CRISPR/Cas9 as a method to generate *myostatin* knocked out rabbit

Research topics and seminar 510

Presented by
Mohamed Abdellatif

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Outline

- The presented paper
- General information on
    - Rabbits
    - *Myostatin* gene
    - CRISPR/Cas9
- Aim
- Methods
- Results
- Discussion
- Implementation in our society

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Efficient Generation of *Myostatin* Gene Mutated Rabbit by CRISPR/Cas9

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Qingyan Lv[1,*], Lin Yuan[1,*], Jichao Deng[1,*], Mao Chen[1], Yong Wang[1], Jian Zeng[1], Zhanjun Li[1] & Liangxue Lai[1,2]

CRISPR/Cas9 has been widely used in generating site-specific genetically modified animal models. *Myostatin (MSTN)* is a negative regulator of muscle mass, related to muscle growth and differentiation. The knockout of *MSTN* with the desired phenotype of double muscle has been successfully generated in mice, goats, pigs and cattle, but not in rabbits. In this study, the *MSTN* knockout (KO) rabbits were generated by co-injection of Cas9 mRNA and sgRNA into zygotes. The typical phenotype of double muscle with hyperplasia or hypertrophy of muscle fiber was observed in *MSTN* KO rabbits. Furthermore, a similar phenotype was found in the F1 generation, suggesting that the mutation of *MSTN* could be stably inherited in the *MSTN* KO rabbits. In summary, we have successfully generated *MSTN* KO rabbits using CRISPR/Cas9 system with high efficiency, which is a reliable and effective animal model for the study of muscle development and related diseases.

Rabbit as an animal model

- Docile and non-aggressive.
- Widely bred.
- Short cycles of gestation, lactation, and puberty.
- Under the small animal category.

