CRISPR-Cas9

Editing of the HBG1 and HBG2 Promoters to Treat Sickle Cell Disease

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ORIGINAL ARTICLE

CRISPR-Cas9 Editing of the *HBG1* and *HBG2* Promoters to Treat Sickle Cell Disease

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Sickle Cell Disease

- ✓ an autosomal recessive disorder

- ✓ caused by mutations in the gene HBB, which encodes the β-globin
- ✓ subunit of adult hemoglobin (α2β2).

- ✓ The mostcommon sickle cell disease mutation causes ahomozygous p.Glu6Val substitution, resulting in the production of sickle hemoglobin (α2βS2)

- ✓ Consquence of Sickle hemoglobin polymerizes at low oxygen concentrations:
- ✓ red cells to become sickle shaped, rigid, and fragile

- ❑ microvascular occlusion
- ❑ hemolysis
- ❑ inflammation

Sickle Cell Disease

- Complication:
  - chronic anemia
  - Recurrent pain
  - progressive multi organ damage
  - increased risk of early death
- Appear Symptoms of sickle cell disease
  - during infancy as γ-globin gene (HBG1 and HBG2) transcription switches to β-globin (HBB), causing a shift from fetal hemoglobin (α2γ2) to adult hemoglobin in red cells.
- Medical therapies:
  - hydroxyurea
  - blood transfusions
  - approved drugs(L-glutamine, crizanlizumab, and voxelotor)
- only potentially curative option: allogeneic hematopoietic stem-cell transplantation
- Induction of fetal hemoglobin is a proven strategy for treating sickle cell disease

CRISPR-Cas9 gene-editing

CRISPR-Cas system relies on two main components:

- A guide RNA (gRNA)

  consisting of a crRNA sequence that is specific to the DNA target, and a tracrRNA sequence that interacts with the Cas9 protein

- CRISPR-associated (Cas) nuclease

- The CRISPR-associated protein is a non-specific endonuclease

  It is directed to the specific DNA locus by a gRNA

  Makes a double-strand break

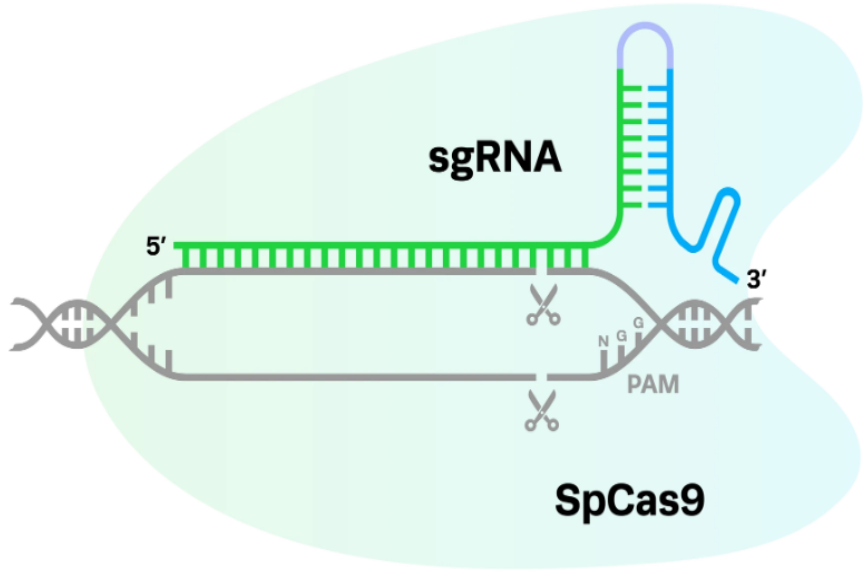
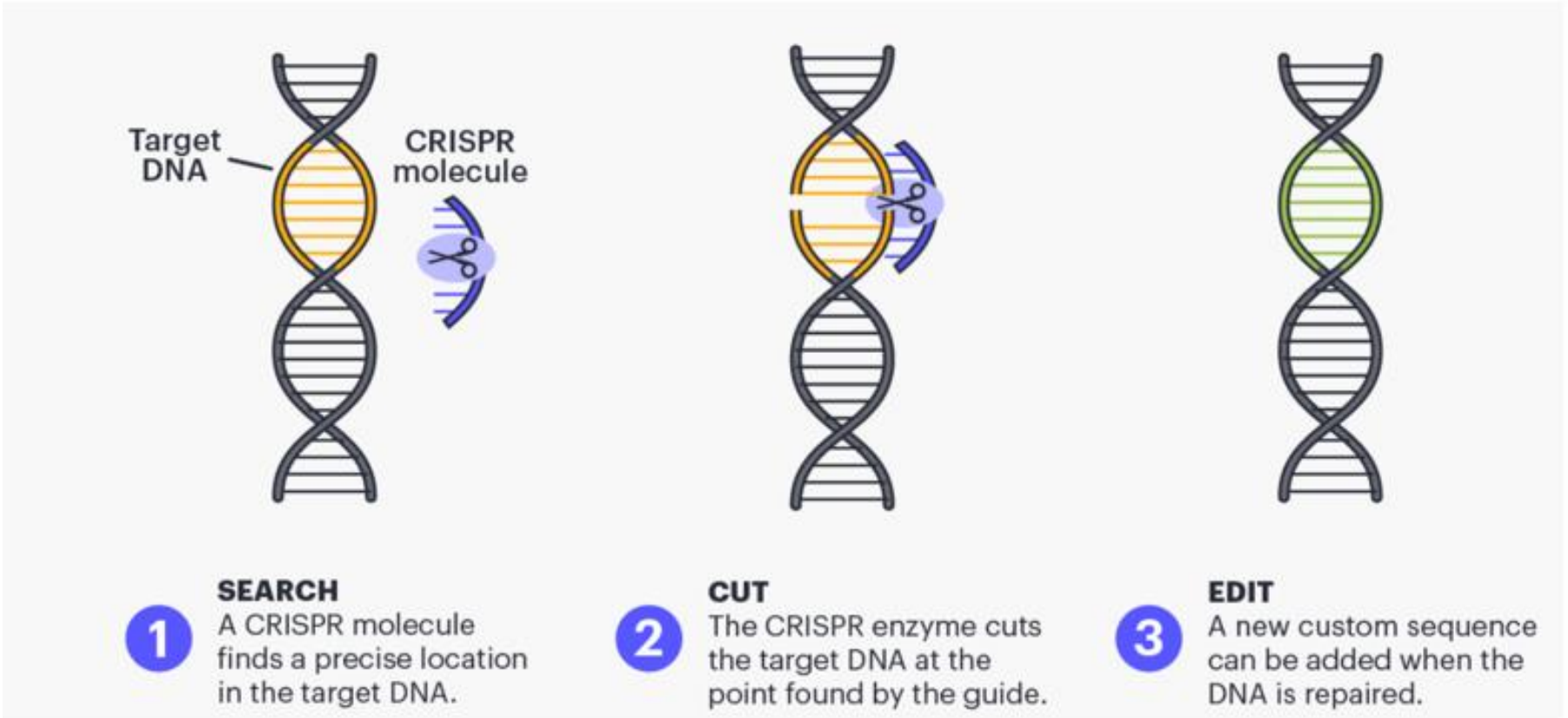


