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LETTER TO THE EDITOR

Heritable gene-targeting with gRNA/Cas9 in rats

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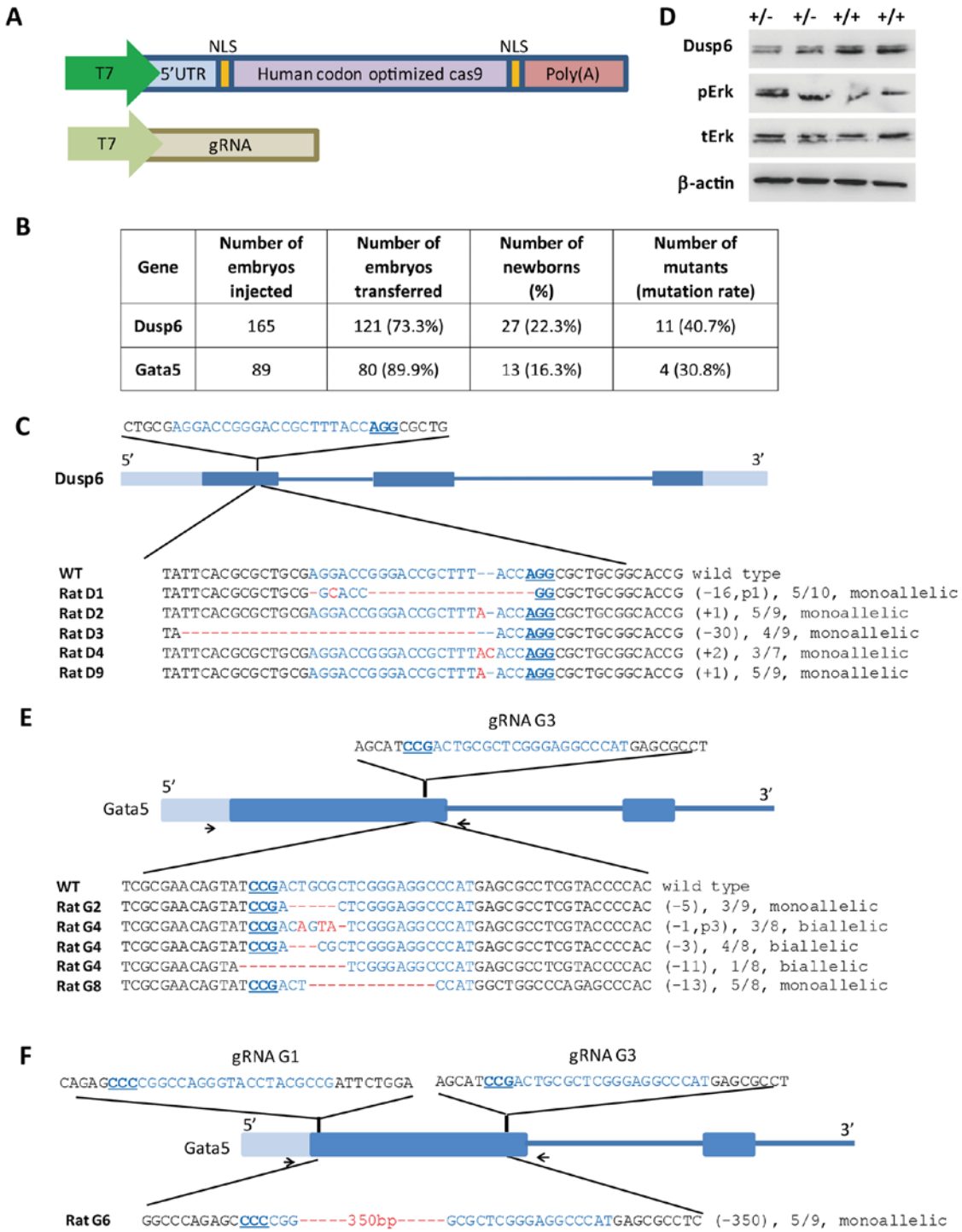
Dear Editor,

The rat is a preferred animal model in many research applications, especially in physiological, behavioral and translational studies [1]. Although the rat embryonic stem (ES) cells were successfully isolated [2, 3], the rat ES cell-based gene-targeting approach has not been widely adopted due to the technical difficulties in manipulating these cells. The first knockout rat was generated via microinjection of zinc finger nuclease (ZFN) into the embryos [4]. In addition to ZFN, new genome-editing tools such as transcription activator-like effector nuclease (TALEN) [5] and the clustered, regularly interspaced, short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system [6] have offered a rapid and efficient means of genome modification in many species. Technologies of ZFN and TALEN have made gene-targeting in the rat genome more convenient and practical [7-9]. The CRISPR/Cas system is the most recently developed technology for targeted genome modification in mammalian cells, bacteria, zebrafish and mice [10-12]. This system requires a locus-specific CRISPR RNA (crRNA), a transactivating crRNA (tracrRNA) and a nuclease Cas9. Previous studies have shown that a chimeric RNA, consisting of a crRNA that recognizes the target sequence and a tracrRNA that recruites Cas9, could fulfill the combined functions of crRNA and tracrRNA [11, 12]. We previously reported highly efficient genome editing with the chimeric RNA-guided Cas9 system in mammalian cells and zebrafish [10]. Here, we successfully extended this simple and efficient gRNA/Cas9 system to modify the rat genome.

