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Using Crisper in the Treatment of Sickle Cell Disease

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ABSTRACT

Individuals with sickle cell disease (SCD) experience many complications and health problems due to their mutated hemoglobin gene; however, these problems could be alleviated by using CRISPR gene editing to alter which hemoglobin variants hematopoietic stem progenitor cells produce. The aim of this review is to determine if it is safe and effective to treat patients with SCD with CRISPR. The findings of "CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β Thalassemia" found an increase in fetal hemoglobin expression in patients after transplanting hematopoietic stem progenitor cells with edits to the BCL11A transcription factor. "Genome editing using CRISPR-Cas9 to create the HPFH genotype in HSPCs: An approach for treating sickle cell disease and β-thalassemia" also raised fetal hemoglobin by editing hematopoietic stem progenitor cells within their β-globin loci to artificially apply a hereditary persistence of fetal hemoglobin deletional genotype to the cells. Researchers in "CRISPR/Cas9-Mediated Correction of the Sickle Mutation in Human CD34+ cells" raised wild-type hemoglobin expression in hematopoietic stem progenitor cells affected with SCD by editing the β-globin loci and "fixing" the point mutation that causes SCD. Target deletion efficiency ranged from 18%-80%, and every study used either SpCas9 or SaCas9. Each study found no punitive off-target effects, and they also succeeded in raising hematopoietic stem progenitor cells levels of their targeted hemoglobin. These results point to using CRISPR as both a safe and effective way to treat SCD.

Sickle Cell Disease

Sickle Cell Disease (SCD) consists of a group of genetic diseases that affect millions of people worldwide, stemming from a single nucleotide mutation to the HbB Gene in chromosome 11. SCD is an autosomal recessive disease meaning an individual needs to receive two copies of the mutant allele in place of HbB genes. People who inherit one copy of the mutated gene have Sickle Cell Trait (SCT), and therefore are only carriers of the disease.[1]

Etiology

The HbB codes for hemoglobin which is a quaternary protein that resides within red blood cells. In humans, hemoglobin has 4 globular subunits which fit together into the globin fold arrangement. It is composed of protein chains that form alpha helixes, and it contains an iron ion that helps with oxygen binding, giving blood its red color. Hemoglobin expressed by humans can vary depending on what stage of life they are in, as we use fetal hemoglobin (HbF) during our development and later switch to β-globin during infancy. The protein most used in adult humans is the β-globin protein (HbB). HbF is used by human cells during fetal stages of life and is specialized in transporting oxygen from the mother's bloodstream to the fetus. The key difference between HbB and HbF is that HbF consists of 2 alpha globular subunits and 2 delta globular subunits,

while HbB has 2 alpha and 2 beta globular subunits. This difference allows HbF to bind more strongly to oxygen molecules than HbB, which is what allows it to take oxygen from the mother's blood cells.[2]

Sickle-hemoglobin (HbS) is an aberrant form of hemoglobin that occurs when there is a mutation in the HbB gene. HbS has a different structure, which causes it to reveal binding sites for other HbS molecules when deoxygenated. The polymerized strands are made up of HbS hemoglobin proteins which clump together once deoxygenated. This allows for the hemoglobin molecules to polymerize and create long protein strands, therefore creating the sickle shape seen in SCD red blood cells. The sickle shape makes it harder to carry oxygen in the cell, exacerbating the process. HbB hemoglobin is capable of being soluble when oxygenated and deoxygenated, unlike HbS which is only soluble when oxygenated. This allows for HbB to be processed by the spleen much easier, making it less energy consuming to recycle.[1] Figure 1[3] demonstrates the difference between HbB molecules on the left and HbS molecules on the right.

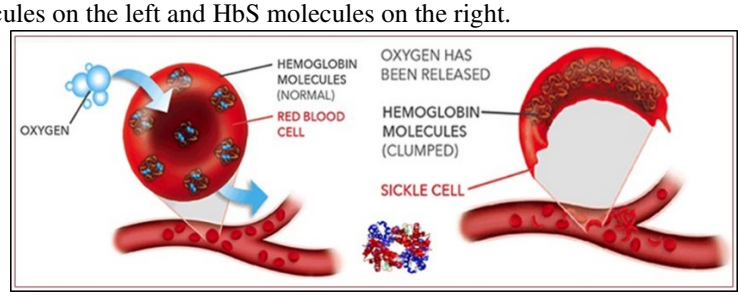

Figure 1. Figure 1 displays the difference between the HbB molecules and how they reside within red blood cells versus HbS and how HbS molecules polymerize and form sickled shapes.3. Source: 3. Esmaeil S.P., 2020. Effect of Hbs on accuracy of pulse oximetry in blood oxygen saturation level measurement among adult patients with sickle cell disease. Eurasian Chemical Communications, 2(3), 296-301.

Pathology

SCD presents with signs and symptoms that affect patients throughout their entire lives as a result of the sickling of the red blood cell. Cell sickling starts when an infant with SCD switches from HbF to HbS, on average at 4-6 months of age, and symptoms present almost immediately after the sickling starts. The symptoms of the disease include anemia, vaso-occlusive crisis, splenic sequestration crisis, acute chest syndrome, aplastic crisis, hemolytic crisis, chronic pain, and dactylitis. Anemia is lack of red blood cells caused by the sickled cells' short life spans-- 10-20 days compared to a regular red blood cell's average of 120 days. Vaso-occlusive crises occur when the sickle-shaped blood cells restrict blood flow to an organ, causing pain, necrosis, and/or ischemia. This often leads to organ damage, and the organs most commonly affected are the penis and lungs. Splenic sequestration crises are caused by infarcted spleens, usually already infarcted by the end of childhood. Patients undergo hypovolemic shock if the crisis is not treated effectively. Acute chest syndrome refers to a group of signs/symptoms: fever, chest pain, hypoxemia, trouble breathing, and pulmonary infiltrates. Aplastic crises occur when the patient's anemia (a symptom every SCD patient experiences) worsens, leading to fatigue, paleness, and elevated heart rate. Hemolytic crises happen when a person with SCD has an acute accelerated drop in hemoglobin levels, leading to red blood cells breaking down faster. Lastly, dactylitis, or inflammation of the digits, is common in all SCD patients, especially among children.[4]

