SUPPLEMENTARY INFORMATION

Targeted mutations in *myostatin* by zinc-finger nucleases result in double-muscled phenotype in Meishan pigs

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7621 aagtta//
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Supplementary Figure S1. Sus scrofa breed Meishan myostatin (*MSTN*) gene sequence (>gi|134244150|gb|EF490988.1) and ZFN targeted knockout site. Red letters stand for specific site targeted by PZFN1/PZFN2. Blue letters stand for ZFN binding sites. Yellow

highlighted sections stands for three exons of *MSTN* gene. Underline stand for the location of the primers of *MSTN*-total and *MSTN*-intact.

Supplementary Table S1. Summary of handmade cloning results using ZFN-mediated porcine fibroblast cells

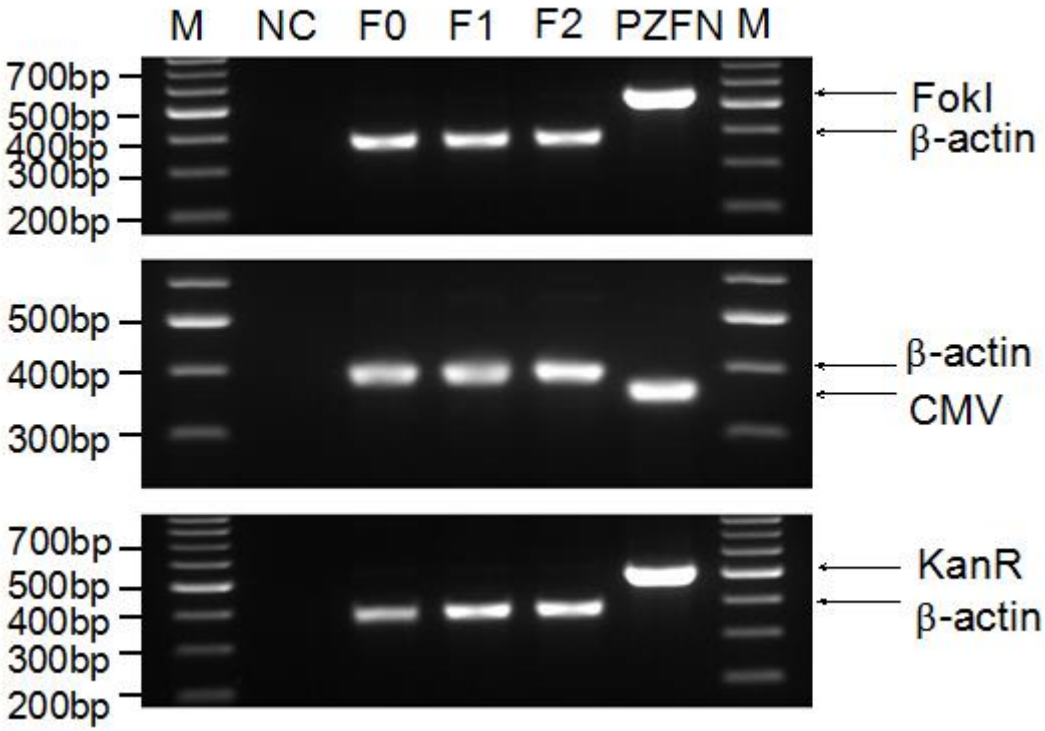
Cloning date	Recipient	Donor cells	No. of transferred reconstructed embryos	Pregnant sows	Stillborn	Liveborn	Cloning efficiency (%)	Piglet ID number and birth weight (kg)
1/2/2012	317	G4	83	—	—	—	0	
	366	G4	103	—	—	—	0	
1/3/2012	269	35/84/166/141	96	—	—	—	0	
1/4/2012	172	G4	94	—	—	—	0	
	205	G4/84	94	—	—	—	0	
1/5/2012	287	113/115/84	89	—	—	—	0	
2/28/2012	283	105	93	—	—	—	0	
	606	105	79	Delivered	3	0	3.8	606-1~606-3[△]
	368	105	79	Delivered	2	8	12.6	105-1[△],105-2[△], 191-198 (08-1.2)
3/5/2012	256	110	99	Delivered	1	0	1.0	256-1[△]
	195	110	125	—	—	—	0	
3/6/2012	269	105	78	—	—	—	0	
	379	110	81	—	—	—	0	
	508	110	90	—	—	—	0	
5/2/12	613	110	84	Delivered	1	2	3.6	613-1[△], T-261 (0.8), T-262 (0.9)

