CRISPR/Cas9 Mediated Gene Therapy via HDR in Stem Cells for the Treatment of Sickle Cell Anemia

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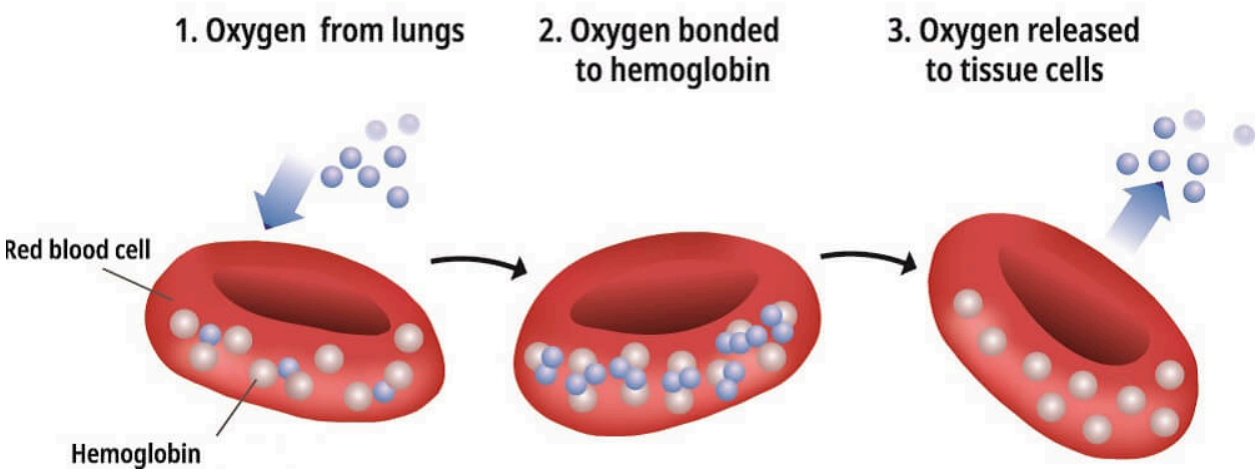
Introduction

Sickle Cell Anemia (SCA) is an autosomal recessive disorder, which means that each parent typically carries a copy of the gene responsible for the disease, but it does not manifest as a dominant trait. The child of two recessive-carrying parents might inherit both copies of the mutated gene in which case the mutated gene is the only gene that will be expressed. Sickle Cell Anemia primarily affects people of Hispanic and African descent. Signs and symptoms of the disease begin to be exhibited in 5-6-month-old infants. In the blood cell's new sickled shape, it has a higher risk of becoming stuck in small arteries and, if this results in blood flow restriction in the brain, or it could also lead to life-threatening strokes. The fragility of the sickled blood cell also often leads to hemolysis or the complete rupture of the blood cell. As blood cells rupture, the net oxygen circulation within the body declines, leading to anemia and hypoxia, or a lack of oxygen in the blood cells, in which patients feel bouts of fatigue, irritability, dizziness, and lightheadedness. Without oxygen transport through the body, patients may even experience organ failure to the heart, kidney, or liver. A single change in the amino acid sequence causes SCA. SCA can also result in each hemoglobin protein aggregating within the blood cell. Instead of maintaining its flattened-disc shape, the red blood cell acquires a stiffened sickle-like shape as the hemoglobin clumps together. This altered shape can be lethal as the blood loses its malleability, often leading to the formation of blood clots and strokes (*Sickle Cell*, n.d.) As of today, Sickle Cell Anemia affects upward of 100,000 people in the United States where the life expectancy of an affected individual is more than 20 years shorter than in those in the average population (*WHAT IS SICKLE*, n.d.). There are current curative treatments.

The hemoglobin protein is composed of four polypeptide subunits (2 alpha subunits and 2 beta subunits), each hemoglobin chain contains a separate molecular component called heme which includes a single iron molecule whose charge is +2. Oxygen is introduced and binds to the iron molecules in our bloodstream through the process of respiration. Our lungs first filter the oxygen we breathe in from our environment through small blood vessels called capillaries. Once in the bloodstream, an individual oxygen molecule, containing a -2 charge, binds to its iron counterpart forming the chemical compound iron oxide. The result is a hemoglobin protein

composed of four iron molecules each bonded ionically to an oxygen molecule.



