PEGGY PETERSON PHOTOGRAPHY

Kiran Musunuru (centre) and his team are using genome editing in the mouse liver to modify enzymes that regulate levels of 'bad' cholesterol.

GENE EDITING

The heart-disease vaccine

Advances in gene editing raise the prospect of a one-off injection that could reduce the risk of cardiovascular disease.

BY ANTHONY KING

Consider this scenario: it's 2037, and a middle-aged person can walk into a health centre to get a vaccination against cardiovascular disease. The injection targets cells in the liver, tweaking a gene that is involved in regulating cholesterol in the blood. The simple procedure trims cholesterol levels and dramatically reduces the person's risk of a heart attack.

According to World Health Organization statistics published in 2015, ischaemic heart disease and stroke are the leading causes of death worldwide. About 17.7 million people died from cardiovascular disease that year, and at least three-quarters of those deaths occurred in low- and middle-income countries. Although antibody-based therapies have been launched to help those most at risk, the cost and complexity of the treatments means that a simpler, one-off fix such as a vaccine would be of benefit to many more people around the world.

The good news is that a combination of gene discovery and the blossoming of genome-editing technologies such as CRISPR–Cas9 has given this vision of a vaccine-led future for tackling heart disease a strong chance of becoming reality. The breakthrough came in 2003, when researchers investigated three French families with members who had potentially lethal levels of low-density lipoprotein (LDL) cholesterol and who harboured a mutation in the gene *PCSK9* (ref. 1). *PCSK9* encodes an enzyme that regulates levels of LDL — or 'bad' — cholesterol. The mutations uncovered in the families increased the enzyme's activity, raising the level of LDL cholesterol in the blood. Breaking *PCSK9*, so that the enzyme it encodes loses its function, might therefore reduce LDL-cholesterol levels.

Sensing the possibilities, investigators at the University of Texas Southwestern Medical Center in Dallas sought to determine whether naturally occurring mutations in *PCSK9* could also have the effect of lowering LDL cholesterol. The researchers interrogated the Dallas Heart Study, a landmark investigation of cardiovascular health carried out from 2000–02 in 6,000 adults living in Dallas County. The participants recruited represent the three main ethnic groups of the United States. After combing the data from about 3,600 individuals who provided a blood sample, the researchers sequenced DNA from the 128 participants with the lowest levels of LDL cholesterol. They discovered that about 2% of African-American participants had one broken copy of *PCSK9*, resulting from one of two inherited mutations[2]. A follow-up study of a different, larger population similarly found mutations in almost 3% of African Americans, which was associated with an 88% reduction in the risk of ischaemic heart disease[3]. "I think of them as having won the genetic lottery," says Kiran Musunuru, who studies human genetic variation and the risk of heart disease at the University of Pennsylvania in Philadelphia.

Musunuru thinks that in the next 20 years, gene editing will enable researchers to confer a mutation in *PCSK9*, or other beneficial mutations, on people who have had less luck in the genetic sense. "They would be dramatically

protected against heart attack and stroke for the rest of their lives," he enthuses.

Others are more bullish. Technologies for delivering gene editing can be safe, effective and work in the long term, says Sander van Deventer, operating partner at investment firm Forbion Capital Partners in Naarden, the Netherlands. van Deventer played an important part at uniQure in Amsterdam, where he supervised the development of alipogene tiparvovec (Glybera), the first gene therapy to gain regulatory approval. He thinks that gene therapy to reduce the risk of cardiovascular disease could become a reality within 5 years — initially targeted to help people with high cholesterol (a condition known as hypercholesterolaemia).

THE GATEKEEPER ORGAN

The liver is a preferred target organ of gene therapy for companies such as Editas Medicine in Cambridge, Massachusetts, Sangamo Therapeutics in Richmond, California, and CRISPR Therapeutics, also in Cambridge; it is straightforward to deliver genes to the liver, and the CRISPR–Cas9 tool is especially efficient in the organ, editing a greater proportion of cells than it does in most other tissues. The liver is also an excellent place from which to tackle cholesterol — it clears LDL cholesterol from the blood and is also a main engine of lipid synthesis. "The liver is the gatekeeper for removal of excess cholesterol from the body," says William Lagor, a molecular biologist at Baylor College of Medicine in Houston, Texas.

