A Conversation with David Bartel

Interviewer: Richard Sever

Assistant Director, Cold Spring Harbor Laboratory Press

David Bartel is a Professor of Biology, a Member of the Whitehead Institute, and an Investigator at the Howard Hughes Medical Institute at the Massachusetts Institute of Technology.

Richard Sever: You spoke yesterday about microRNAs, the small RNAs that regulate the activity of genes post-transcriptionally. You made the point that the effects of knocking down these microRNAs could be really debilitating. That was kind of a shock to me because people are always saying that it's a fine-tuning of gene expression. Can you say a little bit more about that?

Dr. Bartel: I like the term "tuning" rather than "fine-tuning" because it actually is more of a tuning. It turns out that these microRNAs do have very striking phenotypes when you knock them out, but even before we knew the knockout phenotypes, we knew that they recognized many targets. We know that there are 90 families of microRNAs highly conserved from humans to fish. Each of those 90 have on average about 400 preferentially conserved targets. That adds up to more than half of the human genes that are conserved targets of microRNAs. On average, they're targeted by four or five different microRNAs. They [the microRNAs] have these very widespread effects.

It is true that for each microRNA–target interaction, that [regulation] can be a rather subtle—maybe 20%—down-regulation, sometimes 30%, sometimes more. But because they have so many targets, when you knock out the microRNA in mice—which is what many labs have been doing; they've knocked out one or several members of the same family of microRNAs—and when they look at the phenotypes, for these 90 conserved families nearly every one of them where knockouts have been reported on, they certainly do see a phenotype. They've looked at over half of them and seen phenotypes. We already know that 15 of these microRNA families, when you knock them out, you have embryonic lethality or perinatal lethality, so that's pretty severe. And there are others that also have [other] very severe phenotypes like blindness, deafness, infertility, seizures, epilepsy, cancer, etc. There's a huge range of phenotypes—and many of them very severe—from the microRNAs. So, we know that they're playing very important roles.

Richard Sever: Are you saying that the severity of the phenotype is not because of the quantitative effect on any given gene, but just because you're hitting a whole bunch of them?

Dr. Bartel: I think that's the easiest way to think about it. In some cases, people have found single individual targets where that 50% down-regulation has a dramatic effect. But even there, they have hundreds of other targets that are conserved more than they would expect by chance. Certainly, over the course of evolution we know that biology cares about many more than single targets, and, again, each of them is regulated by a relatively small amount of tuning. So, that's our current view of what's occurring.

Richard Sever: For those of us stuck in the '90s, how does that compare with the way we think of transcription factors? Do you think that we shouldn't be singling these out as different in the way they work?

Dr. Bartel: Depending upon the transcription factor, you might also see these minor effects on many targets; it may not be that much different. There are some transcription factors that will have a 40- or 50-fold effect on the transcription of a certain mRNA, but I think, more generally, transcription factors might also have widespread effects—on the level of microRNAs—and may not be a lot different for a lot of those interactions.

Richard Sever: The way the microRNAs act is by exerting their effects on messenger RNA [mRNA]. There's two possibilities there: stopping its translation, and degrading it. You've made the point that actually it's much more of one than the other.

Dr. Bartel: What we see is that—with one exception—every place where we've looked, over two-thirds of the repression could be explained by the microRNA recruiting factors that deadenylate the RNA and then cause the RNA to get destabilized. Often, over 90% can be explained by this degradation mechanism, leaving somewhere between 10% (sometimes less) up to a third with this additional mode of repression that's through translational repression—inhibiting, presumably, translation initiation. So, in general, the majority of the effects that we see are through this mRNA destabilization.

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The exception, which is really interesting, is that in early zebrafish [embryos], you completely miss what the microRNAs are doing by looking at the changes in mRNA levels. Whereas everywhere else—it's kind of nice—you can perturb the microRNA and be able to know what the targets and the effects of the microRNAs [are] just by looking at changes in mRNA, which is much easier to do than looking at changes in protein or ribosome-protected fragments. But, in the zebrafish [embryos], that would not work at all. That was shown by Antonio Giraldez's lab—that in the early zebrafish embryo, the effects of the microRNAs could only be seen when you look at the changes in ribosome-protected fragments.

Richard Sever: That's this early phase of development in zebrafish. Is it early phases of development in other organisms or is it something unique to a particular lineage?

