Precision Genome Editing towards the Treatment of Hemoglobinopathies

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Abstract: Hemoglobinopathies, including sickle cell disease and β – thalassemia, are genetic disorders that cause people to suffer from anemia. Apart from the lifelong therapeutic methods, gene therapy has been introduced in the last decades of research as an efficacious treatment option, supported by various types of delivery methods. In this work, I review the precision genome editing towards the treatment of hemoglobinopathies. With a brief cover of the disease pathology and genome editing tools, special focus has been directed towards the potential editing sites and clinical trials in progress.

1 INTRODUCTION

According to the World Health Organization (WHO), approximately 7% of the global population are carriers of hemoglobinopathies, which are divided into thalassemia syndromes and structural hemoglobin variants. The most common hemoglobinopathies are β-thalassemia and sickle cell disease (SCD). Patients suffering from β-thalassemia and sickle cell disease (SCD) show mutated or low levels of β-globin chain production. The mutated β-globin chains result in the hemoglobin tetramer to polymerize, which causes the red blood cells to create a "sickle-like" shape leading to vaso-occlusive crises and tissue damages. Allogeneic hematopoietic stem cell (HSC) transplantation together with iron chelation is the only approved curative treatment for severe genetic blood disorders. However, this method is constrained by the availability of human leukocyte antigen matching donors and face the limitation of graft rejection. Collectively, these genetic blood disorders are recognized as one of the major health challenges in the world. which results in low flexibility and induces hemolytic anemia and vascular occlusions.

Over the past decade, new technological breakthroughs in genome editing have propelled the potential to cure genetic disorders. A cutting-edge technology adapted from the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated protein) bacterial immune system has been established as a platform for performing more efficient, accurate, and precise genome editing. These fundamental technologies have led to the creation of groundbreaking tools that have the capability to transform biological research and curing genetic diseases. Traditional CRISPR editing employs the creation of double stranded breaks (DSBs) by endonucleases, which subsequently stimulates DNA repair mechanisms. These numerous developments have accelerated the exploration of various curative strategies for the treatment of hemoglobinopathies. Genome editing approaches that generate DNA DSBs can correct the disease-causing mutation or induce higher levels of fetal hemoglobin (HbF) expression. However, nuclease-mediated DSB-induced cytotoxicity and chromosomal rearrangement severly limit these approaches as eventual treatments. Recently developed DSB-free strategies have overcome these issues while maintaining superior clinical outcomes. There are now many parallel clinical trials that explore multiple editing strategies for the treatment of β–hemoglobinopathies. Clinical studies using lentiviral-based gene modifications have proved to be successful at ameliorating the clinical symptoms in patients with hemoglobinopathies. The transplantation of genetically modified autologous hematopoietic stem cells (HSCs) prevents any immunological risks, such as graft reject. The

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majority of the patients receiving edited HSCs demonstrate reduced transfusion requirements while maintaining high levels of fetal hemoglobin expression. In this review, I present a comprehensive overview comparing different types of genome editing technologies and approaches for the treatment of β–hemoglobinopathies.

2 OVERVIEW OF DISEASE PATHOLOGY (SICKLE CELL, BETA-THALASSEMIA, ETC.)

Sickle Cell Disease (SCD) is an autosomal recessive genetic disorder affecting the function of hemoglobin and was first discovered in 1910 by James Bryan Herrick. Hemoglobin is tetrameric molecule that formed by a globin group surrounded by four heme groups and function as carriers of oxygen (O2), carbon dioxide (CO2), and nitric oxide (NO). They are critical in transferring oxygen (O2) throughout the bloodstream from lungs to tissues and cells, which is a major distinction of mammal life. Sickle cell anemia is caused by an inherited, single missense mutation in the β globin chain. The single adenine to thymine base substitution results in an amino acid conversion from glutamine to valine in the globin gene. Normal red blood cells are shaped like a binocave discoid, providing elasticity to the cell and allowing red blood cells to pass through the capillary bed to deliver oxygen. However, the deoxygenation of sickle hemoglobin (Hb S) results in an abnormally shaped erythrocyte with a “sickling” appearance. This results in a polymerization of sickle red blood cell which results in low flexibility and induces hemolytic anemia and vascular occlusions.

