

Generation of heritable double muscle buttocks rabbits via myostatin mutation with CRISPR/Cas9 system

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Research

Keywords: rabbit, MSTN, Double muscle buttocks, Cas9, knockout

Posted Date: December 15th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-125471/v1>

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Abstract

Background

Myostatin (MSTN) is a member of the transforming growth factor (TGF- β) superfamily and is considered to be a negative regulator that inhibits muscle development and regeneration, inactivity of *MSTN* gene may affect the development and regeneration of muscle in a few of animals.

Results

In the present study, the muscle mass negative regulator gene myostatin (MSTN) was knocked out at two novel sites in exon3, and the function of these mutation was determined in the offspring rabbits. The typical double muscle phenotype with hyperplasia and hypertrophy of muscle fiber was observed in the MSTN KO rabbits, and similar phenotype was confirmed in the F1 generation rabbits. The body weight of MSTN-KO⁺⁻ rabbits 3708 ± 43.06 g were significant heavier at 180 days than that of control WT rabbit 3224 ± 48.64 g ($P < 0.001$). Fourteen litters of F1 generation rabbits were produced, and the mutation of MSTN could be stably inherited in the MSTN KO rabbits.

Conclusions

Heritable double muscle buttocks rabbits via myostatin mutation with CRISPR/Cas9 system were generated, which will be valuable in meat rabbits breeding and a useful animal model for the study of human muscle development related diseases.

1 Introduction

Myostatin (*MSTN*) is a member of the transforming growth factor (TGF- β) superfamily and is considered to be a negative regulator that inhibits muscle development and regeneration. Like many other members of the TGF- β family, *MSTN* is an extracellular hormone which signal is transmitted through activin receptors. *MSTN* exists in an inactive state in the skeletal muscle circulatory system and restores its activity by binding to the precursor peptide, follistatin 3 and TGF- β binding protein[1, 2]. The high affinity binding between activated *MSTN* and ActRIIB can lead to the signal chain transmission and then inhibit the myocyte differentiation and proliferation[3–5]. Many evidences have been found that inactivity of *MSTN* gene may affect the development and regeneration of muscle. Mice with *MSTN* gene deletion showed double muscle phenomenon and a significant increase in muscle mass[6]. It was muscle hyperplasia and hypertrophy that caused a significant increase in muscle in mice. Later, natural mutations of *MSTN* were found in cattles[7, 8], dogs[9], sheeps[10, 11] and human[12, 13]. These natural mutations Can cause obvious double muscle trait. Efforts have been done to study the function of *MSTN* in muscle development of animals. The 2020 Nobel Prize in Chemistry has been awarded to Emmanuelle Charpentier and Jennifer A. Doudna[14]. Their discovery of CRISPR/Cas9 enabled scientists to cut a sequence of DNA into two pieces, allowing nature to "repair" it. During the repair process, errors will be introduced to create a different sequence of DNA, thus resulting into an edited gene. "There is enormous power in this genetic tool, which affects us all," said Claes Gustafsson, chair of the Nobel Committee

for Chemistry. "It has not only revolutionized basic science, but also resulted in innovative crops and will lead to groundbreaking new medical treatments." Gustafsson said that, as a result, any genome can now be edited "to fix genetic damage." And now, As the most efficiency genome edit tool, CRISPR/Cas9 system has been widely used to study the role of functional genes. Knock out of *MSTN* with CRISPR/Cas9 system has been processed in rabbits[15, 16], sheep[17, 18], goats[19, 20], and pigs[21, 22] in the last few years. Qian, L et al. knock out the *MSTN* gene of Meishan pig fetal fibroblasts by Engineered zinc-finger proteins, and prepared the *MSTN*-KO pig by somatic cell nuclear transfer technology firstly. Compared with wild-type Meishan pigs, the lean meat rate of Meishan pigs with *MSTN* knockout increased by 11.62%[23]. In addition, the *MSTN*-KO goats obtained by the Transcription activator-like effectors successfully, and *MSTN*-KO goats meat production is 32% higher than wild-type goats.

Efficient TALEN-mediated myostatin gene editing in goats. BMC Dev Biol Zhang et al. Inserted the fat-1 gene to the goat *MSTN* gene by CRISPR/Cas9. While overexpressing fat-1, the *MSTN* gene was knocked out. The results of PCR and gas chromatography analysis showed that the knockout efficiency of *MSTN* was as high as 25.56%, and a significant increase in n-3PUFA level was detected in goat tissues. Zhang et al.[24] used CRISPR/Cas9 to insert the fat-1 gene into the goat *MSTN* gene, While overexpressing fat-1, the *MSTN* gene was knocked out, so this strategy edited both genes simultaneously. PCR and gas chromatography analysis results showed that the *MSTN* knockout efficiency was as high as 25.56%, and a significant increase in the expression level of n-3PUFA was detected in goat tissues. Moreover, further studies found that loss activity of *MSTN* is also relate to the obesity, muscle atrophy and type 2 diabetes[25, 26].

It has a long tradition of rabbit meat production and consumption in France, Italy and Spain, and according to the database of FAOSTAT[27] the world per capita rabbit meat consumption in 0.242 kg, while it is very high in many European countries, especially 4.39 kg per capita in Italy. It presents the stable growth of rabbit meat consumption state from 2000 to 2013 in many European countries[28]. rabbit meat offers excellent nutritive and dietetic properties[29] and contains high-quality protein, low fat, low cholesterol, which is ideal meat of the obese and cardiovascular patients. In the present study, we designed a highly efficient new site, which is different from previous studies, and further verified that the exon 3 region can regulate the *MSTN* gene. At the same time, the double-muscle breech rabbits obtained in this experiment can be normally inherited to the offspring, and have stable double-muscle breech characterization.

2 Materials And Methods

2.1 Animals

In present study, all of the experiments on rabbit were performed by the Principle Guidance for the Use and Care of Laboratory Animals. Rabbits were fed regularly at Laboratory Animal Center of Guangxi University. The rabbits used for ovulation were approximately 6–8 months old and weighed between 3.5 kg to 4.5 kg.

2.2 SgRNA Design and Plasmid Construction

A total of 8 sgRNAs targeting the *MSTN* sites were designed using the website (<http://www.genome-engineering.org/>) and named g1, g2, g3, g4, g5, g6, g7 and g8 (Fig. 1A). The two complementary DNA oligos were annealed to become double-stranded, and cloned into the pMD18-hU6-gRNA vector. Primer T76 and T78

were used as target gene fragments amplified by the two sgRNA targeting vectors. Linearized DNA plasmids and PCR products were extracted and purified using the MEGA shortscript™ T7 kit (Ambion, USA) and sgRNAs were generated according to the manufacturer's recommendations.

2.3 Culture and electroporation of fibroblasts

Primary rabbit fibroblasts were cultured in 10% (v/v) FBS, 35 mm glutamine and 1 × DMEM (Gibco). The plasmid was transfected into fibroblasts by electroporation at a pulse of 225 volts for 10 milliseconds. Briefly, 4 µg of pCMV-T7-NLS-hSpCas9-NLS (hSpCas9) DNA plasmid and 2 µg of gRNA plasmid were used to transfect 5–10 × 10⁵ fibroblasts. the culture medium was changed to DMEM containing 10% FBS 24 hours later. After cultured for 4 days, the DNA was extracted and the *MSTN* was amplified by PCR. Sent to the company for sequencing to analyze the editing effect of the gene fragment. Optimal sgRNA was selected for the following experiment.

2.4 Zygotes collection and Blastocyst genotyping testing

Rabbits of estrus were mated two or three times 16–20 hours before mating, and zygotes were collected from the fallopian tubes with cell culture medium. The cell culture medium was consisted of M199 (Gibco, USA), 3% bovine serum (Gibco, USA), 5 mM Hepes (Sigma, USA) and 5 mM NaHCO₃ (Sigma, USA). The zygotes were microinjected with Cas9 protein and sgRNA and in vitro cultured to blastocyst, and the mutation was analyzed by PCR and T7EI digestion of the target region.

2.5 Generation of transgenic rabbit

The zygotes was injected with 10 pL CRISPR/Cas9 mixture, containing 200 ng/µL Cas9 protein (A36497, Hot Fisher, USA), 20 ng/µL sgRNA6 and 20 ng/µL sgRNA8, in the cytoplasm and the zygotes were transferred back to the oviduct of recipient rabbit. Pregnancy phenomenon was checked on 10 days after the transplantation and the cubs were born about 30 days later.

2.6 T7 Endonuclease I (T7EI) assay

Genome DNA of the cubs were extracted and the mutation induced by CRISPR/Cas9 was analyzed by PCR and T7E1 assay. PCR was performed using primers flanking the target site and the product was sequenced (Shanghai Sangon Company, Shanghai, China) and digested with T7EI (NEB, USA) to characterize the mutation.

2.7 Off-target analysis

Potential off-target sites of the sgRNAs were evaluated using the CRISPR design tool (<http://crispor.tefor.net/>). The top 5 potential off-target sites of each sgRNA were selected and amplified by PCR with specific primers (Table.1), and the PCR products were analyzed by sequencing and T7E1 digestion.

2.8 Western Blot analysis

The gluteus Maximus tissue samples of *MSTN*^{+/−} and WT rabbits (euthanized at 6 months of age) was collected, tissues were ground in liquid nitrogen, and added 2.5 µL / mL protease inhibitor with ice for 30 minutes. Protein concentration was determined using the Bradford method (Bio-Rad). 35 µg of protein sample was subjected to a 5%~12% separation of SDS-polyacrylamide gel. Antibodies used in this study included anti-

MSTN polyclonal antibody (Abcam) and goat anti-rabbit IgG coupled to horseradish peroxidase (HRP) (Santa Cruz, USA). The β-actin antibody (Santa Cruz) was used as an internal control.

Table 1
Primers of off-target detection

<i>Primer</i>	<i>Sequence</i>	<i>Length</i>
POTs6-1F	CAATGGTGTGAGCCTCAAAG	382 bp
POTs6-1R	AGTGGTCGTCTTCTTCATCC	
POTs6-2F	ATGCTCCTGTGTAGTCACTG	373 bp
POTs6-2R	GTTTCCATGTCCAGCTCAC	
POTs6-3F	ATCCAGGTATTAGCAACCGT	292 bp
POTs6-3R	TCTGTGAATGTGCATACATACA	
POTs6-4F	TGACTACTGGCCAAAATGT	450 bp
POTs6-4R	TTCAGTCACAGAGTCGGTT	
POTs6-5F	GAAGGGCCACAAAGAGAAAG	238 bp
POTs6-5R	AAGGCCTTTCCCTCCCAG	
POTs8-1F	GGCGCTCATGATCTCTTGCT	291 bp
POTs8-1R	CCTCACCAATGTCGATGCCT	
POTs8-2F	AGCAGACATTCTGGCGGAAA	348 bp
POTs8-2R	GCCAAATGCAGCCTCAGAAT	
POTs8-3F	TCCAGAGTGCCTGCAGGTA	351 bp
POTs8-3R	TCCAGAAGCTCAAATCTCTTGCT	
POTs8-4F	GTTTGGACACTGCTTGCTGG	291 bp
POTs8-4R	TCATGGTGGATGCCCTCTTG	
POTs8-5F	CACACACATCCTCGGCTCAT	352 bp
POTs8-5R	CATTACCTTCCCTCCCCACCCCC	

2.9 Weight, and sample collection and Histological analysis

In this study, 5 F0 *MSTN*^{+/−} and 5 WT rabbits were feed at the same conditions. All rabbits were weaned at 30 days and were kept in separate cages in the same litter. Body weight was recorded every 10 days up to 200 days. At 2 months of age, rabbits were anesthetized with anesthesia with pentobarbital sodium (1 mL/ kg). The corpses were dissected and the hearts, tongue, gluteus maximus and lateral femoral muscles of the *MSTN* KO and WT groups were weighed.