Dr. Bartel: It's a good question. I think that the same will probably also hold in frogs and in flies, but I'm not sure that people have done those experiments. We do understand what's going on in zebrafish, and we think—based on what we see in these other organisms—that it'll also hold there.

The effects of the microRNAs is that they recruit a protein called TNRC6 [trinucleotide-repeat–containing 6] that in turn recruits the deadenylation complexes, the Pan2–Pan3 or Ccr4–Not complexes, and that causes the poly(A) tail to get shorter. Once the tail gets to be a certain length, you get decapping and decay of the mRNA.

What we see in early zebrafish is that the microRNAs cause the tail to get shorter, and in that developmental context—in that regulatory regime—what we find is that mRNAs with short tails are translated much less efficiently than mRNAs with long tails. There's this very strong coupling in the early zebrafish, before gastrulation, where short-tailed mRNAs are translated much less efficiently than long-tailed [mRNAs]. What's interesting is that that goes away at 6 h postfertilization, which is the [time of] gastrulation of the fish. At that point, the microRNA also causes the tail to get shorter, but in that context, a short tail leads to [mRNA] degradation.

Richard Sever: Sticking with the pregastrulation scenarios, is there an a priori reason for that? Going back to this kind of tunability, if you're stopping a translation rather than degrading the RNA, does that give you more ability to tune? Is it reversible?

Dr. Bartel: I think you're onto it there. I'll just add that we see this same sort of transition in translational control in frogs and in flies, and this coupling between the length of the tail and the efficiency of translation—which is very strong in the early embryo of frogs, flies, fish—is also very strong in oocytes, and then goes away around gastrulation. The reason that we think that there is that coupling between the length of the tail and the efficiency of translation is that this is a time in development—at least up until about 3 h postfertilization—in which there is no transcription. The mRNA is all maternally inherited, and yet the embryo cells still need a way to regulate genes. They can't do it by transcription, and so the way they do it is by changing the tail length. They have this phenomenon called cytoplasmic polyadenylation, which will extend the length of the tail, and that will cause a massive increase in translation. They also have other ways of shortening the tail, so they can adjust the amount of protein output [in either direction].

Richard Sever: Do you get an oscillation there?

Dr. Bartel: Yes, [but more generally,] depending on the gene, you can just get different output at different points in early development through this polyadenylation mechanism.

So, then the question is, "Why does it [this coupling between tail length and translation efficiency] go away?" And the reason we think it goes away is that later in development, transcription is already started up, and that's a great way to regulate genes. So, you don't need this tail-length control to regulate translational output; instead, what the cells do use is transcription, and they also use mRNA stability. That's what the microRNAs are doing; they're changing the stability of the mRNAs—and that's what we see in all these postembryonic contexts.

When the cell is using mRNA stability to regulate genes, then it's not such a great strategy to only translate the mRNAs with long tails, because those are the mRNAs that just came into the cytoplasm, whereas the RNAs that had been there a long time have shorter tails, and the cell would not want to discriminate against them—it wouldn't want to do this "age discrimination" against the older mRNAs. If it's had those RNAs stable, it wants to use them; it wants to use the RNAs with short tails just as much as it wants to use those with long tails—if it's using mRNA stability as a mechanism for gene regulation. So, the idea is that when the cell switches over to this mechanism of using mRNA stability to regulate genes—which they use a lot; you have these massive differences in mRNA stability—then the cells switch away from controlling translation based on the length of the tail.

Richard Sever: When you were looking at the length of the poly(A) tails, you were discriminating between steady state levels and a better experimental approach to make sure you know what the length is and when. Can you tell us a little bit about that?

Dr. Bartel: Sure. What I described so far was in the early zebrafish embryo, and there it's not really a steady state situation. You have development happening, and you can see these tail-length differences very readily for [the targets of] the microRNAs. But interestingly, if you just look in mouse 3T3 cells—the fibroblast cells of mouse—and you're just growing cells at steady state, and you have a microRNA that you've induced that is there at very high levels, and then you just look at the poly(A)-tail lengths of the mRNA targets of that microRNA, surprisingly, at steady state, you see no difference. The distribution of tail lengths is the same whether or not the mRNA is a target of the microRNA or not. That caused us to realize that we shouldn't really be looking at steady state mea-

surements. We need to look at pre-steady state. We need to look at what's happening to the newly transcribed mRNAs and beyond and get measurements of the dynamics that you can't get at steady state.

