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LETTER

One-step generation of myostatin gene knockout sheep via the CRISPR/Cas9 system

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The CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated) adaptive immune system, which was discovered in bacteria and archaea, can specifically degrade invasive viral and plasmid DNA by base pairing between crRNAs (CRISPR RNAs) and the target DNA [1,2]. Recently, the *Streptococcus pyogenes* type II CRISPR system was shown to be able to perform efficient targeted gene disruption by employing three fundamental components: (1) Cas9 endonuclease, which catalyzes DNA cleavage, and (2) crRNAs and (3) tracrRNAs (*trans*-activating crRNAs), which are both crucial for directing Cas9 to target sites and for transforming Cas9 from an inhibited conformation into an active state [3–5]. However, a comparable level of gene targeting can be mediated by a chimeric single-guide RNAs (sgRNAs), which results from the fusion of a crRNA and a tracrRNA, and this system is easier to operate both *in vivo* and *in vitro* [4]. Compared with the complicated design and assembly of ZFNs (zinc finger nucleases) and TALENs (transcription activator-like effector nucleases), redirecting Cas9 to a new target site requires only the alteration of a gene-specific 20-nt DNA sequence in sgRNAs, which can be synthesized on a large scale [6,7]. RNA-guided Cas9 has recently been demonstrated to be a robust tool for genome engineering in many cell lines and organisms [8–13].

Gene targeting in large domestic animals has been considered an intractable task involving screening for gene-targeted cells, the deletion of selective markers, and somatic cell nuclear transfer (SCNT), and it usually results in abortion or unhealthy newborns due to abnormal epigenetic modifications [14,15]. Recently, Zhou and colleagues reported the first gene-knockout pigs generated using a one-step zygote injection of the CRISPR/Cas9 system, demonstrating a highly promising rapid method to create large domestic gene-knockout animals [16]. Here, we report the first successful one-step generation of gene-knockout sheep using the same method.

To test the feasibility of gene targeting in sheep using the CRISPR/Cas9 system, we designed sgRNAs targeting the myostatin (*MSTN*) gene (Supplementary information, Table S1). Myostatin is a transforming growth factor-β family member that negatively regulates muscle mass. Naturally occurring *MSTN* mutations in dogs and Belgian Blue cattle have been found to result in similar double-muscled phenotypes [17,18]. The disruption of the *MSTN* gene in mice has been shown to also cause a pronounced increase in skeletal muscle mass [19]. Thus, animal breeding scientists are highly interested in modifying the *MSTN* gene in large domestic animals, such as pigs and sheep. Further research should be performed to determine whether such genetic modifications could improve meat production in these animals.

We designed sgRNAs targeting the third exon of *MSTN* (Fig. 1a), resulting in out-of-frame indels (insertions or deletions) predicted to abolish normal MSTN function. To determine the working efficiency of the Cas9 system *in vitro*, sheep codon-optimized Cas9 and sgRNA expression plasmids were cotransfected into sheep fibroblast cells. Genomic DNA isolated from the cells 48 h after transfection was subjected to PCR amplification and the Surveyor assay to confirm cleavage. The two expected cleavage bands were observed, suggesting that the *MSTN* gene was mutated in a proportion of the transfected cells.

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The average gene targeting efficiency was estimated to be 19.3% (Supplementary information, Fig. S1).

After confirming that the Cas9 system worked in sheep fibroblast cells *in vitro*, we examined whether it also worked *in vivo*. Briefly, Cas9 mRNA and the sgRNAs were *in vitro* transcribed using T7 RNA polymerase (Supplementary information, Table S2). A mixture of the Cas9 mRNA and the sgRNAs was microinjected into 213 embryos in two independent experiments. The injected embryos were immediately transferred into 55 surrogate females, 31 of which became pregnant, suggesting a low toxicity of the Cas9:sgRNA mixture. Between 140 and 152 days after the uterine transfer, 35 lambs were successfully delivered, resulting in a live birth rate of 16.4% (Supplementary information, Table S3). Tissue samples from the hind leg muscles and ears of all the lambs were dissected for *MSTN* genotyping. The regions surrounding the target site in the *MSTN* gene were amplied by PCR and the Surveyor assay was used to assess cleavage (Supplementary information, Table S4). Apparent cleavage bands were detected in samples from one male and one female lamb (Fig. 1b), suggesting gene disruption by Cas9. To confirm this gene disruption, we cloned the PCR products and randomly selected more than 80 clones derived from each lamb for Sanger sequencing. Consistent with the cleavage assays, five different mutant alleles were found in the two animals, with indels ranging from 0 to 18 bp (Fig. 1c). The female lamb was shown to have the wild-type allele and two mutant alleles, and the male lamb harbored the wild-type allele and five mutant alleles. We thus concluded these two animals as monoallelic mutants. Among the five mutant alleles, three caused out-of-frame mutations that disrupted the coding region; the other two mutant alleles, an in-frame deletion (18 bp) and a substitution of five nucleotides (0 bp), may have had little effect on the function of MSTN.

