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RAPID COMMUNICATION

Development of a high-fidelity Cas9-dependent adenine base editor (ABE) system for genome editing with high-fidelity Cas9 variants

One of the main reasons that hinders the application of base editors in the clinic is the trade-off between editing efficiency and editing fidelity. The off-target base editing includes Cas9-independent and Cas9-dependent manners.[1] The Cas9-independent off-target results from the intrinsic deamination activity of the deaminase domain of a base editor, which affects random RNA or DNA molecules. Meanwhile, the Cas9-dependent off-target results from the binding of Cas9 domain to DNA sequences that show strong similarity to the target sequence. Compared to Cas9-indepedent off-target that has been extensively studied, Cas9-dependent off-target base editing has received much less attention.[1] In principle, Cas9-dependent off-target base editing could be avoided by using a high-fidelity Cas9 in the base editor. Quite a few high-fidelity Cas9 variants have been reported. Among them, several have been tested as a component of a base editor in mammalian cells. However, all these studies focused on cytosine base editor (CBE) with one recent exception in which Sniper-Cas9 is used in an adenosine base editor (Sniper ABE7.10) to reduce off-target editing.[1,2] Hence, it is still unclear how high-fidelity Cas9 variants could improve the performance of ABE.

In this study, we aimed to find which high-fidelity Cas9 variants should be selected as a component of ABE to avoid Cas9-dependent off-target base editing. To this end, we compared the wild-type SpCas9 side by side with four most promising high-fidelity Cas9 variants in ABE. The four high-fidelity Cas9 variants were generated by rational design (eSpCas9(1.1), SpCas9-HF1, HypaCas9) or evolved from yeast-based assay (evoCas9).[1] Specifically, we optimized the codon of ABE7.10 for expression in human embryonic kidney (HEK) 293T and still named it ABE7.10. The wild-type SpCas9 in the ABE7.10 was replaced by each of the four high-fidelity SpCas9 variants (eSpCas9 (1.1), SpCas9-HF1, HypaCas9 and evoCas9) to form high-fidelity ABEs, which were named as e-ABE7.10, HF-ABE7.10, Hypa-ABE7.10 and evo-ABE7.10, respectively (Fig. 1A). Notably, each SpCas9 variant harbors an extra D10A mutation to improve the editing efficiency according to a previous study.[3]

We first selected a well-studied site *HEK4* to evaluate the editing efficiency and fidelity of the five ABEs.[3] We co-transfected HEK293T cells with plasmids that encode either ABE7.10 or one high-fidelity ABE, an on-target sgRNA for the site *HEK4*. We measured the editing efficiency and editing fidelity via targeted sequencing of the PCR amplicons five days after the co-transfection without enrichment for transfected cells. We found that the on-target base editing activity of e-ABE7.10 was similar to that of ABE7.10 (10.61% ± 2.42% *vs.* 9.40% ± 1.75%), while the other three high-fidelity ABEs showed a substantial reduction of the activity (4.25% ± 0.62%, 4.25% ± 0.55% and 1.71% ± 0.21%, respectively) (Fig. 1B). We further tested the off-target base editing activity in the three known off-target sites that all show strong sequence similarity to the *HEK4* site.[3] Importantly, for all high-fidelity ABEs the off-target editing rate was at the background level at the three off-target sites while for ABE7.10 the off-target editing rate was ~10–100-fold higher (Fig. 1B). For each high-fidelity ABE we computed at each site the relative specificity ratio as: (high-fidelity ABE on-target frequency/off-target frequency)/(ABE7.10 on-target frequency/off-target

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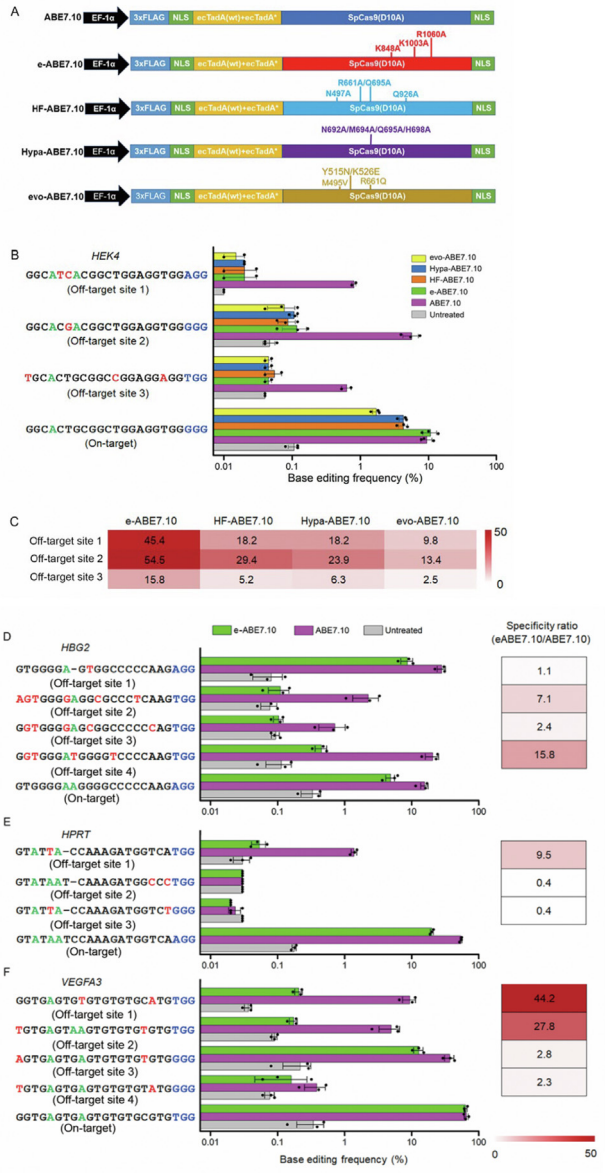

