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Establishment and phenotypic analysis of an *Mstn* knockout rat

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ABSTRACT

Myostatin (*Mstn*) is an inhibitor of myogenesis, regulating the number and size of skeletal myocytes. In addition to its myogenic regulatory function, Mstn plays important roles in the development of adipose tissues and in metabolism. In the present study, an *Mstn* knockout rat model was generated using the zinc finger nuclease (ZFN) technique in order to further investigate the function and mechanism of Mstn in metabolism. The knockout possesses a frame shift mutation resulting in an early termination codon and a truncated peptide of 109 amino acids rather than the full 376 amino acids. The absence of detectable mRNA confirmed successful knockout of *Mstn*. Relative to wild-type (WT) littermates, Knockout (KO) rats exhibited significantly greater body weight, body circumference, and muscle mass. However, no significant differences in grip force was observed, indicating that Mstn deletion results in greater muscle mass but not greater muscle fiber strength. Additionally, KO rats were found to possess less body fat relative to WT littermates, which is consistent with previous studies in mice and cattle. The aforementioned results indicate that *Mstn* knockout increases muscle mass while decreasing fat content, leading to observed increases in body weight and body circumference. The *Mstn* knockout rat model provides a novel means to study the role of Mstn in metabolism and *Mstn*-related muscle hypertrophy.

1. Introduction

Myostatin (*Mstn*), also known as growth and differentiation factor 8 (GDF-8), is a member of the secreted transforming growth factor-β (TGF-β) superfamily of secreted growth factors and is a negative regulator of skeletal muscle development [1]. In mammals, *Mstn* is primarily expressed in skeletal muscle [2], although weak expression in other tissues such as adipose [2], heart [3], mammary gland [4], spleen [5], and placenta [6] have also been observed. The fact that the myostatin protein sequence is highly conserved from rodents to human suggests conservation of function [7,8]. *Mstn* knockout mice exhibit dramatically increased muscle mass from an increased number of muscle fibers (hyperplasia) formed during development and increased fiber size (hypertrophy) in adulthood [2,9]. Likewise, naturally occurring *Mstn* gene mutations generate the similar phenotype of muscular hypertrophy in many different mammalian species including cattle, sheep, dogs, and humans [10]. Mstn regulates muscle homeostasis, as myostatin upregulation is observed in skeletal muscle atrophy and in the pathogenesis of muscle wasting during cachexia associated with different diseases (i.e. cancer, chronic heart failure, AIDS, sarcopenia) [1,11]. What's more, the use of antisense RNA, neutralizing antibodies, or chemical inhibitors against myostatin can improve this observed muscle wasting in animal models of disease [1,12–17].

The human Mstn gene is located on chromosome 2 (2q32.2) and is flanked by a number of binding sites and response elements for transcription factors and hormones, including MyoD (a myogenic regulatory factor), glucocorticoid, and androgen [18,19], suggesting

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myostatin could participate in multiple metabolic pathways. In addition to regulating muscle development and homeostasis, myostatin also exerts effects on adipocyte differentiation, as well as adipose accumulation and turnover. Myostatin has been shown to inhibit or stimulate adipogenesis in various cell culture systems [20], but the reduced expression of the adipogenic markers, such as CCAAT/enhancer binding protein α and peroxisome proliferator-activated receptor γ [21], as well as the reduced fat accumulation in *mstn*-deficient mice [21–23], suggest myostatin promotes adipogenesis *in vivo*. Furthermore, *mstn* deficiency can prevent high-fat diet-induced obesity through enhanced fatty acid oxidation and brown adipose formation in white adipose tissue [24]. Finally, myostatin has been found to reduce insulin sensitivity in response to neuronal stimulation of brown adipose tissue or via degradation of insulin receptor substrate 1 in response to high caloric intake [25,26]. Hence, myostatin appears to play an important role in metabolic pathways not directly related to muscle growth.

Multiple studies have implicated myostatin as a potential target for many human diseases. Most of these studies were performed using mouse models. Given the difficulty of modifying its largely intractable genome, few studies were carried out in rat, another well–established model for the genetic dissection of human disease. From an evolutionary point of view, rats are more genetically similar to humans and possess several genes not found in mice, including genes involved in immunity, chemosensation, detoxification and proteolysis. For proteolysis plays a regulatory function in myostatin signaling [27,28] and myostatin expression has an influence on cytokine secretion within the immune system [5], the activity of myostatin could be quite different between deficient rats and deficient mice. In fact, Mendias et al. recently reported that the muscle and tendon phenotype of *Mstn*-null rats established using the zinc finger nuclease (ZFN) technique was markedly different from that of *Mstn*-null mice, which have impaired contractility and pathological changes to fibers and their extracellular matrix [29]. While that study demonstrated some important distinctions between the mouse and rat models, it examined neither effects on fat content nor sex-specific phenotypes in *Mstn*-null rats.

The present study characterizes a novel *Mstn*-knockout rat model developed using the ZFN technique, examining the muscle mass and fat contents, to provide a foundation for further studies of *Mstn* function.

2. Materials and methods

2.1. Animals

All experimental protocols were reviewed and approved by the ethics committee Institute of Laboratory Animal Science of Peking Union Medical College. Male and female Sprague Dawley rats were purchased from Huafukang Biotech (Beijing, China) and maintained in the animal facility of the Institute of Laboratory Animal Science of Peking Union Medical College. Rats were housed with 12/12-h dark/light cycle and given free access to food and water. Measurements were taken to minimize pain and discomfort of animals during all procedures.

3. Materials

*Cla*I enzyme, *Taq* enzyme, dNTP mixture, polymerase chain reaction (PCR) buffer, SYBR Premix Ex *Taq*II, and PrimeScript 1st Strand cDNA Synthesis Kit were purchased from Takara Co., Ltd (Dalian, China). Diethylpyrocarbonate (DEPC) H_2O and TRIzol reagent were purchased from Invitrogen (Shanghai, China). Proteinase K and agarose were purchased from Sigma (Shanghai, China).

4. Methods

4.1. ZFN construct design and in vitro transcription

ZFN constructs were co-designed by Sigma-Aldrich (MO, USA). The methods have been described in detail elsewhere [30–32]. In brief, ZFNs were designed to target the genomic region around rat *mstn* exon 1 and each were tested for their efficiency in knocking out gene expression in the rat cell line C6. Cells were transfected with the ZFNs and harvested the following day. DNA and RNA were isolated and Surveyor nuclease cleavage analysis was performed according to the manufacturer's protocol (Transgenomic, NE, USA).

