EXPANDING CRISPR CAPABILITIES

How CRISPR technology has advanced HIV, mosquito-borne illness, and cardiovascular disease research

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Knocking out HIV: Two Approaches, One Goal

By Kathryn Loydall, PhD

News spread rapidly in July of 2019 that researchers had eliminated human immunodeficiency virus (HIV) from the genomes of animals for the first time. The accomplishment originated in the laboratories of Howard Gendelman from the University of Nebraska Medical Center (UNMC) and Kamel Khalili from Temple University, Pennsylvania.

Khalili and Gendelman combined forces to use a long-acting therapy to reduce the HIV viral load, followed by gene-editing to remove HIV from infected cells. With this approach, they hoped to come up with a better treatment for those suffering from the viral infection. Even so, Gendelman viewed his data skeptically when initial results showed that some of his HIV-infected humanized mice were no longer infected. “So we repeated the experiments,” he recalled. “After we saw it two or three times, we started incrementally believing that we had stumbled on something that might be important.”

Gendelman and Khalili are among the first researchers using CRISPR-Cas9 to approach HIV. Their efforts focused on removing integrated HIV viral genomes from the cells of animals. Just two months after a paper describing their work was released, another prominent team in the CRISPR-HIV space also announced their results. Hongkui Deng and his team from Peking University in Beijing, along with Hu Chen from the 307 Hospital in Beijing, approached HIV in a different way. They used CRISPR-Cas9 to engineer human stem cells to mimic natural immunity to the virus.

In collaboration with his UNMC colleague Benson Edagwa, Gendelman’s team developed a therapeutic strategy known as long-acting slow-effective release antiretroviral therapy (LASER ART). This approach relies on antiretroviral nanocrystals to keep HIV replication at low levels for a longer period of time than traditional ART. LASER ART targets viral sanctuaries, decreasing the need for typical ART administration. Separately, Khalili’s team developed a novel CRISPR-Cas9 therapy for directly removing HIV DNA in vivo from cells where the viral genome had integrated, which worked after LASER ART had reduced the amount of HIV.

Neither Gendelman’s LASER ART nor Khalili’s gene-therapy independently eliminated HIV from mice. But together, the technologies eliminated HIV infection in more than one third of treated mice. The results show “simple proof-of-concept” that HIV can be eliminated using the dual therapy, Gendelman said.

The goal, of course, is to eventually bring this treatment into the clinic. But the team has a long road ahead before that becomes a reality. What was possible in a humanized mouse (a mouse carrying human bone marrow to imitate the human immune system) treated with an abundance of a vector delivery system may not translate to humans. Researchers will have to conduct extensive testing to determine potential toxicity, develop a delivery system that meets the needs of the human body, and balance the volume of the delivery system with the necessary level of HIV excision.

When HIV is present in the body in a latent state, it usually does not produce symptoms, but it can reactivate at any time. Scientists have to eradicate nearly all—if not all—of the latent virus for genetic HIV therapy to be effective. CRISPR treatment has to be very sensitive and very specific. Khalili’s team designed a CRISPR system that targeted every cell in the body—the majority of which were not infected. This may not be suitable for humans because of the sheer volume of therapy that would have to be administered.

Humanizing HIV Treatment

Because HIV integrates into a host cell’s genome, Khalili and Gendelman approached the problem by viewing the virus as if it were a genetic disorder. “We’re cutting it out,” Gendelman said. “If we are overzealous and deliver a tremendous amount of the excision vectors—in this case we’re using an adeno-associated viral vector—we can cut out HIV to the point that the disease can be eliminated. The disease can be cured.”
The team will also need to explore the possibility of off-target effects. “Even though we didn’t see off-target effects in the mice, it doesn’t mean that we would not, or could not see that in a human. It’s still possible,” Gendelman explained.

Reducing the Burden, Honing the Delivery

To solve these problems, Gendelman’s team plans to alter the ART delivery scheme; delivering the drugs more effectively across cell membranes will reduce latent virus and HIV replication capacity. His team has already gained traction with “next generation LASER ART.” Meanwhile, Khalili’s team is further developing the CRISPR-Cas treatment to target only cells that contain latent virus, with a focus on systems that can target common receptors on CD4+ cells. The team is also looking at new CRISPR delivery systems with greater carrying capacities.

