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Innovation Method to Deliver Gene Editor ABE to Treat Sickle Cell Disease by Nanotechnology Combined with DNA Origami

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Abstract: *Sickle cell disease (SCD) is one of the most common genetic disorders worldwide. In some developing nations, up to 90% of people with such a disorder would die before their fifth birthday. In the contemporary genomic era, an affordable gene editing therapy could make it possible to end this tormont once for all. In this proposal, using a DNA nanoparticle as delivery vehicle formulated with CD34 antibody, we are introducing the CRISPR-deprived, single-base altering system, the adenine-base editor (ABE), to edit the disease genotype CD34+ hematopoietic stem cells (HSC) in patient bone marrow into a non-pathological, naturally occurring genotype.*

Keywords: *Sickle Cell Disease (SCD), DNA Cages Self-assemble, Adenine-Base Editor (ABE), Nanoparticle Drug*

1. Introduction

Sickle cell disease is one of the most commonly occurring monogenic disorders worldwide [1]. CRISPR Adenine base editor (ABE) is the most feasible approach for curing SCD was proposed by Newby and colleagues. ABE has a complex of a deaminase coupled with a partially deactivated Cas9 (dCas9), which could use sgRNA to recognize sequence to cleave but could only introduce single stranded instead of double stranded break. Edited HSC was infused to immunodeficient mice who had undergone bone marrow irradiation to kill endogenous mice HSC in bone marrow so implantation could be more successful.

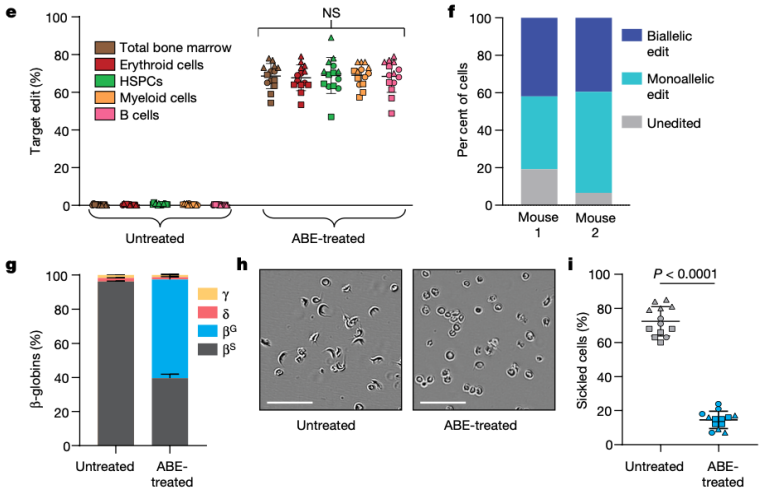

Figure 1: Outcomes of red blood cell population after infusioning of ABE edited pathogenic phenotype HSC (hematopoietic stem cells). e) percentage of cells from different lineages derived from bone marrow. f) allelic editing outcomes determined from single-cell 5' RNA seq using CD235a+ bone marrow cells. g) proportions of pathogenic HBB-S vs non-pathogenic HBB-G in reticulocytes (developing, immature red blood cells) before and after editing as determined by HPLC. h) morphological differences between ABE treated and untreated reticulocytes. i) percentage of HSC exhibiting sickle cell phenotype after treatment with ABE (n = 14, different shapes indicating different donor source of human donor for HSC used in the experiment [2]

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Therapy method proposed by Newby and colleagues is good but the delivery method for the therapy is not so clinically feasible. The recipient mice receiving HSC treatment had to undergo lethal doses of gamma irradiation to eliminate a drastic fraction of bone marrow (which contains the vast majority of hematopoietic stem cells, HSCs), posing significant safety concerns. It may be more desirable to seek methods without the need to destroy original colonies of HSC in bone marrow. An approach would be to formulate the adenine base editor (ADE) and deliver it to the bone marrow, through the intravenous route to achieve in vivo editing. Figure 1 shows some statistics about the treated mice, including the percentage of β globins, cell morphology, etc. It can be clearly seen that the cells of the treated mice have returned to normal morphology, proving the effectiveness of this treatment.