The tongue and gluteus Maximus tissues of F0 generation *MSTN* KO and WT rabbits (6 months old sacrifice) were fixed with 4% paraformaldehyde at 4°C, The increasing concentrations of dehydrated sucrose (30% for 7 h, 40% for 7 h, 45% for 5 h), freezed section at -25°C for histological examination. The tissues which were sectioned into 5 µm thick slices were stained with hematoxylin and eosin (H&E) and analyzed by Fluorescence Inverted Microscope (Nikon, Japan). The integral optical density (IOD) analysis of histological sections was performed by Image-Pro Plus 6.0 software.

2.10 Statistical analysis

All of the data we obtained in this study were analyzed using Graphpad prism software (T test) and one-way ANOVA followed by Duncan's Multiple Comparison Tests in the SPSS 17 statistical software package.

3 Results

3.1 Design and construction of sgRNA

Eight sgRNAs targeting to the *MSTN* exon 1 and exon 3 were designed and cloned into the pMD18T vector, respectively (Fig. 1A). The recombinant vector was named as pMD18-hU6-gRNA and confirmed that the sgRNA has been successfully inserted into the vector by sequence analysis (Fig. 1C).

3.2 Modification efficiency of the sgRNAs in rabbit fibroblasts

The mutation efficiency of these designed sgRNAs was comfirmed in rabbit fibroblasts, and no mutation were generated in g1 to g4, the mutation rates of g5-g8 from 20–45% (Fig. 2). The sgRNAs target to exon 3 is significantly better than that of exon 1. The gene modification rate of sgRNA6 (45%, 9/20) and sgRNA8 (40%, 8/20) were higher than that of the other gRNAs. Thus, sgRNA6 and sgRNA8 were selected in the following experiments.

3.3 CRISPR/Cas9-mediated *MSTN* site-specific KNOCK-OUT in rabbit zygotes

The CRISPR/Cas9 system containing sgRNA6 and sgrNA8 were injected into the zygotes to verify the site-specific deletion of *MSTN* gene. A total of 16 zygotes was injected and 10 of them developed to blastocyst stage in vitro (Fig. 3A). The genome editing efficiency in the blastocysts were analyzed by PCR and Sanger sequence, and results revealed that 7 of them showed genetic mutations (70 %, 7/10) (fig. 3B).

3.4 Generation of *MSTN* knock-out rabbits

A total of 99 cytoplasmic injected zygotes were transplanted to oviduct of 10 receptor female rabbits, and 23 pup rabbits were born after full-term gestation (Table 2). The presence of genome editing was analyzed by T7E1 digestion and Sanger sequencing analysis, and the results showed that 5 of them are *MSTN* knock-out rabbits (Fig. 4). Intrestingly, three *MSTN*^{+/−} rabbits were all males, and two *MSTN*^{−/−} rabbits were all females. The further T7E1 and sequencing analysis showed that 5 *MSTN* knock-out rabbit are all chimeras.

Table 2
Generation of the *MSTN* KO rabbits via CRISPR/Cas9

NO.	SgRNA	gRNA/Cas9 protein(ng/uL)	Embryos Injected	Embryos transferred	Pregnancy	Pups obtained	<i>MSTN</i> KO pups(%pups)
							(%transferred)
M02	Sg6 + Sg8	40/200	14	14	Yes	3(21.4%)	1(33.33%)
M08	Sg6 + Sg8	40/200	12	12	Yes	3(25%)	1(33.33%)
M09	Sg6 + Sg8	40/200	7	7	Yes	4(57.14%)	0(0)
M10	Sg6 + Sg8	40/200	10	10	Yes	4(40)	2(50%)
M12	Sg6 + Sg8	40/200	9	8	Yes	4(50%)	1(25%)
M13	Sg6 + Sg8	40/200	8	8	Yes	3(37.5%)	0(0)
M14	Sg6 + Sg8	40/200	7	14	Yes	2(14.29%)	1(50%)
M15	Sg6 + Sg8	40/200	9	7	Yes	4(57.14%)	1(25%)
M17	Sg6 + Sg8	40/200	11	11	Yes	4(36.36%)	2(50%)

3.5 Off-target analysis of *MSTN* knock-out rabbits

To detect whether off-target mutations were present in those *MSTN* knock-out rabbits, 4 potential off-potsgRNA were designed using the onlineCRISPR Design Tool (<http://crispor.tefor.net/>). The further T7EI digestion and Sangersequencing resultsshowedthatnomutationwasdetected, which mean that no off-targetpresentin the *MSTN*KO rabbits(Fig.5).

3.6 Western Blot analysis of *MSTN* KO rabbits

The expressionof *MSTN*in muscle tissue of *MSTN* KO rabbit was significantly lower than that of WT rabbit based on QRT-PCR analysis ($p<0.05$). And the further western blot analysis results showed that the expression level of *MSTN*protein of the mutant rabbits was lower in the muscle than wild rabbit(Fig. 6).

3.7 Morphological analysis of *MSTN* KO rabbits

The difference in muscle development of the *MSKN* KO rabbit was evaluated by morphological analysis and body weight.The specific data of weight gain are shown in Table 3. No significant difference of the body weight was found in the five *MSTN* KO rabbits with the control group within the early 90 days ($P>0.05$). When the five *MSTN* KO rabbits grown to the third month, the weight of *MSTN* KO rabbits began to appear slightly higher than that of the control group (Fig. 7C). And *MSTN*-KO rabbits appeared "double muscle" phenomenon, rabbit muscle

morphology was more obvious. At 130 days of age, the weight of five *MSTN*-KO rabbits showed a significant heavier than that of wild control rabbits ($P < 0.05$, $n = 5$). And the body weight of *MSTN*-KO rabbits 3708 ± 43.06 g were significant heavier at 180 days than that of control WT rabbit 3224 ± 48.64 g ($P < 0.001$, $n = 5$). The gluteus of the *MSTN*-KO rabbits also looks bigger than WT rabbits, and the further histological analysis showed that the density and diameter of the myofiber was significantly larger in *MSTN*-KO rabbits (Fig. 7B).

Table 3
FO generation rabbits weight data

Age/Day	M2-1	M15-2	WT1	WT2	T2-3	T7-2	T7-3	T2-2	T7-1	T7-4
1	88	85.4	74.2	81.5	73	76.8	70.1	72.5	80.6	58.7
10	165	206	240	218	200	335	320	220	365	160
20	326	360	459	431	410	460	480	430	490	310
30	650	690	770	720	760	680	640	860	720	360
40	1090	1200	1132	1090	1250	1190	1148	1280	1300	1228
50	1450	1690	1680	1620	1560	1330	1355	1435	1470	1250
60	1900	1930	2130	1940	1960	1670	1800	2100	1890	1810
70	2270	2380	2320	2400	2030	1735	2095	2240	2180	2130
80	2510	2660	2656	2595	2385	2080	2250	2415	2285	2390
90	2840	2840	2950	2740	2535	2180	2315	2480	2220	2540
100	3119	2980	3120	2940	2630	2275	2340	2790	2585	2820
110	3420	3100	3160	3090	2980	2380	2460	2980	2600	2880
120	3520	3300	3180	2980	2940	2410	2580	3200	2780	2950
130	3490	3350	2800	3100	2920	2570	2660	3280	2880	3060
140	3448	3670	2960	3200	2980	2630	2750	3430	2960	3130
150	3480	3790	2840	3160	3120	2870	2930	3610	3280	3310
160	3580	3830	3060	3340	3100	2910	3060	3690	3480	3380
170	3530	3750	2980	3300	3270	3110	3200	3750	3650	3440
180	3660	3760	3120	3380	3250	3120	3250	3820	3730	3570
Note:The weight unit is g.										

3.8 Heritability of the *MSTN*-KO rabbits.

To determine whether the *MSTN* KO could be stably transmitted to the offspring, the two female founders *MSTN*^{-/-} were mated with male rabbit, but now offspring pub rabbits were produced. The three *MSTN*^{+/+} F0 male rabbits were mated with wild rabbits, and live F1 offspring pub rabbits generated from all those three rabbits. T-

cloning sequence analysis and T7E1 cleavage assay demonstrated that 28 out of 53 newborn F1 rabbits carried *MSTN* mutations. In these pups, the 23 were monoallelic, while 5 were biallelic *MSTN*-KO rabbits (Fig. 8).

4 Discussion

In this experiment, the Cas9 / sgRNA mixture was injected into the single-cell stage embryos by cytoplasmic injection to delete long fragments and ensure the inactivation of target gene function. The *MSTN* KO rabbit was successfully produced, and the results showed that the system can effectively produce knockout rabbits. The loss of the *MSTN* fragment could leads to muscle growth.

Although the increase in muscle mass makes *MSTN*-KO livestock production more attractive, However, in these *MSTN* mutant animals, calving difficulties are often caused by various reasons. *MSTN* is an important regulator of muscle cell proliferation and differentiation during muscle development. *MSTN* signals can be detected from the early myogenic stage of embryonic sarcomere formation to the adult skeletal muscle development stage[7, 30, 31]. The expression of *MSTN* was observed for the first time during the embryonic period[7, 30, 31]. The birth weight of *MSTN* mutant animals has been observed in sheep, cattle and goats[32, 33].Therefore, the knockout of the *MSTN* gene may affect muscle development in the embryonic stage. The researchers believe that during fetal development, *MSTN* is only expressed in skeletal muscle, thereby controlling the differentiation and proliferation of myoblasts. In our study, *MSTN*-KO rabbits exhibited a typical double-muscle phenotype, but there was no significant difference in body weight at first 60 days and Compared with the wild-type control group, there is almost no difference in body size at birth ($P > 0.05$). It's might beacause IGF-1 is an important positive regulator of muscle cell proliferation and differentiation in skeletal muscle[34]. Despite the inhibition of *MSTN* signal expression, IGF signaling will still upregulate the expression of myostatin in skeletal muscle tissue models, which also indicates that there is an inhibitory autoregulatory loop in the muscular system[35, 36].

