



CRISPR/Cas9 for Sickle Cell Disease: Applications, Future Possibilities, and Challenges

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Abstract

Sickle cell disease (SCD) is an inherited monogenic disorder resulting in serious mortality and morbidity worldwide. Although the disease was characterized more than a century ago, there are only two FDA approved medications to lessen disease severity, and a definitive cure available to all patients with SCD is lacking. Rapid and substantial progress in genome editing approaches have proven valuable as a curative option given plausibility to either correct the underlying mutation in patient-derived hematopoietic stem/progenitor cells (HSPCs), induce fetal hemoglobin expression to circumvent sickling of red blood cells (RBCs), or create corrected

induced pluripotent stem cells (iPSCs) among other approaches. Recent discovery of CRISPR/Cas9 has not only revolutionized genome engineering but has also brought the possibility of translating these concepts into a clinically meaningful reality. Here we summarize genome engineering applications using CRISPR/Cas9, addressing challenges and future perspectives of CRISPR/Cas9 as a curative option for SCD.

Keywords

Gene editing · Gene therapy · Hematopoietic stem cell transplantation · Hemoglobinopathies · Programmable endonucleases

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Abbreviations

AAV	Adeno-associated virus
BM	Bone marrow
Cas9	CRISPR associated protein 9
CRISPR	Clustered regularly interspaced short palindromic repeats
DSB	Double strand breaks
dCas9	Dead Cas9
ddPCR	Droplet digital PCR
eSpCas9	Enhanced specificity <i>Streptococcus pyogenes</i> Cas9
GVHD	Graft-vs-host disease

HbA	Adult hemoglobin
HbF	Fetal hemoglobin
HbS	Hemoglobin S
HDR	Homology directed repair
HLA	Human leukocyte antigen
HPFH	Hereditary persistence of fetal globin
HPLC	High performance liquid chromatography
HRI	Heme-regulated inhibitor
HSCT	Hematopoietic stem cell transplantation
HSPCs	Hematopoietic stem/progenitor cells
HU	Hydroxyurea
INDELS	Insertions/deletions
iPSCs	Induced pluripotent stem cells
LCR	Locus control region
MUD	Matched unrelated donor
NHEJ	Non-homologous end-joining
OTES	Off-target effects
PACE	Phage-assisted continuous evolution
PAM	Protospacer-adjacent motif
QTL	Quantitative trait loci
RBCs	red blood cells
ScCas9	<i>Streptococcus canis</i> Cas9
SCD	Sickle cell disease
shRNA ^{miR}	MicroRNA-adapted small hairpin (sh) RNAs
SpCas9-HF1	high fidelity <i>Streptococcus pyogenes</i> Cas9
TALENs	TAL-effector nucleases
UCBT	Umbilical cord blood transplantation
ZFNs	Zinc finger nucleases

gene, SCD encompasses a group of disorders with variable clinical phenotypes yet share a common pathophysiologic consequence derived from a single monogenic change. The modified β -globin gene produces an abnormal hemoglobin S (HbS) which rapidly polymerizes in the deoxygenated state altering red blood cell (RBC) rheology and lifespan. This single substitution leads to multiple downstream effects and devastating clinical complications including chronic anemia, chronic inflammation, recurrent vaso-occlusion, acute and chronic pain, stroke, organ failure, and early mortality (Paulukonis et al. 2016).

SCD is the most common inherited hemoglobinopathy worldwide, and despite knowledge of the disorder for over 100 years, it remains a life-limiting disease with few therapeutic options to reduce disease severity. Unlike other more recently identified molecular disorders that have benefited from higher federal, foundational, and per person funding (Smith et al. 2006; Lobner et al. 2013), there are only two FDA approved medications to lessen disease severity, hydroxyurea (HU) (approved for adults in 1998; children in 2017) and L-glutamine (approved in 2018). There remains misinformation, poor adherence, and a reluctance to prescribe HU despite benefit (Wang et al. 2011; Zimmerman et al. 2007; Steinberg et al. 2003; Ware 2010), while insurance companies will often not cover the cost of the highly purified form of L-glutamine approved by the FDA. Whereas the two mainstay treatments for SCD, blood transfusions and HU do not fully eliminate the consequences of the disease, simple public health measures such as newborn screening, penicillin prophylaxis, and vaccinations have significantly reduced early childhood mortality. Between 1979 and 2005, childhood mortality for children with SCD decreased by 3% per year; however, a 1% per year increase during the same period was observed for adults (Lanzkron et al. 2013). As more than 94% of children with SCD in well-resourced countries now survive until age 18, and with an expected rise in birth rate for babies with severe hemoglobin disorders to be over 400,000 by 2050 (Piel et al. 2013), disease management needs to shift to a two-tiered system

1 Introduction

Sickle cell disease (SCD) is an inherited monogenic disorder characterized by a single substitution on chromosome 11 where glutamic acid is replaced by valine in the sixth codon of the β -globin gene. Whether inherited either in a homozygous state or with another abnormal β -globin

addressing acute and chronic disease needs while simultaneously searching for curative options to address the global burden and public health issues of the disease. Hematopoietic stem cell transplantation (HSCT) and gene therapy offer a way to reduce disease burden, improve outcomes and quality of life for patients with SCD, and potentially reduce health care costs over the long term (Ballas 2009; Arnold et al. 2017; Saenz and Tisdale 2015; Bhatia et al. 2015).