	363	110	84	—	—	—	0	
	203	217	70	Resorbed	—	—	0	
8/6/12	283	T2-186	87	Delivered	2	0	2.3	283-1[△], 283-2[△]
8/8/12	585	110	123	Resorbed	—	—	0	
8/13/12	426	T2-186	109	—	—	—	0	
8/14/12	395	172	99	—	—	—	0	
8/15/12	477	127	115	—	—	—	0	
8/20/12	441	T2-186	131	—	—	—	0	
8/21/12	470	127	126	Delivered	1	4	3.2	470-1 (0.8), 470-2~470-4 (0.4-1.0)[▼], 470-5[△]
8/22/12	638	110	114	Delivered	0	5	4.4	638-1 (0.9), 638-2~638-5 (0.8-1.1)[▼]
9/4/12	641	127	70	—	—	—	0	
	642	127	66	Resorbed	—	—	0	
9/5/12	367	104	70	—	—	—	0	
Total	n=28		2631	Pregnancy rate 10/28=35.7%	10 10/29=34.5%	19 19/29=65.5%	In average 29/2631=1.1%	

[△] Body weights were not available for stillborn piglets. [▼]These piglets died after birth

Supplementary Table S2. Primers used for analysis of ZFN plasmid DNA integration

	Primers (5'-3')	Size of PCR Product	Tm	Integration (+)/Normal (-)
PZFN-Fok1-F	AAGCAGAAAGCCTGACGG	550bp	56 ℃	-
PZFN-Fok1-R	TTAGGAAAGGACAGTGGGAGT			
PZFN-CMV-F	TGGAGTTCCGCGTTACAT	365bp	58 ℃	-
PZFN-CMV-R	TGAGTCAAACCGCTATCCAC			
PZFN-KanR-F	GGTGCCCTGAATGAACTGC	515bp	58 ℃	-
PZFN-KanR-R	CGGGTAGCCAACGCTATGT			
β -actin-F	GCCAACCGTGAGAAGATG	396bp	58 ℃	-
β -actin-R	TGCAAGGAACACGGCTAA			



Supplementary Figure S2. PCR results of ZFN plasmid DNA in cloned piglets and their offsprings. F0, F1 and F2: PCR products from templates isolated from three generations of cloned founder piglets; PZFN: PCR product of ZFN-plasmid DNA; NC: H_2O; M: Marker. β-actin: PCR product of internal refence gene. None of the analyzed piglets shows FokI , CMV, or KanR integration.

Supplementary Table S3. Off-target analysis of cloned piglets generated from cell lines 105 and 110

Site #	Chr.	Location	Binding Sequence	No. Mismatches	Gene?	Off-target (+)/Normal (-)
1	chr7	5380790	CTACCtGCCCATCTTgGAGCGTcTCCTGGGAAaGC	4	KIAA1856	-
2	chr12	23216526	AtCTTCCCAGGAgAAGTGTGAAGgTGGGCTGGTtT	4		-
3	chrX	9196183	ACCaTaaCAGGACATAGGAGgAGATGGGCTtGTAA	5		-
4	chr2	90396700	ATACCAGCCCcTCTTCAACGCTGTCCTGaGAgacG	5		-
5	chr2	93650711	TtCTgCCCAGGACCACACTGAgGAgGGGCTGGTgT	5		-
6	chr14	1524938	CctCttGCaCATCTTCTGACCTGTCCTGGGAAGGC	5		-
7	chr14	117348558	TgAgCAGCCCAgCTTCCGGCAGGaCCTGGGAAGaG	5		-
8	chr13	15555955	TCtTTCCCAGGAgGGGGTGttGAaGGGCTGGTAG	5		-
9	chr16	30824552	CTACCtGtgCATCTTaGAGGGTcTCCTGGGAAGGC	5		-
10	chr4	2091602	CTAagtGCCCgTCcTCACCACAGTCCTGGGAAGGG	5	POLN	-
11	chr4	96457896	CTcCCAtCCCAcCTTCCTTTCGTCCTGGtAAaGG	5	UNC5C	-

These 11 highly homologous segments were analysed by sequencing PCR products.