The β–thalassemia and α–thalassemia are two major categories of thalassemia syndromes, in which the patient lacks the production of β-globin chain. α–thalassemias are a result of defects in the α–globin chain of adult hemoglobin, including partial (α^+) and complete (α^0) deletions or mutations of the four α–globin genes. α –thalassemia can be further categorized into 4 types based on different genotypes and symptoms. α thalassemia silent carriers are when only one gene is deleted or damaged out of three; because only a single copy is affected, the carrier does not show any clinical symptoms nor a reduction in Hb values, but the affected gene is inheritable through generations. α thalassemina carriers have two missing genes that result in mild anemia. Hemoglobin H disease is caused by three missing genes that may lead to moderate and severe microcytic hydrochronic anemia resulting in impaired hemoglobin production, with symptoms of fatigue, exercise intolerance, and enlarged spleens. α - thalassemia major is a fatal disease without treatment, in which all four genes are missing that cause severe anemia. β - thalassemia are caused by the insufficient (β^+) or absent (β^0) production of β-globin chains in hemoglobin and is highly prevalent in Mediterranean countries. β- thalassemia can be further divided into 3 types. Carriers of β–thalassemia minor are normally clinically asymptomatic. β- thalassemia intermediate cases cause milder anemia and the majority of patients do not require any blood transfusions to reduce chronic anemia due to a compensation mechanism of hypertrophy by erythroid marrow. β- thalassemia major corresponds to symptoms of growth retardation, progressive enlargement of abdomen, and skeletal deformities. Although regular transfusion medications are available, they may result in problems of iron overloading which cause dilated myocardiopathy, fibrosis, diabetes, etc. Cardiac diseases are the most life-threatening complication in patients treated with blood transfusions.

3 OVERVIEW OF GENOME EDITING (ZNF, TALEN, CAS9, BASE EDITING, PRIME EDITING, AND MORE)

Over the decades, the emergence of gene editing technologies has offered scientists the ability to introduce modifications to the genome of various cell types in hopes of creating genetic therapeutics. The use of highly specific and programmable nucleases that introduce double-stranded breaks (DSBs) provides the ability to specifically edit any the region of interest. DSBs are repaired by endogenous cellular mechanisms, either through a non-homologous end-joining (NHEJ) repair pathway that generates insertion and deletions (indel), or homology-directed repair (HDR) pathway that utilizes a genetic donor template to replace the DNA surrounding the DSB. Meganucleases (homing endonucleases), the earliest gene technology, are sequence-specific DNA cleavage enzymes used in targeting, replacing, and modifying the genome. Meganucleases, exist as dimers or single-chain enzymes, bind and cleave sequences that are at least

12 base pairs in length with high specificities. Although effective at manipulating DNA, meganucleases have one major limitation. The biggest limitation is that for a meganucleases to bind to DNA, the protein requires specific sequences upon which only this protein can bind to the matched DNA sequence. If one wishes to change the target DNA sequence, a whole different protein, or a profound protein engineering effort of the meganucleases is needed to evolve and re-engineer the protein, which makes it difficult for widespread usage of these tools across biomedical research and biomedicine.

The next two big classes of gene editing technologies developed are zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN). ZFNs consist of engineered zinc fingers fused to a non-specific, dimeric FokI nuclease domain. DNA-contacting residues can be replaced by connecting individual zinc fingers with novel DNA-binding specificities. However, there is a grand challenge when making multi-finger arrays as the fingers are not always modular. Each individual zinc finger recognizes a specific 3-base pair sequence, but each finger also has context-dependent effects, so it is not always as simple as replacing the finger with the desired targeting sequence. TALENs are modular DNA-binding protein units derived from naturally occurring TALEs (from Xanthomonas). Each monomeric TALE repeat recognizes a specific type of DNA base. Like zinc finger arrays, individual TALE subunits can be simply joined together to recognize longer sequences of DNA. Similar to ZFNs, TALE repeat arrays can be fused to a dimeric FokI nuclease domain to create TALENs. Several differences are noted between ZFNs and TALENs. The biggest distinction between them is that each monomeric TALE subunit binds to only a single nucleotide instead of a nucleotide triplet, which makes TALE targeting simpler than that of ZFNs. From a cost and time perspective, TALENs are cheaper and faster to produce, and are more flexible and easier to design because of their simple DNA recognition properties compared to ZFNs. Immunogenicity is another huge consideration for using ZFNs and TALENs in therapeutic applications. ZFNs display little to no immunogenicity because the sequences are found in all organisms from yeast to humans, while the immunogenicity for TALENs in humans is still unknown. However, it is predicted that the immune response of TALENs may be relatively higher since the sequences of TALE repeats are only found in Xanthomonas plant pathogens.

Although meganucleases, ZFNs, and TALENs all have their distinct advantages, there are still several limitations remaining to their widespread. The primary difficulty in using these technologies is that they are fundamentally dictated by a protein-to-DNA interaction when driving specificity of DNA targeting. Therefore, an extensive protein engineering and optimization effort is needed when one wants to target a new sequence of DNA. Furthermore, the cost and time needed to produce each variant is a huge limitation that needs to be considered. It takes weeks to months to produce one specific agent that then still needs to be optimized for a specific site, and many times prices go beyond the financial capability of people and researchers with goals of routine usage. Therefore, there remains a need for an alternative approach that can overcome the remaining limitations in genome editing.