Previous reports have suggested that a small number of mismatches between sgRNAs and the complementary target DNA are easily tolerated, resulting in a high frequency of off-target mutagenesis in human cells [20,21]. To determine whether there were off-target mutations in the two lambs, we searched the entire sheep genome. We found six potential off-target loci containing a maximum of five mismatches compared with the specific sgRNAs designed for the *MSTN* gene (Supplementary information, Table S5). The genomic regions flanking the putative off-target sites were amplified and examined using both Surveyor assays and the direct sequencing of PCR products. The cleavage bands of off-target (OT-1, Supplementary information, Fig. S2 and Table S5) were found to result from nearby SNPs, but no mutations in any intended loci were revealed. However, we cannot exclude the possibility that an extremely low level of some off-target mutations beyond the sensitivity of the method we

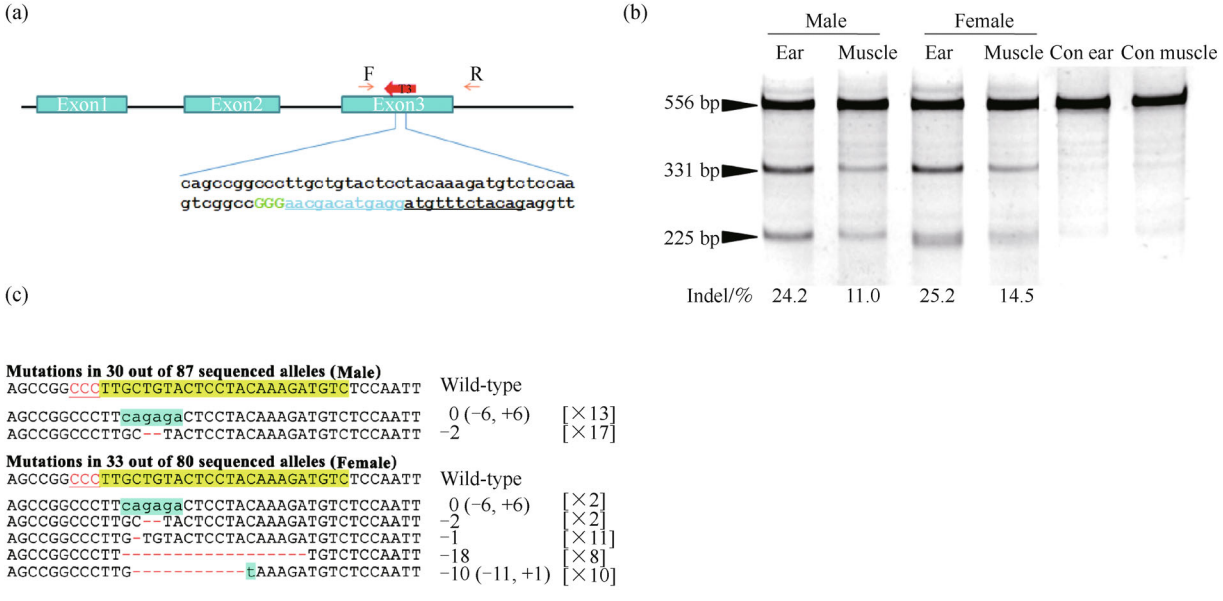

Fig. 1 Generation of *MSTN*-knockout sheep and analysis of the mutant alleles. (a) A schematic of the sgRNAs targeting the third exon of the *MSTN* gene. The PAM motif is shown in green. The target site is underlined, and the 12 bp seed sequence is highlighted in blue. The primers for the PCR analysis are indicated by arrows; (b) indel mutations in the ear and muscle were detected using the Surveyor assay. Ear and muscle tissues from the control group with a wild-type *MSTN* gene produced a 556 bp band. Monoallelic mutant sheep produced multiple bands with lengths of 556 bp, 331 bp, and 225 bp; (c) sequence analysis of the mutations detected in the two lambs. Deletions are indicated by a dashed line, and insertions are shaded in cyan. The numbers following the sequences indicate the specific type of mutation, and the clone numbers are surrounded by brackets.

used may have occurred. Taken together, the results indicate that we have successfully generated *MSTN* genetically modified sheep through the one-step microinjection of a Cas9 RNA:sgRNA mixture into fertilized eggs, although the production efficiency (2/35) of mutant lambs was not as high as those previously reported for other species [11,16].

At least two mutant alleles were identified in each of the two *MSTN*-knockout lambs. The presence of multiple mutant alleles is a common phenomenon, and it has also been observed in other genetically modified species generated by the microinjection of a Cas9 RNA:sgRNA mixture into zygotes [11,13,16]. These observations strongly indicate that when using this method, Cas9-mediated double-stranded DNA breaks (DSBs) could occur many times independently after the one-cell embryo stage, leading to multiple modified alleles and, thus, mosaic animals. Considering the time and efficiency issues, a better method to create gene-targeted large animals might be the injection of a preassembled Cas9:sgRNA protein complex directly into the nucleus of a one-cell-stage embryo.

In summary, we have successfully obtained *MSTN* gene-mutated sheep, demonstrating that the direct injection of Cas9:sgRNA into zygotes can be widely used to create gene knockouts in large domestic animals. This method may greatly facilitate improvements in animal breeding and the application of these animals in biomedical studies.

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Compliance with ethics guidelines Hongbing Han, Yonghe Ma, Tao Wang, Ling Lian, Xiuzhi Tian, Rui Hu, Shoulong Deng, Kongpan Li, Feng Wang, Ning Li, Guoshi Liu, Yaofeng Zhao and Zhengxing Lian declare that they have no conflict of interest or financial conflicts to disclose.

All applicable institutional and national guidelines for the care and use of animals were followed.

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