Studies believe that in most terrestrial vertebrates, the increase in mammalian muscle production is mainly caused by muscle fiber hypertrophy, hyperplasia, or a combination thereof caused by the lack of *MSTN*[21, 37–39]. The number of muscle fibers mainly determined before birth, and the diameter of muscle fibers may expand after birth[40, 41]. The muscle mechanisms of hypertrophy and hyperplasia may not occur simultaneously[42, 43]. But it seems that the mechanism by which *MSTN* mutations enhance muscle mass is different between species. In *MSTN* mutant mice, goats and rabbits, the increase in muscle mass is caused by muscle fiber hyperplasia and diameter hypertrophy[37, 44, 45]. However, only muscle fiber hyperplasia was observed in *MSTN* mutant cattle and pigs[8, 46]. The muscle mass of *MSTN*-deficient mice is 2–3 times than of wild-type mice. The increase in muscle mass is the result of the combined action of muscle fiber hyperplasia and hyperplasia[37]. In contrast, double-muscle cattle show muscle mass increased by 20% -25%, and the increased muscle weight seems to be the result of muscle fiber diameter proliferation, rather than an increase in muscle number[8, 39]. The phenotypic trait of muscle hypertrophy is a quantitative trait. The quantitative trait is the result of the joint action of many independent small-effect multi-genes. The effect of each gene is equal, but in some cases there is a major effect gene Gene is the main effect in this trait, and the other is micro-effect. The muscle growth inhibition is controlled by *MSTN* gene, and other genes are controlled by the micro-effect gene.

In addition, the *MSTN*-KO rabbit exhibits the muscular feature of "double mushy buttocks", and the fetus of the rabbit presents with "big tongue syndrome"[47]. In some live rabbits born, some of the *MSTN*-KO rabbits also have "big tongue syndrome", which causes milk sucking. Success leads to death. Most of the *MSTN*-KO rabbits

show a rapid increase in muscle mass after 60 days of age, and *MSTN*-KO rabbits are healthy and can inherit the knocked-out gene fragments, which shows that the *MSTN*-KO rabbits produced by the CRISPR / Cas9 system are suitable for studying muscle development and Related diseases.

5 Conclusion

In summary, we obtained *MSTN*-KO rabbits with double muscle typically. These *MSTN*-KO rabbits could be a promising tool for studying the development of livestock muscles and improving their important economic traits. Thereby improving the meat production efficiency of rabbits and promoting the development of rabbit industry. We established a CRISPR/Cas9 gene editing system suitable for the laboratory, which is more effectively applied to various model animals and large livestock animals.

Abbreviations

CRISPR: Clustered regularly interspaced short palindromic repeats

Declarations

Ethics approval and consent to participate

All experimental studies were approved by the Experimental Animal Care and Use Committee of Guangxi University (Permit code: GXU2019-029).

Consent for publication

Not applicable.

Availability of data and materials

All datasets for this study are included in the manuscript and the Supplementary Files. The datasets used or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that there is no conflict of interest.

Funding

Present study was granted and supported by the National Natural Science Fund (Grant No. 31760648 and 31860638). and we are also grateful for Guangxi Natural Science Foundation (Grant No. AB16380042).

Authors' contributions

QYL and KQC designed the experiments, YZ and YLZ conceived the experiments and draft the manuscript. LYW, NX, and HLL help performed the experiments. YZ and LYW analyzed the data. ZPL , LA and DSS revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figures

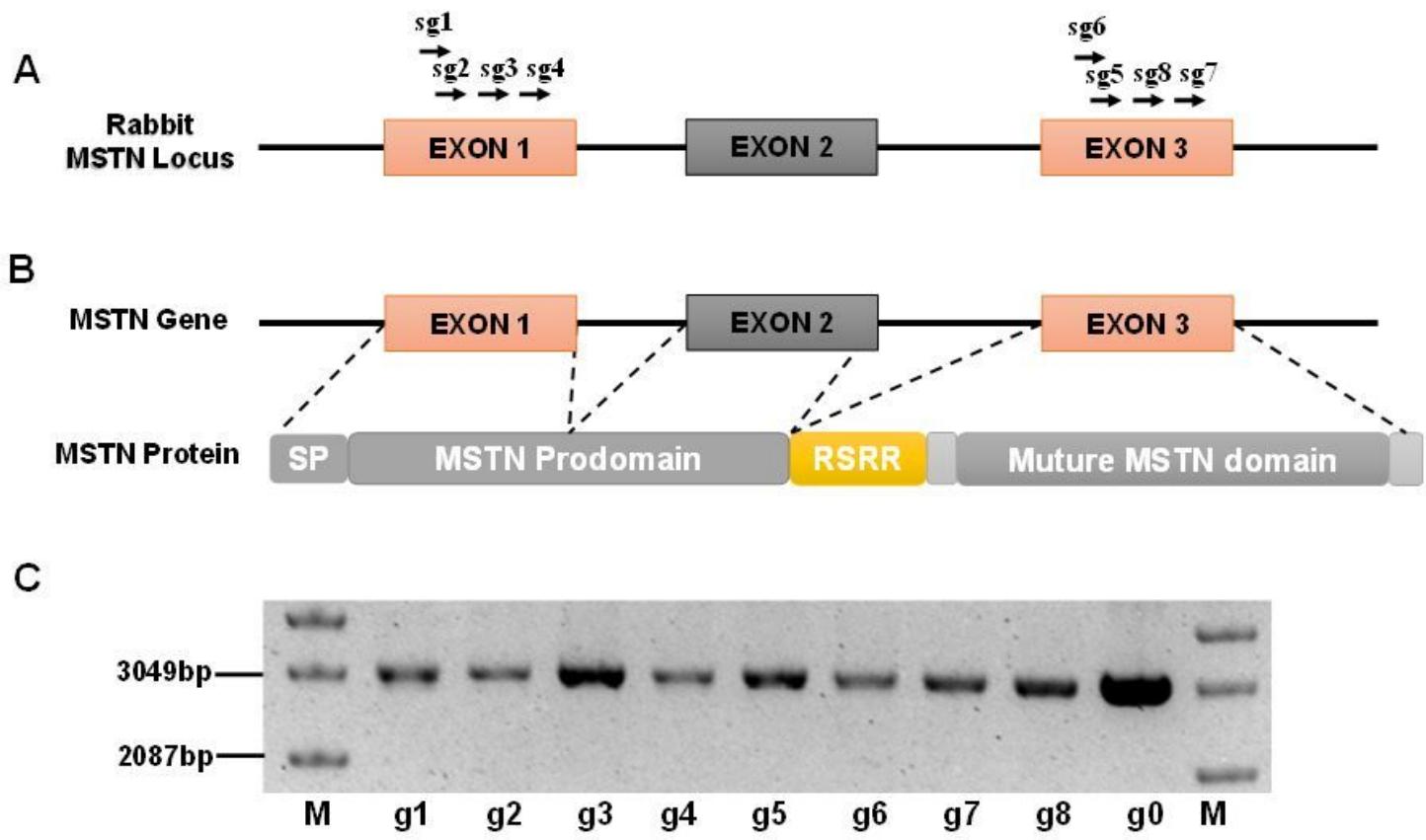


Figure 1

(A) Design and construction of sgRNA Schematic diagram of sgRNA targeting site in the rabbit MSTN gene locus. (B) Structure of GDF8 gene and MSTN protein. (C) Electropherogram of eight sgRNA vectors, M Supercoiled DNA Ladder Marker,g1-g8 refer to phU6-gRNA-Sg1to phU6-gRNA-Sg8.

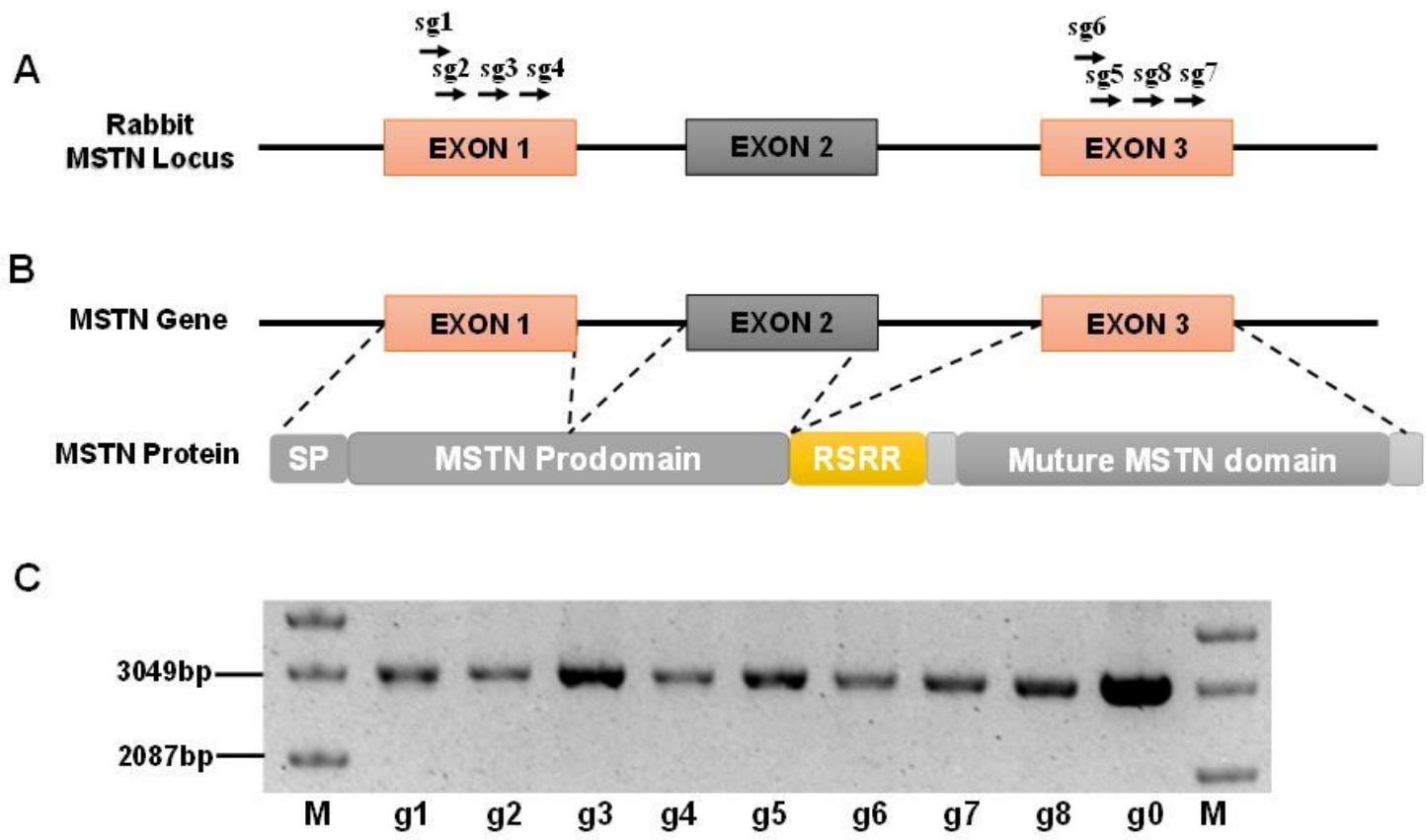
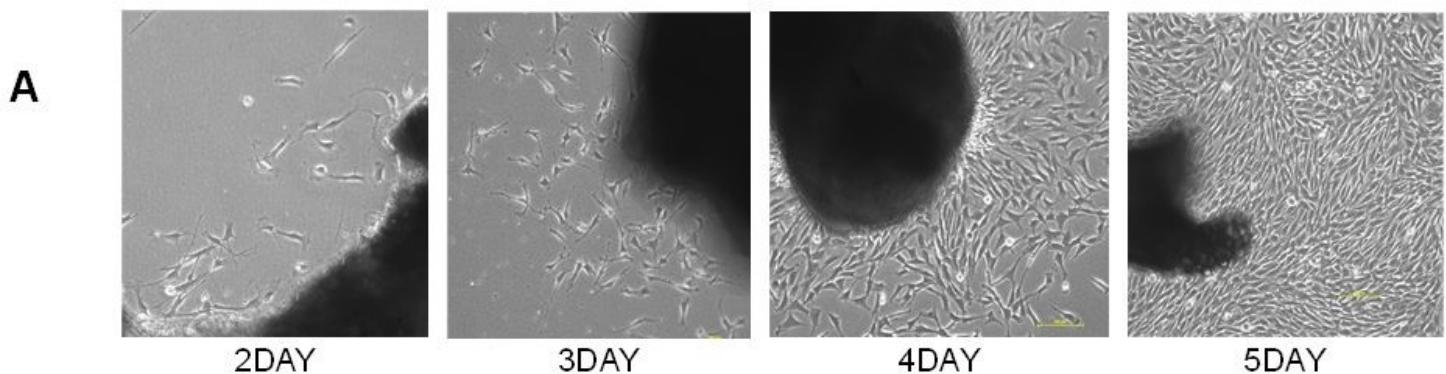


