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Review Article

CRISPR-CAS 9 AND ITS APPLICATION AS THERAPEUTICS FOR β-HAEMOGLOBINOPATHIES

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ABSTRACT: CRISPR/Cas 9 is presently an excellent genome editing tool and is supposed to be instrumental in correcting several genetic diseases. Sickle cell disease (SCD) and β-thalassaemia are important genetic diseases that result from mutations in the β-globin (*HBB*) gene. Lentiviral-mediated gene therapy is an efficacious strategy for the treatment of β-hemoglobinopathies but is quite expensive with variable outcomes. Allogenic HSC transplantation from HLA-matched donors to treat beta-hemoglobinopathies is assessable to limited individuals due to the scarcity of immunologically matched donor sources. One of the strategies to treat β-hemoglobinopathies is the induction of high fetal hemoglobin (HbF) because it is known that high HbF levels are strongly linked with milder disease in SCD and β-thalassemia patients. Amidst several pros and cons of available therapies, CRISPR-mediated therapy is based either on the induction of HbF or correcting defective β-globin gene. The induction of HbF can be achieved by knocking down HbF repressors or editing HbF regulatory elements while the defective β-globin gene is corrected in patient HSPCs and iPSCs and then autologous transplantation is performed. CRISPR/Cas 9 offers an excellent opportunity for scientists in treating the β-hemoglobinopathies.

Key words: CRISPR/Cas 9, Sickle cell disease, β-thalassaemia, Fetal hemoglobin.

INTRODUCTION

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) Cas (CRISPR-associated proteins system) has recently generated considerable excitement among scientists in the field of gene editing. It is currently the excellent genome editing tool, bearing unparallel specificity and precision in addition to its supremacy in simplicity and economy over other previously available similar tools like zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (Xu *et al.* 2019, Tripathi *et al.* 2021). Among three types and ten subtypes of the CRISPR/Cas system, CRISPR/ Cas 9 belongs to type II and is the most widely studied (Chandrasekaran *et al*. 2018). Designing and manipulating CRISPR/ Cas9 is easy and efficient in genome editing as compared to other similar tools (Chandrasekaran *et al.* 2018).

CRISPR/Cas system is naturally present in most archaea and several bacteria to provide adaptive immunity against viruses (Makarova and Koonin 2015). It is an RNA-guided endonuclease in which RNA precisely binds with the target sequence and the Cas9 protein cuts at the specific point of the DNA. Scientists have been able to explore the mechanism of disruption of DNA and eventual DNA repair mechanism to edit the gene of interest by delivering Cas9 protein along with the in vitro synthesized RNA (Single guide RNA or SgRNA) after its *in-silico* designing (Lino *et al.* 2018, Naeem *et al.* 2020). The delivery of the CRISPR/Cas system into the target cells includes CRISPR/Cas9 cargoes and delivery vehicles. There are three types of cargoes that include (1) DNA plasmid encoding guide RNA and Cas9 protein (2) Cas9 mRNA (for translation into the cell) and guide RNA (3) guide RNA and Cas 9 protein in the form of ribonucleoprotein (Lino *et al.* 2018). The vehicle used for CRISPR/Cas9 cargoes into the target cells comprises delivery vectors and physical methods. Delivery vectors include viral-mediated delivery (adenoviral vectors, adeno-

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associated viral vectors, lentiviral vectors), and non-viral vectors (polymeric materials, liposomes, cell-penetrating peptides, and cationic nanocarriers) while physical methods include microinjection and electroporation (Chandrasekaran *et al.* 2018). Every method of delivery of CRISPR/Cas9 to cells has both pros and cons and some methods can be specific or not suitable to certain types of delivery, however, the ribonucleoprotein format is better in gene editing and generally associated with minimum off-target effects as compared to plasmid DNA or mRNA format (Lino *et al.* 2018).

