How CTX001 Therapy for Sickle Cell Disease Overcomes Many Challenges Associated with CRISPR Therapeutics

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Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR) is a new-found rapid and easily programmable genome editing tool that holds potential use to cure genetic diseases. Monogenic sickle cell disease (SCD) is an inherited blood disorder, characterised by abnormal erythrocytes that fail to transport oxygen efficiently, commonly leading to vaso-occlusive crises, serious infections, and anaemia. Stem cell transplant is the only available cure for SCD, for which the majority of SCD patients are not eligible. CTX001 is a novel SCD therapy that employs CRISPR gene editing in autologous hematopoietic stem cells to disrupt the enhancer region required for expression of BCL11A, a repressor of fetal hemoglobin. Consequently, CTX001 induces elevated fetal hemoglobin levels in erythrocytes, diminishing sickled hemoglobin effects and SCD symptoms. CTX001 could be the first curative therapy available to SCD patients and a similar approach could be undertaken to target other hemoglobinopathies or even immune system disorders. This review suggests that SCD and BCL11A present suitable targets for CRISPR, that overcome many of the associated safety, efficacy and ethical challenges hindering clinical use of this technology.

Introduction

Recent discovery of CRISPR technology has “sparked a revolution in genome editing” [1], with its precise and easily programmable nature eliciting widespread preclinical use and potential clinical applications [1,2]. In brief, CRISPR-associated (Cas) proteins, such as Cas9, form complexes with programmable guide RNA (gRNA) to bind DNA (Figure 1). Progressive guide-target base pairing through sequence complementarity causes conformational transformation to activate Cas9 [2,3] and induce a specific DNA double-strand break (DSB) (Figure 1) [2]. Natural pathways for DNA repair, primarily non-homologous end joining (NHEJ) and homology-directed repair (HDR), subsequently facilitate genome editing [4]. CRISPR-Cas9 presents a powerful tool, able to create gene knockouts or knock-ins, precise site modifications, and transcriptome or epigenome alterations [5,6].