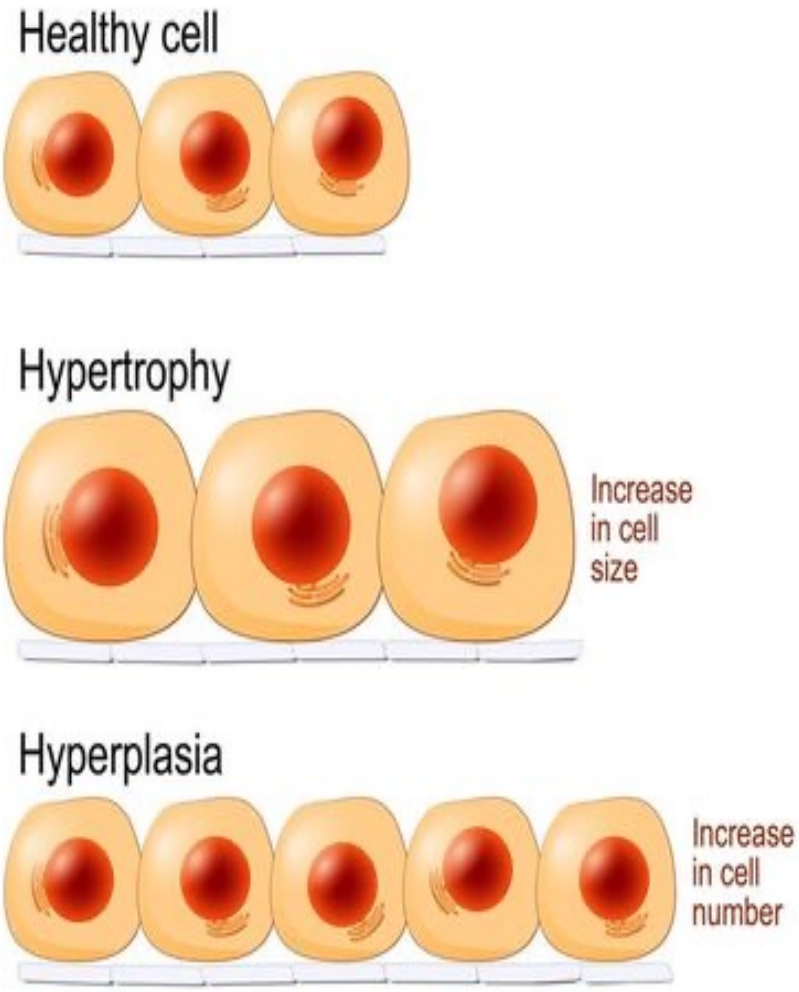
Rabbit as an animal model

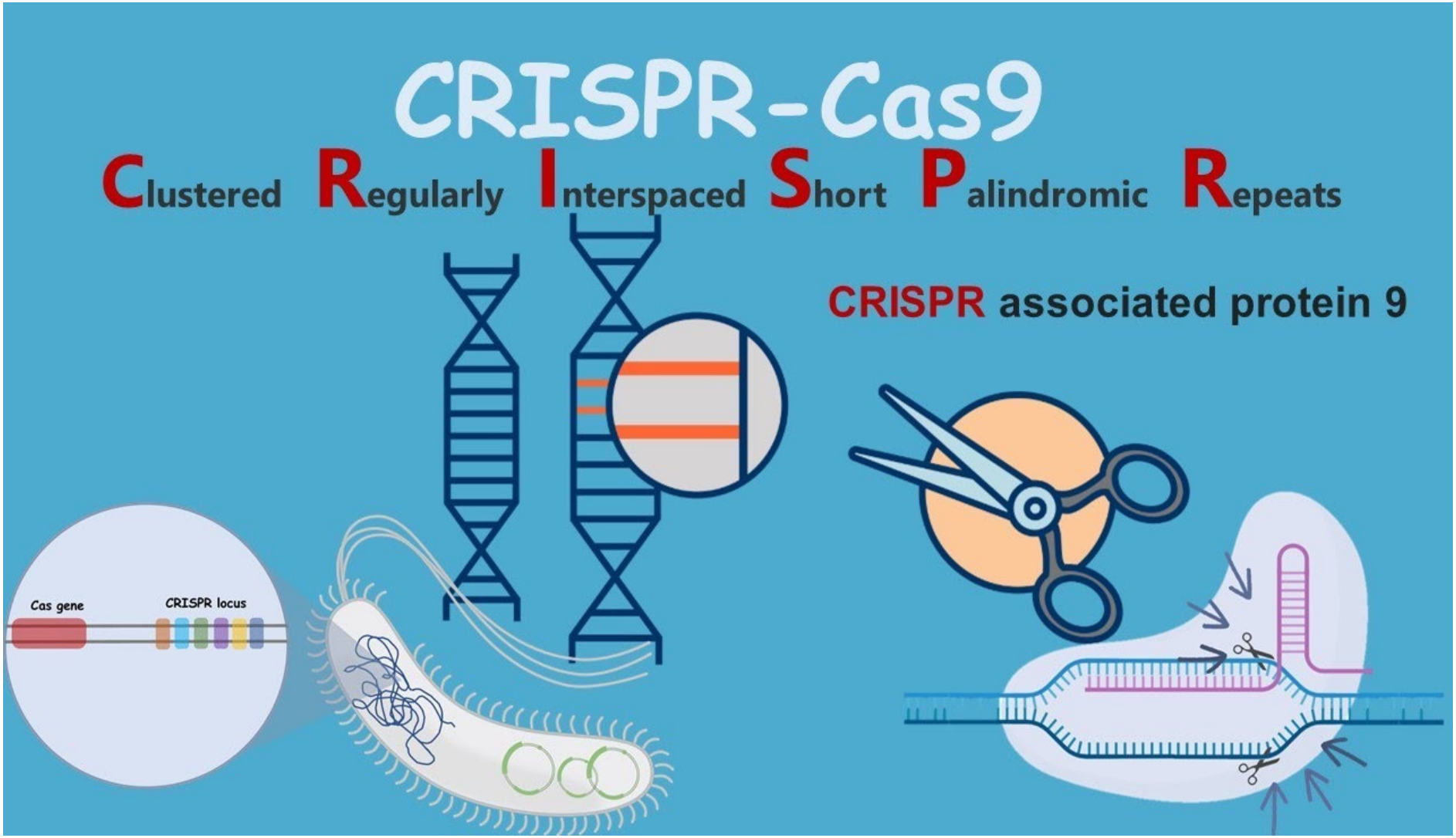
- Similar to human beings in terms of physiology and anatomy.
- Used extensively to study cardiovascular, metabolic and ophthalmic diseases.

