# Analysis of myofiber composition in myostatin monoallelic mutant pigs

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## Abstract

Myostatin (MSTN) is a protein involved in the negative regulation of the growth and development of skeletal muscle. In this study, MSTN-monoallelic mutant pigs were developed to evaluate myofiber composition. MSTN monoallelic mutant zygotes were prepared using oocytes obtained from a local slaughterhouse and epididymal spermatozoa collected from MSTN biallelic mutant boar generated using CRISPR/Cas9-mediated gene editing. After embryo transfer, two MSTN monoallelic mutant piglets were born. Immunohistochemical analysis revealed that type I myofibers were significantly reduced in one of the MSTN monoallelic mutant pigs (female) compared to the wild-type littermates (male), which is consistent with a previous study evaluating MSTN monoallelic mutant pigs. On the other hand, another MSTN monoallelic mutant pig (male) had significantly more type I myofibers than wild-type littermates. In contrast to previous studies, more type I myofibers in MSTN heterozygous mutant pigs were identified. Although further analysis using an increased sample size is required to clarify the phenotype associated with MSTN mutation in pigs, the phenotypes of the MSTN monoallelic mutation may be influenced by differences in sex.

**Keywords**: CRISPR/Cas9-mediated gene editing, *in vitro* fertilization, *MSTN*, myofiber, pig

# Introduction

Myostatin (MSTN) is a protein involved in the negative regulation of skeletal muscle growth and development. *MSTN* inactivation causes the double muscling phenotype in several species, including cattle, dogs, sheep, and humans (Stinckens et al., 2011). In pigs, muscular hypertrophy and decreasing fat mass were also observed phenotypically by the inactivation of *MSTN* (Bi et al., 2016; Cai et al., 2017; Qian et al., 2015; Wang et al., 2015). These studies evaluated phenotypes of *MSTN* mutants carrying homozygous mutations because the majority of *MSTN* mutant pigs have been generated by somatic cell nuclear transfer techniques using *MSTN* biallelic mutant cell lines.

Skeletal muscle fiber is classified into type I (slow-twitch), type IIa and type IIb (fast-twitch) fibers (Lefaucheur et al., 1995). In humans, muscle fiber type is thought to be associated with obesity and weight loss (Tanner et al., 2002). *MSTN* mutants show increasing type II myofiber proportions in pigs (Tanihara et al., 2016), and in mice (Wang et al., 2012). A previous study reported that *MSTN* monoallelic mutant pigs decreased the expression of MSTN protein, enhanced myofiber quantity, increased *longissimus* muscle size, and decreased backfat thickness (Bi et al., 2016). However, few studies have evaluated the myofiber proportion in *MSTN* monoallelic mutant pigs. Pigs are excellent large animal models in biomedical research because of their close similarity with humans in several respects, particularly in terms of anatomy and physiology (Douglas, 1972). Inhibition of MSTN is expected as a therapy for muscle disease (Rodino-Klapac et al., 2009), therefore the analysis of myofiber proportion in pigs will benefits to the studies of aging- and disease-related changes of skeletal muscle in humans.

Gene-edited pigs lacking the function of *MSTN* have been produced previously using the CRISPR/Cas9 (Tanihara et al., 2016). In this study, *MSTN* monoallelic mutant pigs were produced using epididymal spermatozoa collected from *MSTN* biallelic mutant pigs, and myofiber composition of these pigs was evaluated.

# Materials and Methods

**Location of research and ethics**

This study was conducted at the Laboratory of Animal Reproduction, located on the Ishii Campus of Tokushima University (Latitude 34.060719, Longitude 134.452810). The animal experiments were approved by the Institutional Animal Care and Use Committee of Tokushima University (approval number: T28-21). All animal care and experiments were performed in accordance with the Guidelines for Animal Experiments of Tokushima University based on the regulations of the Japanese Standards relating to the care and keeping and reducing pain of laboratory animals.

**Preparation of cryopreserved epididymal spermatozoa**

The collection and cryopreservation of epididymal spermatozoa were conducted as described previously (Kikuchi et al., 1998). Epididymal spermatozoa were collected from epididymides isolated from approximately 12-month-old wild-type (WT) crossbred boar and 7-month-old *MSTN* biallelic mutant boar, which carried a 1 bp insertion allele and a 1 bp insertion with a 2 bp modified allele (Fig. 1A), generated in our previous study (Tanihara et al., 2016). These spermatozoa were cryopreserved and subsequently used for *in vitro* fertilization (IVF).

