACTGC		
<i>laminin</i> -I-R	ACAGTGGCACTGAAGGCAAT	XM_005454536	
M13 ⁺	CGCCAGGGTTTTCCAGTCACG		
M13 ⁻	AGCGGATAACAATTTCACACAG		

F, Forward primer; R, Reverse primer, respectively.

reactions are listed in Table 1.

2.4. Fluorescence in situ hybridization (FISH)

The skeletal muscle and brain partition tissues (medulla, hypothalamus, telencephalon) of adult tilapia of 6 mah tilapia were fixed overnight in 4% paraformaldehyde (Sigma-Aldrich, Germany) in 1 × PBS at 4 °C to check the cellular localization of *mstnb* gene expression in skeletal muscle and *mstn* (*mstna*/*mstnb*) gene expression in brain by FISH. After fixation, tissues were embedded in paraffin and cross-sections of 5–7 μm were cut using microtome (Leica, Germany). Fluorescence multi-color ISH were performed as described previously (Nakamoto et al., 2010; Zhou et al., 2007). Probes of sense and antisense digoxigenin or fluorescein labeled RNA strands were transcribed in vitro with RNA labeling kit (Roche, Germany) from the linearized plasmids DNA of tilapia *mstna*, *mstnb* and *laminin*, respectively. The images of FISH were taken under FV3000 laser confocal scanning microscope (Olympus, Japan). Primer sequences used for FISH are listed in Table 1.

2.5. Knockout of *mstnb* gene by CRISPR/Cas9

To verify negative regulation of *mstnb* gene on muscle growth in tilapia, the CRISPR/Cas9 technology was used to knockout *mstnb* gene in tilapia genome. The guide RNA (gRNA) target site of *mstnb* gene (GCAGCCTTCCGTCAGCACCC) was selected from sequences corresponding to CCN18NCC on the anti-sense strand of DNA (<http://zifit.partners.org/ZiFiT/>) as previously reported (Li et al., 2019).

For gRNA in vitro transcription, the DNA templates were obtained from pMD19-T 122 gRNA scaffold vector (kindly provided by Dr. JW Xiong, Peking University, China) using polymerase chain reaction (PCR) amplification (Chang et al., 2013). Forward primer (*mstnb*-Cas9-F: TAATACGACTCACTATAGCAGCCTTCCGTCAGCACCCCTTTA-GAGCTAGA.

AATAGC) and reverse primer (*mstnb*-Cas9-R: TGTAACATTTATGACAGTGGG) was designed for subsequent PCR amplification. The reaction mixture (25 μl) consisted of 2 μl of gRNA scaffold template, 2.5 μl of 10 × reaction buffer (MgCl₂⁺), 0.8 mM of each dNTPs, 0.2 μM of each primer, and 0.25 unit of TaKaRa Ex Taq DNA polymerase (Takara, Dalian, China). The PCR conditions were 94 °C for 3 min; 35 cycles of 15 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C; and a final extension of 10 min at 72 °C. The PCR products were purified using QIAquick Gel Extraction Kit (Qiagen, Germany) following the manufacturer's instructions. The Cas9 nuclease expression vector pcDNA3.1+ (Invitrogen, USA) was used for mRNA transcription as the protocol described previously (Chang et al., 2013). Plasmids were linearized with *Xba* I and purified by ethanol precipitation. In vitro transcription of both gRNA and Cas9 mRNA were performed with Megascript T7 Kit (Ambion, USA) by incubating at 37 °C for 4 h with 1000 ng of purified DNA as template. The transcribed gRNA and Cas9 mRNA were purified through phenol/chloroform extraction and isopropanol precipitation, and quantified using NanoDrop-2000 (Thermo Scientific, USA) before diluting to 500 ng/μl in RNase free water and then stored at −80 °C until further use.

Subsequently, microinjection, genomic DNA extraction and mutation assay were carried out as follows. Combinations of gRNA (500 ng/μl) with Cas9 mRNA (500 ng/μl) were microinjected into about four hundred one-cell stage embryos. The genomic DNA was extracted from pooled embryos, and DNA fragments spanning the *mstnb* targeting site were amplified using a pair of gene specific primers (Table 1) adjacent to the target site. A restriction enzyme site of *HpyAV* adjacent to the protospacer adjacent motif (PAM) sequence (ACC) was selected to screen the putative mutants of chimeric mutant F0 (XY) by restriction digestion, which was used to produce F1 generations. Subsequently, both XX and XY F1 fish carrying same mutation genotype were reared to produce the homozygous F2 generation. The homozygous *mstnb* mutant fish were screened by genomic PCR amplification with the same pair of gene