4.2. Creation of Mstn KO rat and sequencing confirmation

Embryos and microinjection were performed as previously described [33]. Genomic DNA from the ZFN-modified founder animal was sequenced to confirm the presence of the ZFN-induced mutation. In brief, genomic DNA was extracted from the tails of newborn (10 d) rats using the salting-out method (ref). PCR was performed to amplify the *Mstn* sequence with the primers 5′-GGCATGGTAATGATTGTTTCCGTG-3′ (forward) and 5′-TTTACCTGTTTGTGCTGATTGCTGC-3′ (reverse). Conditions for PCR amplification were set as: 94 °C pre-denaturation for 5 min, 94 °C denaturation for 30 s, 60 °C annealing for 30 s, 72 °C elongation for 1 min, 35 cycles, and 72 °C final elongation for 10 min. Confirmed ZFN-mediated *Mstn* knockout F1 founder rats were backcrossed to WT Sprague Dawley rats. Heterozygous rats were crossed to generate *Mstn* homozygous KO rats. To reduce the probability of nonspecific ZFN-mediated deletion effects, F1 rats were backcrossed with WT Sprague Dawley rats for 5 generations.

4.3. Mstn mutant genotyping

Genomic DNA was extracted from the tails newborn (10 d) rats. Primers and PCR conditions were employed as above. PCR products were digested by *Cla*I restriction endonuclease at 30 °C overnight and fractioned on a 2% agarose gel.

4.4. RNA extraction and real-time PCR

Rats were anesthetized with 0.6% sodium pentobarbital (0.8 ml/100 g) and the trapezius dorsi muscle tissues were collected for RNA extraction using TRIzol. Reverse transcription (RT) was performed on total RNA (500 ng) and the cDNA products were used as a template for quantitative real-time PCR (qRT-PCR) (Applied Biosystems 7500, ABI, CA, USA). SYBR green was used as a fluorescent label and primers specificity was tested by applying the melting curve cycle. qRT-PCR conditions were as follows: 95 °C pre-denaturation for 5 min, 95 °C denaturation for 30 s, 60 °C annealing for 30 s, 72 °C elongation for 30 s, 40 cycles, followed by final denaturation at 95 °C for 15 s and final elongation at 60 °C for 1 min *β-actin* was used as an internal control and the expression of *Mstn* was represented by $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{mutantMstn} - Ct_{mutant\text{-}\beta\text{-}actin}) - (Ct_{wild\ type\text{-}Mstn} - Ct_{wild\ type\text{-}\beta\text{-}actin})$.

4.5. Primer sequences for Mstn

Forward: 5′- GATCGATCAGTACGACGTCCA-3′
Reverse:5′-GAAAGTCAGACTCGGTAGGC-3′

4.6. Primer sequences for β-actin

Forward: 5′- CACCCGCGAGTACAACCTTC-3′
Reverse:5′-CCCATACCCACCATCACACC-3′

4.7. Recording body weights

Male (*Mstn* $^{+/+}$ (n = 7), *Mstn* $^{+/-}$ (n = 5), and *Mstn* $^{-/-}$ (n = 6)) and female (*Mstn* $^{+/+}$ (n = 7), *Mstn* $^{+/-}$ (n = 6), and *Mstn* $^{-/-}$ (n = 6)) rats were selected and weighed weekly from the 2nd to the 17 th week after birth. Body weights were compared via 2-way analysis of variance (ANOVA) and statistical significance was defined as $P < 0.05$.

4.8. Measuring muscle weight

Male rats aged 5-, and 20-weeks-old (n = 3 per group) were anesthetized with 0.6% sodium pentobarbital (0.8 ml/100 g). The trapezius dorsi muscle was collected, weighed, and photographed alongside. A Vernier caliper was used to determine muscle thickness. For inconvenient measurement to muscle volume, we adopted an alternative method to measure muscle weight. ImageJ software was used to calculate the area (cm^2) of the muscle. Muscle weight per unit area (g/cm^2) was calculated by dividing the mass of the muscle (g) by the area of muscle (cm^2).

4.9. Measuring grip strength

Male rats aged 5-months-old, (n = 3 per genotype) were selected and measured for their grip strength using an YLS-13A dynamometer (Jinan Yiyan Technology and Development Co., Ltd., Shandong, China). During measurement, an experimenter grabbed the middle part of the rat tail and horizontally pulled it back with even force, until the rat withdrew its paws from the dynamometer. The dynamometer would automatically record the maximum grip strength in Newtons. The grip strength of each rat was averaged with 6 attempts.

4.10. MRI imaging

Male rats aged 5-months-old, (n = 3 per genotype) were anesthetized with 0.6% sodium pentobarbital (0.8 ml/100 g) and their fat content was assessed using an Agilent 7.0T magnetic resonance imaging (MRI) system for animal imaging (Agilent, 7T/160/AS, USA). The parameters of MRI were set as follows: TR/TE 230 ms/16 ms; FOV 70 mm × 45 mm; slice thickness 1 mm; interslice gap 1 mm; 4 slices; matrix 256 × 256; scanning time 3 min 55 s. The parameters for axial scanning were set as follows: FOV 40 mm × 45 mm. IRW software (Inveon Research Workplace) was used to calculate the fat content in each slice, which was combined to determine the whole-body fat content.

4.11. Statistical analysis

Data are presented as mean ± standard deviation (SD). Two-way analysis of variance (ANOVA) was performed for multi-group comparisons, while a *t*-test was performed for intergroup comparison. Statistical significance was defined as $P < 0.05$.

