Comparative Analysis of Genome Editing for Curing Sickle Cell Disease: Induction of Fetal Hemoglobin and Gene Correction of Sickle Gene

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***Abstract*—Known as a monogenic disease, Sickle Cell Disease (SCD) is a collective term for a series of red blood cell disorders affecting a large population in the world. Though current treatments exist and are proven effective (i.e., bone marrow transplant), accessibility severely limits their impact on most patients. Because the monogenic nature of SCD renders it simple to study, scientists are currently attempting to use gene therapy and genome editing to cure the disease. The genome editing approaches are mainly comprised of two methods: reactivating the previously suppressed fetal hemoglobin or a direct gene correction at the mutated residue in the adult beta (β) globin gene. Each approach is supported by a handful of in vitro experiments demonstrating sufficient levels of fetal hemoglobin activation or production of correct β globin in edited cells to rescue SCD. Although genome editing has shown great potential to treat this deadly disease, there remains much more work in in vivo and clinical trials to be done for further long-term evaluation before these approaches to be approved for use in humans. Safety issues and the long-term effects of the genome editing reagents are key topics that require large efforts to improve upon.**

***Keywords*—Sickle Cell Disease (SCD), genome editing, *BCL11A*, gene correction**

I. Introduction

SCD affects millions of people throughout the world and is one of the most common severe monogenic disorders [1]. It is caused by the production of abnormal hemoglobin that carries oxygen in the bloodstream [2]. The mutated hemoglobin goes through conformational changes that can block blood vessels [3]. Patients usually suffer from the complications such as tissue and organ damage which leads to excruciating attacks of pain, anemia, and thus limited life span [2]. There have been several efficacious FDA-approved drugs such as hydroxycarbamide as well as curative treatment like Hematopoietic Stem Cell Transplant (HSCT) [4]. Alternatively, gene therapy, though showing a great potential in curing the monogenic disorders and under extensive research, is still in an immature stage.

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A. Pathogenic Mechanism of SCD

In a genetic level, the abnormal hemoglobin that causes SCD appear with a single nucleotide substitution in the sixth codon of the *HBB*, which encodes for hemoglobin subunit β (β-globin) [5]. The missense mutation turns GAG into GTG with the change in protein expression from glutamic acid into valine (Glu6Val), and the adult hemoglobin (HbA) turns into sickle hemoglobin (HbS). The change in amino acid sequence causes hemoglobin molecules to stick together when oxygen levels in the blood are low, causing blockages in microcirculation which are referred to as vaso-occlusive crisis damaging vital organs and tissues [6].

Fetal hemoglobin (HbF), composed of 2 adult α-globin polypeptides and 2 fetal γ-globin polypeptides, is produced during embryonic development [7]. During gestation, the γ-globin is suppressed and replaced by β-globin (Fig. 1). HbF has high oxygen infinity so oxygen can be pulled out from the maternal circulation more easily [9]. A few weeks before birth, the fetus starts to make increasing amounts of HbA to adjust to new metabolic needs. Though with lower oxygen affinity, HbA facilitates the transport of oxygen from lungs to tissues as the gas is easier to release [10].

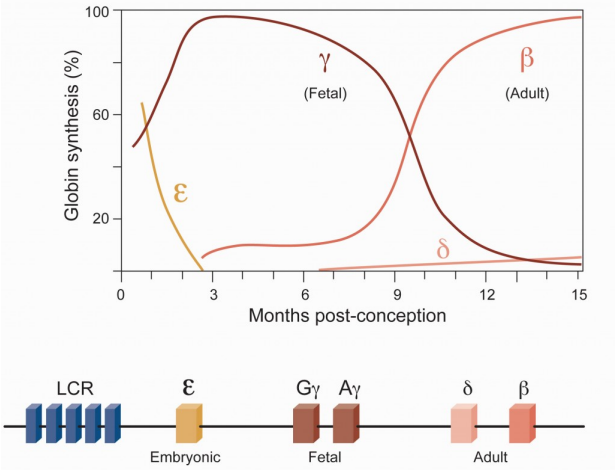

Figure 1. Fetal switch refers to the change in globin expression during gestation. γ-globin decreases while β-globin increasing (adapted from Bauer Lab [8]).