Since the first HSCT in 1984 for a pediatric patient with SCD and acute myelogenous leukemia, numerous patients have successfully undergone bone marrow (BM) HSCT with a human leukocyte antigen (HLA)-matched sibling donor. Whether using a myeloablative or non-myeloablative preparative regimen, greater than 90% of all patients are cured of SCD with a BM HSCT (Walters et al. 1996; Hsieh et al. 2014; Walters et al. 2001; Gluckman et al. 2017). Between 1986 and 2013, over 1,000 patients have received an HLA-matched sibling HSCT with a 5-year event free survival and overall survival of 91.4% and 92.9%, respectively (Gluckman et al. 2017). HSCT should be considered standard of care when a patient has a clinical indication and an HLA-matched sibling donor, yet less than 15% of patients with SCD have an appropriately matched donor (Walters et al. 2001). Furthermore, only 10% of eligible patients have undergone curative HSCT despite patient willingness to consider HSCT morbidity and mortality at the chance for cure (Chakrabarti and Bareford 2007). HLA-matched unrelated donor (MUD) transplantation, umbilical cord blood transplantation (UCBT), and haploidentical transplantation offer more patients the chance for cure, though high rates of complications currently limit the broad use of these therapies. Such complications, including graft rejection and graft-vs-host disease (GVHD), are addressed in gene therapy models where a patient's autologous hematopoietic stem and progenitor cells (HSPCs) are modified thereby eliminating such complications.

The premise of gene therapy either by gene editing or insertion into autologous HSPCs raises the promise of a safer cure for SCD that is available to all patients. Such methodology eliminates

two major barriers in the cure of SCD: the lack of suitable donors, and the morbidity and mortality associated with GVHD. After decades of scientific progress, gene therapy for the cure of SCD is currently in multiple clinical trials with promising initial results. Potential methods for gene therapy in SCD are multiple: (i) addition of therapeutic globin such as β -globin or β^{T87Q} -globin to make adult hemoglobin (HbA), or γ -globin to enhance fetal hemoglobin (HbF) levels, (ii) HbF induction by editing of globin regulatory elements or knock-down of HbF repressors, or (iii) direct gene correction of the SCD mutation with programmable nucleases. Here we focus on the challenges of CRISPR-Cas9 editing, its implications, and future possibilities as a curative option for SCD.

2 Genome Editing in SCD

Given the prospect for genotypic and therefore phenotypic correction in a monogenic disorder like SCD, significant effort has been devoted to find critical genes/chromosomal areas contributing to the pathophysiology of the disease. Antisickling genes such as wild type β -globin, modified β -globin (T87Q) which confers additional antisickling properties, γ -globin or β/γ hybrids have been transferred to sickle HSPCs using various viral constructs; of those, some are currently being tested in clinical trials for both safety and efficacy (reviewed in (Demirci et al. 2018)).

Genome editing is desirable as it leads to permeant removal or correction of a detrimental mutation, or by the creation of protective insertions or deletions. Theoretically, programmable nucleases create double strand breaks (DSB) at a specific genomic locus followed by recruitment of DNA repair mechanism through either non-homologous end-joining (NHEJ) or homology directed repair (HDR) (using homologous sequences found in sister chromatids, homologous chromosomes or extrachromosomal donor DNA sequence provided for correction purposes) to the DSB site. Until 5 years ago, three major nucleases including meganucleases also known as homing endonucleases (reviewed

in (Stoddard 2011)), zinc finger nucleases (ZFNs, reviewed in (Urnov et al. 2010)), and TAL-effector nucleases (TALENs, reviewed in (M Scharenberg et al. 2013)) were introduced for various genome editing purposes. These tools have been successfully used *ex vivo* to correct the SCD mutation and induce fetal globin by editing regulatory sequences such as promoters or other regulatory sequences including *BCL11A*, *KLF1* and *MYB* to circumvent the severity of the mutation in sickle HSPCs (reviewed in (Tasan et al. 2016)). While these nucleases are highly specific thereby diminishing off-target effects (OTEs), programming of these enzymes is difficult, time consuming, and requires significant expertise.

In 2012, Doudna et al. presented a new genome editing technology (Wiedenheft et al. 2012; Jinek et al. 2012), referred to as Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9), in which a specific RNA (guide RNA) sequence recognizes the target DNA region of interest and directs the effector Cas protein there for editing. This strategy not only revolutionized genome editing strategies but also brought forth the improved possibility of translation of genome editing approaches to the clinical setting due to its advantages: easy to design, highly efficient, and inexpensive. Once introduced into target cells, CRISPR/Cas9 directed DSBs result in activation of DNA repair mechanisms. This machinery would lead to either some insertions/deletions (INDELs), which ideally results in loss-of-function for a given gene, or would repair the DNA break using homology strands if HDR is activated. In this manner, CRISPR/Cas9 technology can target correction of the SCD mutation or induce fetal hemoglobin expression by editing chromosomal areas controlling its expression (Fig. 1), yet challenges in the use of this technology remain surrounding efficiency, safety, and delivery.