In addition to its role in genome editing, CRISPR/Cas9 has widespread application in gene silencing, activation of gene expression, disease control, diagnostics, fighting antimicrobial resistance using CRISPR-based antibacterial, vector control, production of disease models, cancer research, and many more (Serajian *et al*. 2021, Rodríguez *et al*. 2019, Gago *et al.* 2021). CRISPR/Cas9 has the potential to treat many diseases caused by a genetic mutation (Khan *et al.* 2016). Sickle cell disease (SCD) and β-thalassaemia are important β-hemoglobinopathies caused by mutations in the β-globin *(HBB)* gene that affect millions of people worldwide. Recent progress in CRISPR/Cas 9 research in reducing its undesirable off-target effects paves the way for its application in therapeutics including the treatment of SCD and â-thalassaemia (Frangoul *et al.* 2021, Demirci *et al.* 2021).

CRISPR/CAS 9 AND ITS ACTION MECHANISM

CRISPR/Cas system (Fig. 1) consists of a set of CRISPR arrays, leader sequence, and cas genes (Richter *et al.* 2012). In the CRISPR array repetitive sequences called repeats are interspaced by spacers. Each spacer bears a unique sequence of similar length (Loureiro and da Silva 2019). The length of each repeat usually ranges between 23 and 47 bp and is arranged in a palindromic fashion while the sequence of many of the spacers matches with the sequences that originate from extra-chromosomal sources like phage or plasmids (Richter *et al.* 2012). Upstream to the first repeat, a leader sequence is present that has a role in the integration of foreign genetic material into the CRISPR array (Richter *et al.* 2012). The AT-rich leader sequence consists of a promoter that is essential for the transcription of the CRISPR array (Loureiro and da Silva 2019). *Cas genes* are present close to the CRISPR array and encode for cas proteins that have endonuclease properties. Among the several different types of CRISPR/Cas systems for genome editing, the simplest and widely used for different applications is the type II-CRISPR/Cas9 discovered in *Streptococcus pyogenes* (spCas9) (Chylinski *et al.* 2014, Adli 2018).

The process of natural adaptive immunity provided by the CRISPR/Cas system in the bacteria is accomplished in three phases. It starts with the acquisition of the foreign genetic material into the bacterial genome. When a phage invades a bacteria part of the DNA of the virus gets integrated into the CRISPR array of the bacterial genome in the form of spacers called the spacer acquisition phase (Hille and Charpentier 2016). In this way, the bacterial genome consists of several spacers acquired by different viruses and interspaced by repeat sequences. When the same virus infects the bacteria again the biogenesis phase starts in which transcription of the CRISPR array occur producing pre-crRNA. In the case of type I and type III CRISPR systems, pre-crRNA is cleaved by Cas6 endonucleases while in type II, as occurs in *Streptococcus pyogenes* an additional trans-activating CRISPR RNA molecule (tracr RNA) and host RNase III is required for maturation of pre-crRNA (Karvelis *et al.* 2013). Following maturation short mature CRISPR-RNA (crRNA) is generated consisting of the sequence of the invader genome (Hille and Charpentier 2016). The 5' of the mature crRNA contains the 20 nucleotide spacer sequence of the foreign phage origin (Jiang and Doudna 2017). 3' of the crRNA bind with the 5' of the tracr RNA to form crispr-tracr RNA. In the third phase (interference phase or target degradation) the crRNA-tracrRNA complex binds with the Cas-9 protein having endonuclease property the resulting ribonucleoprotein then binds and cleaves the invading viral DNA (Hsu *et al.* 2014). In detail, the 20 nucleotide sequence (spacer sequence) of the crRNA guides the CRISPR-Cas9 complex to bind with the foreign DNA having a complementary 20 nucleotide sequence (protospacer sequence) (Jiang and Doudna 2017). This 20 nucleotide sequence in the foreign genome is adjacent to PAM (protospacer adjacent motif) sequence. Recognition of the target depends on both the complementary base pairing between crRNA and DNA as well as the presence of a suitable protospacer adjacent motif (PAM) sequence in the DNA. PAM is usually a 2-6 base pair DNA sequence adjacent to the 20 nucleotide sequence which is targeted by the Cas9 (Molla and Yang 2019). Cas9 cleaves the DNA target sequence following PAM recognition and RNA-DNA duplex formation. (Jiang and Doudna 2017). The PAM sequence for the commonly used SpCas9 is three base pair length 5'-NGG-3' DNA sequence in which N may be any of the four bases present in the DNA (Gleditzsch *et al.* 2019). After recognition of the suitable PAM, Cas9 initiates local DNA