The monogenic mutation and fetal switch have provided two opportunities to cure beta-thalassemia's: reactivating the HbF expression or specifically corrected the mutated amino acid. Induction of re-expressing HbF could be achieved by knocking down the repressor gene directly or disrupting the repressor from binding to the regulatory region of the HbF gene [11]. For gene correction, other than inducing site-specific genome editing event in Hematopoietic Stem/Progenitor Cells (HSPCs), creating correct sickle-cell mutation in Induced Pluripotent Stem Cells (iPSCs) may be a more promising and efficient way to cure sickle cell disorders [11].

B. Genome Editing Tools

Several powerful editing tools have been explored over the past few decades. In 1985, the discovery of Zinc-Finger Nucleases (ZFNs) allowed scientists to design restrictive enzymes that can target a diverse set of sequences in a precise fashion. Each ZFN contain a DNA-binding domain comprised of zinc-finger proteins and a cleavage domain such as FokI endonuclease [12]. Similar to ZFNs, transcription activator-like effectors nucleases (TALENs), discovered in recent years, are also composed of DNA-binding domains and catalytic domains of FokI [12]. TALENs are believed to be more easily engineered to recognize different DNA sites compared to ZFNs, thus TALENs are more popularly applied. Winning the Nobel Prize in Chemistry 2020, Jennifer Doudna and Emmanuelle Charpentier discovered and developed Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas (CRISPR-associated) into an efficient editing tool. Different from ZFN and TALEN which recognize specific sequence by protein-DNA interaction, CRISPR/Cas system depends on RNA to bind with the target DNA sequences, and two RNAs (crRNA and tracrRNA) are both required to complete the site-specific cleavage [13]. Comparison between the most used endonucleases including CRISPR/Cas9, TALEN, and ZFN is shown in Table I. When more tools are developed, the concentration of the studies has been shifted from exploring to more precisely manipulating genomes. Due to its advantage in simplicity, efficiency, and specificity, CRISPR has revolutionized the field of genome editing, making more prospect for research of gene therapy.

TABLE I. COMPARISON OF THREE WIDELY USED ENDONUCLEASES (ADAPTED FROM [14])

Feature	CRISPR/Cas9	TALEN	ZFN
Target	DNA	DNA	DNA
Efficiency	Very High	High	Low to Medium
Simplicity of design	Simple	Moderate	Complex
Synthesis	Easy	Moderate	Difficult
Cellular side-effects	Minimum	Low to Medium	High
"Off-target" activity	Minimum	Low	High

II. METHODOLOGY AND FINDINGS

A. HbF Gene Re-expression

1) Regulating repressor gene BCL11A

Gene *BCL11A* is known to be associated with the production of HbF for fetal switch. Sankaran [7] has investigated the level of HbF production in erythroid cells and found that the alleles coding for "low-HbF" expressed a 3.5-fold higher level of *BCL11A* transcripts than those for the "high-HbF" alleles (Fig. 2). The study attempted to reactivate HbF expression by directly knocking down *BCL11A* gene and it turned out successfully reactivating the production of HbF without affecting the overall erythroid differentiation. The discovery of CRISPR/Cas9 endonucleases allows gene disruption and knockdown/knockout by creating site-specific double-stranded breaks at the target DNA. Thus, disrupting or knocking out the repressor *BCL11A* could be a potential method to reactivate HbF expression.

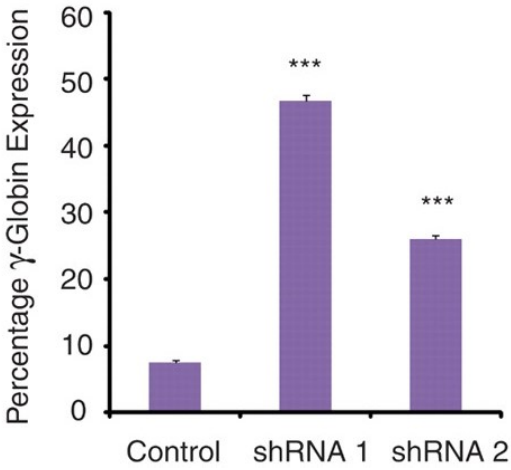

Figure 2. shRNA1 and shRNA2 both had *BCL11A* knocked down. Compared to control, these two shRNA-mediated knockdowns significantly reactivated HbF expression (6.5-fold and 3.5-fold).