2.1 HbF Induction

HbF is the predominant globin type after the first trimester of gestation and is replaced by HbA by

6 months after birth. Both HbA and HbF are maintained on chromosome 11, with switching from HbF to adult globin mainly controlled by a powerful upstream enhancer known as the locus control region (LCR) that loops to each globin promoter to activate their expression (Li et al. 2002). After the switch to HbA, HbF is not entirely suppressed, though it is not evenly distributed among RBCs. When there is not a genotypic cause for persistence of HbF in all RBCs, HbF can be minimal in some cells or concentrated in specific cells referred to as F-cells (Demirci et al. 2018).

After the initial observation by Janet Watson and colleagues that newborn babies do not show SCD complications for a certain period due to high levels of HbF in the infant's blood (Watson et al. 1948), more work has been devoted to increase HbF levels in the adult body. The important role of elevated HbF for SCD protection was further confirmed with the reports showing asymptomatic patients with SCD with elevated HbF as a result of coinheritance of hereditary persistence of fetal globin (HPFH) mutations (Forget 1998; Stamatoyannopoulos et al. 1975). Such mutations occur either in the form of large deletions in the β -globin gene, or smaller deletions/single nucleotide polymorphisms (SNPs) in γ -globin promoter or HbF regulating quantitative trait loci (QTL) (Paikari and Sheehan 2018). In line with these reports, deletion or inversion of 13.6 kb chromosomal region to obtain a HPFH-like phenotype in SCD patient derived HSPCs resulted in elevated levels of HbF in erythroblast and ameliorated the *ex vivo* sickling (Antoniani et al. 2018). Similarly, point mutations created by CRISPR/Cas9 approach in the -115 and -200 clusters of the γ -globin promoter, inhibiting the binding of validated HbF transcriptional repressors *BCL11A* and *LRF* (also known as *ZBTB7A*), respectively (Wang and Thein 2018), de-repressed the expression of HbF (Martyn et al. 2018; Liu et al. 2018). To show the applicability of these approaches, animal models are required for *in vivo* evaluations prior to human studies. Immunodeficient mice are generally used for human cell engraftment studies but are not proper for *in vivo* erythropoiesis. To

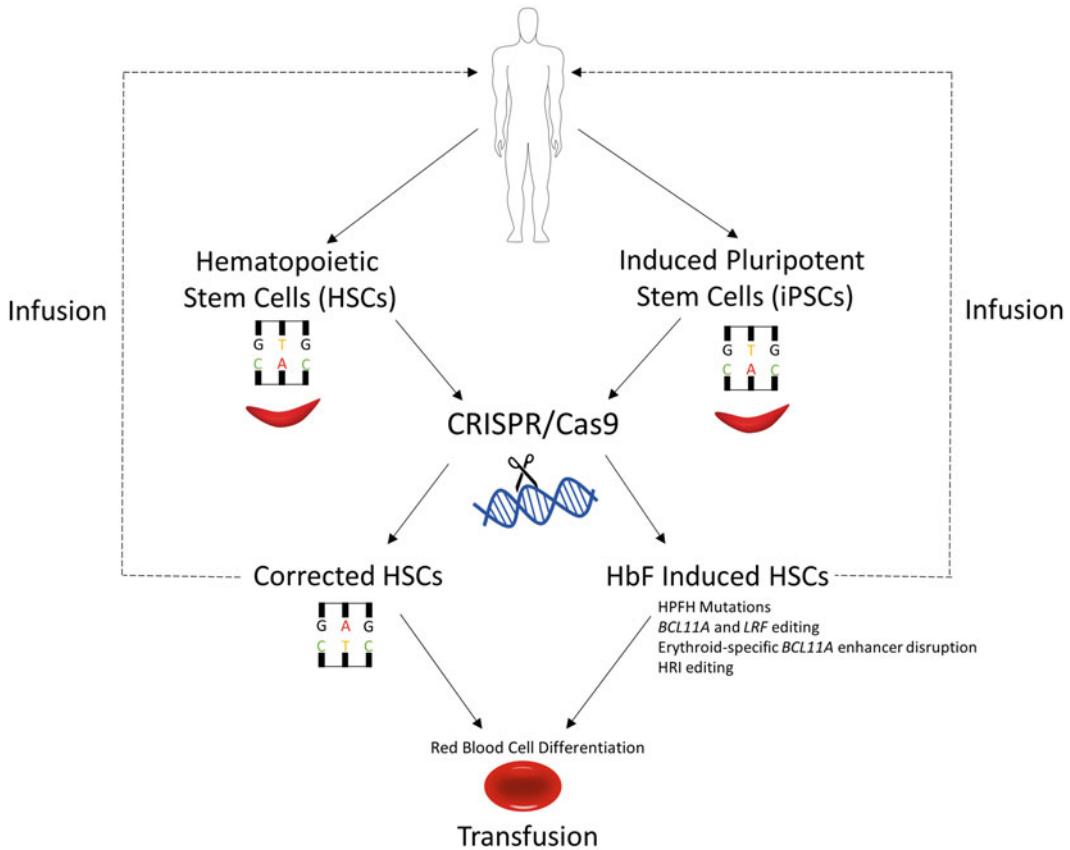


Fig. 1 Potential CRISPR/Cas9 applications for sickle cell disease (SCD). The proof-of-principle experiments have proven the possibility of SCD mutation correction and fetal hemoglobin (HbF) induction in SCD derived HSCs