Supplementary Table S4. Primers used for examining the off-targeting effect of the *MSTN*-specific ZFNs

Similar sequence location	Primer-F	Primer-R	Product size
rorochr7-5380790	AAAATAACTATGGCAGAGGCT	GTATCTGGAGGACTTTGGGT	469bp
chr12-23216526	TCAGCACATCGCTCCAAAT	GTCGTCCTGTCTCCAAGGTTAA	573bp
chrX-9196183	TTAGACTGTCTCAAATGGAAGG	ACTGTTCTGGGGCTTGTCT	479bp
chr2-90396700	AGCAGAAGGCAATGAGGC	CGAACTGGGTCTTGTGGG	465bp
chr2-93650711	CCTGGAGGCTTCTGTGAG	TGTGGGTCTGGAAGTGATG	538bp
chr14-1524938	GGCTTCCTTCCTTGTGCTAC	TGTTGGTGGCTTTGTGGC	511bp
chr14-117348558	CCCATCACTAAGAAAGGCC	CTGCCAGACGATCCACAT	528bp
chr13-15555955	TCTTTCTCAGGCAGGTTTAA	GCAAGGCACTGTGGTTCT	534bp
chr16-30824552	CTGGGAGTCTGGCATTCA	GGTGGAGCTGGGTCTTGT	517bp
chr4-2091602	TGTTTGTCAAGGCCCACG	GAACGAACGGCAACCAGA	481bp
chr4-96457896	CCATACCCTCTAACCCTTT	TCCTGGCAGATTCTTTCTT	574bp

Supplementary Table S5. Average weights of individual muscles from *MSTN*$^{-/-}$ and *MSTN*$^{+/+}$ pigs

	Weights (g)		**Percentage of *MSTN*$^{+/+}$**
	***MSTN*$^{+/+}$ (n = 4)**	***MSTN*$^{-/-}$ (n = 3)**	
Longissimus dorsi	828.30 ±6.19	1629.00 ±99.06	196.67
Semimembranosus	450.90 ±44.01	909.50 ±51.40	201.71
Triceps muscle	402.30 ±17.05	725.00 ±15.12	180.22
Gastrocnemius	218.90 ±9.70	349.90 ±13.46	159.84
Semitendinosus	159.80 ±5.52	262.90 ±21.47	164.52

Supplementary Table S6. Nutrition facts of longissimus dorsi from 8-month-old *MSTN*$^{+/+}$, *MSTN*$^{+/-}$ and *MSTN*$^{-/-}$ Ms pigs

Name	***MSTN*$^{+/+}$**	***MSTN*$^{+/-}$**	***MSTN*$^{-/-}$**
Total protein, %	23.02±0.60	22.54±0.29	22.67±0.01
Total fat, %	1.10±0.16	1.04±0.14	0.61±0.04[a,b]
Moisture, %	74.20±0.70	75.18±0.40	75.07±0.32
Amino acid, %	22.23±0.41	21.94±0.31	22.48±1.15
EAA, %	8.23±0.22	8.19±0.13	8.405±0.53

There is no statistically significant difference in each nutrition ingredient among the *MSTN*$^{+/+}$, *MSTN*$^{+/-}$ and *MSTN*$^{-/-}$ pigs, except for the total fat, which is significantly decreased in *MSTN*$^{-/-}$ pigs compared with *MSTN*$^{+/+}$ and *MSTN*$^{+/-}$ pigs. [a]Statistically significantly different between *MSTN*$^{-/-}$ and *MSTN*$^{+/+}$ pigs ($p<0.05$), [b]Statistically significantly different between *MSTN*$^{-/-}$ and *MSTN*$^{+/-}$ pigs ($p < 0.05$). The “amino acid” including 17 of the 20 common amino acids, except for Trp, Gln and Asn. Essential amino acid (EAA) including 7 of the 8 essential amino-acid, except for Trp. Data are expressed as mean ± SEM.

Supplementary Table S7. Hematology characteristics of 8-month-old *MSTN*$^{+/+}$, *MSTN*$^{+/-}$ and *MSTN*$^{-/-}$ Ms pigs