There are clinical trials [15] in 3 patients with small de novo deletions that only removed *BCL11A* and 1–2 adjacent genes, without deletions or mutations in the the *HBG1/HBG2* or *HBB* genes that encode for HbF production. From the results, the mRNA analysis showed a higher level of expression of HbF-encoding genes, containing *HBG1/HBG2*. Additionally, HbF was substantially elevated at 23.8%, 16.1%, and 29.7% in the blood from patients 1–3 (Fig. 3). The data suggested the levels of HbF regenerated because the deletions of *BCL11A* would be sufficient to ameliorate symptoms for SCD patients. Importantly, the patients have no history in severe or unusual infections suggesting that the deletions of *BCL11A* gene would not impair normal immunological function.

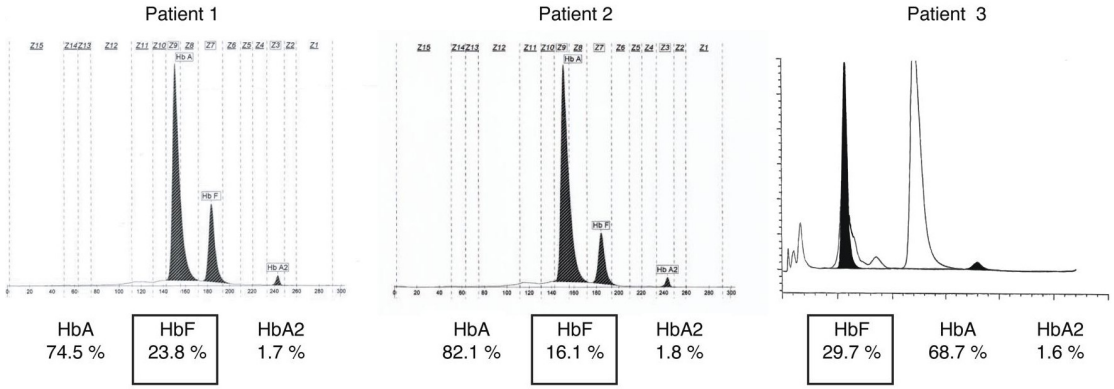

Figure 3. Quantified hemoglobin level in each patient [15]. (In controls, HbF level would be < 1%)

Bjurström [16] has examined the efficiency, specificity, and mutational signatures of ZFNs, TALENs, and CRISPR/Cas9 systems to target *BCL11A* in vitro. The 3 designed endonucleases showed different cleavage efficiency: 27%, 12%, and 4% indels for ZFNs, TALENs, and Cas9. The relatively low efficiency affected the consequence in HbF level. ZFNs have induced cells to display a significant 4.3-fold increase of HbF expression and 3.4-fold increase in γ-globin, while the TALENs and Cas9 were less effective with no significant increase in HbF or γ-globin. The increase in HbF expression and γ-globin generated by ZFNs modification showed certain possibilities for treating SCD with the knockout of *BCL11A*. At the same time, it is significant to consider that knockdown of *BCL11A* might impair human hematopoietic stem cells [17].

Basak & Sankaran [18] discussed possibilities of the regulation of *BCL11A* as a method to induce HbF for therapeutic purposes in vivo in transgenic mice with the human β-globin. The *BCL11A* knockout reactivated the HbF-encoding genes and thus regenerated γ-globin expression with normal erythropoiesis. The potential for clinical benefit included reversing γ-globin silencing and preventing clinical complications in a mouse model of SCD.

Aside, the absence of *BCL11A* in transgenic mice results in a failure to properly silence the γ-globin genes and a correction of the SCD phenotype. More recently, *ZBTB7A* was discovered to be a major repressor of the mouse embryonic globin genes and to be the second major repressor of the human γ-globin genes [19].

