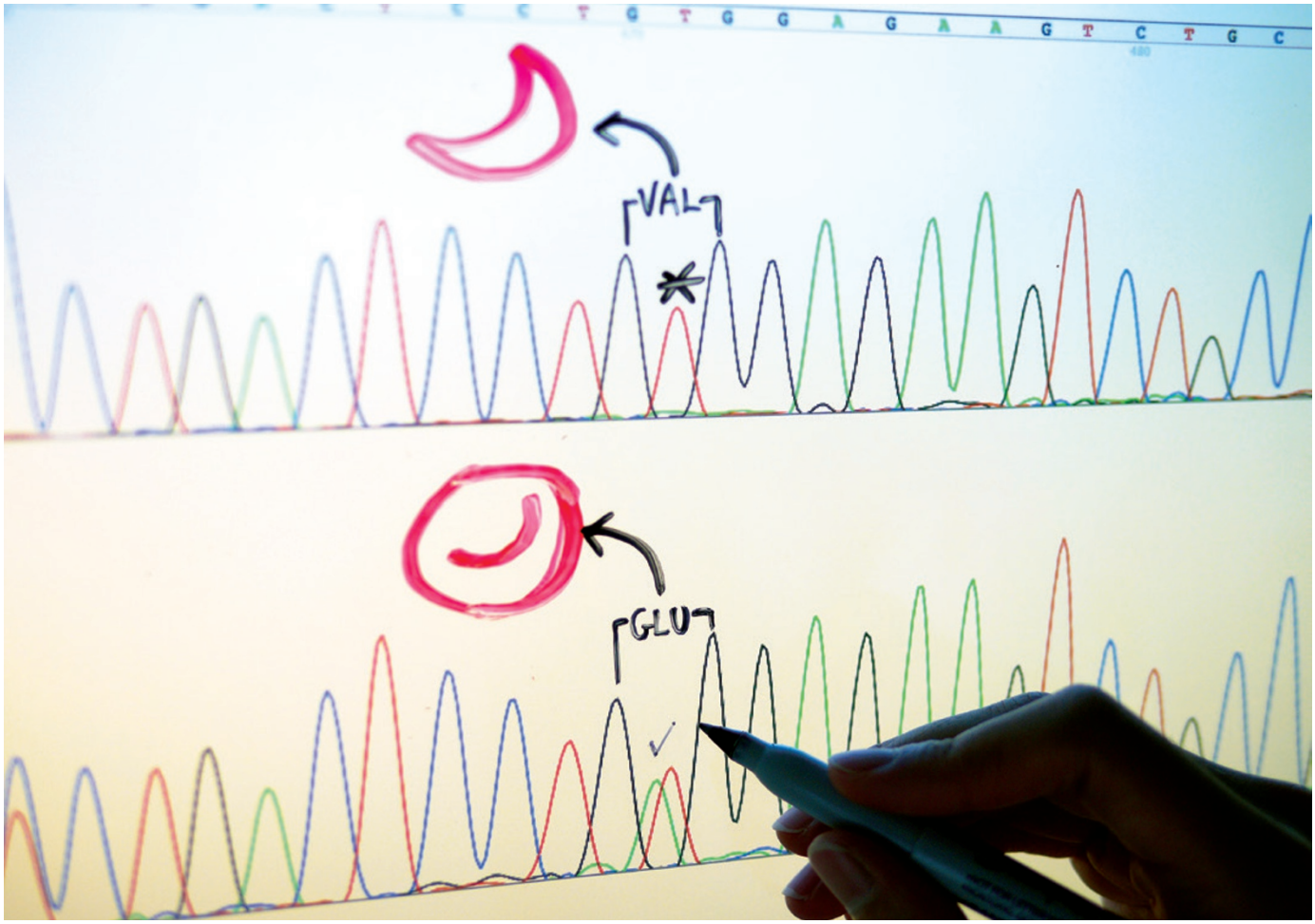
JUAN CARLOS IZPISUA BELMONTE



DNA sequences showing the sickle-cell disease mutation (marked with an asterisk, top) and the sequence corrected (below) using gene-editing technology.

GENE THERAPY

Editorial control

Correcting the genetic error in sickle-cell disease might be as simple as amending text.

BY KATHARINE GAMMON

Tiny changes in DNA can have huge consequences. For years, scientists have been trying to 'fix' these mutations in the hope of treating and potentially curing some of humanity's most devastating genetic diseases. After some tragic early setbacks (see *Nature* **420,** 116–118; 2002), techniques that allow precise genetic manipulation have created a surge of research.

