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Review

Revolutionising healing: Gene Editing's breakthrough against sickle cell disease

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ABSTRACT

Recent advancements in gene editing illuminate new potential therapeutic approaches for Sickle Cell Disease (SCD), a debilitating monogenic disorder caused by a point mutation in the β-globin gene. Despite the availability of several FDA-approved medications for symptomatic relief, allogeneic hematopoietic stem cell transplantation (HSCT) remains the sole curative option, underscoring a persistent need for novel treatments. This review delves into the growing field of gene editing, particularly the extensive research focused on curing haemoglobinopathies like SCD. We examine the use of techniques such as CRISPR-Cas9 and homology-directed repair, base editing, and prime editing to either correct the pathogenic variant into a non-pathogenic or wild-type one or augment fetal haemoglobin (HbF) production. The article elucidates ways to optimize these tools for efficacious gene editing with minimal off-target effects and offers insights into their effective delivery into cells. Furthermore, we explore clinical trials involving alternative SCD treatment strategies, such as LentiGlobin therapy and autologous HSCT, distilling the current findings. This review consolidates vital information for the clinical translation of gene editing for SCD, providing strategic insights for investigators eager to further the development of gene editing for SCD.

1. Introduction

Sickle cell disease (SCD) is an autosomal recessive disorder caused by a missense mutation of the β-globin gene. It is one of the most common severe monogenic disorders worldwide, with an estimated 300,000 infants born with the disease every year, concentrated in the areas of the Mediterranean, Africa, the Middle East, and South Asia [1,2,3]. The global distribution of the disease and the causing allele derives from two main factors: the survival advantage of malaria carriers led to evolutionary selection for these individuals in the mentioned regions, and the subsequent migration led to the presence of the SCD-causing allele in other parts of the world [2,3].

A GAG to GTG point mutation in the 6th codon of the *HBB* gene - which encodes the β-chain of haemoglobin - resulting in glutamine to

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valine substitution, is responsible for causing SCD [4,2] (Fig. 1a). HbF, composed of two α-globin and two γ-globin chains, is usually replaced by adult haemoglobin (HbA) during the first year of life, which occurs due to the γ-globin to β-globin expression switch [5]. Under deoxygenating conditions, hydrophobic motifs on sickle haemoglobin (HbS) tetramers become exposed, prompting β_S-globin chains to bind with adjacent molecules in the cell's aqueous compartment to conceal these motifs. This initiates the formation of HbS polymers, resulting in the generation of long, rigid fibres that distort the red blood cell (RBC) membrane and give rise to the characteristic sickle-like shape [6]. These sickled erythrocytes are dysfunctional and contribute to cellular failure and stress [7]. When oxygen is restored, the HbS fibres can "melt" and revert the RBC shape to their flexible, biconcave form. However, depending on oxygen levels, the continuous cycles of sickling and unsickling lead to irreversible damage to the RBC membrane [8] (Fig. 1b).

Depending on the number of HbS alleles in an individual's genetic makeup, SCD can be classified into various types. The most common and severe form of SCD is sickle cell anaemia. This occurs when an individual inherits two copies of the HbS allele (HbSS), resulting in predominantly HbS in the red blood cells. Sickle cell anaemia is characterised by the typical symptoms and complications associated with SCD [7]. Another type is sickle cell trait (HbAS), where an individual carries one HbS allele and one normal β-globin allele. In this case, the individual usually does not experience symptoms associated with SCD because the healthy β-globin allele compensates for the presence of HbS. Sickle-β-thalassemia (HbSβ-thalassemia) is a rare type of SCD that occurs when an individual inherits one HbS allele and one β-thalassemia allele. β-thalassemia is a blood disorder marked by a diminished or no production of normal β-globin chains due to mutations in the *HBB* gene, with some symptoms such as anaemia, delayed growth and development, splenomegaly, bone deformities and iron overload [9]. Therefore, individuals with HbSβ-thalassemia display typical symptoms of both SCD and beta-thalassemia [10]. Haemoglobin C (HbC) is the second most common variant found in *HBB*, caused by glutamine to lysine substitution at the 6th codon and is associated with distinct characteristics and symptoms. When co-expressed with the HbS allele, sickle-haemoglobin C disease (HbSC) occurs, a form of SCD with a milder phenotype than HbSS [11].

The main pathophysiological features of sickle cell disease are haemolytic anaemia and vaso-occlusion [2]. HbS polymerisation triggers a cascade of cellular abnormalities. The stiffness and fragility of sickle red blood cells (SS-RBCs) causes haemolysis, which results in anaemia. In addition, aberrant expression of adhesion molecules on the SS-RBC membrane due to oxidative stress often causes vaso-occlusion; haemolysis is again responsible for this phenomenon, as the release of heme and ions from dying RBCs induces oxidative stress. The same process causes a reduction of Nitric Oxide (NO), a vasodilatory and anti-inflammatory molecule, which reductions results in inflammation [1] (Fig. 1c). Vasculopathy, ischaemia, infarction, tissue necrosis and organ failure are some disease-related complications [2]. Finally, reperfusion injury can be added to the disease pathophysiology, which can occur when a vaso-occlusive crisis is resolved as a consequence of the rapid influx of oxygen and related increased levels of free radicals, resulting in hypercoagulability and risk of thrombosis [1].

2. Drug therapies

Despite our increasing understanding of SCD pathophysiology, the currently available therapeutic strategies mainly aim at alleviating the disease symptoms. This includes both the prevention of HbS polymerisation and the amelioration of its effects [12].

2.1. Hydroxyurea

Hydroxyurea (or hydroxycarbamide) is an FDA approved HbF inducer for the therapy of SCD [13]. Despite decades of research, the exact mechanism by which hydroxyurea induces HbF production remains unclear and clinical trials and *in vivo* studies suggest a more complex mechanism. The primary proposed mechanism involves hydroxyurea acting as an inhibitor of ribonucleotide reductase, an enzyme required for DNA synthesis, resulting in cell cycle arrest and cell death, subsequently promoting erythroid progenitor cell recruitment and HbF synthesis [14]. It also interferes with repressors and their cofactors at the globin promoters or stabilising activator interactions [15]. Additionally, hydroxyurea has been associated with a reduction of neutrophils and reticulocytes, correlating with reduced morbidity and mortality in SCD patients [16,17]. It improves erythrocyte hydration and deformability [8], and releases nitric oxide (NO) to address NO deficiency due to haemolysis [18,19]. It is considered generally safe for both adults and children, with response rates of up to 70% [17,8,15]. However, its effectiveness varies significantly among patients for unknown reasons, with non-compliance with regular doses and potentially insufficient doses being the most probable reasons for non-responsiveness [20,21]. Hydroxyurea is regarded as the best disease-modifying treatment for SCD, given its effectiveness in reducing symptoms and mortality and worldwide accessibility [17].

2.2. Crizanlizumab

Crizanlizumab is an FDA approved monoclonal anti-P-selectin antibody for SCD that reduces vaso-occlusive episodes by inhibiting a P-selectin on the vascular endothelium. This limits activated neutrophils from trapping sickled erythrocytes, preventing the cascade of events preceding the vaso-occlusive crises [22,23]. As the first FDA-approved therapy for SCD since hydroxyurea, it addresses cell adherence, a problem that hydroxyurea does not tackle [24]. Consequently, the European Commission withdrew its marketing authorisation in 2023, a decision influenced by the STAND trial results, which showed no statistically significant difference in pain crisis rates between the treatment and placebo groups in the first year [25].

2.3. Voxelotor

Voxelotor (GBT440 or Oxbryta) is an oral drug designed to increase the affinity of haemoglobin for oxygen, stabilising its oxyhaemoglobin state, thus reducing deoxy-HbS concentration and decreasing sickled cell polymerisation [22,26]. Moreover, it increases oxygen availability, stabilising the non-polymerising relaxed state of haemoglobin, therefore decreasing disease severity [27,28].

Early clinical trials showed it improves haematological parameters with minimal adverse effects [29], while a phase 3 trial confirmed its effectiveness in increasing haemoglobin levels, reducing anaemia, and preventing haemolysis with a favourable safety profile [30]. Voxelotor is contraindicated for those with a history of drug hypersensitivity or when using CYP3A4 inhibitors and can interfere with Hb subtype identification by high-performance liquid chromatography [31].

2.4. L-Glutamine

L-Glutamine, another FDA-approved drug for reducing the frequency of SCD complications, is a conditionally essential amino acid that may work by producing nicotinamide adenine dinucleotide in RBCs, potentially decreasing oxidative stress and preventing sickling and related crises [22,32,33]. However, phase 3 trials showed no significant change in haemoglobin, haematocrit levels or reticulocyte counts despite reduced pain crisis episodes [33]. Despite FDA approval, its efficacy and safety are less established than for hydroxyurea, and there is limited patient accessibility due to high costs [32,34].