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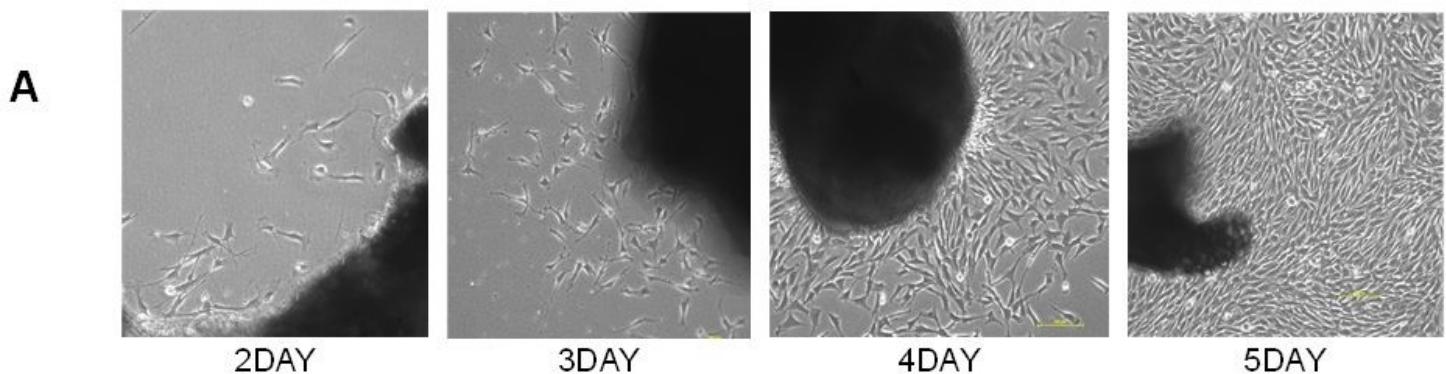


B

WT	AAGACGGGCTGTGAATGCATGCACTTGGAGACAAAACAGTAAATAT	0
g1 (0, 0%)	AAGACGGGCTGTGAATGCATGCACTTGGAGACAAAACAGTAAATAT	0
g2 (0, 0%)	AAGACGGGCTGTGAATGCATGCACTTGGAGACAAAACAGTAAATAT	0
g3 (0, 0%)	AAGACGGGCTGTGAATGCATGCACTTGGAGACAAAACAGTAAATAT	0
g4 (0, 0%)	AAGACGGGCTGTGAATGCATGCACTTGGAGACAAAACAGTAAATAT	0
g5 (5/20, 25%)	AAGAGATACAAGGCCAATTACTGCTCTGGAGAGTGTGAATTGTG 0 AAGAGATACAAGGCCAATTACT--CTCTGGAGAGTGTGAATTGTG -1 AAGAGATACAAGGCCAATTACTGTTCTGGAGAGTGTGAATTGTG C-T	
g6 (9/20, 45%)	TTGGATTATTGCGCCCAAGAGATACAAGGCCAATTACTGCTCTGG 0 TTGGATTATTGCGCCCAAG----TACAAGGCCAATTACTGCTCTGG -3 TTGGATTATTGCGCCCAAGAGATTACAAGGCCAATTACTGCTCTGG +1 TTGGATTATTGCGCCTAACAGAGATACAAGGCCAATTACTGCTCTGG C-T TTGGATTATTGCGCCCAAGAG-TACAAGGCCAATTACTGCTCTGG -1 TTGGATTATTGCGCCCAAGA-----CAAGGCCAATTACTGCTCTGG -4	
g7 (6/20, 30%)	AATAATATATGGAAAATTCCAGCCA TGGTAGTAGACCGCTGTGG 0 AATAATATATGGAAAATTCCA--CCATGGTAGTAGACCGCTGTGG -1 AATAATATATGGAAAATTCCA----TGGTAGTAGACCGCTGTGG -4 AATAATATGTGGAAAATTCCAGCCA TGGTAGTAGACCGCTGTGG A-G AATAATATATGGAAAATTCCAGCCC ATGGTAGTAGACCGCTGTGG +1 AATAATATACGGAAAATTCCAGCCA TGGTAGTAGACCGCTGTGG T-C	
g8 (8/20, 40%)	CATCTTGTACACCAAGCAAACCCAGAGGTTCAGCAGGTCCTTG 0 CATCTTGTACACCAAGCAAACCC--AGAGGTTCAGCAGGTCCTTG -1 CATCTTGTACACCAAGCAA-----AGGTTCAGCAGGTCCTTG -6 CATCTTGTACACCAAGCAAAC--AGAGGTTCAGCAGGTCCTTG -3 CATCTTGTACACCAAGCA-----AGCAGGTCCTTG -14	

Figure 2

The results of sequencing for mutations at the specific sites in rabbit REFs. (A) Rabbit Primary fibroblast cell in culture from 2days to 5days. (B) Sanger sequence analysis of REFs. g1-g4 target to exon1, g5-g8 target to exon 3. gRNA edited were named on the left. g1-g8 represent different gRNA, PAM sequence is marked in green. The sgRNAs sequence is marked in red. The numbers on the right indicated the type of mutation, with “-” represents deletion of the given number of nucleotides, “+” represents insertion of the given number of nucleotides.

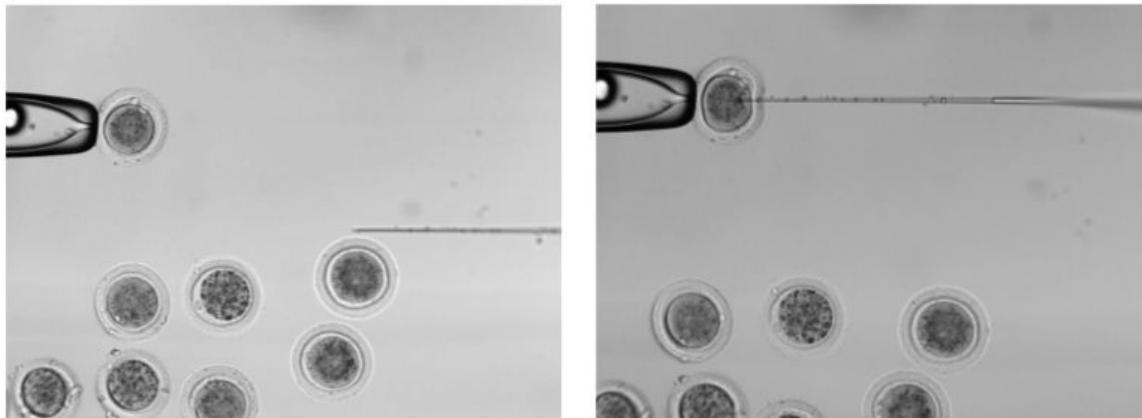


B

	WT	AAGACGGGCTGTGAATGCATGCACTTGGAGACAAAACAGTAAATAT	0
g1 (0, 0%)		AAGACGGGCTGTGAATGCATGCACTTGGAGACAAAACAGTAAATAT	0
g2 (0, 0%)		AAGACGGGCTGTGAATGCATGCACTTGGAGACAAAACAGTAAATAT	0
g3 (0, 0%)		AAGACGGGCTGTGAATGCATGCACTTGGAGACAAAACAGTAAATAT	0
g4 (0, 0%)		AAGACGGGCTGTGAATGCATGCACTTGGAGACAAAACAGTAAATAT	0
g5 (5/20, 25%)	AAGAGATACAAGGCCAATTACTGCTCTGGAGAGTGTGAATTGTG	0	
	AAGAGATACAAGGCCAATTACT--CTCTGGAGAGTGTGAATTGTG	-1	
	AAGAGATACAAGGCCAATTACTGTTCTGGAGAGTGTGAATTGTG	C-T	
g6 (9/20, 45%)	TTGGATTATTGCGCCCAAGAGATACAAGGCCAATTACTGCTCTGG	0	
	TTGGATTATTGCGCCCAAG----TACAAGGCCAATTACTGCTCTGG	-3	
	TTGGATTATTGCGCCCAAGAGATTACAAGGCCAATTACTGCTCTGG	+1	
	TTGGATTATTGCGCCTAACAGAGATACAAGGCCAATTACTGCTCTGG	C-T	
	TTGGATTATTGCGCCCAAGAG-TACAAGGCCAATTACTGCTCTGG	-1	
	TTGGATTATTGCGCCCAAGA-----CAAGGCCAATTACTGCTCTGG	-4	
g7 (6/20, 30%)	AATAATATATGGAAAATTCCAGCCA TGGTAGTAGACCGCTGTGG	0	
	AATAATATATGGAAAATTCCA--CCATGGTAGTAGACCGCTGTGG	-1	
	AATAATATATGGAAAATTCCA-----TGGTAGTAGACCGCTGTGG	-4	
	AATAATATGTGGAAAATTCCAGCCA TGGTAGTAGACCGCTGTGG	A-G	
	AATAATATATGGAAAATTCCAGCCC ATGGTAGTAGACCGCTGTGG	+1	
	AATAATATACGGAAAATTCCAGCCATGGTAGTAGACCGCTGTGG	T-C	
g8 (8/20, 40%)	CATCTTGTACACCAAGCAAACCCAGAGGTTCAGCAGGTCCTTG	0	
	CATCTTGTACACCAAGCAAACCC--AGAGGTTCAGCAGGTCCTTG	-1	
	CATCTTGTACACCAAGCAA-----AGGTTCAGCAGGTCCTTG	-6	
	CATCTTGTACACCAAGCAAAC--AGAGGTTCAGCAGGTCCTTG	-3	
	CATCTTGTACACCAAGCA-----AGCAGGTCCTTG	-14	

