CRISPR Applications Flourish in 2017 Despite Simmering IP Battle

Dec 21, 2017 | Christie Rizk

Premium

NEW YORK (GenomeWeb) – In only a few short years, CRISPR genome editing has gone from being a discovery that had George Church telling *The Independent* in 2013 that he was "jumping out of my skin with excitement," to being discussed as a possible technique for everything from creating gene drives to stop the spread of the Zika virus, to treating various cancers and other diseases, developing sophisticated diagnostic tools, and improving crop yields.

CRISPR's many potential uses and relative newness have prompted researchers to undertake many kinds of studies to learn more about its basic principles, how it can be used, what its limitations are, how to make it safer for use in humans or for human-adjacent purposes, and even how it can be coupled with other technology.

In 2017, GenomeWeb documented several trends and breakthroughs in both the science and business of CRISPR, some of which may indicate where the research community and the industrial sector choose to focus their efforts in 2018.

New CRISPR nucleases

For many genome editing applications, researchers have found the Cas9 enzyme to work well — in most cases, it's considered to be the gold standard. But the past few years have seen the discoveries of several new nucleases — most notably, the Broad Institute's Feng Zhang's bombshell revelation of Cpf1 in 2015 and Jennifer Doudna and Robert Tijan's 2016 study on C2c2 (now called Cas13a).

In the past year, both researchers and companies have made efforts to look for or develop additional Cas9 orthologs or new nucleases that can be used for different purposes. In February, for example, researchers at the Institute for Basic Science in South Korea and their partners at Seoul National University Hospital and South Korean biotechnology company ToolGen engineered what they say is the smallest Cas9 ortholog to date. The CjCas9 ortholog comes from *Campylobacter jejuni* and is about 984 amino acids large. It's smaller than the first and most commonly used Cas9, which is derived from *Streptococcus pyogenes* (SpCas9) and is some 1,370 amino acids large; and even smaller than *Staphylococcus aureus* Cas9 (SaCas9), which has been engineered to fit into certain viral vectors for delivery into cells. The researchers packaged CjCas9 into adeno-associated virus vectors and delivered it to mouse muscle and retinal cells, suggesting it could be used to target genes previously thought to be undruggable.

In May, a team of University of California, Berkeley researchers led by Doudna published a study describing 10 variants of Cas13a. Of those, three differed from the original protein in the way they cut RNA. This led the researchers to conclude that the Cas13a variants can be divided into two distinct functional groups that

recognize orthogonal sets of CRISPR RNA and possess different single strand-RNA cleavage specificities. Importantly, the researchers' identification of two distinct subfamilies of Cas13a suggested that the various enzymes could be multiplexed to serve different functions, including disease diagnosis.

Indeed, Broad researchers this year developed a technology they dubbed Sherlock, which uses Cas13a's RNA cleavage properties to look for and find biological material. In a study, the team described the technology's ability to detect Zika and dengue viruses, bacteria and their antibiotic resistance genes, human SNPs, and mutations in cell-free tumor DNA.

More recently, Inscripta (formerly Muse Biotechnology) announced that it was working on developing a completely new family of novel RNA-guided nucleases called the Madagascar family, or MADzymes. The company released the first of these — MAD7, an enzyme initially characterized in *Saccharomyces cerevisiae* and *Escherichia coli* — for free to the research community.

Stay on target

One major drawback of CRISPR technology is its tendency to induce off-target edits. Indeed, "What about the rate of off-target effects?" has become an easy question to ask when gauging the utility of a given genome editing technique. The detection and/or reduction of these inadvertent alterations has become its own area of CRISPR science, with researchers developing new methods and tools for these twin purposes at a rapid clip.

In June, researchers from Merck KGaA life science business MilliporeSigma published a study describing a tool they had developed called proxy-CRISPR. Taking the Cas9 ortholog approach, it uses a Cas9 enzyme from the *Francisella novicida* bacterium (FnCas9) rather than the more common SpCas9. The team identified the type II-B FnCas9, noting that it possessed a novel enzymatic property that cleaves target DNA in a staggered pattern, exhibiting a higher intrinsic specificity than SpCas9. But they also found that it wasn't as efficient as SpCas9 as it was being blocked from reaching human DNA by human chromatin.

The MilliporeSigma researchers developed a proximal CRISPR targeting method that restores FnCas9 nuclease activity in a target-specific manner. It enhances the usability of native CRISPR proteins without the need to re-engineer them, and importantly, the use of FnCas9 caused no off-target cleavage in the researchers' experiments.

