GENETICS

Missing the target?

Targeted nucleases based on the CRISPR/Cas9 system can in some cases cleave at imperfectly matched 'off-target' sites.

The prospect of being able to target a nuclease to any genomic sequence simply by designing an RNA oligonucleotide that hybridizes to the target site has generated huge excitement about the clustered, regularly interspaced, short palindromic repeat (CRISPR)/Cas9 system (or RGENs, RNA-guided nucleases) for targeted genome modification. The genomes of a whole roster of creatures—bacteria, mice, worms and zebrafish—have already proven amenable to modification with RGENs, and these tools have also been applied in human cells. Some of the previous studies have examined whether potential off-target sites are also cleaved, but those investigations have been so far quite limited in organisms with more complex genomes. The specificity of this exciting new tool has remained an open question.

Keith Joung and colleagues at Harvard Medical School now report that at least some RGENs can cut with high efficiency at off-target sites with up to five mismatches with the target sequence, in human cell lines.

Targeting a specific genome sequence with CRISPR/Cas9 requires expression of the Cas9 endonuclease and a 20-nucleotide guide RNA (gRNA) designed to hybridize to the target sequence. In their studies, Joung and colleagues tested six gRNAs targeting four human genes (three of the gRNAs were directed toward different regions of the gene encoding VEGFA). They then searched the genome sequence for off-target sites with 1– 5 mismatches with each target sequence, and they tested many of these regions (46–64 of them, depending on the RGEN) for evidence of cleavage.

As with other targeted nucleases, the double-strand break made by an RGEN results in small insertions and deletions (indels) upon error-prone nonhomologous end joining–based repair; thus, the presence of these indels is evidence of RGEN cleavage. Even when Joung and colleagues examined potential off-target sites with a relatively insensitive assay, the T7 endonuclease 1 (T7E1) assay, they saw evidence of off-target cleavage for four of the six tested gRNAs in U2OS cells. Strikingly, the off-target sites were modified at 5–125% the efficiency of the target sequence. At a lower but still easily measurable frequency, the researchers observed off-target cleavage in HEK293 and K562 cells as well. With a detection limit of finding mismatches in 2–5% of the population, it should be noted that the T7E1 assay will miss rarer but potentially still relevant cleavage events.

As has been reported before, cleavage is most sensitive to sequence mismatches with the 3′ half (the so-called seed sequence) of a 20-nucleotide target sequence. But some one- or two-nucleotide mismatches at the 3′ end did not affect cleavage, and some changes in the 5′ half did. As already indicated by studies in bacteria, the rules determining RGEN specificity do not appear to be simple.

What is more, the target site does appear to matter. For some sites, there was evidence of off-target cleavage at sites that differed by as many as five nucleotides from the target sequence. For others, no off-target activity could be detected for even single-nucleotide changes, either with the T7E1 assay in this study or by sequencing in previous work. It is also worth noting that previous studies have not reported evidence of undue toxicity upon expressing these tools early in development in several species, including the mouse, a result indicating that, at least for those target sites and gRNAs, the tools are not substantially damaging the genome.

Although the observation of such high off-target cleavage by RGENs should give one pause when considering these tools for some applications—for clinical use in humans, in particular—there will be other applications in which experiments can be designed to control for off-target cleavage, or to work around it, as long as the potential for nonspecificity is understood. As more RGENs targeting different sequences in various genomes are tested, the rules that determine their specificity will hopefully emerge. It is clear, however, that the potential for off-target cleavage with CRISPR/Cas9 cannot be ignored.

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RESEARCH PAPERS

Fu, Y. *et al.* High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* doi:10.1038/nbt.2623 (23 June 2013).