Figure 2

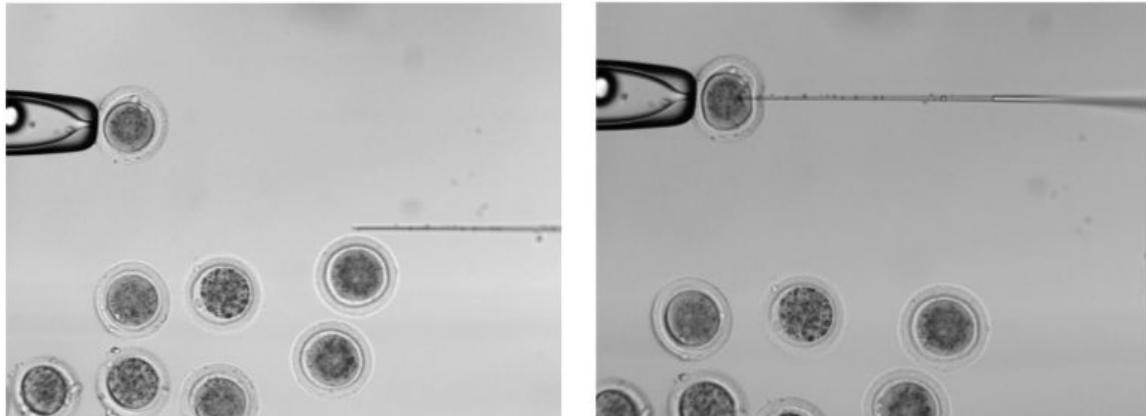
The results of sequencing for mutations at the specific sites in rabbit REFs. (A) Rabbit Primary fibroblast cell in culture from 2days to 5days. (B) Sanger sequence analysis of REFs. g1-g4 target to exon1, g5-g8 target to exon 3. gRNA edited were named on the left. g1-g8 represent different gRNA, PAM sequence is marked in green. The sgRNAs sequence is marked in red. The numbers on the right indicated the type of mutation, with “-” represents deletion of the given number of nucleotides, “+” represents insertion of the given number of nucleotides.

A**B****sgRNA 6****sgRNA8**

WT	GGGATTGGATTATTGCGCCAAGAGATACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC	0
BL1,BL6,BL7	GGGATTGGATTATTGCGCCAAGAGATACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC	0
BL2	GGGATTGGATTATTGCGCCAAGAGATACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAGAGA-ACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAGAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGG-----CCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC	0 -1 -7 -30
BL3	GGGATTGGATTATTGCGCCAAGAGATACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAG-----TAACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAGAGA-ACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAATA-----ACT // TTGTACACCAAGCAAACCC-AGAGGTTCAGC GGGATTGGATTATTGCGCCA-----TAACT // TTGTACACCAAGCA-----GAGGTTCAGC	0 -9 -15 -1 -16 -22
BL4,BL5	GGGATTGGATTATTGCGCCAAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAG-----GCCAATTACT // TTGTACACCAAGCAAAC-CCAGAGGTTCAGC GGGATTGGATTATTGCGCCA-----TTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC	-9 -10 -15
BL8	GGGATTGGATTATTGCGCCAAGAGATACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCA-----TAACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAGAGA-ACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAATA-----ACT // TTGTACACCAAGCAAACCC-AGAGGTTCAGC GGGATTGGATTATTGCGCCA-----TAACT // TTGTACACCAAGCA-----GAGGTTCAGC	0 -9 -15 -1 -16 -22
BL9	GGGATTGGATTATTGCGCCAAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAGAGA-ACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAGAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC	-9 -1 -7
BL10	GGGATTGGATTATTGCGCCAAGAGA-ACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGCAGG GGGATTGGATTATTGCGCCAAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGCAGG GGGATTGGATTATTGCGCCA-----ACT // TTGTACACCAAGCA-----GAGGTTCAGCAGG	-1 -9 -22

Figure 3

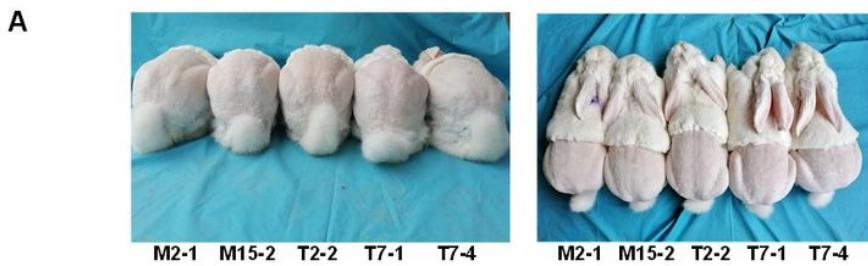
Targeted mutagenesis efficiency of the CRISPR/Cas9 system in rabbit zygotes. (A) Schematic diagram of the microinjection of Cas9 mRNA and sgRNAs into rabbit zygotes. (B) BL1-BL7 represent different blastocysts used in this study. Sanger sequence analysis of zygotes. zygotes edited were named on the left. PAM sequence is marked in green. The sgRNAs sequence is marked in red. The numbers on the right indicated the type of mutation, with “-” represents deletion of the given number of nucleotides.

A**B****sgRNA 6****sgRNA8**

WT	GGGATTGGATTATTGCGCCAAGAGATACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC	0
BL1,BL6,BL7	GGGATTGGATTATTGCGCCAAGAGATACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC	0
BL2	GGGATTGGATTATTGCGCCAAGAGATACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAGAGA-ACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAGAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGG-----CCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC	0 -1 -7 -30
BL3	GGGATTGGATTATTGCGCCAAGAGATACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAG-----TAACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAGAGA-ACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAATA-----ACT // TTGTACACCAAGCAAACCC-AGAGGTTCAGC GGGATTGGATTATTGCGCCA-----TAACT // TTGTACACCAAGCA-----GAGGTTCAGC	0 -9 -15 -1 -16 -22
BL4,BL5	GGGATTGGATTATTGCGCCAAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAG-----GCCAATTACT // TTGTACACCAAGCAAAC-CCAGAGGTTCAGC GGGATTGGATTATTGCGCCA-----TTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC	-9 -10 -15
BL8	GGGATTGGATTATTGCGCCAAGAGATACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCA-----TAACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAGAGA-ACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAATA-----ACT // TTGTACACCAAGCAAACCC-AGAGGTTCAGC GGGATTGGATTATTGCGCCA-----TAACT // TTGTACACCAAGCA-----GAGGTTCAGC	0 -9 -15 -1 -16 -22
BL9	GGGATTGGATTATTGCGCCAAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAGAGA-ACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC GGGATTGGATTATTGCGCCAAGAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGC	-9 -1 -7
BL10	GGGATTGGATTATTGCGCCAAGAGA-ACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGCAGG GGGATTGGATTATTGCGCCAAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTCAGCAGG GGGATTGGATTATTGCGCCA-----ACT // TTGTACACCAAGCA-----GAGGTTCAGCAGG	-1 -9 -22

Figure 3

Targeted mutagenesis efficiency of the CRISPR/Cas9 system in rabbit zygotes. (A) Schematic diagram of the microinjection of Cas9 mRNA and sgRNAs into rabbit zygotes. (B) BL1-BL7 represent different blastocysts used in this study. Sanger sequence analysis of zygotes. zygotes edited were named on the left. PAM sequence is marked in green. The sgRNAs sequence is marked in red. The numbers on the right indicated the type of mutation, with “-” represents deletion of the given number of nucleotides.



		sgRNA 6	sgRNA8
WT	GGGATTGGATTATGCGCCCAAGAGATACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTTCAGC		0
M2-1	GGGATTGGATTATGCGCCCAAGAGATACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTTCAGC GGGATTGGATTATGCGCCCAAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTTCAGC GGGATTGGATTATGCGCCCAAGAGA-ACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTTCAGC		0 -9 -1
M15-2	GGGATTGGATTATGCGCCCAAGAGATACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTTCAGC GGGATTGGATTATGCGCCCAAGAGA-ACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTTCAGC GGGATTGGATTATGCGCCCAAGAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTTCAGC GGGATTGGATTATGCGCCCAAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTTCAGC		0 -1 -7 -9
T2-2	GGGATTGGATTATGCGCCCAAGAGA-ACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTTCAGC GGGATTGGATTATGCGCCCAAGAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTTCAGC GGGATTGGATTATGCGCCCAA-----TTACT // TTGTACACCAAGCAAACCCAGAGGTTTCAGC GGGATTGGATTATGCGCCCAA-----ACT // TTGTACACCAAGCA-----GAGGTTTCAGC GGGATTGGATTATGCGCCCAA-----TAACT // TTGTACACCAAGCA-----GAGGTTTCAGC GGGATTGGATTATGCGCCCAAATA-----ACT // TTGTACACCAAGCAAACCC--AGAGGTTTCAGC		-1 -7 -15 -22 -22 -16
T7-1	GGGATTGGATTATGCGCCCAAGAGATACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTTCAGC GGGATTGGATTATGCGCCCAAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTTCAGC GGGATTGGATTATGCGCCCAA-----TTACT // TTGTACACCAAGCAAACCCAGAGGTTTCAGC GGG-----CCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTTCAGC		0 -9 -15 -30
T7-4	GGGATTGGATTATGCGCCCAAG-----GCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTTCAGC GGGATTGGATTATGCGCCCAAGAGA-ACAAGGCCAATTACT // TTGTACACCAAGCAAACCCAGAGGTTTCAGC		-9 -1

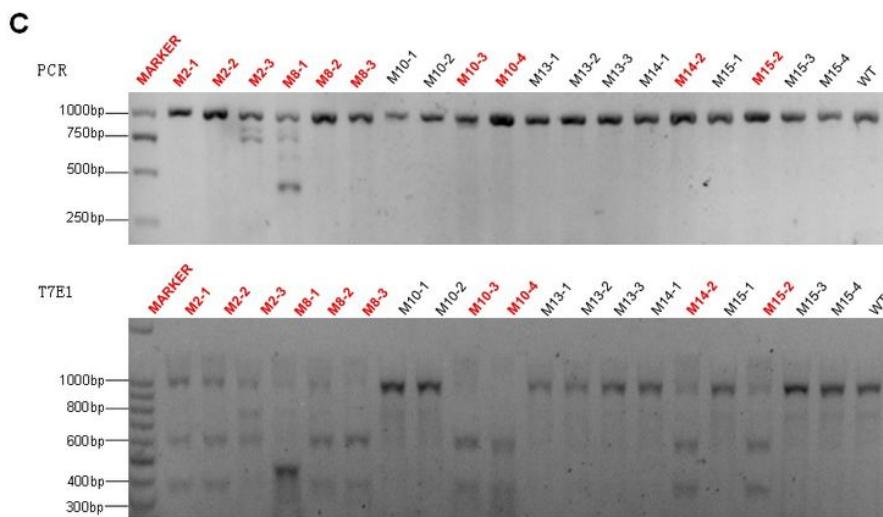


Figure 4

Generation of F0 MSTN KO rabbits via CRISPR/Cas9. (A) Five mutated rabbits of F0. (B) Sanger sequence analysis of mutated rabbits. Five rabbits edited were named on the left. WT stands for wild type control, PAM sequence is marked in green. The sgRNAs sequence is marked in red. The numbers on the right indicated the type of mutation, with “-” represents deletion of the given number of nucleotides. (C) Upper: The results of sequencing for mutations at the specific sites in Agarose gel electrophoresis of PCR products about a part of pups of F0. Lower: T7E1 cleavage assay for the rabbits.