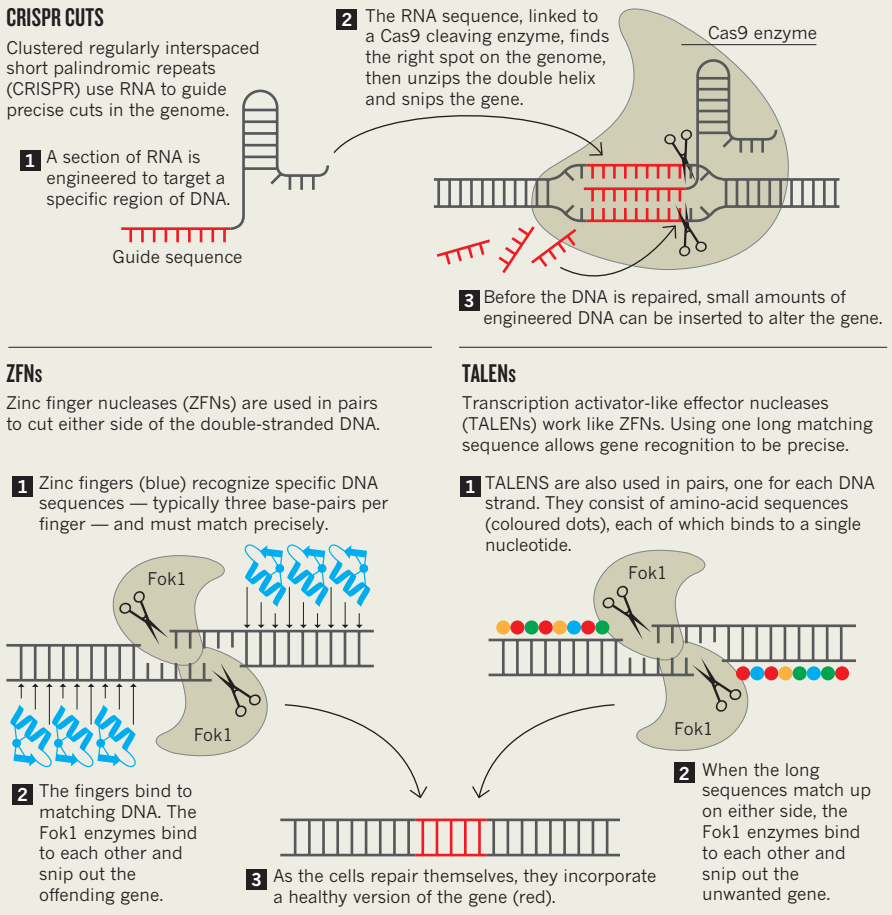
Although most existing treatments for genetic diseases typically only target symptoms, genetic manipulation or 'gene therapy' goes after the cause itself. The approach involves either inserting a functional gene into DNA or editing a faulty one that is already there, so the conditions most likely to prove curable are those caused by a single mutation. Sickle-cell disease is a perfect candidate: it is caused by a change in just one amino acid at a specific site in the β-globin gene. This results in the production of abnormal haemoglobin proteins that cause the red blood cells that house them to twist and become sickle shaped. The distorted cells get sticky, adhere to each other and block blood vessels, preventing oxygenated blood from flowing through.

Gene therapy has been used successfully in a handful of patients with immune disorders, and sickle-cell disease is among researchers' next targets. The most advanced of these projects is slated to begin clinical trials by the end of the year, and other trials are set to follow. The approaches being developed to treat sickle-cell disease take one of two forms. Conventional gene therapy, also known as gene addition, typically involves inserting new genes. Usually, a harmless virus is modified with the gene to be inserted, and this 'viral vector' is mixed with cells from the patient *in vitro*. The virus searches out the cells and inserts the gene into the cells' DNA, after which the cells are transplanted into the patient. Conversely, gene editing is more nuanced: in a molecular cut-and-paste, researchers cut out the faulty DNA sequence and then insert a piece of laboratory-created DNA. In both approaches, the modified DNA dictates the formation of a normal, working protein.

In sickle-cell disease, the only cells that need their DNA edited are blood stem cells — also known as haematopoietic stem cells — which are found in bone marrow. These cells continually form new red blood cells to replace those that are lost, and reprogramming just a small fraction of them will create enough perfectly formed red blood cells to eliminate disease

MOLECULAR CUT-AND-PASTE

Three different gene-editing techniques could allow researchers to fix the single mutation in the haemoglobin gene that causes sickle-cell disease.



symptoms. "Achieving genome editing via direct repair of blood stem cells represents a high hurdle," says George Daley, director of the Stem Cell Transplantation Program at Boston Children's Hospital in Massachusetts, "but perhaps not an impossible one."

Although these approaches are promising, several important issues must be addressed before they can be used to treat patients, such as ensuring that the therapies accurately hit their targets and do not cause irreparable harm to the cells or introduce additional genetic information that could cause problems such as cancer.

