

A detailed 3D rendering of a DNA double helix structure, shown in a dark grey/blue color. A specific segment of the helix is highlighted in a vibrant orange color. This highlighted segment is enclosed within a white outline that forms a stylized, bracket-like shape. Small white circles are placed at the ends of the white outline's lines, suggesting points of interest or specific genetic markers.

GENE EDITING

FDA-Regulated Technologies and Applications

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Summary of the Nov. 1, 2016 workshop

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ACTIVITY OUTLINE

Gene editing is the technique by which DNA is inserted, deleted or replaced in the genome of an organism using an exogenous editing nuclease, often delivered in a gene therapy vector. The most popular technology is built around the CRISPR/Cas9 enzyme system, but there are several other alternative systems such as Zinc finger and TALEN nucleases. The technology is being developed commercially to edit human genes both to correct specific pathogenic mutations, and to create resistance to pathogens like HIV. In this regard it represents a highly personalized medicine. The technology is also being used to design animal models of genetic diseases and to manipulate plants to make them more resistant to pathogens or improve crop yield. Gene editing applications have been approved for use in the agricultural industry, and several companies are advancing rapidly towards proposed clinical trials.

The rapid development of gene editing technology creates new opportunities to develop innovative approaches in biomedical, veterinary and agriculture research, but also poses serious challenges for scientific community, regulators, industry, consumers, ethicists, and policy makers.

LEARNING OBJECTIVES

After completion of this activity, the participant will be able to:

- Describe the latest discoveries related to gene editing technologies and potential applications in biomedical, veterinary, and food industries
- Discuss gene delivery and its implications on gene modification
- Explain applications of gene editing in animals
- Discuss the use of CRISPR for basic plant biology
- Discuss the potential applications of CRISPR for crop improvement

TARGET AUDIENCE

This activity is intended for all FDA staff, including physicians, pharmacists, nurses, reviewers, and scientists.

EXECUTIVE SUMMARY

The FDA is responsible for protecting public health, by assuring the safety and efficacy of medical products and the safety of our nation's food supply. Now for the first time in history, genome editing tools such as CRISPR/Cas9 are available with the great promise of correcting genetic diseases, making xenobiotic organs suitable for transplantation, and rendering plants immune to viruses or tolerant to herbicide. Although the FDA has a regulatory framework in place for guiding gene editing tools to the clinic, at this nascent stage of development we naturally face some uncertainty around how to assess risk while maximizing benefit.

Recently, the National Academies of Sciences, Engineering and Medicine (NAS) produced a consensus study to understand risk and governance issues associated with gene editing technologies including germline approaches. Overlapping with the NAS-led activities, the Health Research Alliance (HRA) and the FDA co-sponsored a gene editing workshop at the FDA's White Oak campus. The event, part of the "New Frontiers in Science Distinguished Lectureship Program" at the FDA, was aimed at educating FDA staff, including reviewers, scientists and physicians. It was particularly unique in that it fostered scientific exchange among multiple divisions of the FDA and the scientific community engaged with human, veterinarian and plant gene editing applications.

With a philanthropic mandate, the workshop represented a neutral exchange between leading scientists and the FDA to help ensure that the FDA is prepared to respond to multiple gene editing applications with the appropriate regulatory pathways that ascertain risk and benefit. Importantly, as Jennifer Doudna concurred in her keynote, the FDA has a strong framework in place for regulating gene editing technologies though it will be important to think about how we can move forward responsibly as the tools she and her colleagues only recently discovered are now widely and internationally available. Other challenges include targeted delivery, and

efficient expression of the CRISPR/Cas9 machinery in the cells and tissues where gene editing is needed, the development of more sensitive readout approaches to detect off-target editing, and the availability of personalized and cost-effective therapies.

Workshop participants discussed methods for overcoming some of these challenges. Keith Joung discussed novel GUIDE- and CIRCLE-seq readout methods for more precisely detecting off-target editing. Matthew Porteus presented disease-specific strategies for delivering stable guide RNA *ex vivo* into CD34+ cells of sickle cell patients. Joseph Tector presented a gene editing strategy to delete multiple antigens in pigs that holds promise in reducing the risk of graft rejection in order to address the shortage of transplantable organs. Paul Nakata illustrated the removal of off-target edits in gene-edited plants via back-crossing to wild type plants in crops with short generation times.

Participants of this workshop were optimistic that some of the CRISPR/Cas9 applications currently under development, such as for sickle cell disease and kidney xenotransplantation from pigs to humans, are ready for clinical investigation. While these therapies will continue to be improved, there was consensus that we shouldn't wait for them to be perfect. We are now at the frontier of understanding how to best apply these tools for specific diseases, including what cell or tissue type to modify when, in what way, and at what frequency to achieve a durable response that collectively maximizes benefit while reducing risk.