Some of the state-of-the-art delivery methods for delivery of CRISPR systems that could be feasible for in systematic delivery includes viral methods (lentivirus, adeno-associated virus, adenovirus), and lipid-based nanoparticle [3]. Viral delivery method is limited by the molecular size of the cargo, potential risks of carcinogenesis caused by random insertion, and potential off-target effect as the result of overexpression of CRISPR system [3]. As of today, research has shown efficient lipid-based nanoparticle in vivo delivery of mRNA of Cas9 and sgRNA targeting PKL1 gene in metastatic ovarian cancer model using OV8 cell line with intraperitoneal pathway [4]. However, these nanoparticles require denaturing agents, such as ethanol, that may affect the stability and catalytic activity of the ribonuclease and deaminase used in editing. Moreover, an issue for base editor is that it is a duplex of protein linked with an amino acid chain. Formulating the ABE into mRNA does not guarantee correct folding and enzymatic activity of the enzymes after transcription, which is the reason why authors of Newby electroporated the ABE-sgRNA complex into the HSCs.

2. Proposed solution

We propose to use a type of DNA RCR-based ligand-conjugated nanoparticle as a vehicle to encapsulate the ABE-sgRNA in its functional form for delivery, through the IV route. By using adenine base editing, the treatment approach is nearly identical to reverting the disease into healthy genotype. Our delivery method (through formulation into enzyme-friendly DNA origami nanoparticle and IV-based delivery) is also safer and cheaper than the method used in previous publication, and potentially confers better targeted delivery profile.

The version of DNA origami nanoparticle used to deliver CRISPR-Cas9 was originally designed by Sun and colleagues [5]. In our formulation, we are replacing the Cas9 enzyme with the ABEe-NRCH protein for single-base editing. Figure 2 shows the basic design of the origami-based DNA nanoparticle system used for delivery for CRISPR-Cas.

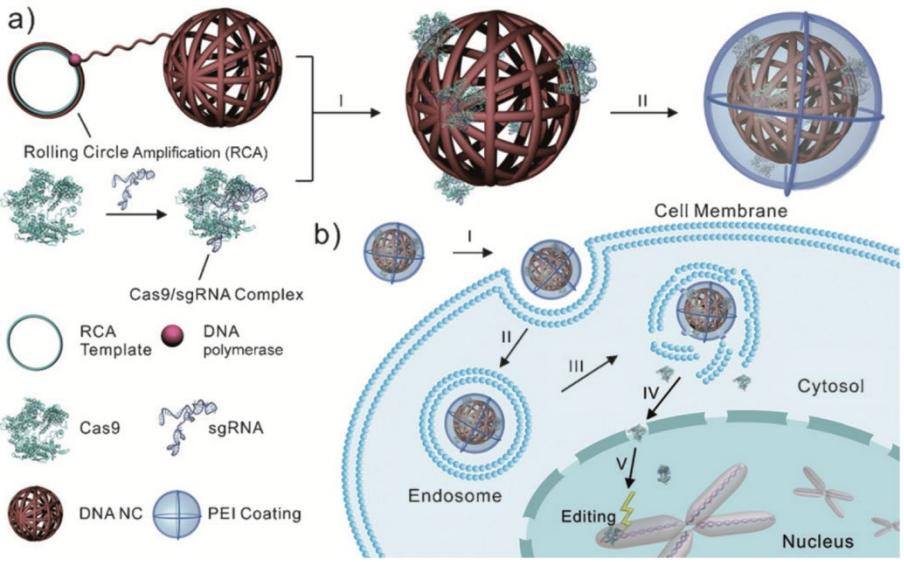

Figure 2: Design of the origami-based DNA nanoparticle system used for delivery for CRISPR-Cas9 [5].

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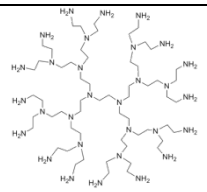
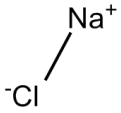
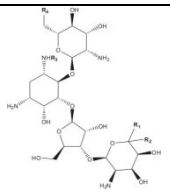
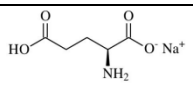
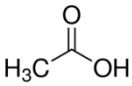
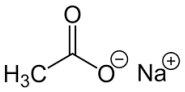
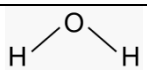
2.1. Drug components (GMP standards will be applied for steps):

Table 1 is a reference for formulation, the actual amount to be added needs to be determined by subsequent experiments.