melting followed by an invasion of the RNA strand, and RNA- DNA hybrid formation occurs. Cas9 then degrades the foreign genetic material 3 base pair upstream to the NGG (PAM) sequence (Jiang and Doudna 2017). Cas9 protein consists of 2 domains having nuclease activity *i.e.* Crossover junction endodeoxyribonuclease RuvC domain and HNH domain (Hsu *et al.* 2014). RuvC domain cuts the PAM-containing noncomplementary strand while the HNH domain cleaves the guide RNA-bound complementary strand of the target DNA thus a double-stranded break (DSB) is created (Nadakuduti *et al.* 2021). This mechanism of the adaptive immune system of bacteria has been harnessed by scientists to cleave any foreign target gene using the *in vitro* synthesized single guide RNA (sgRNA) and Cas9 endonuclease. sgRNA for specific target can be properly designed in-silico using available software like CHOPCHOP, E-CRISP, CRISPR direct, Off-Spotter, sgRNAcas9, CRISPER (Montague *et al*. 2014, Heigwer *et al*. 2014, Xie *et al*. 2014). sgRNA after binding with the cas9 can cleave the target DNA (Wilkinson *et al.* 2019).

Harnessing of repair mechanism for genetic correction

In the mammalian genome self-repair mechanism starts after the formation of DSB. Two important mechanisms have been reported to occur for the repair of DSB, *i.e.* NHEJ (Non-homologous end joining) and homology-directed repair pathways (Sansbury *et al.* 2019, Yang *et al.* 2020). NHEJ is the primary pathway that is utilized by the mammalian cell to repair the broken DNA because it is faster, active during the whole cell cycle, and has the potential to suppress the HDR pathway (Yang *et al.* 2020). However; the introduction of deletions and insertions at the site of repair occurs so the mechanism is error-prone (Sansbury *et al.* 2019). This process has been harnessed by scientists for knocking out any gene. Using CRISPR/Cas technology double-stranded break (DSB) can be created at the desired site (Sansbury *et al*. 2019) and repair by NHEJ occurs subsequently resulting in the knocking out of the gene. A triple target or multiple targets can be adopted to increase the accuracy of the knocking out of the gene. Knocking out of the gene using CRISPR-Cas9 has created an easy way for understanding the functions of the gene and exploring the various biological mechanisms in different animal models (Tschaharganeh *et al.* 2016). HDR, on the other hand, is high fidelity repair mechanism that can take place during the S/G2 stages of the cell cycle. During this process, undamaged sister chromatid or donor DNA containing the desired corrected sequence is utilized for repair. This mechanism has been explored for insertion of mutation of interest at the desired site by using a synthetic DNA donor template (HDR template) comprising the sequences homologous to the target region.

β-HAEMOGLOBINOPATHIES

β-hemoglobinopathies are a group of disorders resulting from the defective synthesis of the β-globin chain of hemoglobin (Papizan *et al*. 2021). It includes β-thalassemias and sickle cell diseases. Sickle cell anemia is the most severe type of sickle cell disease that occurs as a result of a point mutation in the sixth codon of exon 1 of chromosome 11 which is responsible for the synthesis of the β-globin chain of hemoglobin leading to the substitution of glutamic acid for valine amino acid in the beta-globin chain (Cordovil 2018, Papizan *et al.* 2021). Alteration in the single amino acid leads to the formation of hemoglobin S. Deoxygenated sickle hemoglobin undergoes polymerization and sticks with one another to form stiff bundles (Gardner 2018, Luthra *et al*. 2021). Due to the formation of bundles, the shape of the blood cells gets distorted resembling a sickle and the cells become susceptible to being trapped in microcirculation by which the downstream tissues may suffer from ischemia (Gardner 2018, Luthra *et al*. 2021). Sickle cell disease is characterized by vaso-occlusion, anemia, hemolysis, inflammation, hypercoagulability, and increased oxidative stress and the patients suffer from repeated painful episodes, recurrent episodes of vaso-occlusion, and chronic organ damage (Gardner 2018).