Rusty Kelley, PhD, MBA

Member of the HRA Board of Directors and

FDA Lectureship Program Chair

Program Officer, Burroughs Wellcome Fund

AGENDA

Time	Topic	Speaker
9:00 am– 9:30 am	Keynote Introduction	Moderator: Leslie Wheelock, MS, RN
9:30 am– 10:30 am	CRISPR-Cas Genome Engineering: Biology, Technology and Ethics Keynote address	Presenter: Jennifer A. Doudna, PhD
10:30 am– 10:45 am	Break	
10:45 am– 11:30 am	Defining and Optimizing the Genome-wide Specificities of CRISPR-Cas Nucleases	Moderator: Larisa Rudenko PhD, DABT Presenter: J. Keith Joung, MD, PhD
11:30 am– 12:15 am	Nuclease and Gene delivery	Moderator: Brian Mansfield, PhD Presenter: Matthew Porteus, MD, PhD
12:15 pm– 1:15 pm	Lunch	
1:15 pm– 2:00 pm	Gene Editing for Development of Pig Organ Donors	Moderator: Khaled Bouri, PhD; MPH Presenter: Joseph Tector, MD, PhD
2:00 pm– 2:45 pm	CRISPR mediated Genome Editing in Plants	Moderator: Jason Dietz, MS Presenter: Paul A. Nakata, PhD
2:45 pm– 3:00 pm	Break	
3:00 pm– 4:00 pm	Panel Discussion	Moderator: Russell Kelley, PhD Panelists: Peter Marks, Jennifer A. Doudna, J. Keith Joung, Matthew Porteus, Joseph Tector, Paul A. Nakata, Silvana Konermann

GENE EDITING: FDA-REGULATED TECHNOLOGIES AND APPLICATIONS

FDA Opening Statements

The objectives of this workshop were to discuss gene editing technologies including the CRISPR system and their potential applications in biomedical, veterinary and food industries including crop improvement, the FDA's **Leslie Wheelock** said in her introduction.

There are several different approaches to gene editing, including one that uses zinc finger nucleases and another that uses TALEN nuclease, but the workshop focused on the CRISPR/Cas9 approach, which is currently most popular and has shown the most promise in recent years. As a tool, CRISPR/Cas9 is a major advance, said **Peter Marks**, who directs the FDA's Center for Biologics Evaluation and Research. Recalling the days when he spent a lot of time making transgenic mice using homologous recombination, Marks said homologous recombination is to CRISPR/Cas9 like "slide rule is to an advanced electronic calculator. It is quite an amazing advance."

Marks said desirable characteristics of gene editing tools include efficient delivery and targeted and long-lasting expression, while undesirable features include immune responses and off-target editing, expression or insertional mutagenesis. From a regulatory standpoint, he said "we will have to be very careful about" off-target cleavage, adding that it's important to understand what insertions or deletions are introduced into a cell or organism by gene editing.

The FDA, Marks said, intends to take a science-based approach to better understand these technologies and to be able to do a risk/benefit analysis. "We expect to be relatively inundated with submissions in the coming years based on these technologies," he said. "There's probably no such thing as a perfectly safe technology like this, but with benefit/risk [analysis] I think we can hopefully work our way through." To do so, he said, the FDA hired five principle investigators who will study things like off-target effects and how to consistently manufacture gene-edited products.

CRISPR DISCOVERY AND DEVELOPMENT

Keynote: Jennifer Doudna

In her keynote talk, CRISPR co-inventor **Jennifer Doudna** (UC Berkeley) recalled what led her to propose, in a paper in 2012, that CRISPR/Cas9 could be used as a tool for programmable gene editing. It started, she said, 10 years ago with a project to understand how bacteria fight viral infections. In 2005, Jill Banfield, who is also at UC Berkeley and studies the sequences of bacteria that are difficult to culture, told her about findings of unusual bacterial sequences called CRISPRs that are somewhat repetitive and contain spacer sequences that correspond to sequences found in the kinds of viruses that infect these bacteria.

Could it be, Doudna wondered, that bacteria kept some kind of genetic record of the viruses they had been exposed to so that they could fight them once they get reinfected? Indeed, this turned out to be the case: Once a virus injects its DNA into a bacterial cell, the viral DNA is integrated at the CRISPR site and transcribed into an RNA. This “guide” RNA then binds to a protein called Cas9 and guides Cas9 to bind to and cut the matching sequence in the viral DNA, which is chopped up and destroyed, halting the bacterial infection.