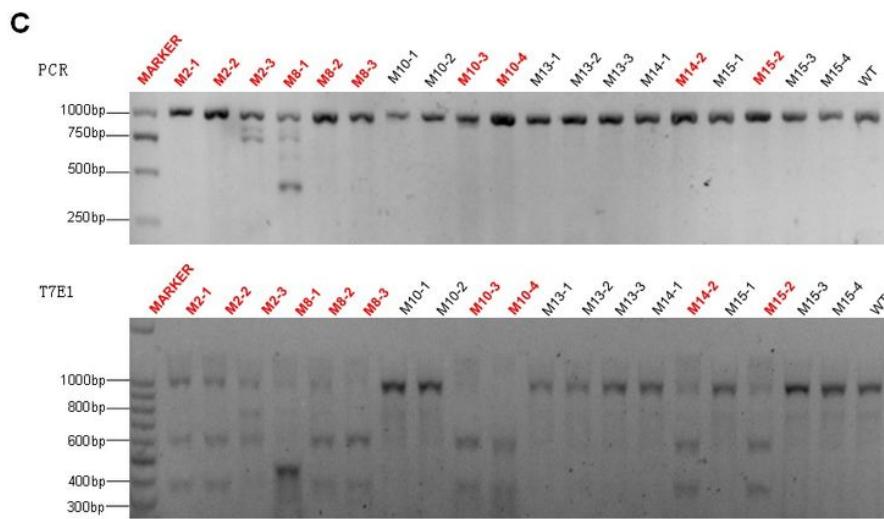
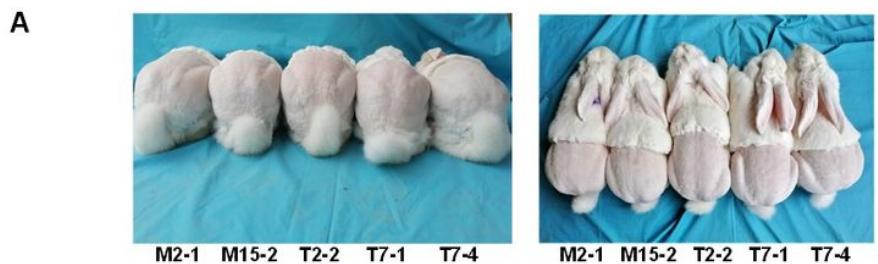


Figure 4

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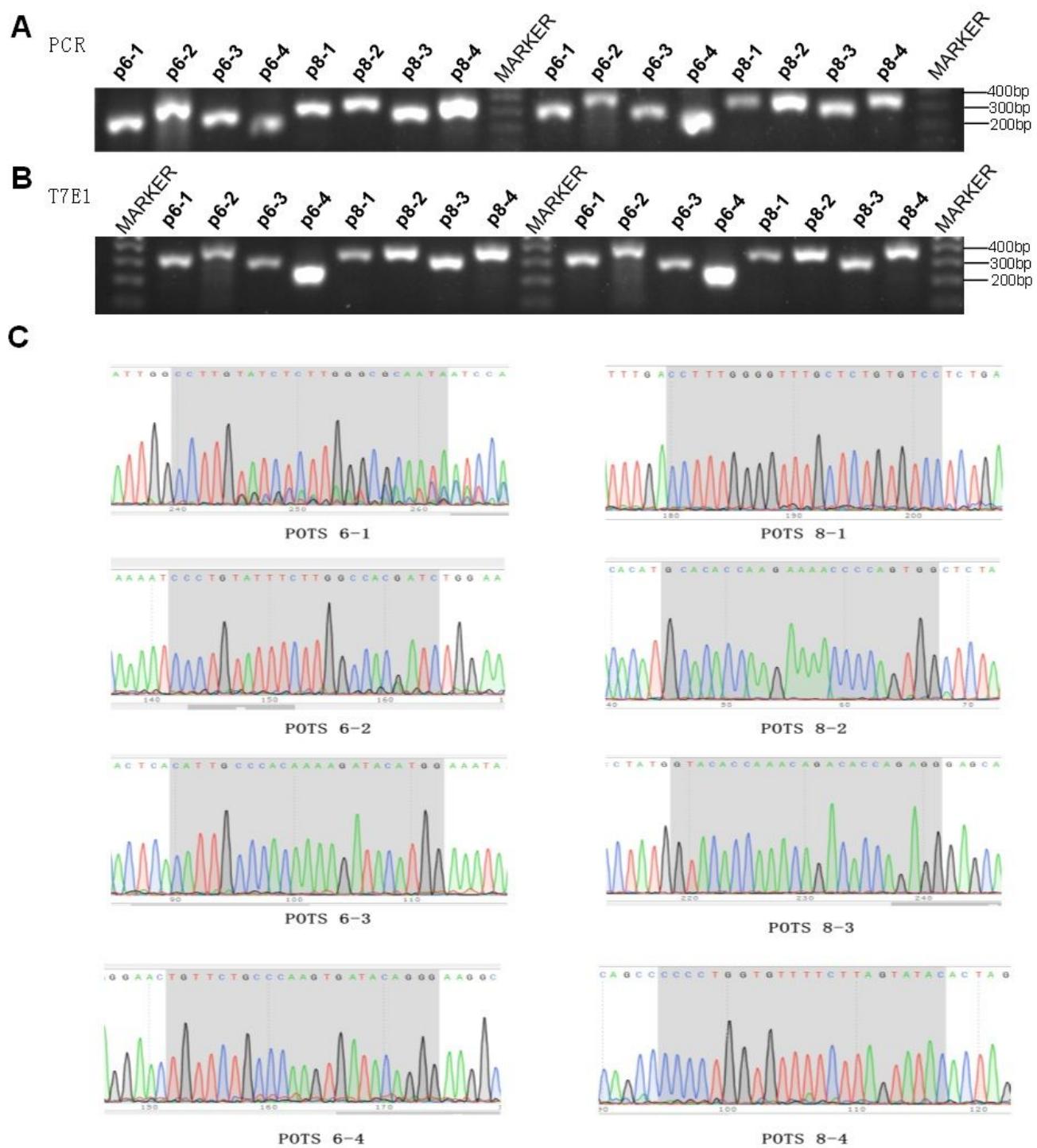


Figure 5

Detection of MSTN modified rabbits. (A) The results of suspicious POTS in Agarose gel electrophoresis of PCR products. M: DL100bp; p6-1 ~ p6-4 represent g6 four suspicious POTS, p8-1 ~ p8-4 represent g8 four suspicious POTS. (B) The results of suspicious POTS in Agarose gel electrophoresis of T7E1. (C) Sanger sequencing for suspicious POTS sites.

