

GENOME ENGINEERING IN HEMATOPOIETIC STEM CELLS FOR THE TREATMENT OF BETA GLOBIN DISORDERS

ANILA GEORGE

PhD THESIS

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SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND
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A THESIS SUBMITTED BY

ANILA GEORGE

TO

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES
AND TECHNOLOGY, TRIVANDRUM.

IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

2024

DECLARATION BY THE STUDENT

CERTIFICATE

I , **ANILA GEORGE** , hereby certify that I had personally carried out the work depicted in the thesis titled, “**GENOME ENGINEERING IN HEMATOPOIETIC STEM CELLS FOR THE TREATMENT OF BETA GLOBIN DISORDERS**”.

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*Clearance was obtained from the Institutional Ethics Committee / Institutional Animal Ethics / Institutional Committee for Stem Cell Research / Other appropriate committees for carrying out the study.

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THE TREATMENT OF BETA GLOBIN
DISORDERS”**

Submitted by

ANILA GEORGE

for the degree of

Doctor of Philosophy

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TABLE OF CONTENTS

Contents	Page
Declaration by student	i
Certificate by guide	ii
Approval of the thesis	iii
Acknowledgements	iv
Table of contents	viii
List of figures	xi
List of tables	xiv
List of abbreviations	xv
Synopsis	xix
1. Introduction	
1.1 Background	1
1.2 Aim and objectives	5
2. Literature Review	
2.1 Hemoglobin	6
2.2 Hemoglobin switching	6
2.3 Hemoglobinopathies	8
2.4 Sickle Cell Disorder	8
2.5 Beta Thalassemia	9
2.6 Hereditary Persistence of Fetal Hemoglobin (HPFH)	10
2.7 Regulation of gene expression.....	11
2.8 Role of introns in gene regulation	11
2.9 Conventional treatment strategies for Beta hemoglobinopathies	12
2.10 Gene Therapy	12
2.11 Programmable Nucleases for gene editing	13
2.12 CRISPR/Cas9 system	13

2.13 Cas9 Variants	14
2.14 Base Editors	15
2.15 Prime Editors	16
2.16 Gene Editing approaches for beta hemoglobinopathies.....	17
2.17 Large interstitial deletions- undesired outcome of Cas9 mediated editing in homologous regions	19
3.Materials and methods	
3.1 Cell culture : Cell lines and primary cells	21
3.2 Cloning	24
3.3 gRNA/pegRNA designing and cloning	27
3.4 Preparation of stable cell lines	30
3.5 gRNA/ pegRNA lentivirus preparation and transduction	31
3.6 mRNA synthesis by in vitro transcription (IVT).....	31
3.7 pegRNA transfection.....	32
3.8 Nucleofection using Lonza electroporator.....	32
3.9 Nucleofection using Maxcyte electroporator	32
3.10 Nucleic acid isolation	33
3.11 Evaluation of editing efficiency	33
3.12 qRT PCR	33
3.13 Vector copy number analysis	35
3.14 MLPA (Multiplex ligation-dependent probe amplification)	35
3.15 HbF intracellular staining	35
3.16 Enucleation and differentiation	35
3.17 CFU assay	35
3.18 Cell sorting	36
3.19 HPLC	36
3.20 Evaluation of 4.9 kb deletion using qRT PCR	36
3.21 Evaluation of 4.9kb deletion using ddPCR	37
3.22 Evaluation of large deletion using conventional PCR	37
3.23 Statistical Analysis	38

4.Results

4.1: Improving the precision of base editing in globin locus using non-nicking approaches	39
4.1.1: Frequency of interstitial deletion varies with gRNAs and the efficiency of base editing	39
4.1.2: dCas9 ABE8e can edit efficiently in cell lines and in primary cells while overcoming large deletions	41
4.1.3: dCas9 ABE8e can prevent the formation of InDels and large deletions during multiplexed editing in HSPCs.....	45
4.1.4: Characterization of the differences in editing by dABE8e and nABE8e in HSPCs	46
4.2: Identifying regulatory elements in the gamma globin gene amenable to genome engineering for elevation of fetal hemoglobin	48
4.2.1 Intron 1 of gamma globin harbors regulatory elements	48
4.2.2 Nicking but not editing is responsible for elevated fetal hemoglobin while targeting gamma globin intron	53
4.2.3 The effect of DNA nicking is not evident in transcription factor binding sites at the promoter	56
4.2.4 Transient nicking is sufficient for altering gene expression for a prolonged period	57
4.2.5 D10A nickase but not H840A nickase elevates fetal hemoglobin	59
4.2.6 Nick mediated HbF elevation is observed in primary HSPCs as well	61
4.3 Correcting the causative point mutation of sickle cell disease in beta globin gene (E6V) using prime editing	64
4.3.1 Generation of a modular lentiviral PE system	64
4.3.2 Lentiviral delivery of PE2 enables efficient editing in HEK293T cells across multiple target sites	65
4.3.3 PE2 mediated editing in human erythroid cell lines	66
4.3.4 Modelling of sickle cell anemia in HUDEP-2 cell using PE2	69
4.3.5 Correction of sickle mutation and reconstitution of adult	

hemoglobin using in HUDEP2 cells using PE2.....	72
4.3.6 pegRNA mediated large deletion can be overcome by careful selection of pegRNAs	74
4.3.7 Evaluation of mRNA mediated delivery of prime editor.....	75
5. Discussion	
5.1 Improving the precision of base editing in globin locus using non-nicking approaches	76
5.2 Identifying regulatory elements in the gamma globin gene amenable to genome engineering for elevation of fetal hemoglobin	78
5.3 Correcting the causative point mutation of sickle cell disease in beta globin gene (E6V) using prime editing	79
6. Summary and conclusion	84
6.1 Future direction	86
7. Bibliography	87
Annexure	
List of publications	
Curriculum vitae	
Appendices	
Appendix a - Ethics committee approval	
Appendix b - Publications	
Appendix c - Plagiarism check report	

LIST OF FIGURES

Figure No	Figure Caption	Page No
Figure 2.1	Developmental switching of hemoglobin production	7
Figure 2.2	Transcription factors involved in regulation of globin gene expression.	10
Figure 2.3	CRISPR Cas9 based genome editing tools	16
Figure 2.4	Genome editing strategies for treatment of hemoglobinopathies	18
Figure 3.1	Strategy for cloning of pegRNA into lentiviral vector	30
Figure 3.2	Strategy for detection of 4.9 kb deletion by qRT PCR	37
Figure 3.3	Strategy for detection of 4.9 kb deletion by ddPCR	37
Figure 3.4	Strategy for detection of 4.9 kb deletion by conventional PCR	38
Figure 4.1.1	Schematic representation of gRNAs in gamma globin promoter used in the study	39
Figure 4.1.2	Evaluation of nick mediated deletion	41
Figure 4.1.3	Base editing in HUDEP 2 cells using ABE8e and dABE8e	42
Figure 4.1.4	Base editing in gamma globin promoter using dABE8e	43
Figure 4.1.5	Base editing in beta globin gene using dABE8e	44
Figure 4.1.6	Multiplexed base editing in beta globin using dABE8e in HSPCs	45
Figure 4.1.7	Characterization of dABE8e mediated base editing in HSPCs	47

Figure 4.2.1	Mutagenesis screening of gamma globin intron 1 using ABE7.10	49
Figure 4.2.2	Evaluation of gRNAs in gamma globin intron 1 using ABE8e	51
Figure 4.2.3	Evaluation of gRNAs in gamma globin intron 1 using CBE	52
Figure 4.2.4	Evaluation of gRNAs in gamma globin intron 1 using dCas9 ABE8e and nCas9	54
Figure 4.2.5	Evaluation of the effect of DNA nicking on HbF levels in gamma globin promoter	56
Figure 4.2.6	Evaluation of genotypic and phenotypic outcomes after transient editing in gamma globin intron.	58
Figure 4.2.7	Comparison of different Cas9 proteins for the HbF regulation potential.	60
Figure 4.2.8	Evaluation of nick mediated HbF elevation in HSPCs.	62
Figure 4.2.9	Representative flow cytometry data of edited HSPCs	63
Figure 4.3.1	Development of modular lentiviral prime editing system	65
Figure 4.3.2	Evaluation of lentiviral prime editing system in cell lines.	67
Figure 4.3.3	Installation of HBB E6V mutation using lentiviral prime editing system in cell lines	69
Figure 4.3.4	Schematic representation of the workflow for creation of sickle mutation in HUDEP2 cell line	70
Figure 4.3.5	Creation of sickle mutation in HUDEP2 cells	71
Figure 4.3.6	Characterization of HUDEP2 sickle cell disease model	72
Figure 4.3.7	Correction of sickle mutation using Prime Editing	73
Figure 4.3.8	Evaluation of alternative pegRNAs for correction of sickle mutation	74
Figure 4.3.9	Evaluation of PE2 mRNA in HUDEP cells	75

LIST OF TABLES

Table No	Table Caption	Page No
Table 3.1	HUDEP Expansion media	21
Table 3.2	HUDEP Erythroid differentiation media	22
Table 3.3	HSPC expansion media	23
Table 3.4	HSPC erythroid differentiation media	23
Table 3.5	Plasmids used in the study	24
Table 3.6	Primers used for Gibson assembly	25
Table 3.7	List of primers used for sequencing in the study	26
Table 3.8	List of gRNAs used in the study	27
Table 3.9	List of pegRNAs used in the study	29
Table 3.10	List of primers used for IVT	31
Table 3.11	List of primers used for qRT PCR/ddPCR	34

LIST OF ABBREVIATIONS

S No	Abbreviation	Full Form
1	CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
2	Cas9	CRISPR-associated protein 9
3	dCas9	Dead Cas9
4	nCas9	Nickase Cas9
5	BE	Base Editor
6	ABE	Adenine Base Editor
7	CBE	Cytosine Base Editor
8	SCD	Sickle Cell Disease
9	sgRNA	Single guide RNA
10	gRNA	Guide RNA
11	PAM	Protospacer Adjacent Motif
12	HBG	Hemoglobin Subunit Gamma
13	HBA	Hemoglobin Subunit Alpha
14	HBB	Hemoglobin Subunit Beta
15	HBD	Hemoglobin Subunit Delta
16	HBZ	Hemoglobin Subunit Zeta
17	HBE	Hemoglobin Subunit Epsilon
18	HbF	Fetal Hemoglobin
19	HbA	Adult Hemoglobin
20	HbS	Sickle Hemoglobin
21	HbE	Hemoglobin E
22	HPFH	Hereditary Persistence of Fetal Hemoglobin
23	spCas9	Streptococcus pyogenes Cas9
24	Indel	Insertion deletion
25	Tad A	tRNA-specific adenosine deaminase
26	HPLC	High-Performance Liquid Chromatography
27	RP-HPLC	Reverse phase-High-Performance Liquid

		Chromatography
28	ddPCR	Digital droplet PCR
29	FACS	Fluorescence-Activated Cell Sorting
30	HDR	Homologous Directed Repair
31	NHEJ	Non-Homologous End Joining
32	MMEJ	Microhomology-Mediated End Joining
33	qRT PCR	quantitative Reverse Transcription PCR
34	DNA	Deoxyribonucleic Acid
35	RNA	Ribonucleic Acid
36	cDNA	Complementary DNA
37	DSB	Double Strand Break
38	SSB	Single Strand Break
39	HEK	Human Embryonic Kidney
40	HSPCs	Hematopoietic Stem and Progenitor Cells
41	HUDEP-2	Human Umbilical Cord Blood-Derived Erythroid Progenitor
42	PBMNCs	Peripheral Blood Mononuclear Cells
43	UGI	Uracil DNA Glycosylase Inhibitor
44	mRNA	Messenger RNA
45	PCR	Polymerase Chain Reaction
46	GFP	Green Fluorescence Protein
47	RFP	Red Fluorescence Protein
48	EF1 α	Elongation Factor 1 Alpha
49	BCL11A	B-cell lymphoma/leukemia 11A
50	KLF	Krüppel-Like Factor
51	TAL	T-cell Acute Lymphoblastic leukemia
52	GATA	GATA binding protein
53	TSS	Transcription Start Site
54	LCR	Locus Control Region
55	AAV	Adeno Associated Virus
56	HLA	Human Leukocyte Antigen

57	NGS	Next Generation Sequencing
58	LV	Lentivirus
59	ZFN	Zinc Finger Nuclease
60	TALEN	Transcription Activator-Like Effector Nuclease
61	SCF	Stem Cell Factor
62	EPO	Erythropoietin
63	TPO	Thrombopoietin
64	FLT3	Fms-like Tyrosine Kinase 3
65	IL	Interleukin
66	IVT	In Vitro Transcription
67	FBS	Fetal Bovine Serum
68	IMDM	Iscoe's Modified Dulbecco's Medium
69	RPMI	Roswell Park Memorial Institute
70	DMEM	Dulbecco's Modified Eagle's Medium
71	Opti MEM	Opti-Minimal Essential Medium
72	PS	Penicillin/Streptomycin
73	PBS	Phosphate-Buffered Saline
74	BSA	Bovine Serum Albumin
75	Edit R	Edit Deconvolution by Inference of Traces in R
76	ICE	Inference of CRISPR Edits
77	GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
78	APC	Allophycocyanin
79	PE	Phycoerythrin
80	FITC	Fluorescein Isothiocyanate
81	BV	Brilliant Violet
82	EDTA	Ethylenediaminetetraacetic Acid
83	LB	Luria-Bertani
84	OT	Off-target
85	CD	Cluster of Differentiation
86	hCD	Human CD

87	mCD	Mice CD
88	RBC	Red Blood Cells
89	ANOVA	Analysis of Variance
90	MOI	Multiplicity of Infection
91	VCN	Vector Copy Number
92	COSMID	CRISPR Search with Mismatches, Insertions and/or Deletions
93	WPRE	Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element

SYNOPSIS

Genome engineering in the hematopoietic stem cells for the treatment of beta globin disorders

Background:

Hemoglobin A or adult hemoglobin is a tetrameric protein consisting of two alpha and two beta subunits($\alpha_2\beta_2$) that functions to transport oxygen in the body. Beta hemoglobinopathies are hereditary disorders caused by mutations affecting the coding or non-coding regions of the beta globin gene (*HBB*), that codes for the beta subunit of hemoglobin. Two major classes of beta hemoglobinopathies are sickle cell anaemia and beta thalassemia. Sickle cell anaemia is caused by a single point mutation in codon 6 of beta globin resulting in a variant of hemoglobin that polymerizes under low oxygen saturation. This causes the RBCs to acquire a characteristic sickle shape making them vulnerable to lysis resulting in anaemia. The obstruction of small blood vessels by the sickled RBCs can also result in severe pain crisis and cause ischemic injury to organs. Beta thalassemia on the other hand is characterized by reduced or absent synthesis of beta globin or the production of a non-functional variant, depending on the causative mutation, resulting in ineffective erythropoiesis and increased haemolysis. Based on the severity of thalassemia, the symptoms vary from mild anaemia to recurrent infections and growth retardation and in severe cases patients fail to survive. Both beta thalassemia and sickle cell anaemia negatively impact the quality of life and imposes significant treatment burden.