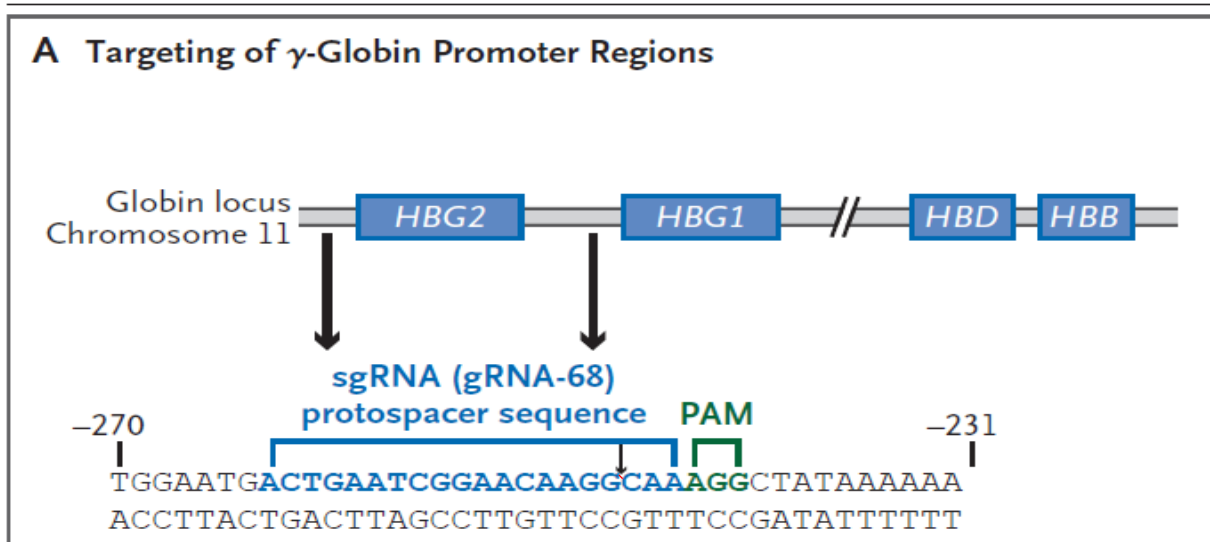
Figure 1. The CRISPR-SpCas9 System. The CRISPR-SpCas9 system comprises a guide RNA (gRNA) and SpCas9 nuclease, which together form a ribonucleoprotein (RNP) complex. The presence of a specific protospacer adjacent motif (PAM) in the genomic DNA is required for the gRNA to bind to the target sequence. The Cas9 nuclease then makes a double-strand break in the DNA (denoted by the scissors). Endogenous repair mechanisms triggered by the double-strand break may result in gene knockout via a frameshift mutation or knock-in of a desired sequence if a DNA template is present.

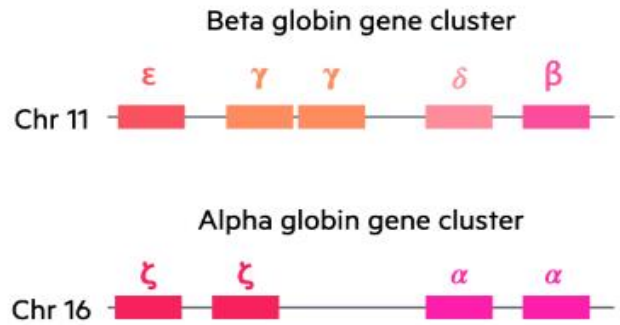

Target DNA
CRISPR molecule
1
SEARCH
A CRISPR molecule finds a precise location in the target DNA.
2
CUT
The CRISPR enzyme cuts the target DNA at the point found by the guide.
3
EDIT
A new custom sequence can be added when the DNA is repaired.

Aplication of CRISPR-Cas9 gene-editing

- Edit the genome of living organisms.
- Replace a disease-causing mutation.
- “knock out” a gene entirely.
- Disrupting gene function.
- causing a cell to stop making the protein.
- The first approved CRISPR therapy for sickle cell disease

Introduction of B –like globin genes





Five paralogous β-like globin genes are located in the β-globin gene cluster on human chromosome 11p

HBE encoding ε-globin
HBG2 (Gγ) and HBG1 (Aγ) encoding γ-globin
HBD encoding δ-globin
HBB encoding β-globin

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Introduction of B –like globin genes

Around the time of birth , the site of red-cell production shifts from the fetal liver to the bone marrow.