We designed 3 guide RNAs (gRNAs) to target exon 1 of the rat *Dusp6* gene (Supplementary information, Table S1). The gRNA molecules were all chimeric RNAs that fused tracrRNA with a *Dusp6*-specific crRNA sequence. To validate the targeting efficiency of these gRNAs, we constructed expression vectors in which a gRNA was driven by the U6 promoter and a Cas9 expression vector in which Cas9 was driven by the CMV promoter (Supplementary information, Figure S1A). Both Cas9 and gRNA expression vectors were co-transfected into the rat embryonic fibroblast cells (REFCs). A GFP expression vector driven by the CMV promoter was also co-transfected to monitor transfection efficiency. Three days after the transfection, REFCs were harvested and the genomic DNA was prepared. Results of genotyping with T7 endonuclease I (T7EI) showed the expected sizes of DNA fragments, suggesting that all the 3 gRNAs efficiently guided Cas9 for genome editing, which led to non-homologous end-joining-mediated insertions and deletions (indels) in the *Dusp6* locus (Supplementary information, Figure S1B). Sequencing results confirmed that the indels were introduced near the corresponding gRNA-targeting sites (Supplementary information, Figure S1C). Based on our previous study showing that a higher genome-editing efficiency was achieved by microinjecting Cas9 mRNA and gRNA into one-cell zebrafish embryos than by plasmid overexpression in cells [10], we chose to inject *in vitro* synthesized Cas9 mRNA (40 ng/μl) and gRNA D3 (20 ng/μl) into the one-cell Sprague-Dawley rat embryos (Figure 1A). Out of the 165 injected embryos, 121 healthy embryos were transferred into surrogate mothers and 27 pups were born (Figure 1B). Out of these 27 pups, 11 showed double peaks in the sequencing of PCR products amplified from genomic DNA (data not shown), suggesting a mixture of mutant and wild-type DNA templates in these rats. Among these 11 pups, we then cloned the PCR products of 5 rats and randomly sequenced several clones from each rat. All these 5 rats had mutations near the targeting site in *Dusp6* locus, and each rat only contained one type of mutation (Figure 1C). About half of the clones sequenced from each rat represented mutant alleles. These results suggest that gRNA/Cas9 can induce mutations in the rat genome with high efficiency.

To determine whether the indels in the *Dusp6* loci are heritable, we bred the mutants D3, D4 and D9 with wild-type rats, and isolated day 10 F1 embryos of founder D4, day 14 F1 embryos of founder D3 and day 1 F1 neonatal pups of founder D9. PCR genotyping revealed that the mutations were present in the F1 of all 3 mutant rats (Supplementary information, Table S3) and the mutations of *Dusp6* in F1 were the same as those in the corresponding founders. These results suggest an efficient germline transmission of the gRNA/Cas9-induced mutations in rats.



Gene	Number of embryos injected	Number of embryos transferred	Number of newborns (%)	Number of mutants (mutation rate)
Dusp6	165	121 (73.3%)	27 (22.3%)	11 (40.7%)
Gata5	89	80 (89.9%)	13 (16.3%)	4 (30.8%)

Figure 1 Generation of mutant rats with the gRNA/Cas9 system. **(A)** Constructs for *in vitro* synthesis of Cas9 mRNA and gRNAs. **(B)** Numbers of injected and transferred embryos, newborns and generated mutants during the establishment of knockout rats using gRNA/Cas9 system. **(C)** Sequencing results of the mutant *Dusp6* alleles in the founder rats D1, D2, D3, D4 and D9. The gRNA-targeting sequences are in blue and the PAM is highlighted and underlined. The mutated sequences are in red. The number of nucleotides of point mutations (p), insertions (+) or deletions (–) are shown to the right of each allele. The fraction number represents number of mutant clones /number of clones sequenced. **(D)** Western blot of brain tissues of F1 embryos from founder D3 that was crossed with a wild-type female rat. Genotyping was performed to identify heterozygous and wild-type embryos. +/–, heterozygote; +/+, wild-type; pErk, phosphorylated Erk; tErk, total Erk. **(E)** Sequencing results of the mutant *Gata5* alleles in the founder rats G2, G4 and G8 with only gRNA G3-guided mutations. **(F)** Sequencing results of rat G6 that contained a 350-bp deletion introduced by gRNAs G1 and G3.