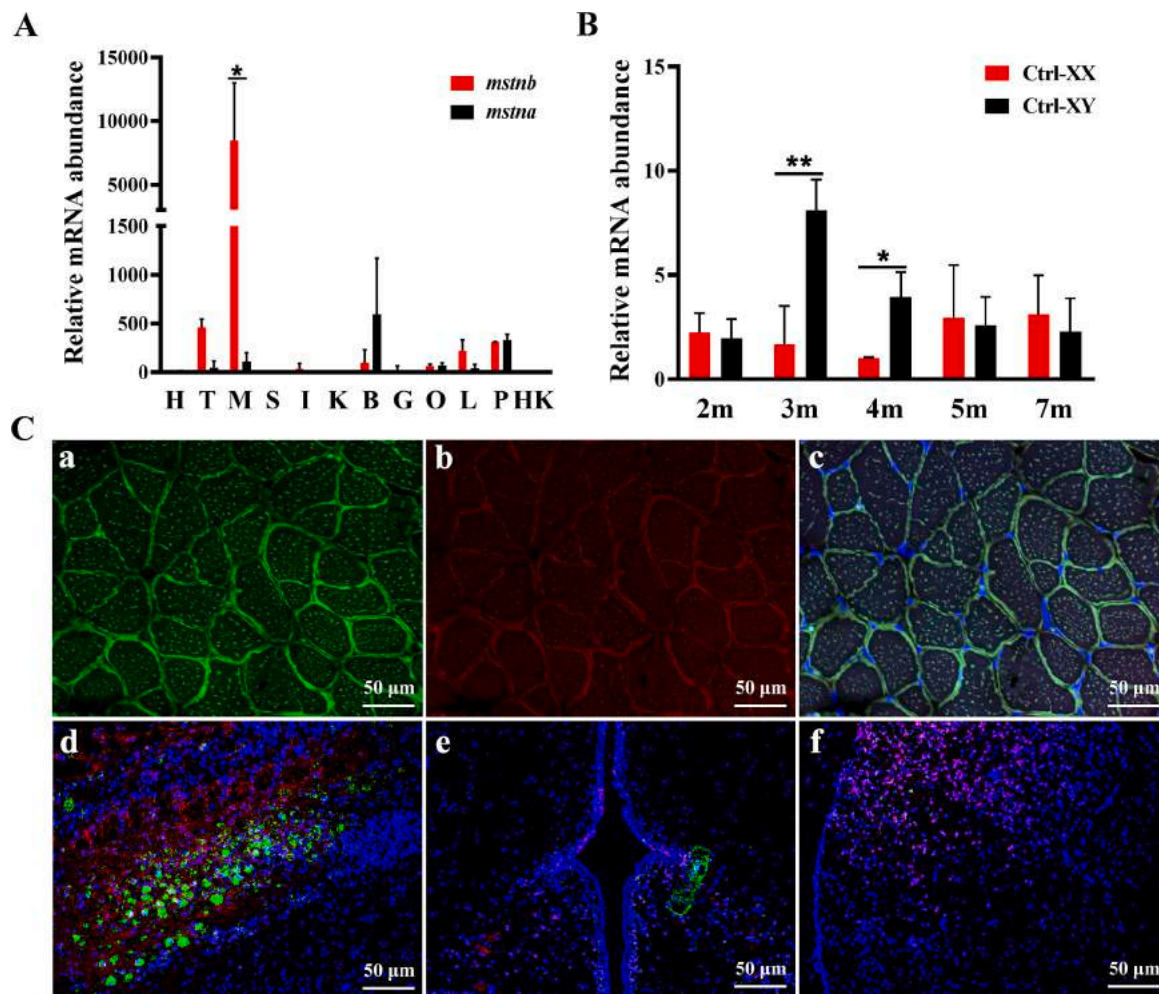


Fig. 1. Expression patterns of *msna* and *mstnb* in Nile tilapia.

(A) Expression of *mstn* genes in different tissues of adult tilapia by qRT-PCR. H, heart; T, testis; M, muscle; S, spleen; I, intestines; K, kidney; B, brain; G, gill; O, ovary; L, liver; P, pituitary; HK, head kidney. (B) Expression of *mstnb* mRNA in the tilapia skeletal muscle at different developmental stages, as determined by qRT-PCR. (C) Fluorescence in situ hybridization analysis of expression patterns of *mstn* in the skeletal muscle and brain of adult tilapia. (a) Green fluorescence represents the *mstnb* signal. (b) Red fluorescence represents the *laminin* signal. (c) Merge of *mstnb* (green) and *laminin* (red) signal. (d) Green and red fluorescence represent the *mstnb* and *msna* signal respectively in medulla. (e) Green and red fluorescence represent the *mstnb* and *msna* signal respectively in hypothalamus. (f) Green and red fluorescence represent the *mstnb* and *msna* signal respectively in telencephalon. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

specific primers (Table 1) and used for subsequent Sanger sequencing.

2.6. Morphological observation and growth performance analysis

To observe the phenotype of *mstnb* null tilapia, the offspring of the tilapia of *mstnb*^{+/+} and *mstnb*^{-/-} were raised together in the lab aquarium under the same conditions. Briefly, The F2 generation fries (*mstnb*^{+/+}; *mstnb*^{+/-}; *mstnb*^{-/-}) were maintained in the aquariums before genotyping analysis. At 2 mah, the F2 generation tilapias were genotyped by polyacrylamide gel electrophoresis (PAGE) with primers (Table 1) as previously reported (Li et al., 2013). Tilapia were then divided into two groups (*mstnb*^{+/+} group and *mstnb*^{-/-} group) and cultured in two rectangular tanks (1.3 m*0.6 m*0.8 m) with 25 fish in each tank and 2 replicates in each group and fed till satiation (3-times feeding per day (09:00, 15:00 and 19:00)) with commercial diets (Shengsuo, Shandong, China). Throughout the entire experiment, the water quality parameters like temperature (26 $\pm$ 2 $^{\circ}$ C), dissolved oxygen (> 5 mg/L), pH (7.05–7.20) and ammonia nitrogen (< 0.02 mg/L) were maintained as per standard breeding protocol of tilapia. When reaching 5 mah and 7 mah, the growth performance of the body length (the anterior tip of the snout to the base of tail fin), the body height (the

vertical distance at the top of a fish), the body width (the maximum distance from left to right of fish body) and the body weight (the weight of fish after wrapping the water) were measured in both wild type (WT) and *mstnb*^{-/-} tilapia. At 5 mah, the growth performance including weight gain rate, condition factor and specific growth rate were calculated in both *mstnb*^{+/+} and *mstnb*^{-/-} fish according to the formula as follows:

$$\text{Weight gain rate (WGR, \%)} = \frac{[(\text{final body weight} - \text{initial body weight}) / \text{initial body weight}] \times 100;}{}$$

$$\text{Condition factor (CF)} = \text{final body weight} \times 100 / \text{body length}^3;$$

$$\text{Specific growth rate (SGR, \% / day)} = \frac{[\ln(\text{final body weight}) - \ln(\text{initial body weight})] / \text{days of feeding}}{\times 100;}$$

2.7. Histological analysis on the skeletal muscle at 7 mah

Three fish from both *mstnb* knockout and the WT tilapia adult (male)

at 7 mah were randomly selected for histological analysis of skeletal muscle. Samples were sectioned transversely in three positions (dorsal fin, ventral fin and adipose fin) of the body. The cross sections at three different position were photoed and the total area of skeletal muscles were quantified by image J (Syed and Khan, 2018). Each sample was fixed in Bouin's solution for 24 h and sectioned (paraffin sectioning) in three different positions (6 μ m). The sections were subjected to H.E (hematoxylin and eosin) staining according to the traditional protocol. The images (40 $\times$) of H&E were taken under Olympus BX5 light microscope (Olympus, Japan).

2.8. Relative quantitative analysis of muscle fibers

To further compare the differences of muscle fiber size and numbers between *mstnb* null and WT tilapia, we analyzed the fiber area of muscle fibers at cephalosome basing on the morphological observations of H.E. staining. In total, three slides and 6 field of vision were randomly selected from three wild types and three *mstnb*^{-/-} tilapia at 7 mah for further quantification by Image J software. The number of skeletal muscle fibers of each field of vision was counted under the microscope.