Together with Emmanuelle Charpentier’s lab, Doudna then developed a programmable two component system—the “guide” RNA matching a 20 base pair target sequence and the Cas9 enzyme—that could be deployed in cells to cut DNA molecules at target sites that are determined by simply changing the guide RNA sequence.

Once DNA is cut in an animal or plant cell, the cell repairs the cut either by nonhomologous end joining (NHEJ) or by homologous recombination. Each of these two repair pathways results in different kinds of edits: NHEJ rejoins the DNA ends in a way that can result in insertions or deletions and is often used to knock out a gene, whereas in homologous recombination, the cell uses another DNA as a template for repair. Homologous recombination can therefore be used to replace genes and make more precise edits, such as introducing point mutations, by supplying an appropriate donor DNA.

Challenges, Doudna said, include controlling which of these pathways a cell uses to repair the breaks; making sure that the cuts happen only at the intended sites; and especially delivery, i.e. how to get the CRISPR/Cas9 machinery into cells and tissues. Delivery is a “big, major challenge,” she said. “If you can’t deliver it, you really can’t ever get started with it in the clinic.” In some cell types like astrocytes, for example, Doudna and her colleagues found that it’s hard to get editing to work, perhaps because the CRISPR/Cas9 machinery can’t get into the cells or because it can’t access the DNA once inside.

To improve delivery, Doudna’s lab modified the surface of the Cas9 protein to enable it to enter cells more easily. Delivery can also be improved by using a higher concentration of Cas9: When injecting mouse brains with CRISPR/Cas9, a larger volume of mouse brain tissue gets edited if Cas9 is more concentrated.



As for applications, CRISPR/Cas9 now enables researchers to genetically manipulate animals where such manipulation hasn't been possible before, Doudna said, adding that one example is work by Michael Perry at NYU, who edited the butterfly genome to change the animal's wing patterns. "This is going on with many different types of organisms," she said. "That's been a very exciting development."

As for humans, she said clinical trials involving Cas9 have already been approved in the U.S. and China to enable the immune system to destroy cancer cells [see for example here: <https://www.chemistryworld.com/news/fears-that-gene-editing-cancer-trials-are-premature/2500206.article>] "Those trials," she said, "are a harbinger of probably a lot of future trials that will be targeting other types of diseases as well."

CRISPR/Cas9 now also enables researchers to introduce transmissible new traits into the germ line. This means that new mouse strains can now be created in a matter of weeks, Doudna said, adding that an early 2014 report of CRISPR/Cas9 germ line editing in monkeys [[http://www.cell.com/abstract/S0092-8674\(14\)00079-8](http://www.cell.com/abstract/S0092-8674(14)00079-8)] prompted her to begin discussing the possibility of germ line editing in humans to correct mutations that cause disease. The fact that such changes would be transmitted to future generations "of course raises a lot of ethical questions," she added.

As for regulation, Doudna mentioned that in the spring of 2016, the USDA announced that CRISPR/Cas9-edited mushrooms can be sold and grown without any regulatory oversight because they don't contain any foreign DNA elements and are therefore not considered to be genetically modified. "This has triggered a lot of discussion globally about, how do we define genetically modified organisms?" Doudna said. (The FDA, in contrast, released draft guidance in January 2017 [<https://www.fda.gov/AnimalVeterinary/NewsEvents/CVMUpdates/ucm536949.htm>] that it intends to regulate edited animal genes as drugs.)

In general, Doudna said the U.S. "already has a very good framework in place for regulating these sorts of technologies," adding that internationally, it will be important to "think about how we can move together responsibly" given that it's "hard for regulation to be global, may be impossible." The fact that the technology is easily available and widely deployable "makes it both wonderful but also really challenging from a regulatory perspective," she said.

Asked if she was aware of any public awareness campaigns to educate congress or the public, Doudna mentioned the Personal Genetics Education Project (pged.org) at Harvard Medical School and the Bay Area-based Innovative Genomics Institute (innovativegenomics.org). She's also doing workshops herself, she said, for students and for people from companies who are looking for hands-on experience in a lab.

OFF-TARGET EVENTS

Keith Joung

Keith Joung (Massachusetts General Hospital and Harvard Medical School) discussed his research trying to assess and reduce off-target editing activity of CRISPR/Cas9. One risk of genome editing with CRISPR, Joung said, comes from off-target cuts, which tend to happen at sites that resemble the target sequence and in some cases might cause cancer by inducing mutations. Ideally, Joung said, any strategy to define off-target events should be genome-wide in scope, unbiased as to where to look in the genome, and as sensitive as possible.