Table 1: Drug components

substance	structure	function	amount to be added
Adenine base editor (ABE8e-NRCH)	See entry 6VPC in protein data bank	API	To be determined
sgRNA	5′-CUAACAGUUGCUUUUAUCAC-3′	API	To be determined
DNA origami	See Sun, et al. 2015 for picture ref.	delivery vehicle	To be determined
PEI		coating surfactant	0.2mg
CD 34 antibody		ligand	
sodium chloride		saline stabilizer	0.9%
Neomycin		preservatives antibiotic	30μg/mL
Aluminum Chloride		adjuvants	0.2mg
Monosodium Glutamate(MSG)		stabilizer	2mg
Acetic acid		buffering	5mg
Solium Acetate		buffering	5mg
Purified water		solvent	1ml

2.2. Production, manufacture, assemble, and characterization

2.2.1. Production and purification of API: ABEe-NRCH Protein [6]

Plasmid pRha-ABE8e-NRCH (Addgene#165417, or ABE plasmid) will be purchased from Addgene. BL21StarDE3 strain from ThermoFisher will be used for production of the protein. After overnight incubation, the inoculum will be added to pre-warmed LB media. Cells will be cultured with shaking

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until the OD600 value reaches around 1.5. The culture will be cold-shocked in a water-ice bath for 1h, then L-rhamnose will be added to reach a final concentration of 0.8% in culture to induce expression. Culture will then be incubated at 18ºC and shaking for 24h for protein induction. Cell pellets will be snap-frozen in liquid nitrogen and stored at -80ªC (if protein extraction is not to be done on the same day).

Cold lysis buffer supplemented with 5 tablets of cOmplete, plus protease inhibitor mix (Millipore Sigma, 4693132001). Cells will be passed three times through a homogenizer at ~18,000 psi to lyse. Centrifuging for 20 min at 4 ªC. The supernatant will be treated with imidazole, then incubated for 1 h with Ni-NTA resin slurry. Protein-bound resin will be washed with lysis buffer at 4 ºC. Elution buffer will be added to elute the protein. Eluted protein will be diluted in a 40 mL low-salt buffer. Ion-exchange chromatography will be used to purify the protein (e.g., Akta Pure25 FPLC), and fractions of protein could be concentrated by first using a 15-mL centrifugal filter then by a 0.5 mL 100-kDa cutoff Pierce concentrator. The concentrated protein could be quantified via a BCA assay kit, and then analyzed by a SDS-Page gel. More details, including the reagents used by the original authors are in the publication [6].

2.2.2. Production of API: guided RNA sequence, sgRNA[6]

The DNA sequence of the sgRNA containing a T7 promoter will be transcribed using a TranscriptAid T7 High Yield Transcription Kit (ThermoFisher). Purification of the sgRNA will be conducted using the MEGAclear Kit (ThermoFisher). All aforementioned protocols were conducted according to the manufacturer's guidelines.

2.2.3. Production of CD34 antibody and conjugation with PEI

To produce CD34 antibody used for human clinical trials, transgenic chicken with human anti CD34 antibody would lay an egg enriched with targeted antibody [7].

PEI solution and CD34 antibody were mixed at a ratio of 1:1, and a mixture of pH 7.5 and MOPS: NaCl=1:5 was added. The mixture was incubated at room temperature for 2 hours by shaking gently. A strong cation exchanger and MOPS: NaCl=2:1 was used to wash away the unbound antibodies. Finally, the conjugated compounds generated by elution of the mixed solution with pH =7.3 and composition ratio of MOPS: NaCl=1:150 were used to obtain the desired PEI/ CD34 mixture[8].

2.3. Industrial/large scale production of ABE, sgRNA, and CD34 antibody

Optimization of expression vectors for the ABE editor and CD34 antibody may pose a challenge. Overexpression of Cas9 protein in bacteria has shown to negatively affect the growth of bacteria, [9], so it would be reasonable to assume that the production of ABE has the same problem. sgRNA could be flanked by two ribozyme sequences, 5' hammerhead and 3' hepatitis delta virus to be induced by a strong RNA polymerase II promoter [9].

Rolling replication (RCR) to synthesize the circular cage-shaped DNA NC, then it will be used as a vehicle for Cas9/ABE delivery. Cas9 binds to selected ABE(ABE8e-NRCH) to form the Cas9/ABE complex. ABE is loaded with part of sgRNA to edit human HSPCs ex vivo. Guide protein sgRNA [5].

As the single stranded DNA (ssDNA) can base pair with the guide portion of sgRNA, which is self-assembled on ABE, we designed DNA NCs to partially supplement sgRNA. After assembling the DNA NCs with the Cas9 / ABE complex, we coated the entire NC with a coating made of the cationic polymer polyethylenimine (PEI), which helped induce the endosomes to escape.

When the entire drug cage is delivered to the cytoplasm, it can be delivered to cells and cells for gene editing by fusing to the cas9 site nuclear localization signaling peptide. The process of drug entry is:

1) binding to cell membrane

2) endocytosis

3) endosomal escape

4) Migration to the nucleus

5) Search for target DNA sites in chromosomes and introduce double strand breaks for genome editing.

