CRISPR: MODIFYING THE LIFE SCIENCE LANDSCAPE

From lampreys to human stem cells, the CRISPR/Cas9 system is challenging our notions of what is possible with genome editing. Nathan Blow talks to researchers pushing the boundaries of CRISPR/Cas9 technology to expand our understanding of biology.

Credit: D. Medeiros

Three years ago, University of Colorado Boulder researcher Daniel Medeiros was at a crossroads in his study of vertebrate evolution. He was interested in a critical period in the history of vertebrate physiology: the transition from jawless vertebrates to jawed vertebrates. For his experiments, he was relying on one species of lamprey—a jawless fish often seen as a parasite of other fish species. Lampreys and hagfish are evolutionarily unique since they represent the only two living groups of jawless vertebrates, providing biologists living subjects for evolutionary studies.

“I was actually thinking about winding down the lamprey work,” says Medeiros. Lamprey would seem like the perfect organism, so why was he thinking about abandoning it? Even though Medeiros knew they could provide important insights into vertebrate evolution, lampreys are very tricky to work with in the lab. They have long life-cycles, and there are very few tools available to manipulate genes in lampreys for functional genetics studies. These characteristics made the lamprey a less than ideal model organism. And for Medeiros, his lamprey experiments had hit a critical point. “We were at the limit of what we could do with lamprey embryos,” he recalls.

Previously, Medeiros’ lab had made some headway using pharmacological inhibitors and morpholinos to study very-early gene function in lamprey, but there are a limited number of chemicals available, and he was really interested in looking at later developmental processes, in particular the formation of the head skeleton. It appeared as if lamprey embryos would not be able to support the long-term research goals Medeiros had in mind. But just as he started to ponder his own future, an encounter with a collaborator from another department,

who was working on frogs, would change everything Medeiros thought was possible in lamprey research.

The “Wild West” of genome engineering

Mike Klymkowsky is a professor in the department of Molecular, Cellular, and Developmental Biology at UC Boulder. Around the time Medeiros was wondering how to move forward with his lamprey work, Klymkowsky was starting an effort in his lab to apply the recently described CRISPR/Cas9 mutagenesis system to his studies of the clawed frog *Xenopus*. For its part, *Xenopus* might be considered a “fringe” model organism—its embryos have frequently been used for patch-clamp studies of ion channels. But Klymkowsky was curious as to whether or not CRISPR could make studies of gene function in *Xenopus* easier and more efficient. He was enthusiastic about the potential of the technology and suggested to Medeiros that it might work for his lamprey embryos too.

Medeiros was very skeptical. The lamprey embryos he uses in his experiments are hard to collect; in fact they can only be harvested at certain times of the year. He wondered if CRISPR would be able to provide the high efficiency required for working with his limited supply of lamprey embryos. Rather than plunging blindly ahead and applying CRISPR/Cas9 in his lamprey work immediately, Medeiros decided to let one of his students work on a project in Klymkowsky’s lab, where they were trying to use CRISPR/Cas9 mutagenesis on one species of clawed frog, *Xenopus laevis*.

It would turn out that those experiments in *X. laevis* did not go according to plan—the efficiency was not very high. But

since his student had CRISPR/Cas9 experience, Medeiros decided it was worth it to try injecting some lamprey embryos using the CRISPR/Cas9 technique. Since the likelihood of success seemed low, the first experiments targeted the tyrosinase gene, which is involved in pigmentation. A deletion knocking out tyrosinase would result in altered pigmentation that could be identified by the naked eye, providing a quick and easy visual readout that would tell the researchers if CRISPR was working.

“I can remember the day when I heard that scream coming from the embryo room,” recalls Medeiros—his student had performed the embryo injections and was seeing white F0 larvae, the sign of a mutant. CRISPR/Cas9 was indeed working in lamprey after a single injection, editing the tyrosinase gene that had been targeted. Better still, in lamprey the CRISPR/Cas9 system was working extremely efficiently—much better than expected.

In the lab’s first experiments with lamprey, synthetic mRNA encoding the Cas9 protein was co-injected with the guide RNA, resulting in a mutagenesis efficiency of 60%–80%. Shortly thereafter, when a purified Cas9 protein became available for injection, Medeiros says their mutagenesis efficiency skyrocketed to >95%.

“Lamprey is a difficult organism to work with, so the efficiency we were seeing was a huge surprise,” he says. It was also hugely encouraging for his research because lamprey embryos can only be collected during a very short 6–8 week period each summer. Without the high efficiency they were seeing in the first experiments, the CRISPR/Cas9 system would not have been feasible as a broader tool for lamprey research.