2.5. Combination drug therapy

Combination drug therapy is a recently developed strategy in which

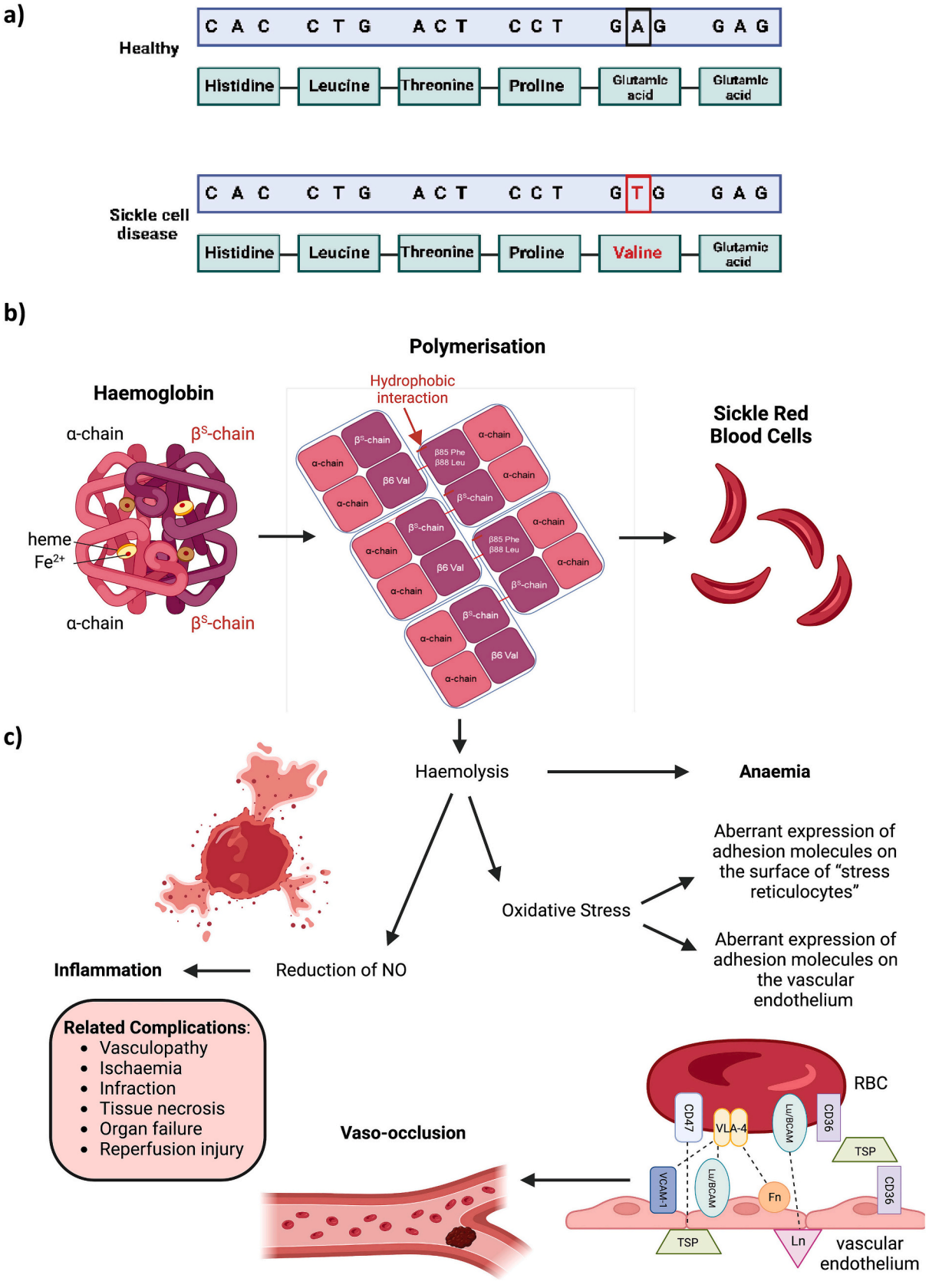

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Fig. 1. Sickle Cell Disease (SCD) mechanisms and pathophysiology a) Schematic diagram of the DNA and amino acid sequences for normal and SCD individuals. A homozygous point mutation (GAG > GTG) is present in the *HBB* gene, which substitutes glutamic acid to valine . b) The basis of SCD pathophysiology. (Left) Haemoglobin is made of 2 α- and 2 β-chains, each containing a heme molecule, used to bind Fe^{2+} ferrous ion. (Middle) The amino acid change (Glu to Val) caused by SCD-causing mutation causes haemoglobin proteins to create hydrophobic interactions during polymerisation under deoxygenated conditions. (Right) The result is the formation of sickle red blood cells. **c)** Pathophysiological features of sickle cell disease. Sickle haemoglobin polymerisation causes a cascade of events: haemolysis directly causes anaemia, while oxidative stress, related to the release of free heme and ions, causes the aberrant expression of adhesion molecules (Fn - fibronectin; TSP – thrombospondin, Ln - laminin, VLA-4 or α4β1 integrin, Lu/BCAM - basal cell adhesion molecule or Lutheran blood group) on the surface of "stress reticulocytes", a population of young RBCs coming out of the bone marrow prematurely, and on the vascular endothelium, causing vaso-occlusion. Finally, hemolysis causes the reduction of Nitric Oxide (NO), resulting in inflammation. A list of related complications is shown in the bottom-left corner. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

different therapeutics are used together, mainly anti-switching and anti-sickling drugs [28]. This approach is the result of a deep understanding of the processes of deoxy-HbS polymerisation and haemoglobin switch, and its proof of concept is found in Atweh et al. study combining hydroxyurea and butyrate [35]. Combination therapeutic regimens will need to be defined for this strategy to enter clinical practice. These will likely be designed based on the severity of clinical phenotypes and will require extensive testing on animal models, finally resulting in the development of new approaches for precision medicine [28].

3. Hematopoietic stem cell transplantation

Current therapeutic strategies for SCD mainly aim at symptom relief, with allogeneic hematopoietic stem cell transplantation (HSCT) being the only exception. It is primarily offered to paediatric patients before irreversible vascular damage occurs [36]. Studies show highly successful results, with >93% overall survival and > 86% event-free survival [37,38,39]. The biggest drawback of this approach is the complications associated with the myeloablative conditioning regimen, like infections and a 12–14% incidence of Graft-*versus*-host disease (GVHD) [40,36,41]. Moreover, finding Human Leukocyte Antigen (HLA) matched sibling donors, crucial for successful transplantation, is a challenge for over 80% of SCD patients [5]. Umbilical cord blood transplantation offers an alternative with less stringent HLA matching but carries higher risks of GVHD, graft rejection, and transplant-related mortality [42,43].

A recent advancement in transplantation for SCD is *in-utero* stem cell transplantation (IUSCT), which aims to cure SCD before birth by leveraging the immature fetal immune system. However, it faces challenges like varying engraftment rates between congenic and allogeneic cells and maternal immune responses [44,45]. Additionally, a significant limitation of this approach is the host cell competition, making it challenging for the donor cells to compete with the rapidly growing and developing fetal compartments, successfully maintaining long-term chimerism [44,46,47]. Different considerations must be made with IUSCT compared to other transplantation approaches, including ethical and safety concerns.

4. Gene therapy

Gene therapy for SCD is a fast-developing area of research driven by the design of tissue-specific and efficient vectors and gene editing technologies. The initial approaches involved β- or γ-globin gene addition, while newer strategies aim to reactivate HbF expression, obtaining similar results to hydroxyurea [48]. The success in β-thalassaemia gene therapy has encouraged many SCD clinical trials using two main approaches: employing lentiviral vectors to carry β- or γ-globin genes and using shRNAs for the suppression of *BCL11A* thereby inducing endogenous HbF [49].

A gene therapy clinical trial (LentiGlobin BB305), based on a lentiviral vector delivering a modified β-globin gene (Thr87Gln substitution), shows excellent potential. This approach involves modifying patient-isolated CD34+ hematopoietic stem and progenitor cells (HSPCs) to produce HbA^{T87Q}, a modified adult haemoglobin designed to inhibit the polymerisation of sickle haemoglobin [50]. Initially FDA-approved in August 2022 for β-thalassemia major as Zynteglo (betibeglogene autotemcel or beti-cel), its efficacy in reducing blood transfusion needs in β-thalassemia has been established [51,52,53]. Bluebird Bio recently obtained a second approval for Lyfgenia (lovotibeglogene autotemcel), based on the same lentiviral vector and gene payload and differing from Zynteglo in small manufacturing features [54].

For SCD, recent Bluebird Bio trials have shown encouraging results, including corrected haematological parameters and reduced vaso-occlusive crises 15 months after treatment [55]. However, challenges include ensuring reproducibility and evaluating the long-term effects of a single-dose gene therapy [50,55]. Drepaglobe (NCT03964792) represents yet one more lentiviral trial, wherein three individuals diagnosed with SCD underwent autologous transplantation of CD34+ cells that were *ex vivo* transduced with the GLOBE1 lentiviral vector, expressing an anti-sickling β-globin protein (AS3) with three amino acid substitutions in the wild-type β-globin gene. Among these patients, two experienced clinical benefits from the treatment. Further investigation and analysis are necessary to determine the crucial factors contributing to its success [56]. Similarly, Aruvant Sciences is assessing ARU-1801 (NCT02186418), a modified γ-globin lentiviral gene-edited cell therapy. At present, four treated patients have shown stable HbF expression, reduced SCD symptoms, and fewer vaso-occlusion events, despite experiencing transient neutropenia and thrombocytopenia [57]. On the other hand, Boston's Children's Hospital has pursued a distinct strategy, transducing CD34+ HSCs with a lentiviral vector, called BCH-BB694. It contains microRNA-adapted short-hairpin RNA (shmiR) which targets the *BCL11A* gene and hence enables specific knockdown in erythroid cells, inducing γ-globin expression while simultaneously repressing β-sickle globin expression [58]. This approach has shown stable induction of HbF with 30.5% HbF of all Hb levels and notable reduction of vaso-occlusive events in severe SCD, except for one patient. Following the promising outcomes of this pilot study, a phase 2 trial is now open for enrolment (NCT05353647). A recent study explored the potential of *in-utero* gene therapy for treating β-thalassemia in a humanised mouse model, indicating promising results in normalising blood parameters and improving cardiac function, emphasizing the ongoing evolution of gene therapies for haematological disorders [59].