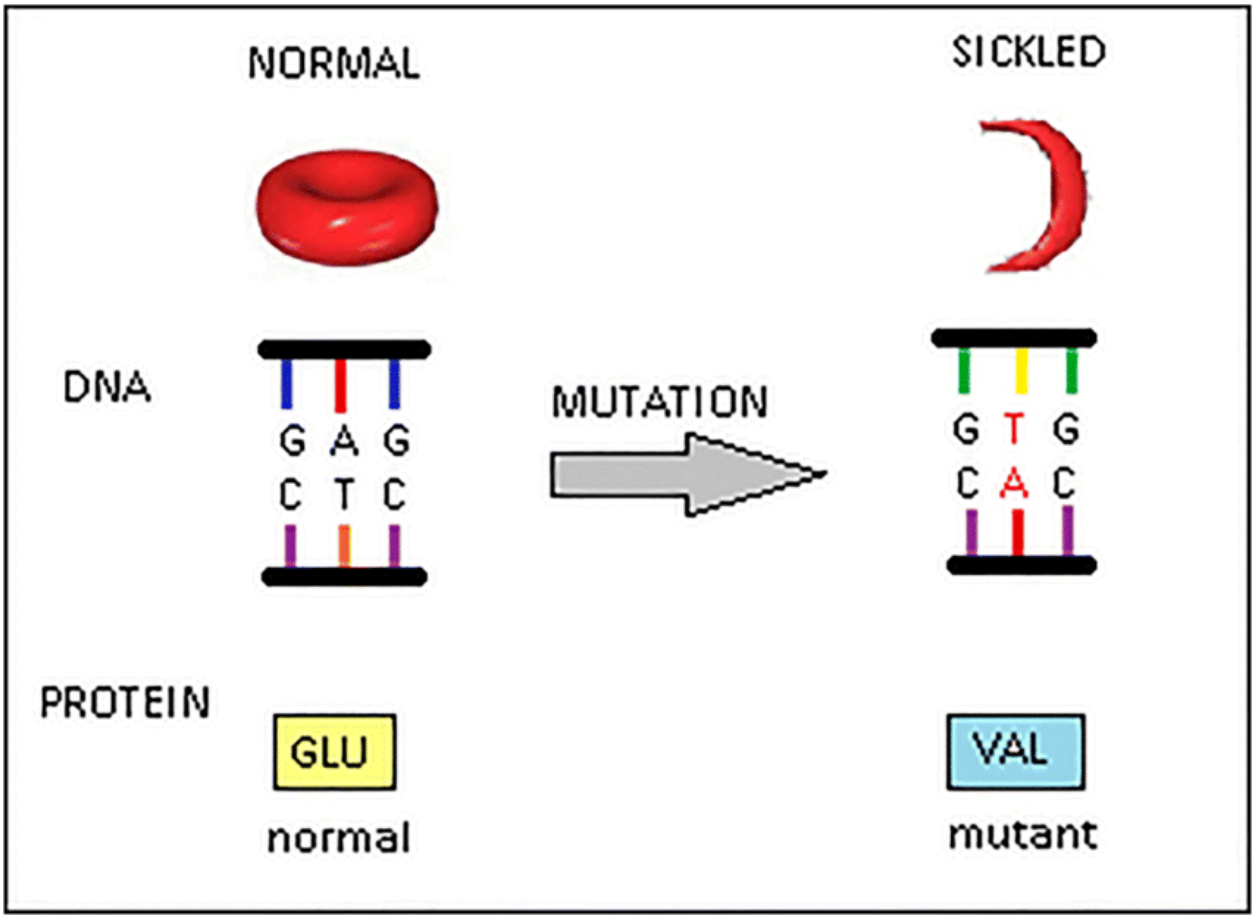
Knapp, S. (2021, May 5). *Red Blood Cell*. Biology Dictionary. Retrieved June 25, 2024, from https://biologydictionary.net/red-blood-cell/

Figure 1. *Process of oxygen transportation via hemoglobin iron bonds.*

Thus, each hemoglobin protein within a blood cell can carry four individual oxygen molecules. In total, a blood cell carries around 270 million of these hemoglobin proteins. Therefore, cumulatively, a single blood cell carries nearly 1 billion oxygen molecules (Rhodes et al., 2022).