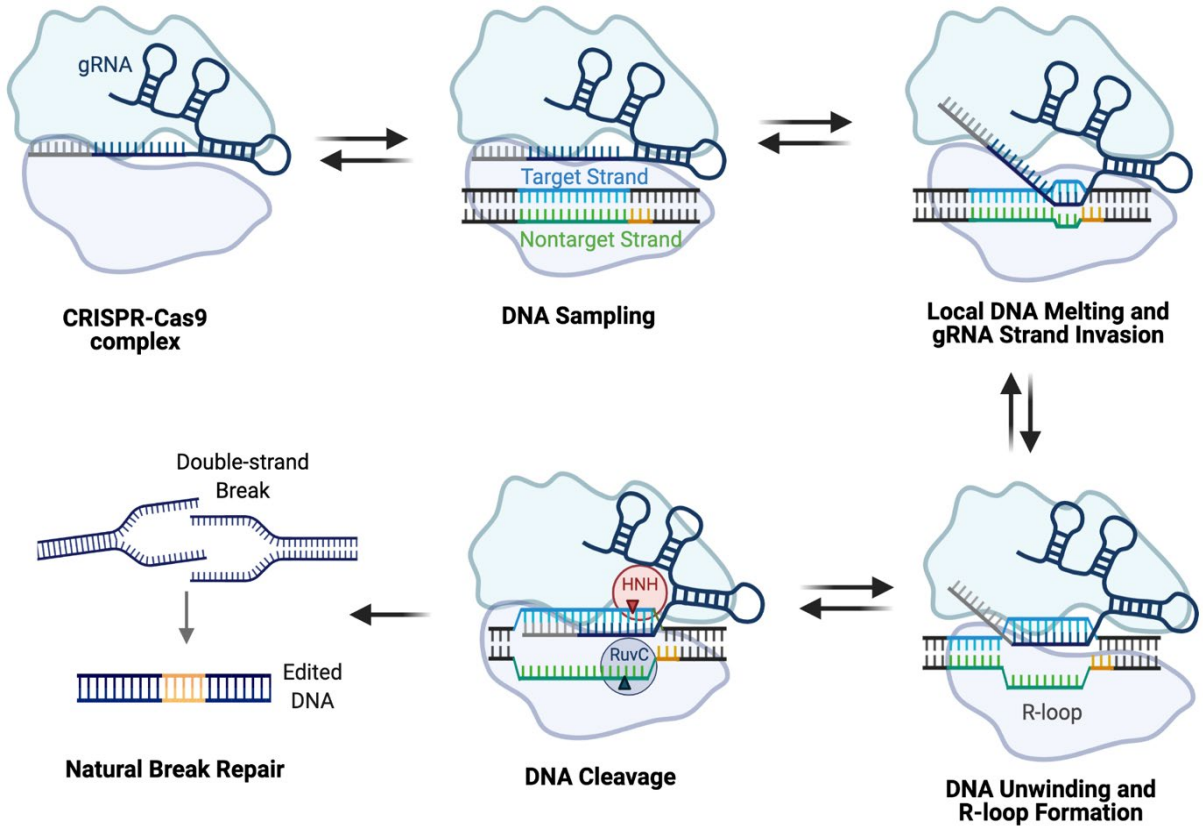

Figure 1: *Mechanism for Genome Editing by CRISPR-Cas9. CRISPR-Cas9 samples DNA for gRNA complementarity, which upon binding and activation, leads to site-specific DNA cleavage. Figure adapted from [7]. Created using BioRender.com.*

Genome editing therapies present novel strategies to cure genetic diseases, such as sickle cell disease (SCD). SCD was the first identified and genetically characterised molecular disease [8,9]; yet 70 years on, treatments remain limited and do not fully remove disease manifestations or correct underlying SCD hematology [10]. The point mutation (T>A) in the β-globin gene and consequent replacement of a hydrophilic glutamic acid with a hydrophobic valine causes sickle cell anemia, the most well-studied and clinically severe hemoglobin genotype in SCD [8,11,12]. Hemoglobin exists as a tetramer of globin subunits, and this modified β-globin gene produces sickle hemoglobin (HbS) that rapidly polymerises and forms stable intermolecular interactions under hypoxic or acidic conditions (Figure 2) [13,14]. These linear polymers exhibit reduced solubility, producing damaged erythrocytes with abnormal rheologic features and adhesion alongside decreased oxygen-binding capacity, leading to painful and life-limiting complications [11,12].

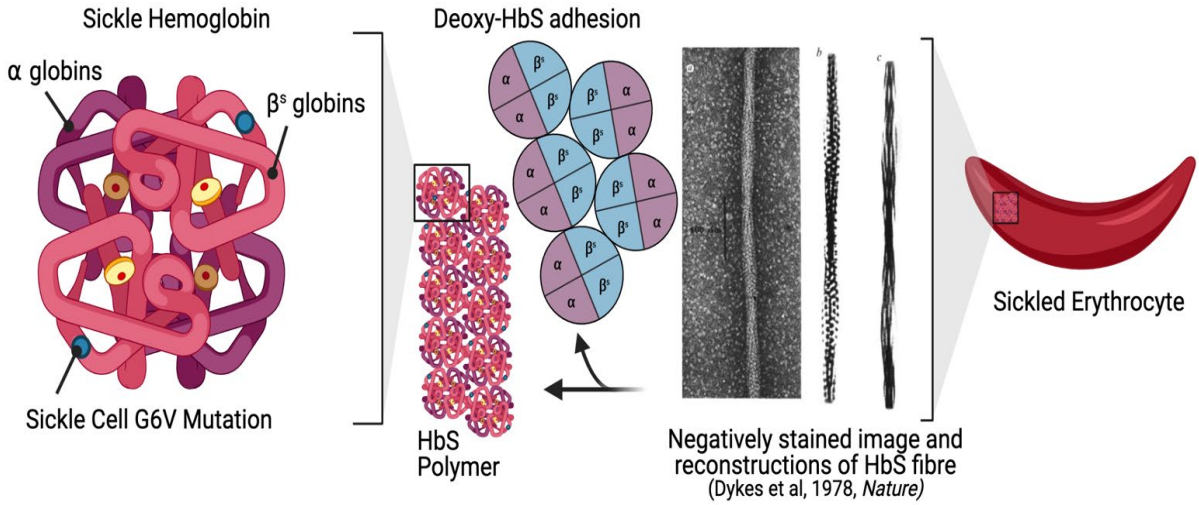

Figure 2: *Sickle Cell Disease Phenotype. □-globin mutation causes HbS polymer formation and sickled erythrocytes. Created using BioRender.com.*

Compared with conventional protein-based gene editing, the RNA-directed approach of CRISPR generates an accessible, easily programmable, and cost-effective technology primed for clinical use. However, CRISPR-Cas therapies generally face three main hurdles surrounding safety, efficacy, and ethics. Broad application of CRISPR-Cas therapies depends on improved safety to diminish off-target effects (OTEs) and immune system interactions, improved efficacy to increase successful editing events, and increased public understanding and careful regulation of use [6,15]. This review will suggest that SCD is amenable to therapeutic genome editing and will focus on how the CRISPR-based therapy CTX001 overcomes many of the efficacy, safety, and ethical challenges this technology faces (Figure 3).