Furthermore, advancements in gene editing technologies offer new therapeutic avenues for hemoglobinopathies, aiming to specifically modify patients' genetic material to correct disease-related genes or act on the disease mechanism at a molecular level [60]. These gene editing strategies, aiming to correct the β-globin gene mutation or induce fetal γ-globin production, could provide a one-time cure for SCD, highlighting the dynamic evolution of gene therapies in treating haematological disorders [61].

5. Gene editing

Gene editing for genetic blood disorders such as SCD is a promising treatment option, resulting in precise correction of pathogenic variants, ameliorating the disease symptoms, or even completely curing the disease [60]. The gene editing technologies are based on targeted nucleases, developed and improved through the years. Table 1 summarises gene therapy and gene editing clinical trials aiming to cure SCD.

The first nucleases used for gene editing are meganucleases, zinc-

Table 1
Gene therapy and gene editing clinical trials.

Gene therapy/editing strategy				Clinical trials	Sponsors	Phase
Gene Therapy	Modified HBB delivery	Zynteglo (BB305)		NCT02151526, NCT04628585	Bluebird Bio	I, II (completed in 2019), long-term follow up (enrolling)
		Lyfgenia (BB305)		NCT02140554, NCT04293185, NCT04628585	Bluebird Bio	I, II (ongoing), III (ongoing), long-term follow up study (enrolling)
		Drepaglobe		NCT03964792	Assistance Publique - Hôpitaux de Paris	I, II (ongoing)
		βAS3-FB Vector Transduced Peripheral Blood CD34+ Cells		NCT02247843	Donald B. Kohn, M.D. (UCLA)	I, II (recruiting)
	shRNA-based gene silencing	BCH-BB694		NCT03282656, NCT05353647	David Williams, M.D. (Boston Children's Hospital)	I (ongoing), II (recruiting)
		CSL200		NCT04091737	CSL Behring	I (terminated in 2021)
	Modified anti-sickling γ-globin gene delivery	ARU-1801		NCT02186418	Aruvant Sciences	I, II (ongoing)
	Zinc Finger Nuclease (ZFN)	PRECIZN-1		NCT03653247, NCT05145062	Sangamo Therapeutics	I, II (ongoing), long-term follow up study (enrolling)
Gene Editing	Crispr-Cas systems	Crispr-Cas9	CTX001	NCT03745287, NCT04208529	Vertex Pharmaceuticals and CRISPR Therapeutics	I, II, III (ongoing), long-term follow up study (ongoing)
			GPH-101	NCT04819841	Graphite Bio	I, II (terminated in 2023)
			OTQ923	NCT04443907, NCT06155500	Novartis Pharmaceuticals	I, II (ongoing), long-term follow up study (not yet recruiting)
			CRISPR_SCD001	NCT04774536	UCSF	I, II (ongoing)
		Crispr-Cas12a	EDIT-301	(RUBY) NCT04853576	Editas Medicine	I, II (recruiting)
	Adenine Base Editor (ABE)	BEAM-101		(BEACON) NCT05456880	Beam Therapeutics	I, II (ongoing)
Allogeneic hematopoietic stem cell transplant (HSCT), gene therapy/editing				(COALESCE) NCT05153967	Vanderbilt University Medical Center	Long-term follow up study (recruiting)

finger nucleases, and transcription activator-like effector nucleases (TALENs). Meganucleases, or homing endonucleases, are naturally occurring monomers that recognise relatively long (14–40 bp) sequences involved in DNA repair [62]. The low frequency of meganuclease target sites in the human genome limits their use as therapeutics; however, target recognition can be engineered by structure-guided design and high-throughput screening [60,62]. Another limitation is their reduced specificity caused by sequence degeneracy [60]. On the other hand, zinc-finger nucleases (ZFNs) are chimeric nucleases made of zinc-finger protein (ZFP) motifs, the most common DNA-binding motif in humans, each able to recognise a short DNA sequence (3–4 bp), therefore put together to increase their DNA recognition ability [60,63]. The main advantage of this technology is its extensive targeting ability, which is achieved thanks to motif engineering by site-directed mutagenesis and rational design or combinatorial library selection [64]. The result is a zinc-finger protein DNA-binding domain fused to the *FokI* endonuclease to make ZFNs [65]. Cleavage activity is only induced when two ZFNs recognise their target sequence, usually separated by a spacer region of 5–7 bp, forming a catalytically active heterodimer complex [65,60,66]. TALENs are another family of chimeric nucleases with a DNA-binding domain and a *FokI* nuclease. The DNA-binding domain differs from the ZFNs, consisting of an array of TALE repeats and 34 amino acid proteins whose residues 12 and 13 determine DNA specificity [67,64]. Specificity is again controlled by a heterodimerisation event, making the *FokI* nucleases of the DNA-bond TALEN monomers pair catalytically active. Target specificity customisation is, in this case, easier than for ZFNs, as TALE repeats can be combined to theoretically target any sequence [68]. An additional advancement is the development of the hybrid nucleases megaTALs, consisting of the easily engineerable TALE DNA-binding domain and the meganuclease site-specific head, active as a monomer, characterised by higher specificity, affinity, and cellular delivery because of its small size (approx. 75 kDa) [69].

When applied in the context of SCD, these nuclease-based strategies are being used in several studies. One such example is the PRECIZN-1 phase I/II clinical trial (NCT03653247) launched by Sangamo Therapeutics, wherein ZFNs were used to disrupt the *BCL11A* erythroid-specific enhancer in autologous hematopoietic stem cells (HSCs). Out of the 8 patients mobilised, a sufficient amount of CD4+ cells were obtained from 5 patients aged 18–35. A successful increase in therapeutic levels of HbF was seen for three out of four patients who received the therapy [70,71]. Similarly, a TALEN-based pre-clinical study observed up to 70% of homology-directed repair (HDR)-mediated *HBB* gene correction in patient HSPCs, while only 20% indels were seen [72]. Therefore, nuclease-based gene editing technologies have great potential for treating SCD, although more data is needed to evaluate its safety and efficacy.

5.1. CRISPR-Cas9 editing

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a recent breakthrough in the field of genetics, revolutionising the way scientists approach gene editing and molecular therapeutics. Initially observed as a peculiar repetitive pattern in bacterial DNA, it was later understood as a form of immune defence against viruses [73]. The CRISPR system is essentially a natural tool bacteria use to protect themselves by 'remembering' parts of invading viral DNA and then targeting it if it enters the bacteria a second time [74]. This discovery has been adapted for use in genetic engineering, enabling targeted modifications in various organisms with high precision. The CRISPR system's ease of use, accuracy, and efficiency have made it a pivotal tool in modern genetic research, driving the development of new gene therapies [75].

The CRISPR-Cas system, first discovered in 1987, serves as an adaptive immune system found in approximately 40% of bacteria and 90% of archaea [76,77]. This system plays a crucial role in creating cellular memory that offers protection against foreign organisms [78]. It consists of a CRISPR locus that includes short repetitive elements known

as repeats, separated by unique sequences called spacers. These CRISPR repeat-spacer arrays undergo transcription to produce CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) [79,80]. Additionally, the system includes a set of CRISPR-associated (*cas*) genes that encode Cas proteins. These proteins play a role in flanking the A-T rich leader sequence preceding the CRISPR array [81]. The typical mechanism of action for the CRISPR-Cas system involves adaptation or integration of spacers, biogenesis of crRNA, and interference of DNA or RNA [82]. CRISPR-Cas systems are classified into two main classes based on their Cas gene structure. Class 1, encompassing Types I, III, and IV, operates with multiple Cas proteins and is more complex. In contrast, Class 2, including Types II, V, and VI, primarily relies on a single Cas protein for its function, making it structurally simpler [83]. Among these classes, the CRISPR-Cas9 system (type IIA) is most commonly used in gene editing. It comprises two main components: single guide RNA (sgRNA) and Cas9 protein [84]. The sgRNA comprises two RNA components: crRNA [85] and tracrRNA [86], which, when fused, can target nearly any gene sequence. The Cas9 protein is a 160 kDa DNA endonuclease responsible for cleaving the target DNA and forming a double-stranded break. It has two lobes: the recognition (REC) lobe involved in binding the sgRNA and the nuclease (NUC) lobe, which plays a role in cutting DNA and conferring specificity for the protospacer adjacent motif (PAM), a short (2–6 bp) DNA sequence found downstream of the target sequence [87]. In the original bacterial Cas9 system (*S. pyogenes* Cas9 or SpCas9), the PAM sequence plays a crucial role, enabling specific recognition of viral DNA integrated into the host bacterial genome, characterised by an NGG PAM sequence, allowing to distinguish the viral DNA from the CRISPR locus [84].