To confirm that the *Dusp6* gene was disrupted, we performed western blot by using the brain tissues isolated from day 14 embryos of founder D3 that was crossed with a wild-type female rat. The heterozygotes showed about 50% decrease in the amounts of Dusp6 proteins (Figure 1D). Accordingly, the level of phosphorylated Erk (pErk), a substrate of Dusp6, was increased by about 60% in the mutants as compared with that in the wild-type littermates (Figure 1D). Thus, we have indeed achieved the disruption of *Dusp6* gene by gRNA/Cas9, which caused a decrease in the Dusp6 phosphatase activity in the mutant rats.

One of the major advantages of the gRNA/Cas9 system is the simple and flexible design of locus-specific gRNA [10-12]. We then tried to determine whether using a pair of gRNAs could cause predictable deletions between 2 targeted gRNA loci. A pair of gRNAs, G1 and G3, were designed to target the 5′ and 3′ regions of rat *Gata5* exon 1, respectively (Supplementary information, Table S1), and co-transfected with Cas9 expression vector into REFCs. A PCR product of 894 bp was expected to be amplified from the wild-type *Gata5* locus. The distance between the 2 targeting sites was about 320 bp. Interestingly, in addition to the 894-bp fragment, a PCR product of ~500 bp was amplified from the genomic DNA of gRNA-transfected REFCs (Supplementary information, Figure S1D; marked with an asterisk). Subsequent sequencing of randomly-selected PCR clones showed that some mutations occurred at only one targeting site, especially at the G3-targeting site (Supplementary information, Figure S1E), suggesting that the gRNA G1 was less effective than the gRNA G3. In addition, we found deletions of ~350 bp between the 2 gRNA-targeting sites (Supplementary information, Figure S1E), suggesting that the simultaneous introduction of 2 gRNAs causes predictable deletions in the targeted loci. We then tested this strategy *in vivo* by microinjecting Cas9 mRNA (40 ng/μl) together with the 2 gRNAs G1/G3 (20 ng/μl each) into the fertilized Sprague-Dawley rat ova. Out of the 89 injected embryos, 80 healthy embryos were transferred into surrogate mothers and 13 pups were born (Figure 1B). Among the 13 pups, 4 showed double peaks in the sequencing of PCR products amplified from genomic DNA. The PCR products from these rats were subsequently cloned and sequenced. The rats G2 and G8 had 5-nt and 13-nt deletions at the gRNA G3-targeting site, respectively (Figure 1E). The Rat G4 contained 3 different kinds of mutations, but they were all introduced by gRNA G3 (Figure 1E). No wild-type allele was identified in the 8 randomly-selected clones from the Rat G4 (Figure 1E), suggesting potential biallelic mutations in this rat. The rat G6 had a 350-bp deletion between the targeting sites of G1 and G3 (Figure 1F). Taken together, these results suggest that large DNA fragment deletions can be generated in the rat genome by using 2 gRNAs simultaneously.

One major concern on genome editing with gRNA/Cas9 is its potential off-target effects. Previous studies have shown that the 8-12 bp "seed sequence" at the immediate 5′ side of the protospacer adjacent motif (PAM) is critical for the specificity of gRNA, while mismatches outside the "seed sequence" can be tolerated [12]. We thus examined the potential off-target sites of the 3 gRNAs (D3, G1, G3) in the rat genome based on this rule. None of these potential sites contain more than 10 nucleotides identical to the gRNA "seed sequence". We then selected 6 potential off-target sites for each gRNA; each site contains a stretch of 9-10 nucleotides identical to the gRNA "seed sequence" (Supplementary information, Table S4). No mutations were observed at these potential off-target sites in the 4 *Gata5* mutant founders, and only 1 site showed an off-target effect in the *Dusp6* mutant founders. These results suggest that few off-target effects were induced by the gRNA/Cas9 system in this work.

In summary, we demonstrated that the gRNA/Cas9 system is a simple and efficient genome-editing tool for generating heritable rat knockouts. While we were writing up this work, similar results were published by 2 independent groups [13, 14]. Our work shows that gRNA/Cas9 efficiently induces indels in 2 gene loci, *Dusp6* and *Gata5*, in rats, and the efficiency is comparable with others [13, 14]. Importantly, we have demonstrated that the *Dusp6* mutations can be transmitted to the next generation with high efficiency. Moreover, we are the first to show that a simultaneous microinjection of 2 gRNA molecules leads to large DNA fragment deletions in the rat genome as reported in zebrafish [15]. The latter enables genetic elimination of a whole exon or even an entire gene including genes for non-coding RNAs, which is particularly useful for studying the functions of non-coding RNAs. Introducing larger deletions will significantly reduce the workload for genotyping as wild-type and mutant alleles can be easily distinguished on regular agarose gels. In addition, we chose to only further analyze the rats, from which the genomic DNA PCR products displayed double-peak sequencing profile. Although we might have overlooked those rats, in which the mutant alleles were not properly amplified by PCR, this double-peak sequencing profile provides a simple and efficient means for identifying mutant rats. Together, the gene-targeting with gRNA/Cas9 in rats described here adds another simple and efficient genetic tool for this important model organism.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)