INJECTING GENES

Gene addition is poised to become the first sickle-cell gene therapy to be tested in humans. At the regenerative medicine and stem cell research centre of the University of California, Los Angeles, molecular geneticist and physician Donald Kohn is developing protocols for a clinical trial of this technique that is due to start enrolling patients by the end of 2014. Doctors will first harvest bone marrow from the hip bones of patients with sickle-cell disease and then extract haematopoietic stem cells from the marrow. Using a viral vector, they will insert a new, working haemoglobin gene into the cells' DNA; the old, faulty haemoglobin gene will still be present, but it will go silent as the new gene takes over. The modified cells will then be infused back into the patient's bloodstream and will migrate to the bone marrow, where they can provide a continual source of healthy red blood cells.

Kohn says that this approach has the potential to cure sickle-cell disease, and with significantly fewer side effects than a bone marrow transplant — currently the only cure (see page S14). He has tested the technique by injecting modified human haematopoietic stem cells into mice, and found that they were free of sickle cells 2 to 3 months later[1]. The limiting factor in mice, Kohn says, is that they can only sustain human grafts for that long. In humans, he thinks the correction should last a lifetime — as long as 50 to 70 years.

One of the challenges in treating sickle-cell disease with gene therapy is that it is necessary to extract bone marrow to retrieve haematopoietic stem cells. With most other diseases, patients can be given drugs that entice these cells to leave the marrow and enter the bloodstream, where they can be easily harvested. But in patients with sickle-cell disease, these drugs can trigger sickle-cell crisis, an acutely painful episode during which the damaged cells stick together and block blood vessels; the crisis can be accompanied by anaemia, chest pain, difficulty breathing, blood trapped in the spleen and liver, even stroke. So researchers must harvest the bone marrow itself, which can be difficult and slow, and limits the number of cells that can be collected at one time. Kohn says that they still do not know whether this approach will yield enough haematopoietic stem cells for reprogramming. And, like other bone marrow transplant procedures, the patient still needs to undergo chemotherapy to kill off the remaining bone-marrow cells to help the genetically altered ones survive once they are reintroduced into the body.

TALENTED FINGERS

Further away from clinical trials, but potentially a lot more exciting, is gene editing. The concept was introduced in the 1990s, when artificial DNA-cutting enzymes known as zinc finger nucleases (ZFNs) were first engineered. ZFNs bind to a specific section of DNA and create a break at both ends (see 'Molecular cut-and-paste'). Cells will start to repair the break, at which point a specific sequence of laboratory-made DNA can be slotted into the gap. After the DNA is repaired, the cells start to create healthy copies of the gene.

In parallel to his work on gene addition, Kohn is exploring the use of ZFNs to edit sickle-cell genes. In collaboration with the firm Sangamo BioSciences in Richmond, California, he has shown that around 7% of haematopoietic cells can be repaired in culture using this technique, using a viral vector to get the ZFNs into the cells. Because the repaired cells continue to replicate, this small proportion could be enough to eventually produce a sufficient amount of working red blood cells. Kohn says that patients have shown major improvements when just 10–20% of their donor cells successfully engrafted and started to make new, healthy cells.

The advantage of gene editing over gene addition (a less complex approach) is that it provides an actual fix rather than a work around. But ZFNs are expensive and difficult to program. In 2010, a gene-editing protein called TALEN (transcription activator-like effector nuclease) was developed, which uses a similar mechanism as ZFNs but is cheaper and easier to work with. It was quickly adopted for use in sickle-cell disease.

At the Salk Institute for Biological Studies, in La Jolla, California, stem-cell biologist Juan Carlos Izpisua Belmonte uses TALENs in concert with viral vectors called HDAdVs (helper-dependent adenoviral vectors) to correct the sickle-cell mutation. Instead of harvesting haematopoietic stem cells from bone marrow, Izpisua Belmonte's team takes easily harvestable cells, such as blood, skin or fat

cells, and then turns them into induced pluripotent stem (iPS) cells, which can be converted into any cell type. The researchers correct the haemoglobin gene defect *in vitro* using gene editing, then differentiate the repaired iPS cells into blood stem cells. From there, the researchers have a couple of choices. The repaired cells could simply be infused into a patient's bloodstream, where they would make their way into the bone marrow and start to make healthy haematopoietic cells.

But Izpisua Belmonte is also working on a cure that could work inside the bone marrow itself. His team is combining TALENs with a different viral vector, HDAdVs, to boost the success rate of gene editing, and the researchers are working on a plan to administer their hybrid vector directly into the bone marrow, so the genetic fix would take place inside the patient's body. Although each infusion into the marrow might correct only 1% of the cells, ten such procedures over the course of several months — something Izpisua Belmonte and his research associate Mo Li think is feasible in terms of time and cost — could alleviate the symptoms of sickle-cell disease. "Little by little, you are correcting the disease *in vivo*," says Izpisua Belmonte. So far, this 'hybrid vector' technique has shown promising efficacy in umbilical-cord blood stem cells.