During gene editing, the CRISPR-Cas9 system follows the recognition, cleavage, and repair process. The CRISPR locus is initially transcribed into a long RNA molecule known as pre-crRNA. Next, the tracrRNA hybridises with the repeat part of the pre-crRNA, resulting in the cleavage within the repeat by RNase III polymerase [86,88]. As a result, mature crRNAs are formed [89]. Cas9 then cleaves and forms double-strand breaks (DSBs) three base pairs upstream of the PAM sequence by using HNH and RuvC domains for cleaving the complimentary and non-complimentary strands, respectively [84,90]. The resultant DSBs are repaired by two mechanisms, namely non-homologous end joining (NHEJ) and HDR pathways. In the NHEJ pathway, which is the dominant repair mechanism, short insertions or deletions (indels) are incorporated, resulting in frameshift or exon-skipping mutations and causing the target sequence to be disrupted [91]. On the other hand, the HDR pathway works by utilization of a DNA donor template that is homologous to the target sequence. Due to HDR's precision in inserting or removing genes, it is widely used in CRISPR-Cas9 gene editing [92]. Nevertheless, it is important to note that HDR occurs less frequently than NHEJ, which can be attributed to various factors. These include: i) NHEJ functioning throughout the cell cycle except during mitosis, while HDR exclusively operates during the S and G2 phases; ii) the swifter repair of DSBs by NHEJ compared to HDR; and iii) the inhibitory effect of NHEJ on HDR, collectively diminishing the editing efficiency of HDR 90 [93].

The Cas12a (also known as Cpf1) protein, a part of the Type V-A CRISPR-Cas9 systems, offers unique features distinct from the more commonly known Cas9 [94]. Unlike Cas9, Cas12a can process its own crRNA due to its RNase site, eliminating the need for tracrRNA. This makes Cas12a a dual-function protein with both endoribonuclease and endonuclease activities [95]. A significant aspect of Cas12a is its recognition of specific PAM sequences (TTTV) and its ability to create staggered DNA breaks downstream of these sites. These breaks result in overhanging ends, which are advantageous for precise gene insertion [96]. Cas12a is noted for its enhanced activity, greater specificity, and reduced off-target effects compared to Cas9 [97]. More recently a new Cas12m base editor has been developed GoCas12, that lacks any DNA cleavage activity, minimizing the possibility of DSBs and large genomic rearrangements [98]. However, the choice between Cas12a and Cas9 should be based on the specific requirements of the gene editing task, as each system has its own strengths and applications [96]. Some relevant CRISPR-Cas variants mentioned here been summarised in Table 2.

The CRISPR-Cas9 system has been utilized to treat SCD using two strategies: i) correcting the mutation to the wild type allele such that the cells express higher levels of HbA, rescuing the SCD phenotype; and ii) induction of HbF production by disrupting gene regulatory elements, therefore decreasing sickling and improving quality of life (Fig. 2a). A 2016 study used Cas9 ribonucleoproteins (RNPs) consisting of the Cas9 protein and single guide RNA and HDR donor template delivered by adeno-associated viral (AAV) vectors in CD34+ HSPCs, achieving promising HDR-mediated gene editing frequencies at the *HBB* locus [112]. Similarly, other studies have shown the *ex vivo* correction of the SCD mutation in CD34+ HSPCs with varying efficiencies, ranging between 18% (Hoban, Lumaquin et al. 2016), 25% [113], 60% [114], 64% [99] and even 94% [115].

In recent years, emphasis has been on inducing high levels of HbF in SCD patients by either regulating transcriptional repressors or generating hereditary persistence of fetal haemoglobin (HPFH) associated mutations. HbF expression and repression is a complex process governed by a multitude of genes and can function through different independent pathways [116,117]. Multiple studies have shown favourable efficiency and safety of increasing γ-globin by disrupting the *BCL11A* gene ([118], Wu, Zeng et al. 2019). On the other hand, CRISPR-Cas9 has also been used to disrupt a 13.6 kb region consisting of δ- and β-globin genes and a putative γ-δ intergenic HbF silencer, which resulted in reactivation of HbF synthesis in erythroblasts and overall amelioration of the SCD phenotype [119]. Disruption of promoter regions for the *HGB1* and *HGB2* genes (which make the two subunits of γ-globin), significantly reduces the ability of transcriptional repressors from binding, therefore increasing the expression of HbF [120,121]. Interestingly, a study comparing the different CRISPR-Cas9 strategies to induce HbF and found that knocking down genes such as *KLF1* and *BCL11A* is a much more clinically relevant approach than disrupting transcription factor binding sites such as *HBG1* and *HBG2*, even though all methods resulted in therapeutic levels of HbF expression [122].

A phase II/III clinical trial (NCT03745287) targeting the *BCL11A* erythroid-specific enhancer in CD34+ HSPCs through CRISPR-Cas9 editing (CTX001) has achieved approximately 80% modification of the target locus, which, when transplanted into SCD patients, showed an increase in HbF levels and reduction of vaso-occlusive episodes (no recorded episodes for a minimum of 12 continuous months within a 24-month follow-up period). Additionally, no graft failure rejection occurred, indicating successful engraftments for all patients. These promising results encouraged the clinical use of an *ex vivo*, autologous gene edited HSPCs for patients suffering from β-thalassemia and SCD [123].

On the 8th of December 2023, the FDA approved this strategy for SCD as the world's first CRISPR-Cas9 gene editing therapy, called Casgevy, developed by Vertex Pharmaceuticals and CRISPR Therapeutics (Table 1). Casgevy is a one-time therapy administered to SCD patients aged 12 years with frequent vaso-occlusive crises, utilized as part of a hematopoietic stem cell transplant [124]. A 15-year multi-site safety study (NCT04208529) started in 2021, on a cohort of paediatric and adult patients from previous CTX001 clinical trials (NCT03745287), including transfusion-dependent β-thalassemia and severe sickle cell disease studies. This trial aims to investigate the long-term effects of Casgevy, and so far no DSB related genotoxicities have been observed [125]. The approval of Casgevy represents a significant milestone in the field of gene therapy and editing as the first of its kind. Addressing both SCD and β-thalassemia, it underscores the potential of the CRISPR-Cas9 technology in treating genetic disorders, paving the way for future developments and applications in precision medicine.

Other Crispr-editing targets include the HBG1/2 genes, with the strategy being the disruption of the promoter region by CRISPR-Cas12a (EDIT-301), as tested in the RUBY clinical trial (NCT04853576). In the

Table 2
Different versions of gene editing technologies.

Gene editing technology		Advantages	Version	Modifications	Reference
Crispr-Cas Systems		Specific gene sequence editing by NHEJ (gene disruption) or HDR (gene modification using DNA template)	Crispr-Cas9	Original CRISPR-Cas technology, based on the Cas9 nuclease	[84]
			Cas9-HF1	Single-point DNA change to reduce off-target effects	[99]
			Cas9n	Conversion of Cas9 nuclease into a DNA nickase, which only cuts one DNA strand to favour high-fidelity homologous directed repair (HDR)	[100]
			CRISPR-PLUS	Fusion of SpCas9 with ReJ exonuclease or GFP to increase editing efficiency	[101]
			Crispr-Cas12a	Based on the Cas12a nuclease. It allows editing of AT-rich regions, and only requires crRNA,resulting in precise insertions	[94]
Base Editors	Adenine base editors (ABEs)	Allows for A•T to G•C transitions	ABE7.10	First ABE version found to be suitable for gene editing. Strategy based on the activity of an adenosine deaminase fused to a catalytically impaired Cas9 (dCas9) to or Cas9n	[102,103]
			ABEmax	Made by modification of the Nuclear Localization Signal (NLS) of ABE7.10 by ancestral recognition to increase editing efficiency	[104]
			ABE8e	Modified eight amino acids in the TadA* domain of ABE7.10 to obtain higher editing efficiency	[105]
			ABE8e-NRCH	Based on a modified SpCas9 that allows for the recognition of the NRCH PAM sequence	[106]
	Cytosine base editors (CBEs)	Allows for C•G to T•A transitions	–	Strategy based on the activity of a cytosine deaminase fused to a catalytically impaired Cas9 (dCas9) or Cas9 nickase	[107]
	Glycosylase base editors	Allows for C•G to A•T and C•G to G•C transversions	–	Base editing strategy based on the fusion of a Cas9 nickase, a cytidine deaminase and a uracil-DNA glycosylase	[108]
	Adenine transversion base editors	Allows for A•T to C•G and A•T to T•A transversions	–	Base editing strategy based on the fusion of an adenine base editor with an *N*-methylpurine DNA glycosylase	[109]
Prime Editors		Allows for precise modifications of the DNA without causing double-strand breaks	PE1	The original prime editor made of M-MLV-RT reverse transcriptase fused with Cas9 and pegRNA	[110]
			PE2	Six single nucleotide changes in the M-MLV-RT gene to improve editing efficiency	
			PE3	The addition of a gRNA to prompt Cas9 to nick the non-edited strand results in the utilization of the edited strand as a template for mismatch repair	
			PE3b	A more careful gRNA design guarantees that mismatch repair follows the editing process, reducing the risk of indel formation	
			PEmax	Improved prime editing system, based on the modification of the PE2 protein	
			PE4	Made of a prime editor enzyme (nickase Cas9–RT fusion), MLH1dn, and pegRNA. The system inhibits mismatch repair to improve the likelihood of retention of edits	[111]
			PE5	Made of a prime editor enzyme, MLH1dn, pegRNA, and nicking sgRNA	
			PE5max	Characterised by the stabilisation of pegRNA and preventing cellular degradation to increase editing efficiency	

