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BRIEF COMMUNICATION

CRISPR/Cas9-mediated knockout of myostatin in Chinese indigenous Erhualian pigs

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Abstract CRISPR/Cas9 has emerged as one of the most popular genome editing tools due to its simple design and high efficiency in multiple species. Myostatin (MSTN) negatively regulates skeletal muscle growth and mutations in myostatin cause double-muscled phenotype in various animals. Here, we generated myostatin mutation in Erhualian pigs using a combination of CRISPR/Cas9 and somatic cell nuclear transfer. The protein level of myostatin precursor decreased dramatically in mutant cloned piglets. Unlike myostatin knockout Landrace, which often encountered health issues and died shortly after birth, Erhualian pigs harboring homozygous mutations were viable. Moreover, myostatin knockout Erhualian pigs exhibited partial double-muscled phenotype such as prominent muscular protrusion, wider back and hip compared with wild-type piglets. Genome editing in Chinese indigenous pig breeds thus holds great promise not only for improving growth performance, but also for protecting endangered genetic resources.

Keywords Erhualian pigs · CRISPR/Cas9 · Myostatin · Double-muscled phenotype · SCNT

Introduction

As a member of transforming growth factor-β superfamily, myostatin acts as a potent negative regulator of skeletal muscle growth. MSTN knockout mice exhibited a 200–300% increase in muscle mass due to muscle fiber hyperplasia and hypertrophy (McPherron et al. 1997). Natural mutations of MSTN have been found in various species including cattle, sheep and dogs (Clop et al. 2006; Fries et al. 1997; Kambadur et al. 1997; McPherron and Lee 1997). The double muscled Belgian Blue cattle are homozygous for an 11 bp deletion in myostatin exon 3(Fries et al. 1997; Kambadur et al. 1997; McPherron and Lee 1997). A point mutation in 3′ UTR of the ovine MSTN gene, which created a new microRNA target site, induced enhanced muscling of the Texel sheep (Clop et al. 2006). Similarly, a two-base deletion in MSTN caused "bully" whippets with better racing performance

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(Mosher et al. 2007). All these observations have made myostatin a strong candidate gene for improving growth performance of livestock. However, side-effects resulted from MSTN mutations also existed. Reduced fertility, low calf viability and dystocia were observed in doubled muscled cattle, and these drawbacks appear more vital in polytocous species such as pigs. Hence, whether MSTN can serve as an ideal locus for porcine gene editing needs further studies.

The advent of programmable endonuclease technique has opened a new era of genome editing. Many myostatin knockout animals such as sheep, cattle and dog have been generated efficiently (Crispo et al. 2015; Luo et al. 2014; Proudfoot et al. 2015; Zhengxing et al. 2014; Zou et al. 2015). These genome-modified animals developed and grew normally, showed several expected phenotypes such as fast growth rate, double muscling, muscle fiber hypertrophy or hyperplasia (Crispo et al. 2015; Luo et al. 2014; Proudfoot et al. 2015). Pig is one of the most important livestock in the world, and pork consumption is huge especially in China. Several studies have created myostatin mutations in European pig breeds (Kang et al. 2017, 2014; Rao et al. 2016; Wang et al. 2015). Heterozygous knockout in pigs caused remarkably increased lean meat proportion and decreased backfat thickness (Kang et al. 2014). However, almost all piglets with homozygous knockout showed some health-associated issues and died within a few days after birth (Kang et al. 2014; Rao et al. 2016; Wang et al. 2015). These dissatisfactory results prompt us to investigate myostatin mutations in other pig breeds.

In the present study, we reported the generation of myostatin homozygous mutations in Erhualian pigs, a Chinese indigenous pig breed that is well known for its large litter size, high fat content and superior meat quality (Zhang 1986). Homozygous knockout piglets were viable and exhibited partial double-muscled phenotype at a very early stage, indicating different effects of the myostatin mutation between European and Chinese pig breeds.