5. Results

5.1. Construction and identification of Mstn knockout rats

A ZFN construct engineered to target bp 277–281 of rat *Mstn* was used for the rat embryo injection (Fig. 1A). After multiple trials, 3 mutant *Mstn* rats were successfully created (Fig. 1B). Gene sequencing results indicated that the #6 ZFN-targeted sequence was changed from GATCA to AGTC, resulting in a frame shift mutation within the open reading frame (Fig. 1C). Comparison to the wild-type gene sequence revealed that the frame shift mutation generated an early termination codon, resulting in a truncated 109 amino acid peptides rather than the full-length 376 amino acid MSTN (Fig. 1C). Another effect of the #6 ZFN-mediated Mstn mutation was the removal of a *Cla*I restriction site (ATCGAT) (Fig. 1A). Thus, *Mstn* $^{+/+}$, *Mstn* $^{\pm}$ and *Mstn* $^{-/-}$ could be distinguished by PCR amplification followed by *Cla*I digestion. After agarose gel electrophoresis, fragment number was used to identify wild-type (2), *Mstn* homozygous knockout (1), and heterozygous rats (3) (Fig. 1D). To further verify functional *Mstn* knockout, qRT-PCR was performed to measure the expression of *Mstn* mRNA in *Mstn* $^{+/+}$, *Mstn* $^{\pm}$ and *Mstn* $^{-/-}$. In homozygous knockout rats, *Mstn* expression was 18.92± 3.33% ($P < 0.001$) of the wild-type expression level (Fig. 1E). This indicated that the open reading frame shift mutation could still be transcribed into mRNA, but the stability of mRNA might be affected.

5.2. The influence of Mstn on rat weight and size

Because *Mstn* is a negative regulatory factor of muscle growth, rats with *Mstn* deletion were expected to demonstrate an increase in muscle mass. To test this, the body weights of male and female *Mstn* $^{+/+}$, *Mstn* $^{\pm}$ and *Mstn* $^{-/-}$ rats were collected weekly from the 2nd to the 17 th week after birth. The results revealed that relative to male WT rats, the weights of male *Mstn* $^{-/-}$ rats were noticeably higher at week 4, gaining significance difference at week 8 (272.92 ± 1.97 g vs. 334.45 ± 18.21 g, $P < 0.05$). By the end of week 17, the difference was even greater (463.55 ± 6.58 g vs. 591.77 ± 36.31 g, $P < 0.001$) (Fig. 2A). While male *Mstn* $^{\pm}$ rats demonstrated body weights slightly higher than WT beginning at week 17, the differences were never statistical significant differences. After 17 weeks of feeding, the difference in the size of male *Mstn* $^{-/-}$ rats compared to *Mstn* (+/+) rats was apparent (Fig. 2C). Interestingly, the body weights of female *Mstn* $^{-/-}$ rats were only slightly higher than that of WT rats, with no statistical significant differences observed ($P > 0.05$) (Fig. 2B). The disparate results between male and female rats suggest that *Mstn* may function in a sex-specific manner. Because *Mstn* deletion did not significantly influence the weight and size of female rats, only male rats were utilized for further experiments.

5.3. The influence of Mstn on muscle development

Because *Mstn* negatively regulates muscle growth, muscle development was compared between WT and *Mstn* knockout rats. The abdominal external oblique muscle of 3-, 5-, and 20-week-old rats were collected, weighed, and measured for size. As predicted, *Mstn* $^{-/-}$ rats in all age groups possessed significantly greater muscle weight/size relative to WT rats (0.885 ± 0.026 g/cm^2 vs. 0.678 ± 0.042 g/cm^2 (30.59%), $P < 0.05$; 2.216 ± 0.049 g/cm^2 vs. 1.823 ± 0.058 g/cm^2 (21.54%), $P < 0.01$; 4.981 ± 0.451 g/cm^2 vs. 4.150 ± 0.466 g/cm^2 (20.04%), $P < 0.01$) (Fig. 3A). The thickness of the back muscle was measured for rats aged 20 weeks, revealing that *Mstn* $^{-/-}$ rats possessed abdominal external oblique muscle thickness significantly greater than WT rats (0.662 ± 0.008 mm vs. 0.502 ± 0.009 mm, $P < 0.0001$) (Fig. 3B). Given the visible difference in forelimb size (Fig. 3C), a dynamometer was used to measure the grip strength of 20-week-old *Mstn* $^{-/-}$ and WT rats. Despite the larger forelimb musculature, the average grip strengths of *Mstn* $^{-/-}$ and WT rats were not significantly different (Fig. 3D).

5.4. The influence of Mstn on rat fat content

To explore the function of *Mstn* in adipose generation and metabolism, differences in fat distribution were measured in 5-month-old *Mstn* knockout and WT rats. Relative to WT rats, *Mstn*

$^{-/-}$ rats displayed lower fat content in multiple regions, including the abdominal cavity, mesentery, and epididymis (Fig. 4A). MRI-generated quantitative measurements revealed that the fat content of *Mstn* $^{-/-}$ rats was 45% lower than of WT rats (14.22300 ± 1.16016 cm^3 vs. 25.91007 ± 1.12717 cm^3, $P < 0.0001$) (Fig 4B, C). Thus *Mstn* deletion not only enhanced rat muscle development but also diminished fat content.

6. Discussion

Using the ZFN technique, this study successfully established an *Mstn* knockout rat model, in which a frame shift mutation in exon 1 of the *Mstn* gene results in a truncated and non-functional MSTN protein.

Body weight comparisons revealed that *Mstn* knockout significantly increases the body weights of male *Mstn* knockout rats by nearly 20% compared with WT rats at 17 weeks of age. Previous studies have reported that *Mstn* naturally mutant animals (cattle, sheep, and humans) [7,34–39] or artificially-generated mutant animals (mice, rats) [29,40] generally demonstrate a 20–40% increase in muscle weights due to muscle hypertrophy or hyperplasia relative to WT animals. Therefore, the observed differences in muscle and body size in male *Mstn* $^{-/-}$ rats are not very surprising. Notably, no significant differences in body weight were found in female *Mstn* $^{-/-}$ rats, a finding inconsistent with studies of *Mstn* knockout mice, where differences between mutant and WT body weights were found to be relatively constant irrespective of age or sex [2,41]. Similarly, both male and female *Mstn* mutant mice were characterized as possessing pronounced musculature [40]. This sex-specific effect of Mstn on body weight might be a novel

