Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems

To the Editor:

CRISPRs are clustered, regularly interspaced, short palindromic repeats present in many bacteria and archaea genomes. Proteins encoded by CRISPR-associated (Cas) genes serve as guardians of the genome, which target foreign DNA at specific sites by means of small CRISPR RNA (crRNA)-guided DNA recognition and degradation[1–4]. Recently, several groups described how CRISPR-Cas systems efficiently create site-specific gene modifications in whole organisms such as *Streptococcus pneumoniae*, *Escherichia coli*, *Danio rerio* (zebrafish) and mice, suggesting its potential application in the production of genetically engineered organisms[5–7], although germline transmission of the mutations remains to be shown. Here, we report the use of CRISPR-Cas systems to generate multiple gene mutations in rats in a germline-competent manner.

The laboratory rat is an important model organism for human disease research. Several technologies including zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN) and isolation of rat embryonic stem cells have enabled targeted gene mutation of the rat genome[8–10]. Compared with these techniques, CRISPR-Cas systems provide a gene editing tool that can more easily be targeted to one or more genomic loci, as targeting is mediated by small RNA that can be simply expressed or transfected with the Cas9 nuclease.

We chose the *Tet* family genes as targets to demonstrate the feasibility of CRISPR-Cas–mediated mutagenesis in rat. The three *Tet* genes encode proteins with DNA hydroxymethylase functions and play key roles in the regulation of many important biological processes[11]. The Cas9 endonuclease functions either with a combination of a short crRNA and a *trans*-activating crRNA (tracrRNA), or with a chimeric single guide RNA (sgRNA). We designed six sgRNAs targeting six different genomic sites encoding rat *Tet1* (sg*Tet1*-1 and sg*Tet2*-1), *Tet2* (sg*Tet2*-1 and sg*Tet2*-2) and *Tet3* (sg*Tet3*-1 and sg*Tet3*-2), respectively. Two sgRNAs were chosen for each gene to increase the likelihood of successful targeting. The sgRNAs were designed to contain 20-nucleotide customized spacer sequences