SCD complications affect the heart, kidneys, spleen, and other organs vulnerable to weak blood supply. SCD can lead to pulmonary hypertension, cardiomyopathy, and left ventricular diastolic dysfunction, all caused by the sickled cells scraping and blocking blood passageways to, from and within the heart. The kidneys can be affected by acute papillary necrosis, which kills the renal papillae (the openings of the kidneys for which urine to flow through to the ureters) due to blood clots in the renal vein and artery, as well as chronic kidney failure. SCD most often negatively impacts the spleen, which is responsible for filtering blood and destroying the HbS cells, by causing hypersplenism—or the overuse of the spleen leading to it indiscriminately destroy-

ing blood cells or destroying them too early and often. More complications can arise from the poor blood supply patients with SCD suffer from, such as hematopoietic ulcers, leg ulcers, avascular necrosis, priapism, osteomyelitis (inflation or swelling of bone tissue), strokes or silent strokes, problems with pregnancy and fertility, the formation of gallstones which leads to inflammation of the gallbladder, and eye diseases that can lead to blindness. Patients typically have weak immune systems due to the loss of functioning spleen tissue, leading to vulnerabilities to many forms of infection, especially bacterial. The most common infections patients with SCD suffer from are caused by *Streptococcus pneumoniae* (pneumonia) and *Haemophilus influenzae*, due to their encapsulated nature. [4,5]

Subtypes of SCD

There are a variety of subtypes of SCD, all having unique versions of hemoglobin expression. The most common type of SCD is sickle cell anemia (SCA) which is characterized by the inheritance of two HbS genes, ergo SCA is also called HbSS. Similar to HbSS, people who have HbSC have inherited one HbS gene but also one HbC gene, both being abnormal copies of hemoglobin. People who have HbSC typically have milder symptoms due to the "C" variant of hemoglobin being less problematic for cell shape. The rarer types of SCD include HbSD, HbSE, and HbSO. These types of SCD are caused by the inheritance of one HbS gene and one abnormal hemoglobin gene (either D, E, or O).[6]

Epidemiology

SCD is found most commonly in sub-Saharan Africa, northern India, the Middle East, and some parts of Southern Europe. Africa has the most cases by far, with over 75% of the world's SCD being found there. SCD is more common in people whose ancestors lived in tropical regions where malaria is prevalent, as when malaria is present, having SCT offers a heterozygote advantage and are therefore more resistant to the disease. Those of African/African-Caribbean descent are at higher risk of inheriting the mutation, and in Nigeria alone, there are approximately 150,000 affected children born each year.[5]

CRISPR

Clustered regularly interspaced short palindromic repeats (CRISPR) is a relatively new gene editing technique that allows for precision cuts in DNA using a guide RNA strand, or gRNA. CRISPR was originally a system of genetic defense used by bacteria against viruses that allowed for the Cas enzymes to scan the genome for viral DNA and delete it using a saved piece of virus gRNA. However, in 2012 researchers discovered that Cas9 could be harnessed by humans, allowing us to build our own gRNA sequences as well as perform more efficient gene editing.[7]

Most gene editing causes double strand breaks (DSB), and CRISPR-Cas9 is no different. DSBs can be fixed by either homologous recombination (HR), where DNA repair systems use an existing copy of the DNA to repair the cleaved site, or non-homologous end-joining (NHEJ). NHEJ is used when there is no DNA template present in the cell to perform HR. NHEJ uses tiny DNA sequences called microhomologies, which are essentially tiny hooks on the end of single-stranded overhangs after a DSB occurs. If these microhomologies are compatible the cell repair happens without flaws; however, when they are not compatible, NHEJ can lead to nucleotide loss, translocations, frame shift mutations, and telomere fusion. This is bad for the cell and it is much more common that these adverse effects happen with NHEJ due to the rarity of microhomology compatibility.[8]

Past Gene-Editing Techniques

Gene editing itself is not new, it has been researched since the 1970s, and scientists did use other gene editing techniques before discovering CRISPR. Zinc finger editing (ZFN) and transcription activator-like effector nucleases (TALEN) were the most effective techniques in the past, and they are still used today.

ZFNs are engineered DNA-binding proteins that create double strand breaks at the user's specification. Structurally, they are composed of a zinc finger protein, a nuclease enzyme that can cut/cleave DNA creating double strand breaks, and a DNA-binding domain (DBD). The DBD usually contains 3-6 zinc finger repeats which can recognize 9-18 base pairs. ZFN uses a restriction enzyme found in *Flavobacterium okeanokoites* that must dimerize to sever DNA, meaning that two ZFNs are required when targeting a non-palindromic DNA sequence. ZFN DBDs can only be so specific, leading to off target cleavages. This problem can be alleviated by adding more zinc fingers, however the structures become more complicated and harder to create as more zinc fingers are added.[9]

TALENs are restriction enzymes that use engineered transcription activator-like effectors (TALEs) to find and cleave specific DNA sequences. TALEs are produced by the *Xanthomonas* bacterium in order to infect plants. TALEs also act as the DBD and contain a repeated 33-34 amino acid sequence with highly variable 12th and 13th positions. These amino acids, called the repeat variable diresidue (RVD), are vital for nucleotide recognition. Changing the RVD can help improve targeting and reduce off-target cleavages. Both ZFNs and TALENs cause double strand breaks, and the repair of these double strand breaks is what causes an edit to the genes.[10]