Current treatment approaches for sickle cell anaemia are mostly symptomatic and are aimed at reducing pain crisis and preventing complications. Beta thalassemia

major often requires repeated blood transfusions along with iron chelation therapy. One of the approved curative approaches is allogenic stem cell transplant from healthy matched donors. This is however limited by the availability of matched donors and the risk of graft versus host disease. Gene therapy approaches aimed at integrating a healthy copy of the gene by viral delivery approaches are also promising but suffers from the risk of insertional mutagenesis.

The past decade witnessed the development of precise genome editing approaches using CRISPR/Cas system for treatment of human diseases. Genome editing is a promising therapeutic strategy for beta hemoglobinopathies because of two reasons. First, being a monogenic disorder, beta globin mutations can be easily targeted using programmable genome editors. Second, being a blood disorder, beta hemoglobinopathies can be corrected by ex-vivo gene editing followed by autologous transplantation of the hematopoietic stem cells. The gene editing strategies for hemoglobinopathies are aimed at either correcting the causative mutations or compensating for defective beta globin by upregulating developmentally silenced fetal hemoglobin. The first cell-based gene therapy, Casgevy, elevating fetal hemoglobin using CRISPR/Cas9 system was approved for use in patients with severe forms of beta hemoglobinopathies by the FDA.

As the evaluation of different strategies for treatment of hemoglobinopathies are underway, several studies have also described the detrimental consequences of current genome engineering approaches. Recent studies report the unintended consequences of double strand break mediated approaches such as large deletions, translocations, chromothripsis, P53 activation etc. This has prompted a shift to DSB free approaches such as base and prime editing for therapeutic gene editing. While

effective base editing is limited to transition mutations, prime editing shows very less editing efficiency compared to base editing in both cell lines and in primary cells. Base editing, although to a lesser extent, also induces the generation of large interstitial deletions while targeting highly homologous regions such as the gamma and beta globin genes. Owing to these limitations of current gene editing approaches, there is a need to develop novel, efficient and precise genome engineering strategies for treatment of beta hemoglobinopathies.

In this work we aimed to develop strategies that could improve and expand the gene editing toolbox for beta hemoglobinopathies. We aimed to achieve this by improving the precision and efficiency of currently available gene editing tools and by identifying novel targets that can be manipulated by gene editing approaches for treatment of beta hemoglobinopathies.

Aim:

To develop CRISPR based precise genome engineering approaches (base and prime editing) for treatment of beta globin disorders

Objectives:

1. Improving the precision of base editing in globin locus using non-nicking approaches
2. Identifying regulatory elements in the gamma globin gene amenable to genome engineering for elevation of fetal hemoglobin
3. Correcting the causative point mutation of sickle cell disease in beta globin gene (E6V) using prime editing

Hypothesis and experimental methods:

As the objectives of the study were limited to the globin locus, most of the experiments were performed in either HUDEP-2 cell line (which express adult hemoglobin and can be manipulated to reactivate fetal hemoglobin) or CD34+ve hematopoietic stem and progenitor cells derived from healthy donors. A summary of methodology used for each objective is given below:

Objective 1:

Hypothesis: Previous generations of base editors used a fusion of nickase Cas9 to deaminase to achieve efficient base conversions. This resulted in large interstitial deletions while performing base editing in highly homologous regions. Recently described hyperactive variant of adenosine base editor (ABE8e) would be able to retain the editing efficiency even when the nickase activity is removed.

Methodology: Two dCas9 ABE8e constructs (for lentiviral delivery and for in vitro transcription) were generated by site directed mutagenesis. The experiments in HUDEP-2 cell lines were performed by lentiviral delivery or mRNA electroporation of base editor and sgRNA. The experiments in HSPCs were performed by mRNA electroporation. The editing efficiency was evaluated by next generation sequencing and sanger sequencing. Deletion frequency was evaluated by qRT PCR, ddPCR or agarose gel electrophoresis.

Objective 2:

Hypothesis: Several studies have reported the presence of elements in the introns of genes that regulate gene expression. We hypothesized that there might be regulatory elements in the first intron of gamma globin gene which can be manipulated for gene therapy applications

Methodology: Gene editor stable cell lines were prepared using lentiviral constructs for the respective editors. An arrayed gRNA screening was performed in these stables by lentiviral delivery of gRNAs. Gene editing was evaluated using NGS and HbF elevation was quantified by flowcytometry and HPLC. The top gRNA was validated in HSPCs by electroporation of the components.

Objective 3:

Hypothesis: Currently available base editors cannot convert Thymine to Adenine which is required for correction of sickle cell mutation. We hypothesized that this could be achieved by prime editing which can introduce precise mutations in the target locus without by-stander edits or DSBs.

Methodology: PE stable cell lines were generated in HUDEP cells and pegRNAs were validated by lentiviral delivery. Genotyping was done by sanger sequencing and functional analysis was performed by HPLC.

Major finding:

Objective 1:

- dCas9 fusion of ABE8e can edit efficiently in cell lines as well as primary cells. Although editing efficiency is slightly lower than nABE8e, the dABE8e construct overcomes the problem of large deletion in homologous genes
- Editing efficiency in HSPCs was improved by optimization of mRNA synthesis and delivery approaches.
- Editing efficiency is not dependent on cell cycle or DNA replication. Editing with dABE8e happens even in primitive CD90⁺ HSPCs.
- dABE8e construct also overcomes the generation of InDels while targeting complementary strands for multiplexed base editing

Objective 2:

- There are regulatory elements in the first intron of gamma globin which can be manipulated for elevation of fetal hemoglobin
- The elevation in HbF levels is mediated by DNA nicking rather than base conversions
- Even transient nicking by Cas9 D10A nickase resulted in persistent elevation of HbF
- The observation is not limited to cell lines but is also true in primary CD34⁺ve HSPCs

Objective 3:

- We achieved efficient creation and correction of sickle mutation in erythroid cell lines using prime editing
- The editing was very precise without any InDels or bystander edits
- Editing efficiency increases over time suggesting that PE might be dependent on cell cycle
- Editing efficiency is more in genes that are actively transcribed
- pegRNAs without homology can overcome nick mediated HBD/HBB interstitial deletions generated during prime editing
- mRNA mediated delivery did not edit in HSPCs suggesting that further optimization might be required to perform prime editing in HSPCs

Implications of the finding:

Objective 1:

The use of dABE8e is possible for therapeutic gene editing for treatment of

genetic disorders. In addition to preventing the generation of large deletions while editing homologous regions, this approach also improves the precision of base editing and limits DNA damage response in the cells. In addition to homologous regions, this construct can also be used for efficient multiplexed editing in complementary strands without the generation of InDels. Taken together, the dABE8e construct adds to the precise genome engineering toolbox which can be used by the broader scientific community for therapeutic and research applications.

Objective 2:

We identified a hitherto undescribed regulatory element of fetal hemoglobin expression within intron 1. Although the mechanism of HbF elevation by DNA nicking in this region is unknown, the region appears to be a plausible target for therapeutic gene modifications. If the mechanism is uncovered, it could also possibly pave way for development of other therapeutic HbF induction approaches and thus benefit patients with hemoglobinopathies

Objective 3:

The modular PE system developed in the study can be used for screening and validation of pegRNAs for other applications. The sickle cell disease model developed in the study can be used for validation of therapeutic approaches for sickle cell anaemia. We uncover some of the factors determining prime editing efficiency which can enable the development of precise prime editing strategy in HSPC

INTRODUCTION

1.1 Background

Hemoglobin is a tetrameric heme containing protein present in the erythrocytes with the primary function of oxygen transport. It is composed of two alpha like and two beta like globin subunits to which heme is bound (Ahmed et al., 2020). In humans, the genes coding for alpha like subunits (*HBZ*, *HBA2* and *HBA1*) are present in chromosome 16 while those for beta like subunits (*HBE*, *HBG2*, *HBG1*, *HBD* and *HBB*) are present in chromosome 11. Chromosome 11 also contains an enhancer region composed of 5 DNase hypersensitive sites and is termed as the locus control region (LCR). The composition of the subunits changes during ontogeny and determines the type of hemoglobin formed. Each hemoglobin has distinct physiochemical and functional properties suiting the changing needs of the body (Sawado et al., 2003).

During development hemoglobin switching happens twice in humans. The first switch from embryonic to fetal hemoglobin occurs in fetal liver after about five weeks of gestation. The second switch from fetal to adult hemoglobin begins in bone marrow towards the end of third trimester and continues till 2 years after birth. A complex interplay of cis and trans acting regulatory elements is proposed to be responsible for the hemoglobin switch (Wilber et al., 2011). With the recent advances in molecular biology techniques, several regulatory elements have been uncovered. However, the exact mechanism and all the factors involved in hemoglobin switch is still unknown (Sankaran and Orkin, 2013; Shen et al., 2021).

Hemoglobinopathies are monogenic disorders caused by mutations affecting the hemoglobin subunits. Beta-hemoglobinopathies - sickle cell anemia and beta

thalassemia – are caused by mutations in the beta subunit. The mutations can result in structural or functional defects of globin chain or reduced/absent production of the subunits (Kato et al., 2018; Thein, 2018). The patients with beta-hemoglobinopathies suffer from severe anemia and associated complications negatively impacting the quality of life. The treatment strategies are mostly symptomatic, and the patients often require repeated blood transfusions. Until recent times the only approved curative therapy was bone marrow transplantation from matched donors, but this is also limited by the availability of donors and risk of GVHD. Hemoglobinopathies have a high prevalence both globally and nationally with populations practicing consanguineous marriages at a higher risk. These diseases not only affect the quality of life but also incur a significant treatment burden (Cao and Galanello, 2010; Kavanagh et al., 2022).

Advances in gene editing has opened new avenues for the cure of monogenic disorders especially beta-hemoglobinopathies. Ex-vivo gene editing of autologous hematopoietic stem cells is especially promising and one such approach has recently been approved by FDA for clinical use (Barbarani et al., 2020; Commissioner, 2024). The therapeutic approaches can be classified into two major groups- those aiming at the correction of causative mutations and those aiming to reactivate fetal hemoglobin. While elevation of fetal hemoglobin can be a universal treatment strategy of all types of beta-hemoglobinopathies, direct correction offers the advantage of retaining adult globin production. CRISPR/Cas 9 system owing to its versatility has been adopted widely for development of treatment strategies over other gene editing approaches (Christakopoulos et al., 2023).

There are however several limitations to the existing strategies. Cas9-nuclease based approaches introduce DSBs that result in uncontrollable and undesired

mutations in the target site. Moreover, there are severe detrimental effects of DNA breaks such as large deletions, chromothripsis, P53 activation etc (Park et al., 2022). The globin locus in particular is prone to large interstitial deletions due to high homology between the genes. Further, precise conversions can only be introduced by HDR approach which shows less efficiency in non-dividing cells (Peterka et al., 2022). Most of these challenges can be addressed by recently developed DSB free approaches for precise editing such as base and prime editing. BE and PE offers better control over the editing outcome and is more efficient than HDR approach. They also generate less frequent large deletions and undesired InDels. However, the well characterized and efficient base editors in use are capable of introducing only transversion mutations. Additionally, base editing suffers from the generation of bystander edits oftentimes masking the desired effect. On the other hand, PE, although precise, is less efficient in introducing the mutations in primary cells as well as in genes that are not actively transcribed (Anzalone et al., 2020a).