2.3.1. DNA cages self-assemble

We synthesized DNA cages using rolling circular replication (RCR) technology.

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Rolling circle amplification (RCA) is a thermostatic DNA amplification technique, which is a simplified version based on the original RCA replication. The RCA process can be found from figure 3. The initiation of replication begins primarily with initiation proteins encoded by the plasmid or phage genome. The starting protein makes a cut at a fixed position in the DNA to form a double-linked DNA molecular chain, known as double strand origin (DSO). The starting protein is bound to the 5' phosphate end of the notch chain, and the free 3' hydroxyl end is released, which acts as a primer for DNA synthesis by DNA polymerase III. We can take an intact DNA strand as a template, replace the broken DNA strand with a single strand, and replicate around the circular DNA molecule. When the plasmid replication initiation protein is present, the notch chain is replaced by a helicase enzyme encoded by the host, called PcrA(short for plasmid replication Reduction pair).

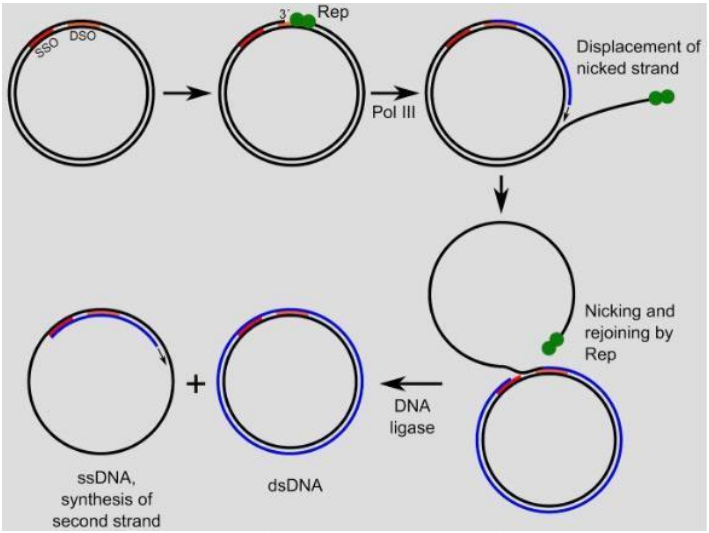

Figure 3: Rolling Circle Replication technology (Figure of courtesy: Wikipedia "Rolling Circle Replication")

First, continuous DNA synthesis can be done in tandem, by producing multiple single-stranded linear copies of the original DNA to form a continuous sequence end to end. We can convert the resulting linear copy into a double-stranded circular molecule by Figure 4:

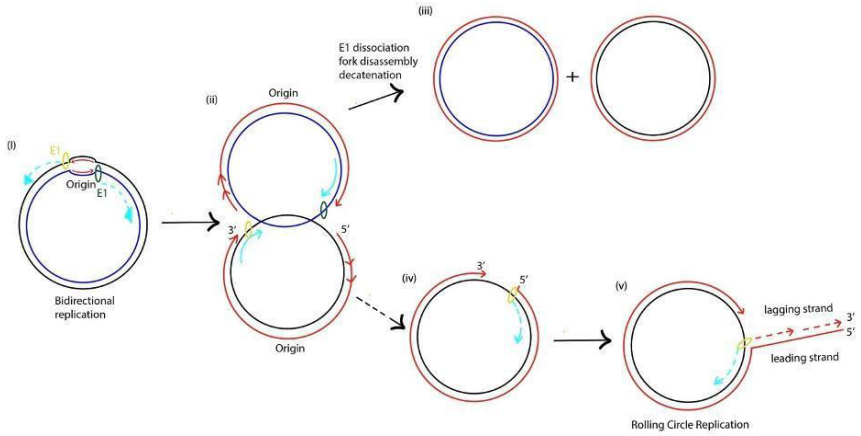

Figure 4: HPV Rolling Circle Replication Model (Figure of courtesy: Wikipedia "Rolling Circle Replication")

Rolling circular replication involves two processes that must take place simultaneously:

1) DNA polymerase must synthesize sequences that are complementary to the circular template

2) During replication, there must be some mechanism to initiate parental duplexing to allow the polymerase to advance.

In general, polymerase is preceded by a helicase or single-stranded DNA binding activity that allows replication to continue.

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The target DNA cage was synthesized by RCR. First, long linear single stranded DNA encoding aptamer complementary sequences was prepared. Since Nc assembly is independent of template sequence, all aptamer complementary sequences can be used in our strategy.