Materials and methods

Ethics statement

All animal studies were approved by the Animal Welfare and Research Ethics Committee of Jilin University, and all procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals. All surgeries were performed under anesthesia, and every effort was made to minimize animal suffering.

Plasmid construction

The plasmid encoding human optimized SpCas9 and the chimeric guide RNA was purchased from Addgene (Plasmid #42230) (Cong et al. 2013). Paired oligonucleotides were designed based on porcine myostatin sequence. A pair of synthesized oligonucleotides was mixed by adding 10× standard Taq buffer (NEB, Beijing, China). The mixture was incubated at 95 °C for 5 min in a 1 L beaker filled with 800 mL of water, and then the water was allowed to come to < 30 °C. The Cas9/gRNA plasmid was digested with BbsI and ligated with annealed oligonucleotides to construct the complete targeting vector.

Cell preparation and culture

Porcine fetal fibroblasts (PFFs) were isolated from 33-day-old Erhualian fetuses as described previously (Wang et al. 2015). The isolated PFFs were cultured in high glucose DMEM (Gibco) supplemented with 10% FBS, Penn/Strep and non-essential amino acids.

Transfection and single-cell colony selection

PFFs at low passages were electroporated with 20 μg Cas9/gRNA-1 and 20 μg Cas9/gRNA-2 using BTX ECM 2001 square-wave electroporator (Harvard Apparatus, Holliston, MA). The detailed parameters for electroporation were described previously (Wang et al. 2015). To acquire single-cell colonies by limited dilution, electroporated PFFs were seeded into 10 cm culture dishes at an appropriate density 72 h post-transfection. After 9 days of culture, single-cell colonies were picked and then expanded in 24-well plates. Once confluent, colonies were trypsinized, and plated in 12-well plates. A small portion of each colony was lysed for PCR screening. The primers used for genotyping were 5′-GGCGAAGACCTCAGG-GAAATTTATATTG-3′, and 5′-ACAGCGATCTAC-TACCATGGCTGGAATT-3′. All PCR products were visualized in 1.0% agarose gels and sequenced. Amplicons with double-peaks were cloned into the

pLB vector (Tiangen, Beijing, China) to examine the accurate sequence for both alleles.

SCNT and embryo transfer

PFF colonies with homozygous mutations were pooled as donor cells for SCNT. The experimental procedures were carried out in accordance with Lai et al.(Lai et al. 2002). Briefly, single donor cell was microinjected into the enucleated pig oocyte. The reconstructed embryos were activated and then transferred into the synchronized recipient pigs. All of the cloned piglets were delivered naturally, and the birth weight was measured immediately after birth. Genomic DNA was extracted from the tail tissue of cloned piglets for genotyping PCR.

Immunoblotting analysis

Fresh muscle samples were collected and ground in liquid nitrogen to a fine powder. After incubation in protein lysis buffer for 1 h on ice, the mixture were centrifuged at 12,000 rpm for 10 min at 4 °C. Protein concentrations were determined using a BCA protein assay kit (Beyotime, Haimen, China) according to the manufacturer's instructions. The protein samples were separated through SDS polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and blocked in 5% skim milk powder for 4 h. The membrane was incubated overnight at 4 °C with a primary antibody to myostatin (Santa Cruz Biotech., Santa Cruz, CA) at 1:200 dilution. After incubation for 1 h with a HRP-labeled anti-goat secondary antibody (dilution 1:1000), the membrane was visualized with the ECL-Plus western blotting kit (Beyotime, Haimen, China).

Off-target analysis

Highly similar sequences in porcine genome were detected by BLAST and three potential off-target sites (OTS) were selected for each gRNA. All OTS were PCR amplified using genomic DNA of the mutant piglets as templates. Sanger sequencing was performed to examine off-target mutagenesis.