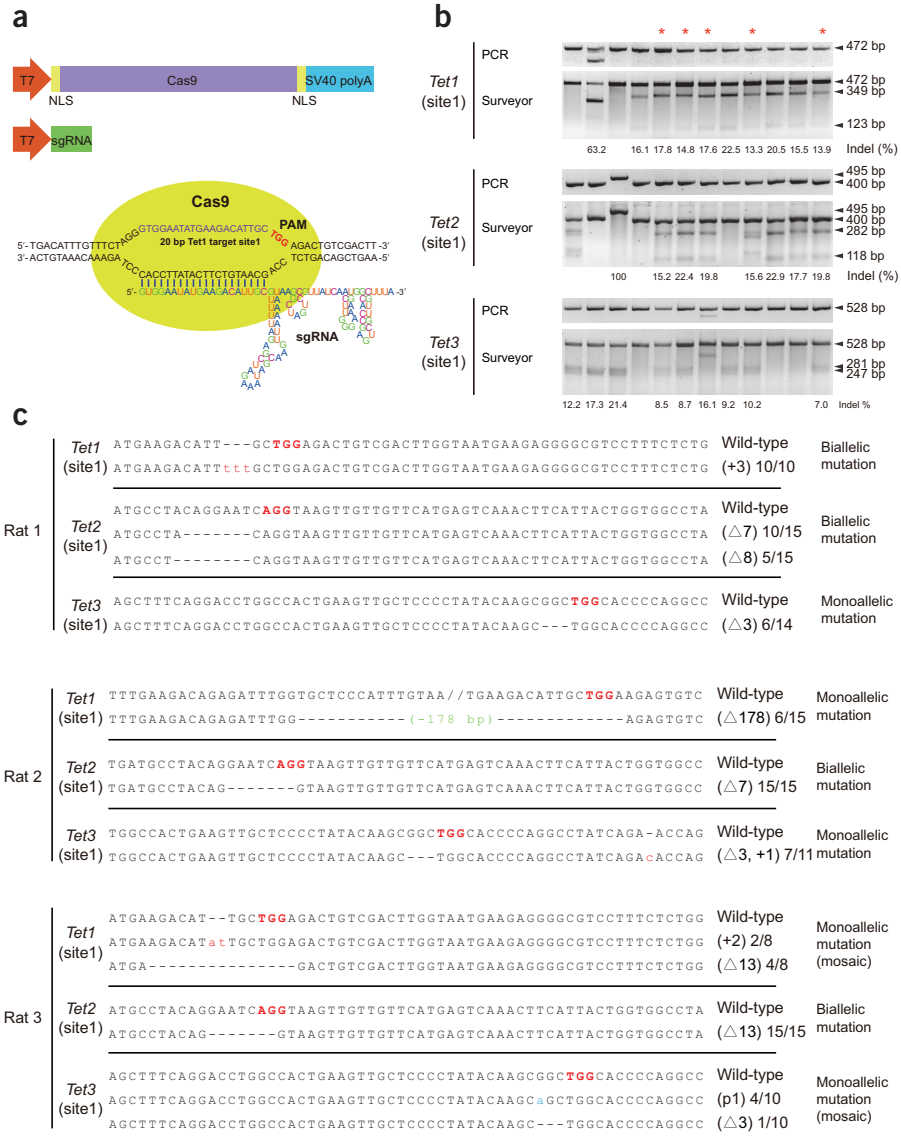

Figure 1 Targeted disruption in the *Tet* gene loci in rat by means of CRISPR-Cas systems. (**a**) Engineered CRISPR-Cas systems and their target *Tet1* gene locus used in this study. The protospacer adjacent motif (PAM) is shown in red letters, and the 20-base-pair spacer sequence is shown in purple letters. NLS, nuclear localization signal. (**b**) The PCR products encompassing the targeted sites and their Surveyor assay for indels in the PCR products. All three Tet genes of each rat are assayed and presented in the same order. Individuals with heteroduplex of wild-type (WT) and mutated alleles have three bands in Surveyor assay, individuals with large indels have PCR and Surveyor products with shifted sizes. Indel rate is calculated and labeled under the lanes with mutation. *, rats with mutations in all three target sites. (**c**) Sanger sequencing of targeted gene alleles in each of the triple-mutated rats induced by injection of Cas9 enzyme together with sg*Tet1*-1/sg*Tet2*-1/sg*Tet3*-1. The PAM sites are red, upper case bold; insertions are red, lower case; point mutations are blue, lower case. The sizes of the point mutations (p), insertions (+) or deletions (Δ) are shown to the right of each allele. The fractions indicate the read number of the mutant allele (numerator) out of total read number (denominator). The targeted locus is labeled as "biallelic mutation" if no wild-type allele reads were recovered, as "monoallelic mutation" if a wild-type allele was recovered, and as "mosaic" if more than two types of alleles were recovered (**Supplementary Discussion**).

Table 1 Multiple gene disruption in rats by means of CRISPR-Cas systems

Injected sgRNAs	Injected embryos	Transferred embryos	Newborns (% of transferred)	Assayed rats	Mutated rats (%)						
					Single (%)			Double (%)			Triple (%)
					Tet1	*Tet2*	*Tet3*	*Tet1/Tet2*	*Tet1/Tet3*	*Tet2/Tet3*	*Tet1/Tet2/Tet3*
sg*Tet3*-1/ sg*Tet3*-2	130	100 (76.9)	42 (42)	18	–	–	18 (100)	–	–	–	–
sg*Tet1*-1/ sg*Tet2*-1	140	105 (75.0)	30 (28.6)	24	16 (67.7)	23 (95.8)	–	15 (62.5)	–	–	–
sg*Tet1*-2/ sg*Tet2*-2	120	80 (66.7)	22 (31.2)	20	16 (80.0)	19 (95.0)	–	15 (75.0)	–	–	–
sg*Tet1*-1/ sg*Tet2*-1/ sg*Tet3*-1	90	70 (77.8)	22 (31.4)	22	15 (68.2)	20 (90.9)	16 (72.7)	15 (68.2)	13 (59.1)	16 (72.7)	13 (59.1)

together with tracrRNA-derived sequences as previously described[1,5] (**Supplementary Table 1** and **Supplementary Sequences**). For the production of mRNA for microinjection, we cloned the Cas9 gene and sgRNA sequences into separate expression vectors with a prokaryotic T7 promoter (**Fig. 1a**).

