Dear Editor,

The clustered regularly interspaced short palindromic repeat (CRISPR) system has been widely adapted to genome editing to either introduce or correct genetic mutations (Wang et al., 2016). However, due to competition with the dominant non-homologous end-joining (NHEJ) pathway, precise genome modifications through Cas9-stimulated homologous recombination (HR) is inefficient. Through fusion of cytidine deaminases, APOBEC1 (apolipoprotein B editing complex 1) or AID (activation-induced deaminase), with Cas9 variants, several groups have developed the cytidine base editor (BE) systems (Komor et al., 2016; Li et al., 2018; Nishida et al., 2016). The BE system achieves programmable conversion of C-G base pairs to T-A without double-stranded DNA cleavage (Zhou et al., 2017). More recently, adenine base editors (ABEs), which efficiently convert A-T base pairs to G-C in genomic DNA, have been developed via fusion of an engineered tRNA adenosine deaminase (ecTadA from *Escherichia coli*) with nCas9 (Gaudelli et al., 2017). The ABE system has quickly been adapted to generate disease models and correction of genetic disease in mice (Ryu et al., 2017; Liu et al., 2018). However, whether the editing efficiency and the targeting scope of ABE could be improved is largely unexplored. In this study, we describe the efficient generation of base-edited mice and rat modeling human diseases through ABEs with highest efficiency up to 100%. We also demonstrate an increase of ABE activity through injection of chemically modified tracrRNA and crRNA in mouse zygotes, and the expansion of editing scope by fusion of an ecTadA mutant to SaCas9n-KKH and Cas9n-VQR variants in both cells and embryos. Our study suggests that the ABE system is a powerful and convenient tool to introduce precise base conversions in rodents.

To test the ABE efficiency in embryos, we injected ABE mRNA (Fig. 1A) together with sgRNA targeting the TATA box of the *Hbb-bs* gene, into C57BL6 strain mouse zygotes (Fig. S1A and Table S1). Overlapping A/G peaks in the target sites were identified in 14/27 of F0 mice as determined by the chromatograms of Sanger sequencing (Figs. 2F and S1B). Further analysis by deep sequencing revealed allelic frequencies from 6%–71% among the founders (Fig. S1C). In individual allele, the editing window was extended from position A$_2$–A$_9$ in mouse embryos, which is broader than the window spanning position A$_3$–A$_7$ observed in mammalian cell lines (Gaudelli et al., 2017) (Fig. S1B and S1C). These data demonstrate that ABE is efficient to generate point mutant mice and its mutation window expands in embryos.

Next, we tested the capability of ABE to precisely mutate A:T pairs for disrupting the stop codon of the gene encoding the fumarylacetoacetate hydrolase (Fah) (Fig. 1B), whose mutations cause hereditary tyrosinemia type I (HTI) in humans. We observed high A>G conversion efficiency (39/47) among F0 mice with allelic frequencies varying from 7%–99% as determined by deep sequencing (Figs. 1B, 1C, 2F and S2A). Increasing the sgRNA concentration from 50 ng/µL to 100 ng/µL results in 100% (13/13) point mutation rate in F0 mice (Figs. 2F and S2B). Since disruption of the stop codon usually affects mRNA stability and protein expression (Frischmeyer et al., 2002), the *Fah* mRNA and protein levels were dramatically impaired (Fig. S2C and S2D). Through immunohistochemistry analysis of the liver tissue from founder F0–F32, Fah protein expression was almost undetectable (Fig. 1D) suggesting this founder was a homozygote (Fig. 1B and 1C). To investigate germline transmission efficiency, founder mice were crossed with wild type or with other founders. We observed high germline transmission efficiency (Fig. S3A and S3B). In homozygous F1 mice, the expression of *Fah* mRNA and protein was lost (Fig. S3C–E). After withdrawal of the 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) treatment, the phenotypes of *Fah* mutant homozygotes were similar to previous HTI model, including loss of body weight and perturbation of serum biomarkers (Shao et al., 2018) (Fig. S3F and S3G). As mutations that generate premature stop codons are common drivers in various genetic diseases (Keeling et al., 2014), ABE has a promising potential for readthrough of premature stop codons in certain genetic diseases as demonstrated in the mouse DMD model (Ryu et al., 2017).