The enzyme produced by *PCSK9* causes receptors for LDL cholesterol, found on the surfaces of cells throughout the body, to move inside the cell. With fewer receptors available to bind such cholesterol, its level in the blood rises. Already, two antibody-based therapies have been developed to inhibit the enzyme PCSK9, increasing the number of LDL-cholesterol receptors and consequently reducing the amount of cholesterol in the blood. One such PCSK9 inhibitor, evolocumab (Repatha), can cut the risk of heart attack by 27% and stroke by 21%, when administered in combination with statins. But the treatment involves regular infusions of drugs for the rest of a patient's life and costs about US$14,500 per year, a price that many commentators have deemed too high.

In 2014, Musunuru and his team showed that more than half of *Pcsk9* genes in the mouse liver could be silenced with a single injection of an adenovirus containing a CRISPR–Cas9 system directed against *Pcsk9*. This led to a roughly 90% decrease in the level of Pcsk9 in the blood and a 35–40% fall in blood LDL cholesterol[4]. Next, they used a mouse engineered to contain human liver cells, and tuned the CRISPR–Cas9 payload to target human *PCSK9* (ref. 5). The team succeeded in showing that the human gene can also be switched off. "I'm convinced that if we gave this therapy to a human, it would work," Musunuru says.

The approach is "absolutely plausible, even feasible", from a technological point of view, says Lagor. But there is also a philosophical barrier to negotiate. "You don't necessarily want to treat people who haven't got a disease yet," he says. Karel Moons, a clinical epidemiologist at University Medical Centre Utrecht in the Netherlands, goes further. "Changing lifestyle may be much more effective for a population than focusing on high-cost interventions," he says. He worries that a gene therapy for individuals at high risk would hinder efforts to help people to help themselves. "It is the way the human mind works. Take a pill and we think we are protected," he warns.

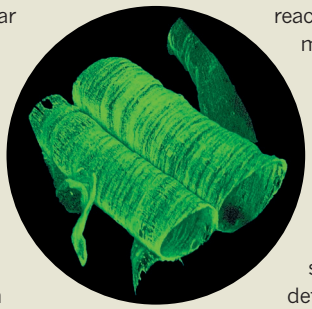
Musunuru accepts that the idea does not have universal approval but thinks that "there will be greater enthusiasm for human trials for common diseases after genome editing has been proven safe in the patients with grievous genetic disorders". Debilitating single-gene conditions such as Duchenne muscular dystrophy are likely to be first to benefit from therapeutic gene editing (see 'Benefits from a partial fix'). Musunuru suggests familial hypercholesterolaemia — the LDL-cholesterol disorder characterized in the three French families — as a similarly logical place to start. The associated mutations in *PCSK9* raise LDL-cholesterol levels from birth, causing premature heart attacks — sometimes in childhood — in those who are worst affected. "It would make a lot of sense to knock out the faulty *PCSK9* gene in those patients," he says.

People with hypercholesterolaemia can make changes to their lifestyle and diet, as well as take statins, but this is often not enough. They might also require treatment with antibodies directed against PCSK9 and frequent cleaning of the blood to remove LDL particles. Those with the most severe disease would receive the greatest benefit from genome editing, says Musunuru, and be the first candidates for therapy. "The strongest rationale for using genome editing is that it would be given just once, whereas patients have to take antibodies every few weeks for the rest of their lives." He views the approach as being particularly useful for people in low-income countries with less-well-funded

DUCHENNE MUSCULAR DYSTROPHY

Benefits from a partial fix

Duchenne muscular dystrophy is a single-gene disorder that will probably be in the vanguard of diseases targeted by gene therapy. The condition affects up to 1 in 3,500 boys and men, and causes the progressive weakening of muscles; heart-muscle failure is the leading cause of death in people with the disorder. "This disease has resisted every therapy applied to it," says Eric Olson, a molecular biologist at the University of Texas Southwestern Medical Center in Dallas. "The only reasonable approach is to go to the root cause of the disease, to the mutated gene. CRISPR seems an ideal approach."