This transition is associated with a switch from γ-globin (HBG1 and HBG2) production to β-globin (HBB) production

The switch of gene expression and is clinically important because β-hemoglobinopathies can be treated by inhibiting this switch

BCL11A and LRF/ZBTB7A

Introduction of B –like globin genes

BCL11A and LRF/ZBTB7A

Perinatal switch from γ-globin to β-globin is mediated by transcriptional repressor proteins, BCL11A and LRF/ ZBTB7A

Bind cognate cis-regulatory elements in the HBG1 and HBG2 promoters

Inhibition of the binding of these repressors to their targets in adult red-cell precursors can reactivate expression of γ-globin and fetal hemoglobin.

Methods

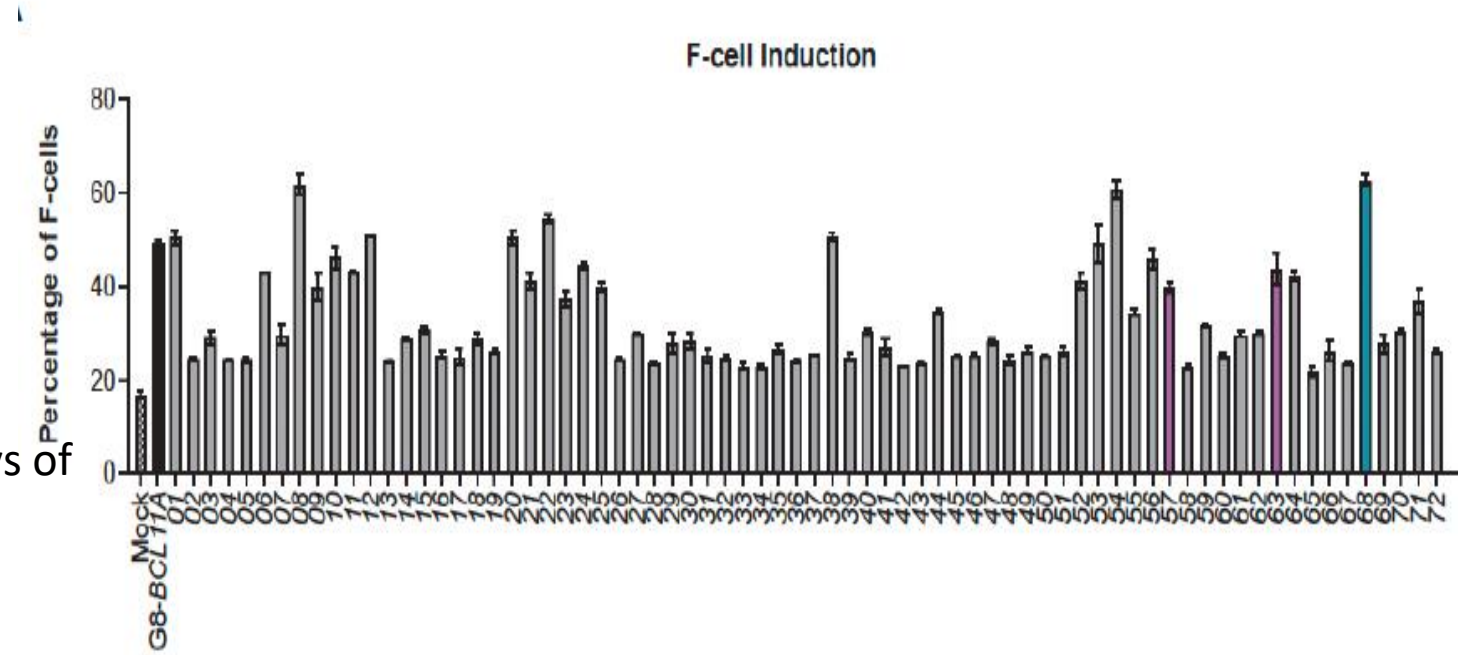
- Preclinical screening
- OTQ923 manufacture
- Electroporating CD34+ cells
- Quantitative polymerase chain reaction(q-pcR)
- Targeted next-generation sequencing(T-NGS)
- Unique identifier tagmentation next-generation sequencing(UnIT) NGS
- Flow cytometry
- Capillary electrophoresis
- HPLC(ion-exchange high-performance liquid Chromatography)

Methods

Preclinical screening

- Seventy-two gRNAs were designed to target the HBG1 and HBG2 promoter regions
- Initial gRNA selection was performed computationally, using the human reference genome
- Each gRNA was chemically synthesized and purified by high-performance liquid chromatography (HPLC).
- Using CRISPR-Cas9 tiling of the HBG1 and HBG2 promoter regions to identify the guide RNA that led to the highest F-cell induction.
- An unbiased guide RNA (gRNA) screen of the HBG1 and HBG2 promoter regions was performed to identify regulatory regions within the HBG1 and HBG2 promoter regions
- Disruption of which led to potent induction of fetal hemoglobin (HbF)-immunostaining erythroblasts (F-cells).

Percentage of HbF-immunostaining cells (F-cells) according to the gRNA used



- Measured by immune flow cytometry after 7 days of erythroid differentiation.

Mock-edited cells (as a negative control):
Received RNP containing Cas9 and tracrRNA only.