β-thalassemias are heterogeneous autosomal recessive hereditary anemias caused mostly by point mutations or rarely by deletions altogether comprising more than 300 mutations of the adult β-globin gene. The disease is characterized by diminished (β+) or absent (β0) synthesis of the β-globin chain which results in decreased adult hemoglobin (HbA) and excess ∝-globin in erythroid cells causing deficient erythropoiesis and apoptosis in the erythroid lineage (Luthra *et al.* 2021, Cosenza *et al*. 2021, Xu *et al.* 2015).

Role of fetal hemoglobin in β-hemoglobinopathies.

The patients with SCD who have hereditary persistence of fetal hemoglobin (HPFH) have milder clinical manifestations or no disease. The improved survival rate and a milder disease course have been noticed in the patients having higher fetal hemoglobin in sickle cell anemia and â-thalassemia (Platt *et al*. 1994, Musallam *et al.* 2012). Fetal hemoglobin (HbF) is the main oxygen transporter having higher oxygen-binding affinity during the last months of gestation and a few months after birth.

It interferes in the process of polymerization of hemoglobin S and thus inhibits sickling (Peralta *et al.* 2013). So the neonates suffering from TDT (Transfusion dependent β-thalassemia) or SCD are asymptomatic till their level of fetal hemoglobin remains high and symptoms appear when the fetal hemoglobin synthesis declines during the first year of life. Fetal hemoglobin consists of two alpha and two gamma chains while adult hemoglobin contains two alpha and two beta chains. The level of γ-globin decreases as there is an increase in β-globin after birth. Individuals with the hereditary persistence of fetal hemoglobin (HPFH) have elevated levels of fetal hemoglobin throughout life.

Treatment of β-hemoglobinopathies

Hydroxyurea and butyric acid have been used in the treatment of sickle cell anemia due to their property of inducing fetal hemoglobin but the side effects caused by them limit their use (Ekman 2010, Gardner 2018).

Recent therapies, including crizanlizumab and luspatercept have been shown to reduce the incidence of vaso-occlusive episodes in SCD patients and the requirements of transfusion in TDT patients respectively (Ataga *et al*. 2017, Cappellini *et al.* 2020) but none of them eliminates the disease manifestation completely. In addition, various other treatments are also being tried but presently the only curative approach for the treatment of β-hemoglobinopathies is allogenic hematopoietic stem cell (HSC) transplantation from Human leukocyte antigen (HLA) matched donors (Gardner 2018). The major drawback of this therapy is its assessability to only a limited number of individuals due to the reduced availability of immunologically matched donor sources (Gardner 2018). In the past several years, many researchers have been focused on achieving the therapeutic levels of exogenous β- globin using lentiviral vectors which is an efficacious strategy for the treatment of β-hemoglobinopathies (Finotti *et al.* 2015). Ribeil *et al* (2017) treated a patient with lentiviral vector–mediated addition of an antisickling β-globin gene into autologous hematopoietic stem cells and after fifteen months of treatment, the therapeutic antisickling β-globin level remained elevated (about 50% of β-like–globin chains) without any recurrence of sickle crises. However, lentiviral-mediated gene therapy has limitations being expensive and variable outcomes (Frati and Miccio 2021).

USE OF CRISPR/CAS9 IN CURING SCD AND TDT

Gene therapy using CRISPR/Cas9 technique may be a valuable tool for the treatment of sickle cell anemia to the majority by transplantation of genetically corrected autologous HSCs (Antoniani *et al.* 2018). A direct approach to corrections of DNA mutations using genome editing tools such as CRISPR/Cas9 in the absence of the off-target effects would avoid the chance of insertional mutagenesis which is inherent in integrating vectors (Finotti *et al.* 2015). The superiority of genetic correction by CRISPR-Cas9 over TALEN (transcription activator-like effector nucleases) can be observed in the experiment of Hoban *et al*. (2016). They targeted β-globin by TALEN and CRISPR-Cas9 in which high rates of *in vitro* gene modification were achieved by the CRISPR/Cas9 as opposed to the TALEN. The correction of SCD patient bone marrow CD34+ cells was found and resulted in the production of wild-type adult hemoglobin. CD34+ HSPCs (hematopoietic stem and progenitor cells) and induced pluripotent stem cells (iPSCs) are the preferred choice for gene therapy in β-hemoglobinopathies (Finotti *et al.* 2015).