CRISPR in Gene Editing

CRISPR-Cas9 is the best gene editing technique found thus far, due to its high specificity and customization, allowing for genes to be targeted with accuracy. CRISPR-Cas9 uses CRISPR-RNA (crRNA) sequences as well as a transactivating crRNA (tracrRNA) to operate. Almost all CRISPR systems works via a guiding strand of RNA (gRNA), composed of both crRNA and tracrRNA when used. Most gene edits using CRISPR induce knock-in mutations, which damage the DNA in a controlled manner that makes it easy to alter the way the DNA is reconstructed. In order to make these changes to the genome, CRISPR technology needs to use all of its parts: the gRNA/crRNA which allows the CRISPR complex to locate the correct DNA binding site, the tracrRNA which binds to the crRNA to form an active complex (when combined the gRNA and the tracrRNA are called the sgRNA or gRNA), the nuclease which binds to the DNA and allows for single or double-stand breaks depending on which Cas enzyme is used, and the repair template which consists of a DNA molecule that is used as the template in HR. This allows for CRISPR systems to locate the correct gene, bind to the target site, employ a form of DNA strand breaking, and use the template DNA to create the desired changes to the broken DNA.[11]

The protospacer adjacent motif (PAM) is a 2-6 base pair DNA sequence that is placed at the end of the targeted DNA for all CRISPR systems. The PAM comes from the plasmid of virus DNA that the cell is attempting to cut but is not actually a part of the virus/plasmid's genome. In bacterium, it is created using the "spacers" in the CRISPR loci, DNA sequences created using the invading DNA of a plasmid of virus that had been previously inserted into the cell. When the DNA is inserted by these invading viruses or plasmids, they are "protospacers". Cas9 knows not to cut spacers because in the CRISPR loci there is no adjacent PAM sequence, however there will be a PAM sequence next to all protospacers, allowing for Cas9 to bind to the DNA. The PAM essentially informs the CRISPR locus what DNA is its own and what DNA is from a foreign source. The PAM is an integral part of CRISPR's workings, as it is also the binding site for the Cas enzyme. The PAM also varies greatly depending on which Cas enzyme you use.[12]

Cas9 has many uses, however new innovations such as the discovery of Cas12a have allowed for CRISPR to use new methods for cleaving DNA. Cas12a was found in the CRISPR system of the bacterium *Francisella novicida* and it allows for DNA to be cut in two different positions, causing a "staggered cut". Cas12a also only needs a gRNA to operate, unlike Cas9 which requires both gRNA and tracrRNA sequences. Cas12a can do this due to its T-rich PAM sequence ("T" in T-rich meaning it has many transactivating agents, the same agent as in tracrRNA). Also, Cas12a cleaves 18-23 base pairs downstream from the PAM's position, allowing for there to be no damage to the crRNA after DNA repair. This means that Cas12a can undergo multiple rounds of DNA cleavage. Cas9, on the other hand, cuts 3 base pairs away from the PAM and uses NHEJ to repair the DNA sequence, causing indel mutations to the recognition sequence and disabling further cutting. Finally, Cas12a employs the "collateral cleavage" property, meaning it stays bound to the target sequence and cleaves other single-stranded DNA (ssDNA) molecules non-discriminately. Another Cas enzyme that has proven useful is Cas13, found in the bacterium *Leptotrichia shahii*. Cas13 is characterized by its unique ability to cleave ssRNA, due to it being an RNA-guided RNA endonuclease. It also has the "collateral cleavage" capability.[13,14]

CRISPR needs a vessel to deliver it to its intended site, especially when used in *in vivo* gene editing. For this purpose, adenovirus serotype 5 (Ad5), a virus that does not affect humans, was engineered to package and deliver CRISPR systems into live cells. This way, the Ad5 can seek out the correct cell, inject CRISPR-Cas systems into the cell, and not cause any damage to the human body after delivery. While this innovation is very useful, it still has limited space, so the Cas enzyme needs to be paired with a relatively short PAM, crRNA, and tracrRNA. Other delivery methods include injection, *ex vivo*, electroporation, and lentiviruses (LVs). However, compared to Ad5, electroporation and LV present problems with health risks and cell death, while injection and *ex vivo* are not as accurate.[15]

There are several studies that have used CRISPR in the treatment of SCD, however three have been selected for review due to their findings being useful for the treatment of SCD in humans. The aim of this review is to use existing studies' findings to create an effective treatment for SCD using gene editing or gene therapy. This review is intended to study whether it is safe and effective to treat SCD with CRISPR.

CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β Thalassemia

BCL11A is regulatory C2H2 type zinc finger protein that plays a role in suppressing fetal hemoglobin (γ-globin) production. When mutated, the mutation hinders the gene's ability to suppress the expression of fetal hemoglobin. This allows for people who suffer from SCD to alleviate their symptoms by producing more cells with γ-globin instead of the mutated HbS hemoglobin. Using CRISPR-Cas9, researchers were able to successfully target and mutate the BCL11A erythroid-specific enhancer locus in healthy donor CD34+ cells. The aim of this study was to increase the effectiveness of hematopoietic stem cell transplants by allowing the transplanted cells to produce not only HbB cells but also HbF cells. Since the BCL11A gene is a relatively short DNA sequence, Cas9 was determined to be the best fit for the edits due to its specificity and low probability of off-target effects with the correct gRNA sequence as Figure 2 shows in panels A and B.[16]

These treatments were tested on 2 patients: one with SCD, and one with β-thalassemia. Both patients used autologous cells for the treatment, making the strategy *ex vivo*. For the purpose of this review, the focus will be placed on the patient with SCD. The patient with SCD who received treatment was a 33-year-old female who averaged 7 severe vaso-occlusive episodes a year. Her fetal hemoglobin expression rose to 99.9 percent 5 months after the treatment, and her expression stayed at nearly 100 percent after 15 months—the latest checkup (see Figure 2, panel C). Approximately 80% of the patient's alleles were modified, and there was no evidence of off-target mutation (see Figure 2, panel D). The treatment resulted in no off-target effects being discovered, however the researchers stated that they had not performed tests on their clinical samples or characterized the patients' clonal diversity. This could mean that off target effects existed but did not present

themselves within the time it took to administer the tests, or that the treatment worked for both patients due to similar genetics but would not work for those with differing genetics. A year later the SCD patient treated with this method showed higher levels of fetal hemoglobin pancellularly as well as transfusion independence. The patient also was cured of vaso-occlusive crises. When the treatment was fully edited and developed, the infusion was named CTX001.[16]