Richard Sever: What's the timeline that you're thinking about them in discriminating the point at which steady state becomes a reasonable measure?

Dr. Bartel: At about 8 h things have pretty much approached steady state in these cells. There aren't that many mRNAs that have half-lives much longer than that. There's some, but you've pretty much approached steady state by 8 h. So, we'll do a metabolic labeling time course that will take a very early time point, and some intermediate ones, and then 8 h, and then just isolate the RNA that was transcribed over those time intervals and measure the tails. When we do that, we can see there are very clear differences in the microRNA effect; we can see that the microRNAs are clearly shortening the tails [of their targets], which we can see at these intermediate time periods.

Richard Sever: When the tails shorten, is there a threshold at which you're basically done: You're going to get degraded? What's the correlation?

Dr. Bartel: Yes. So we set out to do these experiments to look at this microRNA effect, but we can look at these more general principles of tail-length shortening, deadenylation rates, and then the rate of decay once the mRNA gets to be a short tail length. We can get these measurements, which we knew for a very small set of genes previously [from] people in the '90s, like Ann-Bin Shyu's lab and others in mammalian cells, and Roy Parker's lab in yeast. In mammalian cells, we knew from those early experiments the tail-length dynamics and mRNA-decay dynamics that are associated with tail length for [only] four mRNAs. When we realized that we had the data sets now to be able to look at this for thousands of mRNAs, that was very exciting to us. Now we can use these metabolic-labeling data sets to model—for mRNAs from close to 3000 different genes—we can model the initial tail length when the mRNA goes into the cytoplasm; what is the initial tail length at that early point? And then, how rapidly did the tails get shorter? And then, once they get shorter, how rapidly are they degraded? And we get that information now for close to 3000 mRNAs from 3000 genes.

Richard Sever: Is there a broad spectrum of behavior? Do they all look the same? Does this all happen at 30 nt?

Dr. Bartel: What we see is that the deadenylation rates for mRNAs from different genes can vary widely—a 1000-fold. There are some mRNAs that are deadenylating at 30 nt per minute, and there are others where the tail is shortening at 0.03 nt a minute: 1–2 nt per hour, on average. That's one thing we find.

What's also interesting is that once the tails reach a short length, the rate of decay can also vary a 1000-fold. The short-tailed mRNAs from genes where their mRNAs' tails get shorter more rapidly—once they reach a short tail length, the mRNA decays more rapidly. But, if the tail is deadenylating more slowly, once they've reached a short tail, they get decayed much more slowly. This is very interesting to us. What it does is it prevents the mRNAs that have very rapid deadenylation from accumulating in the cell with very short tails. Otherwise, you could have this big buildup of very short-tailed mRNAs. We just don't see that, and this explains why: It's because for the mRNAs where their tails get shorter more rapidly, they also then decay more rapidly once they reach a short tail length. That allows these huge differences in deadenylation rate—a 1000-fold difference—to impart a similarly large difference in decay rate. And so you could have a really broad spectrum of posttranscriptional behavior for mRNAs from different genes, and of course that, together with transcription and other things, is what gives us the regulation that we have.

A Conversation with Adrian Krainer

Interviewer: Anke Sparmann

Senior Editor, Nature Structural & Molecular Biology

Adrian Krainer is the St. Giles Foundation Professor at Cold Spring Harbor Laboratory.

Anke Sparmann: You were awarded the 2019 Breakthrough Prize in Life Sciences together with Dr. Frank Bennett of Ionis Pharmaceuticals for the development of antisense oligonucleotide drugs to target RNA splicing and the incredible success story of SPINRAZA, the first drug approved for spinal muscular atrophy. Can you start by telling us about this devastating disease and the molecular mechanism underlying it that you discovered?

Dr. Krainer: SMA, or spinal muscular atrophy, is a motor neuron disease. It's very severe, and it mainly affects infants and young children. There are milder forms of the disease, with delayed onset, which affect older patients, including adults. Depending on the type of SMA, it leads to progressive muscle weakness and paralysis, and it can be lethal. It's inherited as an autosomal recessive, Mendelian kind of disorder. The disease was well-characterized, and the responsible gene was identified in 1995. Sometime later—4 years or so—it became clear that a defect in splicing is related to the disease. We began to work on that because our interests in my lab have always been on RNA splicing—both the fundamental science and the relationship to disease.