Molecular Driver of Sickle Cell Anemia

Hemoglobin comprises 287 separate amino acids each being assembled through the sequence of nucleic acids (DNA) in the hemoglobin genes. A mutation within the DNA of a single amino acid (glutamic acid) in the beta-globin gene causes the substitution of a different amino acid (valine). The sequence of the nucleic acids responsible for producing glutamic acid is from a change in the codon of GAG to GTG, subsequently replacing adenine with thymine. The new amino acid, valine, naturally has different biophysical properties than glutamic acid. Whereas glutamic acid is polar and negatively charged, valine is non-polar. In the mutated hemoglobin, the two beta-globin subunits now containing valine cause the hemoglobin to clump and aggregate, forming rigid rods otherwise known as hemoglobin-s (Kalkan 2019). Given that the hemoglobin exists within the blood cell itself, the mutation of the hemoglobin gene leads to a change in the blood cell morphology. In its healthy form, the blood cell is circular and shaped in a disk allowing for it to move through tight blood vessels with ease. The polymerization of hemoglobin alters the shape to look like a crest/sickle thereby compromising the cell's malleability.



Ramzan, A. (2022, July 22). Sickle cell anemia. *Getting Rid*. https://amirramzan8.blogspot.com/2022/07/sickle-cell-anemia-introduction-people.html

Figure 2. *Sickle cell causing mutation and the normal vs. mutated genetic sequencing.*

Though the cellular DNA repair system of humans can detect obvious mutations within the genome, it is unable to detect small, more discrete point mutations in which a single nucleotide is substituted with another. Hence, in the case of SCA, the cell is simply following through with the instructions provided by its genetic code (DNA). Furthermore, given the hereditary nature of SCA, the body will produce the mutated hemoglobin-s from birth until death, meaning that within the affected patient, there is no knowledge of another form of hemoglobin that will function correctly. It is for this reason that some technology, such as CRISPR/Cas9, could serve as a cure for SCA.

Description of CRISPR/Cas9

The CRISPR technology was first discovered within the bacterium *Streptococcus pyogenes*, serving as the bacteria's immune system. When infected with a virus, part of the virus's DNA encodes itself into the DNA of the bacterium, in essence providing the bacteria with a memory of the infection. These foreign bits of DNA, referred to as 'spacers', are sandwiched in between repeating parts of the bacteria's DNA that are being infected, otherwise known as palindromic repeats. At this point, the immune response begins with the activation of the Cas9

enzyme (CRISPR-associated nuclease protein) (Baugh, 2022). The Cas9 enzyme binds itself with a tracrRNA: segments of DNA that are complementary to, and can therefore anneal to the palindromic repeats. Along with the tracrRNA, a sequence of the viral DNA is also copied into the Cas9 enzyme, also known as the pre-crRNA. The main function of the Cas9 nuclease is to cleave DNA at precise locations. Thus, with the instruction of the RNA, when the bacteria encounter another viral attack from the same genotype that had initially encoded itself, the Cas9 will be activated to cleave the affected DNA once the RNA recognizes its congruence to the virus. Once cleaved, the virus DNA will no longer be able to express itself, thus terminating its reproduction in the host bacteria. By making modifications to the guide RNA in the lab, CRISPR can be tailored to precisely cleave any sequence ofDNA (Liao, 2021).