In 2015, Medeiros and Klymkowsky reported their successful use of CRISPR/Cas9 for lamprey mutagenesis in the journal *Development* (1). These experiments breathed new life into Medeiros’s evolutionary studies using the lamprey. He says that

Diagram of CRISPR/Cas9 genome editing process. Credit: Reproduced from *Int. J. Mol. Sci.* 2015, 16(11), 26077–26086.

he can now look at his research in a completely different way. Theoretical predictions about the function of genes involved in the transition from jawless to jawed vertebrates can now be tested experimentally—something that would have been impossible 3 years ago. With results in hand, Medeiros’s lab is wasting no time—they are now actively targeting different lamprey transcription factors and regulatory proteins to study genes and networks in this ancient lineage of vertebrates.

Understanding and applying CRISPR

When reading about gene-editing approaches, what always seems to set CRISPR/Cas9 apart from the others is its unique robustness—the technique has been shown to work in many different organisms and for many different applications. Still, there are some basic biological questions about CRISPR/Cas9 that need to be addressed. In the case of the lamprey, it’s curious how there is highly efficient CRISPR editing in this animal, whereas editing is much less efficient in the clawed frog *Xenopus* species Medeiros’s student worked on initially. Why is editing higher in some species than others? Medeiros has one idea.

“The lamprey genome is very GC-rich, with a strong codon bias towards GC,” he explains. And that GC-bias might just lead to stronger interactions with the guide sequences used to target specific genes. The implication is that sequence context might have a significant impact on the ability of CRISPR/Cas9 to edit a specific site in the genome.

William Skarnes works at the Jackson Laboratory for Genomic Medicine in Farmington, Connecticut. Skarnes was part of the International Knockout Mouse Consortium (IKMC), a collaboration between researchers working to generate mutant embryonic stem (ES) cell lines for each of the more than 20,000 genes in the mouse genome. In late 2015, the IKMC reported on how they had applied CRISPR/Cas9 approaches to generate conditional mutants for mouse loci that had previously proven too challenging to successfully mutate.

MIT bioengineer Timothy Lu is creating DNA memory units for cells using CRISPR/Cas9 technology. Credit: T. Lu.

Daniel Medeiros from the University of Colorado Boulder says that CRISPR/Cas9 technology has expanded the possibilities of his evolution studies. Credit: D. Medeiros.

Unlike the lamprey, tools to genetically manipulate mice have been available for decades. In fact, most of these tools are so common and widely used that the IKMC had already generated conditional mutants for the majority of the 20,000-plus mouse genes, but approximately 13% of those genes could not be targeted for mutagenesis, no matter what technique was used. Then, CRISPR/Cas9 entered the picture.

“CRISPR has some real advantages in that it is cheap, easy, and very quick to make mutations,” notes Skarnes. This was the reason IKMC adopted the technique for those loci that had proved challenging using traditional approaches. In a recently published paper, IKMC members showed that CRISPR/Cas9 was effective for conditional mutagenesis of some of those loci (2). But there was a caveat: Generating conditional mutants worked well in mouse ES cell lines but not in live mice.

“Engineering ES or iPS [induced pluripotent stem] cells is still tough, but using CRISPR it actually becomes much easier,” says Skarnes, who himself has moved from mice to human cells for his research efforts.

Skarnes is now using CRISPR/Cas9 to generate knockouts in a diploid cell with only a single round of injections. The system’s high efficiency makes it feasible for the first time to generate libraries of gene knock-outs in human iPS cells in a fairly rapid fashion. And in much the same way Medeiros was able to ask new questions in the lamprey, researchers using human iPS cell libraries can now examine important biochemical and genetic processes in a more direct fashion. Working with stem cells also means that Skarnes can study the process of cell differentiation in greater detail using conditional mutagenesis techniques.

Skarnes says that more work will be needed if conditional mutagenesis is going to become an efficient and accessible tool for mouse models. Unlike Medeiros and his lamprey work,

where deletions were induced to knock out genes of interest, conditional mutagenesis requires the cell to perform the “heavy lifting” of homologous recombination using its own DNA repair machinery, a much more intensive process. Given this, it shouldn’t come as a surprise that the efficiency is so low. Still, Skarnes is optimistic, suggesting that in the future researchers may find ways to improve the process, potentially by supplying some of the components of the cell’s DNA repair machinery directly during transfection.

Engineering cells

As Skarnes works on generating conditional mutants for large numbers of human stem cells, Massachusetts Institute of Technology bioengineering professor Timothy Lu is using CRISPR/Cas9 for a completely different application—to directly record what a cell does or what it encounters (3).