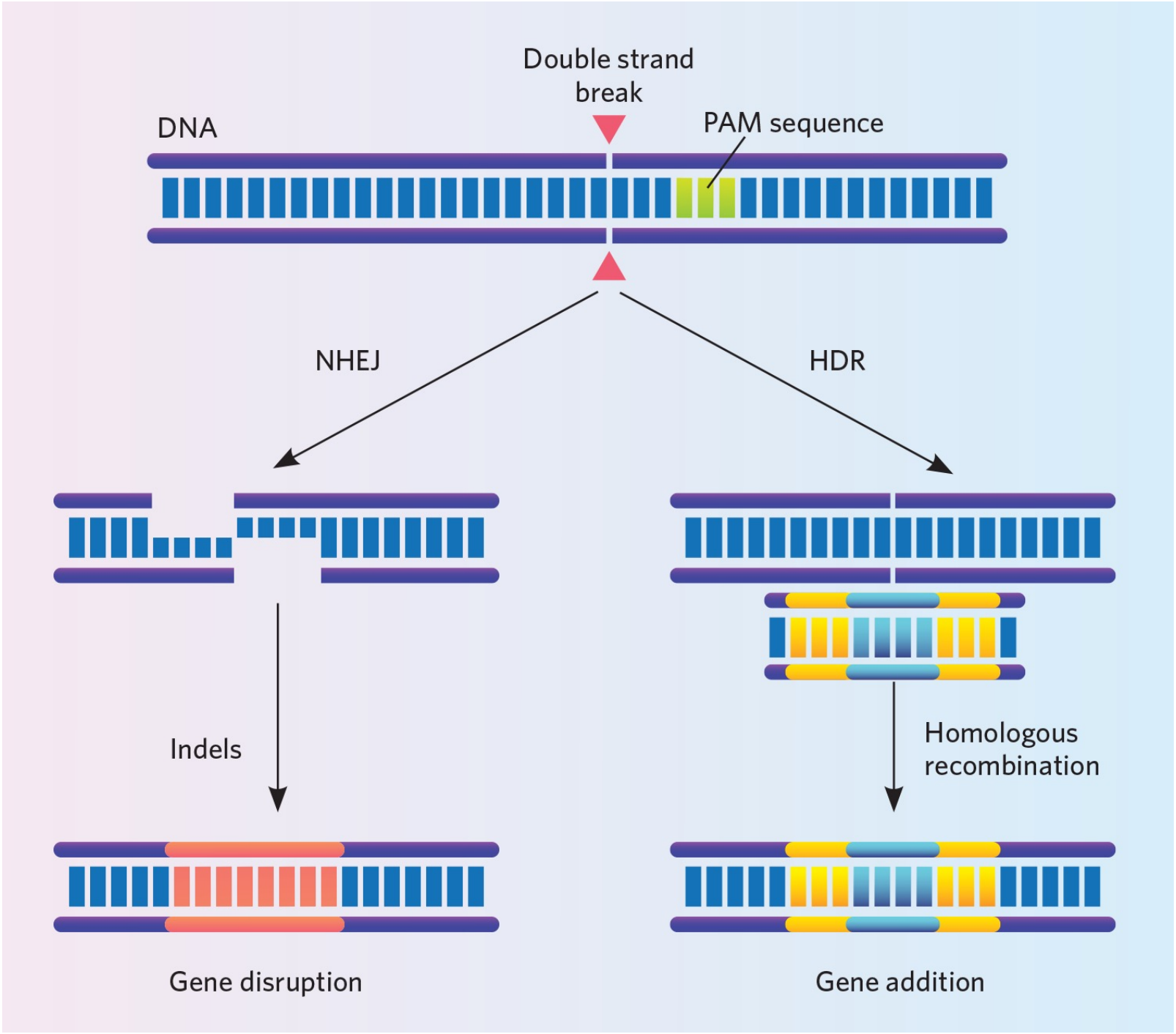
Myostatin gene (*MSTN*)

- Member of transforming growth factor beta (TGF-β).
- Negative regulator of muscle growth.
- Spontanous mutations in cattle and sheep resulted in hypertrophy.

Myostatin gene (*MSTN*)




CRISPR-Cas9
Clustered Regularly Interspaced Short Palindromic Repeats
CRISPR associated protein 9
Cas gene
CRISPR locus


Double strand
break
DNA
PAM sequence
NHEJ
HDR
Indels
Homologous
recombination
Gene disruption
Gene addition