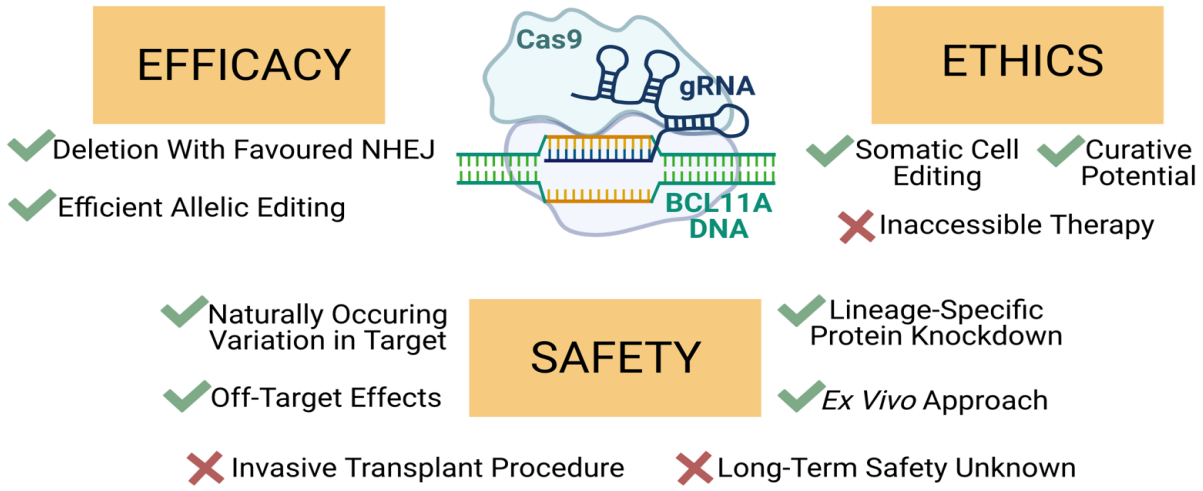

Figure 3: *CTX001 overcomes many CRISPR-associated challenges. Strengths and limitations regarding the safety, efficacy, and ethical issues surrounding CTX001 therapy. Created using BioRender.com.*

CRISPR Therapy for SCD

Three gene editing strategies are under development to cure SCD. Firstly, the mutated β-globin gene can be corrected through repair of the CRISPR-induced DSB using a supplied homology donor of wild-type sequence [16–18]. Secondly, increased fetal hemoglobin (HbF) levels can restore erythrocyte function [19–21]. Expression of the γ-globin subunit of HbF can be induced through reducing γ-globin repressor function [22–24] or introducing mutations recapitulating Hereditary Persistence of Fetal Hemoglobin (HPFH) [25–27]. As sickled globin (β^s-globin) persists, γ-globin must out-compete β^s-globin in hemoglobin formation. Whilst gene correction presents the simplest strategy and would prevent HbS production, it carries risk of β-globin gene deletion and, like HPFH-mimicking mutations, requires unfavourable, precise editing. From these, the only SCD CRISPR-Cas9 therapy in clinical trials is CTX001, which has shown proof-of-principle gene editing in SCD and β-Thalassemia (TDT, another hemoglobinopathy causing ineffective erythropoiesis) to induce HbF expression [24,28].

CTX001 is composed of autologous CD34+ hematopoietic stem and progenitor cells (HSPCs) edited *ex vivo* with a ribonucleoprotein (RNP) of purified Cas9 and gRNA (Figure 4A), targeting the erythroid-specific BCL11A enhancer (Figure 4B) [24]. BCL11A is a master repressor of □-globin, acting to regulate the reciprocal expression of □- and □-globin genes (Figure 5A) [29,30]. BCL11A knockdown (achieved through *BCL11A* enhancer disruption) results in elevated HbF levels, linked to decreased morbidity and mortality in SCD and TDT patients [20,21]. Sufficient HbF levels prevent or prolong deoxy-HbS polymerisation, and as HbF exhibits a high oxygen affinity, healthy erythrocyte phenotype and function can be restored

(Figure 5B) [31]. CTX001-edited HSPCs are thought to confer a selective advantage, as SCD-HSPCs mature inefficiently and display shortened lifespans, implying that correction of a subset of HSPCs will result in a large therapeutic benefit [14,32].