Positive controls:
Included cells treated with gRNA-G8 , which targets the *BCL11A* exon 2, (solid black bar), gRNA-57 or gRNA-63 which target a known BCL11A binding motif(purple colored bars),
The teal colored bar corresponds to induction of F-cells by gRNA-68

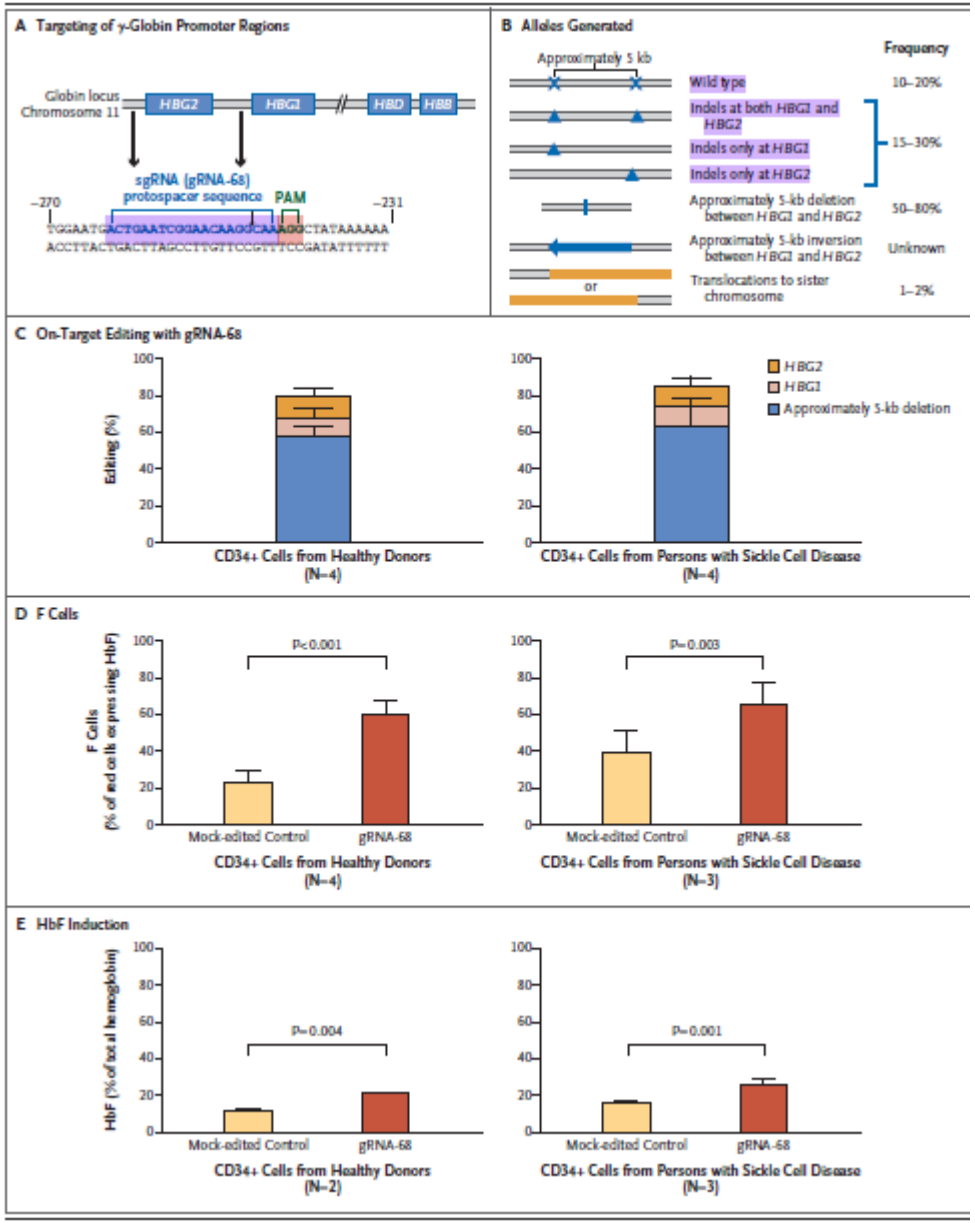
Molecular Approach and Preclinical Characterization of Guide RNA-68 (gRNA-68)–Edited Hematopoietic Stem Cells.

The gRNA-68 targets a site that is 246 bp upstream of the transcriptional start in each of the nearly identical tandem HBG1 and HBG2 genes (Fig.1A)

In HSCs obtained from healthy donors for the preclinical experiments, the mean (±SD) frequency of on-target HBG1 and HBG2 editing was 80.5±9.8% in four participants resulting in 60.5±6.8% F cells in those four participants as compared with 22.9±3.5% F cells in mock edited , control samples (CD34+ cells electroporated with Cas9 protein only).(C-D)

In HSCs obtained from persons with sickle cell disease the frequency of on-target HBG1 and HBG2 editing was 85.8±14.7% in four participants, , resulting in 65.4±12.1% F cells in three participants, as compared with 39.6±12.4% F cells in mock-edited control samples Fig. (1C and 1D).