As the field is nascent there is a need for deeper understanding and a scope for developing better approaches for treatment of beta hemoglobinopathies. Gene editing approaches require refinement in terms of product purity to enable precise and error free gene correction with higher efficiency. Fetal globin reactivation can benefit from better understanding of the globin switching mechanism that would enable development of treatment strategies that are more efficient and with less undesired changes (Zittersteijn et al., 2021).

Keeping in mind the unique challenges associated with the existing approaches, this work was designed to overcome these limitations or to reduce the impact of the drawbacks. As base editing is utilized for both direct correction of

mutations as well as reactivation of gamma globin, we first sought to address the problem of large interstitial deletions generating during base editing using a nick free approach. Additionally, we used prime editing for precise correction of sickle mutation which would not be possible with the existing base editors. Finally, to identify novel regulatory elements we performed mutagenesis screening in the first intron of gamma globin using various Cas9 mutants.

1.2 Aim and Objectives

Aim:

To develop CRISPR based precise genome engineering approaches (base and prime editing) for treatment of beta globin disorders

Objectives:

1. Improving the precision of base editing in globin locus using non-nicking approaches
2. Identifying regulatory elements in the gamma globin gene amenable to genome engineering for elevation of fetal hemoglobin
3. Correcting the causative point mutation of sickle cell disease in beta globin gene (E6V) using prime editing

LITERATURE REVIEW

2.1 Hemoglobin

Hemoglobin is an iron containing protein present in erythrocytes and responsible for the transport of oxygen in vertebrates. It consists of four globin chains (two alpha like and two beta like) each containing a heme moiety. Based on the composition of globin chains there are various types of hemoglobin, the expression of which is developmentally regulated. Chromosomes 16 and 11 harbor the alpha and beta globin locus respectively. Alpha ($\alpha 1$ and $\alpha 2$) and gamma ($G\gamma$ and $A\gamma$) globin genes are duplicated in humans. Similarly, the delta and beta globin genes share sequence homology although they are not duplicated genes (Ahmed et al., 2020; Otto, 2020; Schechter, 2008).

2.2 Hemoglobin switching

Hemoglobin switching refers to the switch in the type of predominant hemoglobin produced in the body. In contrast to other species which have only embryonic to adult hemoglobin switching, humans have two switches. The first switch from embryonic ($\alpha 2\epsilon 2$) to fetal hemoglobin ($\alpha 2\gamma 2$) happens at about 5-10 weeks of gestation. The second switch from fetal to adult hemoglobin ($\alpha 2\beta 2$) begins in the third trimester and would be completed by 2 years of age. In adults the predominant hemoglobin is HBA1($\alpha 2\beta 2$) and a small portion is HBA2 ($\alpha 2\delta 2$) (Hariharan and Nadkarni, 2021; Stamatoyannopoulos et al., 2009).

Although it was assumed previously that different stem cell populations were responsible for production of the two types of hemoglobin, this hypothesis was rejected on the basis of somatic cell hybrid experiments. Another hypothesis was that

the control was exerted by external factors such as hormonal levels, but this too was soon discounted based on experimental evidence. It was concluded that a cell intrinsic clock was responsible for the regulated globin switch and the investigations into the underlying molecular principles began towards the end of 20th century (Bauer et al., 2012; Stamatoyannopoulos et al., 2009).

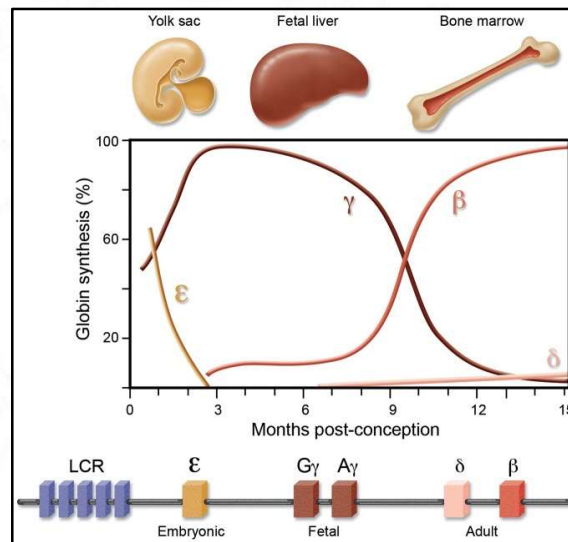


Figure 2.1: Developmental switching of hemoglobin production.

Source: (Bauer et al., 2012)

The advances in molecular biology helped identifying several Cis and Trans regulatory elements that are involved in globin switching. The most important Cis regulatory element is the beta globin locus control region (LCR) located 6-25 kb upstream of *HBE* gene. It contains a series of DNase Hypersensitive sites. HS2 is considered a classical enhancer while HS3 and HS4 are implicated in chromatin remodeling and HS5 is thought to act as an insulator. Other regulatory elements include the promoter elements especially the transcription factor binding sites. Both repressor and activator binding sites have been identified in the globin promoters. KLF-1 was one transcription factor identified to bind to the beta globin promoter

regulate its expression. Subsequently BCL11A and LRF were found to bind to gamma globin promoter acting as its repressor. Additionally, other regulatory elements such as long noncoding RNAs have also been to have role in globin gene regulation and switching (Lettre et al., 2008; Liu et al., 2021; Martyn et al., 2018; Psatha et al., 2018).

2.3 Hemoglobinopathies

The group of disorders involving hemoglobin is termed as hemoglobinopathies and are often monogenic disorders inherited in an autosomal co-dominant pattern. If the mutation affects beta globin gene, the disease is termed as beta hemoglobinopathies which can again be classified based on the type of mutation. Structural variants are qualitative defects and affect the structure of hemoglobin. Common examples include HbE, HbS, HbC etc. They are often benign in heterozygous conditions but can be detrimental if homozygous or compound heterozygous. Quantitative defects on the other hand affect the amount of functional globin chains produced. Examples include alpha and beta thalassemia (Harteveld et al., 2022; Kohne, 2011).

2.4 Sickle Cell Disorder

Sickle cell disorder is the common term for diseases characterized by a point mutation in the sixth codon of beta globin gene. An A>T conversion changes hydrophilic amino acid Glutamine to hydrophobic amino acid Valine. This causes hemoglobin to polymerize under conditions of low oxygen saturation making RBCs acquire the characteristic sickle shape. These sickled RBCs are prone to hemolysis resulting in anemia. The affected individuals suffer from vaso-occlusive crisis causing severe pain crisis and organ damage. The prevalence of SCD in India is very high especially among the tribal population (Kato et al., 2018; Kavanagh et al., 2022; Lettre et al., 2008; Sheth et al., 2013).

2.5 Beta Thalassemia

Beta Thalassemia is caused by mutations that affect the production of functional hemoglobin. The mutations can range from point mutations, small insertions/deletions or large deletions and can be present in any part of the gene including the promoter, UTRs, introns etc (Langer, 1993; Sanchez-Villalobos et al., 2022; Taher et al., 2021). The type of mutation determines the severity of the disease, and the patients are classified into major, intermedia and minor. The complications often arise due to imbalance in the ratio of alpha and beta chains, resulting in the precipitation of free alpha chains within the cell. This causes hemolysis predominantly due to increased production of reactive oxygen species in the affected cells. Premature hemolysis results in accelerated ineffective erythropoiesis. Patients with beta thalassemia major suffer from severe anemia, growth retardation, organ failure etc and require repeated blood transfusions. On the other hand, patients with beta thalassemia intermedia or minor suffer from less severe symptoms and are often not transfusion dependent (Nienhuis and Nathan, 2012; Thein, 2005).

Thus, the complications in SCD arise from the RBC sickling due to Hb aggregation at low oxygen saturation while that in beta thalassemia arises from alpha chain precipitation due to high alpha/beta chain ratio. So, the development of treatment strategies has been focused on preventing sickling or maintaining the alpha/beta chain ratio. Reactivation of fetal hemoglobin can achieve both and has been considered as a universal therapeutic strategy for beta hemoglobinopathies (Bauer et al., 2012; Steinberg, 2020).

2.6 Hereditary Persistence of Fetal Hemoglobin (HPFH)

HPFH is an asymptomatic hereditary condition where the production of fetal hemoglobin continues to adult life without undergoing globin switching. HPFH was identified in individuals with sickle cell anemia who did not show disease phenotype. HPFH is caused by mutations in the globin locus and based on the type of mutation, HPFH is classified into two (Venkatesan et al., 2020; Wienert et al., 2018). Deletional HPFH involves large deletion of varying lengths, most often involving the delta and beta globin. Reorganization of chromatin looping is proposed to be the underlying mechanism of HbF elevation in deletional HPFH. Non-deletional HPFH is characterized by point mutations or small deletions in the gamma globin promoter.

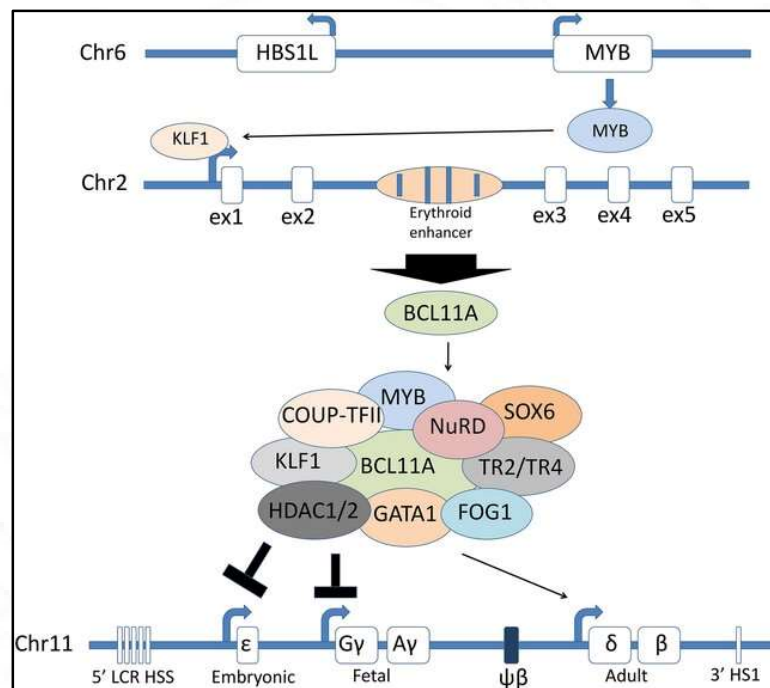


Figure 2.2: Transcription factors involved in regulation of globin gene expression.

(Source: Ugo Testa, 2008)

These mutations often disrupt a repressor binding site or create an activator

binding site. Examples of repressor sites include the binding site for BCL11A at the -115 cluster and of LRF at the -200 cluster. Similarly, -175 T>A mutation creates binding site for TAL1 and -123 T>C conversion creates binding site for KLF1 both of which are activators. GWAS studies have also described polymorphisms in *BCL11A* and *MYB* genes that are responsible for HbF elevation (Amato et al., 2014; Lettre et al., 2008; Ravi et al., 2022; Sharma et al., 2020; Singha et al., 2023).

2.7 Regulation of gene expression

Gene expression is regulated at different levels to achieve homeostasis. The most basic control step is at the level of DNA through the binding of transcription factors. Transcription activators bind to the promoters and enhance gene expression while repressors downregulate gene expression. In eukaryotes transcription factors often form complexes and act in a concerted manner. In addition to transcription factors there are several enhancers often controlling one or more gene clusters. In the human beta globin locus, the LCR serves as a strong enhancer. Chromatin states such as euchromatin and heterochromatin as well as the organization of chromatin domains also play important roles in gene regulation. Histone modifications such as methylation and acetylation also help in fine tuning gene expression. After the transcription several post-transcriptional modifications also help in regulating the abundance of mRNA available for translation (Buccitelli and Selbach, 2020; Cooper, 2000; Jaenisch and Bird, 2003; Lambert et al., 2018).

2.8 Role of introns in gene regulation

Several studies in recent years have attributed regulatory roles for intronic elements both in plants and in animals. Introns have been shown in general to regulate transcription, nuclear export and the stability of mRNA. In the context of globin genes,

the intron of HBB is shown to have elements that enhance gene expression which has been utilized for generation of efficient lentiviral constructs. The intron of BCL11A, which is a repressor of gamma globin harbors an enhancer within its intron which has also been targeted for therapeutic applications. However, no studies till date have investigated the presence of regulatory elements in the introns of gamma globin genes (Buzina et al., 2008; Canver et al., 2015; Jo and Choi, 2015; Rose, 2019).

2.9 Conventional treatment strategies for Beta hemoglobinopathies

The treatment approaches for SCD are often symptomatic and aimed at reducing pain crisis and vaso-occlusion. Hydroxyurea has been the first line management for SCD for a very long time and is known to act by inducing the production of fetal hemoglobin. Other approved drugs include Azacytidine, Pomalidomide, Voxelotor, Crizanlizumab and L-Glutamine. Beta thalassemia major on the other hand requires repeated blood transfusions and iron chelation. However, all these approaches have severe side effects that affect the patient's quality of life (Biswas et al., 2024; Karamperis et al., 2021; Salinas Cisneros and Thein, 2020).

