Molecules and Cells

Rapid Report

Targeted Base Editing via RNA-Guided Cytidine Deaminases in *Xenopus laevis* Embryos

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Genome editing using programmable nucleases such as CRISPR/Cas9 or Cpf1 has emerged as powerful tools for gene knock-out or knock-in in various organisms. While most genetic diseases are caused by point mutations, these genome-editing approaches are inefficient in inducing single-nucleotide substitutions. Recently, Cas9-linked cytidine deaminases, named base editors (BEs), have been shown to convert cytidine to uridine efficiently, leading to targeted single-base pair substitutions in human cells and organisms. Here, we first report on the generation of *Xenopus laevis* mutants with targeted single-base pair substitutions using this RNA-guided programmable deaminase. Injection of base editor 3 (BE3) ribonucleoprotein targeting the *tyrosinase* (*tyr*) gene in early embryos can induce site-specific base conversions with the rates of up to 20.5%, resulting in oculocutaneous albinism phenotypes without off-target mutations. We further test this base-editing system by targeting the *tp53* gene with the result that the expected single-base pair substitutions are observed at the target site. Collectively, these data establish that the programmable deaminases are efficient tools for creating targeted point mutations for human disease modeling in *Xenopus*.

Keywords: base editing, CRISPR/Cas9, genome engineering, *Xenopus laevis*

Xenopus laevis (*X. laevis*) has long been a favored vertebrate model system for studying embryonic development, the cell cycle, nuclear reprogramming, and the mechanisms underlying human diseases (Harland and Grainger, 2011). Although its allotetraploid genome and long generation time have challenged genetic studies on this organism, the loss-of-function analysis using dominant negative mutants or antisense oligonucleotides such as morpholino oligomers has rendered this classical model ideal for elucidating the functions of genes of interest. These methods of which effects are transient, however, cannot be applied to post-embryogenesis events, and hence the development of techniques for a more stable gene knockout is extremely desirable. Recently, programmable nucleases such as zinc finger nucleases (ZFNs), transcription activator-like (TAL) effector nucleases (TALEN), CRISPR/Cas9 or Cpf1 systems have allowed researchers to readily achieve targeted genome engineering in a variety of organisms including *Xenopus* (Aslan et al., 2017; Blitz et al., 2013; Kim and Kim, 2014; Kim et al., 2016; Lei et al., 2012; Nakayama et al., 2013; Young et al., 2011). In the CRISPR/Cas9 system, the Cas9 protein/gRNA complex induces site-specific DNA double-strand breaks (DSBs) in a genome, and most of DSBs are repaired by error-prone non-homologous end-joining (NHEJ) rather than error-free homology-directed repair (HDR) (Kim and Kim, 2014). As a result, small insertions and deletions (indels) occur at

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the target sites with higher frequencies than gene corrections such as single-nucleotide substitution. Thus, it is technically challenging to induce a point mutation in a targeted genomic DNA, even though CRISPR/Cas9-mediated knockout organisms have been generated efficiently.

In recent studies, catalytically inactive Cas9 (dCas9) or Cas9 nickase (nCas9) which is fused with cytidine deaminases has been shown to achieve targeted C-to-T conversion in living cells without generating DSBs (Kim et al., 2017b; Komor et al., 2016; Ma et al., 2016; Nishida et al., 2016; Rees et al., 2017). These base editors induce targeted cytidine (C) to uridine (U) conversion, followed by C-to-T substitution during DNA replication. Recently, several groups have reported base editing system to induce single-base conversion in mice, *zebrafish* and plants for generation of organisms with DNA substitution (Kim et al., 2017a; Liang et al., 2017; Shimatani et al., 2017; Zhang et al., 2017; Zong et al., 2017). In this work, we first describe generation of *X. laevis* mutants with targeted DNA substitutions using RNA-guided programmable deaminases. Base Editor 3 (BE3, rAPOBEC1-nCas9-UGI (uracil glycosylase inhibitor)) could induce C-to-T conversion efficiently in two genes in *X. laevis*, including *tyrosinase (tyr)* and *tp53*, without off-target mutations. Our study highlights the advantages of base editing in *X. laevis* and sheds light on the functional study using *X. laevis* for patient-derived point mutation in human diseases.