2.3.2. PEI preparation and final assembly of the nanoparticle drug complex

Branched structure is chosen because branched structure is more soluble and stable in aqueous solution. Branched PEI formation can be done by ring opening polymerization of aziridine [10]. Branched PEI is highly protonable, and polycationic, keeping them stable over a wide range of pH and positive charge helps PEI bind with DNA molecules.

2.3.3. CD34 antibody

Our drug target is hematopoietic stem cells in bone marrow, so CD34 antibody can be used as a ligand to guide drug particles to the targeting site. CD34 antigen is expressed both in humans and mice, which is a suitable biomarker for clinical trials.

3. Quality control

3.1. CD34 antibody titration, purification and binding effectiveness

Apply BSA conjugated peptide on the ELISA plate and let it stand for 24 hours. Plate was rinsed f20 minutes μ G / ml in PBS for 5 times, then the blocked nonspecific binding site and incubate for 2h. The ascites removed from the mouse abdomen were then added to the 1:4000 diluted HRP combined with Rabbit anti mouse Ig and cultured for 2 hours. Acid solution is used to stop the reaction. Measure reaction optical density by ELISA reader at 450nm [11].

CD34 antigen with 1/32000 shows the highest absorption. Antibody obtained from chromatography was 5.5mg. In reducing SDS-PAGE (figure 5.part a), the light chain and heavy chain of antibody are shown, which were 25kd and 50kd respectively. In non-reducing gel （ figure 5.part b), only one 150KD band represented a purified antibody.

CD34 antibody was labeled with fluorescent dye. The image (figure 5) showed that CD34 antibody could effectively guide particles to hematopoietic stem cells.

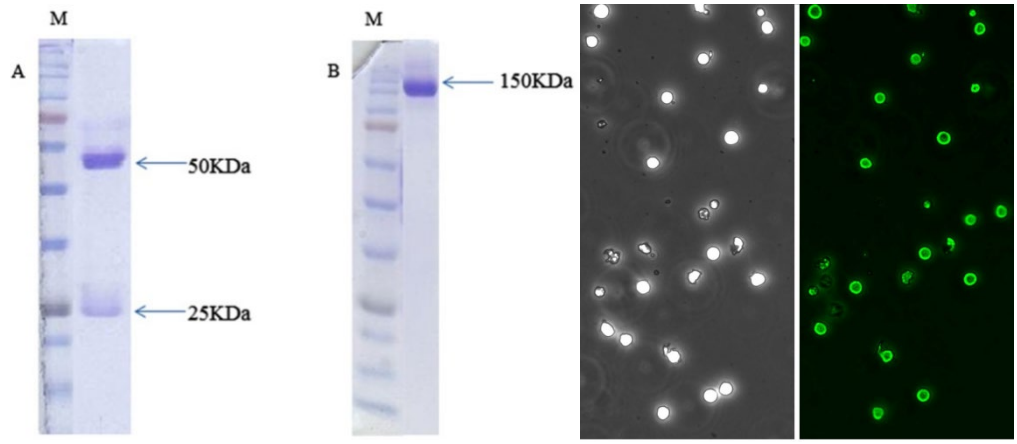

Figure 5: (a). SDS-PAGE result shows antibody purification fraction. (b). CD34 antibody guide particles to hematopoietic stem cells [5].

3.2. ABE Editing Efficiency and Safety

Drug efficiency can be analyzed by measuring the converting rate of HBB^S to HBB^G. We cultured unedited or ABE edited SCDS at 2% oxygen concentration in vitro. The frequency of sickle shaped cells decreased from 47.7% to 16.3%, which proved that this gene editing technology can effectively treat sickle cell anemia. Figure 6 shows that ABE-treated cells show normal cell morphology compared with untreated cells, which proves the efficiency of ABE editing.