Their next step is to look beyond obvious targets, such as common HIV receptors, to find targets for CRISPR that interfere with the latent HIV reservoir, such as co-transcriptional factors and regulatory factors. CRISPR-Cas doesn’t necessarily have to work only on HIV excision—it could work on targets that enhance, extend, or sustain proviral DNA content to excise genes in the host cell that affect that latent HIV reservoir. “Instead of going after the first violin, which is the major part of this symphony, you go after the horn, or the percussion, or other parts of the orchestra that are affecting the latent virus,” Gendelman said.

A Different Slant

While Gendelman and Khalili’s research focuses on direct administration of CRISPR to a patient, Deng’s research concentrates on treating stem cells with CRISPR-Cas to mimic natural immunity to the virus. Deng’s research was inspired by the “Berlin patient,” a man suffering from both HIV and a lethal blood cancer. The man received a bone-marrow transplant in 2007 to treat cancer; this treatment also cured the HIV infection, rendering him the first in the world to be cured of HIV. On a whim, the Berlin patient’s treating doctor had looked for a donor carrying two copies of CCR5 with a delta 32 mutation. Certain strains of HIV use the CCR5 receptor to gain entry to white blood cells, so people carrying the mutated CCR5 receptor are nearly immune to HIV. Deng was a member of the team that discovered this receptor in 1996.

In 2011, Hu Chen from the 307 Hospital in Beijing contacted Deng. Chen had spent decades researching hematopoietic stem cell (HSC) therapy for treating leukemia, and hoped that Deng would collaborate with him to modify HSC therapy to treat HIV. Deng agreed to team up.

Initially, Deng used a TALEN-based gene editing approach to try to insert mutated CCR5 into HSC genomes, but when CRISPR became popular a few years later, he switched tactics. Even with CRISPR’s greater efficiency, HSC genomes are notoriously difficult to work with. The team spent five years improving the efficiency before publishing their first study in 2017. In that paper, the team described a process for inserting mutated CCR5 into human HSCs to mimic natural immunity to the virus. When transplanted into mice, these modified HSCs ablated HIV infection.

In Deng’s latest research, the team transplanted these same modified stem cells into a man with HIV and acute lymphocytic leukemia. The altered stem cells survived in the man’s body for more than a year without causing detectable side effects. Ultimately, however, the number of cells was insufficient to significantly reduce the amount of HIV circulating in the man’s blood.

“It’s encouraging because [CRISPR-edited] stem cells have been in the HIV patient for more than two years now,” Deng said. “And [it] also suggests that we need to improve our editing efficiency, which is what we are currently focused on. We need our treatment for HIV to reproduce like in the Berlin patient case. We need to get better editing efficiency—ideally 100 percent efficiency.”

A Work in Progress

Gendelman and Deng acknowledge that there is still a great deal of research to be done before clinicians can combat HIV using CRISPR. Many groups are working on generating human HSCs via induced pluripotent stem cell technology, which may improve editing efficiency. “The gene editing efficiency of pluripotent stem cells is very high, so you can do very precise gene editing,” Deng said. However, how to go about this is a challenge.

For directly delivering CRISPR to patients, “It’s not until [LASER ART-CRISPR HIV therapy] is reproduced and affirmed by others, and when we extend from a couple of animals to a large number, to the majority, and move this to large animals, that we can really claim success,” Gendelman said. “I look at this as a process, not a Eureka. I hope that makes sense, but healthy skepticism is probably a good part of being a good scientist.”

References

Designing Tools for Disease Eradication

by Niki Spahich, PhD

With millions dying from mosquito-borne diseases every year and more than half of the world’s population at risk, researchers are racing to solve this global health problem. For centuries, people have protected themselves from mosquitoes by using bed nets, eliminating water sources where mosquitoes breed, or employing insecticides to kill the pests. Over the decades, improvements in these methods offered more effective protection, but more recent technological developments have opened new avenues for combatting the problem. With the introduction of CRISPR technology, scientists have introduced new, high-tech gene drives to ward off this ever-growing threat. Whether the public and regulators will accept this technology remains to be seen.