Hematopoietic stem cell transplantation is a curative approach for both SCD and thalassemia. Transplantations from HLA matched related donors have been found to have very high success rates, but it is often difficult to find suitable donors. Transplantation from haploidentical unmatched donors suffer from the risk of graft versus host disease (Anurathapan et al., 2020; Lucarelli et al., 2012; Santarone et al., 2022).

2.10 Gene Therapy

Autologous stem cell transplantation following gene therapy is a promising approach for beta hemoglobinopathies and overcomes the risk of graft rejection.

Lentiviral gene therapies for integration of beta globin have been approved for use in patients with transfusion dependent beta thalassemia and sickle cell disease. In addition to beta globin gene insertion, vectors carrying gamma globin gene have also been tested. Although successful at overcoming disease symptoms, gene therapy carries the risk of insertional mutagenesis due to random integration. Reports of myelodysplastic syndrome after gene therapy was also investigated but was found to be caused by conditioning regimen rather than transgene integration (Biffi, 2018; Christakopoulos et al., 2023; Demirci et al., 2020; Ribeil et al., 2017).

2.11 Programmable Nucleases for gene editing

Detailed understanding of DSB repair pathways in eukaryotes led the scientific quest for programmable nucleases that can be leveraged to introduce targeted mutations in the genome. Maria Jasin was the first to report the use of meganucleases to introduce DSB in the DNA. However, meganucleases are severely limited by the availability of target site and thus was not programmable in the true sense. This was followed by the development of Zinc Finger Nucleases (ZFNs) and Transcription Activator Like Effector Nucleases (TALENs). Both ZFNs and TALENs used FokI endonuclease fusion to programmable DNA Binding Domains to achieve targeted DSBs. While ZFNs used programmable Zinc Fingers to direct DNA binding, TALENs utilized TALEs that can be targeted to unique sites by changing the amino acid sequence of the RVDs (Repeat Variable Di residues). While both the approaches could be programmed to specific regions, each new target site requires protein reengineering making the process cumbersome and expensive (Carroll, 2017; Khalil, 2020).

2.12 CRISPR/Cas9 system

CRISPR/Cas9 system is a bacterial defense mechanism against invading

bacteriophages. Insights into the mechanism of CRISPR/Cas9 activity enabled its quick repurposing as a gene editing tool. This gene editing system consists of two essential elements- a Cas9 nuclease protein and a guide RNA(gRNA). The gRNA has two parts- the ~20 nucleotide spacer sequence at the 5' end that can be programmed to bind complementarily to any site in the genome followed by an ~90 nucleotide long tracrRNA that helps in binding of gRNA to the Cas9 nuclease. Once the gRNA and Cas9 enter the nucleus, the gRNA directs the complex to the target site. The binding of gRNA to the target DNA alters the conformation of Cas9 enabling it to make DNA strand break 3 nucleotides 5' to the PAM sequence. The cellular repair machinery further attempts to correct the DSB by either error prone NHEJ or error free HDR(if repair template is available) thus generating mutations or intended corrections (Prasad et al., 2021).

2.13 Cas9 Variants

Knowledge of the structure of Cas9 has enabled its manipulation to develop different variants with a multitude of functions. Cas9 exerts its nuclease activity with the help of two independent domains that nicks one strand each of the DNA. HNH domain cleaves the target strand while RuvC domain cleaves the non-target strand. Introduction of D10A mutation in the RuvC domain inactivates the nuclease domain and creates a nickase that cleaves only the target strand. Similarly, introduction of H840A mutation in the HNH domain creates a nickase that cleaves the non-target strand. If both the nuclease domains are mutated it yields a catalytically deadCas9 which retains the capacity to bind to the target site but does not introduce any strand break (Doudna and Charpentier, 2014).

deadCas9 has been used in targeted gene regulation by fusing activator or

repressor domains to the Cas9 protein. This approach is termed CRISPRa/CRISPRi and has been a valuable tool for research. Recently several other effector domains have been fused to dCas9 to yield epigenome editors with scientific as well as therapeutic applications. Based on the species from which Cas9 is isolated, the PAM requirement varies. In addition, protein engineering has made it possible to generate Cas9 with no or very minimal PAM constraints (Chen and Doudna, 2017; Nidhi et al., 2021).

2.14 Base Editors

Base editors are precision gene editing tools which consist of two parts- a dCas9/nCas9 domain which recognizes and binds to the target site and a deaminase fused to the Cas9 domain. The deaminase converts the respective nucleotide to its deaminated variant. Cytosine base editors were the first class to be developed and contained a cytosine deaminase (e.g. APOBEC) fusion to nCas9. This enables the deamination of cytosine to uracil which will then be converted to Thymine by the cellular DNA repair pathways. Adenosine base editors were similarly generated by fusing adenosine deaminase to nCas9 that converts Adenine to Guanine with inosine as intermediate. As there were no naturally occurring enzymes that can deaminate adenosine in DNA, tRNA adenosine deaminase was evolved to edit DNA and used in the generation of ABEs (Gaudelli et al., 2017; Komor et al., 2016).

Subsequently, other base editors such as C>G base editors were developed by rational protein engineering. Over the years several modifications were made in BEs and hyperactive variants were evolved with lesser off-target activity, modified editing window and varying PAM requirements (L Chen et al., 2021). This has greatly enhanced the gene editing toolkit. Base editors have several advantages over Cas9 nuclease- the most important being the absence of DSBs and the predictability of

editing outcomes. Due to lack of DSBs, BEs induce far less DNA damage and associated complications than the conventional approaches. However, the biggest drawback is the by-stander effect wherein all the target nucleotides within the editing window will be converted. This often creates undesired mutations while attempting precision genome engineering. Although this can be overcome to a large extent by rational design of gRNAs and use of PAM derestricted Cas variants, it is not possible to avoid by-stander effects in all regions (Anzalone et al., 2020b).

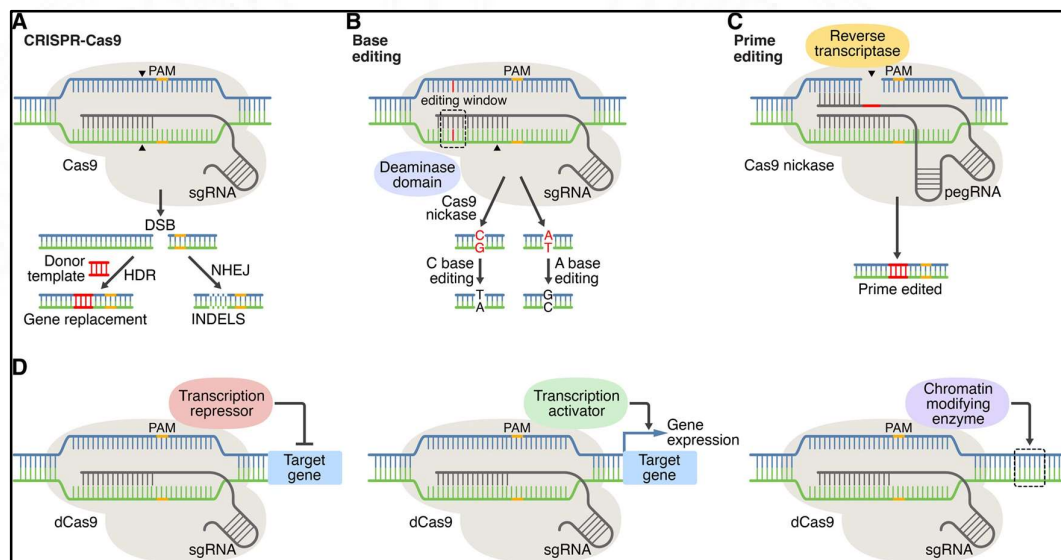


Figure 2.3: CRISPR Cas9 based genome editing tools

(Source: Liu et al., 2022)

2.15 Prime Editors

Prime editor (PE) is yet another nCas9 based gene editing tool that has a fusion of reverse transcriptase to H840A nickase. Prime editing uses a special gRNA termed pegRNA for gene editing that in addition to the spacer and tracrRNA also has a reverse transcriptase template at the 3' end that serves to introduce the desired mutation in the target site. The 3' flap generated by DNA nicking would be reverse transcribed by the enzyme utilizing the pegRNA as template thus installing the desired mutation in the

newly synthesized strand. There would be a flap equilibration that installs either the newly synthesized strand or the existing strand based on the cellular conditions (Anzalone et al., 2019; Doman et al., 2022).

The advantage of PE is its ability to introduce a variety of mutations such as single base pair conversions, insertions, deletions without creating DSB. PE is, however, in its early stages of use and still has several disadvantages. The first drawback of PE is its low efficiency in primary cells. Although different generations of PE have been developed in addition to pegRNA modifications, the editing at most of the target sites and cell types remains relatively low. The editing efficiency also depends on other factors such as gene activity, distance of the target site from TSS, chromatin accessibility etc. It also suffers from a major drawback that multiple pegRNAs with varying RT template length has to be tested to achieve good editing efficiency. All these factors have limited the widespread use of PE in clinical and research applications (PJ Chen et al., 2021; Koepfel et al., 2023; Li et al., 2024).

2.16 Gene Editing approaches for beta hemoglobinopathies

Gene editing is a viable curative approach for hemoglobinopathies predominantly because these are monogenic disorders. Moreover, gene editing can be performed ex-vivo in HSPCs isolated from the patients and the corrected cells can be transplanted to achieve therapeutic benefits without the risk of graft rejection. The gene editing approaches are generally aimed at correcting the mutations or reactivating fetal hemoglobin (Christakopoulos et al., 2023).

Homology directed repair for correction of sickle mutation was one of the initial CRISPR based strategies tested for gene correction. One of the major drawbacks of using Cas9 mediated HDR was the development of thalassemic genotype in cells

where successful correction had not happened. Additionally, the engraftment efficiency of HDR edited cells were reported to be low (DeWitt et al., 2016). As sickle mutation is caused by an A>T transversion, currently available base editors are not capable of correcting the mutation. However base editing has been used for converting the sickle Hb to a benign Hb variant HbG Makassar, that does not sickle thus availing therapeutic benefits (Newby et al., 2021). The recently developed prime editing approach is capable of correcting sickle mutation without DSBs and the ex vivo manipulation of HSPCs with PE has been demonstrated. However, prime editing is less efficient compared to base editing (Everette et al., 2023).

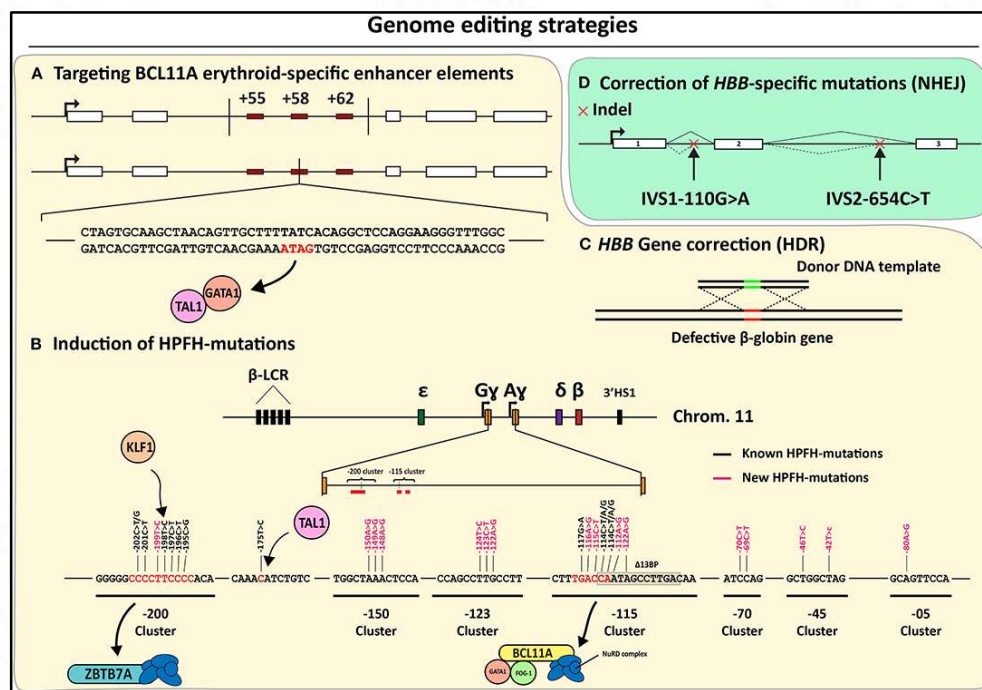


Figure 2.4: Genome editing strategies for treatment of hemoglobinopathies

(Source : Zittersteijn et al., 2021)

Various thalassemic mutations has also been corrected using CRISPR based approaches. Some examples include HDR based correction of β-41/42(TCTT) deletion, NHEJ mediated disruption of aberrant splice sites such as IVS 1-110, IVS 2-

654 etc. Base editing with conventional Cas as well as newly described 'PAM-less' Cas variants has also increased the target scope for direct correction of thalassemic mutations. The major limitation of using gene correction strategies for thalassemia is the need to develop new approaches for each mutation and its extensive preclinical and clinical validation which would incur a heavy cost (Badat et al., 2023; Hardouin et al., 2023; Liang et al., 2017; Prasad et al., 2024; Zhang et al., 2022).