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) is the latest technology that revolutionized the field of genome editing. CRISPR-Cas9 was first identified in 2008 as a bacterial defense mechanism that host bacteria leverage against bacteriophage infections in nature. This was later repurposed as a gene editing tool that uses Cas9 as a nuclease guided by a single guide RNA sequence to bind to a specified target DNA sequence. Although CRISPR-Cas9 is primarily driven by RNA-DNA interactions, there is one remaining protein-DNA interaction known as the protospacer adjacent motif (PAM). The PAM sequence is required for Cas9 binding to a target genomic site. Following Cas9 binding, a small DNA R-loop is formed and a subsequent DNA double-stranded break is generated by the Cas9 endonuclease.

One of the critical aspects of genome editing to evaluate is the off-target propensity of each editing agent. The guide RNA in Cas9 dictates a specific 20 base pair sequence of the targeted DNA sequence. However, Cas9 binding can tolerate small mismatches between the guide RNA and target DNA sequence, meaning that there are possible binding sites that are not the specified genomic site. Each one of these undesired binding sites are known as off-target sites in genome, which may cause undesirable and dangerous negative effects that could lead to additional genetic diseases and unwanted phenotypes, such as cancer. Engineered high-fidelity Cas9 variants have been developed as an effective approach to decrease off-target effects. SpCas9-HF1 is considered to be a high-fidelity variant that maintains high on-target DNA editing

efficiency (at least 70% of the wild-type SpCas9), but dramatically reduced DNA off-target editing efficiencies. The route of delivery into the cell also has a role in the propensity of off-target editing. Protein delivery further reduces off-target effects compared to DNA viral or plasmid delivery since DNA produces transcripts with subsequently product a plethora of editor proteins. Furthermore, the life span of DNA is longer compared to both RNA and protein, which contributes to a longer exposure of editor protein in cells. Because each cell only contains a limited amount of DNA substrate for the editing event, any additional editor protein has the propensity to result in off target editing. Therefore, delivering genome editing protein directly can significantly decrease off target effects.

There are a variety of strategies developed towards detecting Cas9 off target sites, which is vital towards increasing the efficacy and safety of CRISPR-Cas9 genome editing systems. At a single cell level, whole genome sequencing delivers a comprehensive view of the entire genome at a DNA base level. However, off-target effects are ineffective and occur at a relatively low percentage, therefore requiring tens, hundreds, or even thousands of single cell genome sequencing evaluations to thoroughly evaluate all off-target events. Therefore, it is expensive and not effective to rely upon whole genome sequencing to thoroughly evaluate the off-target propensity of every genome editing agent. Alternative approaches to whole genome sequencing were introduced that can enrich for off-target editing events. These rely fundamentally upon next-generation sequencing techniques. GUIDE-seq (Genome-wide, Unbiased Identification of DSBs Enable by Sequencing) and CIRCLE-seq (Circularization for In vitro Reporting of CLeavage Effects by sequencing) are two methods that enrich for Cas9 off-target sites that experience a DSB. There are computational methods that can predict potential off-target sites in the genome, but these computational methods are deemed a preliminary and biased compared to unbiased experimental approaches. GUIDE-seq integrates a dsODN (double-stranded oligodeoxynucleotide) donor template into cleavages sites by non-homologous end joining repair inside a cell's genome directly. However, NHEJ is prone to produce indels rather than incorporating exogenous sequences, which suggests that the sensitivity of GUIDE-seq is one aspect that still needs to be considered. CIRCLE-seq is a highly sensitive in vitro assay that serves as an alternative approach to GUIDE-seq in detecting off target sites. CIRCLE-seq relies on first generating and purifying libraries of circularized genomic DNA, then treating this pool of circles with Cas9 nucleases. Subsequently, nuclease-linearized DNA fragments are ligated with sequencing adapters and sequenced using next generation sequencing to identify genome-wide Cas9 nuclease off target sites.

Base editing is a relatively new genome editing technology that is able to edit DNA without any DSB intermediate. Base editing enables the ability to perform chemistry directly on the genome of living cells. Base editing fundamentally relies upon the Cas9 component from the CRISPR-Cas systems fused together with an enzyme that directly modifies DNA or RNA. Dead Cas9 (dCas9) or nickase Cas9 (nCas9) is first guided by a sgRNA to the targeted region in the genome. Following Cas protein binding, the Cas protein exposes a single-stranded DNA R-loop region that serves as substrate for deamination mediated by deaminases fused to the Cas protein. Base editing is currently largely grouped into either cytosine BEs that can convert cytosine-guanine (C-G) to thymine-adenine (T-A) base pairs, or adenine BEs that can convert adenine-thymine (A-T) to guanine-cytosine (G-C) base pairs. Base editors create precise, predictable, and efficient genetic outcomes at the targeted sequence without any undesired indel byproducts or large genomic perturbations such as p53 activation, DNA rearrangements, or DNA translocations.