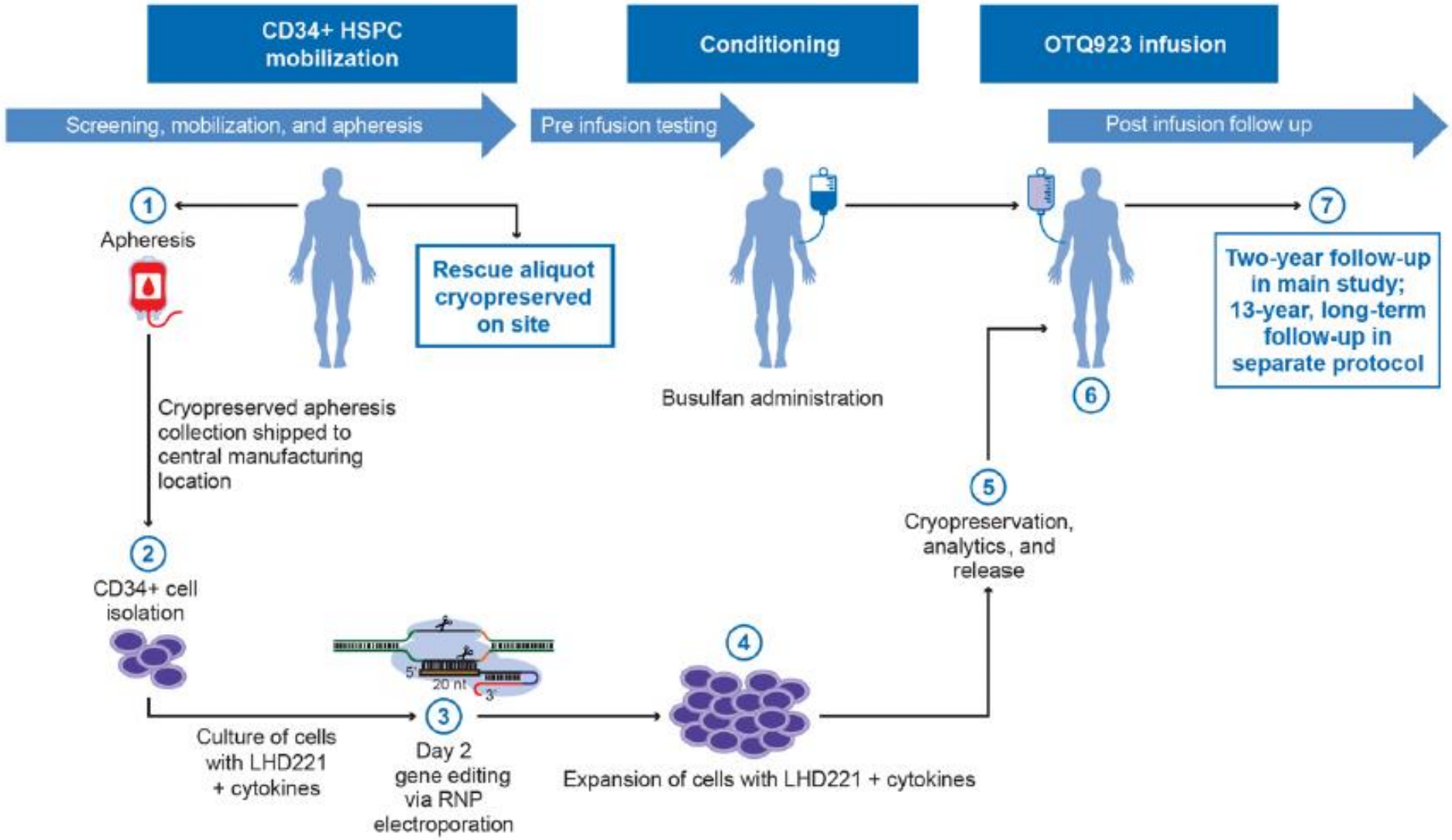
In HSCs obtained from healthy donors , the level of fetal hemoglobin protein after gRNA-68 editing was 20.9±0.2% as compared with 12.0±0.3% in mock edited control samples (Fig. 1E).
In HSCs obtained from persons with sickle cell disease, the level of fetal hemoglobin protein after gRNA-68 editing was 26.2±2.9% as compared with 16.1±2.3% in mock-edited control samples (Fig. 1E).



OTQ923

- An autologous, ex vivo CRISPRCas9–edited, CD34+ HSC product
- Has a targeted disruption in the HBG1 promoter, theHBG2 promoter, or both caused by a ribonucleoprotein complex (RNP)
- Consisting of Streptococcus pyogenes Cas9 protein–single guide RNA (gRNA-68).
- The gRNA-68 targets a site that is 246 bp upstream of the transcriptional start in each of the nearly identical tandem *HBG1* and *HBG2* genes

Schematic of OTQ923 manufacture and treatment approach



Methods

OTQ923 manufacture

- participants received monthly red-cell exchange transfusions for at least 2 months before collection
- **Collection of CD34+ cells**

CD34+ HSCs were mobilized with plerixafor, collected by means of apheresis, cryopreserved, and shipped to a central manufacturing site.

- **immunomagnetic selection**

Upon receipt at the manufacturing site product is thawed and washed, and CD34+ cells are isolated using a closed, sterile, automate, micro-bead system (CliniMACS Prodigy System). (MACS)

- **Incubation of CD34+-enriched cells**

CD34+-enriched cells are incubated in cell culture medium containing: Rh TPO, rh SCF, rh Ft-3L, and human serum albumin (HSA).

