A Conversation with Feng Zhang

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Steve Mao: Would you mind telling us about the new CRISPR [clustered regularly interspaced short palindromic repeats] system that has a great potential to be repurposed as a new tool for genome editing?

Dr. Zhang: Our work looks at the diversity of CRISPR systems. CRISPR is not a single system; there are many different types. This new system is something where a transposable element called Tn7 has, over the course of evolution, co-opted a CRISPR so that it can use the RNA targeting mechanism of CRISPR to spread itself to viruses or plasmids. By studying the molecular mechanism of this, we realized that it's a potentially programmable way to be able to introduce DNA into the genome. One of the major hurdles of gene editing is we can cut DNA, but introducing DNA into the genome in a precise way has been challenging. Using these transposable elements that are RNA-guided, there's the potential to develop a new genome-editing tool.

Steve Mao: Basically, this is a new CRISPR system that is not functioning as an adaptive immune system, but instead it's co-opted by T7-like transposons. It's RNA-guided, and it can insert a large fragment of DNA into a precise location. You've shown that it can be reconstituted in vitro, meaning you only need minimum host factors. You also showed that it can be repurposed in an *E[scherichia] coli* system. Do you think that it will work in mammalian system?

Dr. Zhang: That's a good question. We're now exploring many of these different CRISPR-associated transposase systems, or what we call CASTs. We are very hopeful that we'll have something that can work efficiently in mammalian cells so that we can use it for a broad range of applications.

Steve Mao: What specific application can this system be used for that current systems like Cas9 or Cas12 cannot?

Dr. Zhang: I wanted to have a way to be able to introduce genes into specific positions in the genome so that we can

take advantage of endogenous promoters to drive tissue-specific expression. Before working on gene editing, I worked on a system called optogenetics. Optogenetics allows us to stimulate brain cells using light, but the bottleneck of optogenetics is there are so many different types of brain cells. How do we specifically control one type of cell and not other brain cells? One way to do that is if we are able to introduce these light-sensitive channelrhodopsin protein genes into specific promoter regions so that they're only expressed in the cell type of interest. That has been a major challenge. Neurons are postmitotic, so the traditional way of incorporating DNA through homologous recombination is very inefficient. We needed a new way to do it.

One of the potential applications of CRISPR transposase is to introduce genes into specific sites. If you wanted to control parvalbumin interneurons and not perturb excitatory cells at the same time, you can use CAST to introduce channelrhodopsin into the parvalbumin promoter region. If you want to visualize a particular type of cell in the intestine, you can use CAST to introduce GFP [green fluorescent protein] into the promoter region as a unique marker for that cell type. That's one way to use it.

From a therapeutic perspective, there are also exciting applications. Many genetic diseases are caused by single-nucleotide polymorphisms [SNPs]. What that means is that within some exon of an important gene, there is a small mutation. People are working on ways to use gene editing to correct these mutations, but the way that the existing gene-editing systems work is that you have to introduce specific guide RNAs tailored for individual mutations. Even though conceptually it's all doable, from a practical standpoint in terms of developing drugs, you have to have many different compositions to target the same disease. CAST can provide an alternative approach to treat disease because if there are multiple mutations that affect the same exon, rather than fixing an individual mutation we can use CAST to incorporate an intact exon and that can address any mutations affecting the same exon. You end up with one composition that can treat a number

of different mutations in the same disease group. These are some of the exciting applications.

In agriculture, it's also very exciting: the ability to be able to introduce genes into the same region so that when you are breeding these crops, the genes don't get segregated. We can significantly increase the pace at which we can develop new crops. These are just some of the applications.

Steve Mao: You mentioned that a lot of these diseases are caused by those SNPs. You are one of the developers of the DNA and RNA base editor systems. Can you tell us something about the Cas13 RNA base editor? What's the current progress in this field and what's the future of this field?

Dr. Zhang: Another thing that we have been working on in the lab is to develop new ways to edit RNA. There are a couple of advantages to editing RNA. First of all, there are diseases that are caused by single genetic mutations. For those, the treatment strategy would be to convert that disease-causing variant back to what is found in the majority of healthy people. If we're able to do it precisely and efficiently at a DNA level, then correcting DNA makes a lot of sense.

But then there are other diseases where you may want to introduce a risk-modifying allele. For those, the thinking is a lot more complicated. Even though we know that it confers either increased or reduced risk for some aspect of a disease, those variations can often have other more complicated interactions that we don't know about. For those, putting in a DNA change is probably less ideal, because what if it causes a catastrophe and you need to reverse it? RNA editing provides that possibility. You can reverse the change.

Another really exciting way to use RNA editing is changing proteins transiently so that we can modulate cellular signaling. A number of studies have shown that if you can modulate the Wnt pathway or the Hippo pathway, you can drive regeneration in liver to get hepatocytes to grow, or we can regenerate photoreceptor cells. When modulating these proteins, you don't want the modulation to be permanent because you will probably end up with a tumor. You actually want it to be just for a short enough period of time so that you get enough regeneration, but no more than that. These are the reasons that we're developing RNA-based systems.