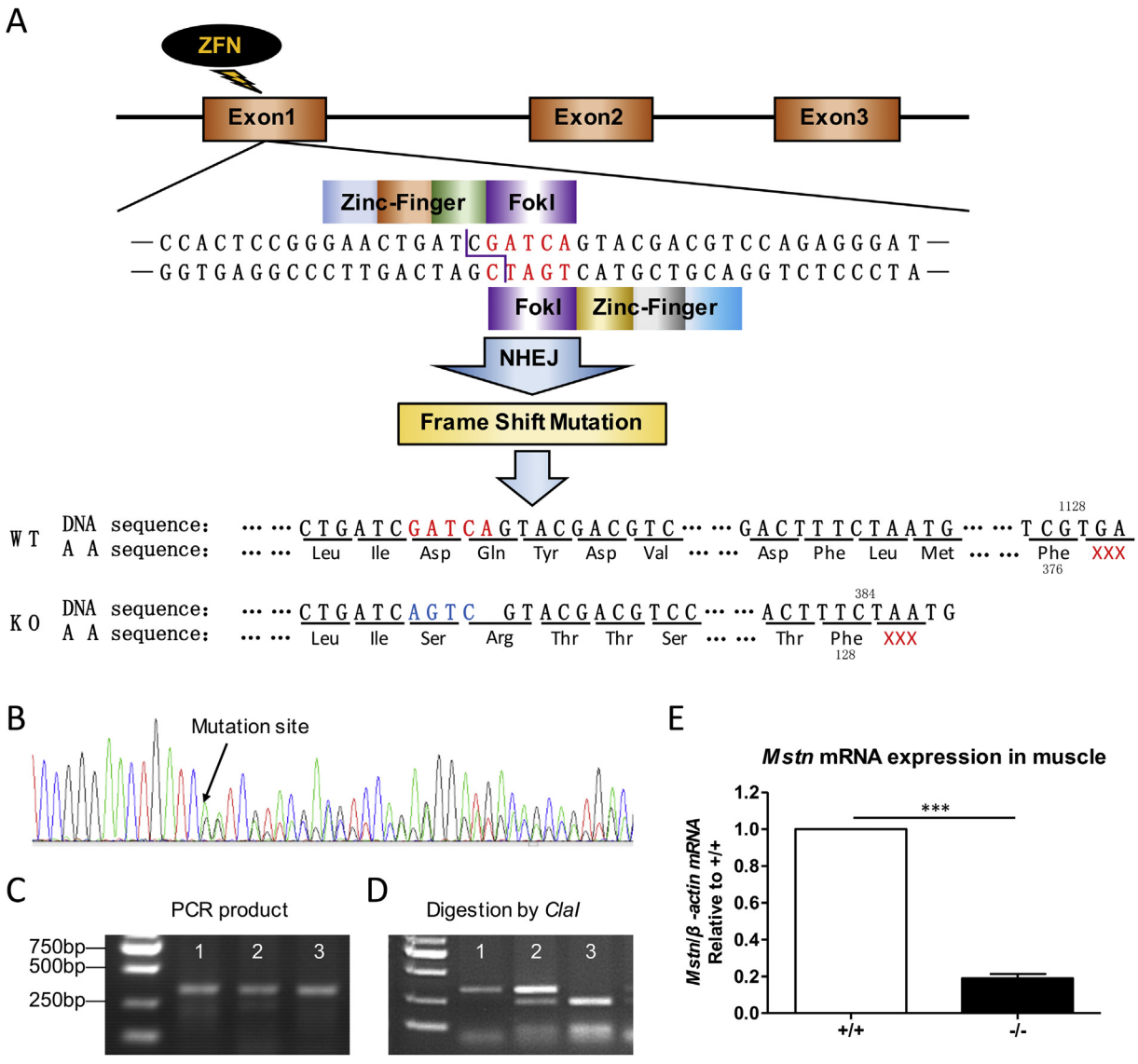

Fig. 1. Design of *Mstn* ZFN constructs and verification of *Mstn* knockout. (A) The design of restriction sites on *Mstn* ZFN constructs: Specific recognition sites were designed to facilitate *Fok*I endonuclease cleavage at a specific site within exon 1 of *Mstn*. The non-homologous recombination repair mechanism induced a frame shift mutation, resulting in an early termination codon and a truncated protein of 109 amino acids. The purple line indicates the *Cla*I restriction site (AT CGAT), which was removed during gene knockout. NHEJ: non-homologous end joining. (B) Sequencing of PCR products obtained from F1 rats: the arrow indicates the mutation site. (C) Electrophoresis of PCR products obtained from F3 rats: the products were indistinguishable at 337 bp each. (D) Electrophoresis of PCR products after *Cla*I digestion: WT PCR product (337 SSbp) predicted digestion products would be 244 bp and 93 bp. Wild-type rats possessed a clear band at 244 bp, *Mstn* knockout rats possessed a single band at 337 bp, and heterozygous rats possessed both the 244 bp and 337 bp band. Lane 1 represents *Mstn* knockout, lane 2 represents heterozygous, and lane 3 represents WT. (E) RT-PCR determination of *Mstn* mRNA expression in WT and *Mstn* knockout of rats. Mean ± SD, $N = 3$. ***$P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

distinction between mouse and rat models. On the other hand, notworthily *Mstn*$^{-/-}$ mice did not continue to gain weight beyond 6 months of age, and the total body weight of *Mstn*$^{+/+}$ mice were comparable to those of *Mstn*$^{-/-}$ mice by 9–10 months of age [22], but to male *Mstn* $^{-/-}$ rats, the difference in body weitht seemed to lifelong. The recent establishment and characterization of another knockout rat model, produced by the ZFN-mediated deletion of the splice acceptor of exon 2 of *Mstn*, did not investigate potential sex-specific differences in body weight [29]. It will be interesting to discover whether such gender differences are present in this model as well. Several lines of evidence support the idea that Mstn function is regulated disparately in males and females. Research on risk factors for sarcopenia among community-dwelling older adults revealed that while age was associated with sarcopenia in both genders, malnutrition conferred significantly higher odds for sarcopenia in women [42]. Hormones possibly account on the differences between two genders, since *Mstn* is flanked by number of binding sites for hormones [18,19]. For example, androgens can induce Mstn signaling in a negative feedback mechanism [18], perhaps the sex-specific differences observed are due to the relative abundance of androgens in males and females. Probably, wild-type male rats maintain proper muscle homeostasis via AR-mediated *Mstn* activation and male *Mstn* knockout rats developed a double-muscle phenotype. While in wild-type female rats, other mechnism not an AR mediated Mstn-dependent manner regulates the muscle homeostasis,, such that female *Mstn* knockout rats would not demonstrate greater body weight or muscle growth. Further research will be required to determine whether there is indeed a sex-specific role for Mstn.