To generate rats with mutations in *Tet3*, we first microinjected the two *Tet3*-targeting sgRNAs together with Cas9 mRNA into the cytoplasm of one-cell-stage rat embryos to test the *in vivo* function of the sgRNA:Cas9 system. We transferred 100 out of 130 injected embryos into pseudopregnant mothers, and 42 pups were obtained (**Table 1**). All but one pup died within 2 days. We randomly chose 18 dead pups and used the SURVEYOR assay to detect insertions or deletions (indels) within the *Tet3* coding region. All of these (18/18, 100%) had mutations within the *Tet3* coding region, with both designed sgRNA targeted sites within *Tet3* containing indels. No wild-type *Tet3* allele was obtained in the sequence reads of any examined dead pups, indicating they had biallelic mutations of *Tet3*, which is consistent with the previously reported neonatal lethality of a *Tet3* knockout[12] (**Supplementary Fig. 1**, **Supplementary Table 2** and **Supplementary Discussion**).

As the target specificity of Cas9 is determined by a small RNA, it should be relatively straightforward to simultaneously target multiple genomic locations at once, a potential advantage of the Cas9 technology. We tested the efficiency of the CRISPR-Cas systems in mutating two genes simultaneously by co-injecting the *Tet1*- and *Tet2*-targeting sgRNAs (only one sgRNA was chosen for each gene) together with the Cas9 mRNA into one-cell-stage rat embryos. We obtained 30 rats from embryos injected with one set of sgRNAs (set 1: sg*Tet1*-1 and sg*Tet2*-1) and 22 rats from embryos injected with another set of sgRNAs (set 2: sg*Tet1*-2 and sg*Tet2*-2) (**Table 1**). We selected 22 and 20 rats from each group, respectively, to examine their mutations. SURVEYOR assays showed that ~70% (set 1, 16/24; set 2, 16/20) of the rats contained *Tet1* mutations and 95% (set 1, 23/24; set 2, 19/20) contained *Tet2* mutations, of which >65% (set 1, 15/24; set 2, 15/20) contained both mutations (**Table 1**). Sanger sequencing confirmed the targeted sites within both *Tet1* and *Tet2* in the double-mutant rats contained indels. Analysis of sequence reads by rat revealed that most double-mutant rats had at least one allele mutated for each target gene, and >40% of the mutated rats of the sg*Tet1*-2 and sg*Tet2*-2 (set 2) group had biallelic mutations of both genes, showing the CRISPR-Cas systems can simultaneously produce two-gene, biallelic mutations in rats (**Supplementary Figs. 2** and **3**, **Supplementary Table 2** and **Supplementary Discussion**). All the mutated rats survived, consistent with a previous functional report of *Tet1* and *Tet2* knockout in mice[13].

Next, we analyzed the triple-gene mutation efficiency of the CRISPR-Cas systems through co-injection of sgRNAs for the three *Tet* genes (sg*Tet1*-1, sg*Tet2*-1 and sg*Tet3*-1) together with Cas9 mRNA into one-cell-stage rat embryos. A total of 22 newborn pups were obtained from the 70 transferred embryos. We examined all the rats and found that 13 rats (13/22, 59.1%) contained mutations of all three *Tet* genes (**Fig. 1b**). All these triple-mutated rats contained site-specific indels in all three gene coding regions, and ~60% of them had a biallelic or monoallelic mutation for each targeted locus (**Fig. 1c**, **Supplementary Fig. 4** and **Supplementary Table 2**). Some of the mutations led to truncated proteins lacking the catalytic domain, potentially disrupting gene function (**Supplementary Fig. 5**). Consequently, we observed reduced 5-hydroxymethyl cytosine levels in some triple-mutated rats (**Supplementary Fig. 6**). These results demonstrate that simultaneous triple-gene mutations can be achieved in rat by the CRISPR-Cas systems.