2.9. Comparison of the growth performance between *mstnb*^{-/-} Nile tilapia and a commercial strain

To further identify the growth advantages of *mstnb*^{-/-} Nile tilapia, comprehensive comparisons of the growth performance metrics of *mstnb*^{+/+}, *mstnb*^{-/-} Nile tilapia and GIFT tilapia were carried out by culturing them in larger scale tanks (20 m³) under a natural photoperiod and an average temperature ranging from 25 to 32 °C. The commercial and healthy GIFT tilapia were purchased from a commercial aquaculture company (Guangdong, Haid) and kept for two weeks before experiments. Five fish (2 mah) from the *mstnb*^{+/+} Nile tilapia, GIFT tilapia and *mstnb*^{-/-} Nile tilapia group were distributed for each into three tanks respectively and set in triplicate for each group. Fish were fed three times daily to apparent satiation using a commercial diet (Shengsuo, Shandong, China) at 09:00, 15:00 and 19:00, from May 10, 2022 to August 10, 2022. The feeding for each tank was conducted in a fixed manner, which constitutes of 10 min of feeding followed by 10 min of non-feeding period. This feeding process was repeated 2 times during each of feedings and the feeding was carefully performed to prevent leftover diet. Total feeding amount was recorded daily as feed intake. Throughout the feeding trial, the water quality parameters were maintained as mentioned in 2.6. When reaching 5 mah, standard growth performance metrics were analyzed by calculating weight gain rate (WGR), condition factor (CF), specific growth rate (SGR) and Feed efficiency (FE) using the following formulae:

The calculating formula of WGR, CF and SGR as mentioned in 2.6.

$$\text{Feed efficiency (FE, \%)} = 100 \times (\text{final body weight} - \text{initial body weight}) / \text{Total feed intake}$$

2.10. Statistical analysis

Data were presented as mean $\pm$ standard deviation (SD). Results were statistically analyzed for significance by using the Student's *t*-test and one-way analysis of variance (ANOVA). Significant differences were considered as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$), respectively.

3. Results

3.1. Phylogenetic and syntenic analyses

Phylogenetic analysis revealed that duplication of *mstn* gene is a unique event only restricted to teleosts (Fig. S1). Furthermore, *mstnb* gene, but not *mstna*, showed conserved gene synteny, i.e. distribution of

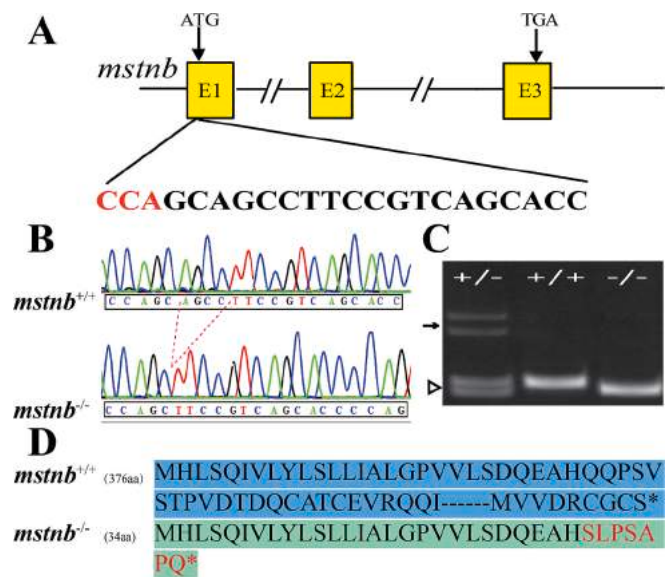


Fig. 2. Establishment of homologous *mstnb* gene mutant line.

(A) Schematic representation of gRNA targeting the *mstnb* locus. The gRNA was designed to target the open reading frame (exon I). The PAM (proto-spacer adjacent motif) site is highlighted in red. (B) A deletion of 4 bp in the genome of *mstnb*^{-/-} fish compared with *mstnb*^{+/+} fish was detected by Sanger sequencing analysis. (C) The genotypes were identified by heteroduplex motility assay (HMA) on PCR amplicons, which shows homoduplexes (open arrowhead) in wild type (WT) (+/+) and homozygous mutant (-/-) and both homoduplexes and heteroduplexes (arrow) in the heterozygous mutant (+/-). (D) In comparison with WT siblings, a putative truncated protein was produced in homologous *mstnb*^{-/-} fish. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

neighbor gene locus (*ORMDL1*, *PMSL* and *HIBCH* genes), between fish *mstnb* and *Mstn* in other vertebrates (Fig. S2A) and thus can be considered as the mammalian ortholog. Propeptide region, proteolytic region and C-terminal region of *mstnb* gene had high amino acid homology with mammals (Fig. S2B). This suggests that the *mstnb* gene might be important for skeletal muscle growth in tilapia, similar to that of mammals *Mstn* gene.

3.2. Difference of *mstna* and *mstnb* gene expression

In adult fish, qRT-PCR showed that *mstna* was mainly expressed in brain and pituitary, while the highest expression of *mstnb* was found in the muscle (Fig. 1A). *mstnb* transcriptions were higher at 3 mah and 4 mah XY fish than XX fish of same age group (Fig. 1B). *FISH* analysis showed that *mstnb* was co-localized with *laminin* in the basement membrane of skeletal muscle (Fig. 1C a-c). In brain, *mstna* was detected in the medulla, hypothalamus and telencephalon, while *mstnb* was mainly localized in medulla (Fig. 1C d-f).

3.3. Generation of *mstnb* gene mutation line by CRISPR/Cas9

To verify negative regulation of *mstnb* gene on muscle growth in tilapia, the homozygous mutant line was produced by CRISPR/Cas9 technology. The target site was selected on the first exon (Fig. 2A). Genotyping of F2 generation by genomic PCR and Sanger sequencing depicted a frame-shift (4 bp deletion) mutation in homozygous *mstnb* gene mutant line (Fig. 2B). In silico analysis showed that 4-bp deletion of *mstnb* caused early occurrence of a premature stop codon which led a truncated *mstnb* protein (34 aa, Fig. 2D). HMA (heteroduplex motility assay) identified the heterozygous *mstnb*^{+/-} individuals with both heteroduplex and homoduplex amplicons, while the *mstnb*^{+/+}, and *mstnb*^{-/-} individuals possessed only homoduplex amplicons (Fig. 2C).

