doi:10.1093/jmcb/mjv061
Published online October 12, 2015

Letter to the Editor

# Generation of gene-target dogs using CRISPR/Cas9 system

**Dear Editor,**

Dogs (*Canis familiaris*) serve as human companions and are raised to herd livestock, aid hunters, guard homes, perform police and rescue work, and guide the blind. Dogs exhibit close similarities to humans in terms of metabolic, physiological, and anatomical characteristics, and thus are ideal genetic and clinical models to study human diseases (Tsai et al., 2007). Gene target technology is a powerful tool to create new strains of animals with favorable traits. However, thus far, gene-target dogs have not been developed due to their unique species-specific reproductive characteristics, which limits the applications of dogs especially in the field of biomedical research. Recently, clustered regularly interspaced short palindromic repeats (CRISPRs)/CRISPR-associated (Cas) 9 system was applied to edit specific genes with a high efficiency (Cong et al., 2013; Mali et al., 2013). Here we attempt to explore the feasibility of producing gene knockout (KO) dogs by using this technology. Beagle dog, the most widely used breed in biomedical research, was used as our animal model. *Myostatin* (MSTN) was chosen as the first gene of interest. MSTN is a negative regulator of skeletal muscle mass (McPherron et al., 1997). Spontaneous mutations of MSTN cause muscle hypertrophy in many species, including dogs (Mosher et al., 2007), without causing severe adverse consequences.

We designed sgRNA targeting the first exon of the dog MSTN gene (Figure 1A). The efficiency of sgRNA was validated by co-transfection with Cas9 vector into canine embryonic fibroblasts (CEFs). Twenty-two colonies were harvested and lysed to test site-specific gene modification by polymerase chain reaction (PCR) amplification. The PCR products were used for T7 endonuclease I (T7E1) cleavage assay. Cleavage bands were found in seven colonies (Figure 1B). Further sequencing showed that 13 colonies (59.1%) were mutated at the cleaved site with various mutation sizes (from $-13$ to $+1$; Figure 1C). Among them, five colonies (22.7%) showed monoallelic mutation. There are eight colonies (36.4%) showing biallelic mutation, in which six colonies had the same mutation in both alleles (homozygous mutation) and two colonies displayed different mutation in each allele (Supplementary Figure S1). There are short repeats (GT, ATGT) around the sgRNA target locus, which probably cause microhomology-mediated end joining contributing to the high homogenous mutation rate (Morton et al., 2006; Qi et al., 2013). The other nine colonies (40.9%) were detected without mutations. These data demonstrated that our sgRNA functioned effectively in canine cells.

We next sought to generate MSTN KO dogs by manipulating dog zygotes. In initial trials, allo-transplantation of embryos was performed. A total of 30 presumptive zygotes were collected. Twenty-five embryos with normal morphology were immediately microinjected with a mixture of Cas9 mRNA and MSTN sgRNA. The injected embryos were transferred into six surrogate mothers with estrus that were presumptively synchronous with zygote donors. Unfortunately, none of the recipient mothers were found pregnant (Figure 1D).

Compared with other mammals, dogs have several unique species-specific reproductive characteristics (Farstad, 2000; Jang et al., 2007), such as mono-oestrus, polyovulatory, polytocous, and non-seasonal reproductive cycle. In addition, in contrast to many mammals that release mature oocytes in the metaphase II stage, dogs release immature oocytes in the germinal vesicle stage, and these oocytes require another 48 – 72 h in the oviduct to reach maturation (Holst and Phemister, 1971). Therefore, the failure of pregnancy by allo-transplantation approach was probably ascribed to the asynchronous reproduction stage in donor zygotes and recipient female dogs. To address these concerns, we designed an auto-transplantation strategy. Briefly, we flushed one side of the canine oviduct corresponding with the ovary with more corpus luteum to collect zygotes. After injected with the mixture of Cas9 mRNA and MSTN sgRNA, these zygotes were immediately transferred back into the other side (not flushed) of the oviduct of the same female dog. A total of 35 injected zygotes were transferred into 10 donor-recipient females. Among them, 8 females were pregnant to term (80.0%) and gave birth to 27 puppies (Figure 1D).

Ear punch tissues were collected from all the puppies for detection of any mutation in MSTN locus. PCR products amplified from the genome of all the 27 puppies were sequenced. Two puppies from different mothers were found with genetic mutations in MSTN locus. The female puppy #5 was named after the heaven dog in Chinese myth as 'Tiangou', and the male puppy #11 was named after the Greek divine hero with powerful strength as Hercules (Supplementary Figure S2A). To determine their mutation patterns, the PCR products were cloned into pMD-18T vector and the generated clones (15 for puppy #5 and 14 for puppy #11) were sequenced. As shown in Figure 1E, puppy #5 had 1-bp insertion in one allele of the sgRNA target site and 8-bp deletion in the other, while puppy #11 had a single mutation of 4-bp deletion.

