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New way of genome editing cures hemophilia in mice; may be safer than older method

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The ability to pop a working copy of a faulty gene into a patient's genome is a tantalizing goal for many clinicians treating genetic diseases. Now, researchers at the Stanford University School of Medicine have devised a new way to carry out this genetic sleight of hand.

The approach differs from that of other hailed techniques because it doesn't require the co-delivery of an enzyme called an endonuclease to clip the recipient's DNA at specific locations. It also doesn't rely on the co-insertion of genetic "on" switches called promoters to activate the new gene's expression.

These differences may make the new approach both safer and longer-lasting. Using the technique, the Stanford researchers were able to cure mice with hemophilia by inserting a gene for a clotting factor missing in the animals.

"It appears that we may be able to achieve lifelong expression of the inserted gene, which is particularly important when treating genetic diseases like hemophilia and severe combined immunodeficiency," said Mark Kay, MD, PhD, professor of pediatrics and of genetics. "We're able to do this without using promoters or nucleases, which significantly reduces the chances of cancers that can result if the new gene inserts itself at random places in the genome."

Using the technique, Kay and his colleagues were able to insert a working copy of a missing blood-clotting factor into the DNA of mice

with hemophilia. Although the insertion was accomplished in only about 1 percent of liver cells, those cells made enough of the missing clotting factor to ameliorate the disorder.

Kay is the senior author of the research, which will be published Oct. 29 in *Nature*. The lead author is postdoctoral scholar Adi Barzel, PhD.

A possible alternative to CRISPR

The Stanford discovery may offer an alternative to a genome-editing technique called CRISPR/Cas9 that relies on an ancient, protective response developed by bacteria to fight off viral attack. Every time a bacterium defeats a virus, it saves a tiny portion of the invader's DNA in its own genome, like a genetic feather in its cap. (Accumulations of these viral regions are called clustered regularly interspaced short palindromic regions, or CRISPR.) When that virus comes around again, the cell uses the snippets of saved genetic material to identify and latch onto matching regions in the viral genome. Once attached, it cuts the viral genes at precise locations with a protein called Cas9.

Researchers around the world have begun to use the CRISPR/Cas9 technique not just to permanently disable genes for study in laboratory animals, but also to insert new, modified genes into the animals' genomes. This allows the rapid creation of genetically modified laboratory animals; what used to take months or years can now take days or weeks.

However, the technique requires not just the gene for the Cas9 nuclease, which itself could integrate into the recipient's genome, but also a promoter to drive the expression of the genes. Researchers are concerned that, if used in humans, Cas9 may cut the DNA at unexpected locations, which could disrupt or kill the cell. Alternatively, the promoter of the new gene could adversely affect the expression of nearby genes,

causing cancers or other diseases. The foreign bacterial proteins could also cause an immune reaction in patients.

"Site-specific gene targeting is one of the fastest growing fields in gene therapy and genome engineering," said Barzel. "But the use of nucleases and promoters may have significant adverse effects. I wanted to come up with a novel gene-targeting scheme that involved no vector-borne promoter and did not require the use of an endonuclease."

The technique devised by the researchers uses neither nucleases to cut the DNA nor a promoter to drive expression of the clotting factor gene. Instead, the researchers hitch the expression of the new gene to that of a highly expressed gene in the liver called albumin. The albumin gene makes the albumin protein, which is the most abundant protein in blood. It helps to regulate blood volume and to allow molecules that don't easily dissolve in water to be transported in the blood.

The researchers used a modified version of a virus commonly used in gene therapy called adeno-associated virus, or AAV. In the modified version, called a viral vector, all viral genes are removed and only the therapeutic genes remain. They also relied on a biological phenomenon known as homologous recombination to insert the clotting factor gene near the albumin gene. By using a special DNA linker between the genes, the researchers were able to ensure that the clotting factor protein was made hand-in-hand with the highly expressed albumin protein.

During homologous recombination, which is a natural repair process, the cell takes advantage of the fact that it has two copies of every chromosome. By lining up the damaged and undamaged chromosomes, the cell can crib off the intact copy to repair the damage without losing vital genetic information. Kay and Barzel used this natural process of recombination to copy sequences from the viral vector into the genome at places designated by the researchers—in this case, after the albumin

gene.

Effective in newborn and adult mice

When they tested their approach in newborn laboratory mice with hemophilia, they found that the animals began to express levels of clotting factor that were between 7 and 20 percent of normal. That amount of clotting factor has been shown in previous studies to be therapeutic in mice. They further showed, surprisingly, that the technique worked as well in adult animals, even though the gene was successfully inserted in fewer than 1 in every 100 liver cells.

"We expected this approach to work best in newborn animals because the liver is still growing," said Kay, who is also the Dennis Farrey Family Professor of Pediatrics. "However, because homologous recombination has been thought to occur mostly in proliferating cells, we didn't expect it to work as well as it did in adult animals."

Kay has been involved in gene therapy for hemophilia for many years. In 2006, he was a leader of a team of investigators that used AAV to provide a clotting factor gene to patients with hemophilia B. This form of hemophilia is less common than hemophilia A, which affects about 1 in every 25,000 newborn boys (because the condition is caused by a faulty gene on the X chromosome, it rarely affects girls). However, hemophilia B is an easier target to treat because the gene for the missing clotting factor is smaller and easier to manipulate with gene therapy.

As with many viruses, there are different strains of AAV. Unfortunately, the expression of the clotting factor with the strain of AAV used in the 2006 study lasted only a few months in humans, in contrast to the long-lived expression in animals. Recently another, similar clinical trial was launched in the United Kingdom using a different strain of AAV, and expression of the clotting factor was still observed in each of six people

treated more than two years after receiving the modified virus. However, in both these trials, the clotting factor gene was not inserted into the genome, but was instead maintained as a separate, free-floating copy.

"The real issue with AAV is that it's unclear how long gene expression will last when the gene is not integrated into the genome," said Kay, who is also a member of the Stanford Cancer Institute, the Stanford Child Health Research Institute and Stanford Bio X. "Infants and children, who would benefit most from treatment, are still growing, and an unintegrated gene could lose its effectiveness because it's not copied from cell to cell. Furthermore, it's not possible to re-administer the treatment because patients develop an immune response to AAV. But with integration we could get lifelong expression without fear of cancers or other DNA damage."

The researchers are now planning to test the technique in mice with livers made up of both human and mouse cells, a model which they recently were able to show may be a good surrogate to further predict what will happen in humans.

More information: Promoterless gene targeting without nucleases ameliorates haemophilia B in mice, *Nature*, DOI: 10.1038/nature13864

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