Frameshift mutation in myostatin gene by zinc-finger nucleases results in a significant increase in muscle mass in Meishan sows

Hanfang Bi[1], Shanshan Xie[1,3], Chunbo Cai[1], Lili Qian[2], Shengwang Jiang[1], Gaojun Xiao[1], Biao Li[1], Xiang Li[1], Wentao Cui[1]*

[1]*Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, P.R. China*
[2]*Institute for Translational Medicine, Qingdao University, Qingdao, P.R. China*
[3]*Beijing Century Legend Bioscience Co., Ltd., Beijing, P.R. China*
*Corresponding author: cuiwentao@caas.cn

Abstract: Myostatin (*MSTN*) is a negative regulator of skeletal muscle growth and development. A significant increase in skeletal muscle was observed in *Mstn*^{−/−} mice compared with wild-type mice. So far, there has been no report on porcine *MSTN* mutations leading to skeletal muscle hypertrophy. In this report a *MSTN* frameshift mutation missing 11 nucleotides in exon 2 was introduced into Meishan pigs by zinc finger nuclease (*ZFN*) technology. *ZFN*-edited *MSTN*^{−/−} Meishan pigs were successfully produced by a cloning method of somatic cell nucleus transfer. Results from slaughter experiments indicated that lean meat yield increased 16.53% in about 80 kg (10-months-old) *MSTN*^{−/−} Meishan sows compared with their corresponding wild-type counterparts. The lean percentage of carcass from *MSTN*^{−/−} sows was 61.20% *vs* 48.25% for *MSTN*^{+/−} sows and 44.67% for wild-type sows. The fat of *MSTN*^{−/−} sows was significantly lower than that of *MSTN*^{+/−} and wild-type sows. The loin eye area of *MSTN*^{−/−} Meishan sows (56.42 cm^2) was greater than that of *MSTN*^{+/−} (37.39 cm^2) and wild-type (26.26 cm^2) sows. The muscle fibre area of *longissimus* muscle in wild-type Meishan sows (1 946 μm^2) was significantly greater than that of *MSTN*^{+/−} (1 324 μm^2) and *MSTN*^{−/−} (1 419 μm^2) sows. Moreover the significantly increased skeletal muscle in these *MSTN*^{−/−} Meishan sows was mainly due to the increase in the number of myofibres rather than to hypertrophy. Compared with wild-type Meishan sows, it was noted that myofibres had transformed from type I to IIB in *MSTN*^{−/−} Meishan sows. Our present study demonstrated that frameshift mutation in *MSTN* by *ZFN* technology led to a significant increase in muscle mass and a significant decrease in fat content in Meishan sows.

Keywords: *MSTN*; *ZFN*-edited; skeletal muscle; lean meat yield; myofibres

Myostatin, also known as growth differentiation factor 8 (*GDF8*), is a transcriptional growth factor that is expressed mainly in skeletal muscle (McPherron et al. 1997). McPherron et al. (1997) observed that the *Mstn*^{−/−} mice had an apparent double-muscled (DM) phenomenon. Later on, other scientists have identified a variety of natural *MSTN* loss-of-function mutations in DM cattle such as Belgian Blue and Piedmontese cattle, which had stronger skeletal muscle (Grobet et al. 1997; McPherron and Lee 1997), and contained much less subcutaneous fat (Smet et al. 2000; Bellinge et al.

2005). It was this *MSTN* loss-of-function mutation that results in double-muscled phenomenon.

Due to the specific function of *MSTN* in skeletal muscle growth and development, *MSTN* gene becomes an important target of gene editing technology in livestock to improve the meat quality with higher percentage of lean yield and lower percentage of fat. Two major strategies can be employed to produce animals containing *MSTN* loss-of-function mutations.

The first antagonistic strategy to inhibit *Mstn* activity was mainly reported in transgenic mice. Wolfman et al. (2003) demonstrated that a mutation of aspartic acid to alanine at position 76 of murine Mstn propeptide resulted in resistance to protease cleavage, which then reduced the mass of the active mature Mstn peptide. Injection of such Mstn propeptide mutant into mice resulted in a significant increase in skeletal muscle (Yang et al. 2001). Some laboratory recently generated transgenic mice expressed porcine *Mstn* mutants (Ma et al. 2015a,b; Qian et al. 2016). These transgenic mice showed an increase in skeletal muscle.

The second strategy is to use a gene editing technology directly introducing mutations into the endogenous *MSTN* gene. There have been many reports on genetic modifications of endogenous *MSTN* introduced in large animals by gene editing technology (Qian et al. 2015; Wang et al. 2015; Bi et al. 2016). Meishan pigs are a locally famous breed in China and are well known for their high prolificacy and early sexual maturity, but the breed has a high percentage of carcass fat and poor feed efficiency (Legault 1985). These unique characteristics make Meishan pigs become a suitable model to test the effect of *MSTN* mutation on skeletal muscle growth and body composition.

In this study, we generated a new type of loss-of-function ($MSTN^{-/-}$) *MSTN* mutant Meishan pigs from cell colony 110 containing an 11-nucleotide deletion in *MSTN* exon 2. Results from separate slaughter experiments show that lean meat yield increased 16.53% in about 80 kg $MSTN^{-/-}$ Meishan sows compared with their corresponding wild-type counterparts. The absolute lean yield in $MSTN^{-/-}$ Meishan sows reached as high as 61.20% when they were slaughtered at body weights of about 80 kg. These high lean meat yields are equivalent to those of the imported foreign breeds. Our results demonstrated that $MSTN^{-/-}$ Meishan sows had a significant increase in skeletal muscle with decreased

intramuscular fat, which was consistent with what was observed for $MSTN^{-/-}$ Meishan boars (Qian et al. 2015).