An Unfair Advantage

Scientists such as Omar Akbari from the University of California, San Diego are using the precise gene editing capabilities of CRISPR technology to create gene drives in mosquitoes that, when spread through a population, will reduce the mosquitoes’ ability to transmit human diseases. When designing a gene drive, Akbari’s team creates genetic elements that skew Mendelian inheritance rules to increase the odds that a desired DNA sequence is inherited. Researchers engineer transgenes that are integrated into chromosomes to express CRISPR-Cas sequences and, in most cases, an antipathogen gene as the cargo. Upon delivery into a cell, the CRISPR machinery causes a targeted DNA break that the cell repairs using the gene drive sequence as a template. As a result, the CRISPR elements and the cargo get copied onto the homologous chromosome, resulting in biased transmission into the subsequent generation, with the majority of offspring inheriting the drive-containing chromosomes. Akbari’s team is using this technology in multiple ways to develop a genetic arsenal that will stop the spread of mosquito-borne diseases.

“One of the major benefits of CRISPR gene drives is the fact that they can autonomously spread invasively into populations. While invasive spreading may serve as a powerful tool for vector control in hard-to-reach locations, it can also act as a double-edged sword by making it difficult to confine the drive and prevent it from spreading uncontrollably into neighboring locations,” Akbari said. “This may pose significant challenges for obtaining regulatory approvals to perform open gene drive field trials, which are necessary to measure efficacy, risks, and assess for unintended consequences.”

Because gene drives are passed down from generation to generation at extremely high levels, their spread throughout a population is quick and often permanent. This could efficiently control the spread of mosquito-borne diseases without consistent human intervention; however, existing populations would be forever altered. “If there were any unintended consequences, then what do you do?” Akbari wondered.

Building a Toolbox

In 2018, Tony Nolan and Andrea Crisanti of Imperial College London used a population suppression gene drive to spread sterility in female mosquitoes by inactivating a gene required for fertility. A single release of engineered mosquitoes reduced mating success in a population and eventually caused a population collapse. Crisanti and Nolan’s suppression gene drive approach succeeded in caged mosquito studies; however, researchers are concerned that resistance will arise in the wild through mutation and selective pressure against these unfavorable alleles.

Akbari’s team used a different approach. Working with Aedes aegypti mosquitoes, the principal vector for Dengue virus, they first engineered a broad neutralizing antibody against the virus in the mosquitoes. This strategy confers disease resistance to the mosquito—a trait that may have better competitive success than sterility in the wild. They found significantly reduced viral loads and transmission rates in the engineered mosquitoes in the lab. In the future, they could use this gene as part of a CRISPR gene drive for rapid spread throughout a mosquito population.

Researchers are also designing alternative gene drives that safeguard against invasive spread and permanent population
alterations. For example, Akbari’s team developed a gene drive for *Ae. aegypti* that is split, or self-limiting. The drive components insert into different regions of the genome rather than at the same locus on the same chromosome. While the Cas drive component is inherited at a normal rate, the guide RNA and linked cargo transmit to the majority of the offspring. Eventually, the normally-inherited Cas component disappears from the population, making the gene drive temporary and, therefore, limited to a local area.

With this approach, scientists would need to release split gene drive organisms on a continuous basis to eradicate disease. Akbari predicts that once releases of his *Ae. aegypti* organisms stop, the population should go back to normal within a few generations. “We have already shown with mathematical modeling that it can spread and maintain itself for quite a period. If it were linked to a Dengue effector, and if all mosquitoes in the population had a copy of it, there would be no local Dengue transmission,” he explained. “This could prevent many infections and save lives.”

With multiple CRISPR gene drive options available, Akbari thinks it’s best to diversify. “Having a split gene drive that is self-limiting provides more tools in the toolbox,” he said. “For the first open field releases, regulators and the public may prefer a technology that’s confineable and controllable, yet still effective. After these initial tests, once all risks are assessed, if everything goes as expected, and more invasive spread is desired and necessary, then perhaps the next step could be to move onto something that is less confineable to spread permanent modifications.”

**The Way Forward**

Scientists and the public alike are concerned about releasing gene drive organisms into the wild. What happens to the ecosystem when you suppress a mosquito species? Even with confineable gene drives, there could be consequences. When spreading disease resistance genes, would parasites evolve to become more pathogenic or to invade another host? What about populations of parasites that are unaffected by the cargo gene?