To determine whether a programmable cytidine deaminase could catalyze site-specific base conversion in the genome of *X. laevis in vivo*, we used BE3 capable of introducing point mutations into its genome and selected the *tyr* gene as a model for targeting. *Tyr* encodes the enzyme tyrosinase that is required for the biosynthesis of melanin during the tailbud and tadpole stages of early *Xenopus* development. As observed in previous works using ZFN, TALEN or CRISPR/Cas9 system, the targeted disruption of *tyr* gene loci results in albinism at the expense of otherwise pigmented melanocytes and retinal pigmented epithelium (RPE). We designed gRNAs to target simultaneously two homeologs for *tyr* gene, *tyra* and *tyrb*, which would induce indels or premature stop codon at the target sites in complex with

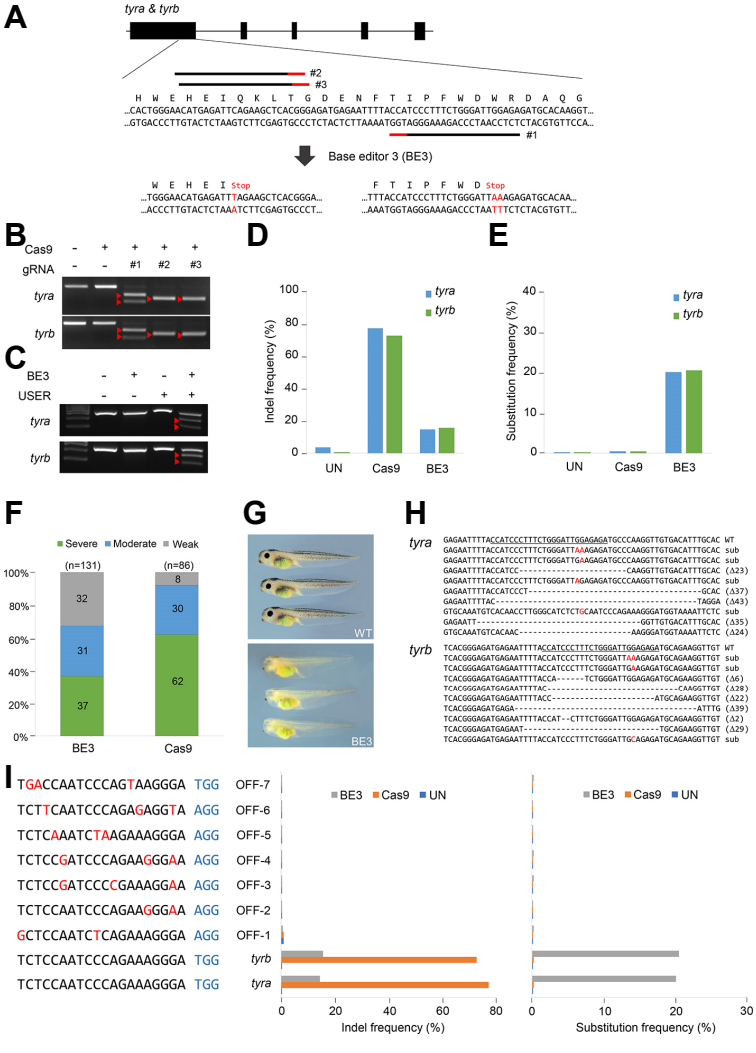

Fig. 1. Generation of tyrosinase-deficient *X. laevis* by a RNA-guided programmable deaminase, BE3. (A) The target sequences at the *tyra* and *tyrb* loci. The PAM and target sequences for gRNAs are shown in red and black bar, respectively. The nucleotides substituted by programmable deaminase are shown in red. (B) *In vitro* assay for Cas9 protein-mediated cleavage of the target sequences at *tyra* and *tyrb*. (C) *In vitro* deamination assay for BE3 RNP with gRNA #1 targeting both *tyra* and *tyrb*. Arrowheads indicate cleavage products for each target site. (D, E) Targeted deep sequencing analysis of frequencies of indel mutation (D) and C-to-T substitution (E) that were induced by Cas9 or BE3, respectively, in *X. laevis*. UN, uninjected control embryos. (F) Percentages of phenotypes of the Cas9 or BE3 RNP-injected embryos. With the phenotypically abnormal (cyclopic, strongly kinked axes) embryos being discarded, the phenotypes were categorized into 3 groups (severe, moderate, weak) according to the degree of loss of pigmentation in melanocytes and RPE. (G) Compared to wild-type (WT) control embryos, the BE3 RNP-injected embryos exhibited significant reduction of pigmentation in the eyes and melanocytes along the antero-posterior body axis. (H) Alignment of the most frequent mutant sequences from the BE3-injected pooled *X. laevis* embryos. Target sequences are underlined. The nucleotides substituted by programmable deaminase are shown in red. Dashes (-) denote gaps. The nature of the mutations is indicated in the right column. WT, wild-type; sub, substitutions; Δ, deletion. The number of deleted base pairs is shown after the symbol, Δ. (I) On-target and off-target sequences of *tyr* gene are shown on the left column. The PAM site and mismatched bases are shown in blue and red, respectively. The UN, uninjected control embryo.