There are two genes that are closely related. One is missing or defective in patients; the other gene functions as a kind of backup. It can express the correct protein, but in fairly low amounts due to the type of splicing that the transcript undergoes. So, we began to characterize that process. We weren't the ones who described this difference in splicing, but we were interested in that general problem. Between the two genes, there are very few nucleotide differences, but one in particular had been pointed out in the exon that is inefficiently spliced. So, we studied that problem: What is it about that nucleotide? What is normally being recognized in the transcript by various factors? We worked on that for a couple of years, and then we began to think about how to correct the splicing of the *SMN2* [Survival of Motor Neurons 2] RNA in order to allow the gene to produce higher levels of functional protein.

Anke Sparmann: How does this drug that was eventually developed actually work? How does it correct the splicing?

Dr. Krainer: It's a kind of drug called an antisense oligonucleotide. Those come in different modalities or "flavors," if you will. They're synthetic short nucleic acids, single-stranded. They have chemical modifications, and they can be designed to destroy the target RNA. They will home in on an RNA through base-pairing interactions, so they can be very specific. If the chemistry is designed in such a way that the duplex is recognized by endogenous RNase H enzymes, then they destroy the RNA target.

In our case, we use a different type of oligonucleotide design that binds to the RNA target by the same sort of physical chemical interactions, but it doesn't lead to its destruction. Instead, it blocks the binding of RNA-binding proteins. If you place an oligonucleotide in the correct place, then you can block the binding of a protein that affects splicing in some way. In our case, we were looking to block the binding of a splicing repressor, so that the exon that's nearby can now be recognized more efficiently by the splicing machinery. Now splicing looks more like it does in the *SMN1* gene, even though we're targeting the *SMN2* gene that is still present in patients. If you deliver the drug to the right cell types, the cell now knows how to allow this gene to express higher levels of functional protein. That's the molecular mechanism of action of the oligonucleotide.

Anke Sparmann: You were involved from the start figuring out how this works, but then also all the way through to actually drug development. What were the major challenges throughout that whole process?

Dr. Krainer: There were many. This started as a very basic science kind of effort, and we made mechanistic observations that inspired a way to try to correct the defect, and we went through successive modalities for doing that. We learned things along the way. The ultimately successful approach was a bit simpler than the way we had started. Importantly, we began to collaborate with Frank Bennett at Ionis Pharmaceuticals in 2004. We had a lot of discussions and decided to use a particular kind of chemistry and to go with the approach that I just described, which is to find oligonucleotides that will block the binding of a repressor.

We did that very systematically. There was a postdoc who joined the lab at that time, Yimin Hua, who did pretty much all the early work, the key preclinical experiments.

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We did have a lot of advice from Ionis people who were doing real pharmacology, but initially we were doing biochemistry, then cell-based experiments. Later, we set up mouse models. This took quite a number of years.

I don't know how to define when exactly we started working on this. We can put the start date when I, and my trainees, began to work on splicing, which is much earlier. On SMA specifically, we began around 2000 or 2001, with the work of a postdoctoral fellow, Luca Cartegni. The antisense screen, as it ultimately was carried out, began in 2004. The SPINRAZA molecule had other names earlier, but we published it in 2008. As things were progressing quite well, particularly when we began to do mouse-model experiments and seeing pretty dramatic results in terms of splicing and protein and phenotype, Ionis got quite serious about undertaking the clinical development and picking among the many different oligonucleotides that were effective, to look for the one that would be most specific and had minimum toxicity at high doses, etc. That's a separate effort in pharmacology, for which they had a lot of experience.

The next step was clinical trials. Those were initially sponsored by Ionis. About a year later, Biogen teamed up with them. The clinical trials were done in a variety of clinical centers and hospitals in several different countries. The key trials—Phases I through III—took about 5 years, which I think all went pretty smoothly. It ended up taking a year less than had been planned, because the results along the way were very encouraging. It was possible to complete the trials so that the patients still remained as part of an open phase extension study, beyond the original clinical trial. There are still ongoing clinical trials, but the ones that were key for obtaining the approval of the drug took about 5 years.

Anke Sparmann: Not that long ago, thinking of RNA as a drug molecule was not really out there. What were the changes that made this possible?