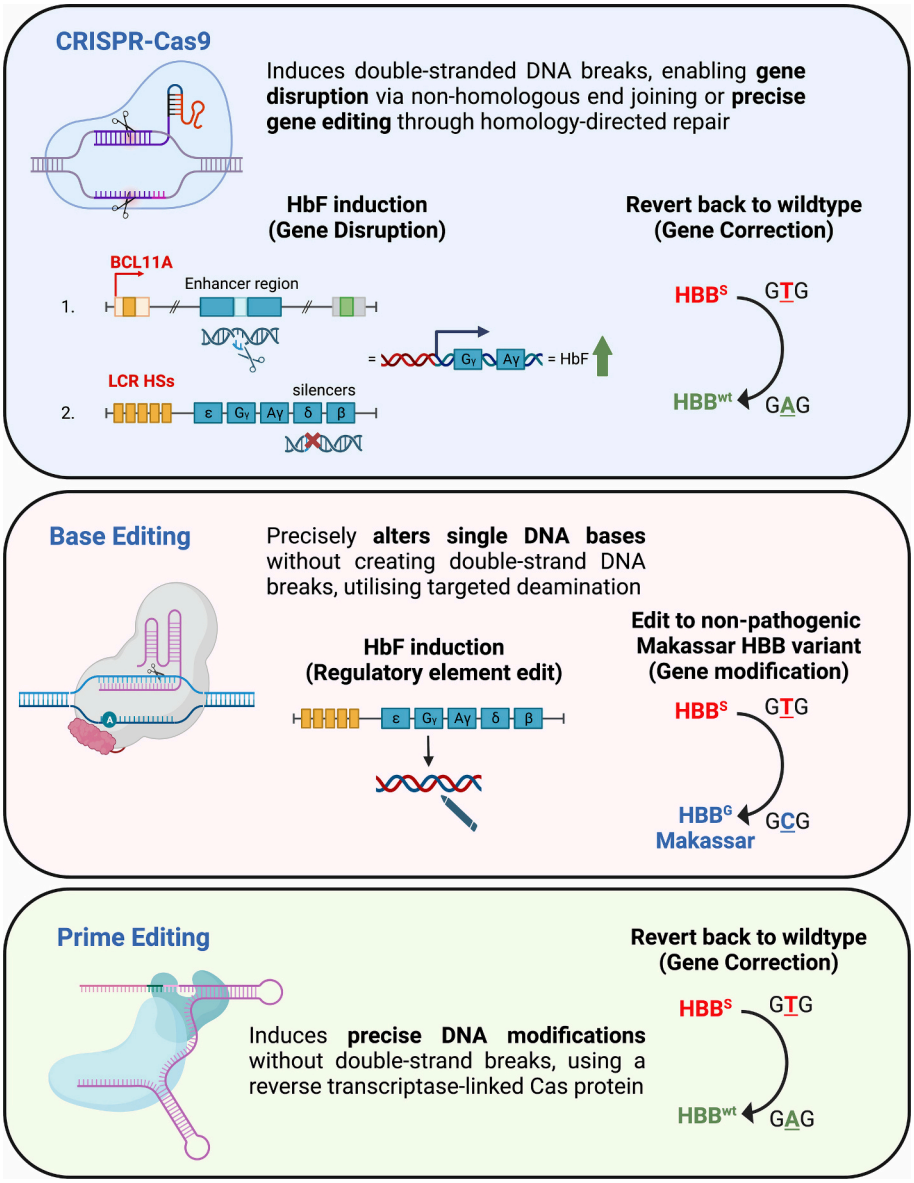

Fig. 2. Gene Editing technologies for SCD: CRISPR-Cas9, Base Editing, and Prime Editing a) CRISPR-Cas9 can be used to either correct the mutation to the wild type or induce fetal haemoglobin (HbF) expression by inducing a change in the *BCL11A* erythroid-specific enhancer or deleting part of the HbF silencers which expression is controlled by LCR hypersensitive sites . b**)** Adenine Base Editor can edit the regulatory elements controlling HbF expression or convert HBBS to a Makassar non-pathogenic variant HBBG. **c)** Prime Editing can be used to correct the HBB mutation back to the wild type without the need for double-stranded breaks.

pre-clinical data, EDIT-301 has demonstrated high efficiency (over 70%) in gene editing, resulting in long-term *in vivo* induction of HbF (40% increase) [126]. Results from the first four patients included in the mentioned phase I, II clinical trial align with the pre-clinical data [127]. However, additional patient recruitment is necessary to establish more robust evidence regarding the efficacy and safety of this strategy. Finally, the CEDAR trial (NCT04819841) utilises CRISPR-Cas9 gene editing to correct the *HBB* mutation and restore the HbA production in erythrocytes, as they have previously shown in their pre-clinical study [114]. Unfortunately, the *ex vivo* strategy used, GPH-101 (nulabeglogene autogedtemcel or nula-cel), caused pancytopenia, prolonged low blood cell counts, to the first administered patients, leading to trial suspension in January 2023 and eventually early termination [128].

5.2. Base editing

In recent years, significant progress has been made in the development of Base Editors (BEs), which can convert specific base pairs into desired forms without creating DSBs and relying on HDR-mediated correction utilizing a donor DNA template [102,107]. As a result, researchers have been exploring their potential for correcting point mutations in various organisms such as animals, plants, bacteria, and human embryos. A typical base editor consists of two key components: a catalytically impaired or "dead" (dCas9) or nickase Cas9 nuclease responsible for programmable DNA binding and a single-stranded DNA (ssDNA) modifying enzyme that targets and modifies nucleotide bases in DNA and RNA sequences [107]. DNA base editors can be broadly classified into two categories: cytosine base editors (CBEs), which convert C•G to T•A, and adenine base editors (ABEs), which convert A•T to G•C base pairs (Table 2). Together, these two types enable four transitions: A to G, T to C, C to T and G to A. The molecular mechanism underlying base editing involves Cas9 identifying a region near the PAM site in the sgRNA, forming a Cas9-sgRNA complex. Subsequently, an R-loop is formed as the Cas9-sgRNA complex invades the DNA strands. This exposes a small window of single-stranded nucleotides on the complementary strand, allowing the BE to deaminate the target base, which,

through mismatch repair, gets converted to desired bases [129,107,130,131].

Base editing offers several advantages, making it a promising gene editing tool. One key advantage is that it does not generate DSBs, which makes it safer and more efficient, especially in non-dividing cells [132]. Additionally, base editing has minimal off-target effects, reducing the risk of unintended genetic alterations at off-target sites. Another advantage is that base editing does not require DNA donor templates, allowing the use of mRNA or RNP editing tools [133,134]. However, when designing experiments, one also needs to consider certain disadvantages associated with base editing. The major limitation is the restricted range of possible base pair conversions with current base editors. To overcome this limitation, the development of glycosylase base editors (C to A and C to G) and adenine transversion base editors (A to C and A to T), which incorporate additional enzyme components to expand the range of targetable base pairs, is being carried out **(**Table 2**)** [109,108]. Another drawback is the possibility of bystander mutations occurring at sites near the intended editing window, which could be approximately five base pairs away from the window [102,107,103,135].

Numerous approaches have been used for treating SCD using base editing, namely: i) correcting the mutation present in the *HBB* gene; ii) increasing the HbF levels by either regulating transcriptional repressors (*BCL11A* and *LRF*); or iii) creating binding sites for transcriptional activators (*GATA1*, *KLF1* and *TAL1*) [136] (Fig. 2b). One such *ex vivo* study demonstrated 80% correction rate in *HBB* gene of HSPCs, while maintaining significant rates when transplanted into mice [4]. Similarly, several studies have been successful in creating *de novo* binding sites for *GATA1*, *KLF1* and *TAL1* by causing −113 A > G, −198 T > C, and 175 A > G *HPFH* mutations, respectively [136,137]. Moreover, two clinical trials have been launched by Beam Therapeutics, wherein *ex vivo* delivery of base editors is to treat SCD. BEAM-101 (NCT05456880) is a phase I/II clinical trial aiming to activate HbF expression by targeting *HBG1/2* promoters [138]. On the other hand, BEAM-102, which is based on preclinical work aiming to change the point mutation (HBBS) in the *HBB* gene to the Makassar non-pathogenic variant (HBBG) [139], is yet to be launched. Despite having certain limitations as a gene editing approach, base editing shows excellent potential as a valuable tool for addressing SCD, as evidenced by the high editing efficiencies achieved and movement towards using it in a clinical context.

5.3. Prime editing

Prime Editing (PE) is a new gene editing technology developed in 2019, which represents an advancement in gene editing strategies aiming to overcome the limitations associated with other editing systems. This new system relied on the PE2 component, a Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) fused to the C terminus of Cas9 (H840A) nickase, to perform a single strand break (SSB) three bases upstream of the PAM site. The real innovation is in the guide RNA, called pegRNA, made of the classical gRNA fragment directing the system to the target DNA and an edit-containing RNA template extension, containing the genetic information that will replace the target DNA [110]. The working mechanism of PE involves the formation of SSB by PE2, leading the resultant 3′ flap of the nicked DNA strand to form a sequence-specific interaction with the primer binding site on the pegRNA. This RNA/DNA hybrid is the primer site for DNA synthesis, while the pegRNA "edit site" is used as a template by the reverse transcriptase to extend the 3′ flap. The original 5′ flap is displaced and degraded by nucleases [140]. A more efficient method called the PE3 system has since been developed, in which a standard gRNA targeting the complementary strand is co-transfected to cause H840A Cas9 to nick the non-edited strand, influencing mismatch DNA repair in favour of the edited sequence **(**Table 2**)**. However, this method increases the chances of indel formation, erasing the advances introduced by SSB-based systems. Therefore, a modified strategy (PE3b) was implemented that allows the non-edited strand nick only after complementary strand editing, thanks to careful gRNA design, resulting in a final system with improved editing efficiency and limited indel risk [110]. The applications of this gene editing system surpass the ones of the previously introduced base editing, as together with higher efficiency for single base substitutions and transversions, prime editing introduces the possibility of producing small deletions and insertions, addressing almost 90% of disease-causing mutations [141,140]. However, it also has certain limitations, mainly the need for multiple components optimisation and careful pegRNA design [140].