Similar to base editing, prime editing is able to perform gene editing without inducing DSBs. But prime editing can generate a wider range of possible alterations as it can perform all twelve possible base-to-base conversions. Prime editing is composed of a Cas9 nickase fused to PE2 (a modified reverse-transcriptase) and a multifunctional prime editing guide RNA (pregRNA). A major advantage of prime editing is its low off-target activity compared to Cas9 as edition of PE2 significantly lowered the off-target effects with fewer indels. Moreover, PE breaks the bottleneck in therapeutic application of gene editing as HDR machinery is not required for prime editing so that post-mitotic cells can be edited.

Table 1: Comparison of four commonly used genome editing biotechnologies.

Technology	Meganuclease	ZFN	TALEN	CRISPR/Cas9
Origin	Microbial genetic elements	Eukaryotic gene regulators	Bacterium Xanthomonas	Adaptive immune system in archaea and bacteria
Targeting	Protein – DNA interaction	Protein – DNA interaction	Protein – DNA interaction	RNA – DNA interaction
Specifi cDNA binding elements		Tripletconfined zinc finger proteins	Single-base recoognition TALE proteins	sgRNA
Off-target Effect	Low	High	Moderate	Variable
Delivery	Major vector systems	Major vector systems	DNA, mRNA, adenovirus, AAV	DNA, mRNA, viral vectors with sufficient packaging capacity
Time		Long (7-15 days)	Relatively long (5-7 days)	Short (1-3 days)
Targeting Efficiency	Low	Moderate	Moderate	High
Size		1kb*2	~3kb*2	4.2kb (Cas9) + 1kb(RNA)

4 INITIAL USES OF DNA DSB CUTTING FOR TREATMENT

Bone marrow and stem cell transplants are currently the primary route of treatment for sickle cell disease and β-thalassemia. In the last decade, the profound discovery and development of genome editing technologies has enabled the ability to use nucleases that create a DNA double stranded breaks (DSBs) in a specific region of the genome to be considered as an initial and effective treatment of β-hemoglobinopathies.

Genes that encode for the β-globin and α-globin are located on chromosomes 11 and 16 in the human genome, respectively. Embryonic hemoglobin, fetal hemoglobin, and adult hemoglobin are three variants of the hemoglobin protein expressed on the erythrocyte at different times during development. Fetal hemoglobin (HbF) is a tetramer consisting of two α-globin chains and two β- globin chains. Following the first three months post-conception, the embryonic globin experiences a dramatic decrease in globin synthesis, while the level of fetal hemoglobin rises rapidly, serving as the primary form of hemoglobin expressed in the fetus while in utero. After the infant is delivered, the level of fetal hemoglobin drops significantly, while the expression of adult hemoglobin becomes the dominant form of hemoglobin.

A regulatory region named as the locus control region, LCR, regulates the expression of the encoded globin genes, and is located 40 to 60 Kb upstream of the beta-globin locus. BCL11A is a protein expressed on the LCR region that binds to two regions on the hemoglobin locus, HBG2 and HBG1, which represses fetal hemoglobin expression, signaling the production of adult globin genes. As the mutation affecting sickle cell is located in the adult globin gene, many therapeutic options being explored using genome editing rely upon upregulation and reactivation of fetal hemoglobin expression to compensate for the sickled adult globin gene. A reasonable approach to elevate the amount of fetal hemoglobin expressed is to reduce the BCL11A repressor activity. Certain mutations found in the erythroid enhancer region can naturally repress the amount of BCL11A being translated, which ameliorates disease symptoms through fetal hemoglobin compensation. Increased expression of fetal hemoglobin is a sufficient at alleviating symptoms of sickle cell because the fetal hemoglobin has a similar to higher oxygen saturation compared to that of adult hemoglobin. It is encouraging that ongoing clinical trials have also demonstrated that the induction of HbF can ameliorate symptoms of sickle cell disease and β-thalassemia.