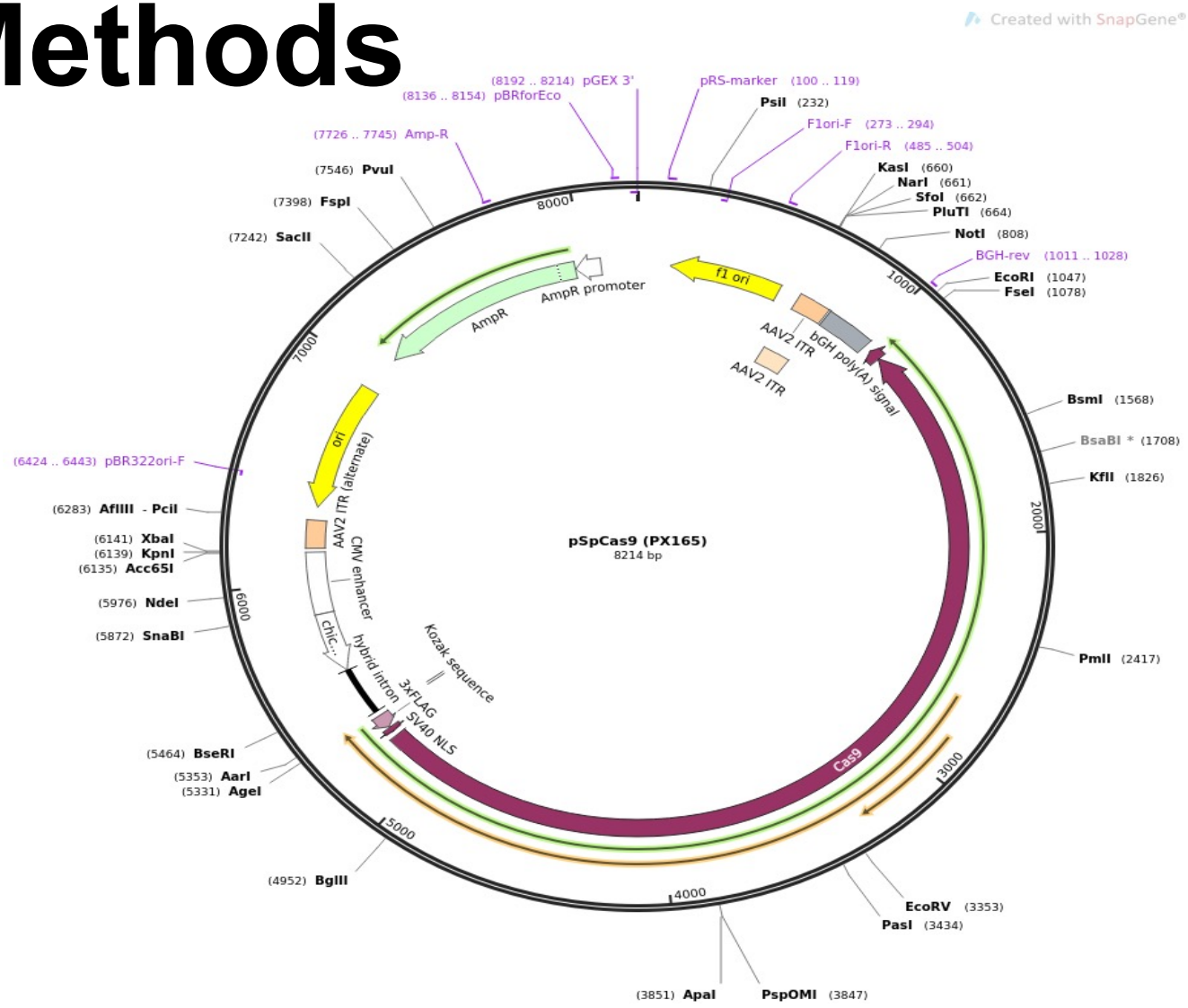
Aim

- Generation of *MSTN* knockout (KO) rabbits by microinjection of transcribed mRNA encoding for Cas9 and sgRNA targeting the *MSTN* gene into the cytoplasm of rabbit embryos.

Aim

- Demonstration of the efficiency of CRISPR/Cas9 as a gene editing tool
- Demonstration of the desired phenotype of double muscle in the *MSTN* KO rabbits.

Methods



- 3× FLAG-NLS-SpCas9-NLS vector

Methods

Vector construction and *in vitro* transcription.

1- The vector was linearized with *NotI.*
2- Products for *in vitro* transcription were amplified by PCR using T7 primers.
3- *In vitro* transcription was carried out T7 RNA Synthesis Kit.
4- The synthesized mRNA was purified using miRNeasy Mini Kit.
5- Qualitative and quantitative assessment.

Methods

Zygote injection with Cas9/sgRNA.

- Mixture of in vitro transcribed sgRNA and Cas9 mRNA were injected into the cytoplasm of pronuclear stage embryos (collected 18-20 hours after mating).
- Kept for 30 – 60 minutes in embryo culture medium.
- Transferred to the oviduct of recipient mother.

Methods

Genotyping of *MSTN* mutation in embryos and pups

- Genomic DNA was extracted from first blastocysts of embrys and later from ear punch tissues from new born pups.
- PCR amplification of the target site.
- T7E1 test was conducted.

Methods

- **Off-target analysis.**
- **Western blotting and histology analysis.**
- **Body weight, carcass dissection, and sample collection.**