Akbari has been considering the ethical questions surrounding gene drives and how to best engage local communities in decision-making with Cinnamon Bloss from the University of California, San Diego. Through her research, Bloss found that, despite the fears connected to genetically modified organisms, the public is surprisingly open to gene drive technology. “Many people recognize that vector-borne disease is a serious global public health problem, and we need novel approaches to address it,” she said.

Moderating the conversation surrounding the release of CRISPR gene drives will be tricky because in Bloss’s view, this is a public health intervention that is not amenable to traditional models of informed consent. Because something that affects the environment also affects all surrounding people, collecting consent from everyone will not be feasible. Also, if a drive is more likely to spread beyond a localized area, scientists and regulators must engage with diverse communities that cross international boundaries. The best approach would be to move forward in a respectful and ethical way. Having more tools in the gene drive toolbox will provide options for how and where these organisms are spread.

Even with the potential for unforeseen effects of altering mosquito populations, the payoffs could be huge. “People are suffering in the world,” said Akbari. “If you have a technology that can make an impact, why not use it?”

**References**

Getting to the Heart of the Matter

By Nathan Ni, PhD

Despite the best efforts of the medical and research communities, the societal impact of cardiovascular disease (CVD) is getting worse, not better. Because of its inherent complexity, CVD research and therapeutics today are largely focused around vigilant screening for known risk factors and managing pathogenic processes. But this approach is not stemming the tide; CVD remains one of the leading causes of death, debilitation, and loss of quality of life.

Scientists and observers alike have hailed CRISPR-Cas gene editing technology as “revolutionary” and “one of the biggest science stories of the decade.” It arrived just in time for researchers like Kiran Musunuru from the University of Pennsylvania. Facing a disease that is projected to double its burden on the health care system in the next twenty years, Musunuru is dedicated to finding a more permanent therapeutic solution for CVD. “A ‘one-and-done’ therapy would be utterly transformational for a chronic disease,” said Musunuru. “This is the biggest advantage that gene editing brings for cardiovascular disease therapeutics.”

A “Cure” for Cardiovascular Disease?

High blood plasma cholesterol levels link to increased CVD risk, and the protein PCSK9 is a major regulator of cholesterol production. In 2014, Musunuru’s research team, then based at the Harvard Stem Cell Institute, used CRISPR-Cas to selectively and permanently knockout the Pcsk9 gene in the mouse liver, significantly decreasing plasma cholesterol levels within four days with no observed unintended mutations. Two years later, they accomplished the same thing in mice bearing transplanted human hepatocytes, demonstrating similar results in terms of efficacy and safety.

In addition to the immediate physiological benefits, one of the biggest advantages for such a therapeutic strategy, according to Musunuru, is that it would take adherence to a medication regimen entirely out of the equation. “If you look at various studies, only about half of the patients are still taking their pills regularly a year after their heart attack, and that’s a huge problem.”

A Game-Changer for Research

CRISPR-Cas gene editing technology is a game-changer for CVD research. Fellow clinician-scientist Liam Brunham, from the University of British Columbia, studies how genetic variations affect lipid and cholesterol levels, as well as responses to therapeutic drugs. For him, CRISPR’s ease of use, precision, efficiency, and ability to alter genes in their genomic contexts give it considerable advantages over existing gene editing strategies when it comes to creating disease models. Where it once took years to create a genetically modified rodent model, now it can be done in as little as three weeks. Moreover, the model creation process has been simplified to take place within a single generation, meaning that it can now be used on animals with longer gestational cycles.

This is critical for CVD research. Right now, rodent models are extremely popular owing to their accessibility, rapid reproductive cycle, and genetic malleability. However, rodent physiology is drastically different from that of humans, and rodent responses to CVD are not the same as those of humans. Larger animals, such as rabbits, dogs, and especially pigs, are more similar to humans physiologically and genetically, but were much more difficult to manipulate from a gene editing perspective—until now.

“CRISPR works just as well in mouse embryos as it does in embryos from pretty much any other species, so now you can make all of these genetically modified animals—rats, pigs, monkeys—really pretty much the entire gamut fairly easily,” said Musunuru. “This diversification of the animal models that we can use to study diseases has been transformational, and...”
we've already gotten more insights into [CVD] using these large animals that we weren't able to get from rodents."