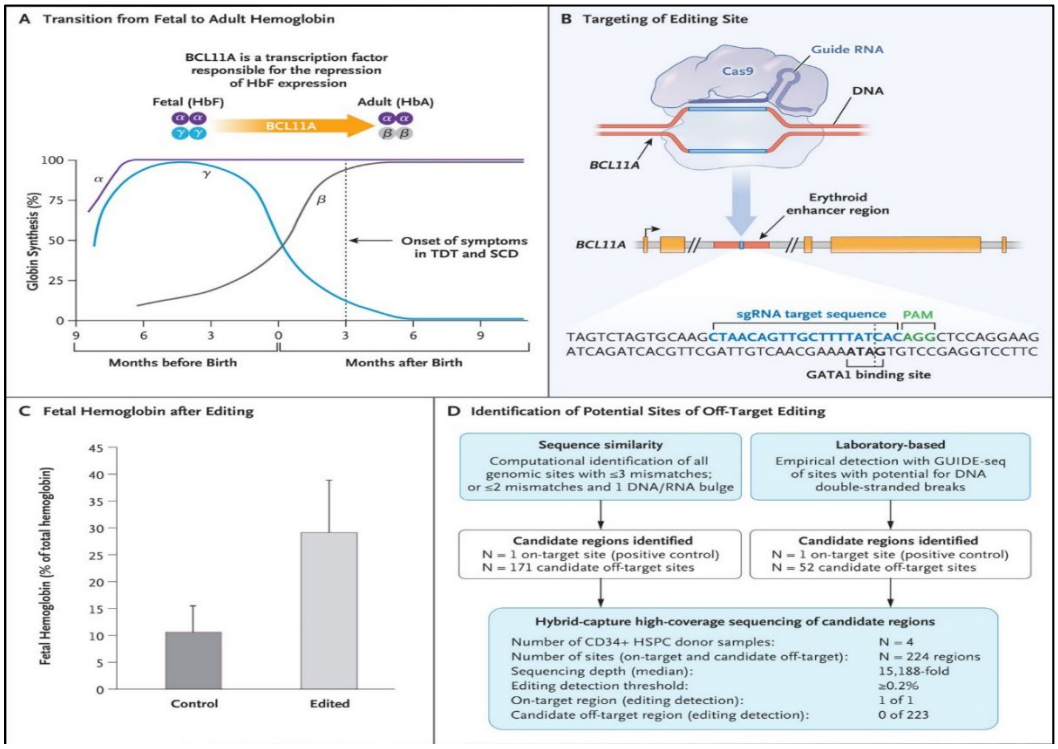

Figure 2. Panel A displays BCL11A's role in the change from HbF to HbB as well as showing the timeframe of the switch. Panel B shows the targeted DNA site in BCL11A as well as how CRISPR will conduct the DNA edit. Panel C is a bar graph that shows the difference in HbF levels in unedited and edited cells. Panel D shows the process of checking for off-target cleavages, as well as some data about the treatment's specificity.16. Source: Frangoul H. 2021. CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β Thalassemia. N Engl J Med, 384, 252-260.

Potential complications could arise from the gene that was chosen to edit, the BCL11A transcription factor. This gene has been found to affect a variety of organs and systems throughout the body depending on which cell is expressing it. For example, BCL11A is expressed in brain neurons where it helps to form a protein complex that regulates axon branching and outgrowth. It has also been found to function as a leukemia disease gene, specifically myeloid leukemia, through its interaction with the BCL6 gene. Furthermore, a link between cognitive dysfunction such as autism, intellectual disability disorder, and global developmental delay has been made with heterozygous *de novo* mutations in BCL11A. BCL11A has even been identified as a gene of interest in regard to type-2 diabetes.[17] With BCL11A regulating all these important factors in our body, tampering with the gene *in vivo* or at an early enough stage in development could lead to health problems later in life. However, both patients were fully developed, and the donor cells were edited *ex vivo,* so this treatment does not raise concerns about disturbing brain function or other systems related to BCL11A.[16]

The greatest success of this experiment was the positive outcome the patient experienced; however, another important aspect is that the operation was performed *ex vivo*. Because the treatment was performed with the patient's own cells, the need for immunosuppressant drugs was bypassed, something that benefits an already immunocompromised person such as one with SCD. The possibility of the body rejecting infused

stem cells or blood transfusions is big risk for those undergoing contemporary forms of treatment for SCD, so this extra layer of added safety increases this treatment's value. While this treatment did help both patients in the long run, they did experience short-term adverse effects such as pneumonia, VOD/SOS, sepsis, cholelithiasis, and abdominal pain. Some of these effects were correlated to neutropenia, like the pneumonia and sepsis, however others were likely caused by a delay in lymphocyte recovery due to CTX001 being enriched in CD34+ cells. The authors of this study presented updated results on 7 patients with SCD treated with CTX001. These patients also demonstrated increased HbF and total hemoglobin levels, similar response patterns, and the elimination of vaso-occlusive crises.[18]

Genome Editing Using CRISPR-Cas9 to Create the HPFH Genotype in HSPCs: An Approach for Treating Sickle Cell Disease and β-Thalassemia

Unlike in "CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β Thalassemia ", this study aimed to change cells' genotypes to a conditional one that increases HbF expression. One of these conditions, hereditary persistence of fetal hemoglobin (HPFH), is a condition that causes patients to contain high levels of γ-globin throughout their lives. HPFH is caused by mutations to the β-globin, α-globin, or δ-globin loci. Since these loci are big, there are 25 known (usually large) mutations that cause it. There are 8 forms of deletional HPFH genotypes, which were the genotypes the experiment was aiming to induce. This normally is considered a disease, as those without any existing hemoglobin production issues become oversaturated with oxygen due to their high levels of γ-globin, but for patients with abnormal hemoglobin like those with SCD, HPFH helps alleviate symptoms. Researchers at the University of California at San Francisco found a way to artificially induce HPFH in patients with SCD by deleting 13 kb of their β-globin locus. Figure 3 highlights the cleavage site used during the experiment.[19]