Results

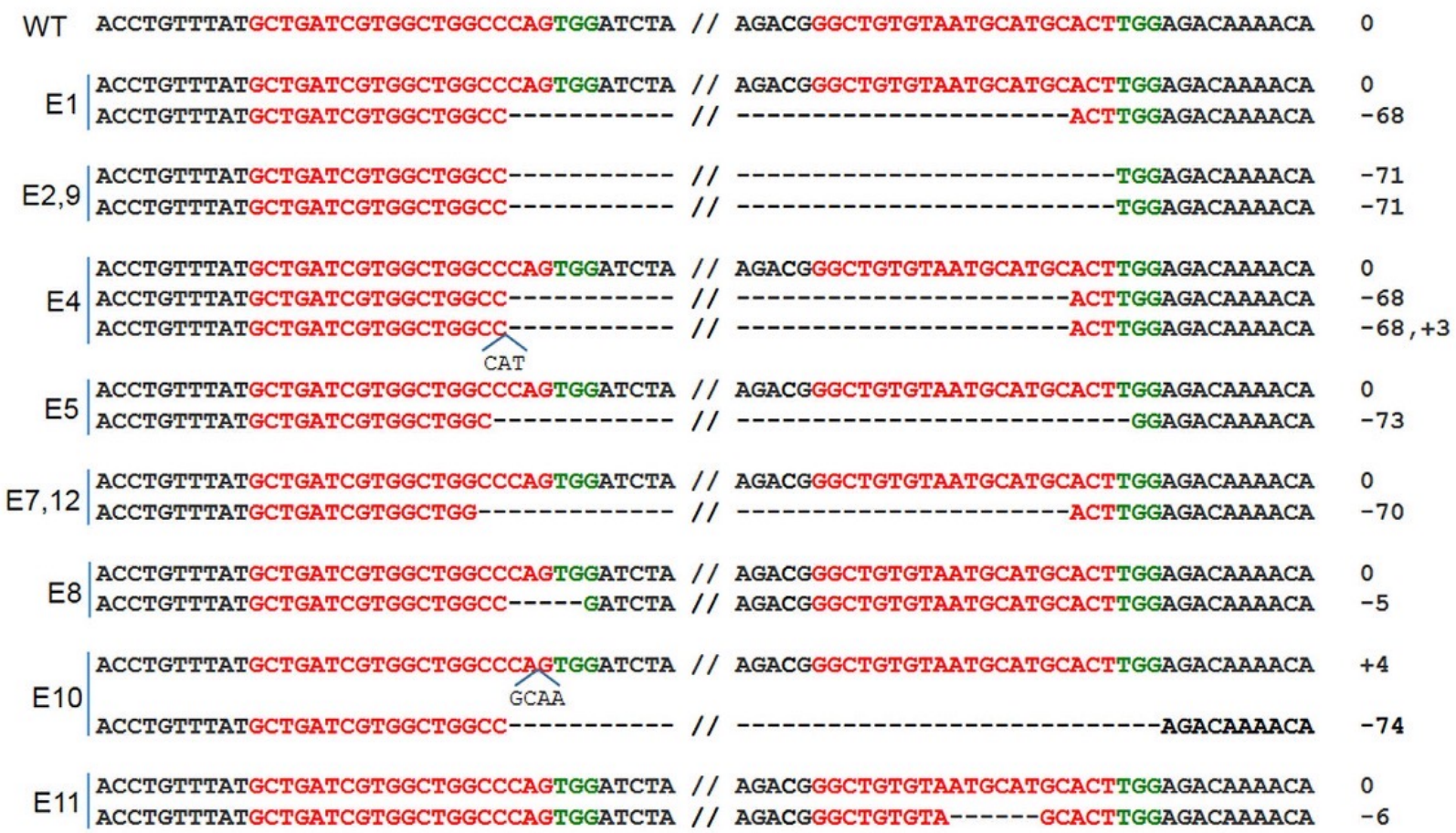


Figure1: represents knocking out of *MSTN* gene by CRISPR/Cas9 activity on exon 1. WT*:* wild type; E: embryos.

Results

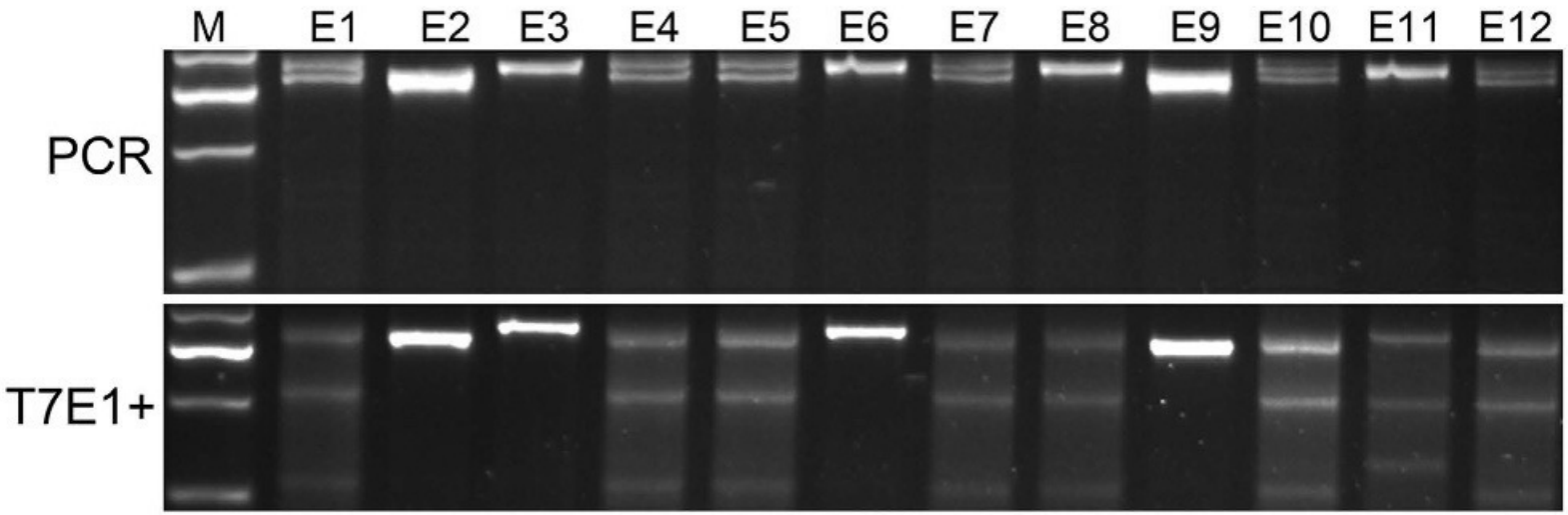


Figure2: represents T7E1 cleavage assay results. M: the marker DL2000; E1-12 represents the different embryos.

Results

Recipients	gRNA/Cas9 mRNA(ng/μL)	Embryos transferred	Pregnancy	Pups obtained (% transferred)	Pups with mutations (% pups)	Bi- allelic modified (% pups)
1	40/180	40	YES	5(12.5%)	4(80%)	0(0%)
2	40/180	38	YES	4(10.5%)	2(50%)	0(0%)
3	40/180	40	YES	5(12.5%)	5(100%)	2(40%)
4	40/180	40	YES	6(15%)	5(83.3%)	4(66.7%)

