From left, former Twitter CEO Dick Costolo, Emmanuelle Charpentier, Jennifer Doudna and Cameron Diaz.

RESEARCH

Biology's big hit

Scientists now have several tools to edit the genomes of living organisms. One of the most recent is revolutionizing research and has thrust two of its creators into the limelight.

BY ZOË CORBYN

Emmanuelle Charpentier's initial ambitions for the genome-editing technique CRISPR–Cas9 were modest. She had originally thought that it might find a practical application in making a virus-resistant yogurt bacterium to help manufacturers create long-lasting cultures. But, as she learned more about how the CRISPR system operates, her plans took a radically different turn. Instead of reporting a potential aid to the dairy industry, the 2012 paper she co-authored¹ introduced CRISPR–Cas9 to the world as a technology that could precisely edit DNA. First her colleagues adopted the platform. Then it spread like wildfire. “I realized that actually people had been a bit desperate for an easy-to-use-tool,” says the microbiologist, now at the Max Planck Institute for Infection Biology in Berlin. “Their hunger was proof that the existing technologies were not that easy to use.” The paper catapulted Charpentier and co-author Jennifer Doudna at the University of California, Berkeley (see page S6), into the realm of science stardom.

A few tools to edit the genomes of living cells already existed when the paper by Charpentier and Doudna came out, most prominently zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). But because CRISPR–Cas9 is much easier to use than either of these options, it has made genome editing, which used to be a specialist process, routine. Many more laboratories have started to edit DNA, and numerous investigators who were previously using ZFNs and TALENs have switched to the new platform (see ‘Popularity of genome-editing kits’), says Dana Carroll, a biochemist at the University of Utah in Salt Lake City, who researches genome-editing tools.

Nevertheless, the mechanistic details of how the three technologies work are remarkably similar in the sense that they all consist of enzymes called programmable nucleases that can be directed to cleave DNA at any specific nucleotide sequence. In all cases, the cell then rushes to repair the double-stranded break, with one of two mending options: non-homologous end joining or homologous recombination (see page S2). The former occurs if restoration is left

entirely to the cell's own machinery, and leads to small, random nucleotide insertions or deletions that often disrupt a gene's activity, effectively turning it off. The other more difficult option allows genes to be corrected or new genes to be inserted as the cell copies a DNA repair template that is delivered alongside the cutting machinery.

As well as improving research tools, genome-editing technologies have advantages over conventional methods for altering gene expression as therapeutics. For example, classic gene therapy uses a vector (such as a virus) to randomly insert a healthy version of a defective gene somewhere in the genome, in the hope that the new gene will correctly perform its function wherever it lands. By contrast, genome editing fixes a faulty gene in its original location. Because there is a very limited chance of altering genomic geography, there is little need to worry that the edit will disrupt other genes. “It is like fixing a deflated tyre rather than attaching a fifth tyre to your car,” sums up Fyodor Urnov, senior scientist at genome-editing biotechnology firm Sangamo Biosciences, based in Richmond, California.

In many circumstances, genome editing also offers an improvement over another therapeutic tool, RNA interference (RNAi), which alters the products of DNA transcription by selectively destroying messenger RNA molecules. This is because genome editing permanently fixes the output of a defective gene for the lifetime of the edited cell and its progeny. With RNAi, changes occur only while a messenger-RNA-destroying agent is present in the cell.

EARLY OPTIONS

Genome-editing technologies came to the fore with the work of Srinivasan Chandrasegaran, a chemist at Johns Hopkins University in Baltimore, Maryland. In the late 1990s, Chandrasegaran was trying to manipulate bacterial enzymes that cut DNA². He realized that the best approach would require an enzyme with both DNA-recognition and cutting domains that did not overlap so he could strip away the recognition part and attach the cutting section to something that could be engineered to locate any nucleotide sequence. Enter an enzyme from the bacterium *Flavobacterium okeanokoites*: FokI. Chandrasegaran fused the enzyme's cutting domain to proteins called zinc fingers. These proteins can be customized to recognize certain three-base-pair codes by changing just a few of the zinc fingers' amino acids. By joining zinc fingers together, longer DNA sequences can be targeted.

Carroll was one of the first to recognize the wider significance of Chandrasegaran's discovery. Together, they showed that ZFNs could edit DNA in living cells (frog oocytes)³. Carroll went on to demonstrate the same thing in a whole organism (the fruit fly)⁴.

And in 2005, Urnov was part of the team that first used ZFNs to edit DNA in human cells⁵.

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As exciting as ZFNs were, they proved tricky to work with. “It was hard to develop zinc fingers for new targets in a really reliable way,” says Carroll. One inconvenience is that FokI only slices through one of the two strands of a DNA double helix. To cut through both strands requires creating zinc fingers that are specific to the target string of nucleotides as well as to its complementary sequence. Worse still, when zinc fingers are linked in a row, they sometimes influence the operation of their neighbour.

TALENs emerged⁶ in 2010, out of work by two groups of plant pathologists — one led by Adam Bogdanove, now at Cornell University in Ithaca, New York, and the other by Ulla Bonas, now at the Martin Luther University in Halle, Germany. These groups were independently trying to ascertain how proteins called TAL effectors recognize DNA.