MATERIAL AND METHODS

Production of MSTN mutant Meishan pigs

A *MSTN* frameshift mutation missing 11 nucleotides of exon 2 was introduced into Meishan pigs by zinc finger nuclease (*ZFN*) technology. In this study, we generated a new type of loss-of-function ($MSTN^{-/-}$) *MSTN* mutant Meishan pigs from cell colony 110 containing an 11-nucleotide deletion in *MSTN* exon 2 using protocols described by Qian et al. (2015).

Ethics statement

All the $MSTN^{-/-}$, $MSTN^{+/-}$ and $MSTN^{+/+}$ sows were fed the same standard diet and raised under the same conditions. In this study 3 $MSTN^{+/+}$ Meishan sows, 3 $MSTN^{+/-}$ Meishan sows, and 3 $MSTN^{-/-}$ Meishan sows were used. All experimental Meishan sows were slaughtered when their weights reached 79.5, 79.2, and 81.3 kg for 3 $MSTN^{+/+}$ Meishan sows; 78.8, 79.6, and 80.5 kg for 3 $MSTN^{+/-}$ Meishan sows; 79.9, 81.1, and 81.8 kg for 3 $MSTN^{-/-}$ Meishan sows. All experiments were performed in accordance with the approved guidelines for animal care and management designed for research projects.

Genotyping of MSTN mutants in pigs

Genomic DNA was extracted from the ear of newborn pigs. PCR was performed and its products were then sequenced. Double peaks indicated the presence of $MSTN^{+/-}$ pigs. Single peaks with no nucleotide deletion indicated the presence of wild-type $MSTN^{+/+}$ pigs. Single peaks with 11-nucleotide deletion indicated the presence of $MSTN^{-/-}$ pigs.

RNA extraction and cDNA synthesis

The methods of RNA extraction and cDNA synthesis were the same as described in the previ-

ous study (Qian et al. 2015). Tissue samples used for molecular detection were collected and snap-frozen in liquid nitrogen.

Quantitative real-time PCR (qPCR)

qPCR was performed by using cDNA as a template and coding sequence primers (Table 1). PCR products were ligated to T vector (Tiangen Biotech, Co. Ltd, Beijing, P.R. China) and sequencing was then performed. qPCR was conducted as previously described (Qian et al. 2015). Reactions were performed on a 7500 FAST Real-Time PCR System (Applied Biosystems, San Francisco, CA, USA). TATA-binding protein gene (*TBP*) was used as an internal reference (Nygard et al. 2007) (Table 1). All data were analyzed by the $2^{-\Delta\Delta CT}$ method using 7500 System SDS Software v1.4.0. qPCR was performed also for genes *MYH7, MYH2, MYH4* and *MYH1*. All sequences of the qPCR primers are listed in Table 1.

Western blot

Total protein was extracted from *longissimus dorsi* muscle and Western blot was performed as previously described (Qian et al. 2015). Protein

concentration was measured with Micro BCA Protein Assay Kit from Thermo Scientific (Cat. No. 23235; Rockford, IL, USA). The mouse primary antibody against human MSTN peptide (ab55106) was from Abcam (Cambridge, UK). Rabbit anti-GAPDH (human peptide) antibody (Cat. No. 2118), horse anti-mouse secondary antibody (HRP-linked) (Cat. No. 7076) and goat anti-rabbit secondary antibody (HRP-linked) (Cat. No. 7074) were from Cell Signaling Technology (Beverly, MA, USA).

Carcass traits

Carcass traits such as body weight and length, backfat thickness, and loin eye areas were measured by methods as previously described (Qian et al. 2015). Experimental pigs were euthanized, and blood samples were collected. Hairs, heads, hoofs and internal organs were removed.

Haematoxylin and eosin (HE) staining

Fresh *longissimus* muscle tissue was fixed in 4% paraformaldehyde for 3 days, and then embedded in paraffin. Fixed tissue was then sliced into 5 μm sections. HE staining was performed by a standard

Table 1. PCR and qPCR primers

Gene	Primer (5' – 3')	GenBank access No.	Amplicon (bp)	Ta (°C)
MSTN-genomic fragment	F: TACAAGGTATACTGGAATCCGATCT R: GCAAAGTAAAAGTATCAAGAGGGTA	NC_010457	397	60
MSTN-coding sequence	F: ATGCTGATTGTTGCTGGTCC R: CCCACAGCGATCTACTACCA	NC_010457	1 080	60
MSTN-intact*	F: GTAACCTTCCCAGGACCAGG R: GGGTAACGACAGCATCGAGA	NC_010457	140	60
MSTN-total#	F: AGTGATGGCTCCTTGGAAGA R: TGTAGGAGTCTTGACGGGT	NC_010457	198	60
MYH7 (fiber of type I)	F: GCACTGAAGAGGCTGACAAG R: ATCCGTGTCACCATCCAGTT	NC_010449	199	60
MYH2 (fiber of type IIA)	F: CTGGCTGGTTGGACAAGAAC R: GACACGGTCTGGAAGGAAGA	NC_010454	175	60
MYH4 (fiber of type IIB)	F: TAGGAAGAAGCACGCAGACA R: GGTTTCCCTTGGCTTTGGAG	NC_010454	158	60
MYH1 (fiber of type IIX)	F: ATTTCTGACCTCACGGAGCA R: ACCTCCGACTTGACTTGGTT	NC_010454	191	60
TBP	F: AACAGTTCAGTAGTTATGAGCCAGA R: AGATGTTCTCAAACGCTTCG	XM_021085497	153	60

MSTN-intact* is used to measure the expression level of intact *MSTN* mRNA; *MSTN*-total# is used to measure the expression level of total *MSTN* mRNA (including wild-type and mutant *MSTN*)

method. Software Image J was used to determine the area of single muscle fibres.