The current study also demonstrated extraordinary muscle development in the back and limbs of male rats with *Mstn* konckout (data not shown). This was consistent with the results obtained in other species, which demonstrated a double-muscle phenotype. Histological staining was utilized in multiple studies to determine that this phenotype is characterized by muscle hypertrophy or hyperplasia [8,10]. Previous studies have analyzed the effects of Mstn on diameter and number of muscle fibers, but no study has evaluated the overall thickness of individual muscles. In this study, trapezius dorsi muscles were collected from wild-type and *Mstn* $^{-/-}$ rats, and the weight per unit area were calculated. The results reveal that *Mstn* knockout significantly increased weight per unit area and thickness of the musculature. However the bulkier muscles failed to translate into greater strength, as revealed using the dynamometer. Although the reason for this is unclear, it may be attributable to an *Mstn* knockout-induced change in the abundance of mitochondria within the myocytes, further decreasing cell phosphorylation capacity [43]. Comparable results were demonstrated in *Mstn* knockout mice, which possessed greater muscle mass, but no elevation in maximum muscle tension. In fact, several measures of strength were lower than that of wild-type mice; muscles from knockout mice contracted and relaxed faster during a single twitch, and were more prone to fatigue [44]. Further investigation revealed that genetic defects of *Mstn* results in a metabolic transition within the muscle cells from an aerobic to an anaerobic condition [43]. These results indicate that although *Mstn* inhibits muscle growth, it endows the skeletal muscle with higher oxidative capacity and fatigue resistance, which requires further examination.

The present study revealed that *Mstn* $^{-/-}$ rats possess significantly less fat than WT rats at 5-month old, as determined by

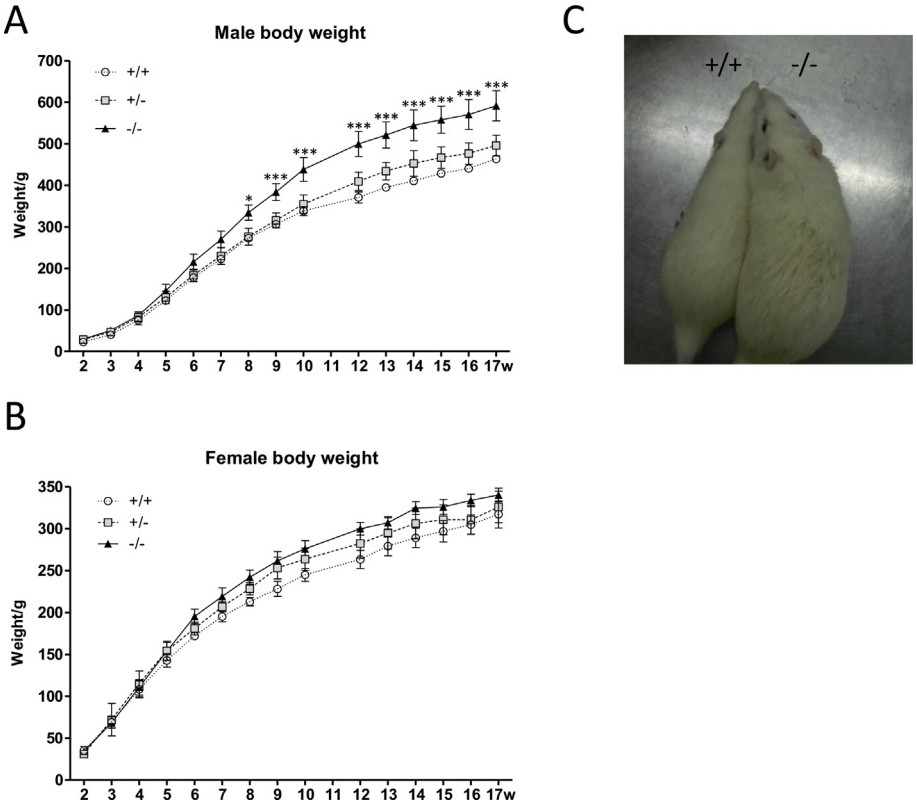

Fig. 2. The influence of *Mstn* deletion on rat weight and size. (A) The influence of *Mstn* deletion on male rat body weight: littermates of *Mstn* $^{+/+}$ ($n = 7$), *Mstn* $^{+/-}$ ($n = 5$), and *Mstn* $^{-/-}$ ($n = 6$) rats were selected and weighed weekly from the 2nd to the 17 th week after birth. (B) The influence of *Mstn* deletion on female rat body weight: littermates of *Mstn* $^{+/+}$ ($n = 7$), *Mstn* $^{+/-}$ ($n = 6$), and *Mstn* $^{-/-}$ ($n = 6$) rats were selected and weighed weekly from the 2nd to the 17 th week after birth. (C) Body size comparison of male littermates of *Mstn* $^{+/+}$ and *Mstn* $^{-/-}$ rats at 17-weeks old age. Mean ± SD. $^{*}P < 0.05$; $^{***}P < 0.001$.

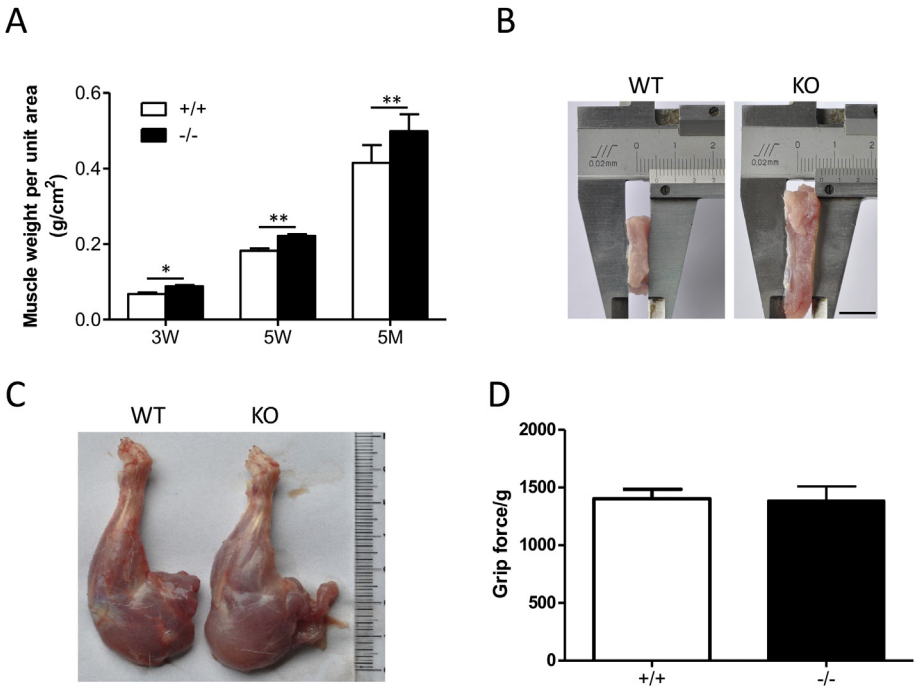

Fig. 3. The influence of *Mstn* on rat muscle development. (A) The weight of trapezius dorsi muscle for rats at different developmental stages. Trapezius dorsi muscles were collected, measured, and weighed to calculate the weight of muscle per unit area (n = 3 per genotype). (B) Measuring the thickness of trapezius dorsi muscle for 5-month-old rats. Bar = 1 cm. (C) Representative comparison of forelimbs collected at 5-month-old. (D) Measuring the grip strength of 5-month-old rats (n = 3 per genotype). Measurements for each rat were collected 6 times. The average was used for statistical analysis. Mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$.