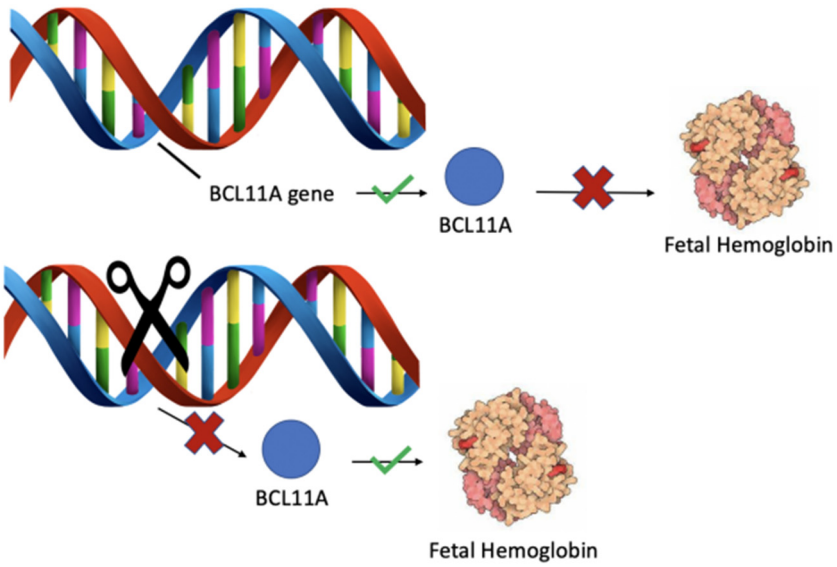

Figure 1: Visual Representation of the Mechanism of Deleting the BCL11A gene.

CRISPR-Cas9 gene editing is helping to tackle sickle-cell disease in many ways. Firstly, Cas9 enzyme can be introduced with a guide RNA to target and repair the faulty β-globin gene through HDR replacement using an exogenous donor sequence as the template to correct the disease-causing mutation. The correction rate ranges from 7% to 50% depending on the editing tool used, such as ZFNs, TALENs, and CRISPR/Cas9, and the delivery method employed when delivering these tools into induced pluripotent stem cell (iPSCs) and HSPCs in vitro. However, xenotransplantation experiments revealed that HDR lacks the generation of long-term engraftable HSCs, shown by a 10% drop in gene correction in vivo. This is a major limitation to this direct replacement strategy so other approaches are needed. Alternatively, a Cas9 enzyme can delete the gene encoding the BCL11A repressors to increase the production of HbF. Like previously discussed, fetal hemoglobin serves as a sufficient replacement of adult globin in alleviating sickle cell disease symptoms. Although these approaches have been demonstrated to be effective, the biggest drawback to using CRISPR-Cas9 nuclease is the creation of double-stranded breaks. These DSBs have the potential to result in large cellular perturbations such as chromathripsis, activation of p53 pathways, and increased cell stress signaling. Therefore, there is an urgent need for alternative approaches of using genome editing without any double-strand DNA break intermediates.

5 PRECISION EDITING USING BASE EDITING, PRIME EDITING

Contrary to CRISPR-Cas9 nuclease editing, base editing can generate direct conversion of one base pair to another at a target site without any double-stranded break intermediates. The DSB-free base and prime editing systems ensure a superior safety aspect of genetic modifications related to the therapeutic treatment for β -hemoglobinopathies. Two major classes of base editing currently exist: A-to-G base editor (ABE) and C-to-T base editor (CBE) that both are mediated by a deamination reaction of either adenine or cytosine, respectively. Base editing has the potential to be one of the most superior gene editing technologies.

Two approaches employing base editing are being explored in treating sickle cell disease: fetal hemoglobin activation and direct correction of the sickle-causing mutation. The first approach relies upon recreating a phenomenon known as hereditary persistence of fetal hemoglobin (HPFH) with base editing. HPFH is a benign genetic condition reflective of high levels of HbF expression in adults, caused by either large deletions or point mutations in the globin locus.

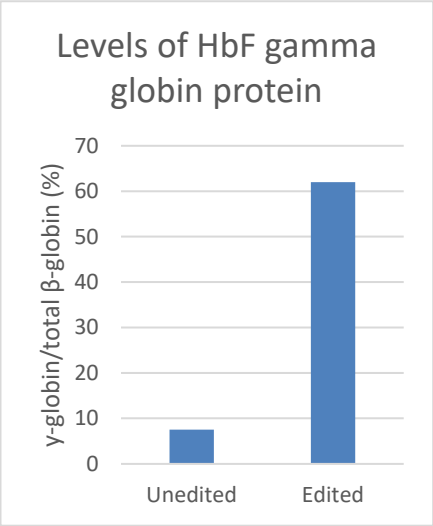

Figure 2: Levels of HbF y-globin protein detected in unedited and edited erythroid cells.

ABE can induce mutations found in HPFH on the fetal hemoglobin genes, HBG1/2, without generating any double-stranded break intermediates or undesired cellular perturbation side effects. Significantly, over 80% base editing was achieved at the HBG1/2 promoters, and this translated to a 60% increase in the expression of fetal hemoglobin in red blood cells. Moreover, high levels of editing and robust HbF induction are maintained after a long-term in vivo engraftment study in mice.