TAL effectors are secreted by pathogenic plant bacteria of the genus *Xanthomonas*, in which their job is to activate plant genes that promote bacterial infection. The two groups found that a special section within a TAL effector’s structure directs the protein to a particular sequence of DNA, each nucleotide of which is specified by a pair of amino acids^{7,8}. Changing the order of these amino-acid pairs directs the TAL effector to different parts of the genome. In other words, these proteins are an alternative to zinc fingers, and, in the same way, can be fused to a FokI cutting enzyme forming TALENs.

CRISPR COMES TO TOWN

Unlike ZFNs and TALENs, CRISPR–Cas9 has nothing to do with FokI. Instead, it cuts DNA with the enzyme Cas9, which snips through both strands of a DNA double helix at once. This platform also differs from its predecessors by using RNA instead of proteins to guide its cutting enzyme to a specific DNA sequence (which is identified by complementary base-pairing between RNA and DNA).

The CRISPR–Cas system is an adaptive immune system that is widely found in bacteria — the reason why Charpentier imagined it might make yogurt bacteria more resilient. CRISPR, or clustered regularly interspaced short palindromic repeats, refers to the small segments of genetic code that bacteria sometimes capture from invading viruses and store in their own genomes for future reference. The term CRISPR was first coined in 2002, although CRISPR systems were observed (without an understanding of their function) in 1987.

Working with *Streptococcus pyogenes*, a component of human skin flora with pathogenic strains, Charpentier’s group ironed out the details of the simplest CRISPR, and of how Cas9 interacts with this reference library. The team showed that when faced with a threatening virus, Cas9 consults the CRISPR array and derives two RNA molecules. One of these, transactivating CRISPR RNA (tracrRNA) changes the shape of Cas9 ready for cutting DNA, and the other, CRISPR RNA (crRNA), defines the

POPULARITY OF GENOME-EDITING KITS

The ease of use of CRISPR–Cas9 has seen a rise in the number of orders for genome-editing kits from Addgene, a supplier of the kits based in Cambridge, Massachusetts (kits are shown because the construction of different tools requires different numbers of plasmids).

cutting site⁹. The group’s collaboration with Doudna’s lab demonstrated that both RNA molecules are needed to lead Cas9 to a particular cutting site on an invading virus’ genome. They also proved that the system still works when the two RNAs are fused into one. And they altered the nucleotide code of this single guide RNA, redirecting Cas9 to cut elsewhere.

Today, when a researcher wants to edit a genome using CRISPR–Cas9, he or she can design a guide RNA, have it made to order, and delivered in the mail. This makes CRISPR–Cas9 less complicated, cheaper and faster to use than the other genome-editing tools.

These advantages aside, the three main technologies each have different strengths. CRISPR–Cas9 is the only one that allows for many DNA sites to be edited simultaneously, using different guide RNA sequences on the same Cas9. TALENs have the longest DNA recognition domain, and therefore tend to have the fewest off-target effects — which occur when parts of a genome with an identical or near-identical nucleotide sequence to the target site are cut unintentionally. And ZFNs are small (one-third of the size of TALENs and much smaller than Cas9 from *S. pyogenes*, the mostly widely used version of Cas9) so they are the only genome-editing tool that can fit comfortably inside the adeno-associated virus, the most promising vector for delivering genome-editing-based therapies.

But there is another issue that influences the technologies’ adoption. Although it is fairly clear who owns the intellectual property for ZFNs and TALENs, the situation with CRISPR–Cas9 is much less certain. This is particularly important for commercial development. “If you are a company it may come down to intellectual property,” says Keith Joung, a pathologist at Harvard Medical School in Boston,

Massachusetts, who both develops genome-editing technologies and applies them in medical research. The key patents covering the use of CRISPR–Cas9 as a genome-editing tool for mammalian cells were awarded to the Broad Institute of Massachusetts Institute of Technology and Harvard beginning in April 2014, based on research by bioengineer Feng Zhang. Zhang’s group at the Broad Institute — as well as that of George Church, a geneticist at Harvard (see page S7) — were the first to show in 2013 that CRISPR–Cas9 works in human cells^{10,11}. The Broad Institute fast-tracked its patent applications, leapfrogging those filed earlier in 2012 by the University of California. The latter are supported by Charpentier and Doudna’s research and are still being examined. In response to the patent awarded to the Broad Institute, the University of California has initiated ‘interference proceedings’ — a patent priority contest — saying that the use of CRISPR–Cas9 in human cells was reasonably self-evident from Charpentier and Doudna’s 2012 paper.

It is a complex situation, but no one has time to sit back and wait for a ruling. Both the Broad Institute and the University of California are issuing licences to companies around the world. Meanwhile, the innovations keep rolling in. In September 2015, Zhang’s group reported a new CRISPR system that avoids using Cas9 altogether. The DNA snipping is instead achieved with another enzyme, Cpf1 (ref. 12). Whether that difference is enough to warrant a new intellectual property ruling, is unknown. But, given the difficulty of fitting Cas9 into an adeno-associated virus vector, the potential advantages of Cpf1, which needs a much smaller RNA guide than Cas9, are clear. And yet, even though Cpf1 was only the second type of CRISPR-cutting enzyme to be characterized, there are already signs that there are many more to come. In late October 2015, Zhang and his collaborators published details of three new CRISPR enzymes¹². Their initial analysis suggests that the new enzymes have distinct properties from Cas9 and Cpf1, and could, therefore, further widen the genome-editing toolbox. “All I can say,” says Charpentier, smiling as she considers the field’s future, “is the principle of RNA programmable enzymes is a very nice one.” ■

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