The two commonly used approaches for gene editing therapy of β-hemoglobinopathies are the correction of defective β-globin gene and the induction of fetal hemoglobin (Papizan *et al.* 2021). The former includes the genetic correction of patient-derived autogenic hemoglobin S gene (causative mutation) in HSPCs and then the transfer of corrected cells into the same patient. Using CRISPR-Cas9 technology the mutated gene sequences can be replaced with normal gene sequences utilizing the homology-directed repair (HDR) mechanism. Due to single-point mutation in SCD, HDR-based corrections have been successfully performed in iPSCs and patient-derived HSPCs (Frati and Miccio 2021). In HSPCs, the efficiency of genetic correction ranged between 7-50% *in vitro* using different gene editing tools which were sufficient for the production of clinically required HbA and in ameliorating SCD *in vitro* but the genetic correction frequencies dipped to 10% *in vivo* following transplantation suggesting the lesser efficiency of HDR repair in vivo (Frati and Miccio 2021). However, encouraging results in improving HDR efficiency in CRISPR experiments are in progress for various clinical applications. In the case of β-thalassemia also HDR-based genetic correction has been tried in patient-derived iPSCs (Frati and Miccio 2021). However; Cas9 base editors as well as prime editors are the newer strategies to correct the single base pair mutations without the generation of DSBs and simultaneously eliminate the necessity of costly donor templates (Papizan *et al.* 2021).

The other method for the genetic correction of β-hemoglobinopathies is the induction of fetal hemoglobin. Induction of HbF expression is easy and effective as

compared to the genetic correction of mutation in SCD (Demirci *et al.* 2021). Induction of fetal hemoglobin occurs either through the knocking down of HbF repressors or editing of HbF regulatory elements (Demirci *et al.* 2021) for which CRISPR/Cas9 has been employed. Clinical trials for gene editing in SCD are focused primarily on the generation of INDEL for the disruption of repressor DNA binding motif to induce HbF expression rather than direct correction of the sickle mutation, as the former does not require repair through the HDR mechanism, which is difficult to achieve in HSCs and HDR is less efficient as compared to NHEJ in long term engrafting stem cells (Genovese *et al.* 2014, Demirci *et al.* 2021). Induction of endogenous fetal hemoglobin is one of the widely used therapeutic strategies for SCD and â-thalassemia (Finotti *et al.* 2015). Elimination of a natural repressor of hemoglobin F by the introduction of mutation in the *BCL11A* gene results in the induction of fetal hemoglobin. B-cell lymphoma/leukemia 11A (BCL11A) is a transcription factor that is expressed in erythroid cells and B cells and represses fetal hemoglobin expression (Peralta *et al.* 2013). Kruppel-like factor (KLF1), an erythroid-specific transcription factor, indirectly regulates γ-globin expression by regulating BCL11A and thus the production of HbF can be promoted by reducing the expression of BCL11A and KLF1 via upregulation of γ-globin expression (Peralta *et al.* 2013).

Naturally occurring large deletions comprising the beta and delta globin genes causes an increase in the fetal hemoglobin expression called hereditary persistence of HbF that ameliorates both thalassemia and SCD (Antoniani *et al*. 2018). To mimic the naturally occurring hereditary persistence of fetal hemoglobin (HPFH) mutation, deletion of 13 kb of the β-globin locus in normal HSPCs (hematopoietic stem and progenitor cells) was achieved using CRISPR/Cas9 (Ye *et al*. 2016). The efficiency of targeted deletion was 31% and significantly higher γ-globin gene expression was recorded in the erythroid colonies differentiated from HSPCs with HPFH deletion as compared to the colonies without deletion (Ye *et al*. 2016). Disruption of the β-globin locus using CRISPR/Cas9 in the SCD patient-derived HSPCs causes reactivation of expression of fetal γ-globin and a concomitant decrease in beta-globin levels in adult

