CRISPR model building

By Chris Cain, Senior Writer

Boston researchers have used the CRISPR-Cas9 genome modification platform to simultaneously engineer mutations into multiple genes in mice.¹ The results from the rapid, one-step process provide the best evidence to date for the potential of this method to revolutionize the creation of complex disease models.

Earlier this year, five separate teams reported on how the CRISPR (clustered, regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated protein 9) system could be adapted to engineer site-specific mutations in the genomes of mammals, bacteria and zebrafish.²⁻⁷ The method was derived from a recently identified acquired immunity-like system in bacteria, in which CRISPR-associated proteins, including Cas9, are guided by short CRISPR-encoded RNAs to cleave homologous foreign DNA contained within plasmids or bacteriophages.

Unlike other approaches that rely on large protein domains such as zinc fingers or transcription activator-like effectors (TALEs) to guide site-specific DNA editing, the specificity of Cas9 is dictated solely by DNA-RNA base pairing, greatly enhancing ease of use. Because of this, researchers and biotech executives told *SciBX* in January that the system was likely to be rapidly developed and widely used for functional genetic analysis.⁸

Now, less than four months after the proof of concept for using the method to modify a single gene at a time in cell lines was published, researchers at the **Whitehead Institute for Biomedical Research** and the **Broad Institute of MIT and Harvard** have used it to simultaneously engineer point mutations into multiple genes in a mouse.

Typically, introducing multiple gene modifications in a mouse requires several rounds of interbreeding that are both time and labor intensive.

The team, led by **Massachusetts Institute of Technology** professor of biology and Whitehead member Rudolf Jaenisch, injected one-cell mouse embryos with different concentrations of *in vitro*-transcribed mRNA encoding Cas9 along with a guide RNA

targeting either *tet methylcytosine dioxygenase 1* (*Tet1*), *Tet2* or *Tet3*.

The Tet proteins convert 5-methylcytosine to 5-hydroxymethylcytosine and are being studied by many labs, including Jaenisch's, because they contribute to embryonic stem cell (ESC) pluripotency.

Using the method, the researchers were able to successfully disrupt both alleles of either *Tet1* or *Tet2* in 50%–90% of mice carried to term. For *Tet3*, only mice carrying one wild-type and one disrupted allele were born, which is consistent with previous studies that have reported that homozygous deletion of *Tet3* is lethal to embryos.

The researchers next attempted to disrupt both *Tet1* and *Tet2* simultaneously by injecting embryos with Cas9 mRNA along with both *Tet1* and *Tet2* guide RNAs. At RNA concentrations chosen to ensure a birth rate of 25%, over half of the mice carried to term had mutations in all four alleles.

In addition, specific point mutations could be simultaneously introduced into both genes by injecting Cas9 mRNA and *Tet1* and *Tet2* guide RNAs along with single-stranded DNA oligonucleotides carrying specific mutations in *Tet1* and *Tet2*.

Finally, the team demonstrated that five genes—*Tet1*, *Tet2*, *Tet3* and the Y-chromosome-encoded *sex-determining region Y* (*Sry*) and *ubiquitously transcribed tetratricopeptide repeat* (*Uty*)—could be simultaneously targeted and disrupted in mouse ESCs.

Results were published in *Cell*.

Jaenisch said in a statement that the technology allows researchers to make mice with five mutations in about three to four weeks. In contrast, he said, conventional interbreeding of multiple, separately generated knockout mouse lines would take three to four years.

Disruptive behavior

Although zinc finger nucleases (ZFNs) and TALE nucleases (TALENs) have previously been injected into embryos to create knockout strains of mammals including mice, rats and pigs, the techniques have never been used to disrupt or modify two or more different genes simultaneously.

Kiran Musunuru, assistant professor of stem cell and regenerative biology at **Harvard University**, said Cas9 editing is having a significant impact on the ease of disease model generation.

"The significantly increased efficiency that we observe with CRISPR-Cas9 in human pluripotent stem cells is making it much easier to generate disease models of all kinds. We have already found that models we were having trouble making with TALENs are now a breeze to make," he said.

In work published in *Cell Stem Cell* last month, Musunuru used Cas9 editing to disrupt disease-associated genomic loci with an efficiency of 51%–79%.⁹ This contrasts with a study published last year by his lab in which TALENs had 0%–34% efficiency in targeting

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—Kiran Musunuru,
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the same genes.¹⁰

When his team attempted to introduce specific point mutations into targets, the Cas9 approach was successful 11% of the time, whereas TALENs had a 1.6% success rate.

He added, “Just as it is now much easier to make disease models, it is likewise much easier to take a patient-specific induced pluripotent stem cell line and correct the disease mutation—which has obvious therapeutic implications.”

The major outstanding question for the therapeutic translation of Cas9 editing is the approach’s accuracy. The *Cell* paper describes a cursory analysis of off-target effects at potential locations with sequence homology to *Tet1* or *Tet2* and found no cleavage. Nevertheless, extensive genomewide analyses of specificity have not yet been conducted.

Musunuru said his team’s work to date has shown that Cas9 can cleave genomic DNA at sites that differ from a given guide RNA by one base pair but to a much lesser degree or not at all at sites that differ by two or more base pairs.

He said another important next step will be to examine Cas9 proteins from different bacterial species, which may have varied efficiencies and specificities.

He expects studies of Cas9 specificity to advance rapidly and said studies including genomewide analysis of cleavage sites are under way by his lab and other groups. “We can expect the specificity of Cas9 to be sorted out by the end of the year,” he said.

Jaenisch could not be reached for comment or to confirm the patent and licensing status of his team’s findings.

Among the paper’s authors was Feng Zhang, a member of the Broad Institute who led one of the first teams to describe a Cas9 editing system earlier this year and has filed a patent on the approach.

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