and iPSCs, and subsequent normal red blood cell derivation for transfusion purposes. However, these advances are waiting to be addressed by clinical trials to explore the full potential

overcome this problem, Li et al. used a human β -globin locus transgenic (β -YAC) mice model to study the *in vivo* effect of disruption of the repressor binding region within the γ -globin promoter (Li et al. 2018). Along with significant target site distribution which was sustained in the secondary transplantation experiments, no hematological abnormality was seen and pronounced switch from human β to γ globin expression in RBCs of adult mice was noted.

Gene edition of transcriptional regulators is an alternative methodology to stimulate rare naturally occurring HPFH mutations to control HbF expression. Several transcription factors including *SCA/TALI*, *GATA1* and *KLF1* are reported to be involved in HbF regulation (Sankaran and Orkin 2013). While all of them could be

considered potential candidates, direct targeting of these factors for HbF induction is challenging as all of them have either broader roles in non-erythroid lineages or have significant roles in normal erythropoiesis. Significant candidates, LFR and BCL11A, are validated HbF silencers (Uda et al. 2008; Menzel et al. 2007), and have been edited in the erythrocyte progenitor cell line (HUDEP-2) leading to robust HbF expression (Masuda et al. 2016). BCL11A is important for HSPC function (Tsang et al. 2015) and normal lymphoid development (Liu et al. 2003), with only one paper demonstrating very low level indels and a slight increase in γ -globin expression in a non-human primate model using TALE nuclease mRNA targeting the *BCL11A* coding sequence with respect to control transplants

(Humbert et al. 2018). The safety and feasibility of the *BCL11A* knockdown is still awaiting to be addressed by large animal models with high indel ratios and subsequent clinical trials with large patients cohorts. The first clinical trial launched in February 2018 uses a lentiviral gene transfer vector encoding a microRNA-adapted small hairpin (sh) RNAs (shRNA^{miR}) targeting *BCL11A* in patients with severe SCD is currently ongoing with the first patient demonstrating 23% HbF (NCT03282656, Shim et al. 2017; Esrick et al. 2018). Recently, Daniel Bauer and colleagues have presented a different approach in which they induce comparable levels of HbF in CD34+ cells by targeting the +58 intronic site of the *BCL11A* gene that acts as an erythroid specific enhancer (Bauer et al. 2013; Canver et al. 2015). They were able to show that while guide RNA directed disruption of the enhancer site provided substantial reduction in *Bcl11a* expression in erythrocyte cells leading to elevated HbF expression in mice, it did not affect the expression in non-erythroid lineages (Smith et al. 2016). The results were extended to erythroid cells derived from progenitor cells of patients with β -Thalassemia major (Psatha et al. 2018), supporting that this enhancer disruption strategy would be favorable for clinical use if it is proven safe with preclinical and clinical studies.

With the establishment of guide RNA screening models, it has become possible to discover novel genomic sites/genes controlling HbF expression. In a recent paper, protein kinase domain-focused CRISPR/Cas9-based genetic screening revealed that heme-regulated inhibitor HRI (also known as EIF2AK1), an erythroid-specific kinase that controls protein translation as an HbF repressor, could be used as a potential candidate for the treatment of hemoglobinopathies (Grevet et al. 2018). Using similar methodology, the same group also identified that SPOP, a substrate adaptor of the CUL3 ubiquitin ligase complex, as a HbF repressor in both HUDEP-2 and CD34+ cells (Lan et al. 2018). Extending these guide RNA screening strategies to non-coding regions and epigenetics would allow identification of stronger candidates or gene combinations to enhance HbF expression

to clinically meaningful levels that reverse the sickling of RBCs and reverse the disease phenotype as seen in patients with HPFH.

2.2 SCD Mutation Correction

As the pathologic mutation for SCD is already clearly identified, correction of the SCD mutation seems the most difficult but potentially the most feasible and promising approach as Cas9 cuts sickle β -globin and this break can be repaired if a normal β -globin sequence flanked with homology arms to the DSB is supplied. Genotypic correction appears possible by targeting the specific locus at the genome and providing the correct sequence for β -globin without the necessity of exogenous transgene activation.