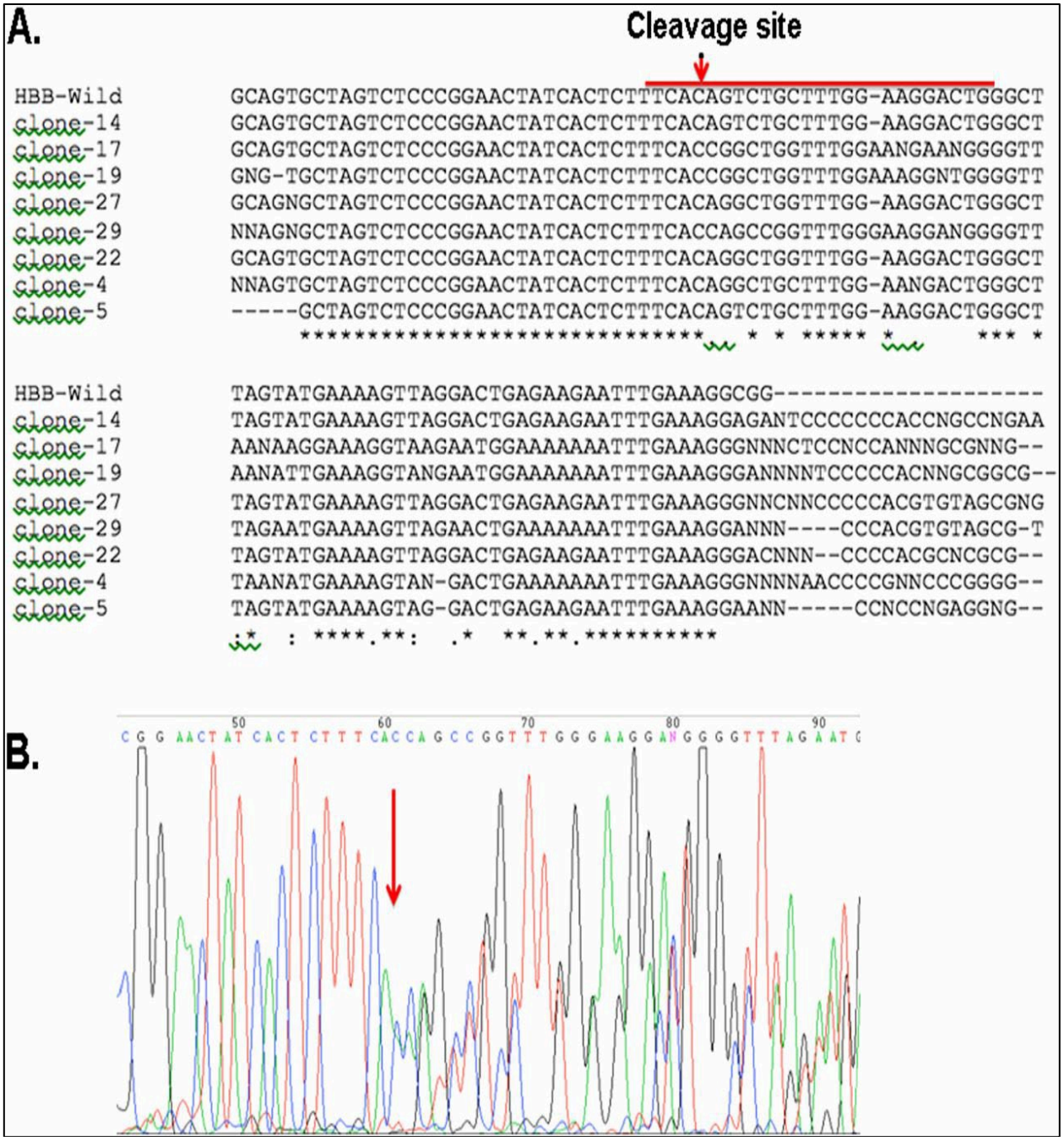

Figure 3. Researchers used Sanger sequencing in cloned HSPCs to detect NHEJ. "A" shows the DNA sequence alignment of the clones, while "B" is a histogram of the sequencing with a red arrow that indicates where the cleavage site is.19. Source: Ye, L. et al., 2016. Genome editing using CRISPR-Cas9 to create the HPFH genotype in hspcs: An approach for treating sickle cell disease and β-thalassemia. Proceedings of the National Academy of Sciences, 113(38), pp.10661–10665.

The researchers used CRISPR-Cas9 to edit normal bone marrow hematopoietic stem and progenitor cells (HSPCs) to encode for the deletional HPFH genotype. They grew a colony of these edited cells along

with a control group of cells taken from the same source to measure the differences between the two. Their process is shown in figure 4.[19]