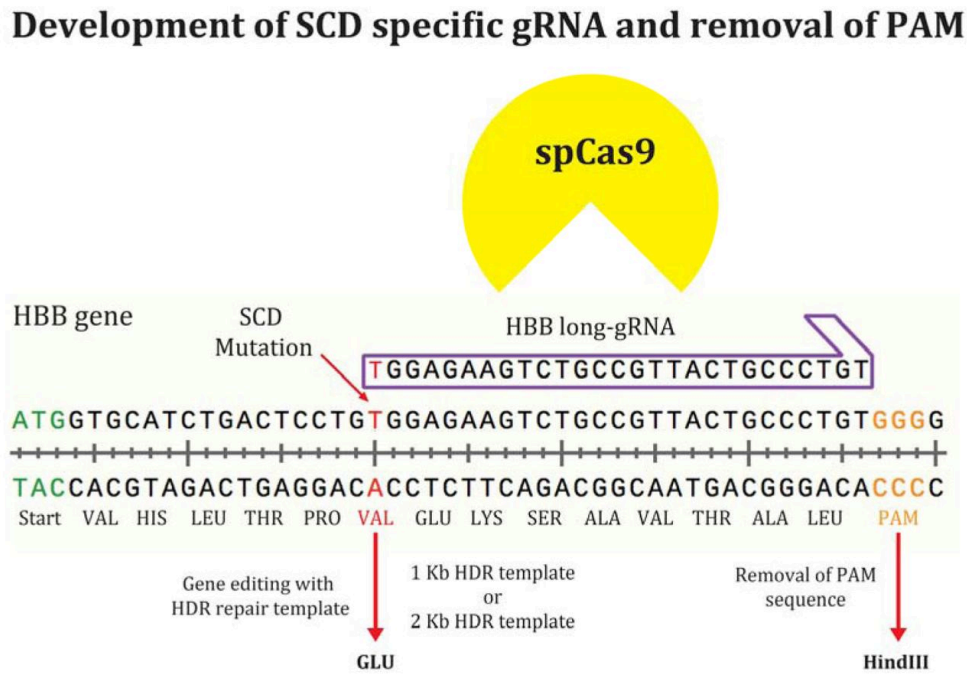


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Figure 3. *The initial step is to design a guide RNA (gRNA) that will guide the Cas9 to the target location and that is complementary to the DNA sequence near the mutation of the HBB gene.*

As seen in Figure 1, the gRNA makes use of the protospacer adjacent motif within the sequence. The design of the gRNA is not included in the PAM sequence. PAM sequences are found directly adjacent to the target sequence and are used to help the Cas9 technology identify its binding site. The sequence is a common sequence within the genome of both the target cell and the Cas9 protein. Depending on the variant of the Cas9 gene, different PAM sequences can initiate the binding of Cas9. The most widely used variant of Cas9 derives from the bacteria in which the technology was first discovered: *Streptococcus pyogenes.* With this variant of the Cas9 gene, an NGG PAM sequence is required for Cas9 to bind to the common sequence. An NGG sequence is where “N” is any nucleobase followed by two consecutive guanine bases. In

the case of the HBB gene, the “N” base would be another guanine base (Anders, 2014). The gRNA is then composed of the sequence that precedes the PAM up to the location of the point mutation. The gRNA is composed of two parts. As seen in *Figure 3*, The crRNA (CRISPR RNA) is the base sequence that is complementary to the target DNA sequence. In the case of SCA, the target sequence is the sequence containing adenosine (“A”) and its complementary sequence is the sequence above it containing thymine (“T”).

The next component is the trans-activating CRISPR RNA (tracrRNA).

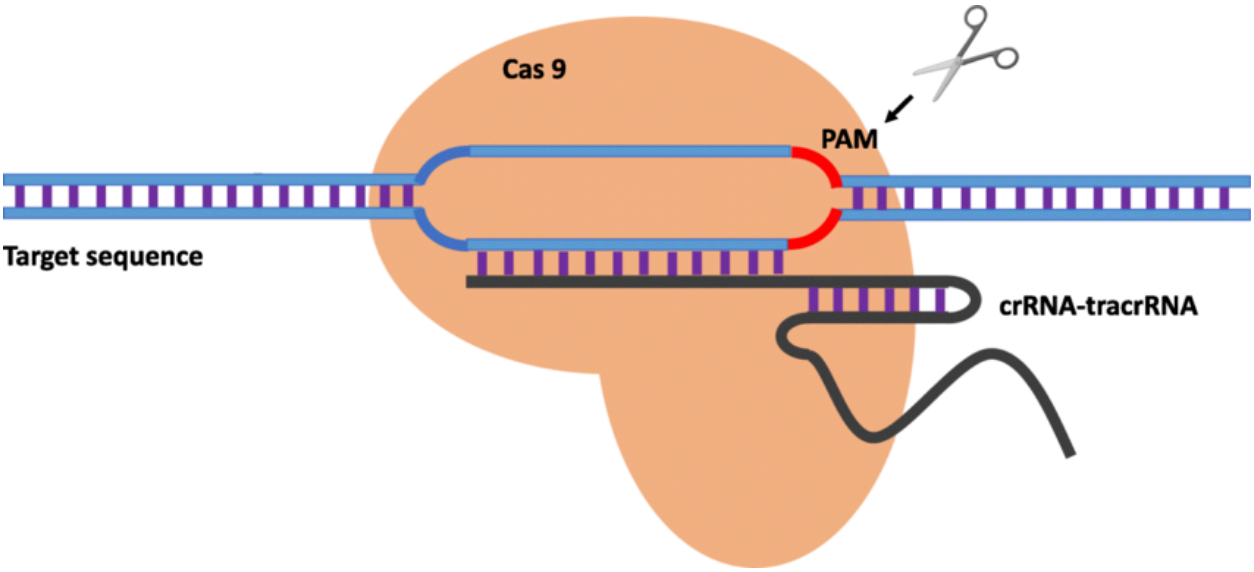

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Figure 4. *Diagram of the location of CRSIPR cleaving relative to PAM sequence and tracrRNA*

The main function of the tracrRNA is to provide overall stability to the binding of crRNA to the Cas9 protein. Its sequencing is complementary to a repeat sequence within the crRNA, allowing it to bind together and form a single guide RNA (sgRNA) that is also bound to the Cas9 protein. Your tracrRNA sequence remains constant and typically follows the target sequence (Genet, 2021).