To ensure proper correction, an increasing number of researchers are using gene editing technologies for correcting the SCD mutation in different cell types (Table 1). Most of these works use the CRISPR/Cas9 system as it has shown better correction efficiency and lower OTEs than other gene editing tools such as TALENs (Bak et al. 2018; Hoban et al. 2016a). The HSPC source is historically bone marrow derived CD34⁺ HSPCs, currently used in the majority of genome editing studies, though recently peripherally mobilized CD34⁺ HSPCs using plerixafor has shown promise in patients with SCD given safety concerns regarding granulocyte colony stimulating factor use in these patients. These CD34+ cells can be modified to be infused back into the patient. However, differences in the cell cycle or the presence of specific nucleases that might disrupt the correction pathways used by the cells after the DSBs offer overall resistance to successful gene editing (Lomova et al. 2018). In order to maximize success, the preferred delivery method in these studies is electroporation with an Adeno-associated virus (AAV)-6 viral vector for the delivery of the CRISPR/Cas9 system with the donor DNA. For evaluating the correction of the SCD mutation, several studies analyzed gene editing at the DNA level using either targeted deep sequencing

Table 1 Selected sickle cell disease (SCD) mutation correction studies using CRISPR/Cas9

Gene	Cell types	Genome editing tool	Outcomes/comments	Mouse transplantation experiments	References
HBB	iPSCs	CRISPR/Cas9	Correction of the SCD mutation detectable at RNA expression and western blot	ND	Huang et al. (2015)
HBB	BM CD34 ⁺	TALEN CRISPR/Cas9	Correction of SCD mutation with higher performance (7.3% HbA production evaluated by HPLC) using CRISPR/Cas9	ND	Hoban et al. (2016a)
HBB	BM CD34 ⁺ Mobilized CD34 ⁺	CRISPR/Cas9	Correction of SCD mutation using an anti-sickling β -globin cDNA donor with a 29% efficiency at the RNA expression level	Long term engraftment of enriched CD34 ⁺ edited population measured by flow cytometry in femur BM (4–30%)	Dever et al. (2016)
HBB	Mobilized CD34 ⁺	CRISPR/Cas9	Correction of SCD mutation up to 33% correction evaluated by RNAseq and up to 29.3% HbA production evaluated by HPLC	Long term engraftment ability of non-enriched CD34 ⁺ edited cells evaluated in BM ($2.3 \pm 1.8\%$) and Spleen ($3.7 \pm 1.4\%$)	DeWitt et al. (2016)
HBB	iPSCs	CRISPR/Cas9	Correction of SCD mutation evaluated by sequencing. No functional studies	ND	Park et al. (2017)
HBB	CD34 ⁺ selected from PBMCs	CRISPR/Cas9	9.4–9.6% genome editing efficiency evaluated by sequencing. Correction also evaluated at protein level by HPLC	ND	Wen et al. (2017)
HBB	iPSCs	CRISPR/Cas9	Up to 67.9% correction efficiency evaluated by sequencing. No functional studies	ND	Li et al. (2016)
HBB	Mobilized CD34 ⁺	CRISPR/Cas9	20% SCD mutation correction evaluated by RNAseq	Long term engraftment of enriched CD34 ⁺ edited cells evaluated in BM (23.4% average)	Magis et al. (2018)
HBB	ESCs iPSCs	CRISPR/Cas9	63% SCD mutation correction evaluated by nested ddPCR	ND	Martin et al. (2018)
HBB	CD34 ⁺	CRISPR/HiFiCas9	More than 50% HbA evaluated by HPLC and nested ddPCR	ND	Vakulskas et al. (2018)
HBB	CD34 ⁺	CRISPR/Cas9	Evaluation of the SCD mutation correction by High-throughput sequencing	Levels of long-term engraftment of non-enriched CD34 ⁺ edited cells up to 2.5% in BM	Lomova et al. (2018)

(Lomova et al. 2018; Wen et al. 2017) or nested droplet digital (dd)PCR (Vakulskas et al. 2018), while others used more functional studies like RNAseq or RNA expression levels (Dever et al. 2016; DeWitt et al. 2016; Chung et al. 2018; Magis et al. 2018) with only three studies using High performance liquid chromatography (HPLC) for measuring protein levels after the

correction of the SCD mutation in the β -globin gene (Hoban et al. 2016a; Vakulskas et al. 2018; DeWitt et al. 2016).

Since the publication in 2008 of a protocol for generating human iPSCs from somatic cells, many groups have developed protocols for the differentiation of iPSCs into different cell lineages such as hematopoietic cells to become another viable source

of autologous HSPCs (Fujita et al. 2016; Ferreira et al. 2018; Sugimura et al. 2017). Currently however, the hematopoietic cells derived from iPSCs are primitive rather than definitive hematopoietic cells and are therefore unable to engraft in a xenograft mouse model. The available protocols for the differentiation of iPSCs towards HSPCs mainly mimic primitive hematopoiesis, which can be noticed when the generated HSPCs are differentiated into erythroid cells containing mainly ϵ -globin and γ -globin, with very low amounts of β -globin if present at all. In order to realize the available gene editing tools to correct the SCD mutation in iPSCs for therapeutic purpose, a proper differentiation protocol is needed to produce engraftable HSPCs from iPSCs. Such therapy, as with other autologous modification strategies, would ultimately eliminate two major hurdles in allogeneic transplantation; rejection and GVHD in transplantation therapies. In addition, as low efficiency of correction is a problem for HSPC studies, cloning corrected cells from a bulk iPSC population would allow derivation of a population with 100% of cells corrected.