electroporation

Methods

Electroporation

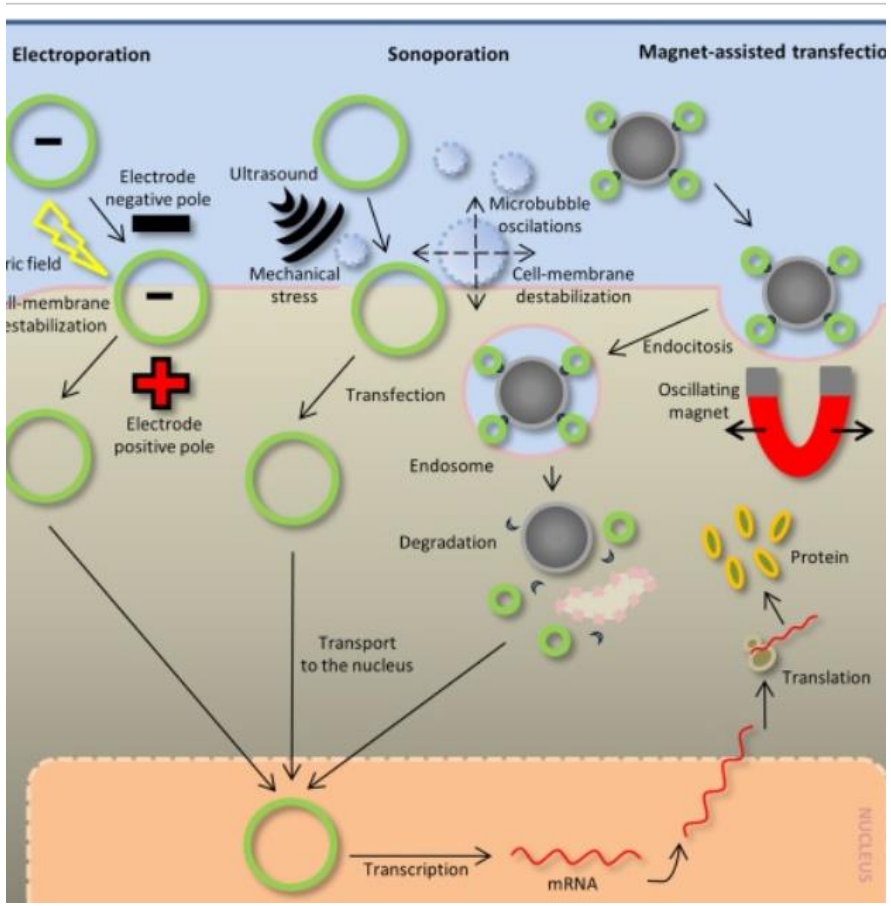
Genome editing performed by electroporation in a buffer containing the RNP complex.

Transduction of hematopoietic stem cells were done by electroporation.

With two way:

- A lentiviral vector encoding an erythroid-expressed short hairpin RNA against BCL11A

- Targeted disruption of a BCL11A erythroid-specific enhancer by (CRISPR)–Cas9



Methods

Erythroid differentiation and HbF induction

Genome-edited CD34+ cells were subjected to a three-stage erythroid differentiation in liquid culture.

The percentage of F-cells was determined at day 14 of erythroid Culture.

Cells were stained intracellularly for HbF, using an anti–human γ-globin antibody directly conjugated with a fluorophore.

HbF protein levels were also determined by ion-exchange HPLC.

Cell pellets were lysed in water at 50,000 cells per microliter for 5 minutes.

The hemolysate was analyzed for HbF protein levels.

Methods

- **Quality control release assays**

  performed on cryopreserved material before batch release

- **Infusion of OTQ923:**

  conditioning: Before infusion of OTQ923, the participants underwent Conditioning with myeloablative busulfan administered IV every 6 hours, with pharmacokinetic monitoring.

- **Assess Edited human HSCs**

Edited human HSCs were xenotransplanted into sublethally irradiated mice to assess the durability of editing and the extent of multilineage reconstitution