“We have been working on creating individual memory units for cells,” Lu explains. Imagine if it were possible to directly record when a certain protein becomes active or when a single neuron fires—without the need for imaging, multiple measurements of cell samples, or any experiments, in fact. This is what Lu and his lab have been working toward in recent years: creating a system where the cell actually records its own actions by making changes to its DNA.

The general concept is not new. Previously, researchers tried to engineer such cellular memories using recombinase proteins as effectors that could produce the needed changes to the cell’s DNA following a specific event.

While that approach worked, Lu came to realize there was a limitation in using recombinases. “There are only a small number of recombinases, and this makes looking at many different events in parallel or over time quite challenging,” he says. His solution to the multiplexing problem came in the form of the CRISPR/Cas9 methodology—more specifically, the variability possible within the CRISPR guide sequences. These sequences can be used to engineer changes all around the genome, there-

Medeiros and Klymkowsky described the use of CRISPR/Cas9 for gene editing in lamprey species (pictured above) in 2015. Credit: D. Medeiros.

by enabling multiplexed records of cellular activity.

While at first glance, it looked as if the CRISPR/Cas9 system could provide Lu the solution to his multiplexing problem, he did not stop there. He wanted a memory system also capable of recording the intensity of a cellular event and even how often that event occurred. This presented a unique technical challenge for Lu's team since recording intensity and frequency would require their system to generate multiple mutations at the same location in a cell's genome, a difficult task as CRISPR normally does not do this. So Lu and his colleagues needed to figure out a way for CRISPR to record multiple mutations at the same locus as a function of the signal to be stored in DNA memory.

The solution would eventually come through another component of the CRISPR system—the PAM sequence. By adding a PAM sequence to the DNA encoding the guide RNA itself, the Cas9 protein targeted the same sequence multiple times for cleavage. This meant that those sequences would now be able to accumulate mutations over time as multiple cellular events took place.

There is more work to be done—Lu acknowledges that his CRISPR/Cas9-based cell memory is still in the early stages; he often refers to it as “Version 1.0.” “We still have a way to go to make the perfect memory system in cells,” he says. Even though they worked out the multiple mutation challenge, the number of possible mutations is still limited by the length of the guide RNA since this molecule will get shorter and shorter as deletions accumulate. This makes it harder to target the correct site in the genome over time and also harder to read out the memory using next-generation sequencing.

Yet another challenge for Lu's system could actually come from the multiplexing problem he solved for Version 1.0. Lu notes that there are reports that in some cells single cleavage events can be toxic—and the current system can cause repeated cleavage events to happen. Thus, alternative strategies for DNA writing may be needed.

Finally, there is the issue of off-target effects. Lu says that in some instances, events might not be written consistently

An example of gene editing in lamprey embryos using CRISPR/Cas9 technology. Here, pigmentation is used as a visual cue for identification of mutants. Credit: D. Medeiros.

into the DNA memory unit, and there is also some worry that off-target modifications could impact the genotypes or phenotypes of the cells.

The good news is that according to Lu, none of the current Version 1.0 issues are insurmountable. He and his team are now working on advancing the system to improve its performance, even as researchers from fields as varied as neuroscience and cancer biology express interest in applying his CRISPR/Cas9 cell memory methods in their own work.

It's hard to appreciate the rapid pace and breadth of development and innovation that has surrounded CRISPR technology in such a short period of time. One needs to step back to realize that the first CRISPR/Cas9 gene-editing articles only came out in 2012 and 2013—yet the explosion of applications

and the impact of the technology in less than half a decade has been nothing short of astounding. From saving careers to recording the inner workings of cells, the uses of CRISPR/Cas9 appear to only be limited by our imagination—and it turns out we have quite the imagination.

References

1. **Square, T. et al.** 2015. CRISPR/Cas9-mediated mutagenesis in the sea lamprey *Petromyzon marinus*: a powerful tool for understanding ancestral gene functions in vertebrates. *Development* 142:4180-7.
2. **Schick, J.A. et al.** 2016. CRISPR-Cas9 enables conditional mutagenesis of challenging loci. *Sci. Rep.* 6:23236.
3. **Perli S. D. et al.** 2016. Continuous genetic recording with self-targeting CRISPR-Cas in human cells. *Science*. 353:pii: aag0511.

Written by Nathan Blow, Ph.D.

BioTechniques 61:225-231 November 2016
doi: 10.2144/000114470

No Drip Design
For Hassle-Free Serological Pipetting

The Better Way to Pipette
Wobble-not™
Low Insertion Force Serological Pipet

2-tier step design=2 zones of contact
max stability, less wobble, better seal

Available through distributors
For more information go to www.vistalab.com/wobble-not

Free Samples! Submit request online at
www.vistalab.com/wobble-not/#get-free-sample-pack