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TALENs-mediated gene disruption of myostatin produces a larger phenotype of medaka with an apparently compromised immune system

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ABSTRACT

Although myostatin, a suppressor of skeletal muscle development and growth, has been well studied in mammals, its function in fish remains unclear. In this study, we used a popular genome editing tool with high efficiency and target specificity (TALENs; transcription activator-like effector nucleases) to mutate the genome sequence of myostatin (MSTN) in medaka (*Oryzias latipes*). After the TALEN pair targeting *Ol*Myostatin was injected into fertilized medaka eggs, mutant G0 fish carrying different TALENs-induced frameshifts in the *Ol*MSTN coding sequence were mated together in order to transmit the mutant sequences to the F1 generation. Two F1 mutants with frameshifted myostatin alleles were then mated to produce the F2 generation, and these F2 *Ol*MSTN null ($MSTN^{-/-}$) medaka were evaluated for growth performance. The F2 fish showed significantly increased body length and weight compared to the wild type fish at the juvenile and post-juvenile stages. At the post-juvenile stage, the average body weight of the $MSTN^{-/-}$ medaka was ~25% greater than the wild type. However, we also found that when the F3 generation were challenged with red spotted grouper nervous necrosis virus (RGNNV), the expression levels of the interferon-stimulated genes were lower than in the wild type, and the virus copy number was maintained at a high level. We therefore conclude that although the $MSTN^{-/-}$ medaka had a larger phenotype, their immune system appeared to be at least partially suppressed or undeveloped.

1. Introduction

Myostatin (MSTN), which is also known as growth differentiation factor-8 (GDF-8), is a member of the transforming growth factor-β (TGF-β) superfamily. After protein secretion and proteolytic processing, a C-terminal active domain is generated and this subsequently forms an MSTN C-terminal homodimer. The MSTN C-terminal dimer then binds to membrane receptors to trigger activation of Smad2/3 [1–3]. The activation of Smad3 leads to the suppression of the expression of downstream myogenic genes [4,5], and MSTN has been shown to be a negative regulator of myogenesis [2,3].

In the MSTNs identified from mammals and fishes, the sequence of the C-terminal active peptide is conserved, with an amino acid identity of nearly 90% [6,7]. However, although a double muscle phenotype is commonly seen in MSTN null mammals, larger phenotypes are not so consistently found in MSTN-deficient higher teleost fish [8–10]. These inconsistencies arise because the *MSTN* gene is often duplicated (or even reduplicated) in fish but not in mammals [6]. Therefore, to investigate the role of MSTN in fish myogenesis, it is important to identify suitable fish species and a methodology which is conveniently able to suppress MSTN in these fish.

A trade-off between growth and immunity is seen in a wide range of animals from mammals through to fish and insects [11–13]. Often, however, the molecular mechanisms that underlie this trade-off remain unclear, and there is therefore a need for good

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model organisms in which these mechanisms can be conveniently studied. Inhibition of the MSTN has been considerable to be tested as a potential therapy for muscle degenerative diseases like muscular dystrophy [14]. Chronic kidney disease and obesity mice increase the MSTN expression which could be related to NF-kB-dependent pathway and p38 MARK pathways [15], suggesting that MSTN expression level is probably important for a host immunity.

Medaka (*Oryzias latipes*), also known as Japanese rice fish, has been widely used as a lower teleost fish model to investigate gene function [16]. We selected this fish for our study because the entire medaka genome sequence is already known [17], and because it containing only a single ortholog of the mammalian MSTN [18]. Knowledge of the entire genome also makes it easy to use genome editing techniques to specifically mutate the *MSTN* gene.

Because genome editing with engineered nucleases (GEEN) is quick and easy compared to traditional knock-out methods, techniques such as transcription activator-like effector nucleases (TALENs) have recently been applied in many studies across a range of species [19-21]. The TALEN backbone contains the FokI nuclease fused with a DNA binding domain derived from the transcription activator-like (TAL) effector of plant pathogenic bacteria in the genus *Xanthomonas* [22]. The target genomic DNA binding specificity of TALENs is determined by a series of tandem repeats, called repeat variable di-residues (RVDs) [23,24]. These series of tandem repeats individually target the sense or antisense strands of the genomic sequence of interest, and after target DNA binding, the DNA double-strand break (DSB) is cleaved by a dimer of fused FokI nucleases that is attached to the TAL effectors [25]. The nonhomologous end-joining (NHEJ) repair system will subsequently be triggered, and the end result will be a disrupted gene with insertion and/or deletion (indel) mutation [26].

In this study, we successfully generated MSTN mutated medaka from the G0 ~ F3 generations by using TALENs-mediated mutagenesis. We investigated the expression of Smad2/3-regulated down-stream MRFs and the growth performance of the TALENs-mediated MSTN-mutated F2 medaka. Further, since recent studies have shown that MSTN null/silencing may alter the immune responses [15,27], we also used a virus challenge test to investigate the effect of TALENs-mediated MSTN-mutation on the expression of interferon-stimulated genes (ISGs) in MSTN-mutated ($MSTN^{-/-}$) F3 medaka. To our knowledge, this is the first report on the growth and immune responses of TALENs-mediated MSTN mutant fish and their offspring.