We observed that all six sgRNA:Cas9 constructs could efficiently introduce targeted indels, including point mutations, insertions and deletions ranging from ~1 bp to ~180 bp (**Supplementary Figs. 1–4**). Previous studies suggested that CRISPR-Cas systems may tolerate sequence mismatches distal from the protospacer adjacent motif (PAM) at the 5′ end of sgRNAs, which would probably induce off-target mutations. To detect off-target effects of the sgRNA:Cas9 constructs *in vivo*, we screened the genome of 13 triple-mutated rats for all off-target sites with more than 14-bp sequence identity to the six sgRNAs. Only four such sites were identified for all six sgRNAs (**Supplementary Fig. 7**). The SURVEYOR assay revealed that only one potential site was mutated by sg*Tet1*-1 in all the examined triple-mutated rats (**Supplementary Fig. 8**). The high birth rate and survival rate of the mutant rats (except for the neonatal lethal gene *Tet3*) indicated that the sgRNA:Cas9 construct had very low toxicity to rat embryos (**Table 1**).

Successful germline transmission of genetic mutations is essential for establishing genetically modified animal models. To test the transmission ability of the mutation, we produced fertilized embryos with sperm from *Tet1*/*Tet2* double-mutant rats and wild-type oocytes. Of seven embryos successfully assayed by Sanger sequencing, we identified five inherited mutations in the *Tet2* site only, and two inherited mutations in both the *Tet1* and *Tet2* sites, indicating that mutations in the founder rats were transmitted to the next generation (**Supplementary Fig. 9**). Because a high ratio of mutant rats had biallelic mutations or nonmosaic monoallelic mutations for all the targeted genes, we expect that the mutations introduced by sgRNA:Cas9 could be efficiently transmitted into further generations (**Supplementary Table 2**).

In summary, our results demonstrate that CRISPR-Cas systems efficiently and simultaneously generated single and multiple gene mutations *in vivo* in rats. During the

revision process of this work, an independent study reported the simultaneous generation of multiple mutations in mice[7]. Our work, together with the mice work, demonstrates that it should be feasible to produce gene-targeted models in rodents and probably other mammalian species using the CRISPR-Cas systems.

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AUTHOR CONTRIBUTIONS
Q.Z. designed the experiments, supervised laboratory work, analyzed and interpreted data; Q.Z. and W.L. wrote the paper; W.L., F.T. and T.L. performed the experiments.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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1. Jinek, M. *et al. Science* **337**, 816–821 (2012).
2. Cho, S.W., Kim, S., Kim, J.M. & Kim, J.S. *Nat. Biotechnol.* **31**, 230–232 (2013).
3. Cong, L. *et al. Science* **339**, 819–823 (2013).
4. Mali, P. *et al. Science* **339**, 823–826 (2013).
5. Hwang, W.Y. *et al. Nat. Biotechnol.* **31**, 227–229 (2013).
6. Jiang, W., Bikard, D., Cox, D., Zhang, F. & Marraffini, L.A. *Nat. Biotechnol.* **31**, 233–239 (2013).
7. Wang, H. *et al. Cell* **153**, 910–918 (2013).
8. Geurts, A.M. *et al. Science* **325**, 433 (2009).
9. Tong, C., Li, P., Wu, N.L., Yan, Y. & Ying, Q.L. *Nature* **467**, 211–213 (2010).
10. Tesson, L. *et al. Nat. Biotechnol.* **29**, 695–696 (2011).
11. Wu, H. & Zhang, Y. *Genes Dev.* **25**, 2436–2452 (2011).
12. Gu, T.P. *et al. Nature* **477**, 606–610 (2011).
13. Dawlaty, M.M. *et al. Dev. Cell* **24**, 310–323 (2013).

Targeted genome modification of crop plants using a CRISPR-Cas system

To the Editor:

Although genome editing technologies using zinc finger nucleases (ZFNs)[1] and transcription activator-like effector nucleases (TALENs)[2] can generate genome modifications, new technologies that are robust, affordable and easy to engineer are needed. Recent advances in the study of the prokaryotic adaptive immune system, involving type II clustered, regularly interspaced, short palindromic repeats (CRISPR), provide an alternative genome editing strategy[3]. Type II CRISPR systems are widespread in bacteria; they use a single endonuclease, a CRISPR-associated protein Cas9, to provide a defense against invading viral and plasmid DNAs[4]. Cas9 can form a complex with a synthetic single-guide RNA (sgRNA), consisting of a fusion of CRISPR RNA (crRNA) and *trans*-activating crRNA. The sgRNA guides Cas9 to recognize and cleave target DNA. Cas9 has a HNH nuclease domain and a RuvC-like domain; each cleaves one strand of a double-stranded DNA. It can be used as an RNA-guided endonuclease to perform sequence-specific genome editing in bacteria, human cells, zebrafish and mice[5–11]. Here we show that customizable sgRNAs can direct Cas9 to induce sequence-specific genome modifications in the two most widely cultivated food crops, rice (*Oryza sativa*) and common wheat (*Triticum aestivum*).