Though recent studies have shown the clinical potentials on regulating *BCL11A* to reactivate the production of HbF, the cytotoxicity from endonucleases and risks of impairment on hematopoietic stem cells still constrains the method to be implemented.

2) Disrupting HBG1/HBG2 promoter motif

An alternative way to disrupt *BCL11A* protein binding event is to manipulate the upstream, which is the promoter region of γ-globin gene (*HBG1* and *HBG2*). In normal adult human erythroid cells, *BCL11A* protein recognizes TGACCA motif and silences the expression of γ-globin. When the promoter region is disrupted by CRISPR editing, *BCL11A* can no longer bind to the promoter. In research from Liu [20], the distal TGACCA motif is edited to destruct the binding site for *BCL11A* in vitro and in chromatin (Fig. 4). Consequently, γ-globin is reactivated while the β-globin is restricted from being transcribed, resulting to an inverse process of fetal switch. Hence, *HBG1/HBG2* promoter motif is significant for *BCL11A* binding event, and down regulatory modification offers a promising way to induce the expression of HbF.

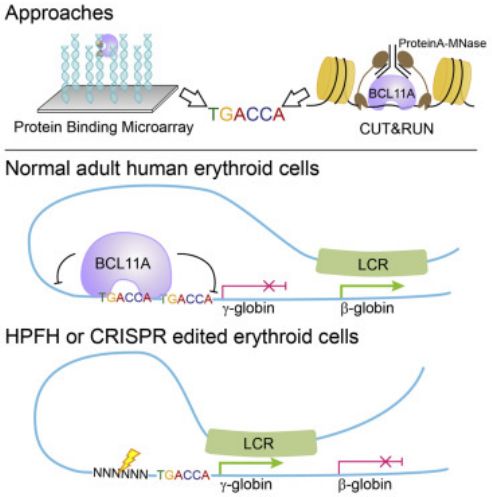

Figure 4. Disrupting *BCL11A* promoter region by editing TGACCA motif. When disturbance appears in that specific motif region, *BCL11A* protein fails to find with HbF promoter region and thus the repression to γ-globin is withdrew.

Editing the *HBG1/HBG2* promoter region has supportive evidence from applications. In the study from Métais [21], in vitro application using Cas9: sgRNA system to edit the *HBG1/HBG2* promoter have been carried out with cultured human CD34+ cells that contain HSPCs. Additionally, in vivo application which transplanted human CD34+ HSPCs in immunodeficient model mice was tested for efficiency. No obvious differences were spotted in morphology and maturation process in late-stage erythroblast, indicating that the editing in *HBG1/HBG2* promoter region does not induce significant effect on the differentiation of HSPCs. Both applications have shown that the editing on promoter has significant increase in the fetal hemoglobin cell and the amount of HbF protein.

Another study by Weber [22] also edited the *HBG1/HBG2* promoters in cultured SCD CD34+ HSPCs and reactivated the HbF expression. The production of γ-globin was positively correlated with the indel rates from Non-homologous End Joining (NHEJ) after the CRISPR/Cas9 site specific cleavage. Previously reported that HbF expression over 30% in 70% of the erythroid cells could mitigate SCD. The results of HbF production around 47% excesses the estimated minimal value (Fig. 5) [22], demonstrating that disrupting the promoter in vitro are able to increase the HbF to a potential effective treatment.

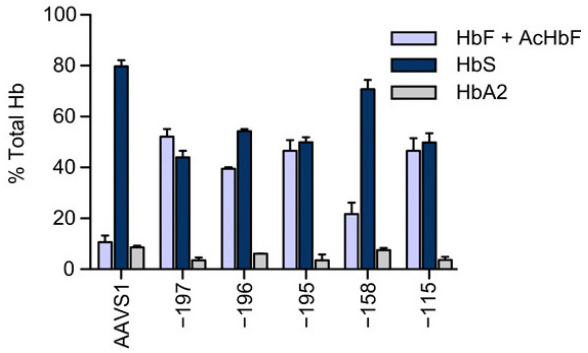

Figure 5. Quantified hemoglobin composition after editing events showing significant boost in HbF expression level. The number in horizontal axis refers to different editing regions.