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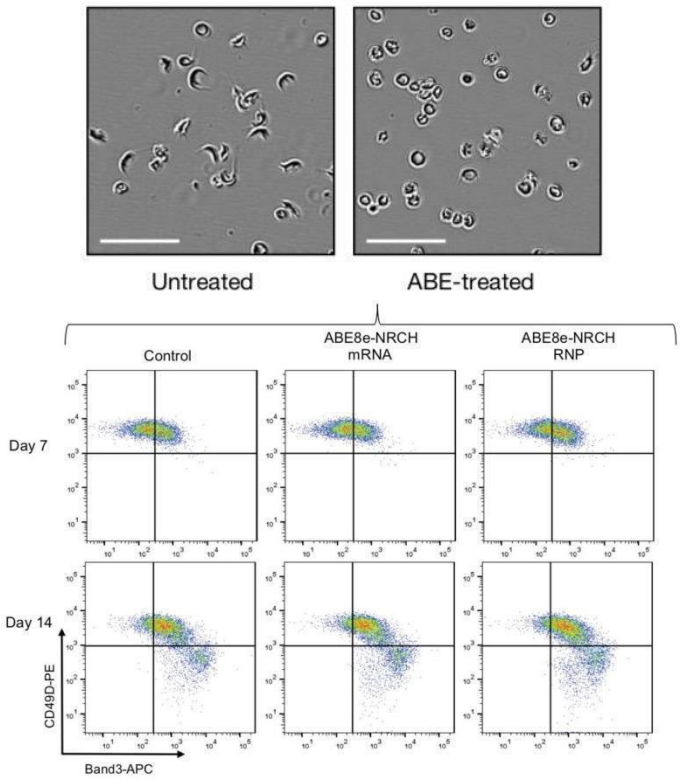

Figure 6: (a). Comparison of untreated and edited cell morphology. (b). flow cytometry for CD49d and BAND3. [6]

4. Preclinical Study

(Unless noted otherwise, each experiment will be repeated for three times for reproducibility)

4.1. In vivo study (All sections of in vivo study has been referred from research by Newby, et al. 2021)

Animal model selection ([6]; Jackson laboratory Website)

Humanized Towners SCD mice from Jackson laboratory (#013071) carry several human hemoglobin knock-in genes in place of endogenous mouse genes (Jackson Laboratory), possible alleles including:

1) Hba-tm1(HBA)Tow), which could heterotetramer in to conjugate with other human origin hemoglobins,

2) Hbb-tm2(HBG1, HBB*) Tow mutation (γ-βS), replacing mouse endogenous major and minor beta-globin with hemoglobin gamma and human sickle cell hemoglobin beta (Beta-S or βS),

3) Hbb-tm3(HBG1, HBB) Tow mutation (383 γ-βA), replacing endogenous mouse major and minor beta-globin with human hemoglobin gamma (Aγ) and and wildtype beta-A (βA)

Mice homozygous for 1) and homozygous for 2) have sickle cell disease phenotype (ha/ha::Beta-S/beta-S).

Mice homozygous for 1) and homozygous for 3) have normal phenotype (ha/ha::beta-A/beta-A), with potential to serve as healthy control.

Mice homologous for 1) and heterozygous with a copy of 2) and 3) is sufficient to rescue sickle cell phenotype (ha/ha: beta-A/beta/S).

4.2. IV administration of the drug (CD34-ABE-nano, ABE-nano, nano) and infusion of the ex-vivo-edited HSC (CD34-ABE-nano, ABE-nano, nano).

In the model of Towners SCD, 1mg/kg, 5mg/kg, 10mg/kg, and 20mg/kg of dose of CD34-ABE-nano,

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or ABE-nano, or nano, will be injected intravenously into ha/ha::beta-A/beta-A (healthy) or ha/ha::beta-S/beta/S (humanized sickle cell disease phenotype). Each treatment group will have a sample size of n=10. Blood will be sampled at time points of 6, 10, 14, 16, 32, 48, 64, 72 weeks and all the way till the mice pass away due to natural death after transplantation via saphenous vein collection. The blood smear in figure 7 is a comparison between unedited and edited HBB. The edited HBB blood smear shows the normal cell phenotype.

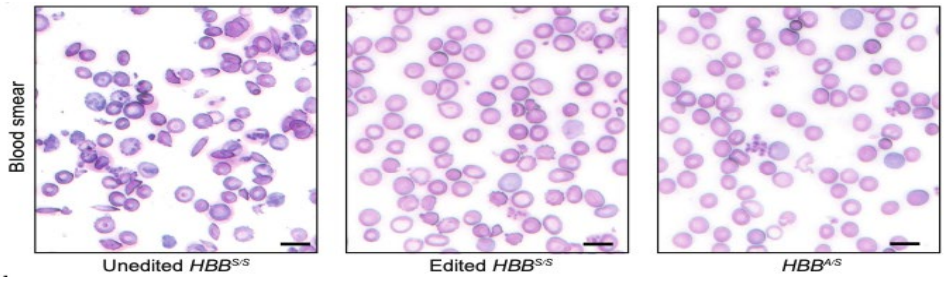

Figure 7: Blood smear analysis of Lin- mouse HSCs with human HBB alleles at week 16 [5].