Results

Dual-sgRNA co-transfection effectively created single cell colonies carrying myostatin deletions

Two verified gRNAs were used to target myostatin exon3 in which the cystine knot structure is required for receptor binding and biologic activity. To obtain MSTN knockout cells, we co-transfected two Cas9/gRNA plasmids into Erhualian PFFs. The edited PFFs were seeded at an appropriate density 3 days after electroporation, and single cell colonies were picked 9 days later. A total of 11 colonies were collected among which 8 were recovered eventually. Each colony was identified by PCR and subsequent sequencing to determine the exact genotype (Fig. 1). The result showed that colony #2 contained heterozygous deletion while colony #5 and #7 harbored homozygous mutations despite the existence of very few redundant cells. Unlike colony #2 and #5, which underwent simultaneous cleavage by two Cas9/gRNAs and formed expected deletion right between the expected cleavage sites, colony #7 harbored indel mutations at both target sites, which indicates that the two Cas9/gRNA complexes worked at the different time points (Fig. 1c).

Generation of MSTN knockout Erhualian pigs via somatic cell nuclear transfer

To create MSTN knockout pigs, single cell colonies with homozygous mutations were pooled as donor cells for SCNT (Table 1). A total of 2290 reconstructed embryos were transferred to ten synchronized recipients. We performed ultrasonography 30 days post-transfer and found that five recipients became pregnant. All pregnancies were maintained to term, and 26 female piglets were delivered (Fig. 2a). We examined the genotype of all cloned piglets by PCR and sequencing using genomic DNA derived from the tail tissue (Fig. 2b, c). The result showed that 23 piglets carried an 104 bp deletion in gRNA-1 target site and a 1 bp insertion in gRNA-2 target site on both alleles, which is consistent with colony #7 (Fig. 2c). Three wild-type piglets were also detected due to very few non-edited cells in the donor cells. Surprisingly, no piglet corresponding to the colony #5 was delivered, which might indicate the embryonic lethality of this kind of donor cell. The off-target effects remain