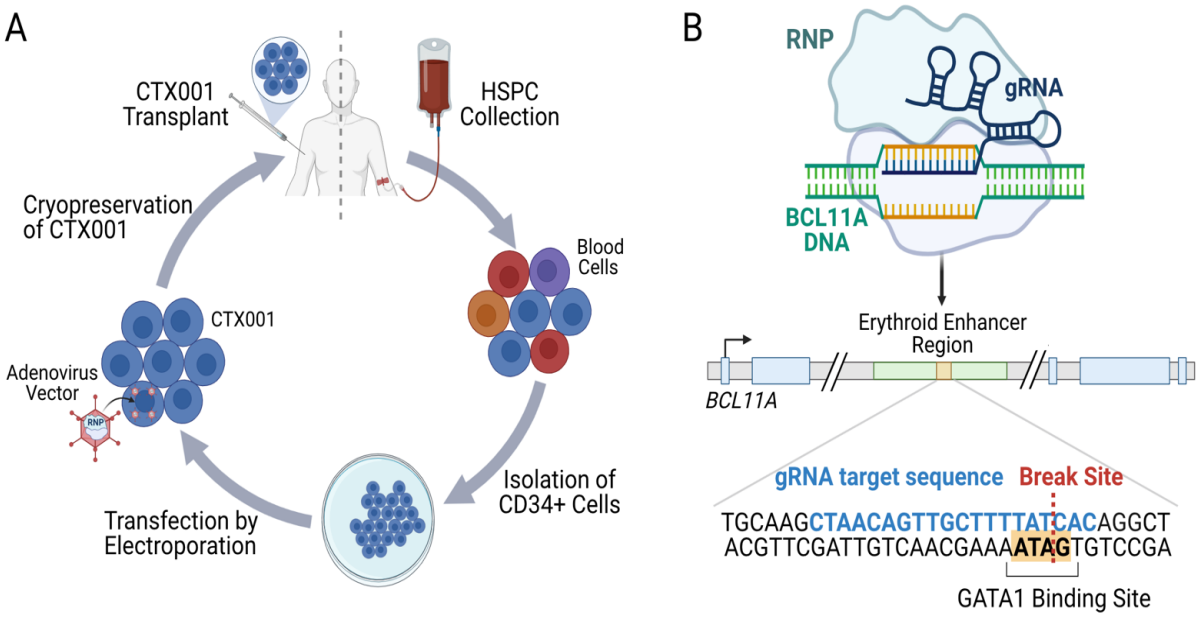

Figure 4: *CTX001 Therapy. (A) Manufacturing and infusion process of CTX001 in autologous cell therapy. (B) CRISPR-Cas9 editing of the erythroid enhancer region of BCL11A, disrupting the GATA1 motif. Figure adapted from [24]. Created using BioRender.com.*

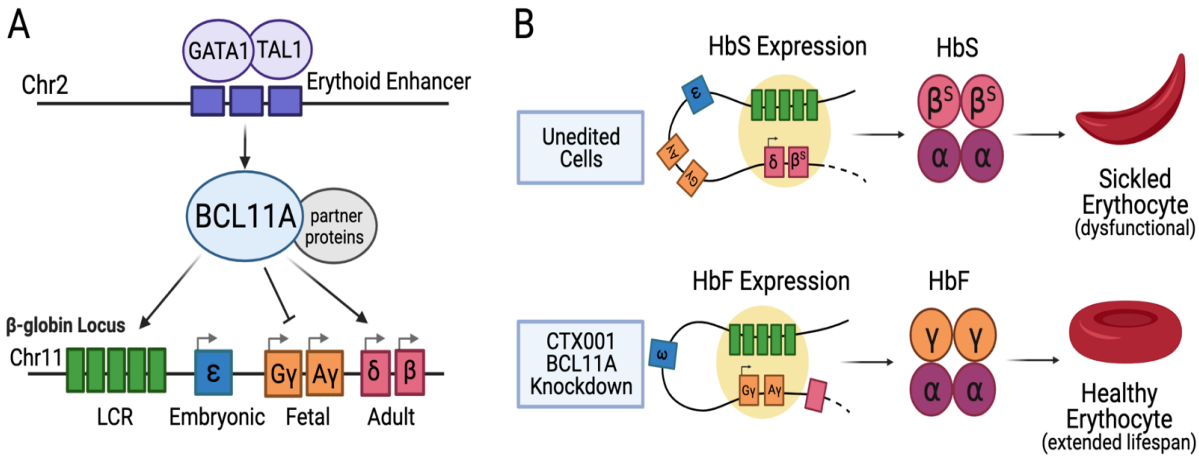

Figure 5: *Effect of CTX001-mediated BCL11A Knockdown. (A) BCL11A controls the competitive association of globin genes with the locus control region (LCR) to promote β-globin expression and inhibit γ-globin expression. (B) Knockdown of BCL11A alters LCR interactions to permit γ-globin expression, HbF production, and healthy erythrocytes. Figure adapted from [29].*