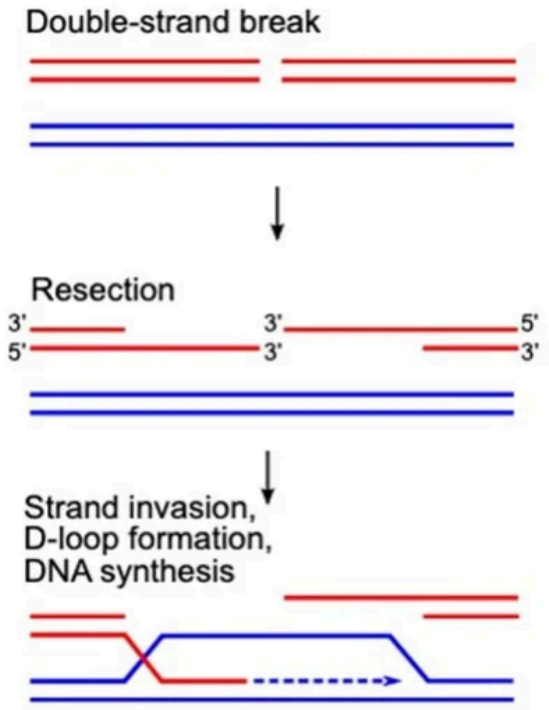
5'-TGGAGAAGTCTGAATGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTT-3'

Pavani, G. (2021, February 26). *Correction of β-thalassemia by CRISPR/Cas9 editing of the α-globin locus in human hematopoietic stem cells*. bloodadvances. Retrieved June 5, 2024, from https://ashpublications.org/bloodadvances/article/5/5/1137/475292/Correction-of-thalassemia-by-CRISPR-Cas9-editi

Figure 5. *Entire gRNA Sequence*

The sequence highlighted yellow in Figure 5 represents crRNA complementary to the target DNA, while the subsequent section represents the sequencing of tracrRNA complementary to the sequencing in the crRNA (an RNA transcript from the main CRISPR locus). With the complementary sequencing of the crRNA bound to the targeted sequencing of the HBB gene, the gRNA is stabilized within the Cas9 protein and functions to specifically target the mutated sequence complementary to the gRNA (Pavani 2021).

Before cleaving, if the gRNA matches sufficiently with the target sequence, the gRNA will begin to anneal itself to the target DNA in a 3' to 5' direction (right to left). Next Cas9 will cleave opposite strands of the target DNA - a double stranded break. This break occurs in 3-4 base sequences to the left of the PAM sequence. After the break, the protein MRX performs a process known as resection: roughly 50-100 nucleotide bases are resected from single strands within the double-strand break, resulting in some sections of sequencing hanging freely without any complementary bases. Supposing the initial CRISPR/Cas9 cleavage occurs in 3-4 base pairs to the left of the PAM sequence, the mutated GTG valine-producing sequence will be removed during resection (Synthego, 2017). The cleavage of the target DNA sequence on both strands allows for various repair mechanisms to engage more freely with the mutated sequencing (Pavani 2021). To create the change in a single base pair, CRISPR/Cas9 can either undergo non-homologous end joining (NHEJ), or homology-directed repair (HDR). When DNA breaks, it undergoes resection in which there is a loss of some nucleotides. A loss of nucleotides can lead to the DNA developing single-strand overhangs in which parts of a strand have lost their complementary nucleotide bases. NHEJ combines various proteins and enzymes to cut the excess nucleotide bases from both sides of the cleavage and then join back the remaining parts of both ends. The biggest caveat to NHEJ is that the repaired DNA sequence will not resemble its original sequence due to the loss of potentially important nucleotides. Furthermore, this new DNA sequence could also potentially give rise to another, more harmful mutation as bases are removed. In the case of a point mutation such as SCA, it is preferable that homology-directed repair is used (HDR).