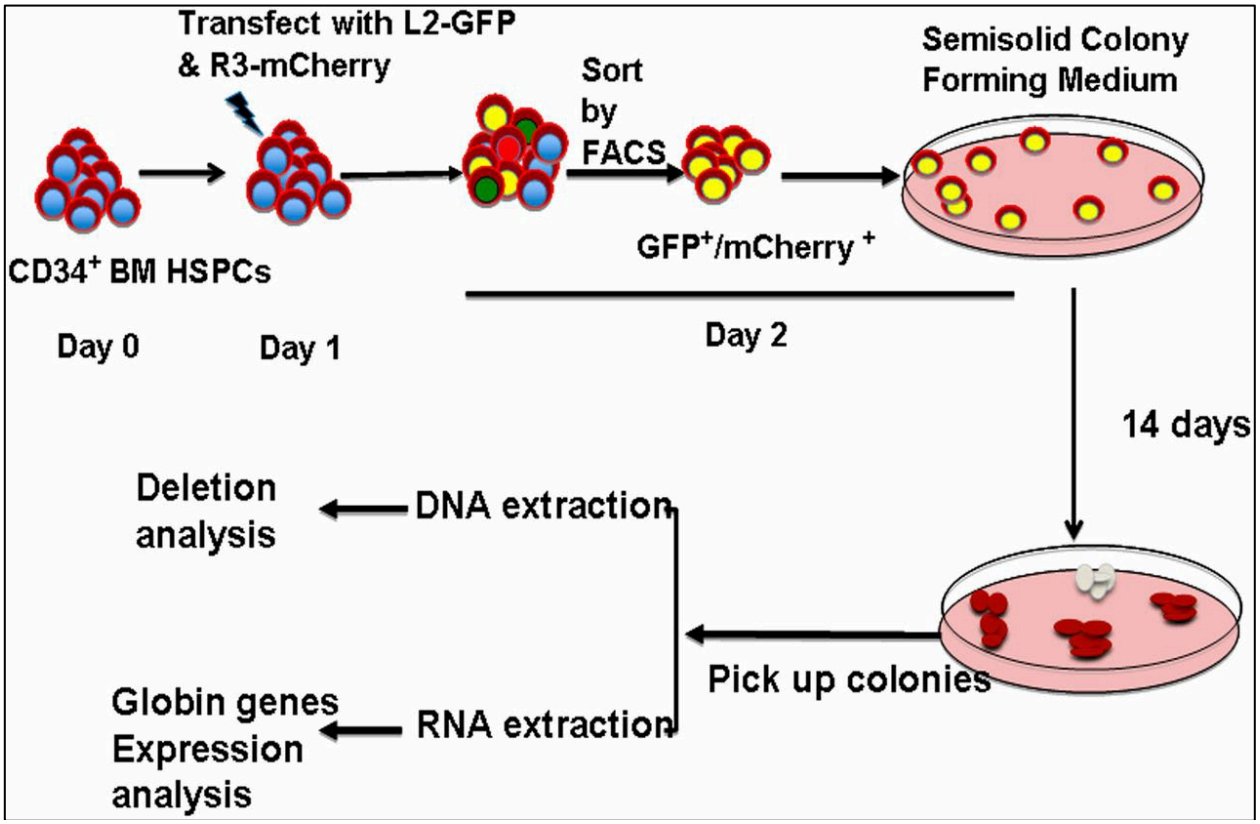

Figure 4. Process used to modify CD34+ HSPCs with the HPFH deletion.19. Source: Ye, L. et al., 2016. Genome editing using CRISPR-Cas9 to create the HPFH genotype in hspcs: An approach for treating sickle cell disease and β-thalassemia. Proceedings of the National Academy of Sciences, 113(38), pp.10661–10665.

This experiment used the *Staphylococcus aureus* Cas9 nuclease, or SaCas9 for short, which was found to reach 31% target deletion efficiency. SaCas9 was chosen due to its smaller size when compared to the generic SpCas9 as well as its need for a longer PAM sequence, reducing the risk for off-target mutations. SaCas9 also can cut longer DNA sequences, making it optimal to cut the 13-kb long sequence targeted in this study. Using T7 endonuclease 1 assay researchers did not discover any off-target effects within the colony of cells with the HPFH deletion. The edited colonies also showed significantly higher γ-globin expression, and due to the HPFH mutation being a deletion, no RNA template was needed to induce homologous recombination, allowing the DNA to undergo nonhomologous end-joining.[19]

While this potential treatment was not tested in humans, it offers another way-- which did not report any adverse effects—to increase γ-globin expression in patients with SCD. One success of this study was its extreme specificity, as researchers not only chose SaCas9, but they also manually picked 4 targeting sites for SaCas9 that showed significantly less homology with other DNA sequences. The entire CRISPR system was even packed into pX601, or Addgene, allowing for all 8 of the gRNAs, tracrRNA sequences, and the Cas nuclease to exist in one vector. The experiment reached 31% gene editing efficiency and using the T7E1 assay researchers found no off-target mutations. It was also found that despite using NHEJ to connect the severed DNA, NHEJ could have occurred perfectly or resulted in one extra base pair deletion. In order to test the accuracy of the engineered gRNAs, researchers used the 10 samples that showed evidence of deletion in at least one allele. Target site accuracy was tested, and the 10 clones (2 clones with homozygous deletions and 8 clones with heterozygous deletions) were found to have 100% target-site cleavage.[19]

While there is not much data about the medical aspects of this experiment besides the outcome, researchers did state that there was work being done to develop a lentiviral delivery system for the corrective β-globin gene at the time of publication. This study was also based off a rare proportion of people who suffer from SCD and have the naturally occurring HPFH mutation, as these people typically experience milder symptoms and less complications with SCD than those without HPFH. The strategy of attempting to artificially change a large enough sequence of nucleotides to mimic an existing genotype is both novel and broaden possibilities. Of course, this study's strategy was only able to succeed due to the extreme specificity of the DNA targeted. Since SaCas9 can stay binded to the target DNA after cleavage, researchers were able to use multiple gRNA sequences during tests until they found one that could create a deletional genotype of HPFH mutations with no evidence of off-target cleavage. Researchers also cloned the SaCas9, gRNA, pX601, cell cultures, and target DNA many times in order to add florescent indicators increasing specificity to ensure their experiment worked as expected.[19]

CRISPR/Cas9-Mediated Correction of the Sickle Mutation in Human CD34+ Cells

Instead of aiming to raise HbF levels like in "CRISPR/Cas9-Mediated Correction of the Sickle Mutation in Human CD34+ cells" and "Genome editing using CRISPR-Cas9 to create the HPFH genotype in HSPCs: An approach for treating sickle cell disease and β-thalassemia", this study tried to correct the mutated HbS gene by using a combined system of both TALENs and CRISPR. Researchers were able to change the β-globin loci from HbS into their wild-type—HbB. The experiment was performed in vitro, so there were no human subjects; however, the results were positive, furthering possible clinical trials in the future. For this purpose, human CD34+ HSPCs were used in the experiment. The aim of this study was to determine whether TALENs or CRISPR could effectively cleave exon 1 of the human β-globin gene, as well as discover their specificity. Six of each type of the two editing techniques were used, each with variations to either their TALE or gRNA. The Cas enzyme used was the traditional SpCas9 and researchers used multiple pairs of TALENs and gRNAs. The TALEN samples showed 10-15% of β-globin cleavage, however 2 samples did not show any signs of DNA cleavage. The CRISPR cleavages were shortened, but 2 CRSIPR units were able to achieve 17-39% β-globin cleavage with all gRNAs. The researchers were able to use half the amount of CRISPR nuclease as they did TALEN nuclease (0.5μg compared to 1.0μg), and the CRISPR system showed higher rates of target nuclease activity. While 2 TALENs showed off-target cleavages in the δ-globin loci, the CRISPR gRNAs did not show any evidence of off-target mutations. Figure 5 shows these results.[10]