Figure 1 Construction of the high-fidelity ABEs and their performances in human endogenous genomic sites. **(A)** Schematic representation of ABE.10, e-ABE7.10, HF-ABE7.10, evo-ABE7.10 and Hypa-ABE7.10. **(B)** Base editing efficiencies of ABEs measured by targeted deep sequencing at *HEK4* on- and off-target sites in HEK293T cell. Mismatched bases, edited bases and PAM sequences are shown in red, green and blue, respectively. **(C)** Relative specificity ratios are shown by the heatmap, calculated by the formula: (high-fidelity ABE7.10 on-target frequency/off-target frequency)/(ABE7.10 on-target frequency/off-target frequency). Means ± SD are from two or three independent experiments. Base editing efficiencies of eABE7.10 and ABE7.10 measured by targeted deep sequencing at *HBG2* **(D)**, *HPRT* **(E)** and *VEGFA3* **(F)** on- and off-target sites in HEK293T cells. Mismatched bases, edited bases and PAM sequences are shown in red, green and blue, respectively. Relative specificity ratios are shown next to the bar-plots. Means ± SD are from three independent experiments.

frequency). As in Figure 1C, the obtained relative specificity ratios are all larger than one, ranging from 2.5 to 54.5, and e-ABE7.10 owns the highest relative specificity ratio at all tested sites (15.8—54.5).

In addition to the *HEK4* site, we further tested *HBG2*, *HPRT* and *VEGFA3*, three additional sites studied in previous studies.[4,5] We focused on e-ABE7.10 since it performed much better than the other high-fidelity ABEs according to above results. The relative specificity ratios of e-ABE7.10 are 1.1—15.8 for *HBG2*, 0.4—9.5 for *HPRT*, and 2.3—44.2 for *VEGFA3*, respectively (Fig. 1D—F). In the case of *HPRT*, both e-ABE7.10 and ABE7.10 had negligible base editing at the *HPRT* off-target site 2 and off-target site 3 (∼0.03% and ∼0.02%, respectively). The low relative specificity ratios (=0.4) are due to the fact that ABE7.10 outperformed e-ABE7.10 at the *HPRT* on-target site (54.32 ± 1.84% versus 20.00 ± 0.98%) (Fig. 1E). In the case of *HBG2*, the editing activity of ABE7.10 at the off-target site 1 and off-target site 4 was higher than that at the on-target site (27.90 ± 3.92%, 20.54 ± 4.57% versus 15.55 ± 1.97% respectively). Meanwhile, e-ABE7.10 showed nearly background editing activity at the off-target sites except off-target site 1 (8.61 ± 1.76%), although there was a reduction of the on-target activity (4.83 ± 0.72%) relative to ABE7.10 (Fig. 1D). In the case of *VEGFA3*, the on-target activity of e-ABE7.10 was equivalent to that of ABE7.10 (65.20 ± 4.51% versus 63.66 ± 3.18%), while the off-target activities were generally much weaker (Fig. 1F). These results together suggest that e-ABE7.10 has a rather high specificity despite in a site dependent manner.

In addition to conventional Cas9-dependent off-target base editing, bystander editing was reported in all base editors when additional cytosines or adenines beyond the desired target base are present in the editing window.[1] To reduce bystander editing many CBE variants with narrowed editing windows have been developed, but such ABE variants are rare. Because the A-to-I deamination catalyzed by TadA occurs when the target DNA is single-stranded,[3] we speculated that a truncated sgRNA should be able to narrow the editing window from the 5′ end because the truncated sgRNA could affect the formation of single strand. To test the speculation, we co-transfected the vector expressing ABE7.10 with a vector expressing a normal sgRNA or a truncated sgRNA (named GX19, GX18 and GX17, respectively) into HEK-293T cells. We examined the base editing efficiency at the site *HPRT* (Supplementary Data). We found the base editing activities were maintained at the positions 3, 5 and 6 using normal sgRNA GX20 (Fig. S1B). Meanwhile, the truncated sgRNA (GX19, GX18 and GX17) reduced the editing efficiency at different levels at the target position 5 while abolishing completely the editing activity at the two surrounding positions 3 and 6 (Fig. S1C—E). Hence, truncated gRNAs could be used to narrow the editing window despite reduced editing efficiency at the target base. Further study is required to test the performance of the combination of e-ABE7.10 with truncated sgRNAs.

In this work, to avoid Cas9-dependent off-target base editing we replaced ABE's original Cas9 with four high-fidelity variants. At the *HEK4* site, e-ABE7.10 outperformed

the other high-fidelity Cas9 variants and showed up to 54.5-fold improvement in specificity ratio compared with ABE7.10. We further compared e-ABE7.10 and ABE7.10 by testing their performance at three additional genomic sites. We confirmed the outperformance of e-ABE7.10 relatives to ABE7.10. Interestingly, it seems there is no clear boundary between Cas9-independent and Cas9-dependent off-target DNA editing. There are reports that catalytically impaired deaminases, which are developed for avoiding Cas9-independent bystander editing, can improve editing specificity at Cas9-dependent off-target sites.[1] In line with this, our research in high-fidelity ABEs and truncated sgRNAs also suggests a way to simultaneously mitigate Cas9-dependent and Cas9-independent off-target DNA editing.

Conflict of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.07.022.

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