Patrick, M., & Gearing, M. (2015, March 12). *CRISPR 101: Homology Directed Repair*. Addgene. Retrieved June 14, 2024, from https://blog.addgene.org/crispr-101-homology-directed-repair

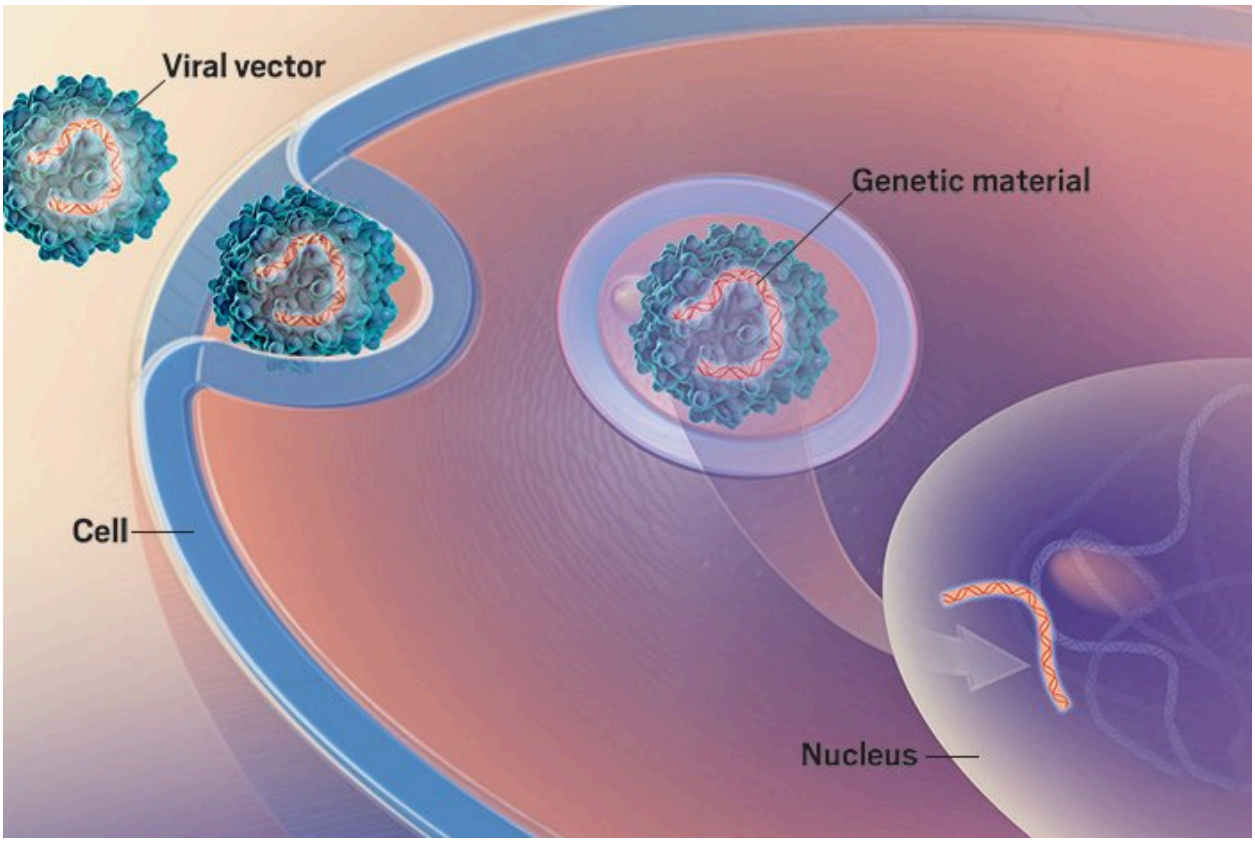
Figure 6. *The crossing of two homologous strands of DNA is known as a Holliday Junction.*

Under the HDR system, the double-strand break is repaired according to a strand of donor DNA - unmutated DNA from another body. Like NHEJ, HDR is one of our bodies' natural repair systems. On every chromosome lies two copies of double-stranded DNA (one inherited from your mother, the other from your father), otherwise known as a 'homologous chromosome'. When a double-stranded break occurs in one of the chromosomes, a protein called Rad51 forms around each single-strand break and pulls it underneath the complementary ssDNA located on its homologous chromosome (Craig, 2014, chapter 1). Once one end of the break is underneath its complementary DNA, the DNA is repaired to connect back with the other part of the broken strand. This repair forms the loop-like structure shown below.