Studies suggest that off-target mutagenesis can even occur at sites with as many as six mismatches to the target site, which means that in theory, up to hundreds of thousands of off-target sites could potentially be affected in the entire genome. Many researchers use software to predict where off-target mutations might occur. The limitation of these programs, however, is that they often only check a limited number of sites and are biased in that they assume that off-target sites will closely resemble the on-target sequence, Joung said.

So Joung and colleagues developed a less biased genome-wide approach called GUIDE-seq (genome-wide unbiased identification of double-stranded breaks enabled by sequencing; see *Nat. Biotechnol.* **33**, 187, 2015: <http://www.nature.com/nbt/journal/v33/n2/full/nbt.3117.html>), where they take a cell population and introduce a guide RNA with a certain target sequence and the Cas9 enzyme and let it make cuts. They also introduce so-called oligonucleotides (short DNA pieces of a known sequence) that can insert anywhere where a break occurred. This enables them to sequence all places in the genome where Cas9 actually cut the DNA. Comparison with a reference genome then makes it possible to check where these cuts generated a mutation. Because this approach looks at a group of cells, it can even tell whether certain off-target edits happen in more cells than others, by counting them.

When Joung and colleagues performed GUIDE-seq with ten different guide RNAs, they found that some led to dozens of off-target cuts (typically ones that targeted more repetitive sequences), while others caused fewer than a dozen and one even no detectable off-target cuts. Importantly, while off-target cuts still occurred in sequences with as many as six mismatches, GUIDE-seq identified far fewer than the tens of thousands of potential sites with that many mismatches across the genome. On the other hand, it identified sites that software programs hadn't predicted. This, Joung said, suggests that it's hard to theoretically predict off-target sites, which is why an experimental approach like GUIDE-seq is better than software programs, at least for now.

Using GUIDE-seq as a readout, Joung and colleagues made versions of Cas9 that cut DNA at fewer off-target sites, by attempting to reduce its nonspecific binding strength to DNA. When they mutated four Cas9 amino acids that interact with DNA in a nonspecific way, they found that most off-target sites seen with the wild-type Cas9 disappeared. This doesn't necessarily mean there were no off-target sites, but rather that these sites show no evidence of off-target cleavage as judged by GUIDE-seq.

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In vitro methods can detect off-target cuts that are even rarer, Joung said, in part because one can add more Cas9 and target genomes than when working inside cells. *In vitro* approaches also avoid the problem that in some cell types like CD34+ cells, GUIDE-seq doesn't appear to work efficiently, at least according to reports by other researchers. So Joung and colleagues developed an *in vitro* approach they call CIRCLE-seq (see *Nat. Methods* **14**, 607, 2017: [<https://www.nature.com/nmeth/journal/v14/n6/full/nmeth.4278.html>]). It involves randomly fragmenting genomic DNA, circularizing it, removing any residual linear DNA, and then treating—and cutting—the circles with CRISPR/Cas9. Any Cas9-induced cut will linearize these circles, allowing the researchers to select only the linearized DNAs for sequencing. This greatly reduces background compared with other *in vitro* methods (like one published in 2015 called Digenome-seq), because sequences not cut by Cas9 are discarded, Joung said.

And indeed, CIRCLE-seq seemed more sensitive than GUIDE-seq, Joung said: It could detect off-target sites GUIDE-seq couldn't, and these were generally sites with more mismatches that were cleaved less often than the sites found by GUIDE-seq, suggesting they were even rarer. At least some of these rare off-target sites were affected in cells as well.

So in practice, Joung suggests a combined approach: First identify all off-target sites *in vitro* with the CIRCLE-seq approach and then confirm them in the cells that will be used for the experiment. Checking for off targets in the same cell type as the one that will be used in the experiment or the gene therapy is important, Joung said, because he observed that different cell lines can have different off-target cuts.

CLINICAL APPLICATION

Matthew Porteus

Matthew Porteus (Stanford University) discussed his progress applying CRISPR/Cas9 to develop a gene therapy for sickle cell disease, one of the most common of at least 6,000 diseases that are caused by mutations in a single gene, which makes them ideal genome editing therapy targets.

Sickle cell disease is caused by a mutation in the beta globin gene, which leads to an abnormal hemoglobin molecule and abnormally shaped red blood cells. Because these cells only live for about 100 days before they are replaced, long-term correction of the defect requires correcting the CD34+ hematopoietic stem progenitor cells (HSPCs) in the bone marrow that give rise to the red blood cells, Porteus said.