2. Materials and methods

2.1. Design and construction of MSTN-TALENs

Medaka *MSTN* was identified from the Ensembl medaka genome browser (Ensembl gene No. ENSORLG00000015057). *Ol*MSTN-TALENs were designed and constructed following the protocol as previously described by Ansai et al. [28]. Briefly, we searched for potential TALENs target sites upstream of the MSTN C-terminal mature domain using the TALE-NT 2.0 program (https://tale-nt.cac.cornell.edu/) [29] with the following parameters: (i) 14–17 bp for spacer length; (ii) 15–18 bp for repeat array length; (iii) only T in the upstream base. The TAL effector modules were assembled and cloned into the array plasmids pFUS by the Golden Gate assembly method [30]. The target sites of the MSTN-TALENs RVD modules for the left and right binding arms were NI-NN-NG-NN-HD-NN-NN-NN-HD-NN-HD-NI-NN-HD-NG and NN-NN-HD-HD-NN-HD-HD-NG-NN-HD-NG-HD-HD-NN-HD, respectively. The assembled repeat arrays were then respectively cloned into the expression vectors pCS2TAL3DD and pCS2TAL3RR [31].

2.2. mRNA synthesis and microinjection of MSTN-TALENs

TALENs mRNA was synthesized using the protocols described in Ansai et al. [28]. Briefly, TALENs mRNA was synthesized *in vitro* from the *Not*I linearized TALEN expression vectors using an mMessage mMachine SP6 kit (Life Technologies). Before microinjection, the transcribed mRNAs were purified using an RNeasy Mini kit (Qiagen) and diluted with Yamamoto's Ringer's solution (0.75% NaCl, 0.02% KCl, 0.02% $CaCl_2$, and 0.002% $NaHCO_3$, pH 7.3) [32]. Finally, MSTN-TALENs mRNA pairs of the same dosage as in Ansai et al. [28] were simultaneously microinjected into Cab strain fertilized eggs at the single cell stage using the medaka microinjection method as previously described in Kinoshita et al. [33].

2.3. Mutation analysis of MSTN-TALENs mediated medaka

To observe the mutation genotypes of the TALENs-mediated MSTN-mutated medaka, genomic DNA was individually extracted from the caudal fin of adult fish by using the methods of alkaline lysis (25 mM NaOH and 0.2 mM EDTA) and neutralization (40 mM Tris–HCl, pH 8.0) as described in Ansai et al. [28]. Subsequently, the primerset *Ol*MSTN-F1/*Ol*MSTN-R1 (Table 1) was used to amplify the target sequence of the MSTN-TALENs. The resulting amplicons were cloned into TA cloning vector (Bioman, Taiwan) and sequenced.

2.4. Quantification of the mRNA expression of myogenic regulatory factors (MRFs) in MSTN-mutated ($MSTN^{-/-}$) F2 medaka using real time PCR

To determine the gene expression of the myogenic regulatory factors, skeletal muscles were taken from four to eleven individual samples of wild type (WT; i.e. non-genome-edited Cab strain) F2 medaka and from MSTN-mutated F2 medaka at the juvenile and post-juvenile stages (2 and 4 weeks post-hatching, respectively). Total RNAs from these skeletal muscle samples were extracted by REzol C&T reagent (Protech Technology, Taiwan), and cDNAs were synthesized by Superscriptase II Reverse Transcriptase (Invitrogen) with Anchor-dTv primer. The relative expression of these MRFs (MyoD, Myf5, and Myogenin) and EF1α housekeeping gene were quantified by real-time PCR with the primer sets MyoD-Q-F/MyoD-Q-R, Myf5-Q-F/Myf5-Q-R, Myogenin-Q-F/Myogenin-Q-R, and EF-1α-Q-F/EF-1α-Q-R. The gene expressions of the MRFs were then normalized with respect to *EF-1α* using the $2^{-\Delta\Delta CT}$ method. Data values were proportionally adjusted to the WT expression level, which was set to 1. Statistically significant differences between WT and $MSTN^{-/-}$ F2 medaka were calculated by student's t-test. Primer sequences are listed in Table 1.

2.5. Growth assessment of MSTN-mutated ($MSTN^{-/-}$) F2 medaka

To observe the phenotype of MSTN-mutated ($MSTN^{-/-}$) F2 medaka, the body weight and standard length were measured using a modified version of the protocol described previously by Chisada et al. [18]. In brief, thirty fertilized eggs of WT and MSTN-mutated ($MSTN^{-/-}$) F2 medaka were incubated in the medaka embryo culture medium (0.0001% methylene blue, 0.1% NaCl, 0.3% KCl, 0.004% $CaCl_2 \cdot 2H_2O$, 0.016% $MgSo_4 \cdot 7H_2O$). After hatching, twenty larvae were randomly chosen from each group, and maintained in 10 L plastic rearing containers under a 14-/10-h day/night cycle at 26 °C. During the measurement of body weight and standard length, fish were anesthetized in 0.003% eugenol (Sigma–Aldrich, Inc., MO, USA). The body weight and standard length were measured in 10 individuals from each group at 2, 3, 4, and 5 weeks post-hatching. Because cumulative mortality increased over time, in week 7, only 7 and 9 WT and $MSTN^{-/-}$ individuals were