Table1: number of *MSTN* KO rabbits generated by CRISPR/Cas9

Results

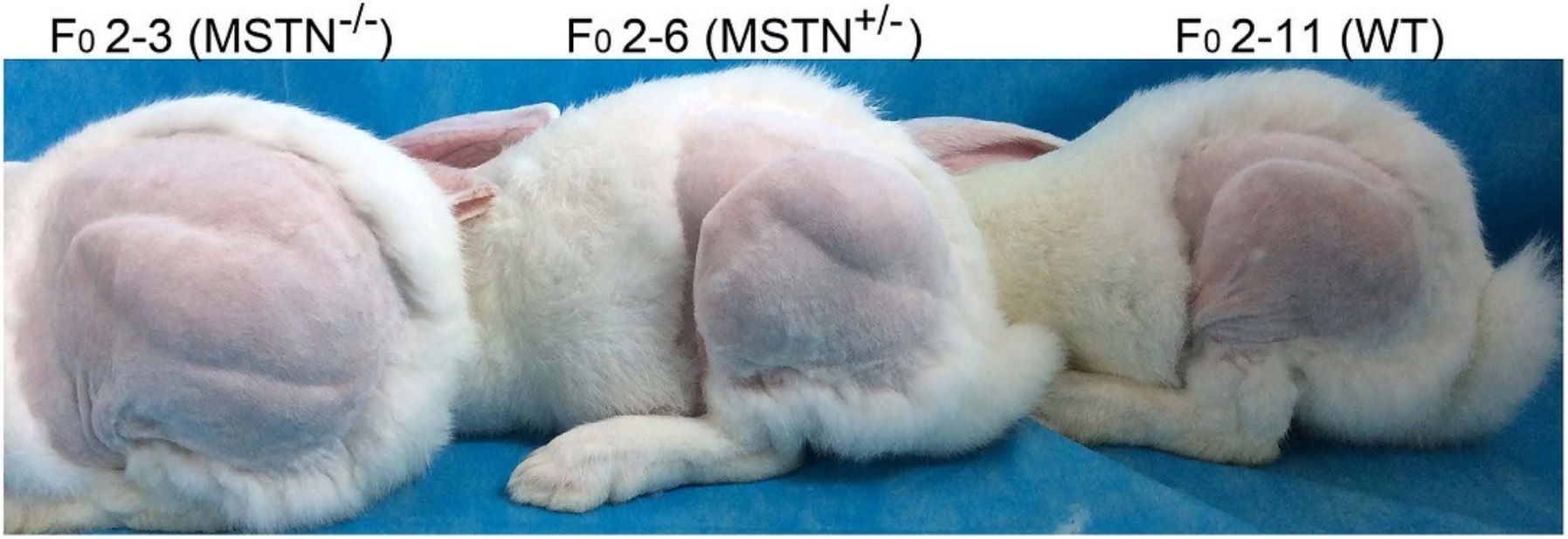


Figure3: photos of 4 months old pubs showing double muscular phenotype in both *MSTN*$^{-/-}$ and *MSTN*$^{+/-}$.