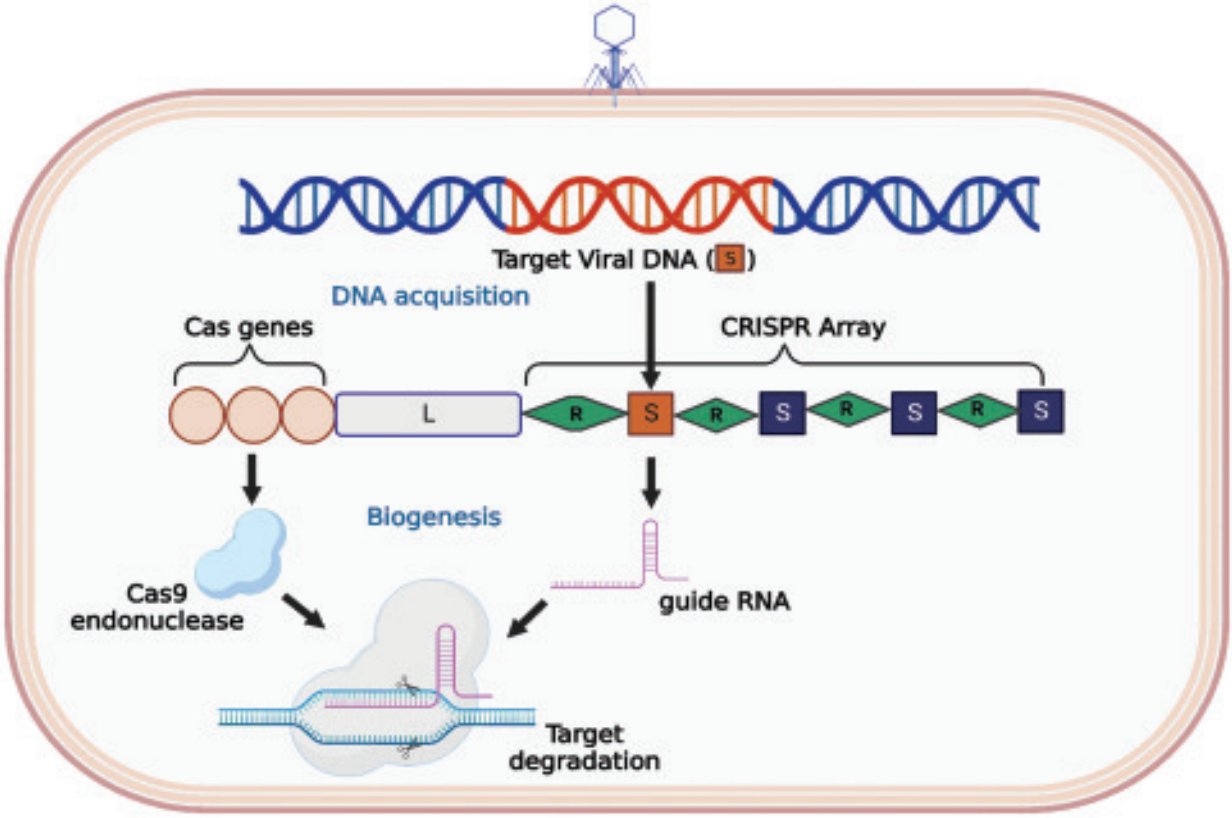

Fig. 1. Bacterial defence against phage DNA.
Bacterial genome consists of Cas genes, Leader sequence (L) and CRISPR array [Repeat (R) and Spacer sequences (S)]; Part of viral genome is acquired as spacer. On subsequent attack by the same virus biogenesis phase starts leading to production of Cas endonuclease and crRNA-tracrRNA complex (guide RNA) which after binding with each other causes target recognition and degradation.