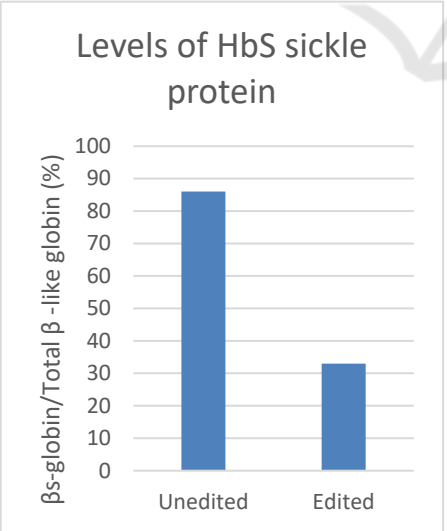

Figure 3: Level of HbS sickle protein detected in unedited and edited erythroid cells.

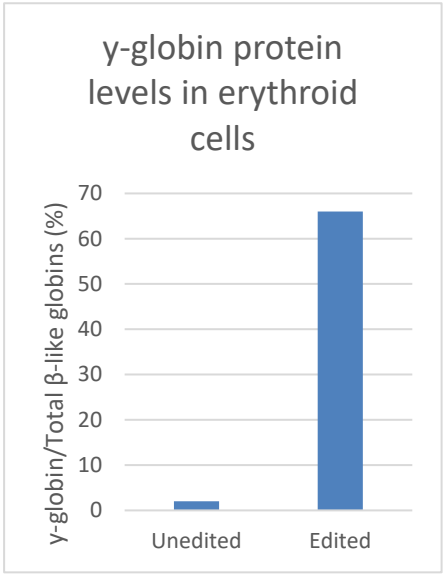

Figure 4: Level of y-globin protein levels in erythroid cells after 16 weeks of editing.

After 16 weeks, 90% base editing at the HBG1/2 promotors was maintained in the erythroid and more than 65% of the cells expressed fetal globin. The second approach in using base editing for sickle cell is the creation of the Makassar mutation. The Makassar variant is a naturally occurring mutation at the sickle site where beta globin has a glutamine instead of alanine. This variant can be found in a portion of the asymptomatic human population in Northern Europe, suggesting that it is an alternative beta globin genotype that maintains life symptom-free. The Makassar mutation allows the β-globin to function normally, which is an important discovery because the sickle cell mutation requires a base edit that converts adenine to thymine, a conversion from purine to pyrimidine that is not currently possible with existing technologies. The Makassar variant relies upon an adenine to guanine mutation at the sickle site, which can be achieved using an adenine base editor. Experimental data showed that 80% of sickle cells can be corrected into this Makassar variant using ABE in cells from a sickle patient. Studies demonstrate that the Makassar mutation is successful in the elimination of the HbS globin. In untreated controls, 100% of cells are classified as sickle cells; in contrast, just 80% successful base editing at the targeted region reduced the amount of sickle cells to only about 10% of the total population.

6 TARGET CELL TYPES AND DELIVERY METHODS

The delivery of genome editing agents to desired cell types is a grand challenge achieving precise and effective gene editing. The molecular weight of Cas9 protein 160kDa and the phosphate backbone of the sgRNA creates an overall negative charge to the Cas9 complex. There are two main pathways being employed for Cas9 delivery: in vivo strategies and ex vivo strategies. Each of these two approaches has its unique advantages and disadvantages so they each can be used in different circumstances. Electroporation is a physical delivery method being explored ex vivo. Electrical currents are used to stimulate cells to create an instantaneous opening of pores in the cell membrane to make it permeable and enable the delivery of surrounding substrates. Electroporation is widely used in ex vivo gene editing since it is capable of being applied to a wide range of cell types. However, the electrical current generated by electroporation results in a high percentage of cell death so it is difficult to scale this method for widespread adaptation.

Lipid delivery is another commonly used delivery approach relying upon the formation of lipid nanoparticles to encapsulate substrate cargo. Lipids are comprised of a hydrophilic polar head and a long hydrophobic nonpolar tail. The encapsulation of negatively charged nucleic acids into positively charged liposomes aids in the entry of these nanoparticles into cells through cellular endocytosis. Once in the cell, the nanoparticle is degraded upon traveling to late endosomes, which releases the cargo and permits downstream genome editing. Previous efforts have demonstrated that these lipid nanoparticles can effectively package DNA, RNA, and ribonucleoprotein complexes. Furthermore, studies have demonstrated that direct injection of these nanoparticles enable effective delivery into the liver of living animals.