Cas9 or BE3, respectively (Fig. 1A) (Sakane et al., 2014). First, we checked the *in vitro* target DNA cleavage efficiencies of gRNAs designated #1, 2 or 3. Of note, each gRNA exhibited a marked ability to cleave target DNA with Cas9 protein *in vitro* (Fig. 1B). To investigate whether a gRNA targeting *tyr* gene could induce deamination of cytidine at the target sites, we also carried out BE3-mediated deamination assay using USER (uracil-specific excision reagent), a mixture of *Escherichia coli* uracil DNA glycosylase (UDG) and DNA glycosylase-lyase endonuclease VIII. The PCR amplicons containing the target sites were treated with BE3 to induce C-to-U conversions and a nick in the Watson and Crick strands and then with USER to remove uracil at the target locus for generating DSBs. DNA cleavage was observed only in the presence of both BE3 and USER, indicating that the gRNA-dependent conversion of C-to-U occurred efficiently *in vitro* (Fig. 1C).

Next, we injected Cas9 or BE3 ribonucleoproteins (RNPs), which consisted of the recombinant Cas9 or BE3 proteins and *in vitro*-transcribed gRNA targeting both *tyra* and *tyrb*, into one-cell stage *X. laevis* embryos, which were subsequently cultured until they reached early tadpole stages and scored for defects in pigmentation. Injection of Cas9 RNPs indeed generated indel mutations with high rates (77.4% at *tyra* and 72.9% at *tyrb*) without inducing C-to-T substitutions as revealed by targeted deep sequencing (Fig. 1D). However, both C-to-T substitutions and indels could be observed at *tyra* and *tyrb* loci in BE3 RNPs-injected embryos (20.1% at *tyra* and 20.5% at *tyrb* for C-to-T conversion; 14.6% at *tyra* and 15.6% at *tyrb* for indel mutations) (Figs. 1D and 1E). As the survival rate of both Cas9- and BE3-injected embryos was approximately 90%, we could not find BE3-specific lethality.

The Cas9 or BE3 RNPs-injected embryos were categorized into three groups according to the degrees of loss of pigmentation in melanocytes and retinal pigmented epithelium (RPE); severe, moderate, and weak (Supplementary Fig. S1). Notably, 62% (53/86) of the Cas9-injected embryos and 37% (48/131) of the BE3-injected embryos displayed severe phenotypes (Figs. 1F and 1G). In support of this, both indels and C-to-T conversions were found in the pooled genomic DNA of the severe mutants as analyzed by targeted deep sequencing (Fig. 1H; Supplementary Fig. S2). We, next, analyzed individual embryos and found that each embryo is mosaicism (Supplementary Fig. S3). These results suggest that BE3 RNP could induce base editing at the target locus with high frequencies in the *X. laevis* embryos.

The specificity of a programmable deaminase is essential for its reliability as a genome engineering tool for *X. laevis*. Previously, BE3 has been highly specific as Cas9 in human cells and mice. To assess the off-target effects of BE3 in *X. laevis*, we first identified, using Cas-OFFinder, potential off-target sites with up to 3-nucleotide mismatches in its genome (X. laevis JGI v7.1) (Bae et al., 2014). We chose 7 potential off-target sites harboring a PAM sequence (5′-NGG-