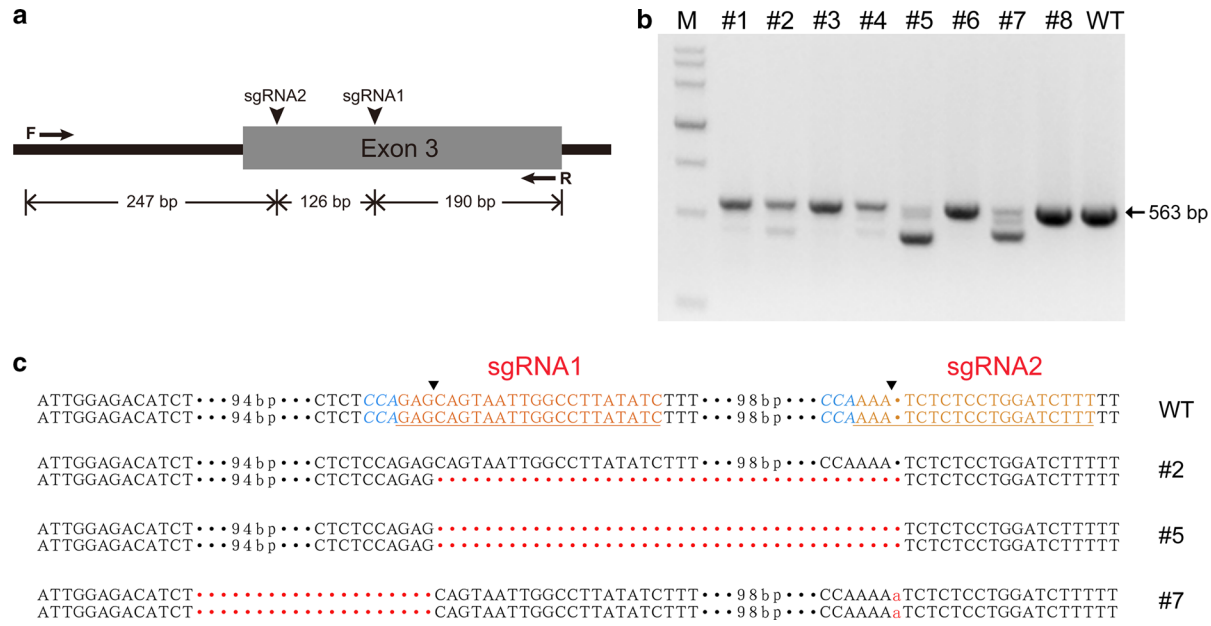

Fig. 1 Dual Cas9/gRNAs induced myostatin gene deletions in Erhualian PFFs. **a** Schematic illustration showing the position of sgRNA target sites and primers. **b** Two CRISPR/Cas9 plasmids targeting porcine myostatin gene were co-transfected into Erhualian PFFs. Eight single-cell colonies were collected after limited dilution and the targeting regions were PCR amplified. Colony #2, #5 and #7 showed smaller bands compared with the wild-type control, indicating deletions resulted from Cas9/gRNAs. **c** The exact genotype of Colony#2, #5 and #7 were determined by sanger sequencing. The targeting sequences of sgRNAs are underlined whereas the PAMs are highlighted in italic type. Potential cleavage sites are labelled with black arrows, and mutant bases are shown with consecutive dots or lower case letters

Table 1 Summary of embryo transfer for the generation of gene-targeted pigs

Surrogate ID	No. embryos transferred	Gestation length (days)	No. pigs born	No. pigs mutant
3214	200	116	8	8
3232	250	115	4	2
3264	215	118	3	3
3360	215	117	7	7
3362	200	116	4	3

one major concern of CRISPR/Cas9-mediated genome editing despite its simple assembly and high efficiency. It is possible that off-target mutagenesis in colony #5 resulted in potential embryonic lethality. We also assessed off-target activity of the mutant piglets by sequencing predicted off-target sites for both gRNAs and no mutation was detected (Supplementary Fig. 1). The average birth weight of $MSTN^{-/-}$ piglets was heavier than $MSTN^{+/+}$ counterparts, although this difference failed to reach significance (Supplementary Fig. 2). To elucidate potential risk of dystocia caused by increased birth weight, further breeding should be performed. To our surprise, several mutant piglets exhibited prominent muscular protrusion with obvious intermuscular grooves in the hind legs, which is not observed in $MSTN^{+/+}$ piglets (Fig. 3a). Moreover, $MSTN^{-/-}$ piglets showed wider backs and hips than wild-type control at 1 month of age, indicating the double-muscled effect induced by myostatin mutation (Fig. 3b, c). At last, western blotting was performed to determine the level of MSTN protein using an antibody specific to the C-terminal domain. The 1 bp insertion at the gRNA-2 target site was predicted to create a premature termination codon and result in a truncated protein which would lack 103 C-terminal