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At the core of the condition lie defects in dystrophin, a long membrane-associated protein that acts as a shock absorber in muscle cells (**pictured**). Dystrophin's central portion comprises 20 or so repetitive sections, which are analogous to the coils of a spring. *DMD*, the gene that encodes dystrophin, is long, containing 79 coding sections, or exons, and Olson says that mutations anywhere along its length can eliminate the production of functional dystrophin.

Rather than correcting specific mutations, he estimates that 80% of patients could benefit from a partial fix. Some of the coils in dystrophin can be deleted without destroying the protein's function. This means that sections of DNA within *DMD* that contain mutations can be removed. The shortened gene will make a working, truncated protein. "One edit can bypass all the mutations," Olson says.

Dystrophin production as low as 5% of the normal level is thought to improve muscle function; Olson thinks that reaching 15% would bring major clinical benefits. In 2017, researchers at the Ohio State University in Columbus blew past that target, restoring dystrophin-expression levels in the heart muscle of mice by up to 40%, simply by slicing out a defective portion of *Dmd* using a CRISPR–Cas9 system delivered by a viral vector[14]. "So long as the gene can still read out, you make a partially functional protein," says Renzhi Han, who led the study. His lab is now evaluating the safety of the strategy in mice. Olson's research group has used the technique to restore up to 90% of normal dystrophin levels[15].

Han and others are optimistic that trials in people can begin in the next five years. "Duchenne is the most devastating muscle disease. There is no escaping the clinical consequences," says Olson. "There is enormous excitement in the Duchenne community about this new technology." A.K.

health-care systems: "I do not see daily pills or monthly injections as being a realistic approach in the developing world." But although a one-off treatment should be cheaper, drug companies could be tempted to charge a high price, on the basis that it achieves the same effect as do decades of expensive antibody-based drugs.

For now, Musunuru says that we need to work out the safest way to perform gene editing in people — not necessarily CRISPR–Cas9 — and also the best way for it to be delivered. Regulatory approval for a clinical trial would then be required, which could take a few years to achieve.

STACKING TARGETS

Since the discovery of *PCSK9*, other variants in genes that alter the risk of cardiovascular disease have emerged. Some affect triglycerides, the main component of fat in the body; high levels of triglycerides in the blood are a known risk factor for heart disease. Apolipoprotein C-III inhibits the breakdown of triglycerides by enzymes; a mutation in *APOC3*, the gene that encodes it, was discovered in a population of Amish people in the United States in 2008 (ref. 6). The 5% of the group who were carriers had lower levels of LDL cholesterol, higher levels of high-density lipoprotein (HDL) — or 'good' — cholesterol and lower levels of triglyceride in the blood, all of which might reduce the risk of cardiovascular disease. A similar pattern has also been found in people who carry the mutation in Crete, Greece.

Musunuru is optimistic that knocking out a gene called *ANGPTL3* can reduce levels of LDL cholesterol and triglycerides. He was part of a team that reported in 2010 on three generations of a family with mutations in *ANGPTL3* and that had no history of heart disease and had low levels of cholesterol and triglycerides in the blood[7]. In 2017, three family members who had a complete loss of function of the protein encoded by *ANGPTL3* were examined[8]. "As far as we can tell, they are substantially protected against cardiovascular disease, but suffer no harmful consequences whatsoever," says Musunuru. At least 1 in 300 people has a broken copy of *ANGPTL3*, which has been shown to reduce the risk of ischaemic heart disease by roughly one-third[9].

Another potential target is the gene *LPA*, which encodes lipoprotein (*a*). High levels of lipoprotein (*a*) are a main risk factor for heart disease and stroke, yet no treatments have been approved by regulators such as the US Food and Drug Administration specifically to lower its levels. "This really is an ideal candidate for disruption with a liver-directed CRISPR gene-editing approach," says Lagor. Initial candidates for the treatment would be people with extremely high levels of lipoprotein (*a*) who also have cardiovascular disease.