Two studies taking different approaches were published simultaneously in *Nature Methods* describing the identification of off-target mutations associated with cell-type-specific SNPs and the detection of potential off-target cleavage sites. The first method, called circularization for *in vitro* reporting of cleavage effects by sequencing (CIRCLE-seq) was described by the authors of the study as an *in vitro* screening method that is highly sensitive and performs better than existing cell-based or biochemical approaches for identifying CRISPR/Cas9 genome-wide off-target mutations. The other, a biochemical method called SITE-Seq, used Cas9 programmed with sgRNAs to identify the sequence of cut sites within genomic DNA.

In July, Berkeley's Doudna made a different suggestion — applying an anti-CRISPR protein from the *Listeria* bacteriophage to block Cas9 from interacting with DNA. Her study in *Science Advances* showed that this inhibitor protein — called AcrIIA4 — could prevent cutting at the wrong sites and therefore reduce off-target effects if applied at the right time to genes that had been treated with CRISPR-Cas9.

CRISPR combos

Companies and institutions also found interesting ways to combine other technologies with CRISPR this year, either to enhance the capabilities of those technologies or to make room for genome editing in their own product pipelines.

In July, UK firm Sphere Fluidics said that it planned to develop an automated, single-cell genome-engineering platform using its existing single-cell analysis and characterization system called Cyto-Mine. The company had won a $1.25 million grant to adapt Cyto-Mine for genome editing purposes, envisioning a benchtop instrument that researchers could use to create edited cell lines in an automated way for whatever purpose they choose.

Bio-Rad Laboratories' intention to create a genome editing application for its popular droplet digital PCR (ddPCR) technology stretches back to 2015, and this July, the company released two of several planned assays that use ddPCR to detect genome edits. The ddPCR technology increases the signal-to-noise ratio, allowing users to quantify extremely rare edits, even those with frequencies of 0.5 percent and from as little as 5 nanograms of genomic DNA, according to the firm. Bio-Rad also said it plans to continue releasing ddPCR-based assays for the detection of genome edits, and is considering how the rest of its tools could fit into a CRISPR research pipeline.

In October, University of Washington researchers tried combining CRISPR with a previously described method called duplex sequencing. Duplex sequencing improves on the accuracy of next-generation sequencing, and the researchers have shown that the method can be used to identify very low-frequency mutations in the cancer gene TP53 in ovarian cancer patients. The problem is that it's not very efficient and requires a lot of input DNA. But when the researchers combined it with CRISPR, they were able to solve those problems.

Their startup company TwinStrand Biosciences, which spun out of UW earlier this year, has licensed the CRISPR-DS method, and they've received a $2.6 million three-year Small Business Innovation Research grant from the National Institutes of Health to develop an assay for the early detection of ovarian cancer.

Early-stage disease research

Of course, the Holy Grail of CRISPR research continues to be finding a way to use genome editing to treat disease. The early-stage research has shown some promising results for several different conditions, including various cancers, sickle-cell disease, and Duchenne muscular dystrophy. Though the studies have yet to advance to human trials, it's possible we may see such research in the coming year.

"Certainly, transitioning into human therapy is where we want to go with this," Eric Kmiec, director of the Gene Editing Institute at Christiana Care Health System, told GenomeWeb. "Even beginning to get into thinking about using CRISPR in human therapy is a huge advance. We worry about problems like immune response, but when you start to worry about these things, you're making quite a bit of progress because you're at the level where you think the technology is going to work. Now it's a matter of making it work safely in patients."

In the meantime, researchers have been busy examining the various angles they could take to use genome editing to correct disease states in human beings.

In July, the Broad's Zhang and his colleague David Scott published an analysis in which they noted that prescreening patients with whole-genome sequencing and then integrating that information with empirical methods for guiding RNA selection could help researchers design CRISPR-based therapeutics that are both efficacious and safe. They cautioned that naturally occurring inter-individual genetic variation could disrupt the targeting of CRISPR therapies, generating off-target effects and reducing the efficiency of the therapy.

Indeed, in a study published this week in *PNAS*, a Harvard University-led team found that human genetic variation can alter the efficacy of CRISPR targeting when it's used for therapeutic purposes, and increase the potential for off-target effects. In particular, the analysis suggested that indels are more likely to create more potent novel off-target sites than SNPs, and the researchers added that genetic variation should be

considered in the design and evaluation of any CRISPR-based therapy in order to minimize the risk of side effects and maximize the potential for treatment success.