**Cautious Optimism**

CRISPR-based gene editing, much like any other nascent therapeutic approach, is not without risk. For example, permanence, which may be its greatest asset, could be devastating if a genetic modification resulted in unforeseen negative side effects. Brunham believes that this problem can be minimized by focusing on somatic mutations rather than germline, but Musunuru is more bullish. "It's only been eight years since CRISPR first came on the scene as a gene editor that can work in human cells," he said. "With newer iterations of the technology, such as base editing, prime editing, and epigenome editing, we may actually develop the ability to reverse what would ordinarily be permanent changes to the genome."

Another potential problem is that the cardiovascular system in general, and the heart in particular, possesses a relatively limited regenerative capacity. As such, any physical damage to cardiovascular system tissues and organs persist chronically, if not permanently. These often prompt additional co-morbidities. Presently, gene editing therapeutics hold greater potential as preventive measures, and neither Brunham nor Musunuru are aware of any studies demonstrating a role for CRISPR in promoting cardiovascular tissue repair and regeneration. "It's much better to prevent cardiovascular diseases than to try to fix the problem afterwards," said Musunuru.

However, Musunuru was unwilling to completely close the door on potentially using gene editing to repair damaged tissue. "I can imagine a scenario where if you have a medication that helps with heart failure or the aftereffects of a heart attack, and you could mimic that genetically, that could be advantageous. I think we'd need to have a better understanding of the genes involved."

**Moving Towards the Clinic**

Gene editing offers real potential for new approaches and strategies when it comes to treating cardiovascular disease. Clinical trials for gene editing-based approaches already exist in other fields, such as chimeric antigen receptor-T cell immunotherapy, sickle cell disease, rare liver disorders, and HIV. As such, Musunuru believes that CVD research will follow suit sooner rather than later, citing eight promising gene targets: PCSK9, NPC1L1, ApoB, Lpa, ApoC3, ANGPTL3, ANGPTL4, and ASGR1.

These eight genes are involved in lipid metabolism, making it easy to determine whether gene editing creates the desired effect by measuring blood lipid and cholesterol levels. Each gene is prominently expressed in the liver, making targeting and delivery more straightforward. Perhaps most importantly, there are documented examples of people who naturally have loss-of-function mutations for one or more of all eight of these genes. These individuals have not shown any serious adverse consequences from lacking these genes, and they possess substantial protection from CVD.

While direct gene editing-based therapeutic approaches are not yet available, gene editing has already found a place in today’s clinic for CVD patients. Genome analysis is starting to play a bigger role in disease screening and diagnosis. “We’re very good at reading the genome, but we’re very bad at interpreting it. You get a lot of information, but you don’t actually necessarily know what to do with it,” said Musunuru, explaining that many mutations detected during genetic screening are classified as variants of uncertain significance (VUSs). “Sometimes, a detected variant falls in a gene that, when mutated, causes diseases in some people. However, your particular variant might be totally benign, or it might be a disease-causing mutation. The problem is, we don’t know, and this uncertainty, this ‘genetic purgatory,’ is very challenging for the patient. It’s challenging for the physician as well—how do I act on this information? How do I manage the patient? And this uncertainty is just going to become an increasingly large problem as more and more people are sequenced.”

In the face of this, Musunuru has developed a way to characterize VUSs as they are discovered. In his lab, his team uses a gene editing platform to introduce identified VUSs into induced pluripotent stem cells from healthy donors, differentiate them into cardiomyocytes or other cardiovascular system cells, and observe whether the cell’s properties have changed. “We are now in a position where a patient will come into our clinic, get genetic testing, and we can find out whether the VUSs they have in CVD-related genes are benign or pathogenic in less than three months, right in time for the patient’s next regularly scheduled visit.”

This capability has tremendous implications not only for patients, but also for their families, explained Musunuru. “There was one patient who needed a heart transplant, but it wasn’t clear what was driving her disease. We were able to take a VUS found during genetic testing and prove that this variant was in fact the driver of this patient’s disease. Now, this patient had been extremely worried about the thought of passing on her disease on to a child, but [this knowledge] allows her to be more open to potentially doing in vitro fertilization and selecting embryos that do not have this mutation, and therefore have children who would not be at risk for her disease.”

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