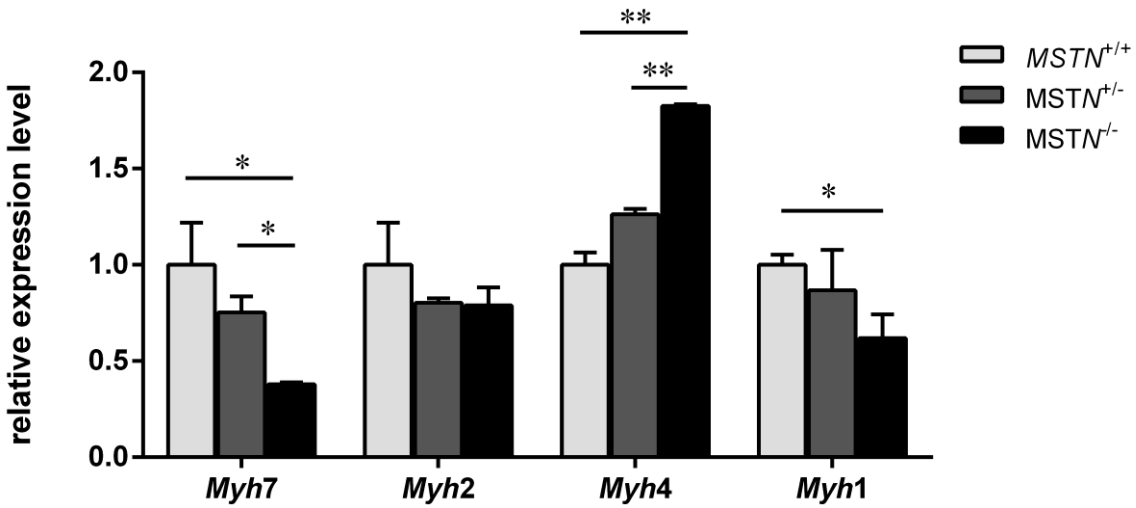
Parameter	***MSTN*$^{+/+}$**	***MSTN*$^{+/-}$**	***MSTN*$^{-/-}$**
RBC (10^{12}/L)	7.72±0.11	7.74±0.54	7.31±0.54
HGB (g/L)	149.00±5.00	141.00±2.50	133.50±2.50
HCT (%)	47.70±2.80	45.20±2.15	42.75±2.15
MCV (fL)	61.50±2.50	58.45±1.40	58.60±1.40
MCH (pg)	19.30±0.40	18.25±1.05	18.35±1.05
MCHC (g/L)	313.00±8.00	312.00±10.00	313.00±10.00
WBC (10^{9}/L)	11.11±4.69	10.02±0.42	10.11±0.42
RDW-CV (%)	17.80±2.60	20.60±2.55	20.85±2.55
RDW-SD (fL)	37.25±0.25	37.75±4.30	39.00±4.30

There is no statistically significant difference in each parameter among the *MSTN*$^{+/+}$, *MSTN*$^{+/-}$ and *MSTN*$^{-/-}$ pigs.

Supplementary Table S8. Biochemical parameters of 8-month-old *MSTN*$^{+/+}$, *MSTN*$^{+/-}$ and *MSTN*$^{-/-}$ Ms pigs

Parameter	*MSTN*$^{+/+}$	*MSTN*$^{+/-}$	*MSTN*$^{-/-}$
ALT (U/L)	41.40±2.54	36.00±2.75	39.00±3.00
AST (U/L)	113.00±15.76	138.20±22.41	90.50±5.50
TP (g/L)	76.46±2.50	79.01±1.15	76.90±5.80
ALB (g/L)	34.70±2.	33.16±1.27	32.15±2.85
GLOB (g/L)	41.76±1.38	45.85±1.73	44.75±2.95
UREA (mmol/L)	3.78±0.12	3.86±0.23	4.43±0.95
CR (μmol/L)	125.60±14.17	117.90±11.79	209.50±12.50[a,b]
GLU (mmol/L)	4.04±0.35	4.02±0.59	6.53±0.48[a]
TG (mmol/L)	0.14±0.02	0.10±0.01	0.05±0.01[a]
TC (mmol/L)	1.47±0.12	1.31±0.10	1.44±0.22
HDL-C (mmol/L)	0.56±0.08	0.45±0.06	0.68±0.16
LDL-C (mmol/L)	0.75±0.04	0.67±0.06	0.68±0.01
INS (pmol/L)	22.26±2.26	22.16±2.10	25.41±2.41

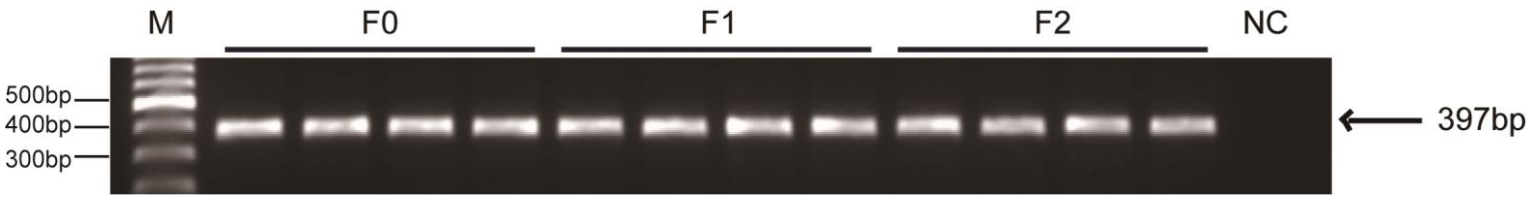
CR and GLU of *MSTN*$^{-/-}$ pigs are significantly increased compared with that of *MSTN*$^{+/+}$ pigs. TG of *MSTN*$^{-/-}$ pigs is significantly decreased compared with that of *MSTN*$^{+/+}$ pigs. There is no statistically significant difference in the other parameters among the *MSTN*$^{+/+}$, *MSTN*$^{+/-}$ and *MSTN*$^{-/-}$ pigs. [a]Statistically significantly different between *MSTN*$^{-/-}$ and *MSTN*$^{+/+}$ pigs ($p < 0.05$). [b]Statistically significantly different between *MSTN*$^{-/-}$ and *MSTN*$^{+/-}$ pigs ($p < 0.01$).