Reactivation of fetal hemoglobin by gene editing is a universal curative approach for beta hemoglobinopathies and has been attempted using various gene editing approaches. This includes recreation of HPFH mutations (both deletional and non-deletional) or introduction of novel HPFH like mutations. Cas9 mediated NHEJ, HDR, as well as base editing has been used for creation of cis mutations in the globin locus that reactivate fetal globin expression. In addition to the globin locus other regulators of gamma globin expression such as transcription factors (BCL11A, LRF etc.) and non-coding RNAs have also been investigated as targets for gene editing to elevate fetal hemoglobin. The major limitation of targeting transcription factors is their involvement in other pathways beyond fetal globin regulation (Antoniou et al., 2022; Li et al., 2021; Ravi et al., 2022; Venkatesan et al., 2023).

2.17 Large interstitial deletions- undesired outcome of Cas9 mediated editing in homologous regions

As Cas9 is an RNA guided endonuclease, that works on the basis of complementarity between the ~20 bp gRNA and the target DNA, there is a high risk of off-target binding and DNA cleavage. Although the number and position of mismatches determine the efficiency of off-target cutting, most of the gRNAs often have several off-target sites. When the off-target site is nearby or in the same

chromosome, it can lead to large deletions because of the double strand breaks happening at two sites simultaneously. This is a major drawback with the use of Cas9 in globin locus as the genes have high homology. The gamma globins, being duplicated genes, are very similar in their sequence. The use of Cas9 nuclease in the gamma globin locus causes DSBs at both sites leading to the deletion of interstitial region and formation of a hybrid gene. The use of base or prime editors where there is only nicking, and not double strand break was expected to overcome this problem. However, there are reports that even these nicking based strategies cause interstitial deletions in this locus even though the frequency is lower than Cas9 nuclease-based strategies. The convergence of replication with nick repair could be the possible explanation for this observed phenomenon (Adikusuma et al., 2018; Kosicki et al., 2022; Park et al., 2022; Prasad et al., 2024; Ravi et al., 2022).

MATERIALS AND METHODS

3.1 Cell culture : Cell lines and primary cells

HEK293T cells were cultured in DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were dissociated using 0.05% Trypsin for passaging. Freezing media used for cryopreservation had the composition FBS:DMSO in 9:1 ratio.

K562 cells were cultured in RPMI media supplemented with 10% FBS and 1% penicillin/ streptomycin. Freezing media used for cryopreservation had the composition RPMI:FBS:DMSO in 7:2:1 ratio.

HUDEP 2 cells were cultured in serum free expansion media with appropriate cytokines as detailed below. Erythroid differentiation was done using a two phase (8 days) protocol with media composition detailed below. Freezing media was prepared using IMDM:FBS:DMSO in 7:2:1 ratio.

Table 3.1 HUDEP Expansion media

Components	From	Working Conc.	For 40 ml
StemSpan™ SFEM II	STEMCELL Technologies		40ml
SCF	Peprotech	(100ng/ml)	20ul
EPO	Zyrop	(3U/ul)	60ul
Dexamethasone	Sigma	(1uM)	400ul
Doxycyclin	Sigma	(1ug/ml)	40ul
Glutamine	Hyclone	(1X)	400ul
Pen/Strep	Gibco	(1X)	400ul

Table 3.2 HUDEP Erythroid differentiation media

Components	From	Working Conc.	For 40 ml
IMDM Glutamax	Gibco		40ml
AB serum	MP	3%	1.2ml
FBS	Gibco	2%	800ul
Insulin	Sigma	1ml/1000ml	40ul
Heparin 110U/mg	Sigma	3U/ml	100ul
EPO	Zyrop	(3U/ul)	60ul
Holo-Transferrin	BB	200ug/ml	100ul
SCF	Peprotech	10ng/ml	8ul
IL3	Peprotech	1ng/ml	8ul
Pen/Strep	Gibco	(1X)	400ul
Doxycyclin	Sigma	(1ug/ml)	40ul

Leftover mobilized blood samples were obtained from CMC, Vellore with informed consent to isolate CD34⁺ve HSPCs. First PBMNCs were separated using density gradient centrifugation after carefully layering diluted blood sample on Lymphoprep (Stemcell Technologies) (2:1) ratio at 600g for 30 minutes with minimum acceleration and brake. The buffy coat was carefully collected and subjected to RBC lysis to remove any contaminating RBCs. CD34⁺ve HSPCs were further purified from these cells using EasySep Human CD34 Positive Selection Kit II (Stemcell Technologies). The percentage of triple positive cells – CD34, CD133 and CD90⁺ve – were evaluated using flowcytometry. The cells were expanded in erythroid expansion media and differentiated into erythroid cells using a 21 days (3 phase) protocol. The media composition is detailed below. Cells were cryopreserved using cryostor (Stemcell Technologies)

Table 3.3 HSPC expansion media

Components	From	Working Conc.	For 40 ml
StemSpan™ SFEM II	STEMCELL Technologies		40ml
SCF	Peprtech	(240ng/ml)	100ul
FLT3	Peprtech	(240ng/ml)	100ul
IL6	Peprtech	(40ng/ml)	16ul
TPO	Peprtech	(75ng/ml)	60ul
Pen/Strep	Gibco	(1X)	400ul

Table 3.4 HSPC erythroid differentiation media

Components	From	Working Conc.	For 40 ml
IMDM glutamax	Gibco		40ml
AB serum	MP	5%	2ml
Insulin	Sigma	1ml/1000ml	40ul
Heparin 100U/mg	Sigma	2U/ml	40ul
EPO	Zyrop	(3U/ul)	60ul
Holo-Transferrin	BB sol	330ug/ml	80ul
SCF	Peprtech	100ng/ml	40ul
IL3	Peprtech	2.5ng/ml	20ul
Pen/Strep	Gibco	(1X)	400ul
Hydrocortisone	Sigma	1uM	40ul

3.2 Cloning

Lentiviral vectors generated in this study (PE_lenti and ABE8e_Lenti) were cloned by amplifying the respective insert plasmids with primers having overhangs for Gibson assembly. Addgene# 112675 digested with *BamHI* and *NheI* was used as the backbone. Briefly the backbone and insert were mixed in equimolar ratio and added along with NEBuilder HiFi DNA Assembly Master Mix as per the manufacturer's instructions for Gibson assembly. The reaction mix was incubated at 50 degree C for 30 minutes and further transformed into DH10B competent cells. The transformed mixture was plated on LB Agar plates and colonies were picked after 16 hours of incubation. Colony PCR was performed using primers listed in Table 3.7.

Table 3.5 Plasmids used in the study:

No	Name	Purpose	Source
1	pLKO5.sgRNA.EFS.GFP/RFP	gRNA cloning	Addgene #57822/57823
2	pU6-Sp-pegRNA-HEK3 CTT ins	pegRNA expression	Addgene #132778
3	lentiCas9n(D10A)-Blast	D10A nickase expression	Addgene #63593
4	lentiCRISPR v2-dCas9	dCas9 expression	Addgene #112233
5	pMD2.G	VSV-G envelope expressing plasmid	Addgene #12259
6	psPAX2	2nd generation lentiviral packaging plasmid	Addgene #12260
7	ABE8e(TadA-8e V106W)	IVT for mRNA synthesis	Addgene #138495
8	pCMV-PE2-P2A-GFP	PE expression	Addgene #132776
9	pU6-pegRNA-GG-acceptor	pegRNA cloning	Addgene #132777
10	pLenti-ABERA-P2A-Puro	Lentiviral construct for stable cell line preparation	Addgene #112675
11	pLenti-FNLS-P2A-Puro	Lentiviral construct for stable cell line preparation	Addgene #110841

12	pCMV–PEmax	IVT for mRNA synthesis	Addgene #174820
13	Plenti.ABE8e-P2A-puro	Lentiviral construct for stable cell line preparation	This study
14	Plenti.dCas9ABE8e-P2A-Puro	Lentiviral construct for stable cell line preparation	This study
15	pCMVdCas9ABE8e(TadA-8e V106W)	IVT for mRNA synthesis	This study
16	pLenti-PE2-P2A-Puro	Lentiviral construct for stable cell line preparation	This study
17	plenti-U6-pegRNA-GG-acceptor	Lentiviral pegRNA acceptor	This study

Table 3.6 Primers used for Gibson assembly:

N	Primer name	Primer sequence 5'- 3'	Purpose
1	ABE 8e lenti F	acacaggtgtcgtgacgcgggatccgccaccatgaaacggacagccgacgg	Cloning of ABE8e/dCas9 ABE8e into lentiviral backbone
2	ABE 8e lenti R	agttggtggcgccgctgccgctagcgactttcctcttctcttgg	
3	PE2 Lenti F	gccagaacacaggtgtcgtgacgcgtctagagccaccatgaaacggacagccgacggaagcg	Cloning of PE2 into lentiviral backbone
4	PE2 Lenti R	gtggcgccgctgccgctagcatgcatgactttcctcttcttcttgg	
5	Peg 57822 F	taaaatggactatcatatgcttacgtaac	Cloning of lentiviral peg RNA acceptor vector
6	Peg 57822 R	ctagaattcaaaaaagagacggtcacgtc	
7	Frag1_F	acgaggacattctggaagatatcgtgctgaccctg	Site directed mutagenesis for creation of dCas9ABE8e
8	Frag1_R	ctctgaggcacgatagcgtccacatcgtagtcgga	
9	Frag2_F	tccgactacgatgtggacgctatcgtgcctcagag	
10	Frag2_R	tcttcttctgggctcgaattcgctgccgtcggcg	

Table 3.7 List of primers used for sequencing in the study

N o	Primer name	Primer sequence 5'- 3'
1	HBG_SAN_F	acaaaagaagtcctggtatc
2	HBG_SAN_R	cttcccagggtttctcctcc
3	HBD_SAN_F	atgtagaggagaacagggtt
4	HBD_SAN_R	ccatcactaaaggcacctag
5	AAVS1_SAN_F	gagatggctccaggaaatgg
6	AAVS1_SAN_R	acctctcactcctttcatttgg
7	HEK3_F	atgtgggctgcctagaaagg
8	HEK3_R	cccagccaaactgtcaacc
9	RNF_SAN_F	acgtctcatatgcccttgg
10	RNF_SAN_R	gtaggaattttggtgggaca
11	HEXA_SAN_F	catacaggtgtggcgagagg
12	HEXA_SAN_R	ccagcctcctttggttagca
13	HBB_SAN_F	gtcatcacttagacctacc
14	HBB_SAN_R	ctgtacctgttacttatcc
15	HBB_CDNA_SAN_F	acatttgcttctgacaca
16	HBB_CDNA_SAN_R	tctggattctgcctaataaa
17	INT1_SAN_F	ggctggctagggatgaagaa
18	INT1_SAN_R	aaaccctttgttgctcatat
19	HBB_NGS_1F	ctattggtctcctaaacctgtcttgaacct
20	HBB_NGS_1R	aggagcagggaggcgagga
21	HBB_NGS_2F	gcttctgacacaactgtgtt
22	HBB_NGS_R	tcttctctgtctccacatgc
23	HBD_NGS(OT_1)F	ccatttgctccttgagcctct
24	HBD_NGS(OT_1)R	tcactggagcaggaggagaca
25	OT_2F_NGS	catatgaacctaatctcccaaaggctc
26	OT_2R_NGS	ctctgctgtaaaaacttgcttgg
27	OT_3F_NGS	cctgctgacacccctatag

28	OT_3R_NGS	ctcttctcttccctccctc
29	OT_4F_NGS	cggaaaatacggcttgaggagag
30	OT_4R_NGS	ctgaggtctaaccagcacttg
31	HBG_NGS_F	tacacgacgctcttccgatctggaatgactgaatcggaacaa
32	HBG_NGS_R	Agacgtgtgctcttccgatctgacgtccagaagcgagtgt

3.3 gRNA/pegRNA designing and cloning

sgRNAs for base editing were designed manually considering the PAM requirements and the editing window. For cloning into lentiviral vectors, gRNAs were obtained as forward and reverse oligonucleotides with appropriate overhangs. The complementary oligonucleotides were annealed and cloned into *BsmBI* digested pLKO5.sgRNA.EFS.GFP/RFP vector (Addgene #57822/#57823) as per established protocols(Ravi et al., 2023).