Statistical analysis

All data are expressed as mean ± SE. All data were analyzed by using unpaired 2-tailed Student's *t*-tests ($P < 0.05$).

RESULTS

All experimental sows were slaughtered when their body weights reached about 80 kg (at the age of 10 months). PCR was performed to amplify genomic *MSTN* (*MSTN*-genomic fragment) fragment using the primers presented in Table 1.

Genotype identification of *MSTN*$^{-/-}$ sows

PCR products were then sequenced (Figure 1A). Using primers of coding sequence (Table 1) and cDNA of skeletal muscle as a template, PCR amplification was performed, followed by sequencing of PCR products (Figure 1B). Sequencing results indicated that mRNA of *MSTN*$^{-/-}$ pigs generated from cell colony 110 indeed contained 11-nucleotide deletion. Alignment of amino acid and mRNA sequences from *MSTN*$^{-/-}$ and wild-type pigs showed that protein translation terminates early at 268 in *MSTN*$^{-/-}$ pigs while it terminates at 375 in wild-type pigs (Figure 1C). Therefore, *MSTN*$^{-/-}$ pigs could not produce an active C-terminal MSTN mature protein due to the early termination. qPCR analysis of total *MSTN* mRNA (for MSTN precursor protein, see primers in Table 1) indicated that total *MSTN* expression in *MSTN*$^{-/-}$ pigs was higher than in *MSTN*$^{+/-}$ and wild-type sows (Figure 1D). On the other hand, qPCR analysis of intact *MSTN* mRNA (which is for mature MSTN protein, see primers in Table 1) indicated that the intact *MSTN* expression in *MSTN*$^{-/-}$ pigs was not detectable while it was clearly expressed in *MSTN*$^{+/-}$ and wild-type pigs (Figure 1E). Western blot analysis further indicated that both MSTN precursor protein and mature MSTN protein could not be detected
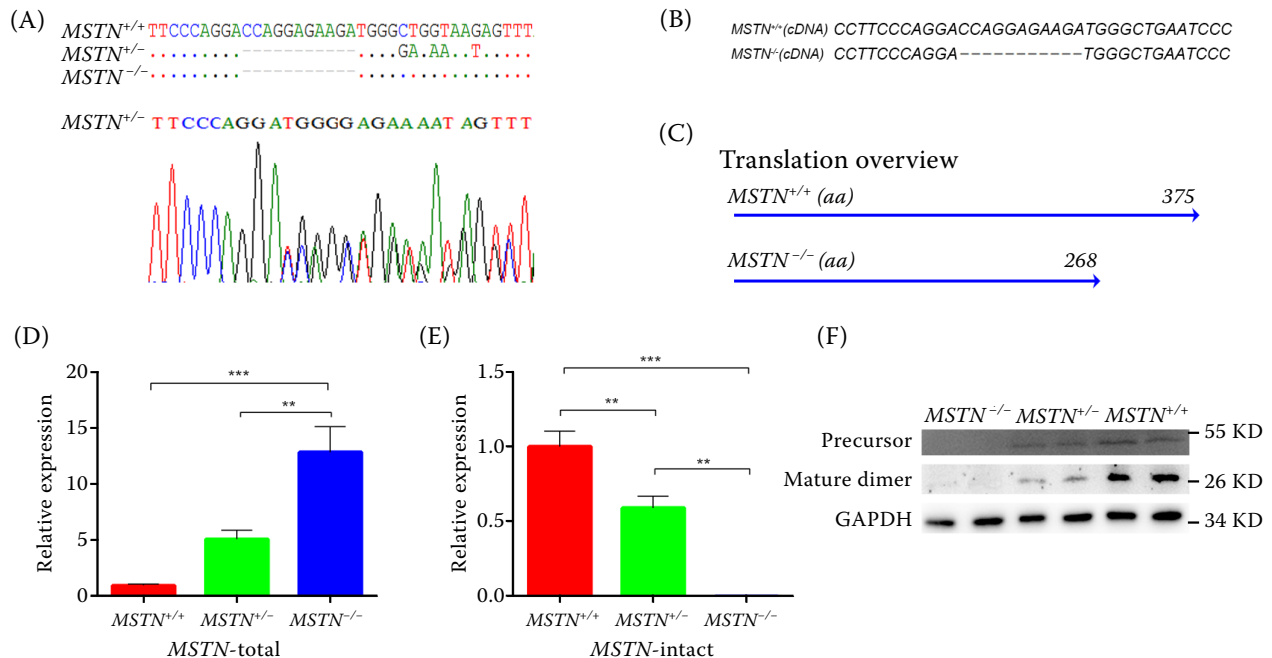

Figure 1. Genotype identification of Meishan sows. (A) Sequence results of PCR products obtained with the genomic primers. (B) Sequence results of PCR products using cDNA of skeletal muscle. (C) Translation overview of intact *MSTN* (*MSTN*$^{+/+}$) and mutant *MSTN* (*MSTN*$^{-/-}$). Mutant *MSTN* shows an early translation termination. (D) and (E) qPCR results of total *MSTN* (D) and intact *MSTN* (E). (F) Detection of MSTN protein in skeletal muscle by Western blot. GAPDH protein was used as an internal reference
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

in $MSTN^{-/-}$ pigs while they were clearly detectable in $MSTN^{+/-}$ and wild-type pigs (Figure 1F). All these data confirmed that $MSTN^{-/-}$ pigs generated from cell colony 110 containing a $MSTN$ mutant with 11-nucleotide deletion could not produce the mature MSTN protein *in vivo*.

