Efficient base editing with expanded targeting scope using an engineered Spy-mac Cas9 variant

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Dear Editor,

The clustered regularly interspaced short palindromic repeat (CRISPR) system, including newly developed base editing technology, has exhibited powerful genome manipulation capability. Base editors that can achieve targeted C-to-T (CBE) or A-to-G (ABE) conversions without generating DNA double-strand breaks (DSBs) or requiring a donor template represent significant advances in both disease modeling and gene therapy. However, the conventional Streptococcus pyogenes Cas9 (Spy Cas9) requires a protospacer adjacent motif (PAM) of NGG, which limits the applicability of base editors that are highly dependent on the PAMs suitably adjacent to target bases. In addition, although some natural and engineered Cas9 variants with different PAM specificities have been utilized in base editors, such as representative Cpf1 (TTTV (V = A/G/C))⁴ and SpCas9-NG (NG)⁵, their targeting scope is still limited to genomic regions rich in G or T bases. Recently, Spy-mac Cas9 was generated by rationally exchanging the PAM-interacting (PI) region of the conventional Spy Cas9 with that of the newly discovered Streptococcus macacae Cas9 (Smac Cas9), showing 5′-NAA-3′ PAM specificity and possessing efficient gene editing in human cells. In this study, we demonstrated the effectiveness of the Spy-mac Cas9-assisted cytidine and adenine base editors Spy-mac BE4max and Spy-mac ABEmax, and found that 5′-TAAA-3′ is the only high-efficiency PAM for Spy-mac Cas9 observed in this study.

To obtain the best efficiency of base editing, the Spy-mac Cas9 system was combined with the current optimal version of the base editors BE4max and ABEmax to generate Spy-mac BE4max and Spy-mac ABEmax, respectively (Fig. 1a). To fully evaluate the PAM specificity and editing efficiency, we first tested the Spy-mac BE4max system in rabbit embryos at 16 target sites, including all NAAN PAMs, as a proof of concept (Supplementary Table S1). Base editing was conducted in rabbit pronuclear-stage embryos by microinjecting of Spy-mac BE4max-encoding mRNA and single-guide RNA (sgRNA). Base editing frequencies were evaluated by Sanger sequencing and T-A cloning. Notably, the average C-to-T editing frequency at the Tyr-1 site with TAAA PAM was high at 86.00 ± 8.72%, while much lower efficiencies ranging from 13.33 ± 6.67% to 26.67 ± 11.74% were observed at the other five sites with AAAT, GAAG, CAAG, AAAC, and CAAC PAMs (Fig. 1b and Supplementary Fig. S1). However, no obvious base editing events were observed at most tested sites (10/16), consistent with variations in the targeting efficiencies of Spy-mac Cas9 at different targeting sites in human cells (Fig. 1b and Supplementary Fig. S1).

Encouraged by the results of the pilot study, we examined whether the Spy-mac Cas9 variant may primarily target TAAA PAM in rabbit embryos. Therefore, another three sites (Tyr-5, Mstn-3, and Dmd-2) with TAAA PAMs were designed to verify our hypothesis (Supplementary Table S1). Remarkably, all sites showed efficient C-to-T conversions, with average editing frequencies ranging from 28.00 ± 13.93% at Dmd-2 to 100.00 ± 0.00% at Tyr-14 (Fig. 1c and Supplementary Fig. S2). Moreover, targeted C-G to T-A conversions were successfully achieved to induce stop codons at all four sites, as expected (Fig. 1d, g). In particular, the homozygous p.W178Stop-
Fig. 1 (See legend on next page.)
or p.W11Stop-targeted mutations were determined at Tyr-1 or Tyr-14 (Fig. 1d, e). In addition, only a few unwanted by-products, including indels and non-C-to-T conversions, were observed in mutant embryos at Tyr-1 (1/10, #3) and Dmd-2 (1/10, #1) using Spy-mac BE4max (Supplementary Figs. S1, 2), which was consistent with the high product purity of the BE4max architecture in human cells. Despite the high efficiency of Spy-mac BE4max-mediated C-to-T conversion, additional base editing tool such as recently reported ABE system was also tested here with TAAAPAMs at the four sites. Notably, site-specific A-T to G-C conversions were observed at all tested sites, with average editing frequencies ranging from 10.00 ± 7.75% to 68.00 ± 9.70% at Tyr-14 (Fig. 1h and Supplementary Figs. S3, 4). Taken together, these results demonstrated that both Spy-mac BE4max and Spy-mac ABEmax could induce efficient C-to-T/A-to-G base editing in rabbit embryos with TAAAPAMs.

Oculocutaneous albinism type 1 (OCA1) in humans results from mutations in the Tyr gene, which encodes tyrosinase, the key enzyme in pigment biosynthesis in mammals. Patients with OCA1 have been reported to have impaired tyrosinase activity and a consequential absence of pigment. Here, two target G-to-A conversions were designed in exon 1 of the rabbit Tyr gene to yield a premature stop codon (p.W178stop) (Fig. 1i). After the microinjection of Spy-mac BE4max-encoding mRNA and sgRNA, the rabbit embryos were transplanted into the surrogate mother, and subsequently five pups were obtained (Supplementary Table S2). Strikingly, T-A cloning showed that four of these pups (80%) were homozygous with nonsense mutations (p.W178stop) at the target site (Fig. 1j, k and Supplementary Fig. S5). In addition, no indels or non-C-to-T mutations were detected in the Founder (F0) rabbits, which demonstrated the high product purity of Spy-mac BE4max (Supplementary Fig. S5). As expected, the mutants exhibited a complete albino phenotype, consistent with their mutant genotype (Fig. 1I). Furthermore, histological haematoxylin-eosin (H&E) staining revealed the absence of melanin in the hair follicles of the T1 mutant, but not in their wild-type (WT) littermates (Fig. 1m). In addition, no obvious off-target mutations were detected at potential off-target sites in mutant rabbits by using Sanger sequencing and T7E1 cleavage assays (Supplementary Fig. S6). Overall, these results demonstrated that Spy-mac BE4max successfully mediated the Tyr-1 p.W178stop mutation in F0 rabbit with high efficiency, which precisely recapitulates the pathological features of human OCA1.

In summary, we first demonstrated that the Spy-mac Cas9-assisted cytidine and adenine base editors Spy-mac BE4max and Spy-mac ABEmax can induce efficient C-to-T/A-to-G conversions in vivo. In addition, the observed PAM scope of Spy-mac Cas9 is not 5′-NAA-3′ as previously reported in human cells but in reality only 5′-TAA-3′ manifests as high-efficiency PAM in this study. Moreover, Spy-mac BE4max can induce targeted base editing in F0 rabbits with high efficiency to precisely mimic human pathology condition. Thus, Spy-mac Cas9-assisted base editors with expanded targeting scopes are promising tools for establishing animal models and developing precise gene therapy in the future.

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Zq.L., L.L., and Zj.L. conceived and designed the experiments. Zq.L. and H.S. performed the experiments. Zq.L., H.S., and S.C. analysed the data. M.C. and Y.S. contributed reagents/materials/analysis tools. Zq.L. and Zj.L. wrote the paper. All authors have read and approved the final manuscript.

Conflict of interest
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