If a matched sibling donor without the disease is available, CRISPR/Cas9 is not necessary, because replacing the patient's bone marrow with the intact bone marrow from the matched donor can cure the disease in most cases. But more than three quarters of patients don't have such a donor, which is why it's important to develop a way to correct the patient's own CD34+ hematopoietic stem cells, for example with CRISPR/Cas9.

One challenge, Porteus said, is that CRISPR/Cas9 editing barely works in CD34+ HSPCs, apparently because introducing naked DNA encoding the CRISPR guide-RNA and the Cas9 enzyme into these cells induces a so-called type I interferon response that makes the cells sick or sometimes even kills them. "[The] cells see it as an invading pathogen," Porteus said.

So instead of DNA, Porteus and colleagues introduced guide RNA and RNA encoding Cas9 (or a “ribonucleoprotein” or RNP containing the guide RNA bound to the Cas9 protein), directly by electroporation into CD34+ cells *ex vivo* (*Nat. Biotechnol.* **33**, 985, 2015). In addition, they chemically modified the guide RNA to make it more stable against degradation. This way, they were able to achieve efficient gene editing in CD34+ HSPCs, Porteus said, adding that using Cas9 protein instead of RNA encoding Cas9 further reduced off-target activity. (Another advantage of using an RNP is that there is no DNA that might integrate randomly into the genome, Joung said during the panel discussion.)

However, to introduce the point mutation required to repair sickle cell disease, Porteus still needed to introduce a donor DNA template with the correct sequence as a template. To do so without using naked DNA, the researchers used adeno-associated virus 6 (AAV6) as a vehicle to get the DNA template into the cells. And indeed, they were able to successfully integrate five different genes into the beta globin locus of about 17% of the CD34+ HSPCs.

Porteus said the mutation remained stably integrated into the target genome for several months after transplantation of the edited cells into mice and even after another transplantation into a second group of mice. This was also the case when the researchers edited CD34+ HSPCs from sickle cell disease patients.

The approach resulted in 50% cells that had at least one allele changed and 20% cells that had both alleles corrected. For sickle cell anemia to become a problem, both alleles have to have the mutation, and 84% of red blood cells that developed from the edited cells had the normal Hemoglobin A, while only 16% had the abnormal Hemoglobin S. If these cells were in patients, they'd be considered cured, Porteus said, because people with Hemoglobin S percentages below 30% are considered cured.

Porteus believes he is ready to design an initial clinical safety trial with this approach, initially in young adults, although the ultimate goal would be to treat babies. "We are hoping to file our pre-Investigational New Drug [application] in the next couple of months and hopefully starting the clinical trial in 2018," he said, adding that sickle cell disease might become the first disease caused by a mutation that will be treated with CRISPR/Cas9 in humans.

It will be important, he added, to make the therapy affordable not only for the 100,000 sickle cell patients in the U.S. and Western Europe but also for the millions of people with the disease in sub-Saharan Africa. "How are you going to reduce the cost so that this can get to the patients who might be living on several dollars a week?" he asked. "I think that that's a real challenge."

One big advantage when working with blood cells, Porteus said, is that they can be edited *ex vivo*, allowing more precise control of delivery and off-target effects than gene editing of tissues *in vivo*.



Porteus noted that off-target activities of CRISPR/Cas9 are lower than what he observed with other commonly used gene editing methods that involve TALENs or zincfinger nucleases. What's more, he added, such off-target activities are actually the smallest mutation-generating factor compared with other factors we are often exposed to or that are involved when preparing cells for treating a sickle cell disease patient. The ideal number of edited CD34+ cells that need to be reintroduced into a sickle cell patient would be about 10 million cells per kilogram, which is 500 million cells for a 50 kilogram person. With that many cells, Porteus said, the Cas9 nuclease might generate 10-150 million off-target insertions or deletions.

While that sounds like a lot, a body CT scan is thought to generate one double strand break per every three cells, so assuming it affects half of the 20 trillion cells we have in the body, that alone could generate trillions of mutations.

Other mutation-generating factors Porteus mentioned include:

- Estimated number of mutations per day in every person just from cell division: 40 trillion
- Estimated number of mutations from growing the cells that will go into the sickle cell patient in a high oxygen environment, where they undergo 1-2 cell divisions: 6,400 million
- Estimated number of mutations from cytotoxic chemotherapy of the sickle cell recipients to prepare their bodies for the gene-edited cells: 1,600 trillion

What's more, Porteus said, the expected mutational load of another gene editing approach that's already in the clinic is higher than the number of Cas9-induced off-target mutations: Lentiviral-based genome modifications of hematopoietic stem cells likely cause 500 million insertions, assuming 500 million cells are inserted into a 50-kilogram patient.