Phenotype characterization of $MSTN^{-/-}$ sows

Figure 2 shows photos of representative $MSTN^{-/-}$, $MSTN^{+/-}$ and wild-type sows. When the body weight reached about 80 kg, selected sows were euthanized to measure the weight of skeletal muscle (Figure 3A). The weight of skeletal muscle from $MSTN^{-/-}$ Meishan sows was significantly greater than that from $MSTN^{+/-}$ and wild-type Meishan sows (Figure 3B). The percentage of lean carcass from $MSTN^{-/-}$ sows was 61.20% ± 0.603% *vs* 48.25% ± 0.8312% in $MSTN^{+/-}$ sows and 44.67% ± 2.104% in wild-type sows. Compared with wild-type sows, the lean meat yield increased 16.53% for $MSTN^{-/-}$ sows ($P = 0.0006$). We also measured weights of skeletal muscle from different parts. Results indicated that the weights of skeletal muscle from all parts of $MSTN^{-/-}$ sows were significantly higher than those of $MSTN^{+/-}$ and wild-type sows (Figure 3C and 3D). Additionally, we also noticed that the fat of $MSTN^{-/-}$ sows was significantly lower than that of $MSTN^{+/-}$ and wild-type sows ($P = 0.0061$, $P = 0.0007$) (Figure 3E and 3F), with 18.39% of fat relative to carcass weight in $MSTN^{-/-}$ sows *vs* 31.76% in $MSTN^{+/-}$ sows and 32.57% in wild-type sows. Therefore, these results clearly indicated

that $MSTN^{-/-}$ Meishan sows derived from cell colony 110 contained a much higher level of skeletal muscle and a lower fat level than both $MSTN^{+/-}$ and wild-type sows.

Muscle fibre hyperplasia is the mechanism for the increased skeletal muscle in $MSTN^{-/-}$ Meishan sows

Skeletal muscle is composed of muscle fibres. We tested the relationship between muscle fibre hyperplasia and hypertrophy in $MSTN^{-/-}$ Meishan sows to investigate the cause for the increased skeletal muscle. Results from the measurement of the loin eye area of *longissimus dorsi* (Figure 4A and 4B) indicated that the loin eye area of $MSTN^{-/-}$ Meishan sows (56.42 ± 1.602 cm²) were greater than that of $MSTN^{+/-}$ (37.39 ± 3.947 cm², $P = 0.0016$) and wild-type (26.26 ± 1.311 cm², $P = 0.0007$) sows. Additionally, the backfat in wild-type sows was significantly thicker than that from $MSTN^{-/-}$ Meishan sows (Figure 4C). HE staining of *longissimus dorsi* revealed that the muscle fibre area of *longissimus* muscle in wild-type Meishan sows (1 946 ± 87.39 µm²) was significantly greater than that of $MSTN^{+/-}$ (1 324 ± 43.93 µm²) and $MSTN^{-/-}$ (1 419 ± 48.66 µm²) sows (Figure 4D and 4E). The myofibre density in *longissimus dorsi* was significantly higher in $MSTN^{-/-}$ and $MSTN^{+/-}$ Meishan sows than in wild-type sows (Figure 4F). We then calculated the number of muscle fibres of *longissimus dorsi* and we found that $MSTN^{-/-}$ and $MSTN^{+/-}$ Meishan sows contained a much high-