Although the application of PE to correct SCD-causing mutation is a relatively new approach, one *ex vivo* study has been successful in correcting sickle *HBB* gene back to its wild type at a frequency of 15–41% by mRNA electroporation of the editing tools into HSPCs (Fig. 2c). The edited cells showed similar engraftment frequencies, hematopoietic differentiation, and lineage maturation to unedited transplanted healthy HSPCs [142]. An *in vivo* study has also been published, which showed ~40% β-globin allele correction with the help of a viral delivery system composed of a non-integrating adenovirus that targets the HSCs. Efficient target site editing accompanied by minimal indels generation and no off-target editing was also observed [143]. Therefore, prime editing can be considered a promising tool for developing treatments for SCD; however, human studies and clinical trials are to be expected in the near future to address the safety concerns that are associated with any new gene editing strategy.

6. Optimal cargo and delivery method for SCD

Efficient cellular delivery is a fundamental aspect of gene editing, dictated by the expression of nucleases needed to produce edits that can create positive changes in the disease phenotype [60]. Targeted nucleases can be delivered mainly as DNA, mRNA, or as proteins or RNPs [144] (Fig. 3a). Each of these cargo types comes with its set of advantages and disadvantages. Plasmid-based DNA is known for its stability, cost-effectiveness, and user-friendly nature. It also allows for multiplexed gene editing. However, the large size of plasmids can hinder the effective delivery of editing tools [145]. Additionally, they are challenging to encapsulate and require transcription before they can act on the target sequence, leading to potentially lower editing efficiencies and cell death [146].

In contrast, mRNA only requires translation before acting on the target sequence, making it much faster [147]. Nevertheless, mRNA is unstable under certain physiological conditions, which may result in inefficient editing. RNP complexes, consisting of pre-assembled sgRNA and Cas9, are considered the fastest method as they do not require transcription or translation. However, their complex distribution of surface charges can pose difficulties in their delivery with certain modes [117,148].

Similarly, the delivery mechanisms used to transport editing tools have their advantages and disadvantages. There are two main ways to deliver gene editing tools: through viral vectors (such as AAV and lentivirus, or non-viral vectors (such as electroporation, microinjection, and nanoparticles) (Fig. 3b). When using viral vectors for delivering editing components into HSCs, the vector's choice depends on the virus's ability to integrate the carrying genetic material, resulting in the transient or permanent introduction of the gene editing machinery and on the different viral vector capacities [149]. In the case of active nucleases, non-integrating vectors may be preferred to avoid insertional mutagenesis, limiting the choice to AAV, characterised by a very low storage capacity, or integrase-defective lentiviruses, with the limitation of low gene expression levels compared to their integrating counterparts [60,150]. However, the best method to achieve transient expression of nucleases in HSCs has been shown to be electroporation [151,152]. In addition, electroporation of mRNA has been related to a reduced risk of integration and cellular toxicity compared to DNA [60]. Electroporation works by applying an electric field that opens transient pores in the cell

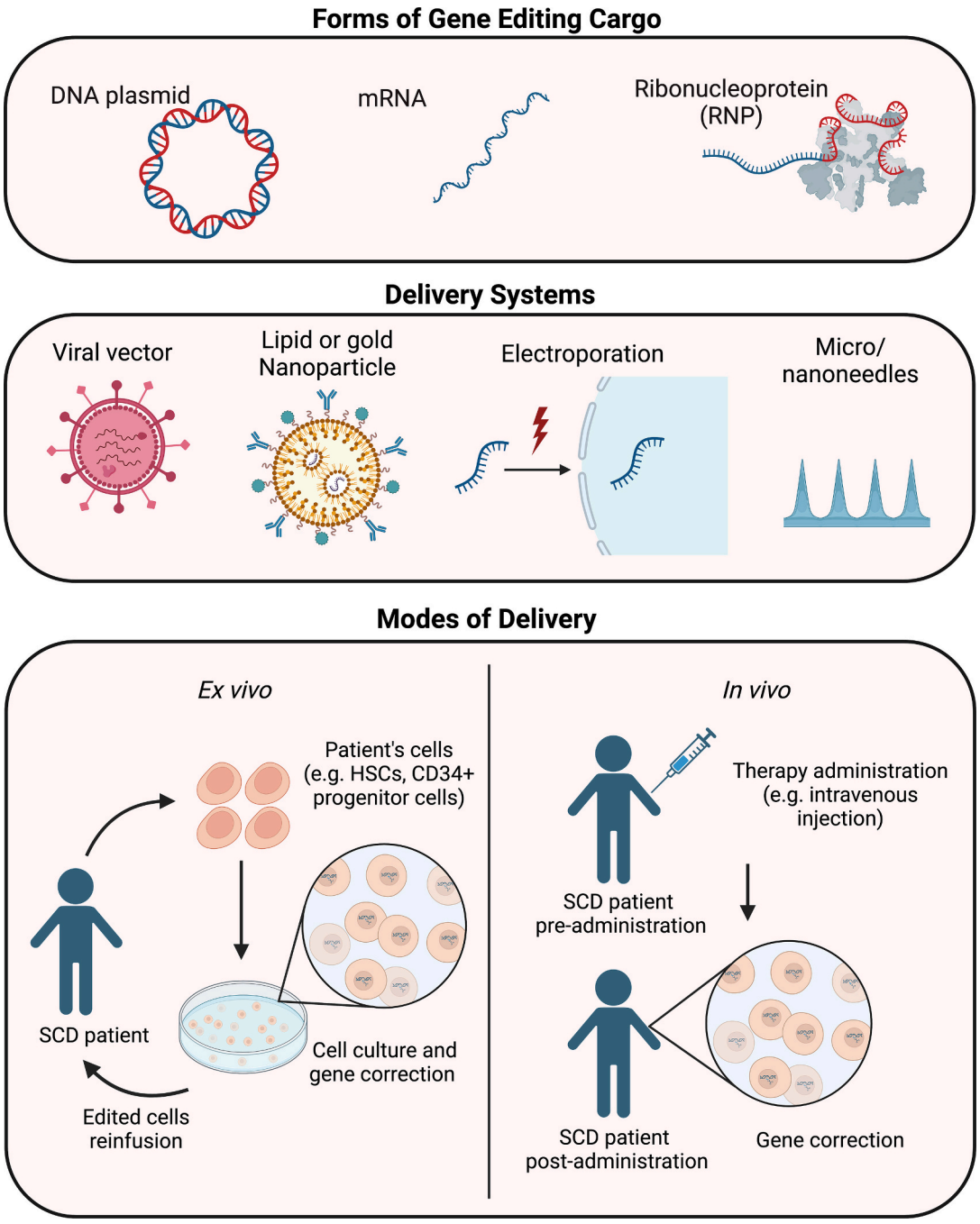

Fig. 3. Gene Editing: delivery methods. Schematic showing different aspects of gene editing delivery method that can be combined to design a gene editing strategy for sickle cell disease. **a)** Forms of gene editing components. Different forms in which targeted nucleases can be delivered . b) Delivery Systems. Different ways to deliver targeted nucleases, guide RNAs (sgRNA, gRNA, pegRNA) and donor templates. **c)** Modes of delivery. *Ex vivo* and *in vivo* strategies.

membrane, facilitating the cell's uptake of gene editing tools [153]. It has become a popular choice for *ex vivo* and *in vitro* gene editing due to its efficient cargo delivery and applicability to various cell types [154]. Although electroporators are being used to efficiently *in vivo* gene edit in animals [155,156], their use in humans is limited due to technical challenges of targeting cell in the human body, delivery of large cargos into the target cell, and increased cell death, particularly in sensitive cell types [157].

Microinjection, a non-viral vector, overcomes some of the limitations associated with viral vectors as it involves direct injection of the editing tools into cells using a needle. This allows for controlled dosing. Nevertheless, microinjection is labour-intensive and challenging to implement [158]. On the other hand, lipid or gold nanoparticles can safely deliver the editing tools without exerting the same stress on the cells as electroporation does and without the chance of integrating into the genome like viral vectors [159,160]. However, they are associated with lower editing efficiencies than other delivery methods and a potential for higher levels of toxicity, especially lipid nanoparticles, depending on the lipid formulation [161].

Additionally, the choice of an *ex vivo* or *in vivo* approach influences the delivery method of the editing tools (Fig. 3c). Many studies propose *ex vivo* gene editing approaches to treat SCD, possible thanks to the availability of HSCs, erythroid progenitor cells, or the recent development of iPSCs, pluripotent cells artificially derived from somatic cells.