Viral vectors are another effective approach being explored for gene delivery due to the ubiquitous nature of viruses in the world. Most commonly used viruses in research include the adeno-associated viruses (AAVs), lentivirus, and adenovirus. AAVs serve as a main vector to deliver Cas9 to differentiated tissues by transduction. Several clinical trials have been approved using AAVs, which are less immunogenic compared with other viruses. Although AAVs are effective at delivery transgenes into cells in animals and humans, a grand challenge for AAVs is the limitation on the carrying capacity. AAV vectors are typically limited to genes smaller than 4.8 kb, so any gene larger would be unable to be delivered using one AAV vector. The delivery of Streptococcus pyogenes Cas9 (SpCas9) by AAVs is challenging due to its large size of 4.2 kb, while the delivery of Staphylococcus aureus Cas9 (SaCas9) is a more feasible approach with a size of 3.15 kb. In addition to the Cas protein, one would also need to package the sgRNA so it is difficult to package everyone onto one AAV vector. Previous studies have used two AAVs to deliver an SpCas9 (4.8kb) and a sgRNA (3.0kb) separately into a mouse brain to target a gene called Mecp2. Furthermore, they demonstrated that they could include two additional sgRNAs into the virus and obtain multiple edits in a single cell. In a subsequent study, they describe that SaCas9, about 1kb smaller than SpCas9, and its sgRNA can fit one a single AAV vector with a size of 4.7kb and demonstrate that this single AAV vector can be delivered into mice liver in vivo.

Lentivirus (LV) is another type of viral vector used to deliver CRISPR-Cas9 ex vivo. LV vectors have a more generous packaging capacity of 8kb, which allows for including both a Cas9 protein and a targeting sgRNA into a single LV vector. Lentiviral vectors are mainly used in ex vivo gene delivery and are currently used as the delivery vector in FDA-approved chimeric antigen receptor-T therapies. These approaches have shown superior delivery efficacy in hematopoietic stem cells and T cells. However, the limitation of the scope of targets for LV is larger than AAV, and previous studies have limited any in vivo delivery prospects of using LVs in preclinical trials. Another challenge to using LVs is the genotoxicity and immunogenicity defects. However, integrase-defective lentiviral vectors (IDLVs) have been introduced to allow for efficient and continual transgene expression in vivo while minimizing any undesired cellular effects.

Adenoviruses (AdVs) are widely used in clinical trials for gene delivery as AVs are able to transduce both dividing and nondividing cells. The genome of AdVs generally range from 34 – 43 kb long and AdVs do not integrate into host cell genomes, which minimizes any potential off-target effects. However, a major concern of using AdVs as a delivery method is that AdVs trigger intense immune responses, which leads to significant inflammation.

Table 2: Delivery Method Comparison Chart.

Method	Delivery Material	Apprpach	Packaging Capacity	Advantages	Disadvantages
Adenoviruses	Double-stranded DNA	In vivo	8-30kb	1.High transfection efficienc 2.Transduce both dividing and non-diving cells	High immunogenicity
Adeno- associated viruses	Single-stranded DNA	In vivo	<4.8kb	1.Low immunogenicity 2.High transfection efficiency	Limited packaging capacity
Lentiviruses	Single-stranded RNA	In vivo	8kb	1.High transfection efficienc 2.Decent packaging capacity	Potential genotoxicity
Electroporation	DNA plasmid, mRNA, RNP	Ex vivo	-	1.Viralfree2.Fast 3High transfection efficiency	Lowcell viability Expensive
Lipid Nanoparticles	DNA plasmid, mRNA, RNP	Ex vivo	-	1.Viral free 2.Cheap	High toxicity

A major challenge to using viruses to deliver genome editing agents is that many newer precision genomes editing approaches are larger than the original Cas9 system. For instance, if using base editing, the fusion of a deaminase with Cas9 enlarges the size of the overall editor complex. A recent study demonstrate that it is possible to split the base editor in half and linking them together using an intein system. Each half could be packaged into two separate AAV systems and can reconstitute into a full-length base editor upon delivery into a target cell. This system was used to demonstrate successful base editing in a mouse brain, and as a result, in both cortex and cerebellum, about 50% of base editing was observed in the targeted cells.

Although most efforts for treating β −globinopathies rely upon ex vivo approaches, a recent study explored the possibility of in vivo genome editing. It is known that editing the autologous HSCs demonstrate a prolonged benefit in treating SCD. Ex vivo delivery of genome editing agents for hemoglobinopathies begin by taking out the stem cells of patients to perform gene editing in the lab, and then reintroduce the edited stem cells back to the patient. A significant limitation is when delivering the edited cells back to the patient, the patients need undergo myeloablation, which is a process of significantly weakening the natural immune response edited stem cells can survive and engraft in the patient. This process in patients can generate other types of detrimental disorders and be very damaging to the patient's overall health. In new data released by Intellia Therapeutics, they demonstrate that certain LNPs can deliver CRISPR-Cas9 mRNA into hematopoietic cells direct in vivo. Lipid nanoparticle with the Cas9 mRNA and the target gRNA were delivered into the cells, and they observed editing in the stem cells of animals treated with these engineered LNPs. Increased editing was observed with repeat multi-dosing. Intellia achieved therapeutic levels of editing in human CD34+ cells in a xenotransplanted mouse. Overall, this data suggests that using engineered LNPs is a safe and effective process to deliver genome editing agents directly into animals with minimal side effect.