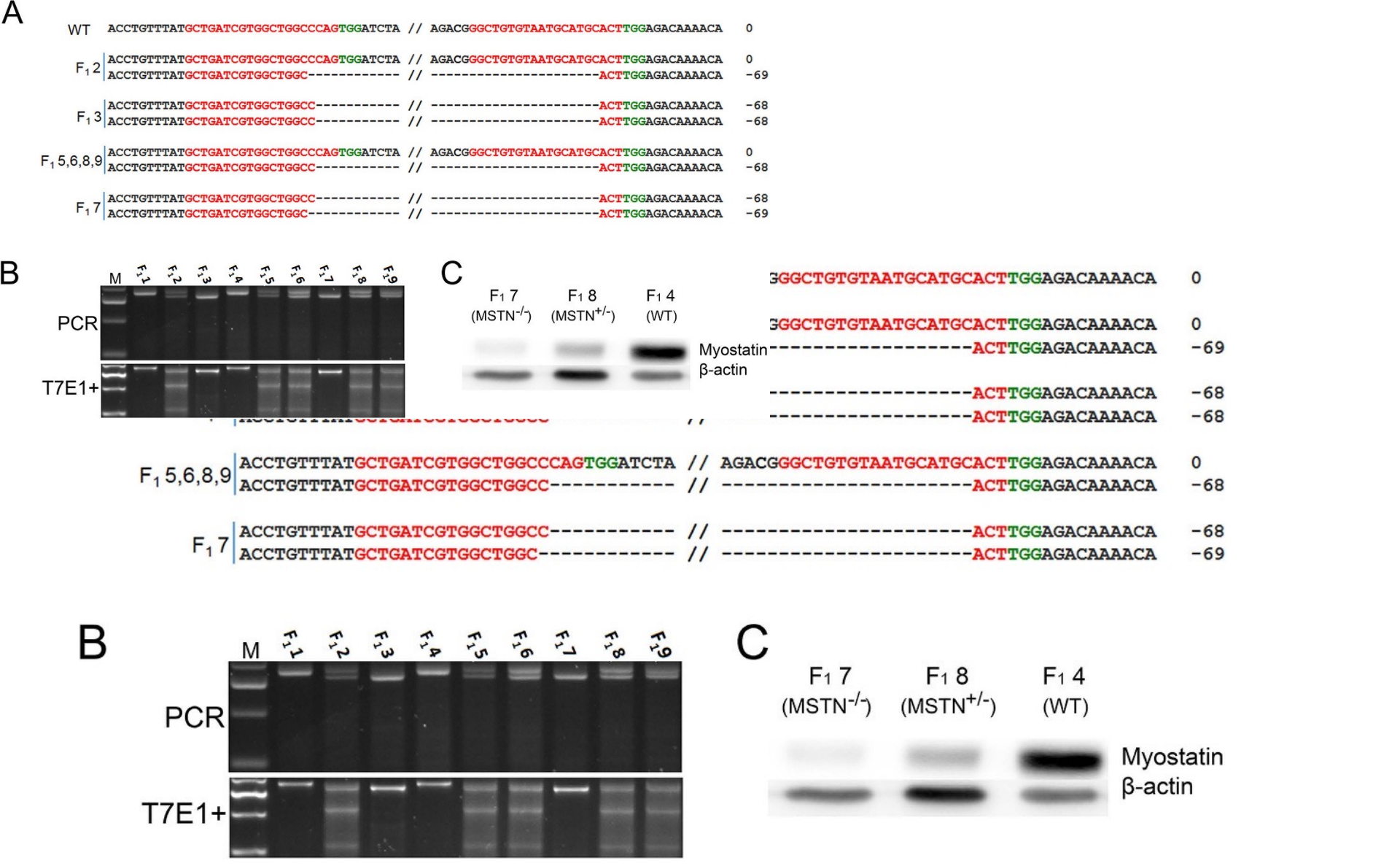


Figure4: Heritability of the *MSTN* KO rabbit. A) sequencing analysis of *MSTN* KO in F1. B) T7E1 cleavage assay to detect mutation. C) western blot of *MSTN* protein in skeletal muscles.

Results

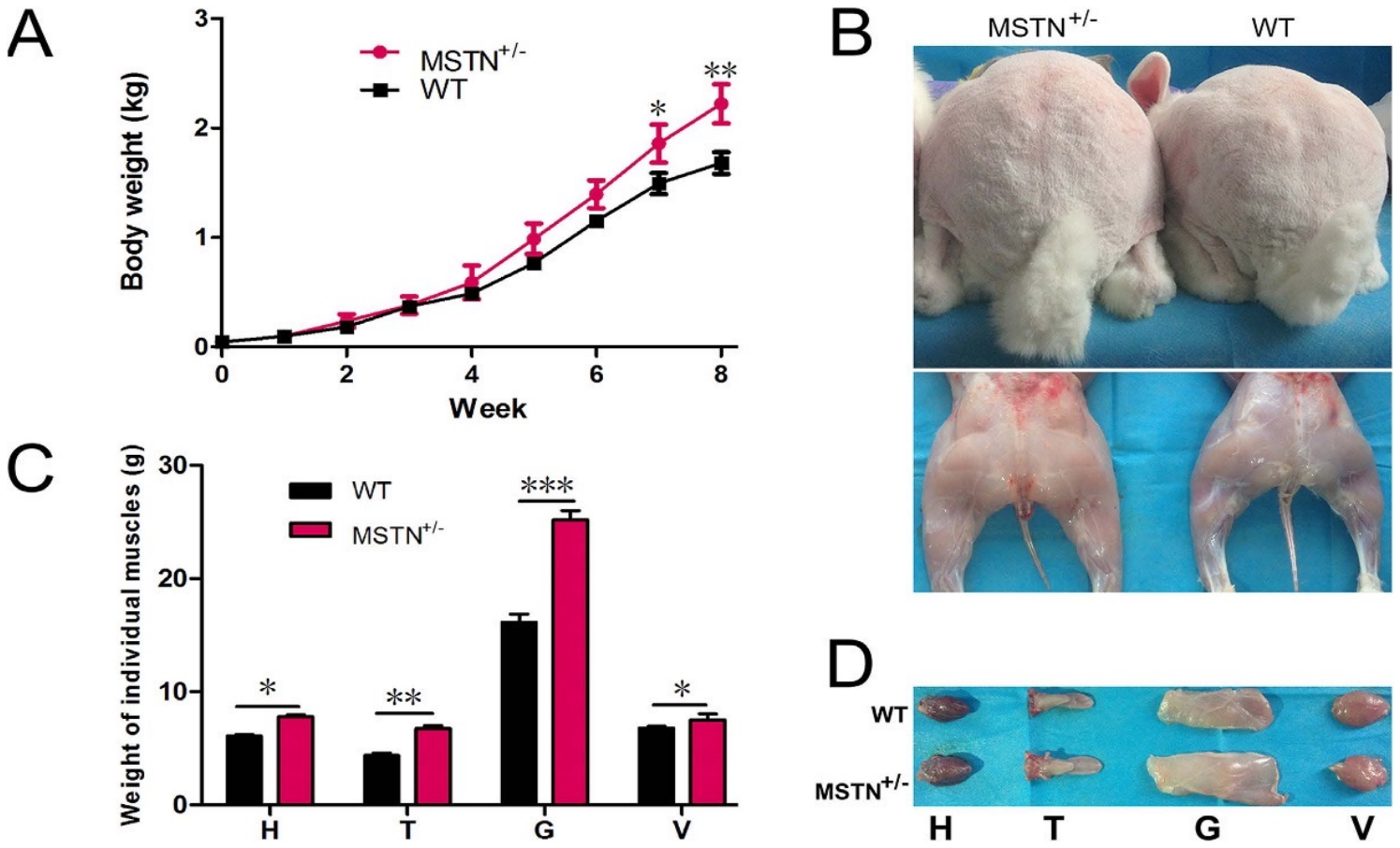


Figure5: Increased body weight and muscle mass in MSTN KO rabbits.