Figure 2. Photos of representatives of $MSTN^{-/-}$, $MSTN^{+/-}$ and $MSTN^{+/+}$ Meishan sows. $MSTN^{-/-}$ Meishan sows with double-muscled (DM) phenomenon (age of 10 months)
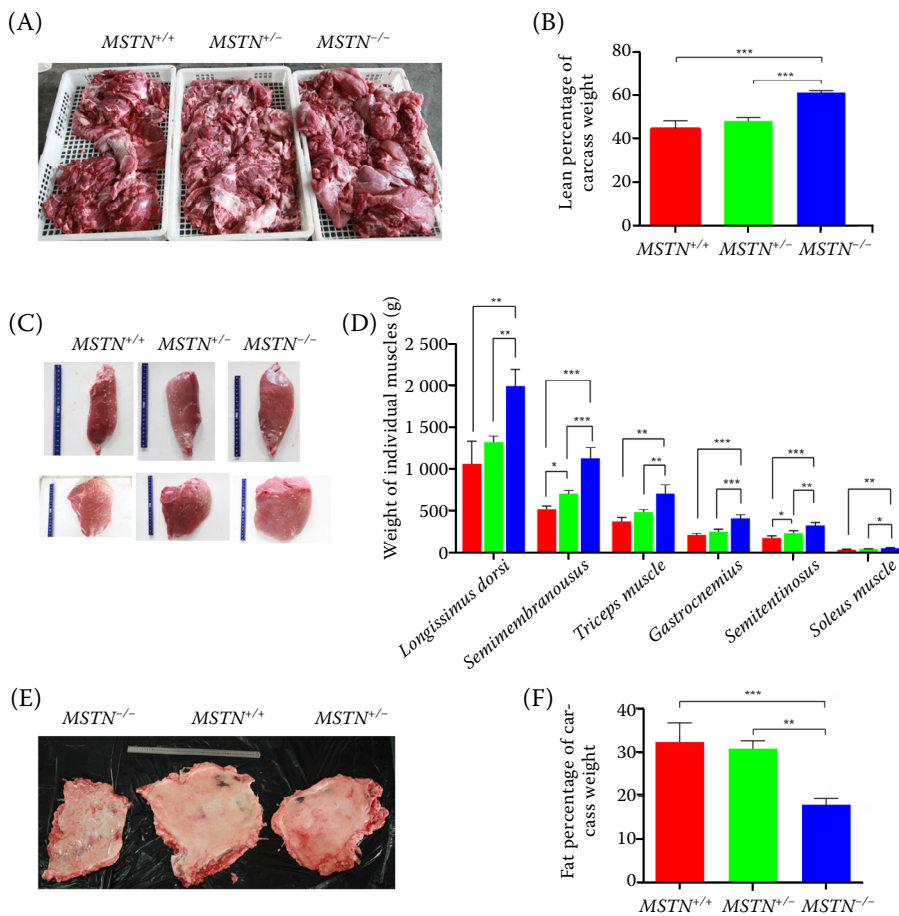
Figure 3. Phenotype characterization of Meishan sows. (A) Photos of all skeletal muscles which were dissected on one side of the body. (B) Percent skeletal muscle of carcass weight. (C) Photos of *semitendinosus* (top) and *semimembranosus* (bottom) muscles of *MSTN$^{-/-}$*, *MSTN$^{+/-}$* and *MSTN$^{+/+}$* Meishan sows. (D) Average weight of individual skeletal muscles which were dissected on one side of the body. (E) Photos of leaf fat of *MSTN$^{-/-}$*, *MSTN$^{+/-}$* and *MSTN$^{+/+}$* Meishan sows. (F) Percent subcutaneous fat and leaf fat of carcass weight
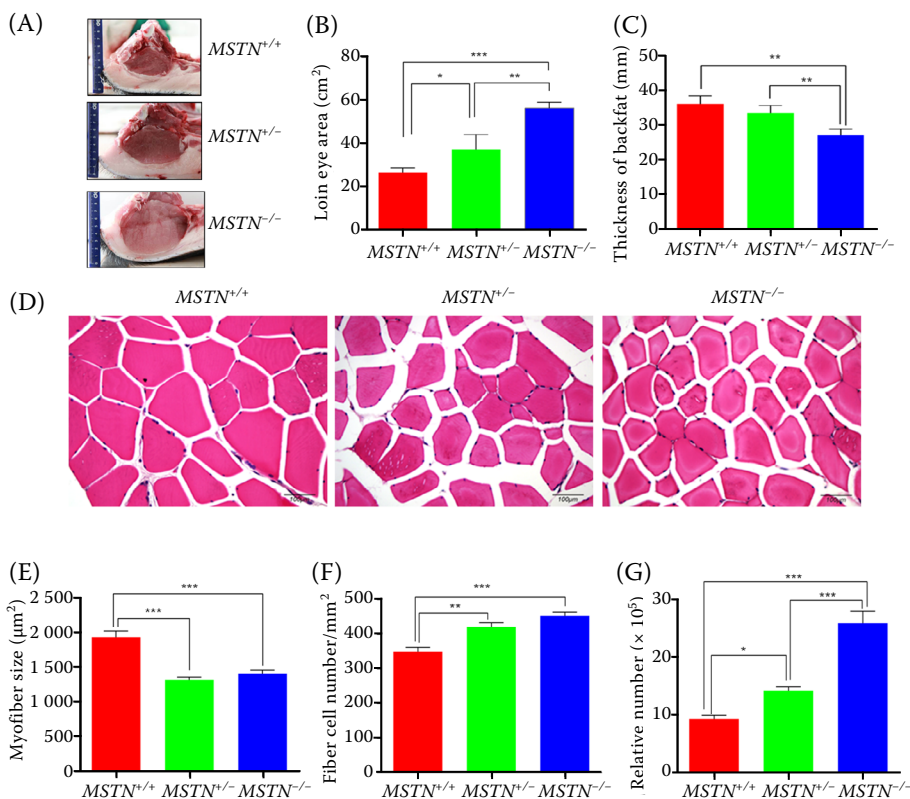
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Figure 4. Muscle fibre hyperplasia is a mechanism for the increased skeletal muscle in *MSTN$^{-/-}$* Meishan sows. (A) The cross-section of *longissimus dorsi* and subcutaneous fat between the 6th and 7th rib. (B) and (C) The loin eye area (B) and backfat (C) of *longissimus dorsi* between the 6th and 7th rib. (D) Histological cross-section of *longissimus dorsi* of *MSTN$^{-/-}$*, *MSTN$^{+/-}$* and *MSTN$^{+/+}$* Meishan sows. (E), (F) and (G) Average size (E), density (F) and number of myofibres (G)

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

er number of muscle fibres than wild-type pigs (Figure 4G). Our results clearly indicated that the increased skeletal muscle in $MSTN^{-/-}$ Meishan sows was mainly due to the increase in the number of muscle fibres rather than to muscle fibre hypertrophy.