Further research has investigated more specific editing using base editors. An improved version of adenine base editors [23] has indicated satisfactory editing efficiency on recreating a natural allele at *HBG1/HBG2* promoter region. Their results showed 60% editing efficiency on human CD34+ cells and over 98% target modification in human T cells. Besides the base editors successfully persisting HbF, they induced no off-target adenine deamination in genomic DNA and only low levels in cellular mRNA. Since base editors can carry out specific conversion without inducing double-stranded breaks in the target genome, Gaudelli [23] opens more possibility and potentiality for HbF to be accurately reactivated without creating undesired modifications.

Studying the effect after genetic interference on both *BCL11A* and *HBG1/HBG2* promoters, researchers have applied qRT-PCR to study the re-expression of HbF [24]. Compared to control samples, up-regulation for HbF showed more than 4-fold in *BCL11A* and 6.5-fold in *HBG1/HBG2* targets in human CD34+ cells. Thus, editing the promoter *HBG1/HBG2* gene shows better performance, which indicates *HBG1/HBG2* is more directly related to the expression of HbF than the repressor gene *BCL11A*.

B. Gene Correction

1) Correcting mutations in HSPCs

Different from gene disruption or deletion, correction requires more specific edits through Homology-Directed Repair (HDR). As HDR process involves with a template strand and competes with the natural repair pathway NHEJ, HDR happens at a much lower frequency. To optimize the effect of HDR, NHEJ can be inhibited by disrupting defined loci [11].

Reviewed by Demirci [25], there are a handful of studies exploring the potential approaches for SCD mutation (Glu6Val) correction, including correcting mutations in Hematopoietic Stem Cells (HSCs) (HSC is included in HSPC) and inducing HbF in HSCs (Fig. 6). HSPCs are usually derived from SCD patients' bone marrow [25]. HSPCs have shown substantial production of HbA around 22.4% of all the hemoglobin after edited with CRISPR/Cas9 in vitro and a yield to 37% of edited HSPCs in vivo [26]. Additionally, Vakulskas [27] used HiFi Cas9 (Cas9 with a single point mutation) to correct *HBB* gene in HSPCs and generated more than 50% HbA in targeted differentiated erythrocytes. In addition, HiFi Cas9 reduces off-target events while maintaining high on-target editing, providing a better choice when considering the precision of endonucleases. With quantified evaluation supported, testing results of the HbA mRNA expression in the erythrocytes differentiated from patient-derived HSPCs showed 56% expression out of total β-globin mRNA (Fig. 7) [28], confirming enhancement in hemoglobin functionality.

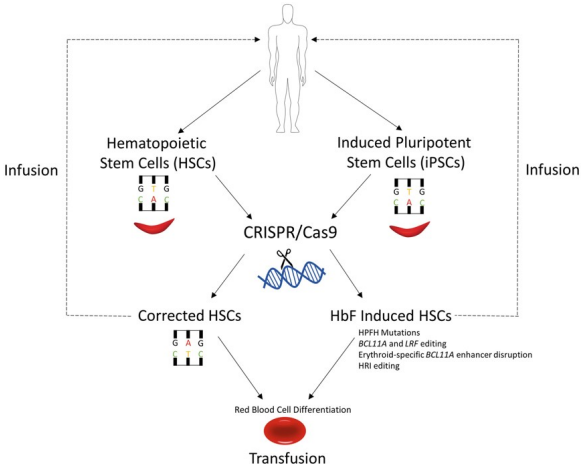

Figure 6. Various approaches to correct SCD mutation. This section mainly discusses corrected HSCs.

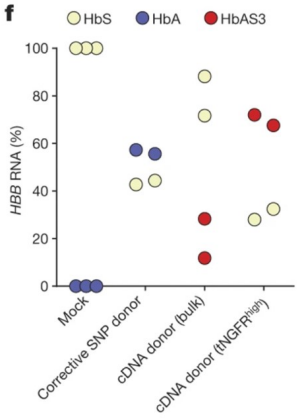

Figure 7. HbA RNA content raised substantially from corrected HSPCs.