In fact, "it's not easy to make cancer," Porteus said, perhaps because cells have a lot of mechanisms to protect themselves from becoming cancerous. For example, when his lab tried to induce cancer by creating translocations in an oncogene called MLL, only a small fraction of cells developed leukemia, and Li Fraumeni syndrome patients who have one mutated copy of the p53 tumor suppressor gene only develop cancer after years.

"How are you going to reduce the cost so that this can get to the patients who might be living on several dollars a week?"

XENOBIOTIC APPLICATIONS

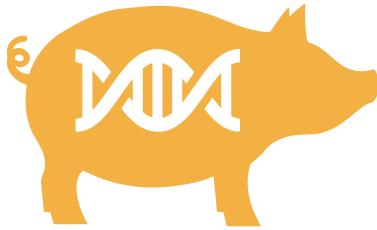
Joseph Tector

Joseph Tector (University of Alabama at Birmingham) discussed his work using gene editing to generate pig organs for transplantation into humans. [see Dec 1, 2016 article in *The Wall Street Journal*: [<https://www.wsj.com/articles/potent-fix-for-transplant-shortage-genetically-modified-pigs-1480604400>]

There is a great need for the approach, he said: More than 100,000 people currently need a kidney in the U.S., and about a quarter of the people in need die before they can get one.

Pigs are a good source for donor organs, but humans make anti-pig antibodies that could cause rejection of pig organs. Selecting recipients with low antibody levels helps: When Tector and colleagues transplanted pig kidneys to rhesus monkeys with high antibody (IgM and IgG) levels, the transplant only survived for 6 days, while it survived for up to 310 days in monkeys with low antibody levels.

Gene editing can be used to remove xeno antigens that eventually lead to this graft rejection, and Tector and colleagues focused on editing out three glycans responsible for this: Galactose alpha-1,3 galactose (alpha Gal, which can be disrupted by eliminating the swine GGTA gene); N-Glycolylneuraminic acid (Neu5GC, which can be disrupted by eliminating the CMAH gene); and beta 1,4 N-acetylgalactosaminyl transferase (which can be disrupted by eliminating the beta4GalNT2 gene).



To create engineered pigs without the genes for the three glycans, the researchers introduced the DNA plasmids encoding the appropriate guide RNAs and Cas9 into pig liver cells with electroporation. They transferred the nuclei of edited cells into an enucleated oocyte, which they placed into a pseudo-pregnant female pig. After 32 days, they terminated the pregnancy, collected and genotyped the fetuses and transplanted the nuclei of the ones that had all three genes knocked out to another enucleated oocyte, which they placed into another pseudo-pregnant female pig to generate the triple-knockout pigs. It takes 135-150 days to generate pigs with multiple genes knocked out, Tector said.

To test the approach in rhesus monkeys, the researchers transplanted kidneys from pigs where two of these antigens were removed (removing Neu5GC wasn't necessary because monkeys also have that gene so their immune system is used to the corresponding antigen). They found that one of the transplants lasted 434 days. When they mixed blood cells from the triple knockout pigs with human serum, they found that most people with low antibody levels were cross match negative, which means that they didn't have elevated antibody responses to the pig cells.

This suggests that the triple knockout pig kidneys could be used for such recipients, and Tector believes he is ready to start a clinical trial with such people. He said he'll probably start with people on the kidney transplant wait list who are at least 65 years old, because their life expectancy on dialysis is typically less than the 8-10 years they usually have to wait until they eventually receive their transplant.

However, about 20% of the population has antibody levels that are too high for them to be able to handle the triple-knockout pig kidneys. These include women who had several babies and developed antibodies to their husband, or people who developed antibodies after they received blood transfusions. For such people, it will be necessary to remove the MHC in the pig genome, something Tector is already working on.

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CROP SCIENCE APPLICATIONS

Paul Nakata

The final speaker, **Paul Nakata** from the USDA Children's Nutrition Research Center in Houston, Texas, discussed manipulating the plant genome, with emphasis on CRISPR/Cas9-mediated genome editing. Dating back thousands of years ago, traditional and then mutational breeding was used to influence, for example, the size of crops including tomato, corn and wheat. While these age-old mechanisms of crop selection are effective at stacking useful traits, gene editing technologies such as CRISPR/Cas9 can be used to more thoroughly determine which genes encode important traits and to make better crops where such genes are changed, Nakata said. This is important given that experts predict that we need to double crop production by the year 2050 to meet growing demand.