Changes in skeletal muscle fibre types

Skeletal muscle fibres are classified into four types: type I, IIA, IIX and IIB. We investigated changes in myofibre types in $MSTN^{-/-}$ Meishan sows by performing qPCR assays to monitor the expression of marker genes for each type of myofibres (Figure 5A). It was noted that the expression of the type I myofibre marker gene myosin heavy chain 7 (*MYH7*) (NCBI ID: 396860) in wild-type sows was significantly higher than that of $MSTN^{+/-}$ and $MSTN^{-/-}$ Meishan sows, indicating a decrease in type I myofibres in $MSTN^{+/-}$ and $MSTN^{-/-}$ Meishan sows. On the other hand, the expression of the type IIB myofibre marker gene *MYH4* in $MSTN^{+/-}$ and $MSTN^{-/-}$ Meishan sows was significantly higher than that of wild-type pigs, indicating an increase in type IIB myofibres in $MSTN^{+/-}$ and $MSTN^{-/-}$ Meishan sows.

The "a" value of meat colour is an indication of meat redness which reflects the level of oxymyoglobin. It was known that the level of oxymyoglobin was higher in type I than in type IIB myofibres

(Wydro et al. 1983). Recent study on *Mstn* knockout mice indicated that there was a decrease in type I myofibres and an increase in type IIB myofibres (Girgenrath et al. 2005).

Our results indicated that the "a" value decreased significantly in $MSTN^{-/-}$ sows compared with wild-type sows ($P = 0.002$) (Figure 5B), suggesting that there was a transition of type I myofibres to type IIB myofibres in $MSTN^{-/-}$ sows. The "a" data was consistent with the change in the expression of the type IIB myofibre marker gene *MYH4* in $MSTN^{-/-}$ sows. We speculate that a decrease in the "a" value of $MSTN^{-/-}$ and $MSTN^{+/-}$ sows has a direct relationship with the increased level of type IIB myofibres.

DISCUSSION

Meishan pig is one of the famous local pig breeds in China. These pigs are well known for their high prolificacy, early sexual maturity, and delicious meat taste. However, this breed has a high percentage of fat and poor feed efficiency (Legault 1985). In this study, we generated a new type of $MSTN^{-/-}$ Meishan pigs from cell colony 110 containing a *MSTN* mutant with 11-nucleotide deletion in exon 2. These changes in lean meat and fat mass induced by *MSTN* loss-of-function mutation are a significant breakthrough in improving the pork quality produced by Meishan pigs.
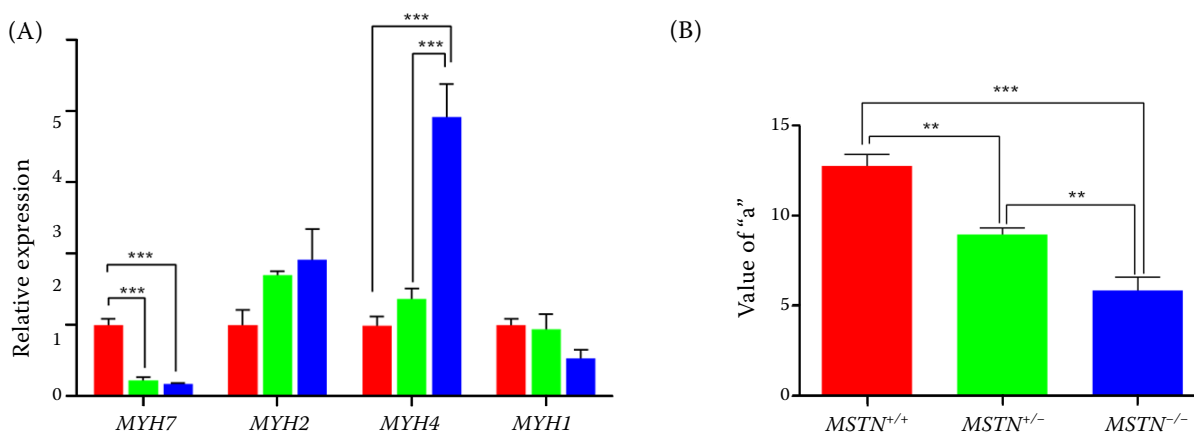

Figure 5. Changes in skeletal muscle fibre types. (A) qPCR results of *MYH7, MYH2, MYH4,* and *MYH1* in *longissimus dorsi. MYH7, MYH2, MYH4,* and *MYH1* are specific marker genes of type I, type IIA, type IIB, and type IIX myofibres, respectively. (B) Average value of "a" for $MSTN^{-/-}$, $MSTN^{+/-}$ and $MSTN^{+/+}$ Meishan sows

All red columns: $MSTN^{+/+}$ Meishan sows; green column: $MSTN^{+/-}$ Meishan sows; blue column: $MSTN^{-/-}$ Meishan sows.

Data are reported as mean ± SEM ($n = 3$)

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

The difference in the increase in lean meat yield between *MSTN*$^{-/-}$ sows generated from cell colony 110 and *MSTN*$^{-/-}$ boars generated from cell colony 105 may be due to the following three reasons. The first reason is that in this study *MSTN*$^{-/-}$ sows from cell colony 110 were slaughtered when their body weight reached about 80 kg (equivalent to the age of 10 months), while in our previous study *MSTN*$^{-/-}$ boars from cell colony 105 were slaughtered when their age reached 8 months (with lower body weights). Thus, they were not in the same stage of development. The second reason is a difference in sexuality. Androgen is known to induce myostatin signalling to regulate the development of skeletal muscle (Claessens et al. 2008; Dubois et al. 2014). In transgenic mice overexpressing porcine loss-of-function myostatin mutant, the increase in skeletal muscle is higher in female mice than in male mice (Ma et al. 2015b). The third reason is that there was no restriction in feeding for *MSTN*$^{-/-}$ sows generated from cell colony 110 for one month prior to their slaughtering, while there was a restriction in feeding for *MSTN*$^{-/-}$ boars generated from cell colony 105. No restriction in feeding is a more natural way, and thus it may explain the higher increase in lean meat yield for *MSTN*$^{-/-}$ sows generated from cell colony 110. No restriction in feeding actually represents the real conditions in the practice of poultry industry.