Dr. Krainer: It's all gradual. Like every new modality, there's a concept, and then there's the problems and reducing it to practice. There are always stumbling blocks. Typically, delivery of a new type of drug is something that requires a lot of effort. We were lucky that by the time we started working on this there were already several years of experience with antisense oligonucleotide pharmacology. They had gone through many chemistries. There was clinical experience, not so much with splice modulation, but nevertheless with the related chemistries. A lot of that knowledge—maybe more than 20 years of accumulated knowledge—is what makes these types of things possible. Monoclonal antibodies went through something similar. There was a description and one could see right away the potential, but to turn those into a drug took many years. Now, it's much more routine.

Anke Sparmann: You're looking at other diseases to target. What are you moving onto?

Dr. Krainer: Part of the lab still continues to study the basic fundamental aspects of splicing mechanisms and regulation, because the way we approach the problem is all based on insights about the mechanism. In this case, we were targeting a splicing repressor binding site. A few years earlier, we didn't even know these molecules existed, so one first had to discover that and understand something about how they work. We continue the basics, but we're also pursuing projects in which we try to apply similar or related approaches—blocking splicing components or RNA-binding proteins—in order to change splicing, and also other RNA processing, such as nonsense-mediated mRNA decay. We're exploring several potential targets that could lead to therapeutics for various diseases.

Anke Sparmann: In this basic kind of research, what is the next thing that's going to happen in splicing?

Dr. Krainer: That's moving along on many different fronts. When I started in this field, we were just doing cell-free splicing. It was all biochemistry. I started working on the development of systems for that as a graduate student. There was a lot to do, a lot of biochemistry to identify components. Of course, other labs were using genetic approaches and model organisms. Nowadays, there are many more disciplines that are contributing to understanding the whole process: quantitative approaches, bioinformatics, genomics, transcriptomics. A lot of techniques have been invented since I started in this field, so there are always new ways to revisit an approach. There are many powerful cell biology approaches, as well.

One sees steady progress on many fronts. Every once in a while, there are breakthroughs, so things advance more rapidly. The structural biology approach has had a tremendous impact in recent years with cryo-EM [electron microscopy]. Seeing snapshots of spliceosomes in action felt like the field suddenly moved forward 10 or 20 years. One could appreciate details, some of which were already known, but now you could really see it in real time. It was no longer an indirect demonstration or hypothesis. There's a lot of work that needs to be done using newer approaches like that to get new insights.

I think there will be a lot of surprises, and all these things inform how you might do therapeutics development where splicing underlies the overall approach. There are efforts to develop small molecules. It's not our work, but developments in the field to also modulate splicing, and so we need to understand better how these molecules are actually doing that. What's the mechanism of action? How specific are they, and how applicable is that approach to other targets, other splicing events? Structural insights from the spliceosome can inform those efforts and vice versa.

Anke Sparmann: Are there any problems with off-target effects of these kinds of drugs?

Dr. Krainer: Any drug obviously has that. With antisense oligos, because they're based on base pairing, you obviously have to pick sequences that are not repetitive. SPINRAZA, in particular, binds to a sequence that's unique: It's only present in intron 7 of the *SMN* genes

and nowhere else in the genome, at least as a perfect match. This doesn't rule out the possibility that imperfect binding—weaker binding, presumably—with one or more mismatches could occur. The question is, just because it binds somewhere else doesn't mean that it's going to perturb splicing or some other process, but there is that possibility. One has to be very careful about looking for adverse effects of drugs. Obviously, that's part of the whole clinical drug development.

With small molecules, it's a completely different mechanism of action. We need to understand better the few examples that we currently know of and how they're actually eliciting the changes in splicing. We understand a limited number of off-target effects and they appear to be quite specific, but maybe that can be improved. If you move to a different target, is it going to be possible to have similar specificity? That's something that the field is going to learn in the next few years.

A Conversation with Feng Zhang

Interviewer: Steve Mao

Senior Editor, Science

Feng Zhang is the James and Patricia Poitras Professor of Neuroscience at the McGovern Institute for Brain Research, Associate Professor in the Departments of Brain & Cognitive Sciences and Biological Engineering at the Massachusetts Institute of Technology, a Core Member at the Broad Institute of Harvard and MIT, and a Howard Hughes Medical Institute Investigator.

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

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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.