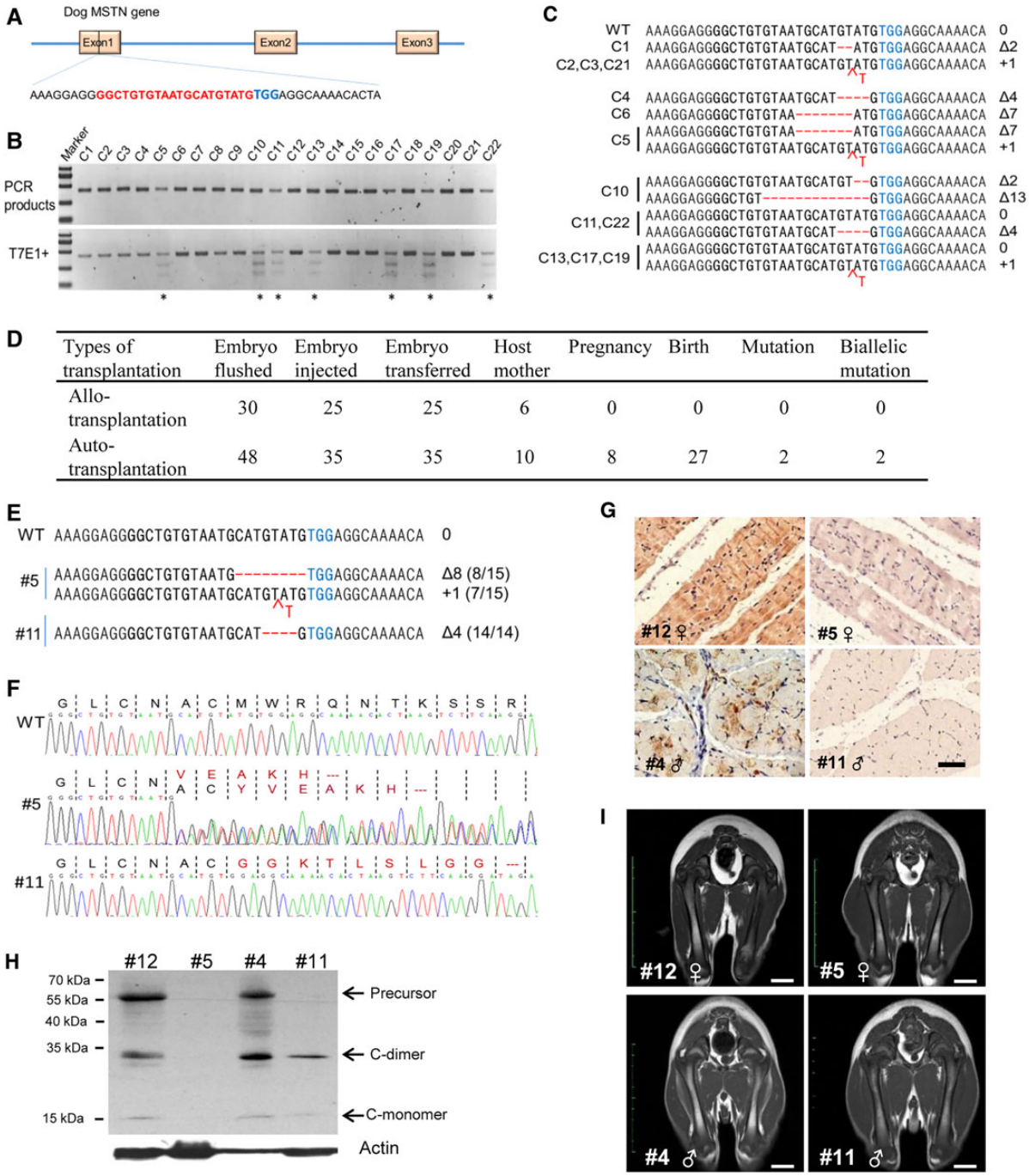


**Figure 1** Generation of MSTN mutant dogs by Cas9/sgRNA. **(A)** Schematic of sgRNA target site within exon 1 of dog MSTN locus. The sgRNA target site and PAM sequence are highlighted in gray. **(B)** Detection of sgRNA/Cas9-mediated cleavage of dog MSTN in CEF colonies by T7E1 cleavage assay. Samples with cleavage bands were marked with an asterisk (*). **(C)** Sequences of modified MSTN locus detected in CEF colonies. Target sequences are shown in boldface; PAM sequence is "TGG", mutations in gray; 'C' stands for clone; 'Δ' stands for deletions; '+' stands for insertions. **(D)** Summary of embryo microinjection of Cas9 mRNA and MSTN sgRNA in beagle dogs. **(E)** Sequencing of target site in both puppies #5 and #11. Puppy #5 had 8 bp deleted in one allele and was inserted with 1 bp in the other. Puppy #11 was deleted with 4 bp in both alleles. **(F)** DNA sequencing diagram and amino acid sequences of MSTN proximal to target site. Sequencing traits of nonsense mutations detected in both puppies #5 and #11. '—' indicates stop codon sites. **(G)** Immunohistochemical analysis of MSTN protein expression in skeletal muscle of puppies #5 and #11 (light gray) and wild-type puppies #12 and #4 (dark gray). Scale bar, 50 $\mu$m. **(H)** Western blot showing the levels of MSTN precursor, C-dimer, and monomer in skeletal muscles of puppies #5 and #11 and wild-type puppies. $\beta$-actin was used as internal control. **(I)** The coronal T1-weighed images of the dog thighs. The sections were crossed the thighbone. The #5 and #11 are MSTN mutant puppies, and #4 and #12 are wild-type puppies. Scale bar, 2 cm.