Overcoming Safety Challenges

Ex Vivo Approach

HSPC extraction allows *in vitro* genome editing (through cell transfection of an RNP) before host transplantation (Figure 4A), increasing control over CRISPR-Cas9 expression and OTEs [24]. Extensive self-renewal of HSPCs upon engraftment results in cell amplification and establishment, meaning that few edited cells need to be transplanted [33]. The likely RNP degradation before CTX001 administration [34] alleviates concerns associated with continual RNP expression from some *in vivo* plasmid- or mRNA-based editing methods. The first concern surrounds potential pre-existing adaptive immune responses against CRISPR-Cas9, as the most commonly used Cas9 sources, *Staphylococcus* aureus and *Streptococcus* pyogenes, frequently infect humans [35]. Charlesworth et al found anti-Cas9 antibodies in 58-79% of healthy human blood samples, alongside Cas9-reactive CD8+ T cells secreting interferon-γ [36]. *Ex vivo* CTX001 is well positioned to mitigate RNP expression in cells in the body, removing risks of detrimental immune responses. Secondly, the transient RNP presence and immediate nuclease activity following transfection may reduce OTE accumulation associated with extended CRISPR-Cas9 exposure [37]. Overall, the circulatory tissue presents an accessible target for CRISPR-Cas9, amenable to safer *ex vivo* editing.

OTE Identification

OTEs produced by CRISPR-Cas9 and associated unpredictable and/or harmful genetic changes are likely to be the main barrier for therapeutic use of this technology. In general, CRISPR-Cas9 can tolerate up to five gRNA-DNA mismatches [38] and induce non-specific cleavage, disrupting gene function and/or generating genome instability through large insertions, deletions, or chromosomal rearrangements [39]. In CTX001 preclinical work, GUIDE-seq mapping of double-stranded oligodeoxynucleotide-tagged (dsOligo) DSBs identified 52 candidate off-target sites (Figure 6) [24]. Computational analysis identified a further 171 genomic sites with ≤3 mismatches or ≤2 mismatches with a DNA/RNA bulge [24]. Despite this, high-coverage sequencing of CTX001-edited healthy CD34+ cells found no off-target edits at these sites (Figure 6) [24]. This highlights the specificity of CTX001, potentially due to appropriate RNP exposure and degree of editing, or loss of mis-edited cells from the population. Given the high mutation resolution available, reducing sequencing cost, and *ex vivo* approach, it would be ideal to sequence CTX001 cells for these candidate off-target sites before patient administration. This would predict safety of therapy in the individual's diseased phenotype, which may differ from results of preclinical work on healthy cells.