Delivery Methods for CRISPR/Cas9

There are two possible infusion methods for CRISPR/Cas9: The first is via a non-viral vector, using a technique such as electroporation, which temporarily permeates the cell membrane of a cell via electric fields allowing for gene therapies to enter. This method, however, hasn't been thoroughly tested in humans and may pose a risk of cell lysis if the cell membrane

is destroyed. The second method is via a viral vector. Viruses are highly efficient in traversing the cell membrane. To enable the safe use of viral vectors, all of the viral genes are removed from the virus in a process similar to that which develops mRNA vaccines (genetic modification of the virus using CRISPR to cleave the viral genetic code) and modified only to deliver therapeutic genes.



Novella, S. (2021, April 21). *Viral Vectors for Gene Therapy*. Science-Based Medicine. Retrieved June 25, 2024, from https://sciencebasedmedicine.org/viral-vectors-for-gene-therapy/

Figure 7. *Depiction of the mechanism of delivery of modified genetic material to the cell nebulous via viral vector.*

There are two types of viral vectors: 1) Non-integrating vectors. These vectors do not typically insert their DNA into the cell genome and instead exist episomally. This means that if a non-integrating vector infiltrates a cell that divides, the therapeutic DNA from the vector will not be copied between each cell division and the therapeutic effect will eventually decline. 2) Integrating vectors. These vectors insert their DNA into the cell's genome, making it the vector of choice for cells that are capable of dividing (*Vectors 101*, 2023). Hematopoietic stem cells divide into myeloid stem cells and lymphoid stem cells within the bone marrow to produce red blood cells and white blood cells, respectively. To ensure effective distribution of the repaired DNA into the red blood cells, integrating vectors, such as lentiviral vectors can be applied in an *ex vivo* treatment before reintroducing the modified stem cells into the bone marrow. The

general effects of using a viral vector on the stem cells include immune responses varying in severity. Additionally, long-term monitoring of the vector after insertion is crucial to ensure that the vector correctly inserts itself into the right location within the cell's genome. Off-target effects can cause unintended genetic modifications.

Extraction of hematopoietic stem cells [HSC] begins by releasing the stem cells into the bloodstream via an injection of a granulocyte colony-stimulating factor. Once in the bloodstream, stem cells are collected by an apheresis machine separating the stem cells from other blood cell components (Moawad, 2024). A sample of the HSC's is exposed to the lentiviral vector and then reintroduced in the body. The process of re-introduction requires patients to first undergo chemotherapy. Radiation from the treatment opens up space within the bone marrow for the modified stem cells. The stem cells are then placed into the bloodstream with the use of a central venous typically via a large vein in the neck. After circulating in the bloodstream, the stem cells will migrate to the bone marrow where they can engraft themselves in the bone marrow and begin producing un-sickled blood cells.

Current Treatments For Sickle Cell Anemia

The first currently available treatment for SCA is a bone marrow transplant in which healthy donor bone marrow is transplanted to the patient via an intravenous catheter. Similar to the delivery process of hematopoietic stem cells, the donor bone marrow is first circulated throughout the bloodstream before integrating itself into the patient's bone. Chemotherapy is frequently used to kill off the stem cells within the bone marrow producing unhealthy red blood cells. Then, the bone marrow producing the unhealthy red blood cells is gradually supplanted (Pruthi, 2024). However, whereas CRISPR directly alters the human genome, bone marrow transplantations lack precision and won't be capable of providing the same effective and long-lasting treatment as CRISPR/Cas9. The *in vivo* treatment of bone marrow transplantation also opens up the risk of complications occurring from the treatment. One example complication is graft-versus-host disease (GVHD), which occurs when the donor cells attack the recipient's healthy cells. Though thorough screening of patients makes such occurrences rare, choosing an incorrect donor can be fatal if enough stem cells are killed. Additionally, other effects of the transplant include bleeding as a result of lost blood cells and risks of infection as the induced chemotherapy heavily weakens the patient's immune system. Therefore, by limiting the amount of transfusion of cells from a foreign body, CRISPR-Cas9 gene editing may be a safer option.