Sickle-cell disease results when both copies of the haemoglobin gene are faulty, and fixing just one of the genes is sufficient to make a big health improvement. As Li points out, people who carry one copy of the mutated gene, a genetic condition referred to as 'sickle-cell trait', do not show symptoms. "In fact, many of the world's best sprinters have the sickle-cell trait, he says. "Our approaches will most likely restore one mutated copy to its wild-type sequence, leaving the other copy untouched."

CRISPRs (clustered regularly interspaced short palindromic repeats) are the most recent addition to the gene-editing toolbox. Whereas ZFNs and TALENs use a protein to lock on to a specific section of DNA, CRISPRs use a 'guide RNA'. These guide RNAs are much easier to program than the proteins in TALENs and ZFNs, as well as being cheaper and more efficient. CRISPRs also make it possible to perform multiple genetic manipulations in one go. CRISPRs work in combination with the Cas9 (CRISPR-associated 9) nuclease: after the CRISPR locks on to the target gene, Cas9 snips both strands of the DNA, disabling the gene. The approach is less than two years old, yet many researchers are now working with CRISPRs in parallel with other *in vitro* techniques.

There are safety hurdles to be overcome before gene editing is used in humans, especially because it involves a permanent change in the genome. The thorniest issue is 'off-target activity' — unintended changes to the genome away from the target gene.

CHRIS CALLERI/GEORGIA INSTITUTE OF TECHNOLOGY

A researcher corrects a mutation in the β-globin gene that causes sickle-cell disease.

Gang Bao, a biomedical engineer at the Georgia Institute of Technology in Atlanta, is developing gene-editing strategies for sickle-cell disease and is paying particular attention to the challenge of limiting off-target effects. He notes that if erroneous cuts happen in a cancer-causing gene, they could potentially trigger tumour growth. Even a rate of off-target activity lower than 1% could still pose serious health risks. So that the technology can move forward, researchers need to have a better understanding of off-target effects. There are two main issues: determining exactly where the off-target cuts occur and at what rate.

Bao's group has created software to predict where the off-target effects might occur for the different gene-editing techniques. In a paper published in May, his team reported that their software predicted 114 potential off-target sites across the whole genome for the CRISPR/Cas9 system, and experiments confirmed 15 of them by sequencing the cleaved DNA[2].

Izpisua Belmonte's team is also looking at the rate of unwanted mutations caused by gene-editing techniques. The group created iPS cell lines and then edited half of the cells using HDAdVs and TALENs[3], but left the other half unedited. The edited cells had no more mutations than the unedited ones, indicating that — in contrast to Bao's findings for CRISPRs — the use of TALENs does not seem to make cells any less safe. Although human testing is still a few years off, they say that these results give them optimism about the potential for gene editing to work.

The other major challenge for gene-therapy researchers is ensuring that the edited stem cells survive and generate healthy red blood cells after they are reinserted into the bone marrow. Edited cells often die because of the amount of stress they undergo during therapy. Researchers might be able to improve the cell-survival rate by delivering other types of cells at the same time, and the speed of gene editing also seems to be important: the longer the cells are cultured *in vitro*, the less likely they are to survive. "Let's see if we can perform the whole procedure in four hours instead of four days," says Bao.

FUTURE REPAIR TOOLBOXES

Based on his research so far, Bao thinks that CRISPRs are the best method for generating DNA breaks, but they are also more likely to cause off-target activity. TALENs are less efficient than CRISPRs, but they seem to have fewer off-target effects. The rate of on-target activity for CRISPRs is between 40% and 80%, whereas the on-target rate for TALENs is between 20% and 50%, Bao says. The rate of off-target activity varies depending on the type of cells and the nuclease used. "If we have a way to overcome the off-target and [cell-survival] problems, CRISPR is a very promising technology," he says.

Kohn has compared ZFNs, TALENs and CRISPRs, and thinks all three have therapeutic potential for patients with sickle-cell disease. The techniques are all good at slicing DNA; now the remaining challenges are delivering them to the target cell and accurately repairing the gene after the break.

Ultimately, for sickle-cell gene therapy to become reality, the details must be sorted out on a large scale. Tinkering with human genes can yield both devastating and remarkable results, and the difference between the two often lies in a single nucleic acid of a single gene. This places a heavy responsibility on the shoulders of every researcher in the field, but the vast potential of gene therapy makes that burden worthwhile. ■

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2. Fine, E. J. *et al. Nucleic Acids Res.* **42,** e42 (2014).
3. Suzuki, K. *et al. Cell Stem Cell* **15,** 31–36 (2014).