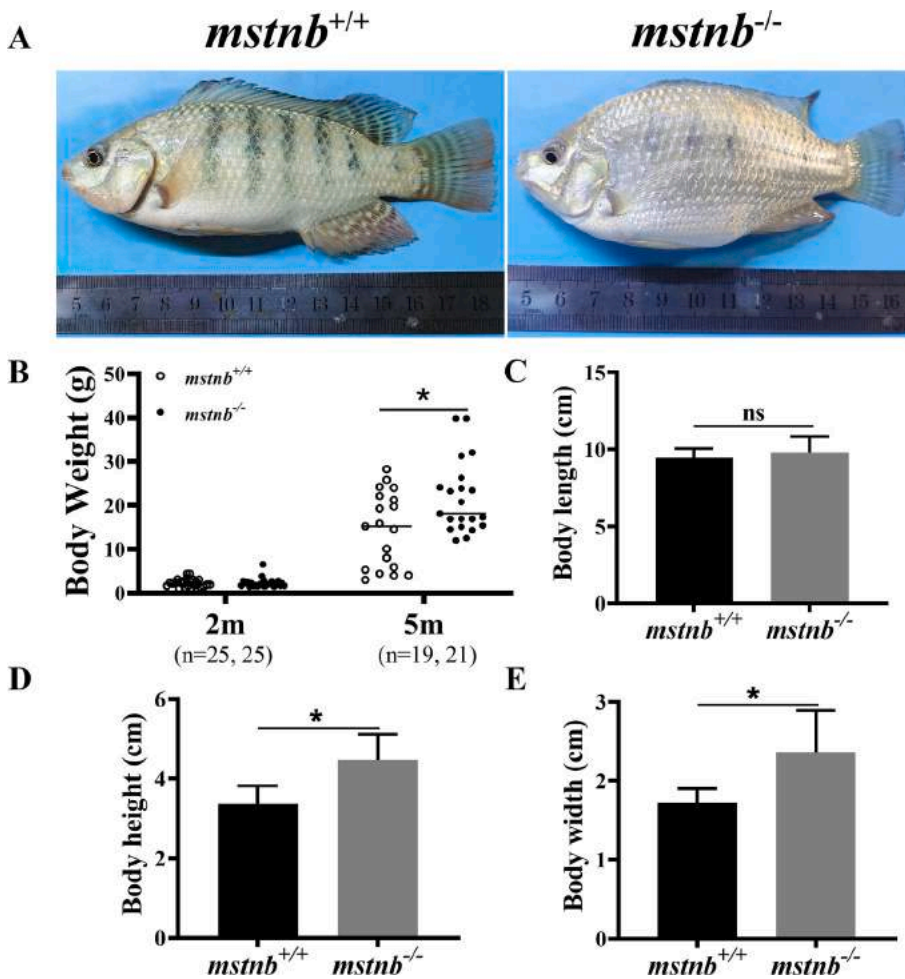


Fig. 3. The morphological and growth alterations in *mstnb*^{-/-} tilapia compared with their wild type (WT) siblings at 5 mah.

(A) Photos of WT and *mstnb* KO tilapia at 5 mah. (B) The average body weights of tilapia with different genotypes at 2 mah and 5 mah. Comparison of the average body lengths (C), body heights (D) and body widths (E) between *mstnb*^{+/+} and *mstnb*^{-/-} tilapia at 5 mah. Data are shown as mean $\pm$ SD. * $p < 0.05$.

Table 2
Growth performance of *mstnb*^{-/-} tilapia at 5 mah.

Parameter	<i>mstnb</i> ^{+/+}	<i>mstnb</i> ^{-/-}
Initial body weight (g)	2.35 $\pm$ 0.91 ^a	2.30 $\pm$ 1.06 ^a
Final body weight (g)	14.50 $\pm$ 8.60 ^a	21.67 $\pm$ 8.26 ^b
Weight gain rate, WGR (%)	400.13 $\pm$ 212.16 ^a	796.24 $\pm$ 116.65 ^b
Condition factor, CF (%)	3.46 $\pm$ 0.20 ^a	4.06 $\pm$ 0.34 ^b
Specific growth rate, SGR (%)	2.02 $\pm$ 0.57 ^a	2.49 $\pm$ 0.15 ^b

Date are shown as mean $\pm$ SD.

^{ab}Means in the same row with different superscripts were significantly different ($p < 0.05$).

3.4. Double-muscle phenotype and growth performance in *mstnb* null alleles at 5 mah

To verify whether the *mstnb* knockout tilapia have double-muscle phenotype, the appearances and growth traits between *mstnb*^{-/-} and *mstnb*^{+/+} tilapia were compared. We found that *mstnb*^{-/-} tilapia exhibited a typical double-muscle phenotype, characterized by obvious muscle hyperplasia mainly in the epaxial muscle at 5 mah. *Mstnb*^{-/-} tilapia has an oval-like body, which are quite distinct from the spindle-shaped *mstnb*^{+/+} tilapia. (Fig. 3A).

The body weight of *mstnb*^{-/-} and *mstnb*^{+/+} tilapia were measured at 5 mah and as expected, the *mstnb*^{-/-} tilapia were heavier (approximately 1.5 times) than their WT siblings. The average body weights of *mstnb*^{-/-} fish (21.67 $\pm$ 8.26 g, $n = 21$) was 49.45% heavier than *mstnb*^{+/+} fish (14.50 $\pm$ 8.60 g, $n = 19$) (Fig. 3B). The average body heights of *mstnb*^{-/-} fish (4.46 $\pm$ 0.65 cm) was 32.74% higher than *mstnb*^{+/+} fish

(3.36 $\pm$ 0.45 cm) (Fig. 3D). The average body widths of *mstnb*^{-/-} fish (2.36 $\pm$ 0.53 cm) was 37.21% wider than *mstnb*^{+/+} fish (1.72 $\pm$ 0.18 cm) (Fig. 3E). However, there was no significant difference in body length between *mstnb*^{-/-} fish (9.46 $\pm$ 1.07 cm) and *mstnb*^{+/+} fish (9.78 $\pm$ 0.60 cm) tilapia (Fig. 3C). These data suggested that the muscle growth of *mstnb*^{-/-} tilapia, showing a typical double-muscle phenotype, is accelerated at 5 mah.

Additionally, the growth performance parameters including WGR, CF, and SGR were significantly increased in *mstnb*^{-/-} fish at 5 mah (Table 2).

3.5. Sexual dimorphism in growth differences in *mstnb* null alleles at 7 mah

At 7 mah, the double muscling phenotype became more prominent (Fig. 4A). The average body weights of male and female *mstnb*^{-/-} tilapia were measured respectively at 7 mah. Interestingly, *mstnb*^{-/-} males had better growth performance than WT XY males, such as being heavier, longer, higher and wider. However, *mstnb*^{-/-} females grew slower than WT XX females. Moreover, sexual dimorphism growth performance was found. Significant differences of growth characteristics between two sexes i.e. male biased growths were recorded in *mstnb*^{-/-} tilapia. The average body weights of XX *mstnb*^{-/-} (27.30 $\pm$ 9.35 g, $n = 7$) was significantly lighter than XX *mstnb*^{+/+} fish (60.42 $\pm$ 14.30 g, $n = 7$) and the average body lengths were 31.55% shorter in XX *mstnb*^{-/-} fish (8.97 $\pm$ 0.78 cm) than their *mstnb*^{+/+} (11.80 $\pm$ 0.92 cm) counterpart (Fig. 4B, C). The average body height of XX *mstnb*^{-/-} fish (3.46 $\pm$ 0.46 cm) were also 35.68% shorter than *mstnb*^{+/+} fish (4.66 $\pm$ 0.45 cm) (Fig. 4E). The average body width of *mstnb*^{-/-} fish (1.46 $\pm$ 0.26 cm) was 27.40%