Was the increase in muscle mass due to hyperplasia or hypertrophy?

Results

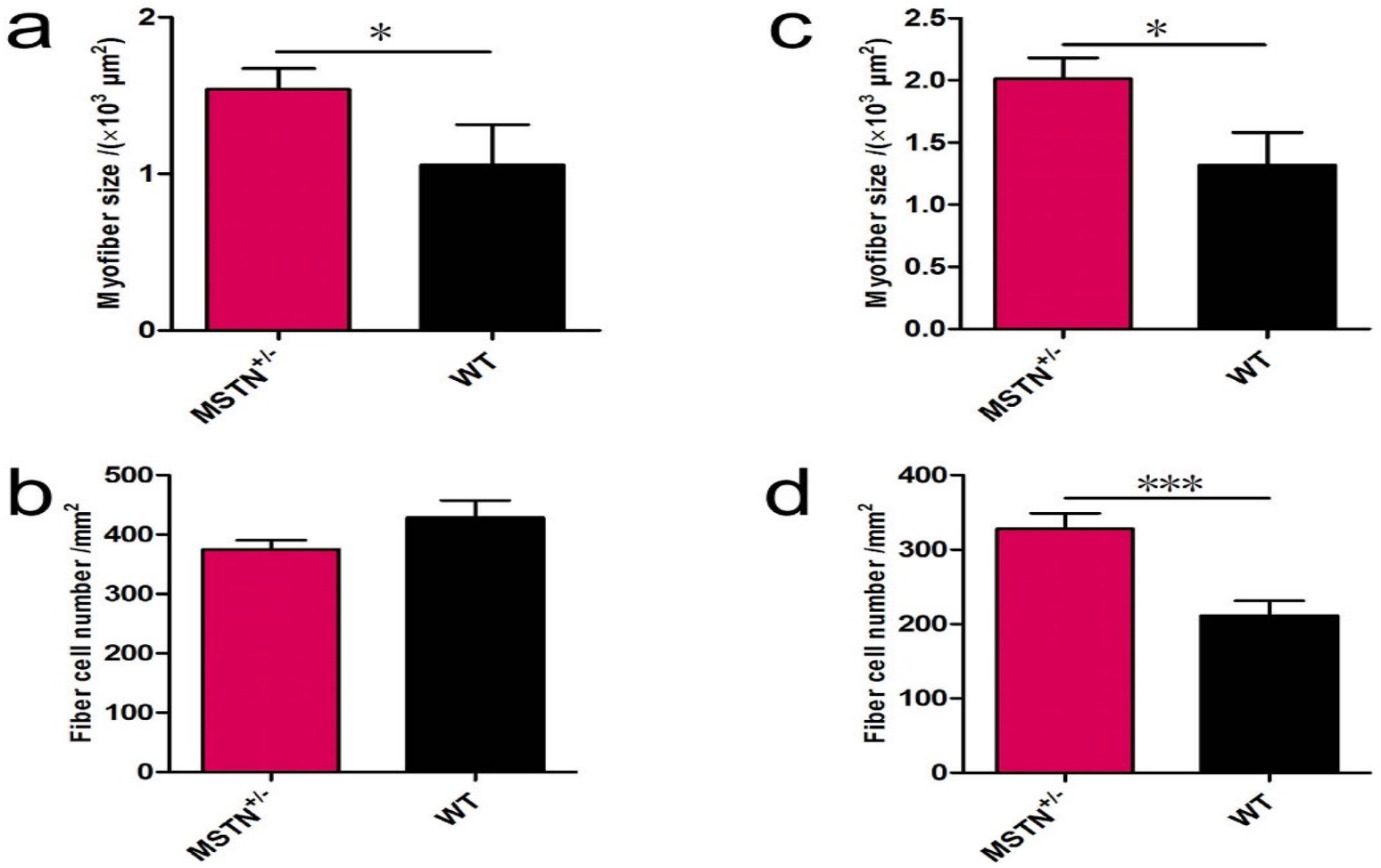


Figure6: A hyperplasia and/or hypertrophy of muscle fibers was observed in *MSTN* KO rabbits.

Discussion

- Microinjection of Cas9/sgRNA into one-cell stage embryos resulted in the successful generation of *MSTN* KO rabbits.

Discussion

- No off-target effect was detected in the *MSTN* KO rabbits.
- When compared to the WT controls, there was no significant change in body size or weight at birth, eliminating the possibility of LOS.

Discussion

- The enlarged muscular phenotype in *MSTN* KO rabbits is attributable to muscle fiber hyperplasia and/or hypertrophy.

Implementation in the society

- Studying muscle development and related disorders is one way of benefitting from this study.

- Another way is improving the quality of livestock traits, mainly increasing the mass of meat in edible animals.

Personal aim of this study

Fetched from YouTube channel Rojeta style