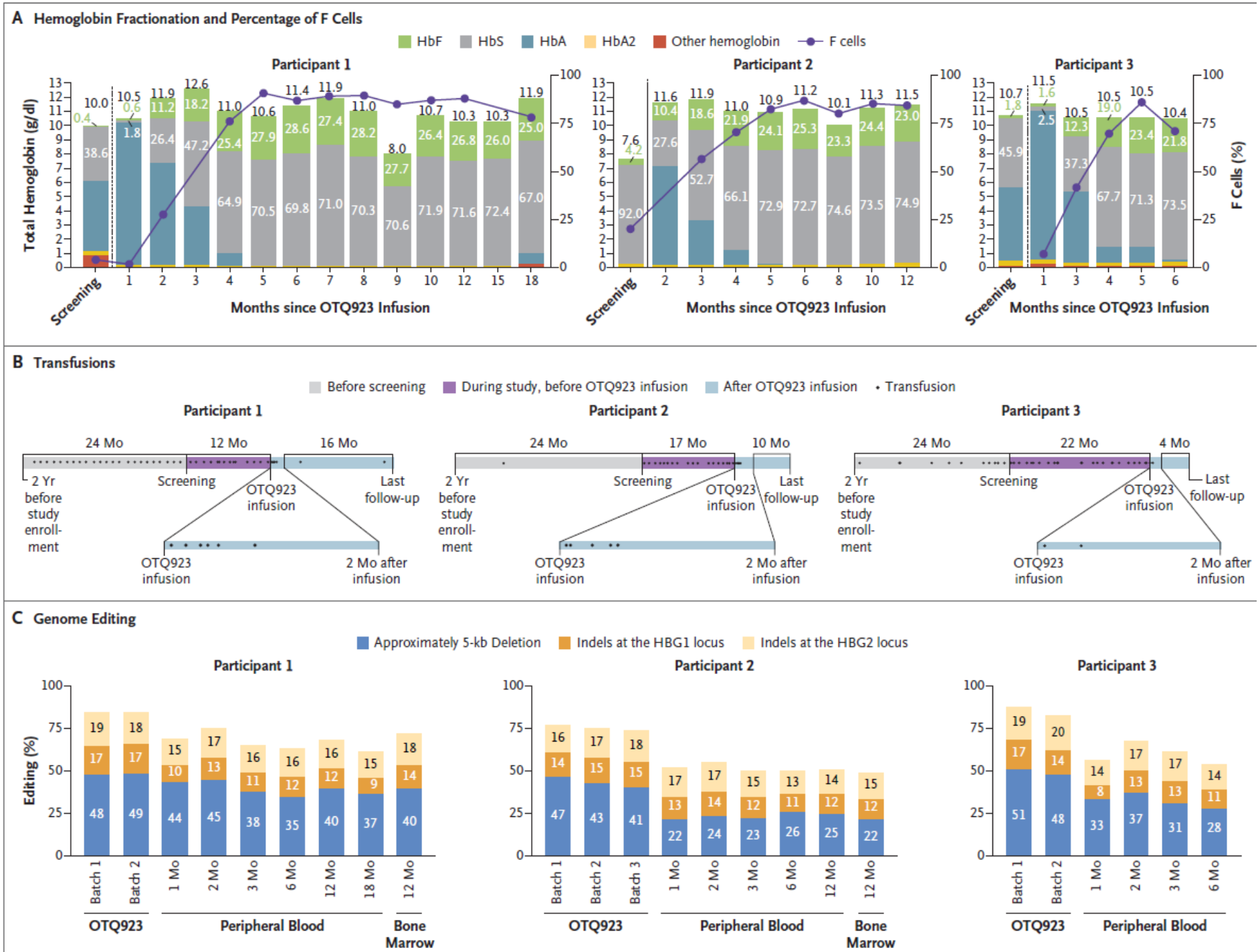
participants

Inclusion criteria

- Between 18 and 40 years of age
- Had a confirmed diagnosis of sickle cell disease
- At least one of the following indicators of disease severity:
  - Three episodes of vaso-occlusive crisis
  - Two episodes of acute chest syndrome within the previous 2years
  - Recent priapism
  - A history of stroke
  - Long- term regular receipt of transfusions
  - Red cell allo immonization

Table 1. Demographic Characteristics of the Participants and Outcomes.

Variable	Participant 1	Participant 2	Participant 3
Demographic characteristics and disease severity			
Age at screening (yr)	22	21	24
Sex	Male	Male	Female
Sickle cell disease genotype	β^S/β^S	β^S/β^S	β^S/β^S
Sickle cell disease–related symptoms before study enrollment	Six episodes of acute chest syndrome over the past 10 yr and a history of a silent cerebral infarction, retinopathy, and priapism	Four episodes of vaso-occlusive crisis, three episodes of acute chest syndrome, and a silent cerebral infarction during the preceding 20 yr	Twenty five episodes of vaso-occlusive pain in the 2 yr before enrollment
Treatment for sickle cell disease ongoing at study enrollment	Regular blood transfusions and hydroxyurea	Hydroxyurea	Regular blood transfusions and hydroxyurea
Apheresis collection and OTQ923 manufacture			
Mobilization cycles lasting 2–3 days each (no.)	3	2	3
Cell dose manufactured (million/kg)	2.80, a combination of two manufacturing batches, each with 84% editing efficiency	5.99, a combination of three batches with editing efficiencies of 78%, 75%, and 73%, respectively	5.04, a combination of two batches with editing efficiencies of 87% and 82%, respectively
Follow-up and outcomes			
Neutrophil engraftment	Day 26	Day 20	Day 18
Platelet engraftment	Day 44	Day 29	Day 29
Adverse events since OTQ923 infusion (no.)	36	16	45
Adverse events considered by investigators to be related to OTQ923 (no.)	0	0	0
Follow-up since OTQ923 infusion (mo)	18	12	6
Sickle cell disease–related events since OTQ923 infusion*	One episode of vaso-occlusive crisis with acute chest syndrome occurred at 17 mo after infusion; recurrent intermittent priapism; no new stroke or silent cerebral infarction; continued mild hemolysis; worsening osteonecrosis of femur	One episode of vaso-occlusive crisis occurred at 12 mo after infusion; no acute chest syndrome, stroke, or priapism; continued mild hemolysis; persistent osteonecrosis of femoral head	One episode of vaso-occlusive crisis occurred at 9 mo after infusion†; continued mild hemolysis; persistent osteonecrosis of femoral head


A Hemoglobin Fractionation and Percentage of F Cells
HbF
HbS
HbA
HbA2
Other hemoglobin
F cells
Participant 1
Participant 2
Participant 3
Total Hemoglobin (g/dl)
F Cells (%)
Screening
Months since OTQ923 Infusion
B Transfusions
Before screening
During study, before OTQ923 infusion
After OTQ923 infusion
Transfusion
24 Mo
12 Mo
16 Mo
17 Mo
10 Mo
22 Mo
4 Mo
2 Yr before study enrollment
Screening
OTQ923 infusion
Last follow-up
2 Mo after infusion
C Genome Editing
Approximately 5-kb Deletion
Indels at the HBG1 locus
Indels at the HBG2 locus
Editing (%)
Batch 1
Batch 2
Batch 3
1 Mo
2 Mo
3 Mo
6 Mo
12 Mo
18 Mo
OTQ923
Peripheral Blood
Bone Marrow

Methods

Hemoglobin fractionation and HbF assessment in clinical samples

Blood samples were collected to assess the hemoglobin fractionation by capillary electrophoresis.

F-cell analysis using flow cytometry in clinical samples

Whole-blood samples were fixed with 0.05% glutaraldehyde, and the cells were permeabilized with detergent

The permeabilized cells were stained with a phycoerythrin (PE)- conjugated monoclonal antibody specific for fetal hemoglobin.

HbF was estimated by fluorescence in the PE channel

Results

The participants had several adverse events:

All were considered by the investigators to be related to either busulfan or underlying sickle cell disease.

None of the adverse events were considered by the investigators to be related to OTQ923

Morphologic assessment of the bone marrow was performed in the first two participants

Before myeloablation and at 1 year after cell infusion.

Before myeloablation showed:

cellular marrow with erythroid-predominant trilineage hematopoiesis.

At 1 year after infusion:

Bone marrow in both participants had cellular marrow with relatively balanced and orderly trilineage hematopoiesis , a normal myeloid–erythroid progeny ratio, and no evidence of increased blasts or dysplasia.