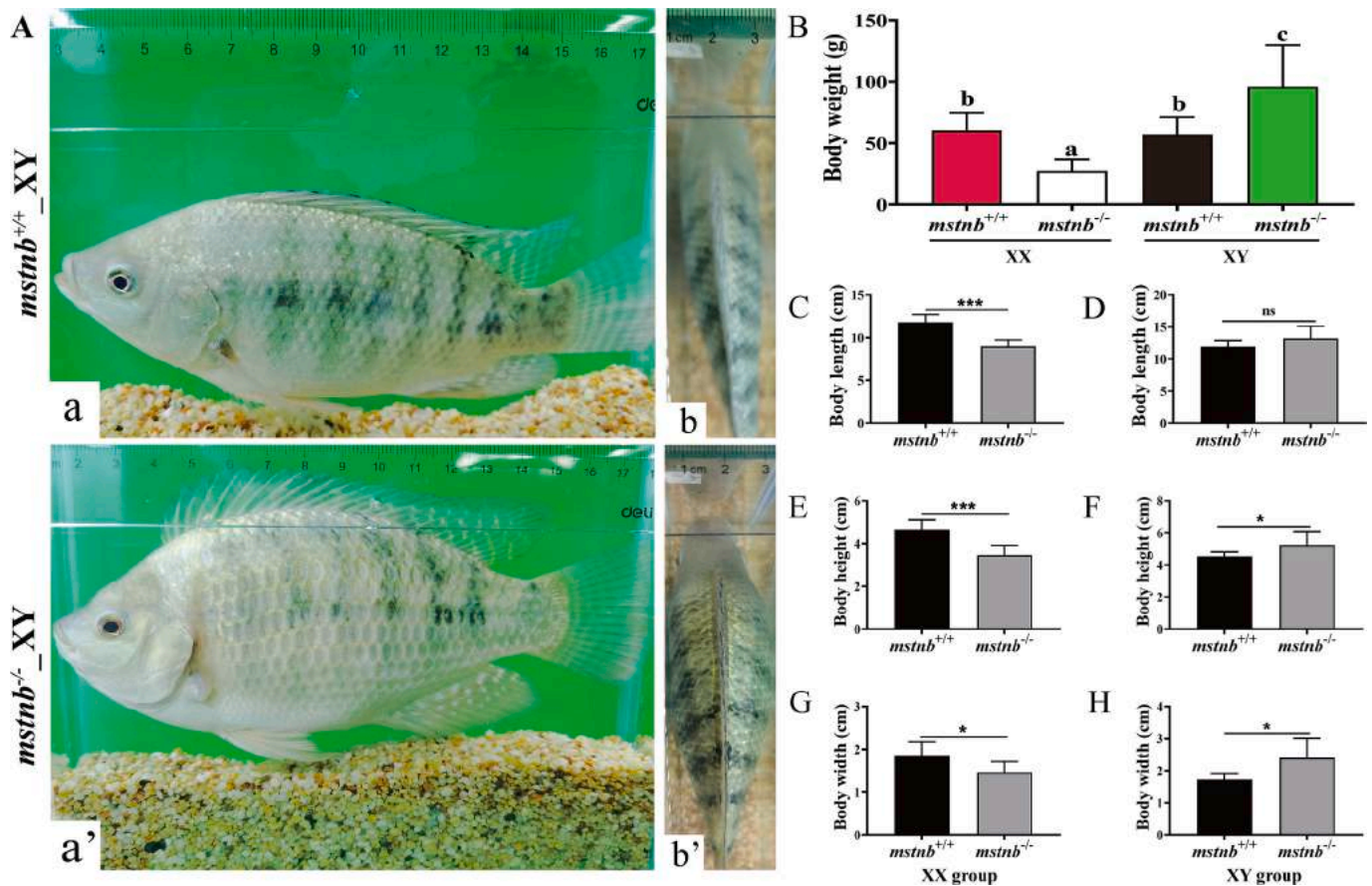


Fig. 4. The morphological and growth alterations in *mstnb*^{-/-} tilapia compared with their wild type (WT) siblings at 7 mah.

(A) Photos of WT (a) and *mstnb* KO (a') tilapia at 7 mah; photos of the back of WT (b) and *mstnb* KO (b') tilapia at 7 mah. (B) The average body weights of XX *mstnb*^{+/+}, XX *mstnb*^{-/-}, XY *mstnb*^{+/+} and XY *mstnb*^{-/-} tilapia at 7 mah. (C) The average body lengths of XX *mstnb*^{+/+} and *mstnb*^{-/-} tilapia at 7 mah. (D) The average body lengths of XY *mstnb*^{+/+} and *mstnb*^{-/-} tilapia at 7 mah. (E) The average body heights of XX *mstnb*^{+/+} and *mstnb*^{-/-} tilapia at 7 mah. (F) The average body heights of XY *mstnb*^{+/+} and *mstnb*^{-/-} tilapia at 7 mah. (G) The average body widths of *mstnb*^{+/+} and *mstnb*^{-/-} tilapia at 7 mah. (H) The average body widths of *mstnb*^{+/+} and *mstnb*^{-/-} tilapia at 7 mah. Data are shown as mean ± SD (*mstnb*^{+/+}_{XX}, n = 7; *mstnb*^{+/+}_{XY}, n = 8; *mstnb*^{-/-}_{XX}, n = 7; *mstnb*^{-/-}_{XY}, n = 8). * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001. mah, months after hatching.

thinner than *mstnb*^{+/+} fish (1.86 ± 0.32 cm) (Fig. 4G). On the other hand, the average body weights of XY *mstnb*^{-/-} fish (95.73 ± 33.70 g, n = 8) was significantly heavier than both XY *mstnb*^{+/+} fish (56.92 ± 14.40 g, n = 8) and XX *mstnb*^{-/-} fish (27.30 ± 9.35 g, n = 7) (Fig. 4B). The average body heights of *mstnb*^{-/-} fish (5.25 ± 0.83 cm) was 15.64% higher than *mstnb*^{+/+} fish (4.54 ± 0.27 cm) (Fig. 4F). Similarly, the average body width of XY *mstnb*^{-/-} fish (2.40 ± 0.62 cm) was 38.73% thicker than XY *mstnb*^{+/+} fish (1.73 ± 0.18 cm) (Fig. 4H). But, the average body length did not differ significantly between XY *mstnb*^{-/-} fish (13.21 ± 1.89 cm) and XY *mstnb*^{+/+} tilapia (11.91 ± 0.98 cm) (Fig. 4D).