However, gene editing can also be performed *in vivo*, directly introducing gene-editing components to the patient instead of transplanting edited cells. However, the main limitation of *in vivo* gene editing is the risk of developing immune responses against the targeted nucleases, which becomes problematic if a second gene editing component administration is needed [60]. The most promising *in vivo* delivery methods are AAV and lipid nanoparticle (LNP). Biological and molecular barriers must be overcome by *in vivo* delivery vehicles that can protect the cargo before entering the cells, bind the target cells, pass through the cell membrane to reach the cytoplasm and release the cargo to the appropriate cellular compartment (*e.g.* nucleus) [144]. When targeting HSCs, lentiviral and non-lentiviral vectors are efficient delivery methods following intraosseous or intravenous administration [60].

Overall, it is essential to keep in mind that the required efficiency is dependent on the target cell type and related gene delivery method; an example is HSCs requiring electroporation as the delivery system [152], and on the form of the delivered gene editor. The different forms of delivery correlate to different gene editing efficiency and insertional mutagenesis risk; costs and safety profiles in the case of *in vivo* approaches, and have to be carefully considered when choosing the appropriate strategy [162].

7. Limitations of gene editing

7.1. Editing efficiency

Gene editing efficiency is essential when designing a therapeutic strategy based on gene correction, as a specific threshold correlates with clinical value. For HSPCs SCD gene-correction *in vitro*, efficiencies ranging from 7 to 50% have been obtained, depending on the gene editing technology used and delivery method, with 50% editing efficiency resulting in a clinically relevant amount of HbA [99]. Methods to improve editing efficiency mainly focus on guide RNA design and gene editing protein optimisation.

7.2. Guide RNA design

Many tools are available when designing gRNAs for CRISPR-Cas9, base or prime editing. CHOPCHOP and CRISPOR are the tools used for CRISPR sgRNA design [163,118,164]. For base editing, BE-designer and BEditor are some of the tools used [165,166]. CRISPick (Broad Institute) can predict the off-target editing derived from sgRNA, allowing for selection of RNA molecules with the best safety profile [167]. Fewer tools are currently available for pegRNA design for prime editing, mainly Prime Design and PnB Designer are available to guide optimal sequence design [168,169].

7.3. Gene editing protein optimisation

Editing protein optimisation to improve efficiency has expanded and keeps expanding the use of gene editing to treat a broader range of diseases. Between the gene-editing proteins, base editors are probably the most fast-evolving technologies. ABE evolution produced advantages for SCD gene editing. The latest optimisation aimed at overcoming the limitation of ABE7.10 SpCas9 base editor, the latest of seven versions introduced by ABE developers in the same paper [102], requiring an NGG PAM sequences approximately 15 bp from the target nucleotides condition met by few pathogenic point mutations [105]. A phage-assisted continuous evolution (PACE) selection system was used to develop a new protein compatible with many Cas homologs, resulting in ABE8e, an evolved ABE with significantly higher editing efficiency [105].

Similarly, using a new SpCas9 variant, ABE8e was modified, causing it to recognise a different PAM site (NRCH *vs* NGG). This technology has subsequently been used in a mouse study to convert the SCD allele (HBBS) to Makassar β-globin (HBBG), a non-pathogenic *HBB* variant, by performing gene editing *ex vivo* on human HSPCs progenitor cells, then transplanting into SCD mouse models [106,4]. A different optimisation route based on the ancestral reconstruction of the deaminase component showed promising results in activating HbF expression in HUDEP-2 cells [170,104]. For CRISPR-Cas9, the optimisation process led to the development of CRISPR-PLUS, fusion SpCas9 proteins with exonuclease Recombination J (RecJ) or with GFP, characterised by high editing efficiency (up to 600%) [101]. This enhanced CRISPR-Cas9 variant has not yet been implemented for gene editing studies. However, the positive results obtained by the developers on cultured cell lines and primary human cells are evidence of their suitability to be used as part of therapeutic strategies for various conditions, including SCD. Prime Editing evolution started from the mentioned changes, culminating in the development of PE3b [110]. Another attempt to improve editing efficiency resulted in a new version called PEmax, characterised by higher indels [111]. Another strategy is that of inhibiting the mismatch repair system to improve the likelihood of the induced edit to be retained, obtained by the development of PE4, which was then combined with PE3 to obtain PE5, characterised by up to 2.5-fold increased editing efficiency over PE3 [111]. A final effort has been made to stabilise pegRNA, preventing intracellular degradation [171], which, in combination with PE5max, resulted in a 12-fold increase in efficiency compared to PE3 [111]. Finally, nuclease specificity and editing efficiency can be tested by assays relying on green fluorescent protein trapping, allowing the selection of edited cells and identifying the nuclease-mediated cleavage sites suitable for SCD studies [172].

7.4. Off target effects

Off-target effects are a known limitation of gene editing, always taken into consideration during the process of strategy design, and that can be limited thanks to prediction tools and experimental methods.

7.5. Prediction tools

In silico prediction relies on bioinformatic algorithms developed through the years. One mentioned example is CRISPOR, based on the 'cutting-frequency determination' (CFD) algorithm, which results in a ranked list of likely off-target sites [163], which has been used by multiple studies [99,173]. However, Elevation and CRISTA tools seem to be the most precise in predicting off-target effects [174,175,176]. Another algorithm that can identify off-target sites is Cas-OFFinder [177], used to identify PAM genomic sites containing mismatches to the target protospacer, such as in the recent SCD base editing [4]. Analysis of amplified genomic regions obtained usually follows, for which Sanger-based methods can be initially used to investigate whether there are changes to the top predicted off-sites. Examples are TIDE [178], ICE [179], and bioinformatic tools to track indels and non-indels off-target events in SCD research [122,115,180,173]. However, for in-depth off-target detection, NGS is more suitable as it has greater coverage compared to Sanger sequencing and can overcome the limitation of the lower sensitivity to detect low-frequency mutations, for example, in the gene editing verification and off-target events analysis of base editing to treat SCD [4].

7.6. Crispr-Cas9 system design

Nucleases and guide RNA design can limit off-target effects, similar to improving their editing efficiency. Another significant advance in sgRNA design is the higher flexibility obtained using Cas9 orthologs recognising different PAM sites downstream of the sgRNA, as mentioned for SpCas9-NRCH [106]. Among the different Cas9 designs, High-fidelity Cas9 (Cas9-HF1) is one of the most specific nucleases that significantly limit off-target editing [99]. SpCas9 is an example of High-fidelity Cas9 that showed evidence of maintaining high gene editing efficiency while reducing off-target events and is also used on HSPCs to

correct the SCD mutation [181]. Another option is to create separate single-strand nicks in each DNA strand using Cas9n nuclease, resulting in high specificity [100]. Delivery methods are the last parameter influencing off-target editing as the different forms of delivery vary in the rate at which they are degraded, resulting in higher off-target activity for the slow-degrading plasmids or viral vectors, compared to mRNA and RNP delivery [182,183].

7.7. Additional off-target events for base and prime editors

Most prediction tools have been designed based on the need to develop a safe gene editing strategy using CRISPR-Cas9. However, as Base and Prime Editors are becoming more and more efficient and clinically used techniques, prediction methods have to be accustomed to their mode of action. Despite sharing the exact mechanism of Cas9-dependent off-target mutations, prediction tools often fail to completely identify Base Editors' off-target events [184,185]. Cas9-independent off-target modifications are an additional cause of off-target events related to deaminases [182]. Two primary recent studies analysed Cas9-independent off-target modifications, observing that most sgRNA-independent off-target editing is to be attributed to Cytosine base editors (CBEs) only, additionally proving the suitability of some new methods to control independent off-target mutations [186,187].

On the other hand, sgRNA-dependent editing, including ABEs, can be predicted by the Endo-Digenome-seq21 and EndoV-seq tools [188]. Ongoing research is focusing on finding a method to efficiently characterise sgRNA-independent off-target editing [182], other than the expensive and time-consuming Whole-genome sequencing (WGS) [189]. Fortunately, gene editing strategies to treat sickle cell disease are not slowed down by this limitation as they mainly rely on ABEs. Still, another limitation involves ABEs: the deamination of RNA molecules nucleotides independently of DNA changes, threatening to cause variations in gene expression and splicing [190,191,192]. To overcome this limitation, modified deaminases have been developed for ABEs, SECURE-ABE is worth mentioning [193]. The transcriptomic editing profile of base editors was further analysed, and ABE variants were characterised by causing fewer RNA changes to develop [194]. A second approach involves modifying sgRNAs to reduce off-target activity, such as designing bubble hairpin sgRNAs [195]. Unfortunately, bystander editing is another mechanism characteristic of base editors and causes off-target events. This term indicates the aberrant conversion of nucleotides adjacent to and of the same type of the target mutation [196]. Detecting these events is easy using NGS, as they only happen within or a couple of bases away from the editing window of base editors. In addition, a machine learning model called BeHIVE was developed to predict the degree of bystander editing [197]. It can be concluded that reducing off-target editing of base editing results in trickier overall results, suggesting the need for a comprehensive off-target analysis before pushing base editing strategies for SCD to the clinic. Reducing base editors' exposure has been shown to be an effective means of reducing gRNA-dependent off-target events [198]. In this case, high editing efficiency becomes fundamental to obtaining a working therapeutic strategy, especially considering the higher concentrations of base editor needed to achieve in-target editing on HSCs compared to Cas9 RNPs [35]. As the most recently developed technology, fewer data on Prime Editing off-target events is available. nDigenome-seq analysis suggests the high accuracy of this tool [199]; however, PE3 pegRNA-independent off-target activity has been recently detected [200].