**Oocyte collection, in vitro maturation and fertilization**

Oocyte collection, *in vitro* maturation, and IVF were conducted as previously described (Do et al., 2015). Briefly, pig ovaries were obtained post mortem from prepubertal crossbred gilts (Landrace × Large White × Duroc breeds) at a local slaughterhouse and were transported within 2 h to the laboratory in physiological saline at 30°C. Cumulus-oocyte complexes with a uniform ooplasm and compact cumulus cell mass were collected, and cultured in maturation medium at 39 °C in a humidified incubator containing 5% CO2. Matured oocytes were subjected to IVF using frozen-thawed *MSTN* biallelic mutant and WT epididymal spermatozoa.

**Embryo transfer**

Two recipient surrogates, whose estrous cycles had been synchronized, were used for embryo transfer as described previously (Onishi et al., 2000). Approximately 24 h after the start of IVF, 150 embryos fertilized by *MSTN* biallelic mutant spermatozoa and 100 embryos fertilized by WT spermatozoa were mixed and transferred to the oviducts of a recipient gilt on the third day after human chorionic gonadotropin injection under anesthesia.

**Analysis of genotypes**

The genotypes of the resulting piglets were analyzed by polymerase chain reaction (PCR). The genomic regions flanking the mutation detected in the *MSTN* biallelic mutant spermatozoa used in this study were amplified by PCR using specific primers (forward, 5′-ATGCAAAAACTGCAAATCTATG-3′ and reverse, 5′- TGTAGGCATGGTAATGATCG-3′) with genomic DNA extracted from ear biopsies. The PCR products were directly sequenced by Sanger sequencing using a BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Thermo Fisher Scientific, Waltham, MA, USA) and an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

**Immunofluorescence staining**

To analyze the distribution of skeletal muscle fiber types, paraffin-embedded sections of *longissimus thoracis* muscle biopsy samples obtained from the 40-day-old piglets were deparaffinized. Antigen retrieval was performed by autoclaving the slides in citrate buffer (pH 6.0) for 15 min. Slow (type I) and fast (type II) myofibers were detected using mouse anti-slow skeletal muscle myosin antibody (ab11083, 1/500, Abcam, Cambridge, UK) and rabbit anti-fast skeletal muscle myosin antibody (ab91506, 1/500, Abcam), respectively. The sections were subsequently incubated for 2 h at RT with Alexa Fluor 594 goat anti-mouse IgG (ab150116, 1/500, Abcam) and Alexa Fluor 488 goat anti-rabbit IgG (ab150077, 1/500, Abcam). After staining, six images were obtained per sample using a BZ-X710 microscope (KEYENCE, Osaka, Japan), and the percentage of slow and fast muscle fiber area was calculated using BZ-X Analyzer software (KEYENCE).

**Statistical analysis**

The percentage data of the proportion of type I myofibers in the total of type I and type II myofibers were subjected to arcsine transformation before performing an analysis of variance (ANOVA). The transformed data were evaluated using ANOVA, followed by protected Fisher's least significant difference tests. The analysis was performed using StatView (Abacus Concepts, Berkeley, CA, USA). Differences with a probability value ($P$) of 0.05 or less were regarded as significant.

# Results

One of the two surrogates that received the zygotes became pregnant, and two *MSTN* monoallelic mutant piglets were obtained that carried the same type of mutation in the *MSTN* gene (+1 bp) (Fig. 1B). These two piglets and one WT littermate were grown for six months under standard protocols, using commercial, phase fed (JA Nishinihon Kumiai Shiryou, Hyogo, Japan), and body weight was measured (Fig. 2A). After six months, the body weight of pig #2 was greater than that of pig #1 and pig #3. Immunohistochemical analysis revealed that the proportion of type I myofibers was significantly reduced in one of the *MSTN* monoallelic mutant pigs (#2, female) compared with the WT (#1, male) ($P < 0.05$). However, another *MSTN* monoallelic mutant pig (#3, male) had a significantly greater proportion of type I myofibers compared with the WT ($P < 0.05$) (Fig. 2BC).

Figure 1

A

| Boar | | Genome sequence† | Indels |
|---|---|---|---|
| *MSTN* biallelic mutant (Tanihara et al., *Science Advances* 2:e1600803. 2016) | target | tgctcgcTGTTCTCATTCAGATCCACGGgaccagc | |
| | | tgctcgcTGTTCTCATTCAGATTCCACGGgaccagc | +1bp |
| | | tgctcgcTGTTCTCATTCAGAGAACACGGgaccagc | +1bp, m2bp |