erythroblasts (Antoniani *et al.* 2018). In genome edited population lowered proportion of sickle cells (30%) was obtained as compared to control SCD cells (65%) and thus reactivation of fetal γ-globin and the downregulation of sickle β-globin led to the amelioration of the SCD cell phenotype (Antoniani *et al.* 2018). Wu *et al.* (2019) utilized the CRISPR-Cas9 mediated cleavage in SCD patient-derived HSCs using RNP format focusing a GATA1 binding site at the +58 *BCL11A* erythroid enhancer leading to disruption of this motif, diminished BCL11A expression as well as induction of fetal γ-globin. Frangoul *et al.* (2021) used CRISPR-Cas9 techniques in CD34+ HSPCs at the erythroid-specific enhancer region of *BCL11A* to reduce the expression of BCL11A in erythroid-lineage cells and thus enhance γ-globin synthesis and the production of fetal hemoglobin. They reported that two patients one each of SCD and TDT who received autologous edited CD34+ HSPCs (CTX001) expressed enhanced levels of allelic editing in bone marrow as well as in blood after more than one year of its infusion and there was an increase in the fetal hemoglobin also. There was the elimination of vaso-occlusive episodes or requirement for transfusion in the patients.

Many of the gene-editing experiments for β-thalassemia are focused either on βIVS2-654 (cytosine to thymine mutation at nucleotide 654 of human β-globin intron 2) or IVS I-110 mutations (IVS I-110 mutation resides 19 nucleotides upstream of the normal intron-1 splice acceptor site) or towards the activation of *HbF gene* expression by disruption of *γ-globin gene* repressor BCL11A (Cosenza *et al.* 2021). Xu *et al.* (2015) utilized TALENs and CRISPR/Cas9 to target the intron 2 mutation site βIVS2-654 in the globin gene in β-thalassemia derived iPSCs and observed higher homologous gene targeting efficiency in TALENs method as compared to CRISPR/Cas9 when combined with the *piggy Bac* transposon donor. Métais *et al* (2019) targeted the disruption of the HBG1/HBG2 gene promoter motif which is bound by the transcriptional repressor BCL11A by electroporating the CRISPR/Cas9 components in ribonucleoprotein (RNP) format into SCD derived donor CD34+ HSPCs and achieved the desired mutation with high frequencies resulting into therapeutic level induction of HbF in the erythroid progeny *in vitro* as well as *in vivo* following transplantation of HSPC mouse models. In an attempt to produce high adult hemoglobin production, Cosenza *et al.* (2021) corrected the β039-thalassemia mutation, in the erythroid precursors cells isolated from the affected patients using CRISPR-Cas9 and the edited cells exhibited a very high production of HbA.

Cai *et al.* (2018) reported a universal strategy for the correction of *HBB* mutations in iPSC lines derived from TDT patients, by targeted insertion of the *HBB* cDNA (that encodes wild-type HBB sequences) at the 52 end of the exon 1 of *the HBB* gene using CRISPR/Cas9. The strategy enables the correction of HBB mutations in the first exon (SCD and β17 mutations) as well as other downstream mutations in exon 2, exon 3, and intron 2 of the β-thalassemia. Hematopoietic progeny derived from human iPSCs may generate an unlimited cell source for autologous transplantation to correct β-hemoglobinopathies. However; the production of long-term engraftable HSPCs derived from human iPSCs is the major limitation (Cai *et al.* 2018).

The combined treatment of erythroid cells with HbF inducers and gene-editing might stimulate the simultaneous increase of both HbF and HbA for the treatment of β-thalassemia (Zuccato *et al.* 2012, Cosenza *et al.* 2021).

CONCLUSION

The treatment of β-hemoglobinopathies is still a concern and the curative approach for the treatment of β-hemoglobinopathies is allogenic HSC transplantation from HLA-matched donors which is assessable to the only a limited number of individuals due to the reduced availability of immunologically matched donor sources. Gene therapy can be a useful tool and hope for the treatment of sickle cell anemia for the majority. The two commonly used approaches for genetic correction of β-hemoglobinopathies are based either on the correction of the defective β-globin gene or the induction of fetal hemoglobin. Induction of fetal hemoglobin occurs either through the knocking down of HbF repressors or the editing of HbF regulatory elements. Genetic correction by editing tools specially CRISPR-Cas9 has enormous potential in the treatment of sickle cell disease (SCD) and â-thalassaemia due to its high specificity, precision, simplicity, and economy over ZFNs (zinc finger nucleases) and TALENs. However, there is still scope for improving the efficiency of gene editing and reducing the off-target effects of this technology.

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