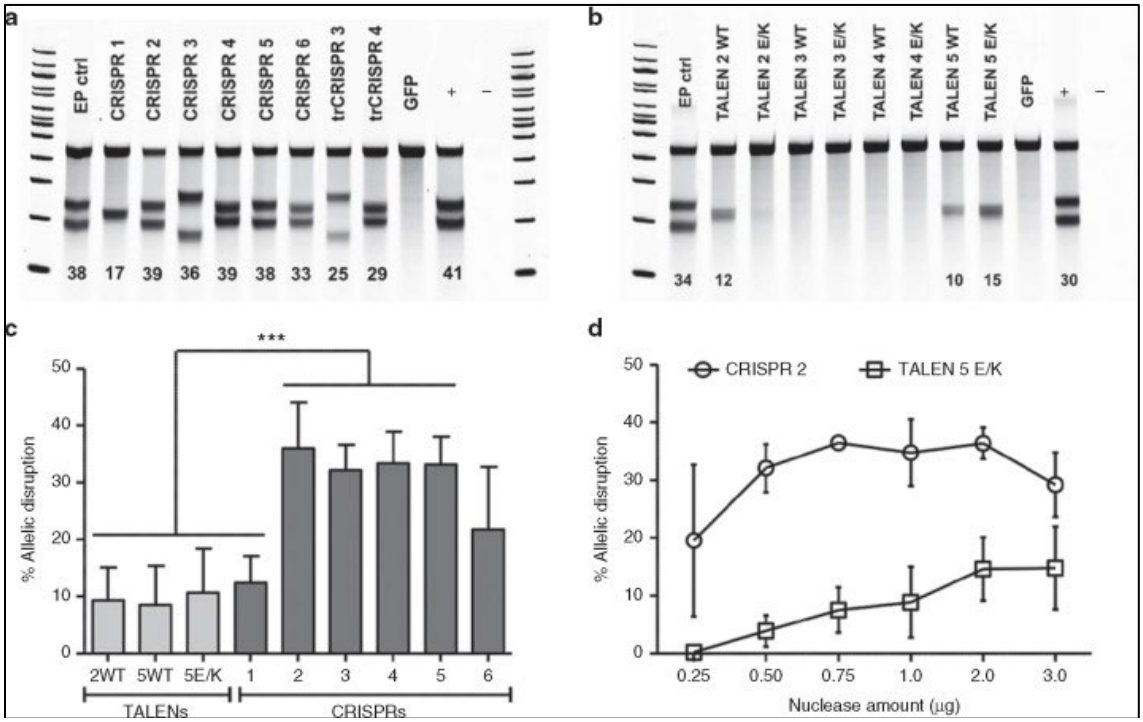

Figure 5. (a) DNA gel showing changes caused by all CRISPR units. **(b)** DNA gel displaying β-globin cleavage using all TALENs. **(c)** The graph represents the average percent of β-globin allelic disruption by all nucleases across multiple experiments. **(d)** Researchers used titration obtain the total plasmid amount by most active nucleases of each type and recorded the data on this line graph10. Source: Hoban M.D. 2016. CRISPR/Cas9-Mediated Correction of the Sickle Mutation in Human CD34+ cells. *Molecular Therapy*, 9(24), 1561-1569,

The specificity of each technique was tested again using the integrase-defective lentiviral vector (IDLV) which traps the nucleases and uses NHEJ to test the extent of off-target mutations. Both 1 CRISPR and 1 TALEN were used, and the TALEN presented evidence of off-target activity in the δ-globin loci again, while the CRISPR system did not have any punitive off-target effects.[10]

Since this study was a test to evaluate the effectiveness of correcting the HbS gene into its wild-type HbB, the biggest concern was off-target mutations. Taking the results at face value, the less effective gene editing technique is TALENs as it provides less specificity and often targeted the homologous δ-globin loci as well. The CRISPR units, on the other hand, provided safe site-specific cleavages that allowed for the use multiple different gRNA strands with only slightly differing results and no off-target mutations.

This study was not a human trial which leads to concerns about potential adverse effects on the immune system as well as potential delivery methods. All gene editing systems were introduced into the cells via electroporation, a technique that often results in cell death around the targeted area. For human use, a viral vector such as lentivirus or adenovirus would be a better option, however both of these techniques also have their own health risks. Another possible complication could be off target effects. Even though the CRISPR systems edited no "punitive off-target sites" these off-target mutations in sequences similar to the HbS gene could result in the mutation of healthy transplanted HSPCs from previous treatments. TALENs presented negative off-target effects when targeting HbS, so it's unsafe to use in the β-globin loci.[10]

The ability to be able to "fix" the HbS gene has great potential, as there would be no need to have people rely on HbF; which can lead to problems with oxygen distribution due to HbF's stronger binding to oxygen molecules compared to HbB. Without the need for HbF patients could rely on their own supply of HbB to deal with symptoms like anemia or hypoxemia. However, while having a supply of HbB would help

patients carry oxygen throughout their body and create more healthy blood cells, patients would not get relief from symptoms like vaso-occlusive crises or any of the complications that arise from blood clots. This is because despite an increase in HbB blood cells throughout the patient's body, there will still be large quantities of sickled blood cells being produced.[10]

Synthesizing Studies' Findings

The first study showed how CRISPR can be used in humans without long-term detrimental effects as well as the importance of using *ex vivo* technique when in clinical trials. It also outlined the importance of the BCL11A gene and how it can be edited to stop repressing HbF expression. The second study introduced combatting SCD with other pre-exiting conditions such as HPFH, as well as using CRISPR nuclease SaCas9. The deletional genotype the researchers induced exists in nature, however the switching of genotype could lead to complications. Also, SaCas9 has useful properties such as allowing for a longer PAM sequence to increase specificity of the target DNA. SaCas9 is smaller than the more commonly used SpCas9, making electroporation (which often results in cell death) unneeded. The final study provided a different approach as researchers edited the mutated HbS gene instead of attempting to raise HbF levels. The experiment used 6 different TALENs and CRISPR with 6 different gRNA units, and 2 of the 6 CRISPR units presented 17-39% β-globin cleavage, with no harmful off-target effects.