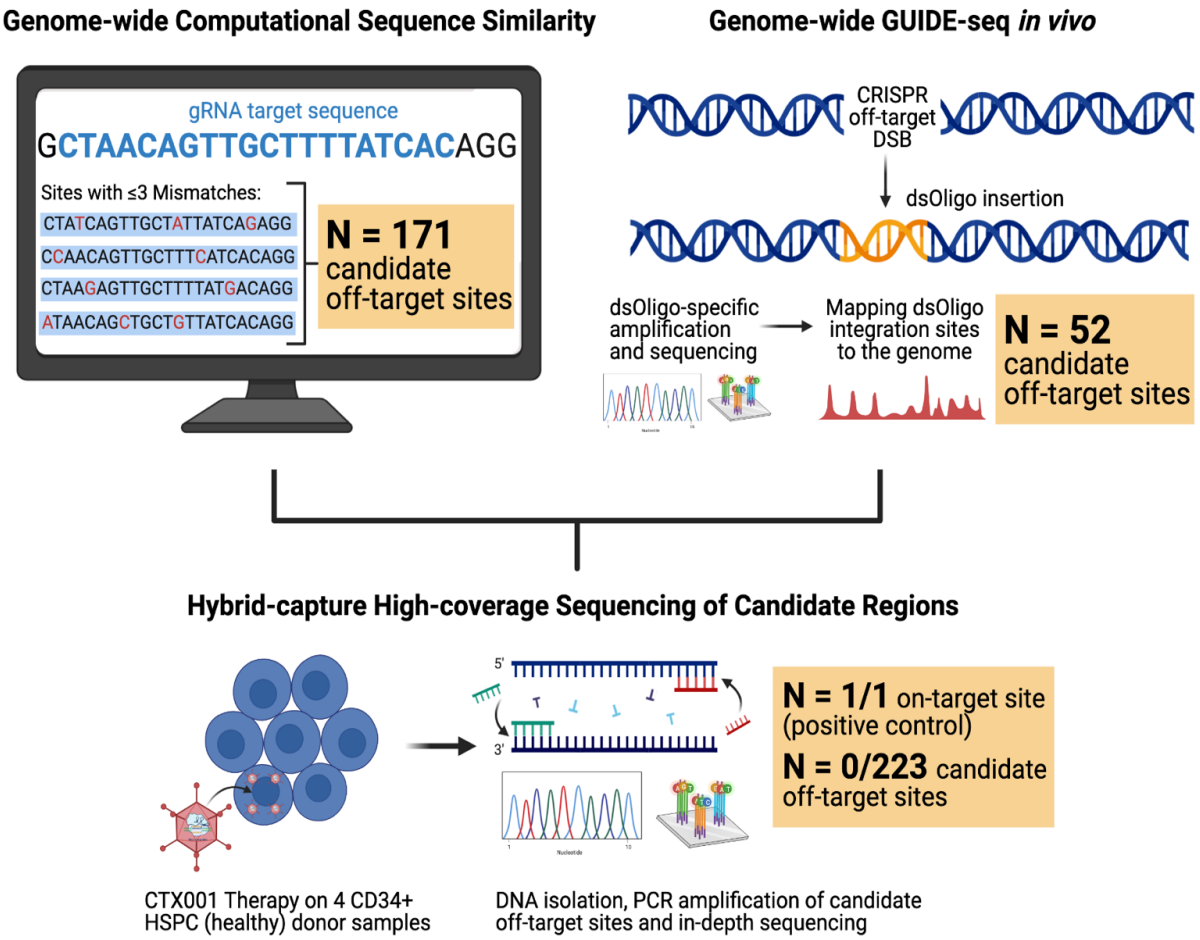

Figure 6: *Off-Target Identification in CTX001 Preclinical Work. Candidate off-target sites were identified using in silico sequence similarity and in vivo GUIDE-seq and validated in CTX001-edited cells using hybrid-capture high-coverage sequencing. Methods detailed in [24]. Created using BioRender.com.*

BCL11A Variation and HPFH

CTX001 intervention is based on natural genetic variation of the *BCL11A* enhancer, and reduced BCL11A expression and elevated HbF levels are well-tolerated and common [40]. The strongest trait-associated variant was found to alter a GATA motif at the erythroid enhancer, leading to a modest reduction in transcription factor binding and BCL11A expression in human erythroid precursors [29,40]. CTX001 disrupts this GATA1 recognition site (Figure 4B), with natural variation at this locus indicating a safe and effective target. Furthermore, γ-globin promoter mutations disrupt repressor binding (Figure 7) and lead to elevated HbF levels, characterising HPFH disorders that are associated with alleviated SCD manifestations [41,42]. CTX001 aims to recapitulate the asymptomatic SCD phenotype of HFPH through reducing BCL11A expression, with the naturally occurring and benign nature of this disorder suggesting a safe and well-tolerated therapeutic outcome of CTX001.

***Figure 7**: γ-Globin promoter and HPFH mutations. Human □globin gene promoter DNA sequence from -100 to -206, showing transcription factor binding sites (blue boxes) and HPFH mutations (red). Figure adapted from [10]. Created using BioRender.com.*

Lineage-Specific BCL11A Knockdown

Selective inhibition of BCL11A can be achieved as the *BCL11A* enhancer is essential for erythroid BCL11A expression, but dispensable in non-erythroid contexts [40]. This erythroid specificity is crucial for safe editing in differentiating HSPCs, where BCL11A functions in other lineages, such as in gene control in B-lymphopoiesis [43]. Not all BLC11A-associated therapeutic targets permit this lineage-specificity and increased safety. Indeed, BCL11A inhibition solely affects the globin gene, contrasting to the impaired erythroid maturation and widespread genetic effects observed with inhibition of BCL11A's protein partners [29]. Taken together, CTX001 target choice and *ex vivo* approach gives rise to erythroid-specific BCL11A depletion with no OTEs, both mimicking HFPH and subsequently increasing the safety of this therapy.

Overcoming Efficacy Challenges

Efficient Allelic Editing

CRISPR efficacy relates to the percentage of successfully edited cells, and insufficient efficacy remains a key barrier to therapeutic use of CRISPR, particularly in primary human cells [37]. CTX001 gives rise to efficient pancellular allelic editing, which elevates HbF levels sufficiently for therapeutic benefit in SCD. 69-83% allelic editing efficiency was achieved in the two patients receiving CTX001, which increased levels of circulating red blood cells expressing HbF (F-cells) to ~100% (Figure 8A) [24]. It has been proposed HbF concentration distribution among F-cells is the most important determinant of SCD phenotype [44]. In HbS-HPFH heterozygous patients above 5 years old, cellular HbF levels are estimated to be 20-30% and distributed evenly among erythrocytes [31,45,46]. Consistent with benign SCD in HPFH patients and computational modelling suggesting an average HbF concentration of 30% protects 70% of erythrocytes, a threshold of 10-30% HbF has been proposed to ameliorate

SCD morbidity [44,47]. The SCD patient surpassed this level 3 months after CTX001 treatment, and HbF concentration remained stable around 42-48% for the following year (Figure 8B) [24]. These results demonstrate high allelic editing and induced HbF levels sufficiently above that required, presenting an efficacious treatment.