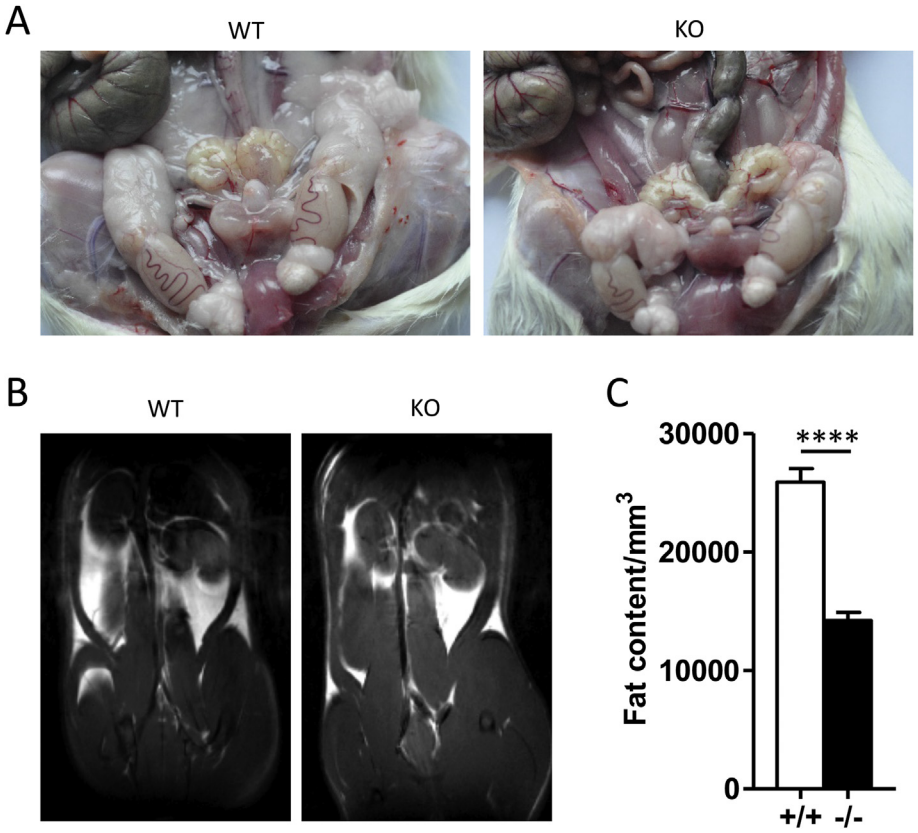

Fig. 4. The influence of *Mstn* on rat fat content. (A) Representative images of fat content in abdominal cavity of *Mstn* $^{+/+}$ and *Mstn* $^{-/-}$ rats. (B) Representative images collected by MRI for determination of fat content in 5-month-old male rats. (C) Statistical analysis of MRI results. $N = 3$. $^{****}P < 0.0001$.

quantitative MRI imaging. This reduction in fat growth is consistent with the results obtained from examples of *Mstn*-mutant cattle [45], humans [17,45], or *Mstn*-knockout mice [21,22] likely due to Mstn decreasing adipogenesis *in vivo* [20,21]. To *Mstn*-knockout mice the results were inconsonant though we accept the conclusion that fat contents is reduction B In *Mstn* knockout mice fat contents was not differences by the age of 11–14-week-old [2] until by 5~6-month-old [22] than in WT mice, while in another paper a significant differences was observed by the age of 12-week-old than that of WT mice [21]. Recently, *Mstn* knockout mice with a high-fat diet revealed a large increase in visceral fat contents than with normal diet, which is incompatible with *Mstn* knockout protecting against diet induced obesity in mice [41]. Therefore, further researchs are needed to explore the mechnism of Mstn affection on fat metabolism.

Currently, *Mstn* is being explored as a target to ameliorate muscular dystrophy or muscle atrophy initiated by multiple factors. Development of Mstn-related molecules or manipulation of endogenous Mstn levels also represent new potential strategies for preventing or treating metabolic diseases such as muscle wasting, obesity, and type 2 diabetes. The establishment of a rat *Mstn* knockout model that more closely mirrors human physiology will hopefully provide a valuable resource for future investigations into *Mstn* function.

