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RESEARCH HIGHLIGHT

In vivo ways to unveil off-targets

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Base editors enable single nucleotide conversion without causing unwanted indels. Two recent studies reveal that cytosine base editors (CBEs), but not adenine base editors (ABEs), induce unexpected genome-wide off-target mutations, calling for high-fidelity CBEs.

The genome editing tools, such as the CRISPR-Cas system, provide unprecedented opportunities for correcting pathogenic mutations in human diseases or obtaining desired traits in cattle and crops. However, potential off-target effects result in irreparable consequences and thwart efforts to move CRISPR therapeutics from bench to bedside. It is thus imperative to develop unbiased and accurate approaches to evaluate the fidelity of genome editing, especially as the CRISPR toolbox is rapidly expanding.

Programmable nucleases induce insertions or deletions (indels) through non-homologous end joining (NHEJ)-mediated repair of DNA double-stranded breaks (DSBs). Derived from the CRISPR-Cas9 system, base editors are widely recognized as more precise tools because they enable single nucleotide conversion within a small editing window without causing DNA cleavage. These tools include cytosine base editors (CBEs, C-to-T conversions) and adenine base editors (ABEs, A-to-G conversions), in which cytosine and adenine deaminases are fused to nickase Cas9, respectively.[1–4]

Multiple in vitro methods have been developed to evaluate CRISPR-Cas9 mediated off-targets, however, they rely on labeling of DSBs caused by Cas nucleases and thus are not applicable for detecting mutations mediated by base editors. Whole genome sequencing (WGS) offers a direct and unbiased option to assess mutations, however, the small percentages of off-targets would be readily averaged out from the bulk cells subjected to genome editing, making it unrealistic to distinguish single nucleotide variants (SNVs) from those of naturally occurring and sequencing errors. It is therefore critical to carefully calibrate genetic background before assessing off-targets using WGS.

Two groups of researchers have recently achieved genome-wide and unbiased off-target assessment of several CRISPR editors through analyzing samples from clonally derived materials by WGS. Using different model systems (mouse embryo and rice), they have come to the same conclusion that cytosine-to-thymine editor, but not adenine-to-guanine editor, induces abundant off-target mutations in vivo.[5,6] Zuo et al. devised a novel method called GOTI (Genome-wide Off-target analysis by Two-cell embryo Injection) to assess off-targets in vivo. They edited one blastomere of two-cell embryos of mice by injecting CRISPR-Cas9, BE3 (a CBE), or ABE7.10 (an ABE), together with Cre mRNA whose expression results in tdTomato fluorescence. At embryonic day 14.5, cells were harvested and sorted into edited (tdTomato+) and non-edited (tdTomato−) populations by FACS. WGS was subsequently performed to determine SNVs in two separate populations. This strategy successfully eliminated colony-related variations because the first two blastomeres derived from the same zygote have the identical genetic background. They demonstrated that CRISPR-Cas and ABE7.10 generated rare SNVs while BE3 induced overt SNVs in the embryo. A similar study in rice echoed Zuo et al.'s findings. Jin et al. transformed BE3, HF1-BE3 (a high-fidelity BE3), or ABE7.10 into clonally derived calli, followed by WGS analysis in regenerated T0 plants. This study also found that CBEs (BE3 and HF1-BE3) induced significantly higher SNVs than ABE7.10.

Intriguingly, SNVs induced by CBEs in mice and rice share the common traits: (1) the majority of the SNVs are C-to-T conversions; (2) the distribution of SNVs is sgRNA independent; and (3) SNVs are significantly enriched in transcribed regions. Because APOBEC1 prefers ssDNA as its deamination substrate, it is reasonable to speculate that most off-target SNVs are derived from APOBEC1-catalyzed C-to-T conversion during DNA replication and transcription, wherein the ssDNA regions are exposed. Another difference between CBE and ABE lies in the fact that CBE carries the uracil glycosylase inhibitor (UGI) while ABE does not. As UGI is reported to induce genome-wide C-to-T conversions,[7] it is also possible that UGI expression contributes to CBE-mediated off-targets.

It has been previously reported that CBE is highly specific based on in vitro analysis using a modified version of Digenome-seq,[8] while ABE7.10 is more specific than Cas9 in a new study using a similar approach.[9] Similarly, a recent study using EndoV-seq reveals that ABE is highly specific and exhibits fewer off-target mutations than Cas9.[10] However, the limitation of these approaches is that they could only detect dominant sgRNA-dependent off-targets from bulk cells. The two *Science* reports have shown that the induction of off-target mutations is mainly due to the overexpression of APOBEC1 and/or UGI. The fact that very few or none off-target mutations concurred to the predicted off-target sites suggests that in silico prediction of off-target sites for base editors does not suffice. Encouragingly, Yang and his colleagues observed that Cas9/sgRNA or Cas9 alone did not generate substantial off-target mutations in mice.

Tremendous efforts have been made to reduce the off-target effect of CRISPR-Cas, either by engineering Cas proteins or optimizing sgRNAs. Now, it is time to seek better cytosine deaminase. As the APOBEC family contains multiple members, including activation-induced deaminase (AID),[2] it is tempting to test APOBEC proteins from different species. The alternative is to develop high-fidelity CBEs by reducing DNA binding activity of cytosine deaminase, possibly through protein engineering.

Both studies successfully established methods for detecting off-targets in vivo, offering valuable approaches for evaluating editing fidelity of programmable nucleases. These in vivo methods are

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applicable in systematic assessment of off-target mutations induced by other programmable nucleases. Nevertheless, there are still unanswered questions. Because APOBEC1 is reported to mediate C-to-T editing in RNA transcripts[11] and TadA is evolved from a transfer RNA adenine deaminase, it is possible that overexpression of these RNA deaminases might induce unwanted SNVs in RNA transcripts. In addition, the sum of off-targets may still be underestimated. In GOTI analysis,[5] although off-targets induced during two-cell editing could be disseminated to the entire embryo and detected at day 14.5, mutations generated during embryogenesis would be diluted during clonal expansion and become indiscernible in the final WGS analysis. Similarly, in the rice study,[6] only the SNVs generated at the early stages after T-DNA integration could be detected in the final WGS analysis. Transient expression of editing nuclease via mRNA[5] or ribonucleoprotein (RNP) could reduce but not eliminate such effects.

The development of high-fidelity single-cell WGS is still desirable to capture any underrepresented SNVs. Precise genome manipulation is a long-searched-for goal especially for gene therapy. Leveraging enzymes acting on DNA, such as the DNA recombinase, may help develop novel editing tools with much improved specificity.

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