The mentioned studies highlight how reducing off-target editing is essential to move genome editing-based SCD treatment into clinical practice. Despite this joint effort, the results are not yet ideal, as off-target mutations can only be introduced in a minimal number of cells without transplanting in safety issues for patients. Even so, clinical trials for sickle cell disease are ongoing and show promising safety results to date.

8. Animal models

Animal models play a crucial role in therapeutic research, as they help researchers better understand diseases' pathophysiology and, more importantly, investigate the efficacy and safety of potential therapeutics in preclinical studies.

8.1. Mouse model

Often, genetic similarity and phenotypic disease expression comparable to the human condition are the criteria determining the choice of animal model; in the case of sickle cell disease, the absence of a natural disease animal model led to the development of transgenic mice that allowed disease investigations: Berkeley and Townes SCD mice [201]. These two models succeeded in solving the issue of preventing viable anaemic mouse models from being obtained. The advance of the Berkeley and Townes mouse models is in modifying γ-globin gene regulatory sequences. Berkeley model is characterised by genetic disruption of endogenous adult α- and β-globin genes and induction of human globin gene expression *via* three DNA transgenes: one including the human α-globin gene, a second containing γ-globin, δ-globin and sickle β-globin genes, and the last one being a 'mini-locus control region (LCR)' [202]. The Townes mouse was obtained using homologous recombination to replace adult mouse α-globin genes with the human homologue and mouse β-globin genes with segments of the human γ-globin and β-globin genes, obtaining an LCR transgene designed to induce haemoglobin switch after birth. The result is an animal that is healthy at birth and develops severe anaemia after the switch [203]. These two mouse models have been widely used to study gene editing on HSCs to treat sickle cell disease [4,204] and have been further modified to simulate different disease conditions such as sickle cell trait, the heterozygous genotype characterised by only one mutated allele [205]. However, data on the structure and regulation of the human globin genes characterising these mouse strains is not easy to access, as their development dates to 25 and 15 years ago. The knowledge gap on genome manipulation and regulation has not yet been addressed by modern researchers, leaving some uncertainty on the possible limitations of these animal models in preclinical studies [206]. Some light on the matter has been recently offered by Woodard et al., who performed physical and functional characterisation of the human globin transgenes of Berkeley and Townes mouse models and highlighted the unsuitability of their genomic structures in mimicking the human developmental regulation of β-like globin gene expression [206].

Furthermore, studies on achieving therapeutic levels of HbF in the Berkely mouse model have encountered challenges. One key issue is the inconsistency and uncertainty in the results of these studies. In particular, the application of genome editing techniques, especially those involving the Cas9 system applied to these mice, often leads to unintended and problematic side effects, such as triggering a strong DNA damage response and subsequent cell death. This adverse effect is attributed to the accumulation of numerous DSBs within the genome. On a contrasting note, studies involving Townes mice have revealed suboptimal levels of HbF, potentially arising from the absence of critical cis-acting distant DNA elements responsible for the regulation of globin genes in this particular model [4,207,208,204,206].

An alternative to transgenic mouse models is the use of NBSGW immunodeficient mice, a mouse model that allows engraftment of human HSCs in the absence of irradiation [209], allowing to study for example, the effects of Cas9-mediated HbF induction in xenotransplanted human HSCs [210] or the ability of *HBB* gene-corrected SCD patient CD34+ cells to repopulate the mouse bone marrow [4]. However, the rapid clearance of human RBCs from the mouse circulation keeps this approach used to analyse long-term therapeutic effects [211].

8.2. Pig model

A final model to mention is the recently developed Yucatan miniature pig model, which promises to offer a more accurate model characterised by higher similarity in anatomy and physiology to humans than mice, useful for pathophysiological and translational studies. This animal model expresses human α-, β- and γ-globin genes under the control of the respective endogenous porcine LCR, similar to the knock-in Townes mouse model, and was obtained using somatic cell nuclear transfer (SCNT). The results from the first 9 months of this model are very positive and the authors have already observed sickling of RBCs. However, it should be noted that full HbF to HbS has not yet been observed and is expected to happen by 12 months of age. At this point, the HbF expressed offers a protective effect and outward signs of SCD are absent. This animal model is under a 24-month natural history study aiming at observing SCD manifestation as the animal ages, and it is still far from being used in research. However, analysis of protein expression and blood morphology is evidence of the suitability of this model for translational SCD studies [212].

8.3. Non-human primate model

Non-human primates (NHPs) and canines play a pivotal role in connecting rodents and clinical studies, given their similarities to humans regarding anatomy, physiology, and metabolism. This resemblance allows for more extended research, enabling a comprehensive assessment of the safety and effectiveness of gene editing interventions. Although specific models of SCD are not yet available, successful endeavours have been made in inducing HbF in NHP models. This success is attributed to the evolutionary conservation of γ-globin genes, which are shared between NHPs and humans [213]. In a particular study, a transplantation model involving NHP, specifically *rhesus macaques*, was established. This study utilized CRISPR-Cas9 to reproduce a 13-nucleotide deletion found in individuals with hereditary persistence of HPFH. This deletion disrupts the *BCL11A* binding site within the γ-globin promoter regions. The study successfully recapitulated the HPFH deletion, achieving up to 39% in CD34+ cells or the refined CD90+ HSC enriched subpopulation. Following this, the edited cells exhibited stable and multilineage engraftment in the NHP model, persisting for over one year [214]. Similarly, a different research study reinstated HbF expression in *rhesus macaques* by disrupting the erythroid-specific *BCL11A* enhancer using CRISPR-Cas9 editing [215,198]. Despite being in the early stages of research and demanding further in-depth studies, these findings hold the promise of becoming a significant and optimistic approach for the future treatment of SCD.

9. Future considerations

In addressing sickle cell disease (SCD), two promising strategies stand out: prenatal therapy and cord blood hematopoietic stem cell (HSC) treatments, especially utilizing CRISPR-Cas12 gene editing [216]. Non-invasive prenatal diagnostics could enable early, safer interventions, with CRISPR-Cas12 offering specificity and efficiency in gene editing. This area requires thorough ethical examination, particularly regarding consent and the long-term impact of fetal gene editing.

Cord blood HSCs present a unique dual opportunity: as a source for both allogeneic transplantation, necessitating improved collection and preservation methods, and autologous transplantation after CRISPR-Cas12 editing to correct the HBB gene mutation. Such treatments, especially autologous transplantation, need rigorous preclinical and clinical testing for safety and efficacy.

CRISPR-Cas12's potential over other gene-editing tools like Cas9 lies in its specificity and efficiency [94,216], warranting research into its application in editing the HBB gene in cord blood stem cells and exploring innovative delivery mechanisms.

Addressing the implementation of these therapies in underdeveloped regions, particularly in Africa where SCD is prevalent, highlights the need for affordable, culturally sensitive, and ethically sound approaches. This includes improving healthcare infrastructure, addressing cultural and ethical concerns, and focusing on preventive measures and early diagnosis to manage SCD effectively.

Overall, advancing SCD therapy through these avenues requires comprehensive research, ethical diligence, and collaborative interdisciplinary efforts to ensure safe, effective, and accessible treatments.

Practice points

1. **Early Diagnosis**: Emphasize early detection through non-invasive prenatal testing to enable prompt intervention.
2. **Informed Consent**: Ensure clear informed consent protocols for prenatal therapies, outlining all potential risks and benefits.
3. **Safety Protocols**: Implement strict safety measures for gene editing, focusing on minimizing off-target effects.
4. **Collaboration**: Foster teamwork among various specialists for a comprehensive treatment approach.
5. **Clinical Trials**: Conduct thorough clinical trials to assess CRISPR-Cas12's safety and efficacy before general application.
6. **Regulatory Compliance**: Follow regulatory guidelines to ensure ethical and safe gene editing practices.
7. **Monitoring**: Establish long-term follow-up for gene-edited patients to monitor treatment outcomes and effects.
8. **Cost Analysis**: Evaluate the economic feasibility of gene editing, considering both costs and potential savings.
9. **Public Engagement**: Engage with the public to understand perceptions and acceptance of gene editing.
10. **Funding**: Seek diverse funding sources to support research.
11. **CRISPR-Cas12 Research**: Develop specific guidelines for CRISPR-Cas12, addressing its unique features and application.
12. **Data Sharing**: Encourage sharing of research findings to foster advancements.

9.1. Research agenda

The research agenda for employing gene editing in treating sickle cell disease is intricate and comprehensive, aiming to address a multitude of avenues for therapeutic possibilities.

Key areas include:

- **Prenatal Therapy**: Investigate the safety and timing for gene editing interventions during pregnancy, alongside developing non-invasive diagnostics for early disease detection.
- **Cord Blood Editing**: Optimize cord blood collection and editing processes, aiming for efficient HBB gene correction. Establish safe transplantation protocols and conduct safety evaluations.
- **CRISPR-Cas12 Advancements**: Focus on the specificity and efficiency of CRISPR-Cas12 [94], exploring innovative delivery mechanisms and assessing off-target effects through computational methods.
- **Ethics and Public Perception**: Delve into the ethical considerations of gene editing and engage public discussions to gauge societal acceptance.
- **Interdisciplinary Collaboration and Funding**: Foster collaboration and secure funding to translate research into clinical practice efficiently.

This streamlined agenda targets advancing gene editing as a viable and ethically sound treatment for sickle cell disease through rigorous research, collaboration, and public engagement.

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Declaration of competing interest

The authors have no conflicts of interest to declare.

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n/a.

Appendix A. Supplementary data

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