Knock-in on CRISPR's door

Interest is growing in genome-editing tools that can insert large chunks of DNA into the genome — and avoid the double-strand breaks associated with CRISPR–Cas9 genotoxicity.

Ten years after its initial description, CRISPR–Cas9 has made remarkable progress as a research reagent and molecular medicine. The bacterial endonuclease has proven a versatile gene-editing tool for introducing frameshift mutations into target genes. Since first entering human testing in 2016, CRISPR–Cas9 has been employed ex vivo in about 46 trials of chimeric antigen receptor (CAR)-T cell therapies against malignancies, in 8 trials of engineered CD34 hematopoietic stem cells against sickle cell disease and β-thalassemia, and in one trial of allogeneic beta-like progenitor cells for type 1 diabetes. In vivo therapies have also been tested in patients with Leber's congenital amaurosis, hereditary transthyretin amyloidosis and hereditary angioedema. In many of these cases, Cas9 has proven adept at ablating aberrant gene function. But increasingly, attention is turning to gene-editing tools that can introduce DNA changes without CRISPR–Cas9's Achilles' heel: repair byproducts at double-strand breaks (DSBs) that give rise to genotoxic changes. In addition to better safety, these newer tools also promise to enable genome engineers to address a wider range of genetic diseases.

CRISPR–Cas9 has proven a versatile gene-editing tool with a dedicated user base to complement zinc finger nucleases, transcriptional activator–like and effector nucleases (TALENs) and meganucleases. Directed to a target locus via a reprogrammable single guide RNA (sgRNA), Cas9 endonuclease makes a DSB in the genome, which is then repaired by endogenous cellular enzymes. In the absence of a DNA donor template, non-homologous end-joining (NHEJ) can inactivate genes at the targeted site by generating insertion and deletion (indel) byproducts via non-productive processes. In the presence of a donor template, homology-directed repair (HDR) enables selective symmetric or asymmetric correction of a genomic site, albeit only in actively dividing cells. The mixture of NHEJ and HDR outcomes from a DSB means that Cas9 often can generate a substantial number of undesirable indels (for example, translocations and large deletions) at either on- or off-target sites, which can lead to p53 activation. And HDR's low efficiency and level in postmitotic cells like neurons means that inserting, rather than disrupting, genes still poses a formidable challenge.

In contrast, base-editing systems, which are slated to enter clinical testing later this year, rarely make DSBs and do not require donor DNA templates. In these systems, an sgRNA directs a Cas9 nickase to a 3- to 5-bp editing window containing the target DNA nucleobase, which is then chemically changed to another base via a deaminase. Adenine and cytosine base editors efficiently mediate all four possible transition mutations (C→T, A→G, T→C, G→A), work in both quiescent and dividing cells, and are associated with low indel frequencies (with uracil glycosylase inhibitor domains increasing fidelity for the latter). However, base-editing efficiency varies in different cell types; some deaminases also generate undesirable bystander DNA mutations or edit RNA. As yet, low-efficiency C→G conversion is the only transversion mutation (that is, a purine to pyrimidine or vice versa) amenable to such enzymes, and transversions account for 30% of genetic disease.

Another system making recent strides toward the clinic is prime editing. Prime editors comprise a reverse transcriptase fused to Cas9 nickase, which is directed by a prime-editing sgRNA (pegRNA) to bind and nick genomic DNA, leaving a single-stranded DNA sequence that then primes reverse transcription of an extended part of the pegRNA — both a template and guide, all in one. Direct synthesis from the genomic DNA strand results in an extended 3′ DNA flap that contains the edited nucleotides, which subsequently incorporates into the genome via 5′ flap excision, ligation and heteroduplex resolution. Incorporating an additional sgRNA to nick and modify the unedited target DNA strand can increase editing efficiency, but also increases indel byproducts. Overall, the approach enables not only precise insertion of small insertions and targeted deletions, but also all 12 base conversions, with no DSBs and low levels of indel byproducts. On the down side, editing efficiency varies widely across different cell types, loci and edit sequences, although first optimization steps are underway.

Finally, Cas engineered recombinase/integrase and transposase systems are attracting interest because of their potential to insert kilobase-sized fragments into genomic DNA. As early as 2016, dead Cas (dCas)-fused versions of Gin recombinase were shown to have low activity in mammalian cells. However, their stringent genome sequence requirements (a recombinase cutting-site motif and flanking targets for two sgRNAs) means they could be applied to only a few genetic loci. An intriguing alternative is CRISPR–Cas12k-associated transposases, which support insertion of up to 10 kb of DNA; however, as yet, these do not work in mammalian cells. Yet another as yet unpublished approach may be to engineer retrotransposases to insert RNA sequences into 'safe harbor' retrotransposons in the genome.

As engineers refine the editing tools (for example, by searching for Cas variants with different activities or size; engineering enzymes tailored for specific targets; or altering RNA guides through chemical, structural (for example, epegRNAs) or nucleotide modification), editing accuracy and efficiency will increase. Similarly, a greater understanding of the biological context where edits are being made (for example, the role of mismatch repair enzymes in determining outcomes in different cells for prime editing or the effect of genome sequence context, structure and chromatin state) will increase the reproducibility of editing in different cellular contexts.

Editing strategies are also likely to converge; for example, the pairing of prime editing with recombinase technology allows recombinase motifs to be introduced potentially anywhere in the human genome, heralding an era where >5,000-bp DNA inserts without DSBs could be made at any desired locus.

CRISPR–Cas9's power to disrupt genes and to engineer cell therapies with exquisite precision ex vivo has transformed molecular medicine. As new editing modalities mature and it becomes possible to insert large DNA fragments with precision, genome engineers will be able to tackle an even greater proportion of the >75,000 pathogenic genetic variants associated with human disease. ❐

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