3.6. Increase of skeletal muscle mass and muscle fiber numbers at 7 mah

To elucidate the effects of *mstnb* deficiency on skeletal muscle development of male tilapia, we compare the histological features of the skeletal muscle of cephalosome, trunk and rump regions of *mstnb*^{-/-} male tilapia with their WT male siblings at 7 mah. We performed H.E. staining and quantified the numbers of muscle fiber at 7 mah (Fig. 5 A). The muscle area in the cross sections of cephalosome, trunk and rump of *mstnb*^{-/-} tilapia (n = 3) were significantly larger than those of their WT siblings (n = 3) (Fig. 5 B, D). However, the number of muscle fibers per unit in *mstnb*^{-/-} male (68.25 ± 16.09, n = 3) were similar to *mstnb*^{+/+} male (73.51 ± 11.50, n = 3) (Fig. 5E). Additionally, the muscle fiber diameter also remained unaltered among WT and mutated groups

(Fig. 5C, F). Therefore, these data indicated that muscle cell hyperplasia not the hypertrophy, led to skeletal muscle growth and increased muscle mass.

3.7. Gene expression analysis of MRFs of *mstnb* KO tilapia at 5 mah and 7 mah

We investigated mRNA levels of MRFs (*myod1*, *myf5*, and *myogenin*) in skeletal muscle between *mstnb*^{+/+} and *mstnb*^{-/-} tilapia. At the juvenile stage (at 5 mah and 7 mah), the expression of *myod1* was upregulated in *mstnb*^{-/-} than that of *mstnb*^{+/+}. But the mRNA levels of *myf5* and *myogenin* were not significantly different between the two groups (Fig. 6A). Additionally, expression of genes concerning muscle fiber proliferation, such as *panx3*, *vegfa* and *fgf11b*, were significantly upregulated in *mstnb*^{-/-} fish than that of *mstnb*^{+/+} fish at 7 mah (Fig. 6B).

3.8. Achievement of better growth performance and feed efficiency by *mstnb* gene mutation

Consistently, the results showed that *mstnb*^{-/-} tilapia demonstrated a better growth performance in the wild natural environment comparing with the commercial strain. Furthermore, the parameters of WGR, CF, SGR and FE were significantly increased in *mstnb*^{-/-} Nile tilapia than those of *mstnb*^{+/+} Nile tilapia and GIFT tilapia (Table 3).

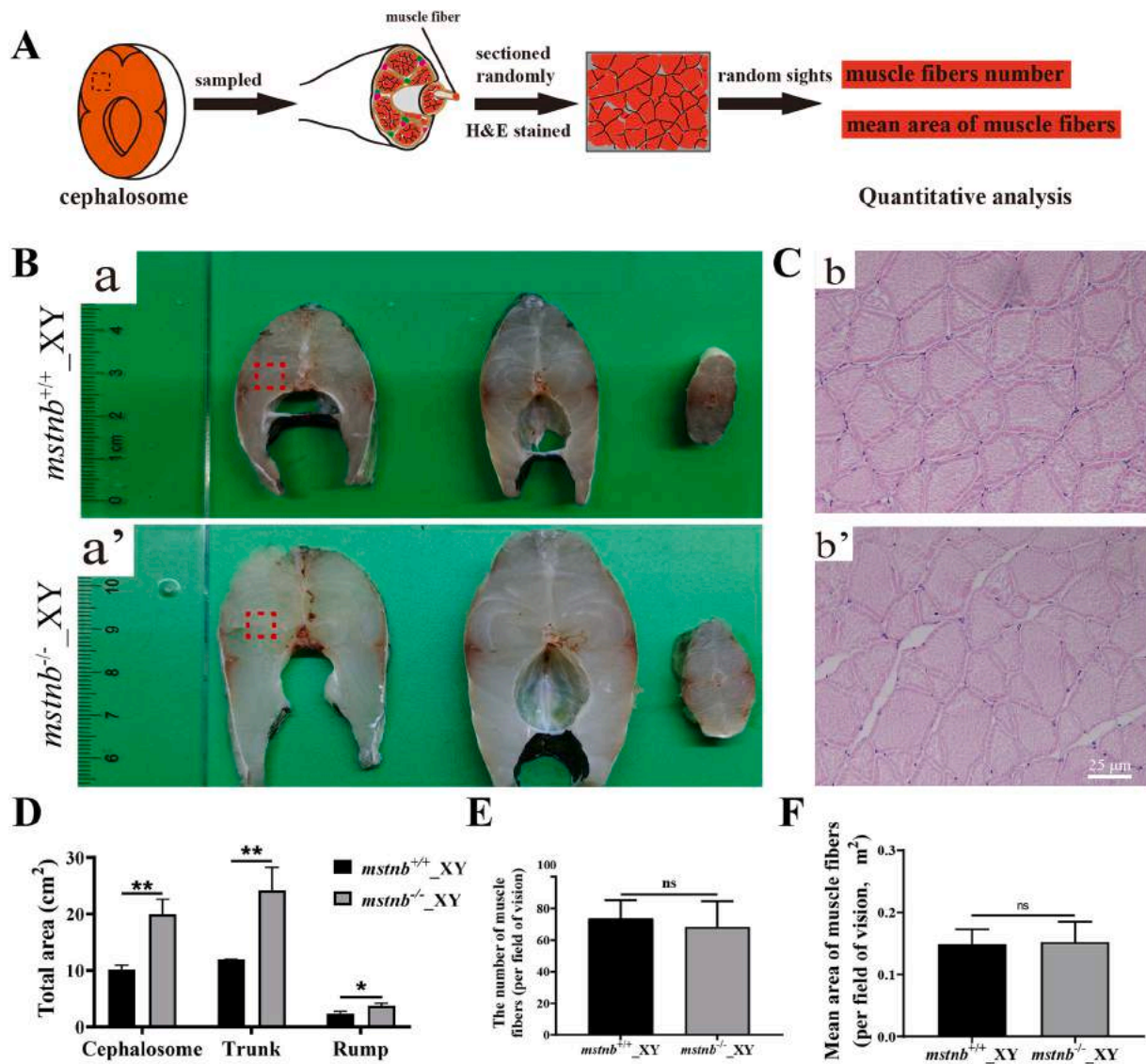


Fig. 5. Histological analysis and quantification on the skeletal muscle fibers of *mstnb* null tilapia and their wild type (WT) siblings at 7 mah.

(A) Schematic representation of quantitative analysis on muscle fibers between *mstnb*^{+/+} and *mstnb*^{-/-} XY tilapia. (B) Photos of the cross sections at the cephalosome, trunk and rump of *mstnb*^{+/+} (a) and *mstnb*^{-/-} (a') tilapia, respectively. (C) Histological observation (hematoxylin and eosin staining) showing the size of muscle fibers were similar between *mstnb*^{+/+} and *mstnb*^{-/-} XY tilapia. (D) Comparison of the cross sections between *mstnb*^{+/+} (a) and *mstnb*^{-/-} (a') XY tilapia. (E) Quantitative analysis of muscle fiber number within per field of vision at 7 mah. (F) Quantitative analysis of mean area of muscle fiber number within per field of vision at 7 mah. Statistics of skeletal muscle fibers in the skeletal muscle tissue section of *mstnb*^{-/-} (n = 3) and *mstnb*^{+/+} (n = 3) XY fish. Data are shown as mean ± SD. * p < 0.05, ** p < 0.01, *** p < 0.001. mah, months after hatching.