7 DISCUSS CLINICAL TRIALS IN PROGRESS

There are many approaches in which genome editing is being explored for the treatment of hemoglobinopathies. In this final section, I will

discuss ongoing clinical trials conducted by Editas Medicine, Beam Therapeutics, CRISPR Therapeutics and Vertex Pharmaceuticals.

CRISPR-Cas9 mediated nuclease genome editing through double-strand break intermediates is considered as an initial and effective approach to treat hemoglobinopathies. EDIT-301, introduced by EDITAS, is an approach of editing HBG1/2 to increase fetal hemoglobin expression as a compensatory mechanism for sickled adult globin. Key regulatory regions in the β-globin locus are shown to be edited by SpCas9 and Cas12a. They demonstrate that Cas9 and Cas12a editing allows for a durable maintenance of indels at the target site. After editing the HBG1/2 region, they demonstrate that there is a constant amount of erythroid and caspases produced, and there is no significant increase in cell death. They evaluated that stem blood cells treated with HBG1/2 editing displayed a 52% increase in the expression of fetal hemoglobin, and that 89% of red blood cells will carry the fetal hemoglobin compared to only 4% of red blood cells when unedited.

Beam Therapeutics is a biotechnology company that uses base editing as a therapeutic approach to treat patients suffering from serious diseases. Beam Therapeutics is exploring two uses of base editing towards the treatment of hemoglobinopathies. First, they use base editors to induce single base changes in the regulatory regions of HBG1 and HBG2 to disrupt repressor binding binds, which results in an increased expression of fetal hemoglobin (HbF). Second, they are exploring the use of adenine base editing to directly edit the adenine implicated in sickle cell disease to correct the E6V mutation into a glutamic acid to reflect the Makassar variant. Their initial data demonstrates that the Makassar program is able to achieve 0% to 70% direct editing of the sickle cell point mutation, which is sufficient towards the alleviation of sickle cell symptoms.

A collaborative clinical trial between CRISPR Therapeutics and Vertex Pharmaceuticals has released preliminary findings on their therapeutic program, CTX001, that targets BCL11A to result in increased fetal hemoglobin expression. They demonstrate that by editing patients' own blood stem cells with CRISPR-Cas9, they can achieve elevated levels of HbF in red blood cells. The most recent data reflect that five patients with beta thalassemia and two patients with sickle cell disease treated under CTX001 all have experienced successful engraftment of edited blood stem cells and that they had no vaso-occlusive crises (VOCs) during the follow-up after the CTX001 infusion. Importantly, all patients maintained near-normal hemoglobin levels and showed drastic alleviation of hemoglobinopathy symptoms.

It is exciting to continue witnessing the rapid development of genome editing towards the treatment of detrimental human disorders.

8 CONCLUSIONS

The rapid development of new genetic therapies support a prosperous future for curing intractable genetic disease. In the last few years, we have witnessed the development of new technologies, experimental models, and pre-clinical and clinical studies. Insights into mechanism of action demonstrated many possibilities of editing the β–globin gene to introduce effective therapeutic strategies that treat β–hemoglobinopathies.

While many encouraging clinical trials have been released, many challenges still exist until we can fully appreciate the full potential of these approaches for curing these blood disorders. Firstly, the off-target effects on DNA can potentially cause irreversible damages, such as large genomic rearrangements. Thus, there is a need to carefully monitor these undesired events in clinical trials to precisely plan for and adjust for any detected effects. Specifically, the off-target activity of BEs, including both sgRNA-independent and sgRNA-dependent events require close monitoring. Fortunately, engineering modifications into the deaminase have lowered sgRNA-independent DNA off-target activity while maintaining highly efficient on-target DNA editing. Secondly, even though BE and PE have overcome cytotoxicity events caused by DSBs-induced indels, proper delivery methods are still needed, especially for primary cells. However, it remains a challenge to develop efficient methods that can deliver the large complex sizes of BE and PE technologies. Thirdly, the current pool of BEs only enable C-T, C-G, and A-G conversions; therefore, more optimizations and technologies are required to enable other types of conversions. Prime editing is a new approach that can overcome many of the editing types possible; however, further optimizations are needed to realize higher editing outcomes. Despite these challenges, gene and cell therapy hold great promise for providing proper treatment approaches for patients diagnosed with β–hemoglobinopathies.

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If any, should be placed before the references section without numbering.

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