With the target genes from the "CRISPR/Cas9-Mediated Correction of the Sickle Mutation in Human CD34+ cells" and "CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β Thalassemia" studies as well as the Cas enzyme from "Genome editing using CRISPR-Cas9 to create the HPFH genotype in HSPCs: An approach for treating sickle cell disease and β-thalassemia" the possibility of creating a new treatment for SCD using CRISPR gene editing arises. In order to make this treatment as effective as possible and as low risk as possible, future studies should take the best aspects of each technique while trying to address short comings. This potential treatment would use SaCas9, the same nuclease used to induce the HPFH deletional genotype, due to its small size and easy delivery via lentivirus. Done in *ex vivo* using the patient's own CD34+ HSPCs, the operation would have two pairs of SaCas9s with distinct gRNAs in order to target both the BCL11A gene as well as the HbS gene. The reasoning behind choosing the BCL11A gene instead of the globin loci to induce HPFH is because repressing BCL11A is a much smaller deletion compared to the induced HPFH mutation. Also, engineering the longer gRNA sequences that can target multiple loci like the ones needed to induce HPFH is more expensive than the shorter gRNA needed for BCL11A. After the operation is concluded the researchers would conduct a T7 endonuclease 1 assay to determine any presence of off-target effects. If there are any punitive mutations the patient will have to supplied with healthy donor HSPCs, however if there are no off-target cleavages the HSPCs will be put back in place. Once the cells are returned to the body the patient will most likely have a pancellular increase in HbF while also producing more HbB blood cells. If the BCL11A transcription factor is successfully repressed then the patient should show no sign of vaso-occlusive crises, while simultaneously having their anemia, hypoxemia, and ulcers alleviated due to increase HbB presence. This would be an expensive operation due to having to use multiple SaCas9s as well as engineer very specific gRNA sequences (especially the gRNA engineered to target the β-globin loci).

Conclusion

When evaluating the safety of potential genetic treatments, the patient's immune response is always a concern. The first study had clinical trials which showed potential complications that could arise from invasive treatments like bone marrow transplants in patients with SCD. Other safety hazards could come from the researchers' delivery methods, like in "Genome editing using CRISPR-Cas9 to create the HPFH genotype in

HSPCs: An approach for treating sickle cell disease and β-thalassemia" where researchers tested using a lentivirus delivery system. This could be dangerous due to the health risks of using viral delivery vector. Of course, with genetic engineering the biggest danger is off-target mutations, so each study conducted tests to measure their treatment's specificity. While the researchers of "CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β Thalassemia" and "Genome editing using CRISPR-Cas9 to create the HPFH genotype in HSPCs: An approach for treating sickle cell disease and β-thalassemia" reported no off-target mutations "CRISPR/Cas9-Mediated Correction of the Sickle Mutation in Human CD34+ cells" researchers' stated that there were no "punitive" off-target mutations—suggesting there were off-target cleavages that didn't affect the cells' ability to survive.

The effectiveness of the treatments of 2 of the 3 review studies is hard to measure in "CRISPR/Cas9-Mediated Correction of the Sickle Mutation in Human CD34+ cells" and "Genome editing using CRISPR-Cas9 to create the HPFH genotype in HSPCs: An approach for treating sickle cell disease and β-thalassemia" since human trials or animal testing were not conducted, however both treatments did succeed in obtaining their desired effects on cells with SCD using CRISPR. When the first study repressed the BCL11A transcription factor, as well as when the second study deleted parts of the β-globin loci to mimic HPFH, the edited cells from both studies produced higher levels of HbF which would be beneficial in sickle cell disease. The study "CRISPR/Cas9-Mediated Correction of the Sickle Mutation in Human CD34+ cells" researchers were able "correct" the HbS mutation within cells' β-globin loci, resulting the production of HbB. All of these edits were performed on HSPCs, exactly the type used during bone marrow transplants in SCD treatments. These successes provide evidence to back up the effectiveness of treating SCD with CRISPR.

Of the selected studies, the first has human sujects which shows evidence of the treatment working outside of a petri dish, and it provides a gene target—BCL11A—for future studies. The second and third study did not include human subjects, however they both did perform *in vitro* CRISPR gene editing with little to no off-target cleavages. In the second study, researchers were able to induce an already existing genotype (HPFH) within CD34+ HSPCs, allowing for an increase in HbF level. The third study successfully used CRISPR on SCD patients' CD34+ HSPCs to change 1 axon mutation within the β-globin loci that was causing the cell to produce HbS red blood cells. The scope of the first experiment is larger than the other two, however there were only two patients treated and their genetic similarity is unsure. The scope of these three studies only extend to small scale clinical trials and cell experiments, however they each provide viable options for further study—using larger scale experiments, mice, or even clinical trials if the treatments become legally approved.

Like all things, the findings in these experiments needs to be further researched, so some potential areas for future study will be highlighted. The first study showed positive results; however, the patients suffered many negative adverse effects. During the clinical trial patients were only being treated for their ailments as a result of the experiment, and the reasons behind their adverse effects were not studied. The second study is attempting to mimic a naturally occurring genotype in patients with SCD to alleviate their symptoms, however, how would the immune system respond to a small proportion of their cells suddenly developing a new genotype? Could the body potentially recognize these cells even if the operation is *ex vivo*? Finally, the third study uses CRISPR to fix the point mutation within the β-globin locus that changes HbB to HbS. Since this study reported "no punitive off-target mutations", the effects of non-punitive off-target mutations on an organism as a whole could be studied using appropriate animal studies. All three studies showcase mostly safe and certainly effective ways to treat SCD with CRISPR.

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