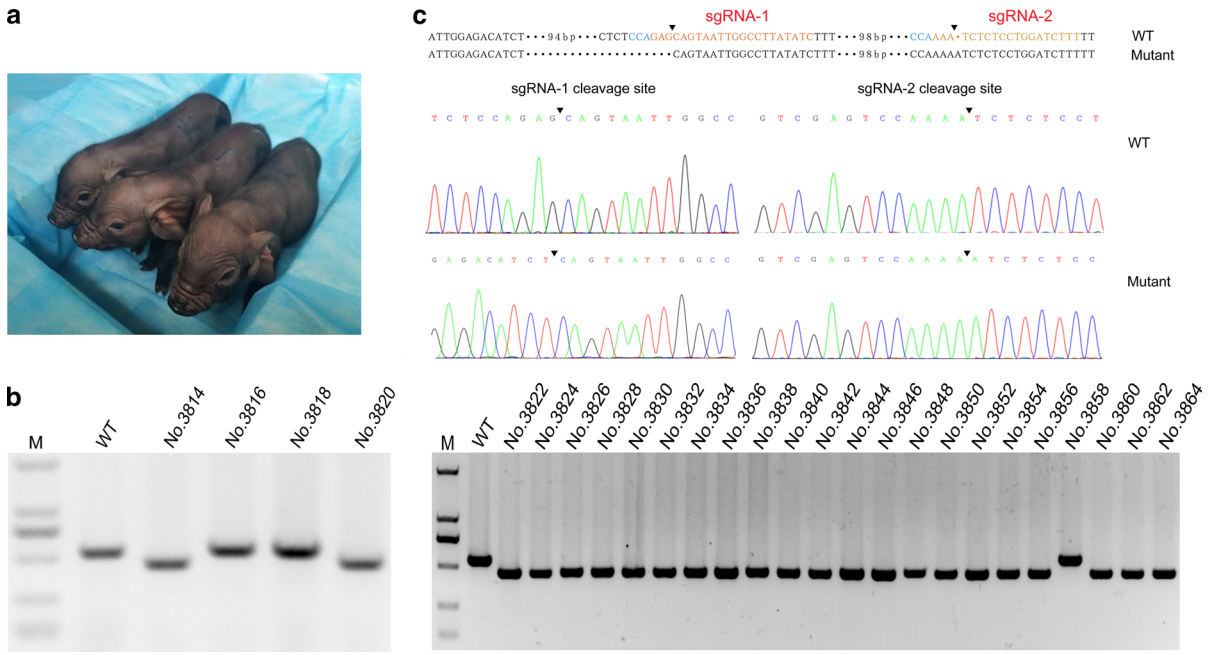

Fig. 2 Generation of cloned Erhualian pigs via SCNT. **a** Photographs of newborn cloned piglets. **b** The targeting regions were amplified using genomic DNA derived from all cloned piglets. Twenty-three piglets presented single deletion bands whereas the other three piglets remained wild-type. **c** Sanger sequencing was performed for genotyping. The mutant sequence and corresponding sequencing chromatogram are shown. All mutant cloned piglets contained the same homozygous mutation as colony #7. Specifically, mutant cloned piglets harbored an 104 bp deletion at gRNA-1 target site and a 1 bp insertion at gRNA-2 target site

amino acids (Fig. 3d). Indeed, we found that the protein level of myostatin precursor decreased dramatically in MSTN$^{-/-}$ piglets (Fig. 3e). The result suggests that functional myostatin was disrupted by this mutation, which is responsible for the double-muscled phenotype of mutant cloned pigs. We will perform a detailed phenotypic analysis for offspring of these pigs in the future.

Discussion

In this study, the dual sgRNAs strategy was used to target myostatin gene in PFFs. On one hand, two sgRNAs targeting the same gene can induce long fragment deletions, which is more likely to disrupt gene function. On the other hand, simultaneous use of dual sgRNAs has been reported to significantly improve Cas9-mediated gene targeting (Zhou et al. 2014). We mixed two different single-cell colonies as donor cells for SCNT. However, all mutant cloned piglets carried only one kind of genotype. It is thus necessary to pool several mutant colonies when performing SCNT, eliminating the risk of embryonic lethality induced by potential off-target mutagenesis.

Myostatin homozygous knockout Landrace, Duroc and Large White pigs showed abnormal legs and died within 10 days after birth (Kang et al. 2014). Our previous study also found that Landrace piglets with myostatin homozygous mutations died within a few days (Wang et al. 2015). In contrast, the work described here and a previous study demonstrate that a few Chinese native pig breeds harboring homozygous mutations in myostatin developed and grew normally (Qian et al. 2015). Partly due to differences in genetic background, an identical genetic mutation can lead to phenotypes with diverse severities in different individuals of the same specie (Threadgill et al. 1995; Vu et al. 2015). Of note, relatively different genetic backgrounds existed between European and Chinese pig breeds because of independent origins and domestication events (Ai et al. 2013; Megens et al. 2008). It is thus possible that different genetic backgrounds contribute to the diverse mutant