In a completely different approach, an international team of researchers reported in August on using a CRISPR-Cas9-based technique to visualize and eliminate pathogenic RNA species produced by microsatellite repeat expansions (MREs) in DNA that cause dominantly inherited diseases such as Huntington's disease and amyotrophic lateral sclerosis (ALS). The RNA-targeting Cas9 (RCas9) CRISPR system was originally developed by University of California, San Diego researchers in March 2016 to track specific RNA sequences and processes *in vivo*. Those researchers along with collaborators in Florida and Singapore aimed to turn the RCas9 system into a diagnostic and therapeutic tool, and were able to target and destroy MRE RNAs both when exogenously expressed and in cells of patients suffering from myotonic dystrophytype 1 and 2 (DM1/2), Huntington's, andC9orf72-linked ALS (C9-ALS).

Why target a gene when you can eliminate a whole chromosome? In November, researchers at the Chinese Academy of Sciences and their collaborators developed a CRISPR-Cas9 system to eliminate targeted chromosomes from the human genome. Their results indicated that eliminating a whole chromosome did not induce significant off-target effects, and concluded that the study was the first step in a potential therapeutic strategy for human aneuploidy diseases involving additional chromosomes.

And in December, a team led by Salk Institute researchers used CRISPR to epigenetically activate target genes in order to alter disease phenotypes in mice without inducing double-strand DNA breaks (DSBs) at all. They developed a system for *in vivo* activation of endogenous target genes through trans-epigenetic remodeling, and successfully tested it on mouse models of diabetes, acute kidney disease, and muscular dystrophy.

"It's variations on a theme," Kmiec said. "Whether it's RNA editing, base editing, or CRISPR-directed gene knockout for lung cancer, they're all going to be useful, and it doesn't have to be one way."

And although he believes it's likely we'll see the first human studies of CRISPR-based therapies in 2018, it's hard to say which disease that will be for. "Sickle-cell disease has been the brass ring for inherited diseases because the mutation is universal," Kmiec said. "What our work has shown is that while we can get correction of the mutation, you leave behind some mutagenesis at the site. But that is the disease that is considered to be low-hanging fruit, so the answer is maybe, if that mutagenesis problem can be resolved."

Kmiec believes it may be more likely to see CRISPR trials for liquid tumors or even lung cancer, and said his group has engaged in discussions with the US Food and Drug Administration and the National Center for Advancing Translational Sciences.

"I think the cancer applications might come sooner because there, you're just destroying a gene to make the chemotherapy or immunotherapy work better," he said. "Destroying something is a lot easier than repairing it. Fixing a mutation is challenging, but if you can use CRISPR to efficiently knock a gene out, I think those are the therapies that will be [easier to develop] because that's what it does in real life. We're using CRISPR in its natural way — that, to me, is a bit of an easier path because you're not trying to balance the cleavage and the repair."

Major breakthroughs

There were also some breakthroughs of incredible significance this year.

In August, a team led by researchers at Oregon State Health and Science University rocked the research community when they published a study in *Nature* reporting that they had used CRISPR-Cas9 gene editing to correct a heart disease mutation in viable human embryos.

Researchers from Sun Yat-sen University in China had previously reported using CRISPR to modify the gene that causes beta-thalassemia in non-viable human zygotes, while a team at Guangzhou Medical University reported using it to inactivate the CCR5 gene to make embryos HIV-resistant. But this was the first time ever that such editing work had been done in viable embryos.

The OHSU-led team used CRISPR to correct mutations within MYBPC3 in the embryos. They targeted an autosomal dominant mutation in MYBPC3 that leads to hypertrophic cardiomyopathy, which affects about one in every 500 people and is a common cause of sudden death in young people. The researchers said they were able to edit the embryos with high efficiency, while largely avoiding mosaicism and off-target cleavage.

There were some doubts later raised by Memorial Sloan Kettering Cancer Center's Maria Jasin, Harvard's Church, and others in a preprint paper published on BioRxiv, in which they suggested the OHSU researchers' conclusions of gene correction in the embryos required direct verification. They have submitted their own paper for publication.

Meanwhile, in October, the Broad's Zhang and David Liu published their own blockbuster papers, describing entirely new CRISPR editing systems.

Liu's work, published in *Nature*, used a guide RNA and catalytically impaired CRISPR-Cas9 to convert A-T base pairs to G-C base pairs in the genome, enabling the editing of single point mutations without the induction of DSBs. And in *Science*, Zhang and his coauthors described their new CRISPR-based RNA editing system, called RNA Editing for Programmable A to I Replacement (REPAIR), which allows for the temporary repair of single RNA nucleotides in mammalian cells without permanently altering the genome.

CRISPR in industry

On the industry side, the business of CRISPR also continues apace. Despite the continuing battle over intellectual property, companies are continuing to make licensing deals for technology, taking a make-deals-now-and-sort-the-patents-out-later attitude.