Table 3.8 List of gRNAs used in the study

Sl No	Name	5'-3' Sequence excluding PAM
1	HBG G2	cttgaccaatagccttgaca
2	HBG G3	atatattgcattgagatagtg
3	HBG G4	gtggggaaggggcccccaag
4	HBG G11	ggcaaggctggccaacccat
5	AAVS1	ggggccactaggacaggat
6	HBB1	catggtgcatctgactcctg
7	HBB2	gtaacggcagacttctctc
8	HBB3	ccacgttcaccttgccccaca
9	HBB4	cgtggatgaagttggtggtg

10	KAG1	gaaggtaggctctggtgacc
11	KAG2	aggctctggtgaccaggaca
12	KAG3	ggctctggtgaccaggacaa
13	KAG4	tctggtgaccaggacaaggg
14	KAG5	caggacaagggagggaagga
15	KAG6	ggaaggaaggaccctgtgcc
16	KAG7	cctgtgcctggcaaaagtcc
17	KAG8	aaaagtccaggtcgcttctc
18	KAG9	aggtcgcttctcaggatttg
19	KAG10	actgttcttgtaattcac
20	KAG11	agaacagtttgacagtcaga
21	KAG12	acaaatcctgagaagcgacc
22	KAG13	aagcgacctggacttttgcc
23	KAG14	cctggacttttgccaggcac
24	KAG15	ctggacttttgccaggcaca
25	KAG16	cttcttccttccttgctcc
26	KAG17	tgaccaggacaaggaggga
27	KAG18	ctggtgaccaggacaaggga
28	KAG19	tcaccagagcctaccttccc

pegRNAs were designed using PrimeDesign software. The components were obtained as oligonucleotides with appropriate overhangs for golden gate assembly. 1 μ L each of 1:10 diluted annealed oligos (total 3 μ L), 50 ng of digested vector, 0.5 μ L

of T4 DNA ligase, 0.25 μ L of *BsmBI* V2 enzyme and 1 μ L of T4 DNA ligase was mixed and volume was made up to 10 μ L using nuclease-free water. The golden gate assembly reaction was carried out as follows: (42°C, 3 min $\rightarrow$ 16°C, 3 min) x 30 cycles, 60°C, 5 min $\rightarrow$ 4°C. The reaction product was then transformed into DH10B competent cells and plated in LB agar. For both sgRNA and pegRNAs lentiviral constructs, colony PCR followed by sanger sequencing using primers listed in Table 3.7 was done to confirm the cloning.

Table 3.9 List of pegRNAs used in the study

N	Name	Spacer (5' TO 3')	Extension
0			
1	HEK_CTT	caccggcccagactgagcacgtgagttt t	gtgctctgcatcaaagcgtg ctcagtctg
2	RNF_C>G	caccgtcatcttagtcattacctggttt	gtgcaacgaacacctcacgta atgactaagatg
3	HEXA_TATC	caccgtacctgaaccgtatacttagttt	gtgcagtcaggccataggat agatatacggtc
4	HEK_HBB_INS	caccgcatgggtgcacctgactcctggttt t	gtgcagacttctccacaggag tcaggtgcac
5	HUDEP_HBB_INS	caccgcatgggtgcatctgactcctggttt	gtgcagacttctccacaggag tcagatgcac
6	HBB_COR	caccgcatgggtgcatctgactcctggttt	gtgcagacttctctcaggagt cagatgcac
7	HBB_COR_2	caccgtaacggcagacttctccacgttt	gtgctctgactcctgaggaga agtctgccg

Positive clones were inoculated in LB Broth and plasmid isolation was performed after 16hrs incubation using MN NucleoBond Mini Plasmid kit as per manufacturer's instructions.

Lentivirus was prepared similarly for gRNAs/pegRNAs. 100ul of virus was transduced in 0.2 million cells in 12 well plate. Media was changed 48hrs after transduction and the transduction efficiency was evaluated using GFP/RFP using flow cytometry. Only cells showing >90% transduction efficiency was taken forward for further studies.

3.6 mRNA synthesis by in vitro transcription (IVT)

ABE8e, dABE8e and PE mRNAs were prepared using plasmids described in Table 3.5 . Briefly, the constructs were amplified with forward primer containing T7 promoter and the reverse primer to install poly A tail. The primers are listed in Table 3.10. 1ug of amplified template was used for IVT using Takara IVTpro™ mRNA Synthesis System as per manufacturer's instructions. ARCA capping was incorporated into the reaction and complete substitution of pseudouridine instead of uridine was carried out. The mRNA was purified using LiCl and stored in -80 as small aliquots until use.

the 8-12 μg of retinoids as preparation and transfection

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Table 3.10 List of primers used for IVT

N o	Primer name	Primer sequence 5'- 3'	Purpose
1	PE IVT F	tcgagctcggtagcttaataacgactcactataagg	IVT of PE max for mRNA synthesis
2	PE IVT R	ttt tttttttttttttttttttttttttttttttcctcctactcaggcttatcaaa agacca	
3	ABE8e IVT F	agctgggttagtggaaccgcgatccgcta	IVT of ABE for mRNA synthesis
4	ABE8e IVT R	ttt tttttttttgtttaaacatggatggatgatgattgccgg	

3.7 pegRNA transfection

2×10^5 HEK293T cells were seeded in a 12-well cell culture dish. Upon reaching 80% confluency, the cells were transfected with the appropriate plasmids using FuGENE-HD (Promega) as per the manufacturer's protocol. Briefly, 100 μ L OptiMem, 1,000 ng of plasmids and 3 μ L of FuGENE-HD (1:3 ratio; scaled up when required) was mixed well, incubated for 15 min, and added to the cells. Media was changed 24 h later and cells were dissociated and taken for analysis from 72 h onwards. Prime Editor to pegRNA ratio was maintained at 3:1.

3.8 Nucleofection using Lonza electroporator

pegRNA was electroporated using Lonza 4D electroporation system as per manufacturer's protocol using EN138 program to K562/HUDEP 2 PE stables. Cells were allowed to recover for 5 minutes in the cuvette in plain IMDM before transferring to prewarmed media in 6 well plate.

gRNA was electroporated similarly to stable cell lines using CA137 pulse code. gRNA/mRNA complex was prepared 5 minutes before electroporation while Cas9 RNP was prepared 15 minutes in advance. The complexes were also similarly electroporated.

3.9 Nucleofection using Maxcyte electroporator

CD34⁺ HSPCs were electroporated using Maxcyte electroporation system. Cells were pelleted, washed in 1x PBS and resuspended in optiMEM media. Cells were mixed with Cas9 RNP complex or gRNA/mRNA complex and transferred to the processing assembly OC 25. The cells were electroporated using the program HSC3 and were allowed to recover for 30 minutes in the cuvette at 37° C before transferring to prewarmed media.

3.10 Nucleic acid isolation

When sufficient cells were available, DNA was isolated using DNA isolation kit (Qiagen) as per the manufacturer's instructions. DNA was eluted in water and stored in 4° C. In experiments involving repeated sampling, DNA was isolated using Quick extract as per the manufacturer's protocol.

RNA isolation was carried out using either MN RNA kit(for cell lines) or MN RNA xs kit (for HSPCs) as per the manufacturers protocol. cDNA conversion was carried out using cDNA Synthesis Kit (iScript™ Bio-Rad). RNA was stored at -80 and cDNA was stored at -20° C.

3.11 Evaluation of editing efficiency

DNA or cDNA was amplified using GoTaq® Hot Start Polymerase premix (Promega) with appropriate primers. Either sanger sequencing or NGS was performed as appropriate. For sanger sequencing, Brilliant Dye Terminator V2 (Nimagen) was used. For NGS, ready to run libraries were generated by adaptor ligation and sequencing was carried out using Illumina Novaseq 6000 platform. The sanger sequencing data was analyzed using EditR(Kluesner et al., 2018) or ICE (Conant et al., 2022)and NGS data was analyzed using CRISPResso 2(Clement et al., 2019) to evaluate editing efficiency.

3.12 qRT PCR

Relative gene expression ($\Delta\Delta CT$) was measured using primers listed in table 2. GAPDH was used as internal control for normalizing gene expression. Commercially available primers targeting GAPDH and CDKN1a were purchased from Qiagen (QuantiTect primer assay) for the assay and used with TB green premix (Takara) for quantifying gene expression. For all other genes primers listed in table

3.11 were used.

Table 3.11 List of primers used for qRT PCR/ddPCR

N o	Primer name	Primer sequence 5'- 3'
1	HBB E2E3 RT F	acctttgccacactgagtgag
2	HBB E2E3 RT R	tttgccaaagtgatgggcca
3	HBA RT F	cgacaagaccaacgtcaagg
4	HBA RT R	gtggggaaggacaggaacat
5	HBf E2E3 RT F	cttccttgggagatgccata
6	HBf E2E3 RT R	aaaacggtcaccagcacatt
7	GAPDH E7E8 RT F	ctgcaccaccaactgcttag
8	GAPDH E7E8 RT R	gtcttctgggtggcagtgat
9	CAS 9 VAR RT F	ccgaagaggtcgtgaagaag
10	CAS 9 VAR RT R	gccttatccagttcgctcag
11	U6 RT F	agggcctatttcccatgatt
12	U6 RT R	aaactgcaactaccaagaaa
13	WPRE RT F	caccacctgtcagctcctt
14	WPRE RT R	acaacaccacggaattgtca
15	FIX_RT_F	acattgcccttctggaactg
16	FIX_RT_R	tccccagccacttacatagc
17	RT_GADD45_F	gagagcagaagaccgaaagga
18	RT_GADD45_R	cagtgatcgtgcgctgact
19	HBG_RT_DEL_F	aggggctcaacgaagaaaaagtgt
20	HBG_RT_DEL_R	cacttcattgtagtaccgtggaaga
21	HBG_RT_LC_F	aatgaatcagcagaggctcac
22	HBG_RT_LC_R	atgcactaacatccaactatacaaaa
23	HBB_RT_DEL_F	cactttcttctgacataaca
24	HBB_RT_DEL_R	cacagggcagtaacggcaga
25	DD_HBG_DEL_F	acggataagtagatattgaggtgaagc
26	DD_HBG_DEL_R	gtctctttagcttagcagtg

3.13 Vector copy number

Vector copy number was analysed in genomic DNA isolated from the stable cell lines. Primers targeting the Cas9 gene were used to detect the integration of prime editor. A primer set targeting Factor IX gene was used as an internal reference. pCMV-PE2 (Addgene #132775) and an in-house plasmid construct with Factor IX CDS were used as standards.

3.14 MLPA (Multiplex ligation-dependent probe amplification)

MLPA was performed using SALSA MLPA probe mix P102 HBB with 100 ng/μL DNA as per the manufacturer's protocol. The data was analyzed and visualized using Coffalyser software.

3.15 HbF intracellular staining

To evaluate the frequency of HbF-positive cells, the cells were fixed, permeabilized, and intracellular staining was performed using Fetal Haemoglobin Monoclonal Antibody (HBF-1), APC (Invitrogen) as previously described (Canver et al., 2015). The stained cells were analysed by Flow cytometry (CytoFLEX LX Flow Cytometer—BC) to measure the number of HbF-positive cells.

3.16 Enucleation and differentiation

HSPCs were differentiated in erythroid media for 21 days and taken for enucleation and differentiation analysis. 0.5 million cells were stained with 5ul of CD235 PE antibody (1:40 diluted with BSA PBS) and 5 ul of NucRed (Invitrogen). Cells positive for CD235 and negative for NucRed was considered as enucleated. Differentiation was evaluated by staining for CD235 and CD71 in a similar manner.

3.17 CFU assay

Colony forming assay was performed using Methocult Medium (Stem Cell

technologies). 500 cells per sample were seeded in the medium as per the manufacturer's protocol. Colonies were enumerated after 14 days.

3.18 Cell sorting

Single cell, one way or two-way sorting were performed with or without staining as appropriate using BD FACS ARIA III.

3.19 HPLC

HPLC was used to quantify haemoglobin variants and individual globin chains in differentiated HUDEP2 cells. Cells were collected, washed with 1x PBS, and resuspended in double distilled water. The cells were then subjected to sonication following which the suspension was centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was collected and stored at -80 until analysis. The samples were analyzed using VARIANT II Haemoglobin Testing System (Bio-Rad) for haemoglobin variants and data was quantified by the Bio-Rad's Clinical Data Management (CDMTM) Software. Individual globin chains were quantified using Reverse phase HPLC (Shimadzu Corporation-Phenomenex).

3.20 Evaluation of 4.9 kb deletion using qRT PCR

Primers were designed targeting deleted intervening region (on target) as well as the locus control. qRT PCR was performed as described previously using QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). SsoFast EvaGreen Supermix (Takara) was used along with 5 pmol each of forward and reverse primer and 50ng of DNA for the PCR.

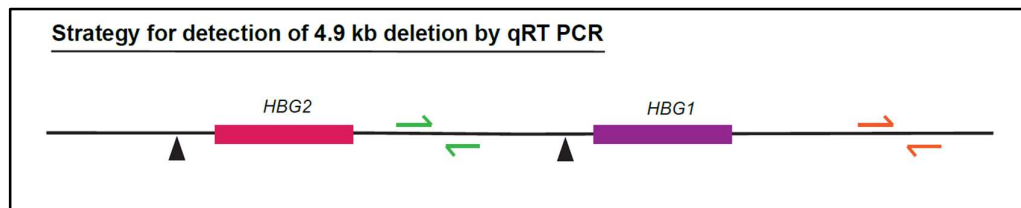


Figure 3.2: Strategy for detection of 4.9 kb deletion by qRT PCR

3.21 Evaluation of 4.9kb deletion using ddPCR

BioRad ddPCR system was used for quantification of 4.9kb deletion using ddPCR supermix for probes. The primers and probes used are listed in table 3.11. The frequency of deletion was calculated by normalizing the ratio of the deletion probe with locus control probe against the average ratio observed in unedited control samples

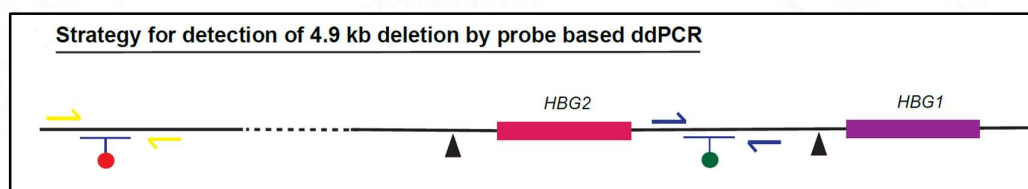


Figure 3.3: Strategy for detection of 4.9 kb deletion by ddPCR

3.22 Evaluation of large deletion using conventional PCR

Forward primer specific to HBG2/HBD and reverse primer specific to HBG1/HBB was designed to specifically amplify the fusion gene product generated after large deletion. 100 ng of DNA was amplified using the appropriate primer set using GoTaq Hotstart master mix (Promega) as per the manufacturer's protocol.