One example is cold tolerance, which determines when and where certain crops can be grown. About 20 years ago, researchers screened a protein library made from the plant *Arabidopsis thaliana* for factors that could bind a promoter of cold-inducible genes. They found a protein called C-repeat Binding Factor 1 (CBF 1) that bound, and identified two related, similar proteins called CBF 2 and 3. The genes encoding all three were later shown to be inducible by cold.

This made them strong candidates for genes required for cold tolerance, but until recently, Nakata said, researchers couldn't find knockout mutations for each of them to test whether this was the case. "They were stuck," he said, adding that CRISPR/Cas9 finally enabled researchers to knock out each of these genes and show that only when all three are knocked out does a plant lose its cold tolerance. "[This] illustrates how this technology is changing what's possible in the plant sciences in terms of gene discovery," Nakata said. These recent studies also identified new roles for CBF, in that the triple knockouts showed reduced seed germination and salt tolerance (*Plant Physiol.* **171**, 2744, 2016).



A second example Nakata mentioned is herbicide tolerance. This is important, Nakata said, in that by 2012, most U.S.-grown soybean, corn and cotton plant varieties were herbicide tolerant so that they can be grown in the absence of weeds. Some herbicides inhibit ALS, an enzyme that's important for synthesis of certain amino acids and is only found in plants. CRISPR/Cas9 has given researchers a way to change the ALS gene to render plants resistant to such herbicides. To do so, they mutagenize plants, spray them with herbicide and sequence the ALS genes of the survivors to find which mutations made them resistant. CRISPR/Cas9 can then be used to replace the endogenous ALS gene with a version containing the mutation(s) by co-introducing the appropriate donor DNA. This has been done with rice, corn and soybean plants, Nakata said.

A third example for the use of CRISPR/Cas9 in plants is rendering them immune to Gemini viruses. Infection with these viruses harms many different crop plants including tomato, cassava, eggplant, cotton and cucumber. Gemini viruses are transmitted by insects and form a double-stranded DNA intermediate in infected cells that serves as the basis for viral replication. Recently, researchers used CRISPR/Cas9 in plants such as cucumber to introduce breaks in this double stranded Gemini virus intermediate to inhibit viral replication. In one study, researchers targeted sequences shared by three different viruses with one guide RNA, Nakata said. Researchers have also used Gemini viruses as a tool, Nakata said: They recently improved gene editing efficiency in plants tenfold, by using the Geminivirus replication machinery to amplify a DNA encoding the guide RNA and Cas9.

Importantly, CRISPR/Cas9 enables researchers to mutagenize an endogenous gene like ALS without introducing foreign DNA, Nakata said. This is because the DNA with the CRISPR/Cas9 machinery integrates at a different place of the plant genome than the target site that's edited. It can therefore be crossed out of the plants by back crossing them with wild type plants, leaving behind only the edits in the endogenous gene, whereas the previously used strategy to create transgenic plants left behind foreign DNA. This back crossing also enables researchers to remove off-target edits, Nakata said, which is why such edits are less of a concern in plants. "In most cases we can back cross to remove any secondary mutations that might have occurred," Nakata said.

While these age-old mechanisms of crop selection are effective at stacking useful traits, gene editing technologies such as CRISPR/Cas9 can be used to more thoroughly determine which genes encode important traits and to make better crops where such genes are changed.

One limitation is that crossing out the CRISPR/Cas9 elements is only easily possible in plants with short generation times such as rice, wheat, soybean and corn, while it's more difficult in plants with longer generation times like avocado, and not possible in plants that are propagated asexually such as potatoes.

In such cases, however, one can still avoid introducing foreign DNA into the plant by introducing an RNP version of CRISPR/Cas9 into plant cells after enzymatically degrading their cell wall. That creates another challenge, however: Regenerating the cell wall and growing plants from such transformed cells has only been done with a handful of plants so far, Nakata said, adding that applying this approach to the major crop plants “would be a really big step forward.”

Another limitation of CRISPR in plants is that it often takes a long time (6-12 months) to regenerate plants from edited cells. “If we could reduce this [time], [it] would be a great help,” he said.

PANEL DISCUSSION

Summary

The **panel discussion** touched on several aspects. One was the extent of easy access to CRISPR technology. For example, organizations like the Cambridge, MA-based non-profit Addgene have made it very easy to exchange research materials, Doudna said: For U.S. \$65, she said, one can get access to these tools from anywhere in the world.

That doesn't mean it's easy to do something with it, Joung said, because getting the components into a cell or an organism still requires some additional expertise. "I think things have gotten dramatically easier in that first step" of making the nuclease, Joung said, "but I still think you need to have some expertise in order to be able to do experiments with it." Still, Porteus added, one worry is that this could be done in secret in some underground lab by people trained in molecular biology at the Master's level.