Seokjoong Kim, director of research at ToolGen, told GenomeWeb he believes it will likely take a long time for the CRISPR IP situation to be completely sorted out, especially once patents in Europe and Asia are also considered.

"One of the things I noticed this year is that there are some startups that have been able to get some investments and seed [money] without having the ground IP, but having some interesting IP, or achievement in the [CRISPR] field, or specific applications like using CRISPR in drug discovery," he said. "So outside of core IP, IP in specific applications is being valued enough to draw investment. [Companies] might need to clear up the IP situation later, but I think it shows even small applications or specific indications with CRISPR could be already valuable enough to make a company and push investment, even with the risk of not having core IP at first. People are not waiting for the [patent] situation to clear up. They are starting important work."

In the latest salvo of the patent fight, the Broad Institute filed a brief in October in the United States Court of Appeals for the Federal Circuit in response to an appeal filed by the University of California, Berkeley in April that aimed to continue the fight for rights to IP underpinning the most lucrative applications of genome editing.

In January, the US Patent and Trademark Office declared an interference proceeding to settle certain claims related to the patent fight. In February, however, the three-judge panel from the Patent Trial and Appeal Board (PTAB) hearing the interference case issued a judgement of no interference-in-fact, stopping Berkeley's bid for the IP, and leaving the Broad to control the key IP estate for companies pursuing

targeted genome editing applications in several areas, especially gene therapy, drug discovery and development, and ag-bio, which rely on editing in eukaryotic cells.

Berkeley's April appeal asserted that the PTAB's decision was based on several errors, but the Broad is disputing those assertions and is asking the appeals court to affirm the PTAB's judgment of no interference-in-fact.

In November, Duke University law professor Arti Rai and Arizona State University professor Robert Cook-Deegan argued in a column in *Science* that overly broad CRISPR patents are contrary to the public benefit, and that the courts must rein them in. In the end, the best thing that could happen in the CRISPR case would be narrow patents that would prevent anyone from exercising too much control over downstream research, they added.

License to edit

While the courts are deciding, companies are open for business. ERS Genomics, a company set up to commercialize the CRISPR-Cas9 intellectual property rights assigned to Emmanuelle Charpentier of the Max Planck Institute for Infection Biology in Berlin, has been especially prolific when it comes to signing licensing deals for the IP it holds.

In only the past few months, in has inked deals with Charles River Laboratories, Oxford Genetics, Taconic Biosciences, DuPont Pioneer, and Cellecta. The firm also announced a deal with Horizon Discovery Group to expand their pre-existing, non-exclusive, worldwide license agreement covering Horizon's use of ERS' CRISPR technology.

For its part, Horizon has been busily acquiring licenses and rights to various genome editing technologies, building a portfolio that includes everything from recombinant adeno-associated virus editing technology from the University of Washington for non-therapeutic applications to a worldwide exclusive license from Sigma-Aldrich for the use of zinc-finger nuclease technology to engineer *in vivo* disease models. In July, Horizon acquired the exclusive rights to a transposon-based platform with applications in bioproduction, reference standards, and therapeutic development. The intellectual property covering the technology is held by inventors from the Max Delbrück Center for Molecular Medicine, the Genetic Information Research Institute, the Paul Ehrlich Institute, and Horizon. The technology is based on helitrons, a type of eukaryotic transposon that can incorporate multiple copies of a DNA sequence into a genome either immediately or at a later time by reactivating the transposon machinery.

And in what is merely a small example of an industry-wide trend, New England Biolabs and startup company CasZyme announced this month that they have formed a multi-year collaboration to identify and commercialize CRISPR-Cas nucleases. Such deals are becoming more and more frequent.

CRISPR in agriculture

In what could be termed a trend-within-a-trend, a large proportion of the licensing deals being signed are for ag-bio purposes.

In August, Monsanto announced that it had licensed a CRISPR technology platform from ToolGen with the intention of developing agricultural products. In September, ag-bio firm Arcadia Biosciences said that had it acquired a worldwide, nonexclusive license to the Broad's IP on CRISPR-Cas9, allowing it to use the technology to develop nutritional and agricultural productivity traits in its core crops. And in October, DuPont Pioneer and the Broad said that they would jointly provide non-exclusive licenses to CRISPR-Cas9 intellectual property under their respective control to any entities wanting to use the technology for commercial agricultural research and product development.

"Agriculture companies are realizing what CRISPR can do, and there are companies that may have genome-edited prototypes or even products that are preparing for commercialization, and they're trying to clear up their IP situation by [signing licensing deals] before the actual commercialization process," Kim said. He also noted that unlike on the therapeutic side, agriculture companies are much more likely to sign non-exclusive deals for CRISPR technology, as they can still develop products that will enhance their value chains.

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