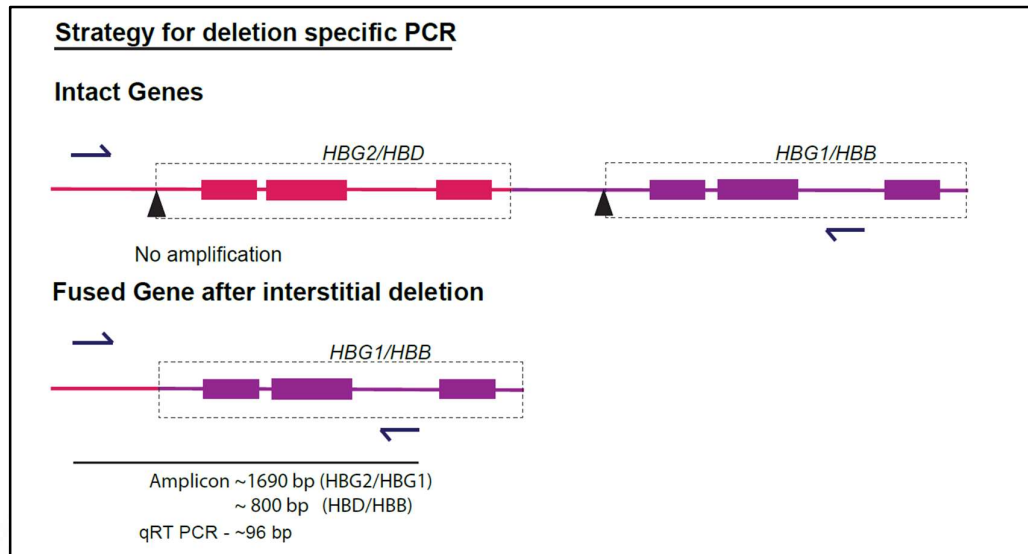


Figure 3.4: Strategy for detection of 4.9 kb deletion by conventional PCR

3.23 Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 8.1. Normal distribution of all data allowed for the use of appropriate statistical tests, such as unpaired two-sided t-tests or one-way ANOVA. Statistical significance was considered at $p < 0.05$. All data are represented as Mean $\pm$ SEM wherever multiple samples are present.

RESULTS

4.1: Improving the precision of base editing in globin locus using non-nicking approaches

Globin locus, owing to its high sequence homology between the genes, is prone to interstitial deletions when targeted using CRISPR Cas9 system. Although base editors were expected to overcome this limitation, we have observed that large intergenic deletions up to 20% occur with both adenosine and cytosine base editors while targeting the gamma globin region (Antoniou et al., 2022; Prasad et al., 2024; Ravi et al., 2022). It is speculated that the convergence of replication fork with the DNA nick results in DSB, and when occurring simultaneously at homologous genes, would result in interstitial deletion. We sought to understand and devise strategies to overcome these large deletions. The experiments were performed using previously validated gRNAs in the gamma and beta globin locus (Fig 4.1.1).

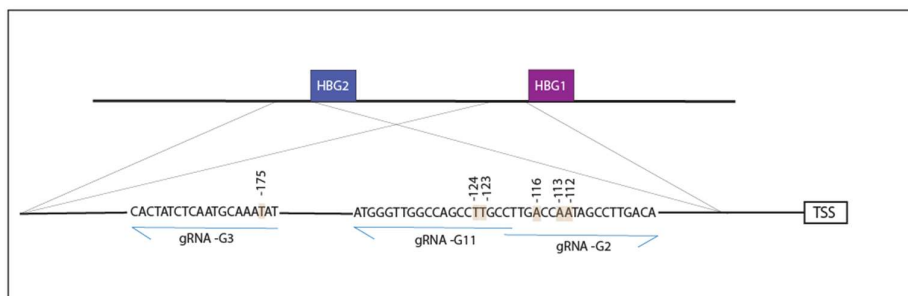


Figure 4.1.1: Schematic representation of gRNAs in gamma globin promoter used in the study

4.1.1: Frequency of interstitial deletion varies with gRNAs and the efficiency of base editing

We had previously observed that the deletion frequency in gamma globin promoter varies with gRNA sequence and the type of base editor used. We first sought

to understand the role of gRNAs in mediating these deletions. To obscure the effect of base conversion in determining deletion frequency, we generated HUDEP2 D10A nickase stable cell lines that would just nick the target region. Three previously validated gRNAs targeting the gamma globin promoter were delivered as lentivirus to these stables and the deletion frequency was calculated by qRT PCR. This would give maximum chance for the gRNAs to bind and simultaneously nick the homologous globin genes.

We observed that G11 gave the highest frequency of deletion while G2 gave lesser and G3 did not show any deletion (Fig 4.1.2 A). To test the gRNA properties affecting large deletion, we assessed the efficiency scores of the gRNAs using two tools- E CRISP and DeepSpCas9 – a hypothesis driven tool and a machine learning tool respectively (Fig 4.1.2 B). G11 had high efficiency while G3 had low efficiency as per both the tools. We also evaluated the MMEJ score of each gRNA using CRISPR RGEN tool and found that G11 exhibited a high MMEJ score which might be responsible for the high frequency of observed deletion (Fig 4.1.2 C). Although a pattern showing the dependence of gRNA efficiency and the target region is seen in this experiment, generalization of this would require extensive studies covering multiple gRNAs in different target regions.

We also tested if quick and efficient base editing can overcome these deletions. We delivered the same gRNAs to HUDEP2 cells stably expressing ABE8e, the hyperactive adenosine base editor. We noted that the deletion frequency was very minimal, not detectable by qRT PCR, and detected only by a conventional deletion specific PCR followed by agarose gel electrophoresis (Fig 4.1.3 A, C, E). Here as well, G3 did not show any deletion suggesting that gRNA plays an important role in

determining deletion frequency.

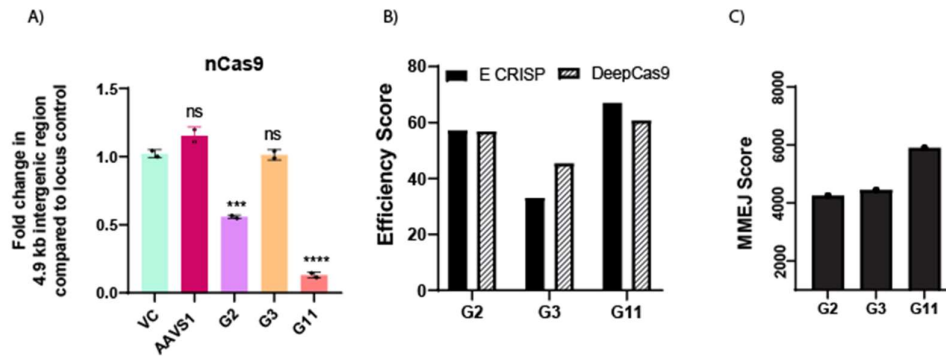


Figure 4.1.2: Evaluation of nick mediated deletion A) Frequency of 4.9kb large deletion in HUDEP2 cells with the use of nCas9. One-way ANOVA with multiple comparisons was used to calculate statistical significance.(ns-not significant, ***- p value <0.001, **** - p value <0.0001) B) *in-silico* predicted efficiency scores of gRNAs C) MMEJ scores of gRNAs used in the study.

Although editing with ABE8e in cell line via lentiviral approach reduced deletion frequency, it might not be possible to achieve similar editing frequency in primary cells and that can lead to increased incidence of interstitial deletions. There might also be not many choices for gRNAs when attempting to introduce precise mutations such as correction of disease-causing mutation or disrupting TF binding sites. This requires the development of methods to overcome large deletion while maintaining editing efficiency.

4.1.2: dCas9 ABE8e can edit efficiently in cell lines and in primary cells while overcoming large deletions

CBE was initially designed with a dCas9 fusion to deaminase. However, it was inefficient in introducing the desired mutation. The introduction of D10A nickase cas9 helped in tricking the cellular DNA repair machinery into efficiently installing the base conversions created by CBE. Subsequently developed base editors were made as

fusions of D10A nickase to deaminase including the hyperactive variant ABE8e. We hypothesized that with its very high activity, ABE8e could do away with nickase and a dCas9 fusion would be sufficient for its activity. This would also prevent the formation of large interstitial deletions.

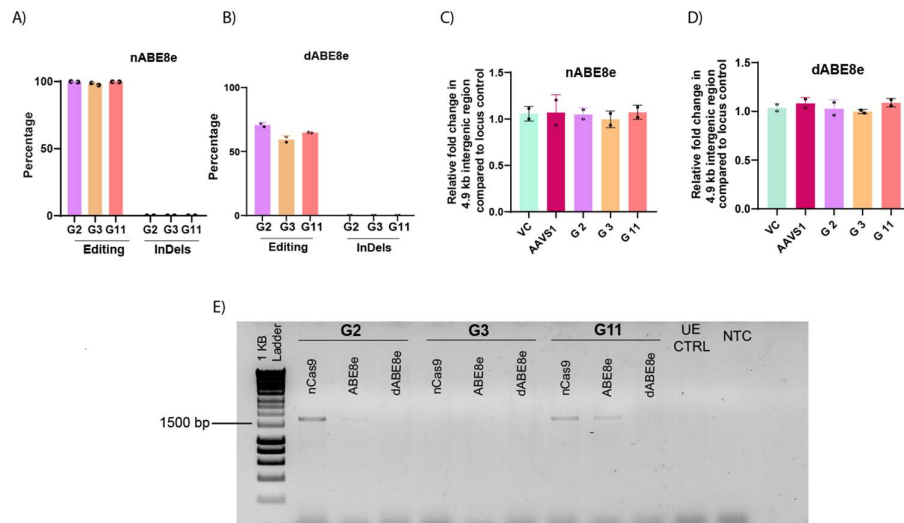


Figure 4.1.3: Base editing in HUDEP 2 cells using ABE8e and dABE8e. Editing efficiency and InDel formation in HUDEP2 cells with the use of nABE8e (A) and dABE8e (B). Frequency of 4.9kb large deletion detected by qRT PCR in nABE8e (C) and dABE8e (D) edited cells. E) Detection of the formation of fusion gene after 4.9kb deletion using conventional PCR.

To test this, we generated dCas9 ABE8e constructs by site directed mutagenesis in nCas9 ABE8e constructs to introduce H840A mutation. We first tested this in HUDEP2 cell line via lentiviral delivery of gRNAs into dCas9 ABE8e stables and evaluated the editing efficiency as well as deletion frequency. We found the dCas9 ABE8e was able to overcome large deletions while maintaining good editing efficiency (Fig 4.1.3 B,D). As expected, the editing levels were slightly lower than what was observed with nABE8e.

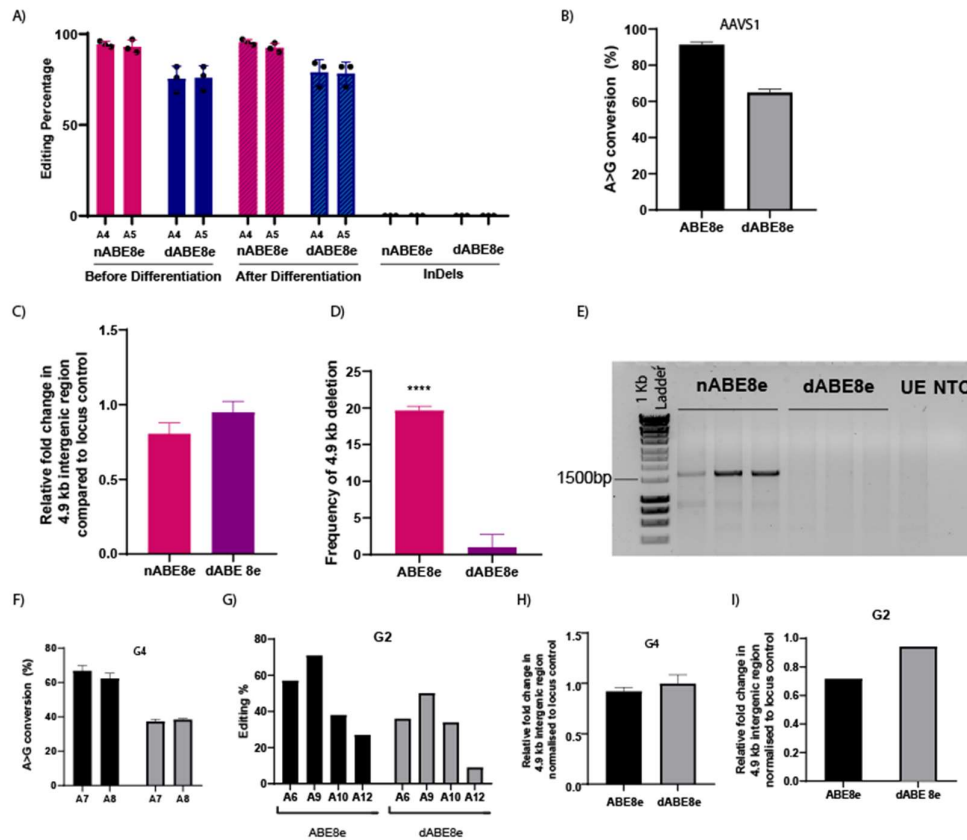


Figure 4.1.4: Base editing in gamma globin promoter using dABE8e. Editing efficiency of nABE8e and dABE8e using gRNAs G11 (A), AAVS1 (B), G4 (F) and G2(G) in Cd34+ve HSPCs. Frequency of large deletion in edited samples detected by qRT PCR(C, H, I) and ddPCR (D) (Student T test was used to determine the statistical significance. **** - p Value <0.0001). Detection of fusion gene formation in HSPCs after 4.9kb deletion detected by conventional PCR.