Previous study demonstrated that 2′-O-methyl-3′-phosphorothioate (MS) modification on each ends of RNA can increase its stability, thus enhancing the Cas9 genome
Increasing targeting scope of adenosine base editors in mouse and rat embryos

LETTER

© The Author(s) 2018 815

Protein & Cell
editing efficiency (Hendel et al., 2015). To explore whether MS modification on RNA also increases ABE activity, MS modified crRNAs and tracrRNAs were directly compared with in vitro transcribed sgRNAs. Indeed, in all three targets tested, the ABE editing efficiencies were higher when injected with MS modified crRNAs and tracrRNAs (Figs. 1E and S4). Moreover, the mutation efficiencies in individual mice were also increased in the group that received chemically modified RNAs (Fig. S4A–D). It suggests that increasing of sgRNA stability is an efficient strategy to increase ABE induced editing in embryos.

To investigate the activity of ABE in rats, we aimed to target the acid alpha-glucosidase (Gaa) gene to mutate aspartic acid (Asp) at codon 645 in exon 13, which is a mutation identified in glycogen storage disease type II (GSDII; Pompe disease) patients (Kroos et al., 2004) (Fig. 1F). GSDII is a fatal disorder characterized by progressive loss of skeletal and/or heart muscle function. Sanger sequencing data suggested that 85% (28/33) of rats tested were increased in the group that received chemically modified sgRNAs. After microinjection of SaKKH-ABE mRNA and sgRNA targeting Hbb-bs (position A3–A14 on EMX1 site 2) compared to ABE. Our preliminary data also suggested that the highly active position of SaKKH-ABE in the target was A9–A13 which was closer to the PAM sequence compared to ABE.

To test whether these two ABEs function in mouse embryos, we injected mRNA of ABE variants with individual sgRNAs. After microinjection of SaKKH-ABE mRNA and sgRNA, 16% (3 out of 19) of the mice carried a single mutation in the Otc locus with an editing rate ranging from 30%–54% in single founders as determined by deep sequencing (Fig. 2D and 2F). For VQR-ABE, we also directly injected VQR-ABE mRNA and sgRNA targeting Hbb-bs into mouse embryos. The editing efficiency was 20% (6 out of 30) at the Hbb-bs locus with the A>G conversion efficiency ranging from 2%–52% as determined by deep sequencing (Fig. 2E and 2F). These data suggest that expansion of the ABE editing scope through fusion with Cas9 variants is efficient in both cell lines and mouse embryos.

To evaluate the off-target effects of this ABE, we predicted the potential off-target sites of sgRNA targeting the Fah stop codon based on sequence similarity through the on-line target prediction program (http://crispr.mit.edu/). 20 predicted off-target sites of 3 highly edited founders for each sgRNA
**Increased sgRNA concentration to 100 ng/μL for microinjection.**

**SaKKH-ABE mRNA is used instead of ABE mRNA.**

**VQR-ABE mRNA is used instead of ABE mRNA.**

---

**Strain** | **Gene** | **Target site** | **mRNA/sgRNA (ng/μL)** | **Injected/Transplanted embryos** | **No. of mutants/offspring**
---|---|---|---|---|---
C57/BL6 | *Hbb-bs* | TATA box | 50/100 | 190/190 | 14/27 (52)
Fah | Stop codon | 50/50 | 105/104 | 39/47 (83)
**Otc** | Stop codon | 50/100 | 46/45 | 13/13 (100)
***Hbb-bs*** | Exon 4 | 50/50 | 47/45 | 3/19 (16)
Sprague-Dawley | Gaa | D645 | 50/100 | 104/102 | 28/33 (85)

* Increased sgRNA concentration to 100 ng/μL for microinjection.