B

| Piglet | Gender | Mutation | | Genome sequence† | Indels |
|---|---|---|---|---|---|
| #1 | ♂ | WT | target | tgctcgcTGTTCTCATTCAGATCCACGGgaccagc | |
| | | | | tgctcgcTGTTCTCATTCAGATCCACGGgaccagc | none |
| | | | | tgctcgcTGTTCTCATTCAGATCCACGGgaccagc | none |
| #2 | ♀ | Mono allelic | target | tgctcgcTGTTCTCATTCAGATCCACGGgaccagc | |
| | | | | tgctcgcTGTTCTCATTCAGATTCCACGGgaccagc | +1bp |
| | | | | tgctcgcTGTTCTCATTCAGATCCACGGgaccagc | none |
| #3 | ♂ | Mono allelic | target | tgctcgcTGTTCTCATTCAGATCCACGGgaccagc | |
| | | | | tgctcgcTGTTCTCATTCAGATTCCACGGgaccagc | +1bp |
| | | | | tgctcgcTGTTCTCATTCAGATCCACGGgaccagc | none |

**Figure 1**. Generation of *MSTN* monoallelic mutant pigs. (**A**) Genotype of *MSTN* biallelic mutant boar that provided epididymal spermatozoa used in this study. (**B**) Analysis of the genome sequence of piglets #1, #2 and #3.

Figure 2

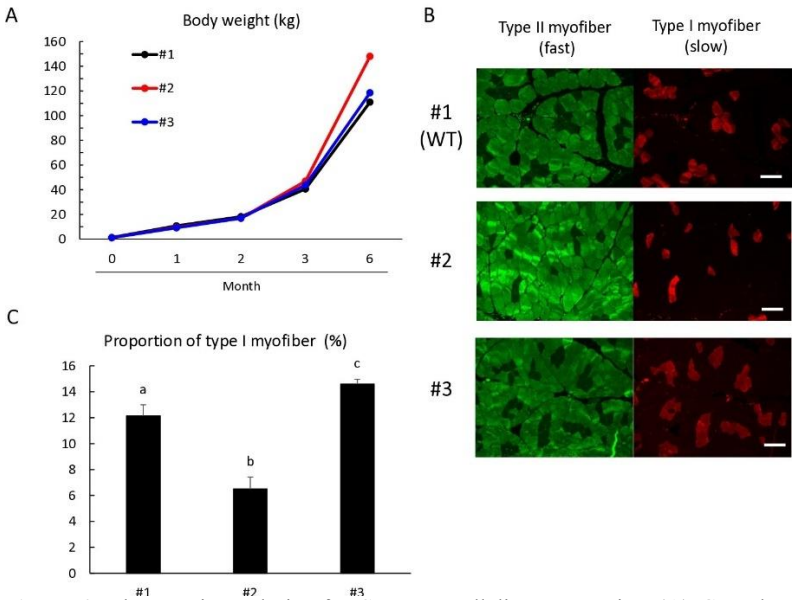


**Figure 2**. Phenotypic analysis of *MSTN* monoallelic mutant pigs. (**A**) Growth curve of the *MSTN* monoallelic mutants (#2 and #3) and wild-type (WT) pig (#1). (**B**) Immunohistochemical analysis of myofiber composition in the *longissimus thoracis* muscle. The scale bar in each panel indicates 100 μm. (**C**) The proportion of type I muscle fibers of the total of type I and type II muscle fibers. a–cValues with different superscripts differ significantly ($p < 0.05$). Error bars indicate mean ± standard error of mean (SEM).

# Discussion

In this study, one piglet carrying a *MSTN* heterozygous mutation were observed to produce a decreasing proportion of type I myofibers in the *longissimus thoracis* muscle, which is consistent with a previous study evaluating *MSTN* monoallelic mutant pigs (Xing et al., 2017). However, the proportion of type I myofibers in one *MSTN* heterozygous pig was significantly higher than that in the WT. The pigs analyzed in this study were produced using oocytes derived from a local slaughterhouse, indicating that the genetic background was different. Furthermore, differences between the sexes have a possibility to affect the phenotypes of *MSTN* mutants. By knockout of the *MSTN* gene in rats, body weight and size were significantly influenced only in male (Gu et al., 2016). On the other hand, the body weight of *MSTN* heterozygous Duroc × Meishan hybrid pigs was statistically similar in males and females (Li et al., 2020). In a previous study that evaluated myofiber composition in pigs, *MSTN* mutants were generated by the somatic cell nuclear transfer technique (Xing et al., 2017), therefore, knowledge of the influences of sex-specific factors and genetic background was quite limited.

**Conclusion**

In contrast to previous studies, an increasing proportion of type I myofibers in *MSTN* heterozygous mutant pigs was found, which may have been affected by sex-specific factors and/or genetic background. Further analysis using an increased sample size is required to clarify the phenotypes of *MSTN* mutation in pigs as animal models.

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**Conflict of interest**

All authors declare that they have no competing interests.

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