4. Discussion

Numerous efforts had been made to identify the negative effects of Mstn on muscle growth in vertebrates. It is well accepted that mutation of *mstn* gene improve the muscle mass and produce double-muscling phenotype in human, mouse, livestock, birds, as well as fish. In this study, characterization of two *mstn* genes, their expression profiles, and functions in muscle growth were extensively examined in tilapia.

In mammals, a single *mstn* gene had been identified in different species. Different from mammalian species, two *mstn* genes, designated as *mstna* (*mstn1*) and *mstnb* (*mstn2*) in teleost due to fish specific genome duplication (Roberts and Goetz, 2001). In tetraploid fish, four *mstn* genes had been found (Liu et al., 2012). Therefore, it is necessary to clarify the distinct expression profiles and possible roles of different isoforms in fish. Previous reports revealed that *mstna* is mainly detected in the brain, while *mstnb* is dominantly expressed in the muscle in Qi river crucian carp (*Carassius auratus*) (Wu et al., 2020), Helmet catfish

(*Cranoglanis boudierus*) (Xie et al., 2019), zebrafish (Wang et al., 2018). In zebrafish, deletion of *mstnb*, but not *mstna*, enhanced the muscle growth with muscle fiber hyperplasia (Gao et al., 2016). Only a single copy of *mstn* gene was isolated from medaka genome. Mutation of medaka *mstn* gene by CRISPR/cas9 technology resulted in the significant increase of both body length and weight, but decrease of muscle fiber density in the skeletal muscles, suggesting that *mstn* null mutation induces muscle hypertrophy in medaka (Chiang et al., 2016). Previous report revealed that mutation of *mstna*, but not *mstnb*, exhibited obvious double muscling phenotype with hyperplasia (Dong et al., 2014), indicating that different Mstn isoform might be involved in different physiological pathway in a species specific manner. In our present study, consistent profile of *mstn* genes expression in brain, pituitary and muscle, were detected in tilapia by qRT-PCR. Furthermore, FISH indicated that *mstnb* is abundantly expressed in the basement membrane of muscle fibers in white muscle, while *mstna* is detected in the brain regions medulla, hypothalamus and telencephalon. The expression profiles

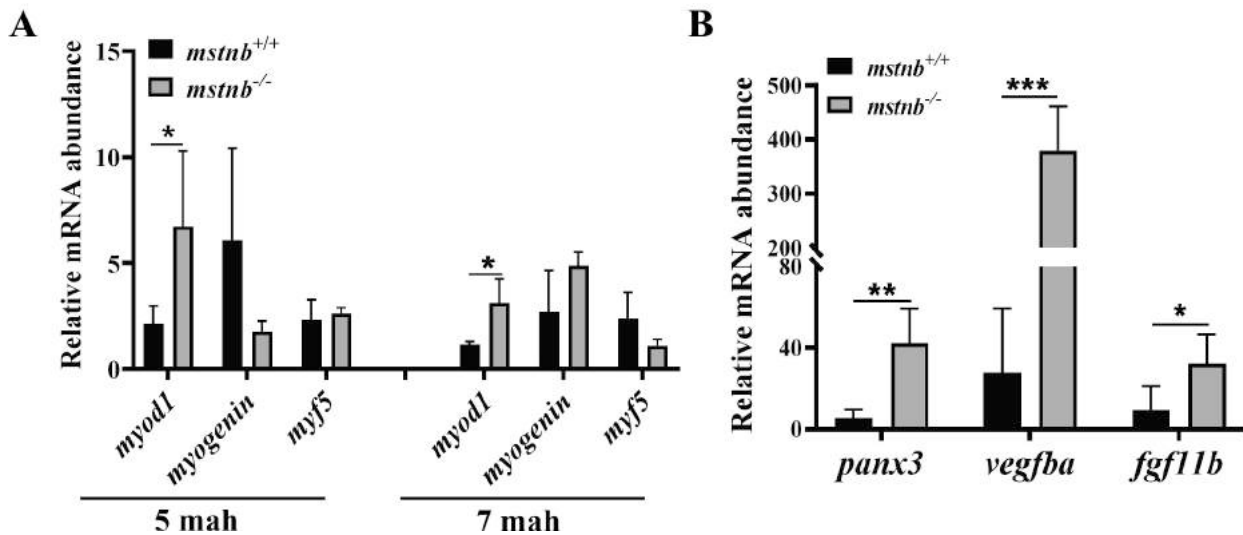


Fig. 6. Gene expression analysis in skeletal muscle of *mstnb*^{+/+} and *mstnb*^{-/-} tilapia at different developmental stages.

(A) MRFs expression at 5 mah and 7 mah in the skeletal muscle. (B) Changes of the expression level in the skeletal muscle of genes in relation to proliferation of muscle fibers were checked at 7 mah. Data are shown as mean $\pm$ SD ($n = 5$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. MRFs, myogenic regulatory factors. Mah, months after hatching.

Table 3

Growth performance and feed utilization of GIFT tilapia, *mstnb*^{+/+} and *mstnb*^{-/-} Nile tilapia.

Parameter	<i>mstnb</i> ^{+/+} Nile tilapia	GIFT tilapia	<i>mstnb</i> ^{-/-} Nile tilapia
Initial body weight (g)	2.06 $\pm$ 0.51	2.32 $\pm$ 0.68	2.12 $\pm$ 0.56
Final body weight (g)	47.49 $\pm$ 1.16 ^a	63.31 $\pm$ 0.20 ^b	78.73 $\pm$ 0.62 ^c
Weight gain rate, WGR (%)	2205.18 $\pm$ 56.53 ^a	2628.79 $\pm$ 8.45 ^b	3613.65 $\pm$ 29.08 ^c
Condition factor, CF (%)	4.04 $\pm$ 0.14 ^a	4.46 $\pm$ 0.64 ^a	5.81 $\pm$ 0.20 ^b
Specific growth rate, SGR (%)	3.49 $\pm$ 0.03 ^a	3.67 $\pm$ 0.01 ^b	4.02 $\pm$ 0.02 ^c
Feed efficiency, FE (%)	70.38 $\pm$ 0.52 ^a	74.07 $\pm$ 1.03 ^b	79.62 $\pm$ 0.81 ^c

Date are shown as mean $\pm$ SD.