**SaKKH-ABE mRNA is used instead of ABE mRNA.**

***VQR-ABE mRNA is used instead of ABE mRNA.***
Figure 2. Fusion of the adenosine deaminase with Cas9 variants. (A) A schematic view of SaKKH-ABE and VQR-ABE vectors. (B) Frequencies of A>G conversions induced by SaKKH-ABE in HEK293T cell line. (C) Frequencies of A>G conversions induced by VQR-ABE in HEK293T cell line. (D) Genotypes of the founders produced by the SaKKH-ABE system in the Otc locus. Target sequence is underlined. Arrow head indicates the targeted thymine. PAM sequence is labeled in blue. Base substitutions are labeled in red. Allele frequencies are listed to the right. (E) Genotypes of the founders produced by the VQR-ABE system in the Hbb-bs locus. Target sequence is underlined. Arrow head indicates the targeted thymine. PAM sequence is labeled in blue. Base substitutions are labeled in red. Allele frequencies are listed to the right. (F) Summary of the targeted sites and injection parameters used to generate the point mutant rodents in the study.

were selected, and PCR products were amplified and subjected to deep sequencing. We found that the frequency of off-target mutation was below 0.2% (due to the threshold of Hi-Tom method) which is similar to wild-type controls by analyzing a total of 50,000–100,000 reads/site via the web site (http://www.hi-tom.net/hi-tom/), demonstrating that ABE might have very few or no off-target effects at these tested sites (Fig. S6). It suggests that ABE is an accurate base editing tool for generation of mouse and rat point mutant strains.

In summary, we demonstrated that ABE and its variants efficiently generate site-specific A:T→G:C conversions in cell lines, mouse and rat embryos. We found that the editing window of ABE7.10 in rodent embryos is from position 2–9. To the best of our knowledge, this is the first report to demonstrate efficient generation of point mutations through base editors in rats. The SaKKH-ABE and VQR-ABE system will be important tools to diversify the range of ABE targets in the genome. As A→G conversion may correct 48% of the pathogenic human SNPs (Gaudelli et al., 2017), in combination with BEs, these base editing systems have promising potential not only for generation of disease models, but more importantly for therapy of hereditary diseases caused by point substitutions.

FOOTNOTES
This work was partially supported by grants from the National Natural Science Foundation of China (Nos. 81670470 and 81600149), a grant from the Shanghai Municipal Commission for Science and Technology (14140901600, 18411953500 and 15JC1400201) and a grant from National Key Research and Development Program (2016YFC0905100).

L. Yang, M. Liu and D. Li designed the experiments; L. Yang, X. Zhang, L. Wang, S. Yin, B. Zhu, L. Xie, Q. Duan, H. Han, L. Peng, Y. Wei, M. Liu, J. Zhang, H. Hu, W. Qiu and H. Geng performed the experiments; L. Yang, X. Zhang, L. Wang, S. Siwko and D. Li wrote the manuscript. D. Li supervised the research.

The authors have submitted a patent application (Application Number 2018101425473) based on the results reported in this study.

Lei Yang1, Xiaohui Zhang1, Liren Wang1, Shuming Yin1, Biyun Zhu1, Ling Xie1, Qihui Duan1, Huiqiong Hu1,2, Rui Zheng3, Yu Wei1, Liangyue Peng1,4, Honghui Han5, Jiqin Zhang1, Wenjuan Qiu3, Hongquan Geng3, Stefan Siwko6, Xuei Zhang1,2, Mingyao Liu1,6, Dali Li1,10

1 East China Normal University and Shanghai Fengxian District Central Hospital Joint Center for Translational Medicine, Shanghai Key Laboratory of Regulatory Biology, School of Life Sciences, East China Normal University, Shanghai 200241, China
2 Fengxian Hospital Affiliated to Southern Medical University, Shanghai 201499, China
3 Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China
4 School of Life Sciences, Hunan Normal University, Changsha 410081, China
5 Bioray Laboratories Inc., Shanghai 200241, China
6 Department of Molecular and Cellular Medicine, The Institute of Biosciences and Technology, Texas AM University Health Science Center, Houston, TX 77030, USA