As for regulation, Porteus said he didn't think the FDA needs any new regulatory structures to analyze and assess these new technologies, with one exception, he added after the workshop: The FDA should be given the authority to evaluate potential heritable editing if that gets developed. "Right now, law says that the FDA can't even evaluate such a protocol, which I think risks driving the process underground," he said.

One important issue is educating the public about the technology, something Joung has been involved in through the American Society of Gene and Cell Therapy. The society is putting together an educational white paper and is reaching out to government representatives to educate them about the technology, he said. Doudna said she recently did a six-hour workshop at UCSF where the participants, technologists, did a gene editing experiment and used a simple method to detect the edited DNA.

She is now working to turn this into a two-week workshop for college students and even wants to do it in high schools. Joung said he has organized a lecture- and lab-based course where he taught CRISPR to graduate students, and Tector said he had four high school students create different strains of pigs.

What's more, discussion of the technology in popular culture "can actually be a great way to engage the public in understanding and thinking about science and the importance of science in our society," Doudna said, "even if we are making up stories, as long as there is a basis in fact." For example, Jennifer Lopez will produce a CRISPR-based TV series called C.R.I.S.P.R. (see <http://www.sciencemag.org/news/sifter/jennifer-lopez-set-produce-nbc-bio-terror-drama-crispr>). "These are not bad things, it draws attention," said Joung. "I think there is the potential for good, but we do need to keep up the education and making sure that there is an accurate understanding of what the technology can and can't do."

As for the risks of cancer being generated by off-target effects, Joung said, a personalized assessment of off-target effects in each individual might become important before performing gene therapy in the future, given that different cell lines can show differences in off-target effects. That's not a reason to not develop CRISPR/Cas9-based gene therapy approaches now (even without personalized assessment) as soon as possible, he added.

Still, Doudna said, off-target prediction will improve and off-target effects in cell lines are likely more serious than what might be observed inside the body where Cas9 and guide RNAs are less concentrated. "I think a combination of looking in actual primary cells as well as algorithms for good target prediction is going to go a very long way towards ensuring that you have the lowest possible levels of off targeting," she said.

Another potential risk, immune responses to the gene editing machinery *in vivo*, Porteus said, could be minimized by expressing the CRISPR/Cas9 system only transiently, which would also further reduce off-target effects. “A transient way of expressing the CRISPR/Cas9 system *in vivo* is I think going to be essential,” Porteus said.

Because any issues like cancer resulting from insertion into a gene might take years to develop, Porteus said gene therapy samples should be banked so researchers can go back in case something goes wrong to look for early signals that indicate potential problems to prevent them from happening again. Tector said he has plans for a tissue bank to store tissues, adding that in the case of problems after kidney xenotransplantation from pigs to humans, it’s always possible to remove the graft and put the person back on dialysis. Still, he noted, we shouldn’t wait until the therapy is perfect. “We should strive to introduce these therapies and then continue to work on them and improve them,” Tector said. “If you think you’re going to have no risk and have it be perfect, then we’re never going to implement any of these things.”

Public acceptance of the technology, Doudna said, differs between people with genetic diseases, almost all of whom want this technology as soon as possible, versus people who think someone might be manipulating their food with the technology in ways that might not be transparent. “Even though it’s exactly the same technology,” she said, “in one case people view it as something very positive, and on the other hand they view it as something very negative or dangerous.” She said she hopes that explaining that this is the same technology in both cases might enable people to make an informed rational decision about it.

As for commercialization, Porteus found it encouraging that big companies are active in the gene therapy space. He said they seem to believe that they need to develop expertise in the technology and the regulatory path by addressing rare diseases first, even though that's not the way they will make money. He added that the three genome editing companies Intellia, Crispr and Editas probably already raised U.S. \$500 million to 1 billion to bring CRISPR-based therapeutics to patients.

As for challenges, delivery—a highly specific and efficient way of getting it only into the cells that you want it to get into—is going to be absolutely key,” Joung said. Beyond that the hardest part, Porteus said, isn't improving genome editing itself anymore, but working on the applications. “The genome editing mechanics [have] gotten streamlined enough with enough people doing it,” he said. “The hardest part is to figure out for your disease, what cell type do you need to modify, at what frequency, in what way, when in development, [and] how long will that last? That is expertise that comes from a deep understanding of the specific disease. The challenge is actually taking the tools we have and applying them and pushing them as far as they can go for that disease.”

“The challenge is actually taking the tools we have and applying them and pushing them as far as they can go for that disease.”

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