^{abc} Means in the same row with different superscripts were significantly different ($p < 0.05$).

indicated that *mstnb* might be essential in regulating the inhibition of muscle growth, similar to zebrafish. However, for the first time we detected a male biased *mstnb* transcription in skeletal muscles. Given the importance of *mstn* in skeletal muscle growth and overall improvement of aquaculture (Kinoshita group reference, *Pagrus major*), it is essential to determine the sex-responsive *mstn* regulation in tilapia.

Mstn function in skeletal muscle growth regulation, especially in repression of muscle growth, is quite conserved among vertebrates. For instance, in mammalian species, MSTN mutation led to the more weight gain and increase of muscle mass in human beings (*Homo sapiens*) (McFarlane et al., 2011), mice (McPherron et al., 1997), cattle (McPherron and Lee, 1997), sheep (Crispo et al., 2015), dogs (*Canis lupus familiaris*) (Mosher et al., 2007). So far, *mstn*^{-/-} mutation strains had been generated in medaka, zebrafish and two aquaculture fish including red sea bream and yellow catfish (Chisada et al., 2011; Gao et al., 2016; Kishimoto et al., 2018; Zhang et al., 2020). In zebrafish, homozygous *mstnb*-deficient zebrafish exhibited 21% of weight increase compared with their control siblings after 80 dpf (Gao et al., 2016). In medaka, at the post-juvenile stage, the average body weight of the *mstn*^{-/-} increased by 25% than WT fish (Chiang et al., 2016). The breed carrying *mstn* gene mutation exhibited a 16% increase of skeletal muscle in red sea bream (Kishimoto et al., 2018). Further investigations in aquaculture fish are required to identify the functions of *mstn* gene in

muscle growth, which will contribute to the aquaculture profitability. In tilapia, we found that mutation of *mstn* gene exhibited double-muscling phenotype with a whopping 49.45% increase of body weight and body heights at both 5 mah and 7 mah. Contrasting to available information in zebrafish and yellow catfish where both females and males demonstrated higher growth, *mstnb*^{-/-} male tilapia had a much better growth performance than *mstnb*^{-/-} female tilapia at 7 mah. Therefore, male-specific growth dominance in *mstnb*^{-/-} in tilapia indicates that the mechanisms of the negative regulation of Mstn on skeletal muscle growth vary among different species in fish. Further investigations are required to explore the functions of Mstn in more fish species. In medaka, at the juvenile stage, only *myod1* gene expression was significantly increased in the *mstn*^{-/-} F2 fish relative to the WT. At the post-juvenile stage, in the *mstn*^{-/-} F2 fish, the expression levels of *myod1*, *myf5* and *myogenin* were significantly upregulated (Chiang et al., 2016). Similarly, in juvenile tilapia, only *myod1* gene expression was significantly upregulated in the skeletal muscle *mstn*^{-/-} strain comparing with the WT. Unfortunately, we haven't checked the expression of these genes during post-juvenile stages. Moreover, the significant increase of growth characteristics under laboratory conditions (WGR, CF, SGR) and under in the wild natural environment (WGR, CF, SGR, and FE) indicated that *mstnb*^{-/-} strain has the evident growth advantages than WT in Nile tilapia. Similar results were also obtained in red sea bream (Ohama et al., 2020). In addition, the growth performance and feed efficiency of GIFT tilapia, as a widely farmed tilapia strain on the market, is between *mstnb*^{+/+} and *mstnb*^{-/-} Nile tilapia under wild environment. Undoubtedly, our data also found GIFT tilapia strain does have a better growth performance and higher feed utilization than wild type Nile tilapia. Importantly, this work also revealed that the growth advantages obtained by *mstnb* knockout is much higher than GIFT tilapia strain screened by the traditional breeding methods. These experiments suggest gene editing is one of the promising tools for genetic breeding for aquaculture in future.

The mechanisms of fish Mstn involved in muscle growth are not fully understood. Previous reports revealed that the muscle cells in *mstn*^{-/-} fish are characterized by both hyperplasia and/or hypertrophy morphology in a species and platform dependent manner (Goossens et al., 2020). In zebrafish, *mstnb* deficient mutants showed an increased muscle fiber hyperplasia at the adult stage. However, the sizes of the muscle fibers in the *mstnb* deficient zebrafish decreased significantly compared with the WT control fish (Gao et al., 2016). In *mstn*^{-/-} F4

medaka, inactivation of *mstn* active domain by CRISPR/Cas9 technology led to increase of body length and muscle fiber hypertrophy, while no hyperplasia was detected (Yeh et al., 2017). Definitely, mutation of *mstn* caused the increase of growth performance and muscle mass in XY tilapia due to the significant increase of the cross-sectional areas of different part of skeletal muscle at trunk, even though no significant difference of density of muscle fibers were found between the *mstnb* null tilapia and their WT siblings. Therefore, these data indicated that the muscle fiber development in *mstnb* null tilapia did exhibit marked hyperplasia but not hypertrophy compared to that in their WT siblings at 7 mah, which showed the similar phenotypes as *mstn*^{-/-} yellow catfish and zebrafish.

Taken together, different expression profiles of two *mstn* gene revealed that *mstnb*, but not *mstna* gene, might be involved in the regulation of the muscle growth in tilapia. Furthermore, null mutation of *mstn* gene led to the significant increase of muscle mass in male fish via muscle fiber hyperplasia, but not hypertrophy. Therefore, production of fast growth and sterile *mstn*^{-/-} male tilapia is a good choice to explore new technology for gene editing breeding in tilapia.

5. Conclusions

Tilapia aquaculture accompanies propagation of all male fish for better productivity and economic return throughout the globe. In this study, using molecular analysis and CRISPR/CAS9 genome editing we determined the male biased negative regulatory roles of *mstnb* in tilapia skeletal muscle growth. Specifically, null mutation of *mstn* gene induced muscle fiber hyperplasia, but not hypertrophy, to increase the muscle mass by 150% in male tilapia. Though much research is required, our data and *mstn*^{-/-} gene editing technology might be a valuable addition towards the goal of profitable and sustainable tilapia aquaculture under the strict guidelines of ethical scrutiny and high standards of animal welfare.

Author contributions

Zhou L.Y., Dai X.Y. and Wu Y. conceived and designed the experiments; Wu Y. and Wu T.F. performed most of the experiments. Yang L.Y. and Zhao C.H. conducted the growth experiments. Li L. and Cai J. maintained the fish stocks. Zhou L.Y. and Wu Y. analyzed the data, interpreted the results, and drafted the manuscript; Zhou L.Y., Wang D. S. and Dai X.Y. edited the manuscript. All authors read and approved the final version of this manuscript.

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Author statement

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. All authors agree on the final version and agree to publish it.

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

No data was used for the research described in the article.

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