We first codon-optimized *Streptococcus pyogenes Cas9* (*SpCas9*), attached nuclear localization signals (NLSs) at both ends (**Fig. 1a** and **Supplementary Fig. 5**) and expressed sgRNA transcripts (**Fig. 1a**, **Supplementary Methods** and **Supplementary Fig. 4**). To disrupt endogenous genes in rice protoplasts, we designed two sgRNA, SP1 and SP2, which target different DNA strands of the rice phytoene desaturase gene *OsPDS* (**Fig. 1b** and **Supplementary Table 4**). Efficient, targeted mutagenesis (15%) was detected starting at 18 h of protoplast cultivation, and similar, if not higher, efficiencies were observed from 24 h through 72 h (**Supplementary Fig. 1a**,**b**). PCR/restriction enzyme (PCR/RE) assays were carried out to detect mutations in both target regions (**Supplementary Methods** and **Supplementary Table 5**). Digestion-resistant bands were detected in both sgRNA targets with efficiencies ranging from 14.5% to 20.0%, as estimated by band intensities (**Fig. 1c** and **Supplementary Methods**). Cloning and sequencing of these uncut bands revealed indels in the targeted *OsPDS* gene. The highest frequency of mutations was obtained with an sgRNA with 20 nucleotides (nts) of sequence complementary to the *OsPDS*-SP1 target site ($P = 0.039$) (**Supplementary Fig. 1c**,**d**).

We targeted another three rice genes (*OsBADH2*, Os02g23823 and *OsMPK2*) and one wheat gene (*TaMLO*) (**Supplementary Tables 1** and **4**) in protoplasts, with indel frequencies of 26.5–38.0% (**Supplementary Fig. 2** and **Supplementary Table 2**). The frequency of mutations induced by sgRNA:Cas9 in Os02g23823 was lower (26.0%) than that induced by TALENs (36.5%) (**Supplementary Fig. 1e**), whereas, in *OsBADH2*, it was considerably higher[12] (26.5% versus 8.0%) (**Supplementary Fig. 2** and **Supplementary Table 7**). Our results suggest that a customized sgRNA:Cas9 efficiently induces sequence-specific modifications in plants. Moreover, only a single customized sgRNA, encoded by a sequence of ~100 nt, is required to target a specific sequence, and Cas9 does not have to be reengineered for each new target site. The sgRNA:Cas9 system is therefore much more straightforward than ZFNs or TALENs.

To test whether sgRNA:Cas9 can induce gene knockouts in rice plants, we bombarded rice callus cells with Cas9 plasmid and sgRNA expression plasmids designed to cleave either *OsPDS* or *OsBADH2* (**Supplementary Methods**). Transformed, hygromycin-tolerant calli were grown into whole plants. Mutations in *OsPDS*-SP1 were identified in 9 of 96 independent transgenic plants (9.4%), and mutations in *OsBADH2* in 7 of 98 transgenic plants (7.1%) (**Fig. 1d** and **Supplementary Table 2**). In addition, biallelic mutations were identified in 3 of the 9 plants mutated in *OsPDS*-SP1. Two of them were homozygous for the same one-nucleotide insertion (**Fig. 1d**), and all three had the albino and dwarf phenotype (**Fig. 1e**), showing that the rice phytoene desaturase gene had been disrupted.

To examine homology-directed repair (HDR), we designed a single-stranded oligo with a KpnI + EcoRI site to be introduced into *OsPDS* (**Fig. 2a** and **Supplementary Table 6**). To detect such mutations, we used a PCR/RE assay that preferentially amplifies mutated DNA sequences. Protoplast genomic DNA was cleaved with PstI before PCR amplification to enrich for sgRNA:Cas9-induced mutations.