So far we have developed this one system that we call REPAIR [RNA editing for precise A-to-I replacement], which allows us to convert adenosines into inosines. Inosine is an RNA base that basically functions in the same way as a guanosine in splicing and also translation. That means if we can reverse specific adenosines into a guanosine-like behavior, then we can correct the protein product or the splicing result that comes from a specific variant.

We're continuing to work on other types of editors. One of the things that we have been putting the most effort into recently is making a C-to-U editor. C-to-U editors allow us to address a different set of changes, but also are very applicable for modulating protein phosphorylation states.

We decided not to use naturally existing cytosine deaminases because most of the known cytosine deaminases work on single-stranded substrates. RNA is naturally single-stranded. If you have a cytosine deaminase, it will be hard to achieve specificity on a targeted RNA.

Instead, we took a directed evolution approach. We hypothesized that maybe you can turn ADAR [adenosine deaminase, RNA-specific], which normally deaminates adenosine, into a cytosine deaminase. ADAR works on double-stranded RNA and only deaminates adenosine that's mismatched in a bubble, mispaired with a cytosine. That's how you get single-base specificity. We found out that you can actually do that with ADAR. You can turn it into a cytosine deaminase by just having a mispair with a cytosine. You get a C-C bubble. After 16 cycles of directed evolution, we were able to get a CDAR [cytosine deaminase, RNA-specific] that has a similar level of activity as a natural ADAR. We're pretty excited about that.

Steve Mao: A lot of your systems have very cool names, like REPAIR. Do you have a cool name for the C-to-U system?

Dr. Zhang: Yeah. We're calling it RESCUE: RNA editing for specific C-to-U exchange.

Steve Mao: There's another acronym from your lab. It's a system called SHERLOCK [specific high-sensitivity enzymatic reporter unlocking]. That's actually a slightly different system. It's not trying to repair, manipulate, or edit the DNA or RNA. Instead, you're trying to detect the nucleic acids. Can you tell us something about that, especially its potential for diagnosis?

Dr. Zhang: SHERLOCK is a diagnostic system that we developed by taking advantage of a property of Cas13. Cas13 is an RNA-guided RNA nuclease, but unlike Cas9, it doesn't cleave just the target nucleic acid. Once it recognizes the target RNA, it can then also go and cleave many other RNAs. What that means is that there is amplification in the nuclease activity of this enzyme. We thought maybe you can use this as a way to develop an amplifying diagnostic.

One of the latest iterations of the technology we developed is a paper strip test. You can use urine, saliva, or blood, and then you just put in Cas13 protein and the RNA guide that you designed to recognize a Zika virus or Ebola or influenza or a bacterial pathogen. Within the same reaction, there's a shorter reporter RNA that has a biotin and another molecule called FAM attached to the two ends. If Cas13 found the virus or the pathogenic sequence, it will cleave the pathogenic sequence but it will also activate this collateral activity to cleave these reporter RNAs. Then you have biotin and the FAM separated from that RNA linker.

Then you flow this reaction on a paper strip. It's not too different from a pregnancy test strip. It's got two lines. The first line has streptavidin on it, so you'll bind to biotin. Then the other line has an antibody that binds to FAM. When you flow the reaction, if the pathogenic sequence you're trying to find is not there, then the reporter is going

to be intact; the biotin stays linked to FAM. When you flow it, biotin will be captured by streptavidin, so you see one line on this paper strip. If the pathogen is present, the reporter will get cleaved. Biotin will get bound by streptavidin, but FAM is now separated from biotin. It will keep flowing and then it will get captured by the antibody. Just by seeing whether you have one line or two lines, you can get a very quick and also low-cost readout for a disease.

Steve Mao: What do you think are the biggest challenges in the genome editing or gene therapy fields?

Dr. Zhang: One of the challenges remaining is how to precisely introduce DNA. Related to that is, how do you precisely delete DNA? A lot of diseases are caused by nucleotide expansion. Huntington's disease is caused by a trinucleotide expansion in the huntingtin gene. There

isn't a good way to be able to contract those expanded regions. Generally speaking, new capabilities to manipulate DNA are still very much needed.

The second—and probably even bigger—challenge is how do you deliver these molecules into the body? So far, people have been able to do *ex vivo* cell manipulation. You take blood cells or immune cells out and they modify them and you can put them back into the patient, but you can't really do that for the vast majority of organs in the body. You can't take out the heart, fix it, and put it back. Ways to be able to deliver a therapeutic agent into the right organ with enough efficiency into enough cells, but also having enough safety so that you're not also causing toxicity in the body, is very important. Those are probably the two major challenges, but there are a lot of other smaller challenges for developing applications for many research needs.

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