The most effective treatments will probably disrupt several of these genes at once to provide the greatest benefit. "Since PCSK9 and ANGPTL3 work by different mechanisms, in principle they should be additive," says Musunuru. Lagor agrees, adding that there are also economic upsides. "It is likely that the cost of targeting two genes, or perhaps even three or four, would be the same as for one gene."

REASONABLE OPTIMISM

Before gene-editing therapy can become routine, two main safety concerns must be addressed. First, off-target effects can occur when the RNA molecule that guides the Cas9 cutting enzyme into position misidentifies its complementary sequence of DNA, resulting in cuts being made in the wrong place. Second, the cellular machinery that repairs the double-strand breaks created in the DNA during gene editing might make an unexpected deletion or addition. Such mishaps could lead to the development of cancer. And although a considerable degree of risk might be acceptable for seriously ill patients with no other option, preventive gene therapy must clear a higher bar. "If the vaccine is being envisioned for the general population, then it needs to be essentially 100% safe," says Musunuru, "at least to the same degree as the infectious-disease vaccinations that are routinely given to infants and children."

A new technology from chemical biologist David Liu's laboratory at Harvard University in Cambridge, Massachusetts, has therefore excited those in the gene-editing field. Liu has developed a technique that uses a modified CRISPR–Cas9 system to alter individual pairs of bases in cells without having to break the DNA double strand[10]. His team was able to chemically change the DNA base cytosine (C) into uracil (a base found in RNA), which the cell later replaced with thymine (T). In 2017, Liu's team created another tool that could rearrange an adenine (A) so that it resembled a guanine (G), and then hoodwinked the cell into fixing the complementary strand of DNA to make the edit permanent, therefore changing an A•T pair into a G•C (ref. 11).

"Base editing is as big a development as the original introduction of CRISPR–Cas9 to the genome-editing field," says Musunuru. "It's totally changed how I've been thinking about tackling cardiovascular disease — in a positive way." He is planning to test Liu's A-to-G base editor in mice to see how well it works.

Gene-editing researchers have embraced targeted base editing to install precise changes without the uncertainty that accompanies a double-strand break. The technique has been used in labs to correct genes in yeast, plants, zebrafish, mice and even human embryos. A proof-of-concept study by Alexandra Chadwick, a postdoctoral researcher in Musunuru's lab, delivered a base editor into the livers of adult mice to disable *Pcsk9*, halving the level of Pcsk9 and cutting LDL cholesterol by almost one-third[12]. Musunuru adds that he has preliminary results showing base editing of *Angptl3* in mice using Liu's C-to-T method.

The pace of innovation in gene editing has created an aura of optimism, particularly around the treatment of people with genetic disorders who have few or no other options. "It makes sense to begin therapeutic efforts with such diseases, even if the understanding of all potential risks is imperfect," says Liu. But there is the potential for the technique's use in the clinic to spread beyond these testing grounds. van Deventer has successfully lowered LDL cholesterol in mice by silencing apolipoprotein B-100 using a method called RNA interference[13]; he sees great potential in using the microRNAs that underpin the technique and, eventually, gene editing to address heart disease. "ANGPTL3, PCSK9 and APOC3 are targets not easily addressed by small molecules or antibodies," he says. And the one-off nature of gene-editing treatments cuts down on issues with patients not following advice about when to take a drug — a perennial problem concerning people on long-term medication.

"The strongest rationale for using genome editing is that it would be given just once."

"If you are talking about cardiovascular disease as a global health threat, which it undoubtedly is, then protecting the entire population is what we need," says Musunuru. Lifestyle changes are important, but a substantial portion of the risk of heart failure and stroke comes from the genome. "You don't need to choose between medicine and lifestyle. You should be doing both," says Liu, citing people with diabetes, who fare best when they take medication and adjust their lifestyle.

"To vaccinate large numbers of people, that is some way off," says Musunuru. But gene editing could reset the odds for those who didn't win the genetic lottery, he predicts. "One way or another, genome editing is going to underlie a host of new types of cardiovascular therapies over the next 25 years." ■

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