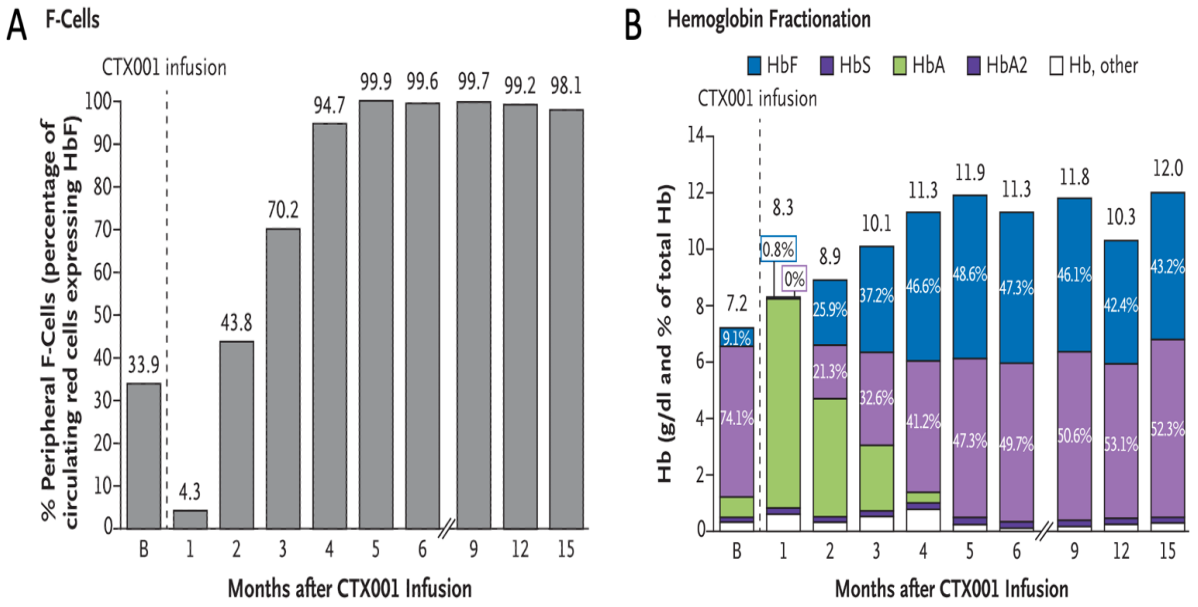

Figure 8: *CTX001 Phase I Trial Results for SCD Patient. Proportion of (A) F-cells and (B) hemoglobin subtypes in a SCD disease patient following CTX001 therapy. Graphs from [24]. Created using BioRender.com.*

BCL11A Deletion Requires NHEJ

Cellular repair of CRISPR-induced DSBs also influences efficacy. NHEJ is the default DNA repair pathway in higher eukaryotes and is intrinsically error-prone, as DNA end resection results in small deletions at the break site leading to mutations and/or frame shifts in coding sequences [4]. CRISPR-Cas9-mediated alteration or addition of genetic information requires HDR, a high-fidelity repair mechanism using homologous donor sequences as templates [4]. Human cells favour NHEJ over HDR for many reasons, including faster completion of NHEJ and repression of HDR by NHEJ [4]. Also, NHEJ has a higher efficiency, and is active, at all cell cycle stages compared to G2/S phase-restricted HDR [48]. HSPCs have been demonstrated to confer limited HDR proficiency and forcibly use NHEJ due to their largely quiescent state and DNA repair machinery composition [49,50]. Mutagenesis by NHEJ in *BCL11A* enhancer disruption is therefore well-suited to HSPCs, avoiding requirement for disfavoured HDR pathways. Use of advanced CRISPR technologies that don't require DSB creation could alleviate efficacy concerns associated with natural repair of DNA [6].