Besides, gene correction can be achieved by restoring the mutated residue to normal function without converting to the precise genotype. Hemoglobin G

Makassar (HbG) has same base pair mutation as HbS but appears to function normally as HbA [29]. Newby [30] hence converted a sickle gene to the non-pathogenic Makassar gene using base editors. Not only they obtained an 80% conversion rate in patient derived HSPCs, but also found that 79% of HbG expression of all hemoglobin in the transplanted mice had reduced pathological morphologies, indicating a successful rescue of SCD.

Furthermore, secondary transplants have indicated a durable effect from the direct gene correction. Human HSPCs labelled with GFP on the *HBB* gene reached 49% after the first transplant and 90% after the secondary transplant, reflecting an efficiency of targeting the *HBB* gene [28]. In Newby [30], a secondary transplantation was applied using a gradient of edited cells from the bone marrow of mice receiving the primary transplant. Mice that received more than 60% of bone marrow that contained corrective cells generated 20% of HBB^G allele frequencies. Additionally, secondary transplants of corrected HSPCs showed a durable effect with more than 70% containing β^G protein in all hemoglobin, suggesting that there exist long-term repopulating abilities of these corrected cells to rescue SCD.

Interestingly, there had been different, innovative approach on modifying HSCs back in 2001. Pawliuk [31] constructed a β-globin gene variant that can prevent HbS polymerization and transferred it into HSCs which were later transplanted in two different mouse models. Long-term expression (10 months) of anti-sickling protein had reached 52% of total hemoglobin and 99% in circulating red blood cells, thus rescued SCD. This kind of genome editing, though not gene correction, has yielded to satisfactory level of well-functioned hemoglobin proteins. However, it has been already two decades since this study published and gene therapy has not been put into wide range of use, especially considering accessibility.

A major limitation of HSPC gene therapy is the incapability of replicating the niche condition to preserve the HSPCs in an undifferentiated state for an ideal period. Papapetrou [32] has discussed the inability to maintain HSPCs ex vivo for more than 72h, which restricts gene modification strategies such as delivery methods and quality control. Moreover, off-target editing events generated by double-stranded breaks in HSPCs has not yet been assessed adequately, indicating that further cautious evaluations on safety and editing precision in HSPCs and their lineages are required.

2) Creating corrected iPSCs

Kazutoshi Takahashi and Shinya Yamanaka studied and successfully developed a protocol for turning somatic cell into induced Pluripotent Stem Cells (iPSCs) in 2006. Regarding the pluripotency, iPSCs are seen as a new promising source for HSPCs in addition to bone marrow extraction. Compared to HSPCs, iPSCs can be maintained ex vivo indefinitely for a longer time due to its extensive self-renewal ability and are able to create more differentiated progeny for theoretically all cell types [32]. Thus, these properties provide researchers more possibilities to manipulate, produce, and select cells with desired gene modifications.

After the protocol became available, iPSCs were adopted for research in curing SCD. Electroporated with a wild-type HbA gene targeting construct, 1 out of 72 iPSC clones in vitro were screened to be modified correctly [33]. Further in vivo experiment detected 65% of HbA among all hemoglobin protein and a remarkable reduction of HbS in mice models with homozygous sickle gene [33]. Using corrective SCD donor template and CRISPR/Cas9, 67.9% allele correction of iPSC clones (50% correct clones) has been achieved without any off-target modifications in 1467 potential off-target sites (Fig. 8) [34]. Similarly, SCD iPSC line showed 63% allele correction in 15 out of 22 corrected clones, with off-targets mutation at predicted sites without eliciting cellular effect [35]. Clearly, CRISPR/Cas9 boosted the efficiency in precise editing of iPSCs of sickle cell defect, but off-target events remain as a limitation for iPSCs to be put into actual treatment.

However, there are still few data for in vivo application of corrected iPSCs compared to that of HPSCs treatment in mice models, which may be due to the inability of iPSCs to robust derivation of hematopoietic stem cells in terms of long-term engraftment and differentiation into all hematopoietic lineages although pluripotency [32]. Overall, though obtaining pluripotency and offering more possibilities to be manipulated ex vivo, iPSCs are more difficult to be controlled in specific differentiated cell lines such as hematopoietic cells. Reviewed by Yamanaka [36], each pluripotent stem cell lineage is not identical, such as in the propensity to differentiate into various cell line, with epigenetic variations. In addition, targeting stem cells instead of differentiated cell types imposes a higher burden when considering safety issues [32].