In addition to the difficulties differentiating iPSCs towards HSPCs, only one study has presented the correction of the SCD mutation in SCD-derived iPSCs at the RNA and protein levels by qPCR and Western blot analyses, respectively (Huang et al. 2015), though several groups have shown the correction of the SCD mutation at the DNA level using nested ddPCR or DNA sequencing (Park et al. 2017; Li et al. 2016; Martin et al. 2018). Correction of underlying mutation in both CD34⁺ and iPSCs seems promising, yet while significant correction rates are reported in *ex vivo* conditions, limited corrected human cell engraftment are reported in immunodeficient mouse models (Table 1). While immunodeficient mice transplantation models for human cell engraftment studies are being widely accepted, it is not clear that whether these results completely reflect the clinical outcome of these approaches. After optimization of the correction methodologies, larger animal models are necessary to explore the potential of the application.

Though editing of CD34⁺ cells is possible, multiple genotypic outcomes are possible and

editing of long-term engrafting HSPCs are not yet fully explored. Treating cells with CRISPR/Cas9 and a β -globin donor might result with cells in their native state (uncorrected), as sickle trait (one allele corrected), as healthy (both alleles corrected), as β -thalassemia major (both alleles disrupted), as β -thalassemia trait (one allele corrected and other disrupted), and/or sickle/ β -thalassemia (one allele disrupted) due to NHEJ/HDR machinery of the cells (Esrick and Bauer 2018). As precise correction in long-term HSPCs is not yet efficient and editing results in reduction in engrafting HSPCs (Hoban et al. 2016a; Dever et al. 2016), transplantation of mixed culture could be clinically problematic and possible unintended consequences should be addressed before clinical trials.

3 Challenges

Genome editing has been the most attractive tool for scientists seeking to correct genetic mutations either as gene knockout or knock-in. Conventional methods for genome engineering, however, are costly, time-consuming, labor-intensive, and require expertise in protein engineering to design specific nucleases (Roy et al. 2018). On the contrary, CRISPR/Cas9 genome editing is a system that is relatively easier, cheaper and more efficient, and is being used in a large variety of model cells and species. It has not only led to easier and cheaper development of knock-out animal models but has also contributed to the establishment of whole-genome screening libraries that identify therapeutic genes/chromosomal regions that may directly affect a targeted phenotype. While there is a huge international interest in CRISPR/Cas9-based editing approaches, there is still much to improve upon such as the efficiency of cutting and editing (both NHEJ and HDR), improving specificity, and improving delivery methods. Lastly, there is a world-wide concern about safety, particularly as it relates to OTEs, that needs to be clarified and addressed before transferring this approach into routine clinical care.

3.1 Efficiency of Editing

The limiting factor for diverse application of a given CRISPR/Cas9 system has been the dependency on a protospacer-adjacent motif (PAM) sequence flanking the target. For instance, as SCD mutation correction studies need to target a specific chromosomal area, there are not many guide RNA options for different Cas proteins. Therefore, substantial effort has been made to engineer various Cas effector proteins for the recognition of different PAM sequence (Kleinstiver et al. 2015a; Nishimasu et al. 2018; Kim et al. 2017). While the introduction of 19 subtypes of CRISPR systems with various Cas effector proteins recognizing different PAM sites have extended targetable genomic loci (Leenay and Beisel 2017), not all of them have been widely studied in terms of efficacy and safety. Therefore, scientists still tend to use well-established Cas types (*i.e.* *Streptococcus pyogenes* Cas9-SpCas9 or Cpf1-Cas12a) in their research. SpCas9 has a PAM recognition of 5' NGG 3', while some other Cas9 orthologs have been reported to require longer PAM sites (Fonfara et al. 2013; Ran et al. 2015). While these have some advantages over classical SpCas9, their longer PAM sites restrict their use despite potentially more efficient delivery. For example, smaller Cas effector proteins such as *Staphylococcus aureus* derived Cas9 (SaCas9) with a PAM site of NNGRRT, are more efficient for viral delivery systems (Kleinstiver et al. 2015b). To extend the boundaries of targeting range for Cas9 proteins, PAM preference can successfully be altered by targeted mutations to residues near the PAM DNA duplex (Anders et al. 2016; Hirano et al. 2016).

Understanding the subunits of Cas effector proteins have allowed the modification of PAM specificity. In a recent report, Chatterjee *et al.* characterized *Streptococcus canis* Cas9 (ScCas9) displaying 5'-NNG-3' PAM, reporting an 89.2% sequence similarity to SpCas9 (Chatterjee et al. 2018). Structural analysis showed that two distinct mutational areas [a positive-charged insertion in the REC domain (at 367–376) and a KQ insertion in the PAM-interacting domain (at 1337 and 1338)] are responsible for having the specificity for a minimal PAM sequence. Another group has

recently generated Cas9 variants with various PAM compatibilities (including NG, GAA and GAT) using phage-assisted continuous evolution (PACE) approach (Hu et al. 2018). But more intriguingly, although extending PAM recognition capacity of Cas9 variants would be assumed to augment OTE (Hu et al. 2018; Tsai et al. 2015), they reported greater DNA specificity for Cas9 variants with respect to canonical SpCas9 along with lower genome-wide off-target. In a different approach, Sniper-Cas9 (F539S/M763I/K890 N variant) was successfully obtained using directed evolution, and characterized with high on-target and reduced OTEs (Lee et al. 2018). These studies illustrate the potential and the need for further improvements in targetable loci on the genome for various Cas effector proteins. While improving the efficiency, safety should also be parallelly taken into account to realize the approaches in routine clinical applications.