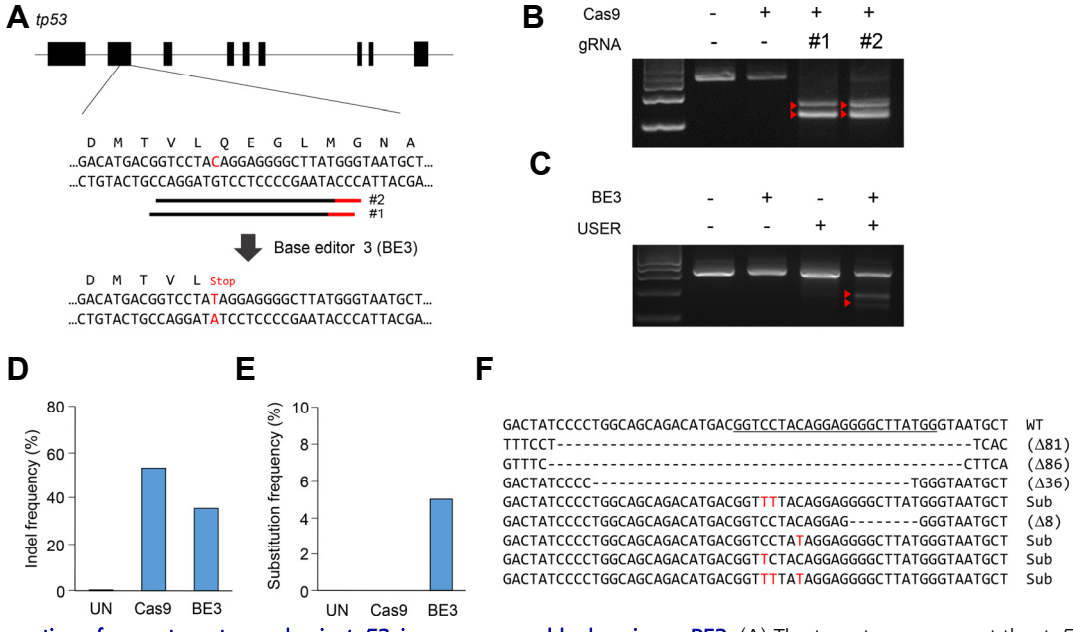

Fig. 2. Generation of premature stop codon in *tp53* via a programmable deaminase, BE3. (A) The target sequences at the *tp53* locus. The PAM and target sequences for gRNAs #1 and #2 are shown in red and black bar, respectively. The nucleotides substituted by programmable deaminase are shown in red. (B) *In vitro* cleavage and (C) *in vitro* deamination assays for the target sequences in PCR amplicons from *X. laevis* genomic DNA. Arrowheads indicate cleavage products for each target site. (D, E) Targeted deep sequencing analysis of frequencies of indel mutations (D) and C-to-T substitution (E) in Cas9 or BE3 RNP-injected *X. laevis* embryos. UN, uninjected control embryo. (F) Alignment of mutant sequences from the BE3-injected pooled *X. laevis* embryos. The target sequences are underlined. The nucleotides substituted by programmable deaminase are shown in red. The nature of the mutations is shown in the right column. WT, wild-type; Δ, deletion; sub, substitutions. The number of deleted base pairs is indicated after the symbol, Δ.

3′), which is critical for the binding and cleavage of Cas9 or BE3 enzymes. To check off-target effects at these loci, we generated PCR amplicons containing the off-target sites from the same genomic DNA used to evaluate mutations in *tyr* gene and measured the frequency of BE3-induced substitution or Cas9-induced indel mutation by targeted deep sequencing. BE3-induced point mutations or Cas9-indued indels were not detected at all potential off-target sites (Fig. 1I). These results support that BE3 is a highly specific programmable cytidine deaminase, being a reliable strategy for targeted base editing in *X. laevis*.

To further test whether the programmable deaminase is a robust tool for base editing in *X. laevis*, we prepared gRNAs in complex with BE3, which would generate premature stop codon at the target site in *tp53* (Fig. 2A). *In vitro* cleavage assays showed that the designed gRNAs in complex with Cas9 could digest efficiently the target sequence in each gene (Fig. 2B). In addition, they could induce deamination of cytidine at the target sites *in vitro* with BE3 enzyme (Fig. 2C). We also injected Cas9 or BE3 RNPs into one-cell stage embryos and subsequently, they were cultured to the mid-gastrula stages at which mutations in *tp53* were assessed. Notably, with no base conversion, indel mutations occurred at the target site in each gene with high frequencies (53.3%) in the Cas9 RNP-injected embryos (Fig. 2D). Injection of BE3 RNPs produced not only indel mutations (35.9%) but C-to-T substitutions (5.0%), albeit with low rates, at the target sites (Figs. 2D and 2E). This low frequency of base conversion may be due to the limited design of target sites. Other types of programmable deaminases may be good alternatives to overcome this limitation and extend the range of base conversions.

In summary, we could generate *tp53* or tyr knockout *X. laevis* by introducing early stop codon using programmable deaminases. We also confirmed that programmable deaminase did not induce unwanted mutations at potential off-target sites. Compared with the previous study in which *tyr* knockout was performed using TALEN or Cas9, the ratio of the loss-of-pigmentation phenotype in embryos injected with BE3 was similar to that of embryos injected with TALEN or Cas9 (Lei et al., 2012; Sakane et al., 2014). Programmable deaminase which induce base editing without DNA double strand breaks is safer genetic engineering tool compared with TALEN or Cas9. Taken together, the programmable deaminase can be used to generate various *X. laevis* models by introducing early stop codon or single amino acid substitutions.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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