No wild-type sequence was detected in both puppies (Figure 1E). Other available tissues, including tail skin, blood, adipose tissue, and skeletal muscle, were also collected for sequencing and T7E1 assay. For each tissue, 10 − 15 clones of PCR products were sequenced. All tissues from the same dog demonstrated the same mutant sequences (data not shown) and cleavage bands (Supplementary Figure S2B) as ear punch tissues. To test whether the puppies were chimeras, we performed deep sequencing of the captured targeted region of MSTN locus on all tissues. Almost all of the sequencing reads had mutations (86.8% + 1 bp and 13.1% − 8 bp) at the MSTN locus in puppy #5, and 99.2% of the sequencing reads had the deletion of 4 bp in puppy #11 (Supplementary Figure S2C). No wild-type sequences were found in puppy #5, while ∼0.5% wild-type sequences were detected in puppy #11, suggesting that puppy #5 might be a biallelic mutant whereas puppy #11 was a chimera.

The insertion or deletion of the targeted gene leads to premature termination of MSTN translation at the first exon (TAA for − 8 bp and + 1 bp, TAG for − 4 bp; Figure 1F). Next, MSTN protein expression in skeletal muscles were analyzed by immunohistochemistry and western blot. As shown in Figure 1G, MSTN protein was not detected by immunohistochemistry in the muscles of puppies #5 and #11. Similarly, both full-length unprocessed MSTN precursor protein and the processed dimer or monomer were undetectable in skeletal muscles of puppy #5 by western blot (Figure 1H), indicating that puppy #5 was a MSTN biallelic mutant dog and no other chimeric mutation existed in its tissues. However, a small amount of MSTN processed forms were found in puppy #11, although the precursor protein was still undetectable (Figure 1H), confirming that puppy #11 was a chimeric mutant dog. Moreover, after 4 months, puppy #5 displayed obvious muscular phenotype on thighs compared with its wild-type littermate sister #12, while puppy #11 did not show notable difference compared with its wild-type littermate brother #4 (Supplementary Figure S2D). Magnetic resonance images (MRI) of the coronal and axial of thighs further confirmed that puppy #5 displayed obvious double-muscled phenotype on thighs, but puppy #11 had similar muscle mass as the wild-type littermate brother (Figure 1I and Supplementary Figure S2E).

To examine the germline transmission potential of the mutations, semen samples were collected from the male dog #11 when it reached sexual maturity at 10 months after birth. Sperm genotype was analyzed by sequencing. Ten clones of PCR products were sequenced and only a single mutant genotype ( − 4 bp) was found, consistent with that detected in tissues from puppy #11 (Supplementary Figure S3). This indicates that the mutation can be transmitted to the next generation, which is essential for the expansion of the gene-target dog population.

To test the specificity of Cas9/sgRNA cleavage in these genetically modified dogs, we screened the dog genome and predicted a total of 16 potential off-target sites (OTSs) with 2 − 4 mismatch oligodeoxynucleotides in the 20-bp sgRNA target sequence (Supplementary Table S1). All OTSs were amplified and then subjected to T7E1 cleavage assay. Four out of the 16 PCR products yielded the same cleavage bands as wild-type control (Supplementary Figure S4). All PCR products were further cloned into pMD-18T vector and each clone was sequenced for 5 − 10 times. All potential OTS-generated clones had the same sequence as wild-type, and no authentic mutation was detected (Supplementary Table S1), suggesting that the Cas9/sgRNA did not induce detectable off-target mutation in our study.

In summary, our study demonstrated for the first time that a single injection of Cas9 mRNA and sgRNA corresponding to a specific gene into zygotes, combined with an auto-embryo transfer strategy, can efficiently generate site-specific genome-modified dogs. This approach may not only greatly facilitate the generation of novel dog models for biomedical research, but also promote the creation of new strains of dogs with favorable traits for other purposes.

*[Supplementary material is available at Journal of Molecular Cell Biology online. We thank Guanwen Shi, Zhaowei Chen, Hualin Zhang, and Zhenhua Lin from Guangzhou General Pharmaceutical Research Institute for suggestions and assistance. This work was supported in part by grants from the National 973 Basic Research Program of China (2011CB944203, 2011CB944104) and the Ministry of Science and Technology of China (2011ZX09307-304, 2011BAI15B02, 2013BAK11B02, 2012BAI39B01).]*

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