3.2 Potential Immunogenicity of Editing Tools or Edited Cells

The ultimate goal of CRISPR technology is to edit mutations related with disorders or control disease associated gene expressions in patient-derived specific stem/progenitor cells. However, *in vivo* effects of CRISPR/Cas9 systems have a lot of unanswered questions. In 2019, there are open clinical trials in the United States and abroad using CRISPR/Cas9 for a potential treatment of SCD, Thalassemia, HIV-1, and several cancer types (<https://clinicaltrials.gov/keyword/CRISPR>). Though hope remains for these clinical trials, *ex vivo* work conducted thus far, demonstrate preliminary data pointing toward possible adverse effects of the technology. The first question is whether guide RNAs or Cas9 itself has any effects on the immune system. To partially address this uncertainty, Kim *et al.* demonstrated that *in vitro* transcribed guide RNAs with a 5'-triphosphate group (5'-ppp) leads to cytotoxicity due to the activation of innate immune system in human and mouse cells (Kim et al. 2018). The authors also reported that removal of triphosphate resulted in high mutation rate in primary human

CD4+ cells thus avoiding the innate immune system. In a recent pre-print article, Charlesworth et al. showed pre-existing antibodies against Cas9 derived from *Staphylococcus aureus* (79%) or *Streptococcus pyogenes* (65%) in a small group of healthy volunteers (Charlesworth et al. 2018). In a follow up work performed with 200 blood samples, prevalence of antibodies against SaCas9 and SpCas9 were reported to be 10% and 2.5%, respectively (Simhadri et al. 2018). While these results are not unexpected, triggering of the immune system by CRISPR/Cas9 is potentially problematic and harmful in vivo. While these observations and potential immune response are awaiting to be addressed by large animal models and clinical studies, Cas9 expression levels, delivery methods, vector types in case of transduction routes, and target cells populations should be optimized in any capacity to diminish a severe immune response.

3.3 Specificity of Editing

Other than a potential immune response, OTEs are one of the biggest challenges of CRISPR/Cas9 system. As Cas9-guide RNA complex can recognize sequences with up to 5 mismatched bases (Fu et al. 2013), the possibility of OTE for a given guide RNA cannot be ignored. A number of advances have been taken to increase the specificity of CRISPR/Cas system, but the guide RNA design is the first critical process for reduction of OTEs. There are vast guide RNA design tools available; of those, newer ones include supplementary algorithms evaluating on-target cutting efficiency other than selectivity for the target. During the synthesis of guide RNA, additional modifications on the guide RNA structure including truncation of spacer RNA (Fu et al. 2014) and chemical modifications (Cromwell et al. 2018) have been reported to increase Cas9 endonuclease specificity. In addition, chemical modifications with 2'-O-methyl 3' phosphorothioate (Hendel et al. 2015) and 2'-fluoro-ribose (Rahdar et al. 2015) improve the editing efficiency via increasing the stability of guide RNAs in cells.

The second important aspect to reduce OTEs is to enhance Cas9 specificity. A mutated variant of Cas9, nickase (Cas9n), can only cut a single DNA strand such that two close recognition sites in the DNA are required for a double strand break and thus OTEs are drastically reduced (50–1500 fold in human cells) (Ran et al. 2013). However, as some single nicks can be converted to double strand breaks, this approach was further improved with introduction of a catalytically inactive Cas9 (dead (d)Cas9) and Fok1 fusion protein (Tsai et al. 2014). In this approach, recognition of guide RNAs by dCas9 brings Fok1 enzyme the close proximity that is required for active dimerized Fok1 nuclease. While these approaches provided significant reduction in the off-target issues, requirement for a double recognition site might result in less editing efficiencies, and the necessity for double guide RNA usage might limit viral delivery approaches. To therefore keep editing efficiency high enough for clinical application, active nucleases are being engineered for higher specificities. The initial idea for high specificity nucleases was to decrease the interactions of Cas9 with its DNA target to lessen OTEs while keeping enough energy for on-target recognition. With the introduction of high fidelity Cas9 (SpCas9-HF1, N497A/R661A/Q695A/Q926A) (Kleinstiver et al. 2016) and enhanced specificity Cas9 (eSpCas9 (1.1), K848A/K1003A/R1060A) (Slaymaker et al. 2016), there are no or significantly reduced OTEs compared to wild type nucleases while maintaining robust on-target activities. Recently, Doudna *et al.* has published that both SpCas9-HF1 and eSpCas9(1.1) are trapped in an inactive state when bound to mismatched targets and that the non-catalytic domain of Cas9, REC3, is responsible for target recognition and direction of nuclease activity (Chen et al. 2017). Using these observations, they were able to create hyper-accurate Cas9 variant (HypaCas9) with wide-range genome specificity without compromising any detectable OTEs.