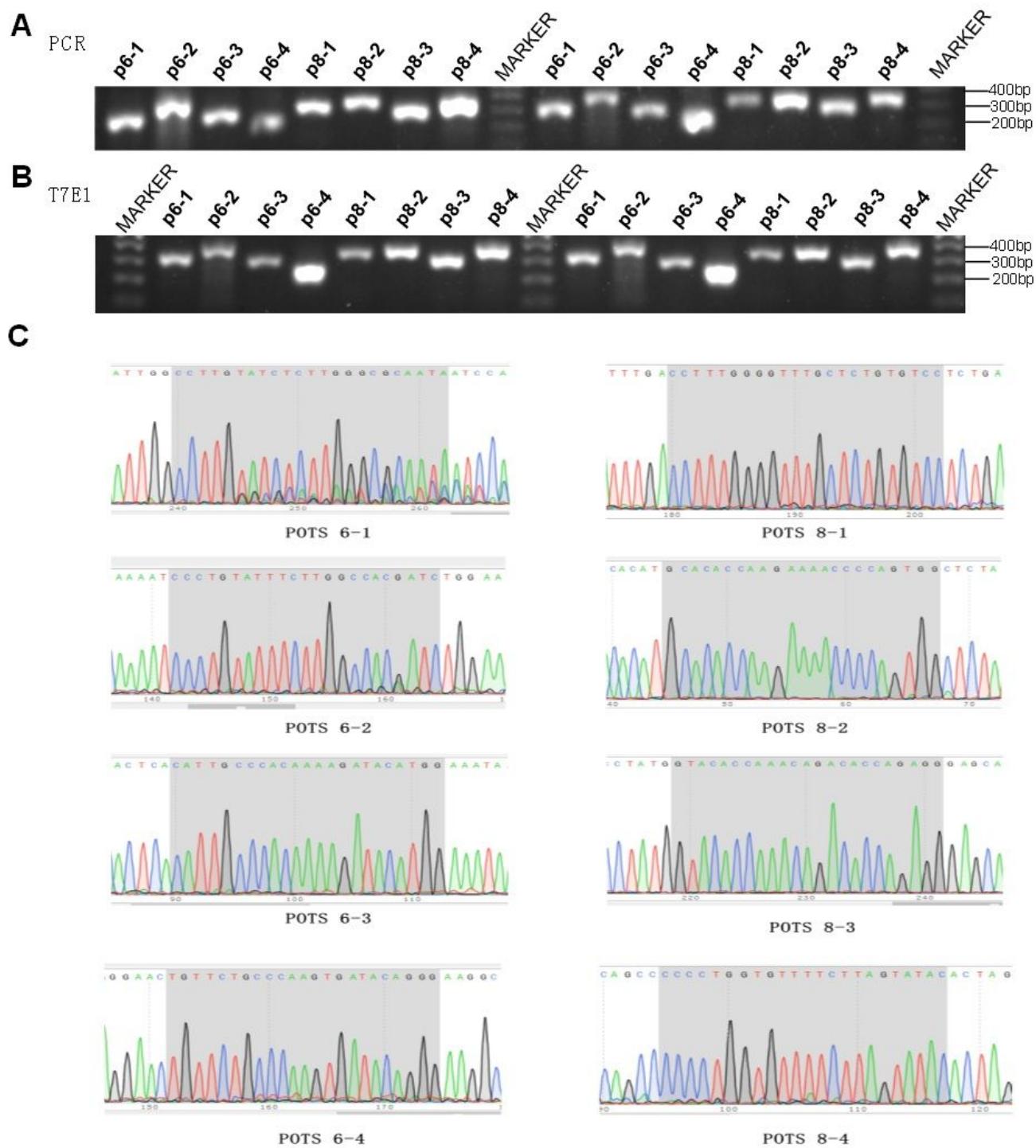


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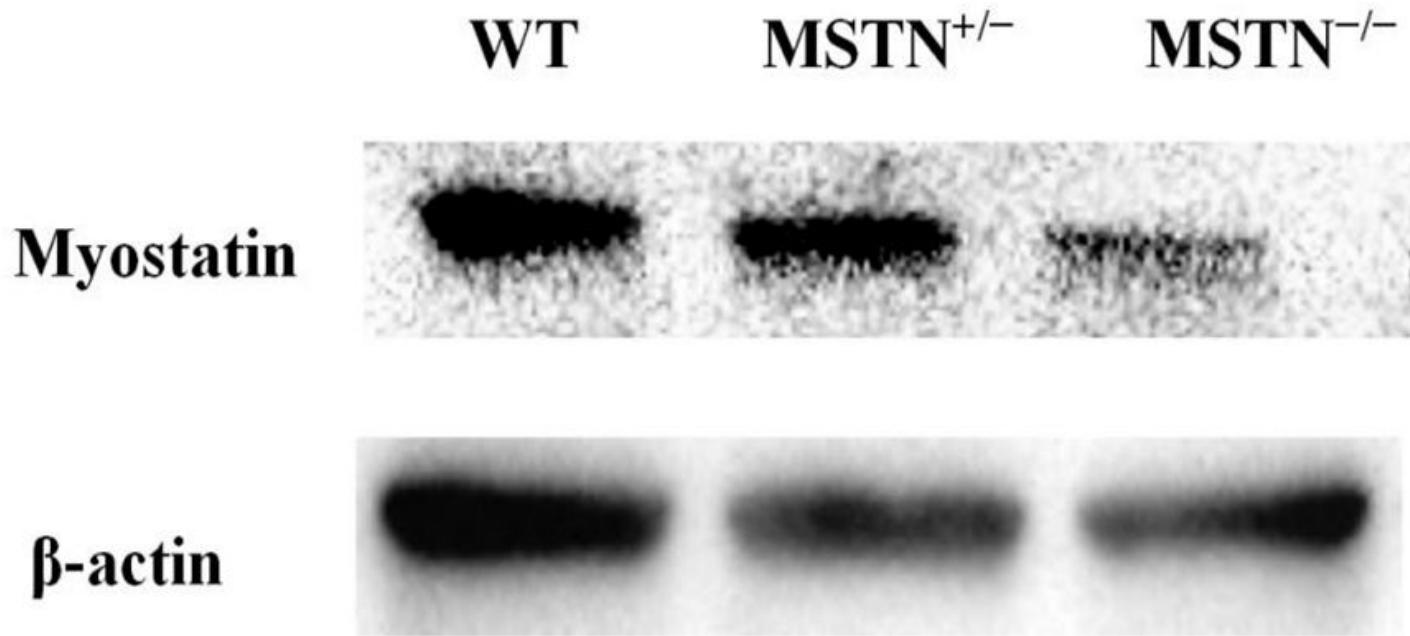


Figure 6

Result of MSTN^{+/-}, MSTN^{-/-} and WT rabbits in Western Blot. Beta-actin served as a loading

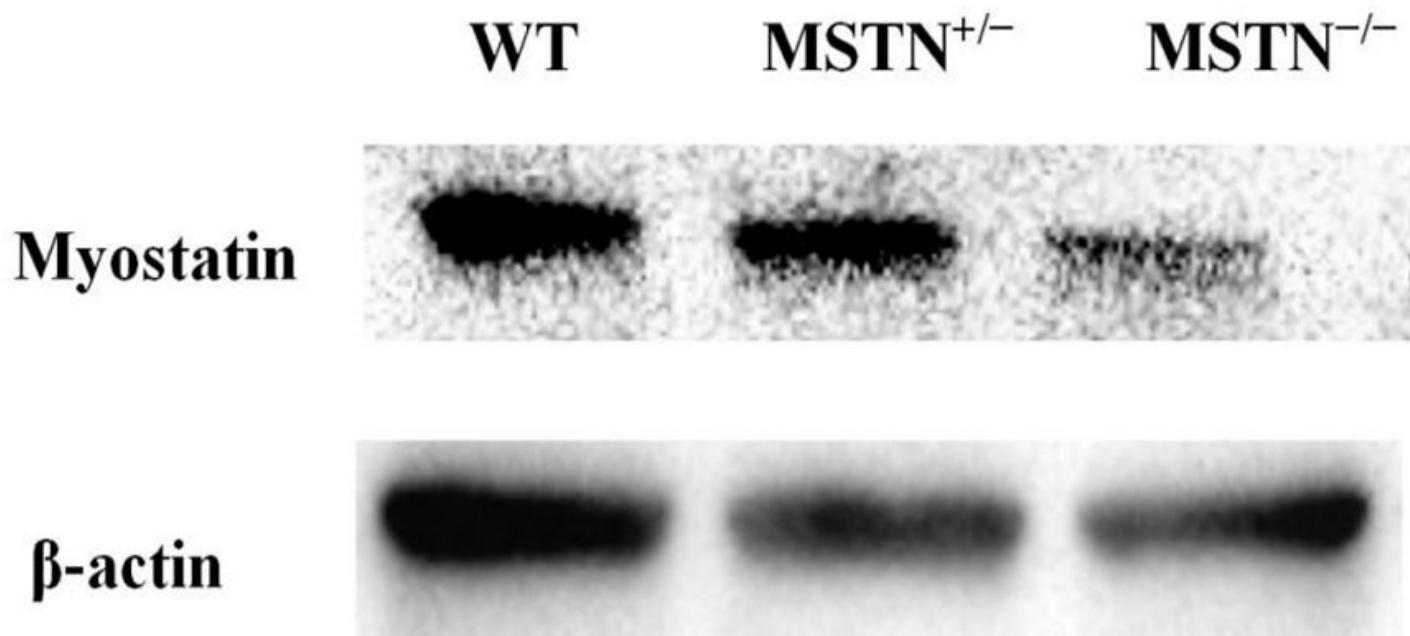


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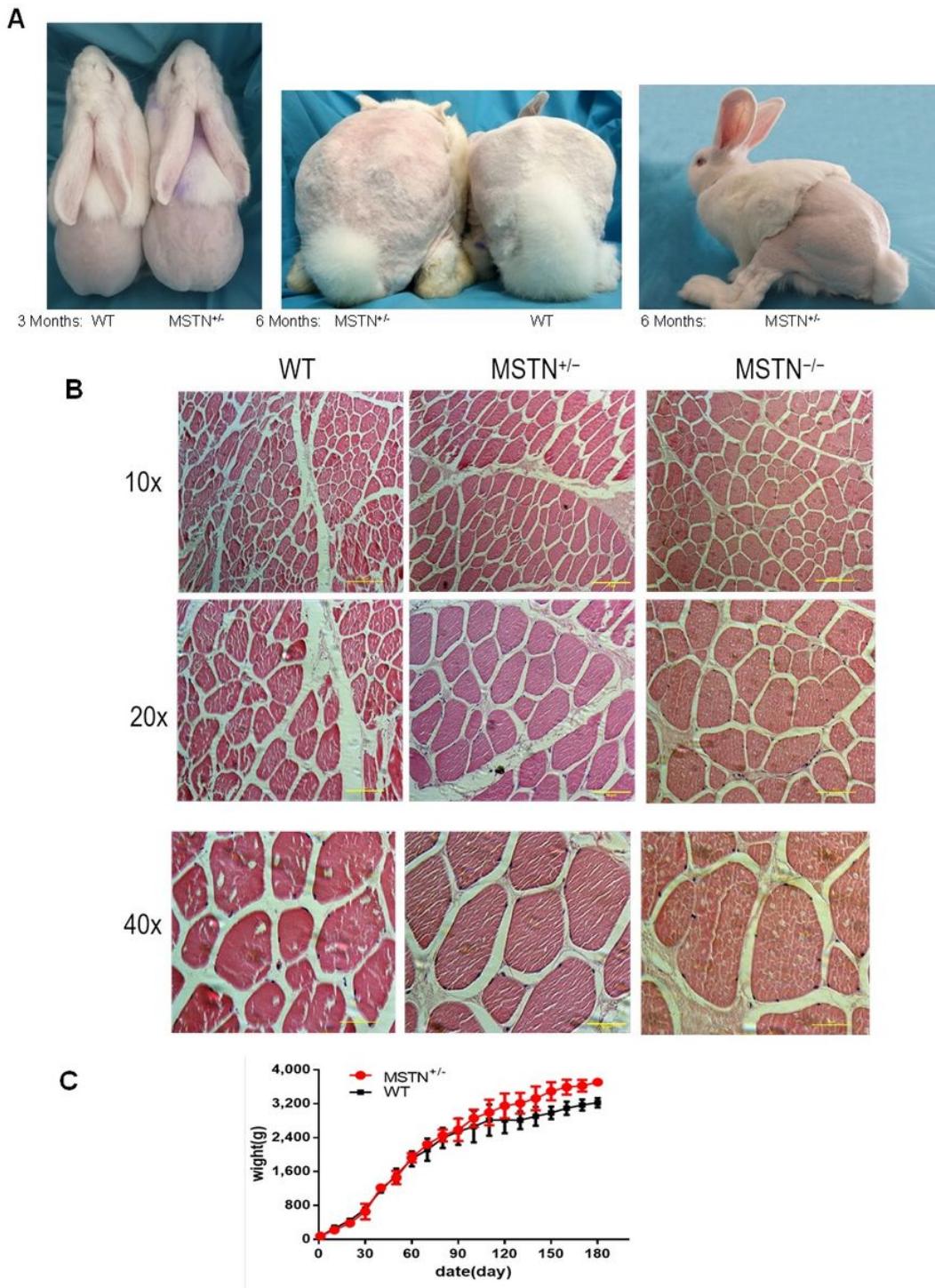


Figure 7

Detection and observation of MSTN-KO rabbits. (A) Comparison of mediated and wild type Rabbits at 3 and 6 months of age. (B) H&E staining of the muscle fibers from gluteus maximus. (C) The average body weight of MSTN^{+/-} and WT rabbits from F0 ($n = 5$).