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Table 2. Markers Suggestive of Ongoing Hemolysis and Hematopoietic Recovery in the Study Participants after OTQ923 Infusion.

Marker	Normal Range	Participant 1					Participant 2				Participant 3		
		Screening	3 Mo	6 Mo	12 Mo	18 Mo	Screening	3 Mo	6 Mo	12 Mo	Screening	3 Mo	6 Mo
Absolute reticulocyte count (million/mm^3)	0.021–0.085	0.3364	0.4025	0.2419	0.2721	0.1943	0.3034	0.3478	0.3951	0.3802	0.3444	0.1841	0.2321
Lactate dehydrogenase level (U/liter)	94–260	345	265	263	217	292*	397	387	284	243	359	341	325
Total serum bilirubin level (μmol/liter)	3–21	26	24	48	43	56*	60	32	65	72	67	31	31

* These laboratory tests were performed a few weeks after the participant had a motor vehicle accident and sustained a liver laceration. Hence their clinical significance should be interpreted in conjunction with the participant's clinical status.

Results

- After transplantation into immunodeficient nonobese diabetic mice with severe combined immunodeficiency (NOD SCID), healthy donor–derived gRNA-68–edited CD34+ HSCs showed durable long-term engraftment and multilineage reconstitution similar to that of mock-edited control cells. These findings indicated that genome editing with gRNA-68 did not impair the developmental potential of long-term repopulating HSCs.

- **Potential off-target editing sites:**
  Identified with the use of computational methods and SITE-Seq, which measures off-target indels caused by incubating purified DNA with ribonucleoprotein.(RNP)

- Of the 279 unique potential off-target sites identified in these screening assays, none were detected in gRNA-68–edited CD34+ HSCs by means of PCR and next-generation sequencing

- No abnormalities were identified in gRNA-68–edited CD34+ HSCs with a targeted next-generation sequencing cancer gene panel

Results

- On flow cytometry, the tiling CRISPR-Cas9 screen revealed several gRNAs that increased levels of F cells in erythroid progeny of edited CD34+ CELLS

- Editing with gRNA-68 increased the percentage of F cells to levels equal to or higher than those achieved with other gRNAs.

Discussion

Genetic modification of autologous HSCs has shown great promise for the treatment of sickle cell disease , although the best approaches are not yet established.

The lentiviral vector–mediated addition of an anti-sickling β-like globin is effective, although this approach does not eliminate βS-globin expression and creates a potentially toxic excess of β-like globin chains which can lead to ineffective erythropoiesis and erythroid dysplasia.

Induction of endogenous γ-globin transcription concomitantly reduces βS-globin, thereby maintaining the balance of α-globin and β-like globin chains

Disrupting the BCL11A erythroid-specific enhancer with CRISPR-Cas9 can increase fetal hemoglobin levels and alleviate symptoms of sickle cell disease

Disrupting a repressor element in the HBG1 and HBG2 promoters is a more targeted approach than eliminating erythroid BCL11A expression because the latter may impair erythropoiesis.

Discussion

- Through a CRISPR-Cas9 screen, They identified a repressor element in HBG1 and HBG2 promoters. In their preclinical testing, on disruption, this repressor element caused F-cell induction with an efficacy similar to that of disruption of the BCL11A erythroid-specific enhancer.

- . Although all three participants had at least one episode of vaso-occlusive crisis after infusion, the frequency of such episodes was very limited

- In Participants 1 and 2, cardiac, pulmonary, and renal function appeared to be stable or had improved at 12 months after infusion. This preservation of organ function — at least in the short term — raises hope that sickle cell disease–induced organ dysfunction may be prevented by this therapy.

- Osteonecrosis persisted and perhaps even worsened after treatment. Although exposure to busulfan could have contributed to worsening bone health, continued sickle cell disease–related damage cannot be ruled out.

Discussion

Despite improved hematologic levels and a reduction in the incidence of symptoms of sickle cell diseas
all the participants had ongoing mild hemolysis, findings that indicate that their red-cell fetal hemoglobin
levels were insufficient to inhibit sickle hemoglobin polymerization completely.

Hemolysis appeared to be somewhat improved in all the participants, as suggested by improved biochemical markers of hemolysis such as lactate dehydrogenase and serum bilirubin, but it was still
persistent as evidenced by an elevated reticulocyte count

the bone marrow assessments showing balanced and orderly trilineage hematopoiesis without any evidence of dysplasia.

Although the degree of fetal hemoglobin induction seen in these participants may be enough for attenuation of sickle cell disease, it is not sufficient for complete amelioration of disease.

Discussion

Previous studies of autologous HSCT for sickle cell disease used freshly collected cells to manufacture the cellular drug product.

The current study, we used a cryopreserved apheresis product to facilitate future access to OTQ923 therapy for the majority of patients with sickle cell disease who reside in low-resource settings.

CONCLUSION

They found that Cas9 disruption of a negative regulatory region in the HBG1 and HBG2 promoters of autologous HSCs obtained from participants with sickle cell disease resulted in induction of red-cell fetal hemoglobin and a partial correction of sickle cell disease.

Busulfan was used as a myeloablative agent in this study, as has been the case in most other ongoing studies.

Given the associated toxicity profile of busulfan, alternatives such as reduced-intensity melphalan, alternative reduced-toxicity alkylating agents such as treosulfan and nongenotoxic antibody -drug conjugates should be evaluated in future clinical trials.

Although the long-term durability of the response and the safety of this genetically modified product continue to be evaluated,

Their data suggest that this approach offers a safe and potentially disease-attenuating option for patients with severe sickle cell disease.

Thanks for Attention