4.3. Pharmacokinetic analysis

In a separate group of SCD mice receiving the treatments, 100 uL blood of the mice will be collected at different timepoints after the IV administration via saphenous vein collection. The RNA in the of the serum will be extracted from the blood using a QIAGEN miRNeasy Serum/Plasma Kit and subject to Northern Blot analysis for quantification of the sgRNA used for the ABE. The ratio of sgRNA between each sample from blood could be a proxy for the amount of nanoparticles in the blood at the given time. A calibration between the milligrams of nanoparticles and the corresponding Northern Blot signal intensity will be performed.

4.4. Post-necropsy analysis of mouse samples: Bone marrow analysis [6]

4.4.1. HTS for examining editing outcome in red blood cells (CD235+)

CD235+ red blood cells will be subjected to magnetic -activated cell sorting (MACS), and the purified CD235+ cells will be subjected to single-cell RNA-seq.

4.4.2. Ex vivo sickling assay

Isolated CD235+ red blood cells from mice will be cultured in a 2% oxygen environment to induce sickling. In study by Newby et al., RBC from unedited mice had shown 86.3+/-3% sickling, whereas mice infused with ABE edited HSC had 29.8+/-6.5% of sickling, yielding a 2.9 fold decrement of sickling.

4.4.3. Splenomegaly and spleen histology

Spleen will of mice from each group will be weighed. As SCD is known to induce splenomegaly, it was reported by Newby et al. that mice received edited HSC has spleen weight of 0.22+/-0.043g, mice received unedited HSC (beta-S/beta-S) have spleen weight of 0.39+/-0.016g, and mice with received normal phenotype HSC (beta-S/beta-A) have spleen weight of 0.11+/-0.007 g.

Spleen of mice will be stained with H&E. SCD could induce extramedullary erythropoiesis and vascular congestion. From figure 8, the reduction of red pulp and induction of white pulp could be seen as indication of improvements.

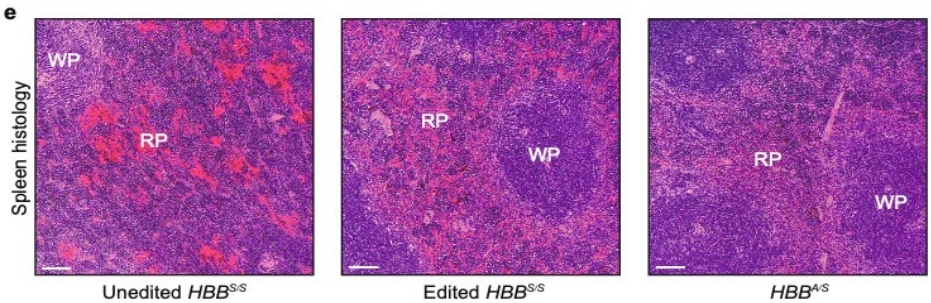

Figure 8: Histological staining showing amelioration of extramedullary erythropoiesis (red).

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5. Sample Clinical Study Description for Phase I

5.1. Study population

Patients with SCD, ages 18 and older who have SCD.

5.2. Eligibility Criteria

a. inclusion criteria

1) Patients with no more than two single nucleotide polymorphisms in the DNA region flanking the base pair to be edited as indicated by next generation sequencing (NGS) results.

2) Per NGS result, patients who have DNA sequences elsewhere with high similarity toward the sgRNA complementary sequence (e.g., more than or equal to 15 base pairs out of total 20) will be excluded due to high risk of off-targeting effect.

3) Patients requiring infusion therapy

b. Exclusion criteria

1) Patients with more than two single nucleotide polymorphism in the genomic region indicated above (may experience significant off target effect)

2) Informed consent form not signed

3) patients not hospitalized in a ward that is participating in the study.

4) patients participating in another clinical study or clinical field test

5) Active, acute manifestations of sickle cell disease including painful crisis, acute chest syndrome, cerebrovascular events or active infection.

6) Pregnant women will not be eligible for study enrollment.

Biomarkers for drug response: from patient blood samples

1) Decrement of fractions of pathogenic Beta-S protein in the patient blood sample (initially 100%; in vivo endpoint 40%) by HPLC analysis

2) Percentage of sickling cells via blood smearing, record % of patient cells with sickling before treatment and after treatment (from 60-80% in vivo before to 5-20% in vivo after treatment).

3) High throughput sequencing for blood cell genotype (per vivo result from [6]: 20% unedited in vivo, 40% monoallelic, 20% biallelic).