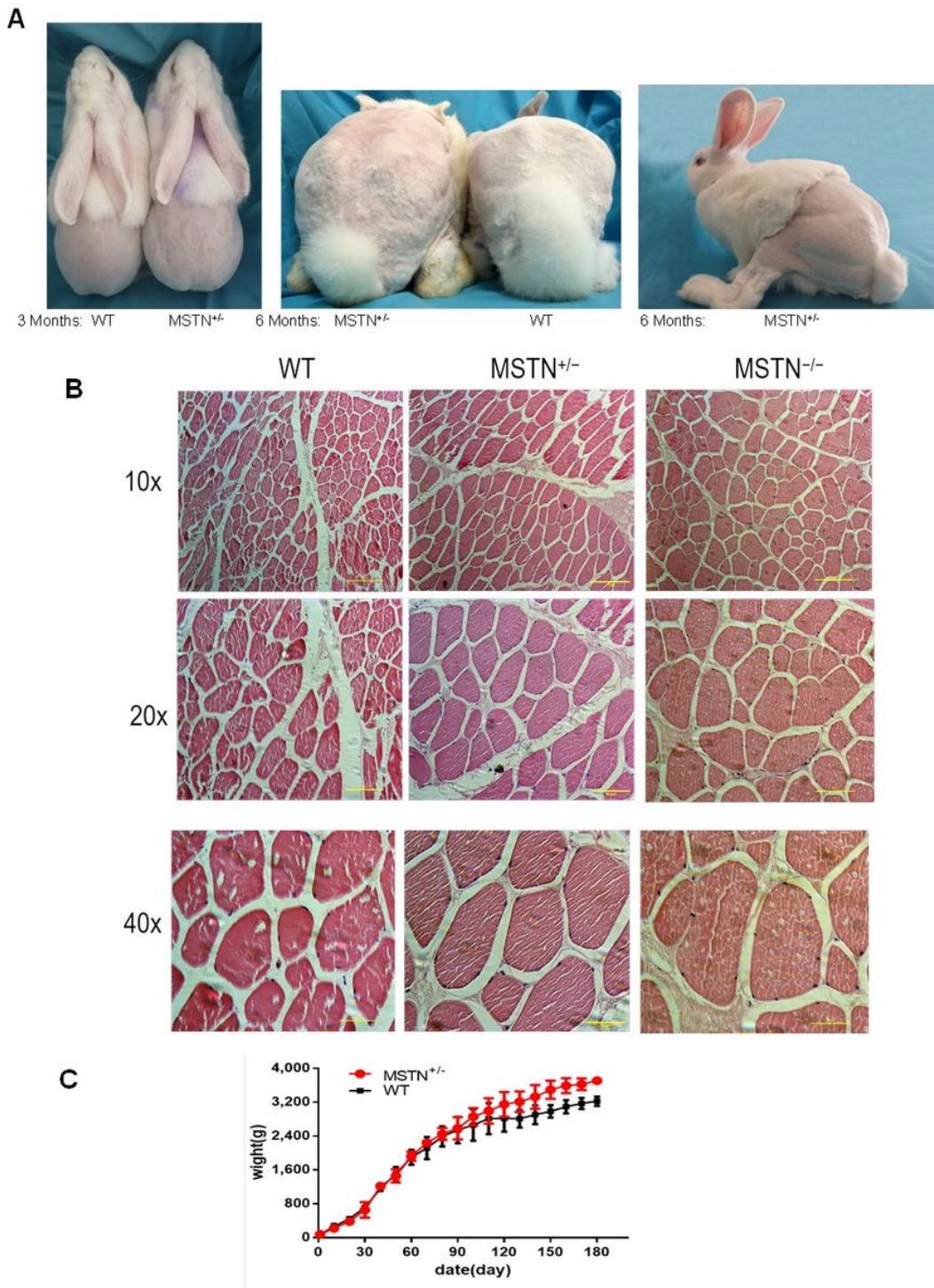
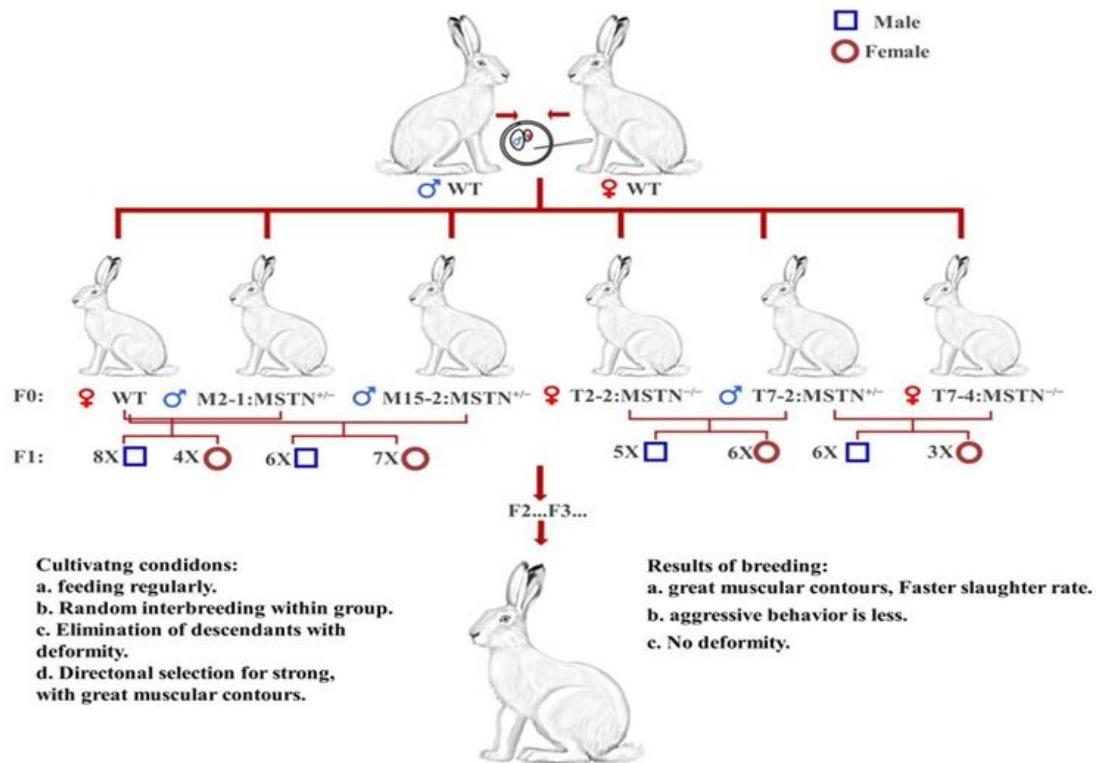


Figure 7

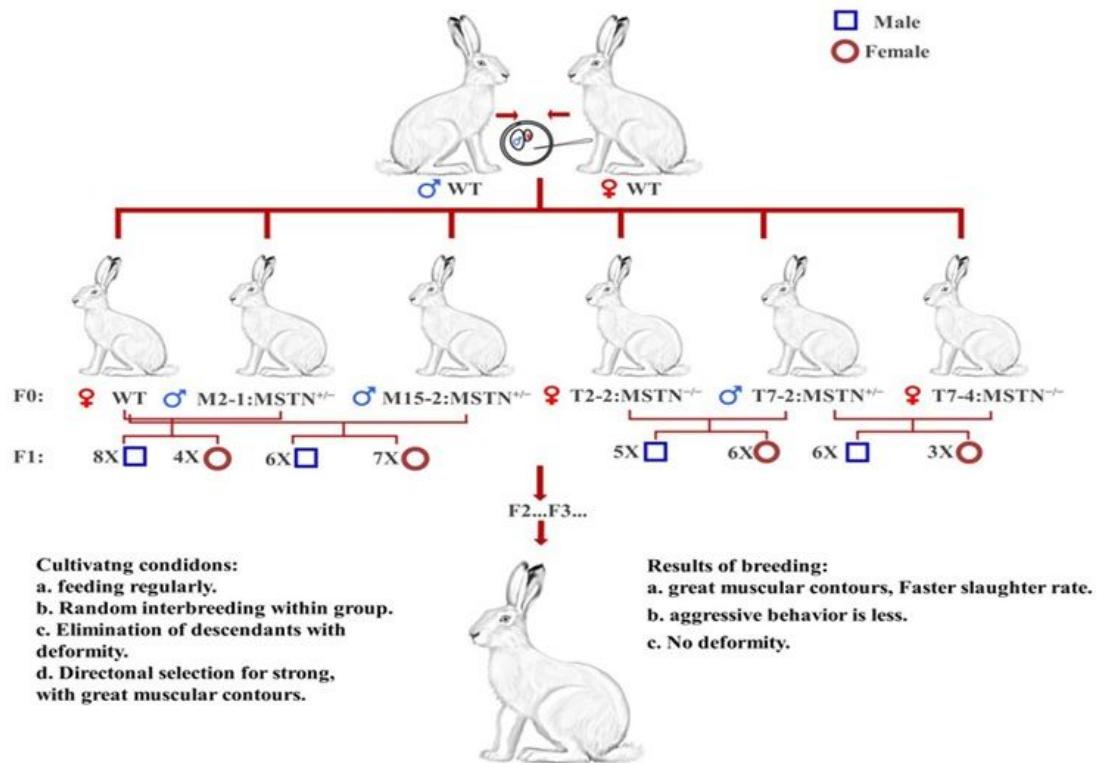
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A**B****C**

WT	GGGATTGGAT TATTGCGCCAAGAGATACAAGGCCAATTACT // TT GTACACCAAGCAAACCCAGAGGTTTCAGC 0
1	GGGATTGGAT TATTGCGCCAAGAGATACAAGGCCAATTACT // TT GTACACCAAGCAAACCCAGAGGTTTCAGC 0 GGGATTGGAT TATTGCGCCAAGAGATACAAGGCCAATTACT // TT GTACACCAAGCAAACCCAGAGGTTTCAGC +1(T)
2	GGGATTGGAT TATTGCGCCAAGAGATACAAGGCCAATTACT // TT GTACACCAAGCAAACCCAGAGGTTTCAGC 0 GGGATTGGAT TATTGCGCCAAG ----- GCCAATTACT // TT GTACACCAAGCAAACCCAGAGGTTTCAGC -9

Figure 8

(A) Schematic diagram of MSTN KO Rabbits breeding family. (B) Left: Pups with big tongue. Right: Dystocia due to the large size of the pups. (C) Rabbits of F1 were named on the left. 1-2 represent different rabbit, PAM sequence is marked in green. The sgRNAs sequence is marked in red. The numbers on the right indicated the type of mutation, with “-” represents deletion of the given number of nucleotides, “+” represents insertion of the given number of nucleotides.

A**B****C**

WT	GGGATTGGAT TATTGCGCCAAGAGATACAAGGCCAATTACT // TT GTACACCAAGCAAACCCAGAGGTTTCAGC 0
1	GGGATTGGAT TATTGCGCCAAGAGATACAAGGCCAATTACT // TT GTACACCAAGCAAACCCAGAGGTTTCAGC 0 GGGATTGGAT TATTGCGCCAAGAGATACAAGGCCAATTACT // TT GTACACCAAGCAAACCCAGAGGTTTCAGC +1(T)
2	GGGATTGGAT TATTGCGCCAAGAGATACAAGGCCAATTACT // TT GTACACCAAGCAAACCCAGAGGTTTCAGC 0 GGGATTGGAT TATTGCGCCAAG ----- GCCAATTACT // TT GTACACCAAGCAAACCCAGAGGTTTCAGC -9

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