Supplementary Figure S3. Relative expression level of *Myh*7, *Myh*2, *Myh*4, *Myh*1 in longissimus dorsi from 8-month-old male *MSTN*$^{+/+}$, *MSTN*$^{+/-}$ and *MSTN*$^{-/-}$ Ms pigs. *Myh*7, *Myh*2, *Myh*4, *Myh*1 is the specific gene of type Ⅰ, type ⅡA, type ⅡB, and type ⅡX myofibers, respectively. The relative expression level of *Myh*4 (the specific gene of type ⅡB) is significantly higher in *MSTN*$^{-/-}$ pigs than in *MSTN*$^{+/-}$ and *MSTN*$^{+/+}$ pigs.

Supplementary Table S9. Primer pairs used for sequence analysis of the targeted region of *MSTN* gene

	Primer pair sequences (5'-3')	Tm	Size of PCR Product
PZFN1/PZFN2	TACAAGGTATACTGGAATCCGATCT GCAAAGTAAAAGTATCAAGAGGGTA	58°C	397 bp



Supplementary Figure S4. PCR products of the targeted region of *MSTN* which were used for sequencing analysis of cloned piglets and their offsprings. F0, F1 and F2: three generations of cloned founder piglets; NC: H_2O; M: Marker (0.1 kb–1 kb in 0.1-kb steps).

Supplementary Table S10. Primers used for RT-PCR and real time quantitative PCR

Gene	Primer-F	Primer-R
MSTN（coding sequence）	ATGCTGATTGTTGCTGGTCC	CCCACAGCGATCTACTACCA
GDF11（coding sequence）	GTCATTAGCATGGCCCAGGA	ACTTGAGTGCTCATCGCAGT
MSTN-intact	TGAGAATGGTCATGATCTTGCTG	TCCAGTCCCATCCAAAAGCT
MSTN-total	AGTGATGGCTCCTTGGAAGA	TGTAGGAGTCTTGACGGGT
*Myh*7(fiber of type Ⅰ)	GCACTGAAGAGGCTGACAAG	ATCCGTGTCACCATCCAGTT
*Myh*2(fiber of type ⅡA)	CTGGCTGGTTGGACAAGAAC	GACACGGTCTGGAAGGAAGA
*Myh*4(fiber of type ⅡB)	TAGGAAGAAGCACGCAGACA	GGTTTCCCTTGGCTTTGGAG
*Myh*1(fiber of type ⅡX)	ATTTCTGACCTCACGGAGCA	ACCTCCGACTTGACTTGGTT
GAPDH	GTGAAGGTCGGAGTGAACG	CTCGCTCCTGGAAGATGGTG

Methods:

Analysis of Off-Target and ZFN Plasmid Integration into Porcine Genome

ZFN off-target sites were identified by ZFN manufacturer using bioinformatics tools. PCR primers were designed to flank the 11 most likely off-target sites (2 mismatches containing 4 bp and 9 mismatches containing 5bp) as detailed in Supplementary Table S3 and S4. These target regions were amplified in the knockout founder animals and the PCR products were directly analyzed for ZFN cleavage by sequencing. To test the possibility of ZFN plasmid integration into MSTN mutant piglets, Fok1 nuclease-specific PCR was performed using primers ZFN Fok1-F and ZFNFok1-R (Supplementary Table S2 and Fig. S2). PCR product of 550 bp length (obtained with an annealing temperature of 56 °C) was separated on an agarose gel (2%).

Hematology and blood biochemical analysis

Hematology was performed in a local hospital, including the following parameters: red blood cells (RBC), haemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), White blood cells (WBC), red blood cell volume distribution width- coefficient of variation (RDW-CV), red blood cell volume distribution width-standard deviation (RDW-SD). Biochemical parameters were performed in Beijing CIC clinical laboratory using a biochemical analyzer (Vital Selectra E 2000, Holland). The following tests were performed: alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein(TP), albumin(ALB), globin(GLOB), urea, creatinine (CR), glucose (GLU), triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C). Insulin (INS) was detected using an ELISA kit.