The increase in skeletal muscle mass can be caused by fibre hyperplasia and/or hypertrophy of skeletal muscle fibres. In *Mstn*$^{-/-}$ mutant mice it was noted that there was an increase in both the number and the size of myofibres (McPherron et al. 1997). In this study, we only observed an increase in the number of myofibres for *MSTN*$^{+/-}$ and *MSTN*$^{-/-}$ Meishan sows derived from cell colony 110. On the other hand, it was noticed that the myofibre sizes actually decreased in *MSTN*$^{+/-}$ and *MSTN*$^{-/-}$ Meishan sows derived from cell colony 110, which was not totally consistent with the observations of our previous study with *MSTN*$^{+/-}$ Meishan boars derived from cell colony 105. *MSTN* plays an important regulatory role in the growth and development of myoblasts (Lee 2004; Manceau et al. 2008; Lee and Lee 2013). The number of muscle fibres typically remains unchanged in the late embryonic development and will not change after birth (Swatland 1973; Wigmore and Stickland 1983; Wegner et al. 2000). However, the area of muscle fibre can keep increasing after birth. We believe that there is a sig-

nificantly higher increase in myoblast proliferation at the embryonic stage in *MSTN*$^{-/-}$ and *MSTN*$^{+/-}$ sows compared with wild-type sows, leading to an obvious increase in the number of myofibres. After birth, the enlargement of myofibre sizes is limited by the increase in the number of myofibres in *MSTN*$^{-/-}$ and *MSTN*$^{+/-}$ sows.

Skeletal muscle is formed by the polymerization of muscle fibres. The number of muscle fibres is a key factor in determining the mass of skeletal muscle. It is reported that the number of muscle fibres in skeletal muscle of *Mstn*$^{-/-}$ mice increased significantly (McPherron et al. 1997). Results from our present study indicated that the number of skeletal muscle fibres in *longissimus dorsi* of *MSTN*$^{-/-}$ Meishan sows was significantly higher than that of the wild-type Meishan sows. During embryonic development, there is a significant difference in the *MSTN* regulation of the number of skeletal muscle fibres between Meishan boars and Meishan sows. The number of myofibres in *longissimus dorsi* of *MSTN*$^{-/-}$ Meishan sows is significantly higher than that of *MSTN*$^{-/-}$ Meishan boars (Qian et al. 2015). Ma et al. (2015a) reported that inhibiting *Mstn* activity can promote the expression of androgen receptor (*Ar*), resulting in less enhanced muscle growth in male mice. We believe that the *Ar* level is lower in Meishan sows than in Meishan boars, and thus, the inhibition or loss of *MSTN* activity could lead to a significant increase in the number of skeletal muscle fibres in Meishan sows compared with boars.

Higher lean meat yield in combination with lower body fat has long been one of the ultimate goals in the livestock industry. In this study, we generated *ZFN*-edited *MSTN*-mutant sows with double-muscled (DM) phenomenon that are very similar to DM cattle which also contain 11-nucleotide deletion. Therefore it is expected that our *ZFN*-edited *MSTN*-mutant pigs should be safe to enter commercial food supply following the review and approval process by government regulatory agencies.

When specific primers (see Table 1) for total mRNA were used, qPCR results indicate that total *MSTN* expression in *MSTN*$^{-/-}$ pigs was higher than in *MSTN*$^{+/-}$ and wild-type sows (Figure 1D). This is mainly due to the fact that the forward primer is located at 304–322 bp and the reverse primer is located at 483–500 bp, while the deletion site is located at 729–740 bp in *MSTN*$^{-/-}$ sows. Thus, PCR products can be obtained in both wild-type

and *MSTN*s$^{-/-}$ ows using the pair of primers designed to detect total *MSTN* mRNA. The reason why total *MSTN* transcripts in *MSTN*$^{-/-}$ sows are significantly higher than those in wild-type sows may be that the mutated *MSTN* gene is being transcribed but cannot be translated into the active myostatin protein, which then may enhance the transcription level through feedback regulation. On the other hand, when specific primers (Table 1) for intact *MSTN* were used, qPCR results indicate that the expression of intact *MSTN* mRNA was not detectable in *MSTN*$^{-/-}$ sows, but was clearly detectable in *MSTN*$^{+/-}$ and wild-type sows (Figure 1E). This is mainly due to the fact that forward primer is located at 715–733 bp and reverse primer is located at 835–853 bp. Since the deletion site is located at 729–740 bp in *MSTN*$^{-/-}$ sows, the primers designed for intact *MSTN* mRNA amplify *MSTN* mRNA in the wild-type and *MSTN*$^{+/-}$ sows, but not in *MSTN*$^{-/-}$ sows.

CONCLUSION

In conclusion, we have successfully generated *MSTN*$^{-/-}$ mutant Meishan sows by using *ZFN* technology in combination with somatic cell nucleus transfer. These *MSTN*-mutant sows produce higher lean meat yield and lower body fat by a mechanism of fibre hyperplasia. The effect of gene editing technology on growth, reproduction, muscle quality, and safety remains to be further studied.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

Bellinge RH, Liberles DA, Iaschi SP, O'Brien PA, Tay GK. Myostatin and its implications on animal breeding: A review. Anim Genet. 2005 Feb;36(1):1-6.