In patients suffering from SCA, oxygen levels within the blood are heavily depleted. Oxygen therapy helps these patients receive more oxygen than they would receive otherwise. Hemoglobin proteins in patients with SCA often experience polymerization of their hemoglobin which in turn reduces the potential for oxygen to bind to each protein. Supplemental oxygen via compressed gas helps to increase oxygen concentration within the blood to be delivered to vital organs and tissues. Consequently, by ensuring that more oxygen is present in the bloodstream, more hemoglobin can remain in its normal oxygenated form which is less likely to polymerize (Rhodes et al., 2022). Patients who experience frequent episodes of pain and vaso-occlusive

crisis are recommended to take some form of oxygen therapy. This may involve multiple visits to a medical facility and overexposure to oxygen can even lead to oxygen toxicity which can lead to the production of reactive oxygen species (ROS) capable of destroying cell membranes in the body (Whetherspoon, 2017). Aside from the risks, oxygen therapy does provide effective temporary relief to patients suffering from the symptoms of SCA. However, as a long-term treatment that attacks the root of the mutation, CRISPR/Cas9's potential efficacy outweighs that of oxygen therapy. Instead of making multiple visits to a facility to acquire this therapy, CRISPR/Cas9 precisely targets the point mutation to permanently return the patient's genetic sequencing to its healthy state. Oxygen therapy should instead be used in tandem with permanent treatments to provide relief for patients through the course of their treatment.

CRISPR/Cas9 Application To Sickle Cell Anemia

Ideally, CRISPR/Cas9 must be delivered to the patient to change the point mutation and restore the beta-globin gene to its functional state. Specifically, CRISPR/Cas9 must target the mutated sequencing of the HBB (beta-globin) gene and replace the thymine in the GTG sequence with adenosine to establish a GAG sequence to produce glutamic acid at that position. To ensure the complete replacement of all mutated cells, CRISPR/Cas9 would target hematopoietic stem cells, located within the bone marrow and responsible for the production of all blood cells. Hematopoietic stem cells can be extracted from the patient via apheresis, allowing for an *ex vitro* sample to be infused with CRISPR/Cas9 from a petri dish.

DNA synthesis copies the DNA sequencing from its healthy homologous counterpart until it joins back to its other broken strand. The same process is followed for all single-strand DNA (ssDNA) breaks. By this logic, supposing that the copy of DNA homologous to the mutated DNA functions by producing glutamine, then by the process of HDR, the sequencing from the glutamine-producing DNA will be transcribed into the broken DNA. This treatment is promising as it may lead to a potential cure for SCA.

However, the reality of CRSIPR's application to SCA is very different, and only a few clinical trials have been performed in this area. Currently, research for other forms of treatments aside from the gene therapy outlined above has opened other avenues for potential cures for SCA. Trials performed by Vertex Pharmaceuticals Inc. and CRISPR Therapeutics have revealed that complete replacement of the mutated b-globin gene can also occur by replacing the adult b-globin with unmutated fetal hemoglobin (Huang, 2023). As blood cells develop postnatally, the production of fetal hemoglobin is gradually repressed by the BCL11A gene as the patient ages. Once in adulthood, gamma globin is replaced with the production of beta globin, increasing the presence of adult hemoglobin in the body. Considering SCA specifically targets the b-globin-producing HBB gene, the reintroduction of gamma-globin and fetal hemoglobin would thereby repress the presence of the mutated adult hemoglobin. To achieve this, a particular variation of the CRISPR/Cas9 technology labeled CTX001 would need to be infused intravenously into the hematopoietic stem cells [HSPC] of the patient located within the bone marrow. CTX001 is first manufactured by collecting healthy HSPC from the patient via apheresis

(Yin, 2019). Within the HSCP cell itself, CRISPR/Cas9 is used to suppress the expression of the BCL11A gene, and the production of fetal hemoglobin is increased.

Conclusion

Although research on CRISPR/Cas9 technologies is still in the nascent phase, and applications of CRISPR on the human genome have yet to be seen, the dynamic mechanisms of CRISPR/Cas9 exudes an immense amount of potential to cure fatal genetic and hereditary diseases such as SCA. For patients who endure the disease into adulthood, their quality of life is severely restricted due to the painful symptoms that come along with the disease. However, mortality due to SCA expands beyond just able-bodied adults, even taking the lives of young infants who experience fatal tumors or clots. Furthermore, the constant monitoring of their complete blood count (CBC), hemoglobin levels and cardiac function requires frequent visits to the hospital which are often expensive. Developing a cure for SCA with the use of CRISPR/Cas9 will not only revitalize the health of millions of people worldwide as well as be economically beneficial for patients, but it will also open discussion for CRISPR's applications in other adjacent disorders and diseases. As for now, the medical community can only remain steadfast in their research to further develop CRISPR/Cas9 to be compatible with the human genome.

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