OPEN ACCESS
This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

REFERENCES
Frischmeyer PA, van Hoof A, O’Donnell K, Guererro AL, Parker R, Dietz HC (2002) An mRNA surveillance mechanism that eliminates transcripts lacking termination codons. Science 295:2258–2261. https://doi.org/10.1126/science.1067338
Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI, Liu DR (2017) Programmable base editing of A→T to G→C in genomic DNA without DNA cleavage. Nature 551:464–471. https://doi.org/10.1038/nature24644
Hendel A, Bak RO, Clark JT, Kennedy AB, Ryan DE, Roy S, Steinfeld I, Lunstad BD, Kaiser RJ, Wilkens AB et al (2015) Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. Nat Biotechnol 33:985–989. https://doi.org/10.1038/nbt.3290
Keeling KM, Xue X, Gunn G, Bedwell DM (2014) Therapeutics based on stop codon readthrough. Annu Rev Genomics Hum Genet 15:371–394. https://doi.org/10.1146/annurev-genom-091212-153527
Kleinstiver BP, Prew MS, Tsai SQ, Nguyen NT, Topkar VV, Zheng Z, Joung JK (2015a) Broadening the targeting range of Staphylococcus aureus CRISPR-Cas9 by modifying PAM recognition. Nat Biotechnol 33:1293–1298. https://doi.org/10.1038/nbt.3404
Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, Zheng Z, Gonzales APW, Li Z, Peterson RT, Yeh J-RJ et al (2015b) Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature 523:481–485. https://doi.org/10.1038/nature14592
Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature 533:420–424. https://doi.org/10.1038/nature17946
Kroos MA, Kirchner J, Gellerich FN, Hermans MMP, Van der Ploeg AT, Reuser AJJ, Korinthenberg R (2004) A case of childhood Pompe disease demonstrating phenotypic variability of p. Asp645Asn. Neuromuscul Disord 14:371–374. https://doi.org/10.1016/j.nmd.2004.02.012
Li X, Wang Y, Liu Y, Yang B, Wang X, Wei J, Lu Z, Zhang Y, Wu J, Huang X et al (2018) Base editing with a Cpf1-cytidine deaminase fusion. Nat Biotechnol 36:324–327. https://doi.org/10.1038/nbt.4102
Liu Z, Lu Z, Yang G, Yang G, Li G, Feng S, Liu Y, Li J, Yu W, Zhang Y et al (2018) Efficient generation of mouse models of human diseases via ABE- and BE-mediated base editing. Nat Commun 9:2338. https://doi.org/10.1038/s41467-018-04768-7
Nishida K, Arazoe T, Yachie N, Banno S, Kakimoto M, Tabata M, Mochizuki M, Miyabe A, Araki M, Hara KY et al (2016) Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. Science. https://doi.org/10.1126/science.aaf8729
Ryu S-M, Koo T, Kim K, Lim K, Baek G, Kim S-T, Kim HS, Kim D, Lee H, Chung E et al (2017) Adenine base editing in mouse embryos and an adult mouse model of Duchenne muscular dystrophy. Nat Biotechnol. https://doi.org/10.1038/nbt.4148
Shao Y, Wang L, Guo N, Wang S, Yang L, Li Y, Wang M, Yin S, Han H, Zeng L et al (2018) Cas9-nickase-mediated genome editing corrects hereditary tyrosinemia in rats. J Biol Chem. https://doi.org/10.1074/jbc.RA117.000347
Wang H, La Russa M, Qi LS (2016) CRISPR/Cas9 in genome editing and beyond. Annu Rev Biochem. https://doi.org/10.1146/annurev-biochem-060815-014607
Zhou C, Zhang M, Wei Y, Sun Y, Sun Y, Pan H, Yao N, Zhong W, Li Y, Li W et al (2017) Highly efficient base editing in human tripronuclear zygotes. Protein Cell 8(10):772–775

Lei Yang, Xiaohui Zhang, Liren Wang, and Shuming Yin have contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13238-018-0568-x) contains supplementary material, which is available to authorized users.