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Transparency document

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References

[1] J.M. Argiles, M. Orpi, S. Busquets, F.J. Lopez-Soriano, Myostatin: more than just a regulator of muscle mass, Drug Discov. Today 17 (2012) 702–709.
[2] A.C. McPherron, A.M. Lawler, S.J. Lee, Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member, Nature 387 (1997) 83–90.
[3] M. Sharma, R. Kambadur, K.G. Matthews, W.G. Somers, G.P. Devlin, J.V. Conaglen, P.J. Fowke, J.J. Bass, Myostatin, a transforming growth factor-beta superfamily member, is expressed in heart muscle and is upregulated in cardiomyocytes after infarct, J. Cell. Physiol. 180 (1999) 1–9.
[4] S. Ji, R.L. Losinski, S.G. Cornelius, G.R. Frank, G.M. Willis, D.E. Gerrard, F.F. Depreux, M.E. Spurlock, Myostatin expression in porcine tissues: tissue specificity and developmental and postnatal regulation, Am. J. Physiol. 275 (1998) R1265–R1273.
[5] J.A. Lyons, J.S. Haring, P.R. Biga, Myostatin expression, lymphocyte population, and potential cytokine production correlate with predisposition to high-fat diet induced obesity in mice, PloS One 5 (2010) e12928.
[6] M.D. Mitchell, C.C. Osepchook, K.C. Leung, C.D. McMahon, J.J. Bass, Myostatin is a human placental product that regulates glucose uptake, J. Clin. Endocrinol. Metab. 91 (2006) 1434–1437.
[7] M. Schuelke, K.R. Wagner, L.E. Stolz, C. Hubner, T. Riebel, W. Komen, T. Braun, J.F. Tobin, S.J. Lee, Myostatin mutation associated with gross muscle hypertrophy in a child, N. Engl. J. Med. 350 (2004) 2682–2688.
[8] B.D. Rodgers, D.K. Garikipati, Clinical, agricultural, and evolutionary biology of myostatin: a comparative review, Endocr. Rev. 29 (2008) 513–534.
[9] J.L. Chen, T.D. Colgan, K.L. Walton, P. Gregorevic, C.A. Harrison, The TGF-beta signalling network in muscle development, adaptation and disease, Adv. Exp. Med. Biol. 900 (2016) 97–131.
[10] A. Stinckens, M. Georges, N. Buys, Mutations in the myostatin gene leading to hypermuscularity in mammals: indications for a similar mechanism in fish? Anim. Genet. 42 (2011) 229–234.
[11] J. Jespersen, M. Kjaer, P. Schjerling, The possible role of myostatin in skeletal muscle atrophy and cachexia, Scand. J. Med. Sci. Sports 16 (2006) 74–82.
[12] C.M. Liu, Z. Yang, C.W. Liu, R. Wang, P. Tien, R. Dale, L.Q. Sun, Myostatin antisense RNA-mediated muscle growth in normal and cancer cachexia mice, Gene Ther. 15 (2008) 155–160.
[13] X. Zhou, J.L. Wang, J. Lu, Y. Song, K.S. Kwak, Q. Jiao, R. Rosenfeld, Q. Chen, T. Boone, W.S. Simonet, D.L. Lacey, A.L. Goldberg, H.Q. Han, Reversal of cancer cachexia and muscle wasting by ActRIIB antagonism leads to prolonged survival, Cell 142 (2010) 531–543.
[14] Z. Aversa, A. Bonetto, F. Penna, P. Costelli, G. Di Rienzo, A. Lacitignola, F.M. Baccino, V. Ziparo, P. Mercantini, F. Rossi Fanelli, M. Muscaritoli, Changes in myostatin signaling in non-weight-losing cancer patients, Ann. Surg. Oncol. 19 (2012) 1350–1356.
[15] J. Heineke, M. Auger-Messier, J. Xu, M. Sargent, A. York, S. Welle, J.D. Molkentin, Genetic deletion of myostatin from the heart prevents skeletal muscle atrophy in heart failure, Circulation 121 (2010) 419–425.
[16] V. Siriett, L. Platt, M.S. Salerno, N. Ling, R. Kambadur, M. Sharma, Prolonged absence of myostatin reduces sarcopenia, J. Cell. Physiol. 209 (2006) 866–873.
[17] D. Padhi, C.S. Higano, N.D. Shore, P. Sieber, E. Rasmussen, M.R. Smith, Pharmacological inhibition of myostatin and changes in lean body mass and lower extremity muscle size in patients receiving androgen deprivation therapy for prostate cancer, J. Clin. Endocrinol. Metab. 99 (2014) E1967–E1975.
[18] V. Dubois, M.R. Laurent, M. Sinnesael, N. Cielen, C. Helsen, L. Clinckemalie, L. Spans, G. Gayan-Ramirez, L. Deldicque, P. Hespel, G. Carmeliet, D. Vanderschueren, F. Claessens, A satellite cell-specific knockout of the androgen receptor reveals myostatin as a direct androgen target in skeletal muscle, FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 28 (2014) 2979–2994.
[19] K. Ma, C. Mallidis, J. Artaza, W. Taylor, N. Gonzalez-Cadavid, S. Bhasin, Characterization of 5'-regulatory region of human myostatin gene: regulation by dexamethasone in vitro, American journal of physiology, Endocrinol. Metab. 281 (2001) E1128–E1136.
[20] N. Li, Q. Yang, R.G. Walker, T.B. Thompson, M. Du, B.D. Rodgers, Myostatin attenuation in vivo reduces adiposity, but activates adipogenesis, Endocrinology 157 (2016) 282–291.
[21] J. Lin, H.B. Arnold, M.A. Della-Fera, M.J. Azain, D.L. Hartzell, C.A. Baile, Myostatin knockout in mice increases myogenesis and decreases adipogenesis, Biochem. Biophys. Res. Commun. 291 (2002) 701–706.
[22] A.C. McPherron, S.J. Lee, Suppression of body fat accumulation in myostatin-deficient mice, J. Clin. Investig. 109 (2002) 595–601.
[23] M.F. Jackson, D. Luong, D.D. Vang, D.K. Garikipati, J.B. Stanton, O.L. Nelson, B.D. Rodgers, The aging myostatin null phenotype: reduced adiposity, cardiac hypertrophy, enhanced cardiac stress response, and sexual dimorphism, J. Endocrinol. 213 (2012) 263–275.
[24] C. Zhang, C. McFarlane, S. Lokireddy, S. Masuda, X. Ge, P.D. Gluckman, M. Sharma, R. Kambadur, Inhibition of myostatin protects against diet-induced obesity by enhancing fatty acid oxidation and promoting a brown adipose phenotype in mice, Diabetologia 55 (2012) 183–193.
[25] S. Bonala, S. Lokireddy, C. McFarlane, S. Patnam, M. Sharma, R. Kambadur, Myostatin induces insulin resistance via Casitas B-lineage lymphoma b (Cblb)-mediated degradation of insulin receptor substrate 1 (IRS1) protein in response to high calorie diet intake, J. Biol. Chem. 289 (2014) 7654–7670.
[26] S.M. Steculorum, J. Ruud, I. Karakasilioti, H. Backes, L. Engstrom Ruud, K. Timper, M.E. Hess, E. Tsaousidou, J. Mauer, M.C. Vogt, L. Paeger, S. Bremser, A.C. Klein, D.A. Morgan, P. Frommolt, P.T. Brinkkotter, P. Hammerschmidt, T. Benzing, K. Rahmouni, F.T. Wunderlich, P. Kloppenburg, J.C. Bruning, AgRP neurons control systemic insulin sensitivity via myostatin expression in brown adipose tissue, Cell 165 (2016) 125–138.
[27] C. Rommel, S.C. Bodine, B.A. Clarke, R. Rossman, L. Nunez, T.N. Stitt, G.D. Yancopoulos, D.J. Glass, Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways, Nat. cell Biol. 3 (2001) 1009–1013.
[28] S. Welle, K. Bhatt, C.A. Pinkert, R. Tawil, C.A. Thornton, Muscle growth after postdevelopmental myostatin gene knockout, American journal of physiology, Endocrinol. Metab. 292 (2007) E985–E991.
[29] C.L. Mendias, E.B. Lynch, J.P. Gumucio, M.D. Flood, D.S. Rittman, D.W. Van Pelt, S.M. Roche, C.S. Davis, Changes in skeletal muscle and tendon structure and function following genetic inactivation of myostatin in rats, J. Physiol. 593 (2015) 2037–2052.
[30] M.L. Maeder, S. Thibodeau-Beganny, A. Osiak, D.A. Wright, R.M. Anthony, M. Eichtinger, T. Jiang, J.E. Foley, R.J. Winfrey, J.A. Townsend, E. Unger-Wallace, J.D. Sander, F. Muller-Lerch, F. Fu, J. Pearlberg, C. Gobel, J.P. Dassie, S.M. Pruett-Miller, M.H. Porteus, D.C. Sgroi, A.J. Iafrate, D. Dobbs, P.B. McCray Jr., T. Cathomen, D.F. Voytas, J.K. Joung, Rapid "open-source" engineering of customized zinc-finger nucleases for highly efficient gene modification, Mol. Cell 31 (2008) 294–301.
[31] T. Cathomen, J.K. Joung, Zinc-finger nucleases: the next generation emerges, Mol. Ther. 16 (2008) 1200–1207.
[32] I.D. Carbery, D. Ji, A. Harrington, V. Brown, E.J. Weinstein, L. Liaw, X. Cui, Targeted genome modification in mice using zinc-finger nucleases, Genetics 186 (2010) 451–459.
[33] A.M. Geurts, G.J. Cost, Y. Freyvert, B. Zeitler, J.C. Miller, V.M. Choi, S.S. Jenkins, A. Wood, X. Cui, X. Meng, A. Vincent, S. Lam, M. Michalkiewicz, R. Schilling, J. Foeckler, S. Kalloway, H. Weiler, S. Menoret, I. Anegon, G.D. Davis, L. Zhang, E.J. Rebar, P.D. Gregory, F.D. Urnov, H.J. Jacob, R. Buelow, Knockout rats via embryo microinjection of zinc-finger nucleases, Science 325 (2009) 433.
[34] I.A. Boman, D.I. Vage, An insertion in the coding region of the myostatin