Overcoming Ethical Challenges

Somatic Cell Editing

Therapeutic genome editing carries serious ethical implications, and it is widely accepted that the public must be engaged in discussions shaping the application and restrictions of this technology [51]. The public predominantly support somatic genome editing to prevent disease or disability, although resistance to altering non-disease characteristics and germline editing is apparent [51–53]. Current regulation and guidance reflects this view, as clinical applications of somatic cell gene editing for improving health are generally supported [54], whereas germline editing requires “a stringent oversight system able to limit use to specified criteria” [55]. CTX001 therapy on somatic cells of a consenting patient may circumvent many ethical, legal, and societal problems associated with germline modifications and trait enhancement, especially given the well-defined, diagnosable SCD phenotype. This results in SCD as a widely non-contentious target for CRISPR-Cas9 genome editing.

Curative Potential

Currently incurable or untreatable diseases present a strong ethical case for use of CRISPR-based therapies and could be viewed as a priority over diseases with existing effective treatments for all patients. Only four FDA-approved drugs exist to lessen SCD acute complications, three of which were approved in the last three years [14]. Hematopoietic stem cell transplant (HSCT) is currently the only cure for SCD, and although HLA-identical sibling HSCT offers excellent long-term survival, less than 20% of patients have appropriately matched donors [56–59]. Use of matched but unrelated or haploidentical donors is linked with graft rejection, graft-vs-host disease, and increased patient morbidity and mortality [58,60]. Considering these limitations of current treatments, the FDA granted CTX001 ‘Regenerative Medicine Advanced Therapy (RMAT) status as it fills an unmet medical need for a disease with no cure. Therefore, CTX001 is eligible for priority review and accelerated approval and so offers a strong ethical case for use of CRISPR-Cas9.

Accessibility

Tropical regions exhibit the highest frequencies of SCD, due to the malaria protection afforded by SCD conferring a selective advantage. Globally by 2050, ~400,000 newborns are predicted to inherit sickle cell anemia, 85% of whom will be born in sub-Saharan Africa where access to health clinics and prophylactic care is minimal or non-existent [62–64]. Some argue basic interventions (vaccinations, penicillin prophylaxis, and prenatal diagnosis) should be prioritised, that once targeted to the most affected countries, have been predicted to save ~10

million children's lives over the next 35 years [64]. Similarly, others suggest that efforts should focus on improving HSCT prospects, due to greater experience and excellent demonstrated outcomes [31]. Another important and poorly understood consideration is malaria susceptibility following treatment, and whether CTX001 will require administration alongside malaria prevention strategies in certain areas. If CTX001 was proven effective, its use would likely be limited to affluent nations. CRISPR-based therapies are expensive, resource-intensive and require specialist expertise and facilities, rendering this treatment inaccessible to the countries in most need. *In vivo* approaches could remove the invasive procedures and high costs associated with CTX001, however numerous barriers exist to clinical viability of this method, most notably delivery and editing efficacy [6].

Conclusion

Despite promising initial outcomes, CTX001 faces limitations that may hinder its therapeutic use, most notably concerning invasive therapy, long-term safety, and accessibility. *Ex vivo* editing imposes inherent challenges, largely due to the rebuilding of the hematopoietic and immune system. Myeloablative conditioning for stem cell transplantation can induce chemotherapy-related adverse effects like infections and/or low blood counts, exemplified by the pneumonia or sepsis in the presence of neutropenia observed in patients following CTX001 treatment [14,24]. Furthermore, harvesting HSPCs is invasive and yields small percentages of CD34+ HSPC cells from SCD patients [14]. Secondly, the long-term safety of CTX001 is unknown. Particular uncertainty surrounds potential effects of SCD-associated chronic inflammation and ineffective erythropoiesis on HSPC viability/engraftment or treatment efficacy as preclinical studies used healthy cells. Phase I/II clinical trials are recruiting 90 SCD and TDT patients for long-term longevity and safety investigations (ClinicalTrials.gov Identifier: NCT03745287/NCT03655678). Robust, rapid, and high-throughput detection systems will be required to monitor toxicity, as detection limits of current sequencing methods limit OTE identification [14,61].

A permanent cure for SCD available to all patients from CTX001 therapy could fulfil the large unmet medical need for this largely neglected disease. SCD presents a good candidate for one of the first CRISPR-Cas9 therapeutics, with a target profile that exhibits efficient allelic editing and exploits default cellular repair pathways. Additionally, BCL11A and HbF are subject to natural variation, increasing confidence in a well-tolerated outcome of CTX001. The *ex vivo* technique and lineage-specific protein knockdown further improves safety, resulting in no OTE identification in CTX001 preclinical work. Looking ahead, this HbF induction strategy could apply to other hemoglobinopathies, where similar disease phenotypes arise from diverse

genotypes [14]. Given the existing limitations for CRISPR therapeutics, great interest will be taken in the efficacy and long-term safety of CTX001 to draw learnings for CRISPR-Cas9 use in diseases of the blood and immune systems.

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