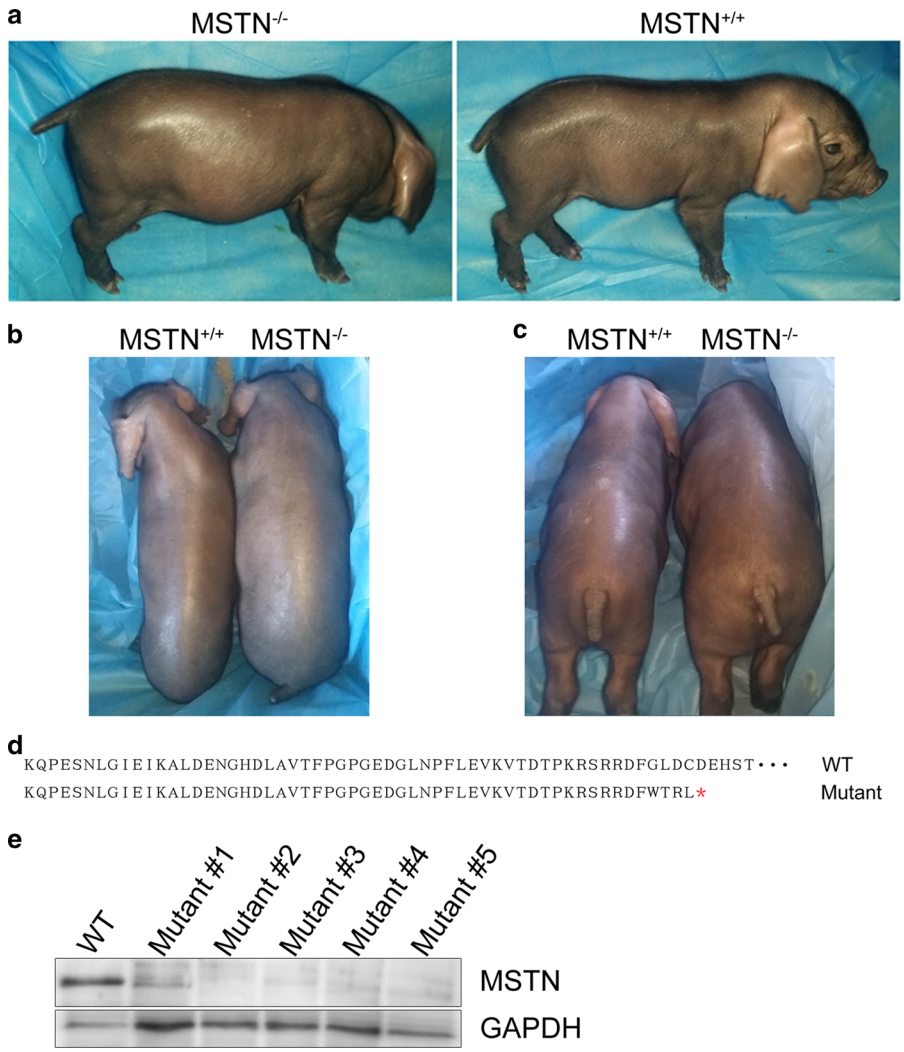

Fig. 3 Preliminary phenotypic confirmation of myostatin knockout Erhualian pigs. **a** Several mutant piglets exhibited prominent muscular protrusions with clearly visible intermuscular boundaries at 1-week-old. **b** Mutant pigs showed wider back compared with wild-type pigs. **c** Mutant pigs also showed plumper rump at 1-month-old. **d** The predicted myostatin translations from the mutation in cloned pigs. The premature stop codon is indicated with an asterisk. **e** The protein level of myostatin precursor in mutant pigs was analyzed by western blotting

effects among different pig breeds. The molecular mechanism underlying the different phenotypes is quite interesting.

With the introduction of European commercial pig breeds, some Chinese indigenous pig breeds, such as Erhualian and Minzhu, are increasingly endangered due to slow growth rate and low lean meat percentage. In this study, myostatin knockout Erhualian pigs showed obvious muscular protrusion, wider back and fuller hip compared with wild-type control. In this regard, myostatin knockout may contribute to the preservation of Chinese native pig breeds, although further selective breeding is still needed.

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Compliance with ethical standards

Conflicts of interest The authors declare no conflicts of interest.

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