6. Conclusion

After reviewing existing treatment options and the one proposed by Newby and colleagues, we proposed an improved approach, to formulate the adenine base editor (ADE) and deliver to the bone marrow, through the intravenous route to achieve in vivo editing. This innovation method is to deliver gene editor ABE to treat sickle cell disease by nanotechnology combined with DNA Origami. The article introduces the production of ABE, sgRNA, the process of DNA cages self-assemble, and PEI preparation and final assembly of the nanoparticle drug complex. Next, we conducted some in vivo experiments to prove the feasibility of this new method. From the experimental data and blood smear, the treated cells have restored their normal cell morphology, thus proving the effectiveness of this approach. To help companies and research institutions interested in this program conduct further research, this article also designs a clinical study model for phase I. Although the article provides reference for the synthesis of each part, the actual amount added to the formulation still needs further experimental verification.

References

[1] Rees DC. Williams TN. Gladwin MT. Sickle-cell disease. Lancet. 2010 Dec 11; 376(9757): 2018-31. doi: 10. 1016/S0140-6736(10)61029-X. Epub 2010 Dec 3. PMID: 21131035.
[2] Piel FB. Steinberg MH. Rees DC. Sickle Cell Disease. N Engl J Med. 2017 Jul 20; 377(3): 305. doi:

ISSN 2616-5791 Vol.5, Issue 6: 45-54, DOI: 10.25236/AJMHS.2024.050607

10. 1056/NEJMc1706325. PMID: 28723338. Vaccine excipients summary https: //www. pharmaexcipients. com/stabilizer/vaccine-excipient-summary/

[3] Yip BH. Recent Advances in CRISPR/Cas9 Delivery Strategies. Biomolecules. 2020 May 30; 10(6): 839. doi: 10. 3390/biom10060839. PMID: 32486234; PMCID: PMC7356196.

[4] Rosenblum D. Gutkin A. Kedmi R. et al. CRISPR-Cas9 genome editing using targeted lipid nanoparticles for cancer therapy. Sci Adv. 2020; 6(47): eabc9450. Published 2020 Nov 18. doi: 10. 1126/sciadv. abc9450

[5] Sun W. Ji W. Hall JM. Hu Q. Wang C. Beisel CL. Gu Z. Self-assembled DNA nanoclews for the efficient delivery of CRISPR-Cas9 for genome editing. Angew Chem Int Ed Engl. 2015 Oct 5; 54(41): 12029-33. doi: 10. 1002/anie. 201506030. Epub 2015 Aug 27. PMID: 26310292; PMCID: PMC4677991.

[6] Newby. G. A. Yen. J. S. Woodard. K. J. et al. Base editing of haematopoietic stem cells rescues sickle cell disease in mice. Nature 595. 295–302 (2021). https: //doi. org/10. 1038/s41586-021-03609-w

[7] Frenzel A. Hust M. Schirrmann T. Expression of recombinant antibodies. Front Immunol. 2013; 4: 217. Published 2013 Jul 29. doi: 10. 3389/fimmu. 2013. 00217

[8] Strehblow C. Schuster M. Moritz T. Kirch HC. Opalka B. Petri JB. Monoclonal antibody-polyethyleneimine conjugates targeting Her-2/neu or CD90 allow cell type-specific nonviral gene delivery. J Control Release. 2005 Feb 16; 102(3): 737-47. doi: 10. 1016/j. jconrel. 2004. 10. 033. PMID: 15681094.

[9] Zhang S. Guo F. Yan W. Dai Z. Dong W. Zhou J. Zhang W. Xin F. Jiang M. Recent Advances of CRISPR/Cas9-Based Genetic Engineering and Transcriptional Regulation in Industrial Biology. Front Bioeng Biotechnol. 2020 Jan 28; 7: 459. doi: 10. 3389/fbioe. 2019. 00459. PMID: 32047743; PMCID: PMC6997136.

[10] W. T. Godbey. K. K. W. Antonios G. Mikos. Poly (ethylenimine) and its role in gene delivery. Controlled Release. 1999. 60(2-3): p. 149-60.

[11] Sahoko Matsuoka. Yasuhiro Ebihara. Ming-jiang Xu. Takefumi Ishii. Daisuke Sugiyama. Hiroshi Yoshino. Takahiro Ueda. Atsushi Manabe. Ryuhei Tanaka. Yasuo Ikeda. Tatsutoshi Nakahata. Kohichiro Tsuji; CD34 expression on long-term repopulating hematopoietic stem cells changes during developmental stages. Blood 2001; 97 (2): 419–425. doi: doi: 10. 1182/blood. V97. 2. 41