Bi Y, Hua Z, Liu X, Hua W, Ren H, Xiao H, Zhang L, Li L, Wang Z, Laible G, Wang Y, Dong F, Zheng X. Isozygous and selectable marker-free MSTN knockout cloned pigs generated by the combined use of CRISPR/Cas9 and Cre/LoxP. Sci Rep. 2016 Aug 17;6:31729.

Claessens F, Denayer S, Van Tilborgh N, Kerkhofs S, Helsen C, Haelens A. Diverse roles of androgen receptor (AR) domains in AR-mediated signaling. Nucl Recept Signal. 2008 Jun 27;6:e008.

Dubois V, Laurent MR, Sinnesael M, Cielen N, Helsen C, Clinckemalie L, Spans L, Gayan-Ramirez G, Deldicque L, Hespel P, Carmeliet G, Vanderschueren D, Claessens F. A satellite cell-specific knockout of the androgen receptor reveals myostatin as a direct androgen target in skeletal muscle. Faseb J. 2014 Jul;28(7):2979-94.

Girgenrath S, Song K, Whittemore LA. Loss of myostatin expression alters fiber-type distribution and expression of myosin heavy chain isoforms in slow- and fast-type skeletal muscle. Muscle Nerve. 2005 Jan;31(1):34-40.

Grobet L, Martin LJ, Poncelet D, Pirottin D, Brouwers B, Riquet J, Schoeberlein A, Dunner S, Menissier F, Massabanda J, Fries R, Hanset R, Georges M. A deletion in the bovine myostatin gene causes the double-muscled phenotype In cattle. Nat Genet. 1997 Sep;17(1):71-4.

Lee SJ. Regulation of muscle mass by myostatin. Annu Rev Cell Dev Biol. 2004;20:61-86.

Lee YS, Lee SJ. Regulation of GDF-11 and myostatin activity by GASP-1 and GASP-2. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(39):E3713-22.

Legault C. Selection of breeds, strains and individual pigs for prolificacy. J Reprod Fertil Suppl. 1985;33:151-66.

Ma D, Gao P, Qian L, Wang Q, Cai C, Jiang S, Xiao G, Cui W. Over-expression of porcine myostatin missense mutant leads to a gender difference in skeletal muscle growth between transgenic male and female mice. Int J Mol Sci. 2015a Aug;16(8):20020-32.

Ma D, Jiang S, Gao P, Qian L, Wang Q, Cai C, Xiao G, Jiang S, Cui W. Functional verification of a porcine myostatin propeptide mutant. Transgenic Res. 2015b Oct;24(5):837-45.

Manceau M, Gros J, Savage K, Thome V, McPherron A, Paterson B, Marcelle C. Myostatin promotes the terminal differentiation of embryonic muscle progenitors. Genes Dev. 2008 Mar 1;22(5):668-81.

McPherron AC, Lawler AM, Lee SJ. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature. 1997 May 1;387(6628):83-90.

McPherron AC, Lee SJ. Double muscling in cattle due to mutations in the myostatin gene. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(23):12457-61.

Nygard AB, Jorgensen CB, Cirera S, Fredholm M. Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. BMC Molecular Biology. 2007 Aug;8(1):67.

Qian L, Tang M, Yang J, Wang Q, Cai C, Jiang S, Li H, Jiang K, Gao P, Ma D. Targeted mutations in myostatin by zinc-

finger nucleases result in double-muscled phenotype In Meishan pigs. Sci Rep. 2015 Sep 24;5:14435.

Qian L, Ma D, Gao P, Jiang S, Wang Q, Cai C, Xiao G. Muscle hypertrophy in transgenic mice due to over-expression of porcine myostatin mutated at its cleavage site. J Integr Agric. 2016 Nov 1;15(11):2571-7.

Smet SD, Webb EC, Claeys E, Uytterhaegen L, Demeyer DI. Effect of dietary energy and protein levels on fatty acid composition of intramuscular fat in double-muscled Belgian Blue bulls. Meat Sci. 2000 Sep;56(1):73-9.

Swatland HJ. Muscle growth in the fetal and neonatal pig. J Anim Sci. 1973 Aug;37(2):536-45.

Wang X, Yu H, Lei A, Zhou J, Zeng W, Zhu H, Dong Z, Niu Y, Shi B, Cai B, Liu J, Huang S, Yan H, Zhao X, Zhou G, He X. Generation of gene-modified goats targeting MSTN and FGF5 via zygote injection of CRISPR/Cas9 system. Sci Rep. 2015 Sep 10;5:13878.

Wegner J, Albrecht E, Fiedler I, Teuscher F, Papstein HJ, Ender K. Growth- and breed-related changes of muscle fiber characteristics in cattle. J Anim Sci. 2000 Jun;78 (6):1485-96.

Wigmore PM, Stickland NC. Muscle development in large and small pig fetuses. J Anat. 1983 Sep;137 (Pt 2):235-45.

Wolfman NM, McPherron AC, Pappano WN, Davies MV, Song K, Tomkinson KN, Wright JF, Zhao L, Sebald SM, Greenspan DS, Lee SJ. Activation of latent myostatin by the BMP-1/tolloid family of metalloproteinases. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(26):15842-6.

Wydro RM, Nguyen HT, Gubits RM, Nadal-Ginard B. Characterization of sarcomeric myosin heavy chain genes. J Biol Chem. 1983 Jan 10;258(1):670-8.

Yang J, Ratovitski T, Brady JP, Solomon MB, Wells KD, Wall RJ. Expression of myostatin pro domain results in muscular transgenic mice. Mol Reprod Dev. 2001 Nov;60 (3):351-61.