Recently, several publications have raised appropriate concern about the CRISPR/Cas system showing unintended consequences such as large deletions, insertions, and rearrangement of the chromosome when used in clinical trials

(Kosicki et al. 2018; Shin et al. 2017; Adikusuma et al. 2018). It is not clear that this uncertainty is going to be elucidated, or is clinically relevant, but it is sensible to urge more pre-clinical studies addressing these valid safety concerns.

3.4 Delivery

To apply CRISPR/Cas9 system to a given cell type/organism, the structure and vehicle of the components should be determined based on requirements for protein amount, exposure time, efficiency, and restrictions for OTEs and other safety issues. For the structure of the system, it could be (i) integrating/non-integrating viral vectors/plasmids expressing both mRNAs for guide RNA and Cas9, (ii) Cas9 mRNA and guide RNA, or (iii) ribonucleoprotein complex (RNP) constituting Cas9 protein and guide RNA. A short time after the discovery that CRISPR/Cas9 system could be used in human cells for genome editing purposes, viral constructs providing continuous expression of Cas9 and guide RNAs were used to explore this potential. However, while it might be advantageous for gene editing approaches requiring long-term expression, it was also recognized that sustained expression of guide RNAs and Cas9 augmented the possibility of mismatch bindings and OTEs (Pattanayak et al. 2013). For precise temporal control of expression, several inducible systems have been presented (Nihongaki et al. 2015; Zetsche et al. 2015). Using vectoral delivery in the lab is stable and cheap; however, there is still ongoing debate about the problems of viral systems with the immune system (Yin et al. 2014) and insertional mutagenesis (Hoban et al. 2016b). An alternative method to plasmids/vectors carrying Cas9 sequence is the introduction of mRNA for Cas9 that is translated to active protein once it is transferred to the cell. While this system avoids the time needed for transcription for Cas9 transferred with plasmids, it is also applicable only for genome editing approaches doable with transient Cas9 expressions. In addition, as mRNAs are not as stable as DNAs, delivery time of RNAs for Cas9 and guide RNA would

be critical. Jiang *et al.* showed that Cas9 protein was at maximum level 6 h after delivering Cas9 mRNA and not detectable after 24 h in mice (Jiang et al. 2017). One way to optimize efficiency would be different delivery times or chemical modifications to provide the stability of RNAs as was mentioned earlier (Safety section).

The RNP complex is another alternative in which native Cas9 protein and guide RNA form a single complex that is readily active once it is in the cell. Other than the question of whether native foreign protein to the human cells is significantly immunogenic to hinder the potential of RNP usage, the main drawback of this application is that Cas9-guide RNA structure is a relatively large complex. Non-viral delivery systems including electroporation, encapsulation, and delivery by modification are trending for not only transferring this large cargo but also for other DNA and RNA systems (reviewed in (Glass et al. 2018)). Electroporation, a non-selective delivery method, has been used for a long time for various DNA, RNA, and protein transfers through the cell membrane by enlarging the pores on the cell membrane via a strong electric field. While this method is highly efficient in transferring Cas9 and guide RNA to HSPCs for an aim of correction of SCD mutation (Hoban et al. 2016a; Dever et al. 2016; Magis et al. 2018), toxicity and the long-term viability issue of electroporation for a clinical setting is still being questioned. From a clinical point of view, huge quantities of Cas9 protein might be required for a clinical setting, and purification of endotoxin-free Cas9 protein is not economically feasible at this time. More industrial work is warranted to explore feasible ways for GMP grade Cas9 production in order for this technique to be practical in a clinical setting.

4 Future Perspective and Directions

While SCD was characterized more than century ago, definitive treatment for all patients is not currently available given a lack of suitable donors for curative HSCT. As monogenic disease, SCD is

one of the most important candidates for programmable nucleases, particularly CRISPR/Cas9 due to being cost-effective, easily applicable, and highly efficient. Proof-of-principle studies have shown that CRISPR/Cas9 can efficiently be used to correct the SCD mutation or induce HbF expression in *ex vivo* cell culture conditions and mouse models. However, there is still concerns about the safety due to random off-target effect and subtherapeutic efficiency. More work should be conducted in larger animal models to demonstrate the safety of the approach along with optimization studies in *ex vivo* conditions.

Clinical trials investigating the prospective of CRISPR/Cas9 for SCD are in progress or are starting soon, which will certainly direct the future of this approach. The application itself is promising but it is not currently feasible for translation into routine use especially for less developed countries such as Africa where prevalence of SCD is high. Additional cost-effective manufacturing processes for clinical grade guide RNAs and Cas9 proteins should be implemented to extend the use, and ensure a safer, more efficient product. The premise of gene therapy for the cure of SCD is moving closer to reality, though questions and challenges remain to ensure this as a feasible, safe, and lifelong curative strategy.

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