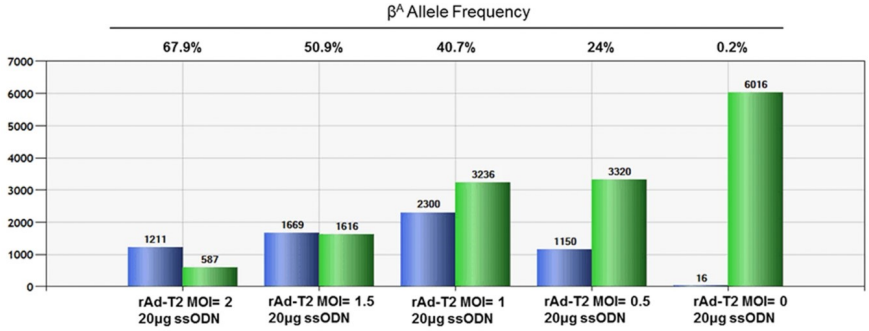

Figure 8. HbA allele frequencies under different MOI. HbA allele frequencies measure the constitution of HbA gene in either or both DNA strands. Green bar: HbS-positive; blue bar: HbA-positive.

III. Discussion

This paper mainly discusses two approaches in rescuing SCD, including the re-activation of HbF to offset the negative impacts from HbS and the correction of the mutated residue in HbS to convert the sickle gene back to normal. For induction of HbF, both disrupting of HBB repressor gene BCL11A and binding site for the repressor HBG1/HBG2 have shown positive results both in vitro and in vivo. Higher efficiency in up-regulating HbF after editing promoter region HBG1/HBG2 than disrupting BCL11A suggests the promoter can be a better target for re-expressing HbF. Down-regulating repressing events successfully released HbF to reverse the effect of natural fetal switch, which elevates the normal-functioning hemoglobin level in the blood to ameliorate SCD symptoms.

On the other hand, gene correction through HDR has demonstrated promising editing efficiencies for cultured cells in vitro for both HPSCs and iPSCs. Following the same approach, in vivo implementation of corrected HPSCs in mice models indicated long-term restorative effect based on the analysis of repopulating hemoglobin levels in primary and secondary transplants.

All the above approaches are largely dependent on CRISPR/Cas9, an efficient endonuclease to make gene modifications. However, off-target editing events are almost unavoidable because of the nature of inducing double-stranded cleavage. Additionally, because the repair mechanism NHEJ happens more naturally than HDR, precise gene corrections are usually achieved at a lower level. Base editors, consisting of a partially inactive Cas9 nickase, avoid generating a double-stranded break, and thus lead to more efficient editing with fewer errors.

IV. Conclusion

Gene therapy has shown great potential in curing SCD given its single base pair mutation in nature. Significant advances in sequencing, genome editing tools, and protein detection have paved the way forward for research in SCD therapeutics. Reversing the fetal switch process and correcting the mutated base pair are both promising in ameliorating SCD pathogenic symptoms. However, limitations remain where CRISPR editing reagents can still generate off-target events, HSPCs cannot be maintained undifferentiated for an ideal period, and iPSCs are difficult to control in a particular differentiated cell lineage. Future research is recommended in improving editing precision including the efficacy of base editors and evaluating long-term rescuing effects.

Furthermore, most studies focusing on curing SCD with genome editing are still in the early in vitro stages. Besides the technicality of gene modifications mentioned above, further studies are required to thoroughly evaluate critical aspects of realizing these efforts as an eventual therapy, such as delivery methods and immunogenicity. Moreover, the issue of accessibility and cost is also an important part of the discussion for gene therapy. The question of how to make these treatments feasible, especially for less developed regions where SCD is prevalent, should be a major goal in the future study. The advancement in gene modification tools has made gene therapy appear more and more approachable as turning into a reality, though future efforts are expected to improve efficiency, specificity, safety, and feasibility.

Conflict of Interest

The author declares no conflict of interest.

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