(MSTN) gene affects carcass conformation and fatness in the Norwegian Spaelsau (Ovis aries), BMC Res. Notes 2 (2009) 98.
[35] S. Dunner, M.E. Miranda, Y. Amigues, J. Canon, M. Georges, R. Hanset, J. Williams, F. Menissier, Haplotype diversity of the myostatin gene among beef cattle breeds, Genetics, selection, evolution, GSE 35 (2003) 103–118.
[36] L. Grobet, D. Poncelet, L.J. Royo, B. Brouwers, D. Pirottin, C. Michaux, F. Menissier, M. Zanotti, S. Dunner, M. Georges, Molecular definition of an allelic series of mutations disrupting the myostatin function and causing double-muscling in cattle, Mammalian genome, Off. J. Int. Mamm. Genome Soc. 9 (1998) 210–213.
[37] C. Marchitelli, M.C. Savarese, A. Crisa, A. Nardone, P.A. Marsan, A. Valentini, Double muscling in Marchigiana beef breed is caused by a stop codon in the third exon of myostatin gene, Mammalian genome, Off. J. Int. Mamm. Genome Soc. 14 (2003) 392–395.
[38] D.S. Mosher, P. Quignon, C.D. Bustamante, N.B. Sutter, C.S. Mellersh, H.G. Parker, E.A. Ostrander, A mutation in the myostatin gene increases muscle mass and enhances racing performance in heterozygote dogs, PLoS Genet. 3 (2007) e79.
[39] G.D. Shelton, E. Engvall, Gross muscle hypertrophy in whippet dogs is caused by a mutation in the myostatin gene, Neuromuscular disorders, NMD 17 (2007) 721–722.
[40] X. Zhu, M. Hadhazy, M. Wehling, J.G. Tidball, E.M. McNally, Dominant negative myostatin produces hypertrophy without hyperplasia in muscle, FEBS Lett. 474 (2000) 71–75.
[41] A. Matsakas, D.A. Prosdocimo, R. Mitchell, H. Collins-Hooper, N. Giallourou, J.R. Swann, P. Potter, T. Epting, M.K. Jain, K. Patel, Investigating mechanisms underpinning the detrimental impact of a high-fat diet in the developing and adult hypermuscular myostatin null mouse, Skelet. Muscle 5 (2015) 38.
[42] L. Tay, Y.Y. Ding, B.P. Leung, N.H. Ismail, A. Yeo, S. Yew, K.S. Tay, C.H. Tan, M.S. Chong, Sex-specific differences in risk factors for sarcopenia amongst community-dwelling older adults, Age 37 (2015) 121.
[43] E. Mouisel, K. Relizani, L. Mille-Hamard, R. Denis, C. Hourde, O. Agbulut, K. Patel, L. Arandel, S. Morales-Gonzalez, A. Vignaud, L. Garcia, A. Ferry, S. Luquet, V. Billat, R. Ventura-Clapier, M. Schuelke, H. Amthor, Myostatin is a key mediator between energy metabolism and endurance capacity of skeletal muscle, Am. J. Physiol. Regul. Integr. Comp. Physiol. 307 (2014) R444–R454.
[44] H. Amthor, R. Macharia, R. Navarrete, M. Schuelke, S.C. Brown, A. Otto, T. Voit, F. Muntoni, G. Vrbova, T. Partridge, P. Zammit, L. Bunger, K. Patel, Lack of myostatin results in excessive muscle growth but impaired force generation, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 1835–1840.
[45] A.K. Esmailizadeh, C.D. Bottema, G.S. Sellick, A.P. Verbyla, C.A. Morris, N.G. Cullen, W.S. Pitchford, Effects of the myostatin F94L substitution on beef traits, J. animal Sci. 86 (2008) 1038–1046.