We further evaluated dABE8e in parallel with nABE8e in terms of editing efficiency and frequency of large deletions in human CD34+ve HSPCs. The base editors were synthesized as mRNA and was delivered to the cells via electroporation along with synthetic gRNAs. Editing efficiency and deletion frequency evaluated after 5 days. In all the three gRNAs tested in gamma globin promoter, we found that the editing efficiency of dABE8e was slightly lower than that of nABE8e however, the editing was completely devoid of large deletions or InDels (Fig 4.1.4). We further

tested dABE8e in the beta globin gene using a gRNA having sequence homology to delta globin and here also we found that the dABE8e edited without generating large deletion. We further tested a gRNA that lacks homology and binds only to the beta globin gene (Fig 4.1.5). Here we did not find large deletions even with ABE8e suggesting that simultaneous binding and nicking at the homologous regions is responsible for interstitial deletions. As the editing efficiency is higher with nABE8e by ~20-30% in all target sites tested, it would be advisable to use nABE8e in non-homologous regions. However, dABE8e efficiently prevents the generation of large deletions in homologous regions thus compensating for the slightly lower efficiency.

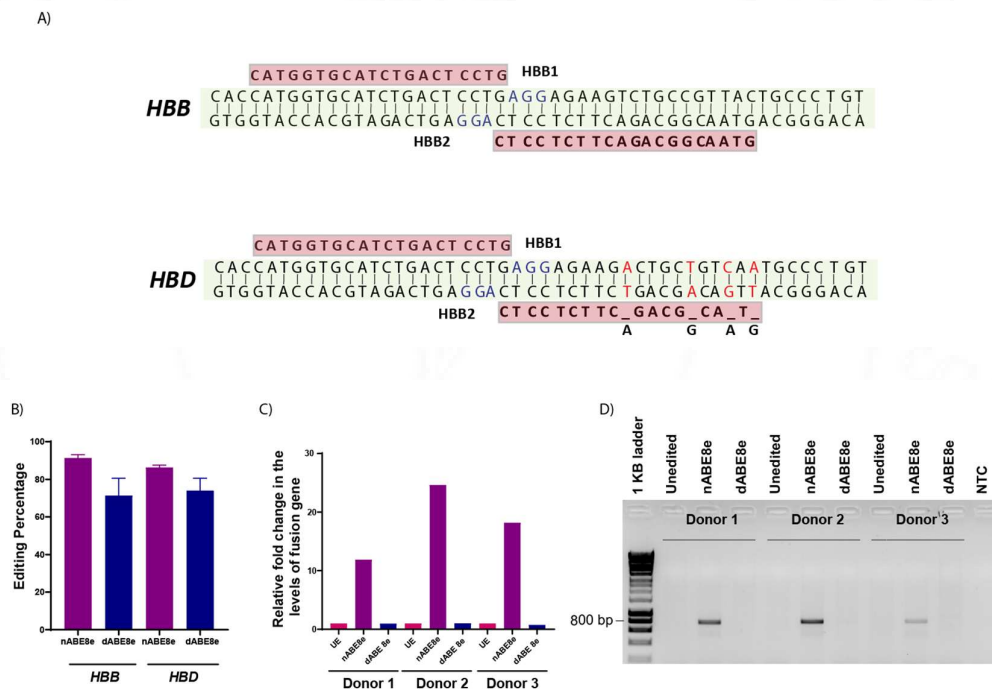


Figure 4.1.5 : Base editing in beta globin gene using dABE8e. A) schematic representation of gRNAs used in the study. B) editing efficiency in HSPCs using nABE8e and dABE8e evaluated using sanger sequencing. C) quantification of large deletion by qRT PCR. D) Detection of fusion gene formed after large deletion using conventional PCR

4.1.3: dCas9 ABE8e can prevent the formation of InDels and large deletions during multiplexed editing in HSPCs

As dCas9 ABE8e was validated to not generate nicks, we tested its utility in multiplexed editing. Often therapeutic base editing requires multiplexed editing using two gRNAs targeting the complementary strands. This would result in DNA nicking on the opposite strands leading to DSBs and consequent InDel formation if the gRNAs are in proximity. This in turn can diminish the therapeutic output and can be detrimental in the coding regions.

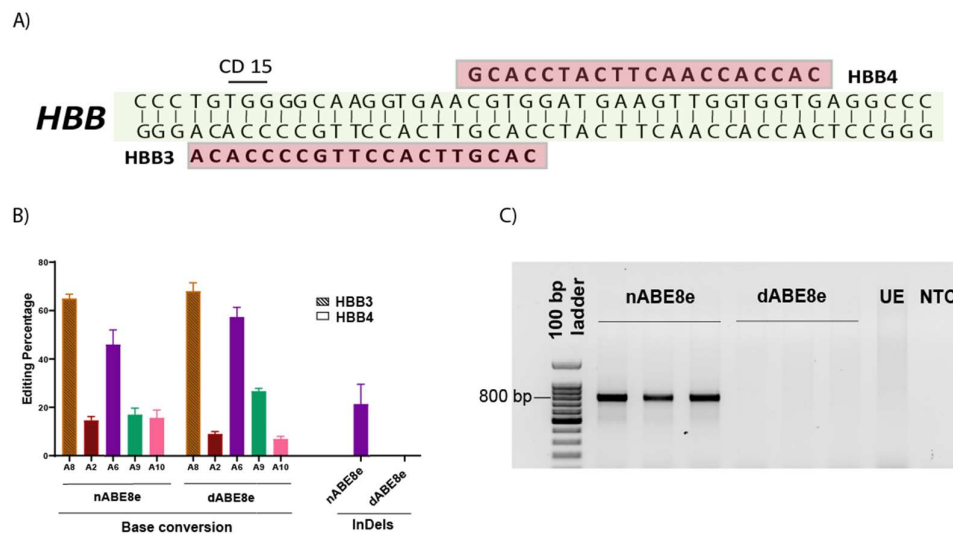


Figure 4.1.6 : Multiplexed base editing in beta globin using dABE8e in HSPCs. A) Schematic representation of gRNAs used in the study. B) Editing efficiency and InDel formation at the target site measured by sanger sequencing. C) Detection of large deletion after multiplexed editing by conventional PCR.

We tested the utility of dABE8e in overcoming these InDels by using two gRNAs targeting the beta globin exon on the complementary strands. We observed close to 20% InDel formation with nABE8e which was completely absent in dABE8e (Fig 4.1.6). To our surprise we also found that the base editing efficiency was higher in dABE8e corresponding to absence of InDels. Taken together, dABE8e would be a

better choice for multiplexed editing in terms of editing efficiency as well as product purity.

4.1.4: Characterization of the differences in editing by dABE8e and nABE8e in HSPCs

Since there is a complete lack of DNA nicking with dABE8e, we sought to evaluate the differences in HSPC editing between nABE8e and dABE8e. First, we tested the editing kinetics from 3 hours to 24 hours after electroporation of gRNAs targeting the beta globin exon as well as gamma globin promoter. We found that the editing efficiency was similar for both editors at 3 hours but the increase in editing overtime was more pronounced in nABE8e. We also noted that this kinetics varied between the two target sites (Fig 4.1.7 D-E). To see if the increase in editing with nABE8e was accounted for by the editing of non-actively dividing primitive HSPCs, which was lacking in dABE8e, we sorted the CD90+ve population after nucleofection and checked the editing efficiency. We did not see any significant difference between the sorted population and the bulk edited population. This suggests that the editing by dABE8e might not be cell cycle dependent (Fig 4.1.7 A).

We also performed a clonogenic assay and found that dABE8e edited cells were able to differentiate into all lineages (Fig 4.1.7 C). However, further experiments such as long-term engraftment in vivo are required to definitely prove the cell cycle dependency and multilineage repopulation potential of cells edited with dABE8e. We further evaluated if the editing efficiency of dABE8e could be increased by increasing the dose of base editor cargo delivered to the cells. There was no significant increase in editing efficiency even with 4 fold increase in cargo suggesting that the availability of components is not the limiting factor in editing with dABE8e (Fig 4.1.7 B) .

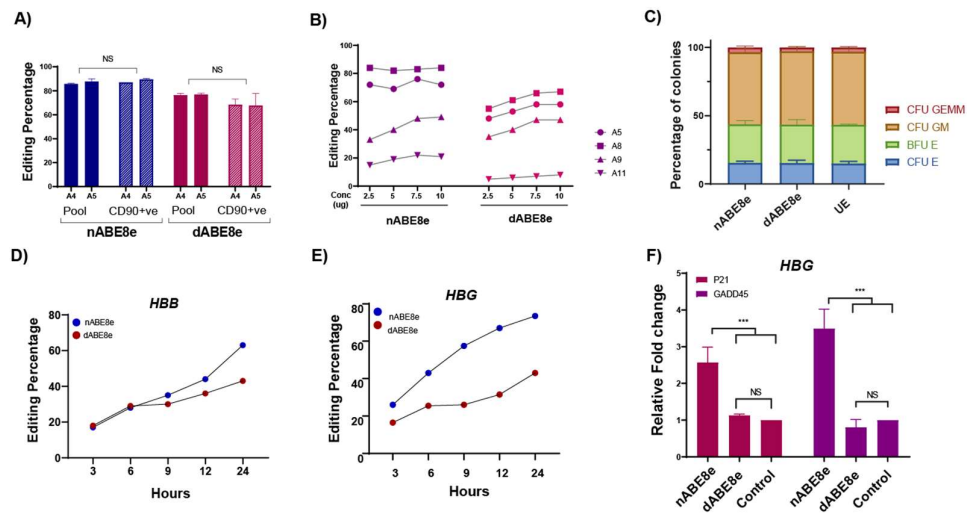


Figure 4.1.7: Characterization of dABE8e mediated base editing in HSPCs. A) comparison of editing efficiency in bulk vs CD90+ve population in HSPCs. B) evaluation of editing efficiency with escalating doses of base editors. C) clonogenic assay following base editing in HSPCs. D, E) editing kinetics in HSPCs at beta and gamma globin genes in HSPCs. F) Relative fold change in DNA damage response genes P21 and GADD45 after base editing in HSPCs

We also evaluated the DNA damage response induced by nABE8e and dABE8e by measuring the gene expression of P21 and GADD45. While nABE8e showed slight elevation in the levels of both the markers, dABE8e showed levels similar to mock electroporated samples (Fig 4.1.7 F). This suggests that dABE8e, due to its lack of nicking, induces less DNA damage response which would improve the overall health of the edited cells.

4.2: Identifying regulatory elements in the gamma globin gene amenable to genome engineering for elevation of fetal hemoglobin

Several studies have reported the presence of regulatory elements in introns including transcription factor binding sites and sequences coding for regulatory RNAs. This study was an attempt to explore if any regulatory elements are present in the intron of gamma globin which can be manipulated by genome engineering approaches to elevate fetal hemoglobin expression.

4.2.1 Intron 1 of gamma globin harbors regulatory elements.

Previously by an arrayed base editor screening we had identified novel regulatory clusters in the gamma globin promoter (Ravi et al., 2022). In a similar approach, to identify genetic regulatory elements, we performed a mutagenesis screening in the first intron of gamma globin using adenosine base editor (ABE7.10). Of the 19 possible sgRNAs with NGG PAM in this region, all but one had at least one 'A' within the gRNA sequence and hence were included in the screening. Base editor stable was prepared in HUDEP-2 cells and the gRNA lentiviruses were transduced to these stables. Transduction efficiency of >80% were achieved in most of samples which rules out any bias from gRNA availability. The gRNA targeting BCL11A binding site in the gamma globin promoter was included as a positive control.

We evaluated the editing efficiency and HbF levels for each of these gRNAs by sanger sequencing and intracellular staining, respectively. Although the editing efficiency varied considerably between the gRNAs, we noted that those targeting the initial one third of the intron (~40bp) upregulated fetal globin to a significant level, some even higher than the positive control. Interestingly, gRNAs KAG2 and KAG3

elevated HbF even without any significant base conversions (Fig 4.2.1). This absence of editing would be due to the lack of adenosines within the preferred editing window for ABE7.10 but the elevation in HbF levels were perplexing. One possible explanation for this elevated fetal globin levels even in the absence of editing would be base editor mediated large 4.9kb deletion altering the chromatin looping in the globin locus or removing any intergenic regulatory elements.

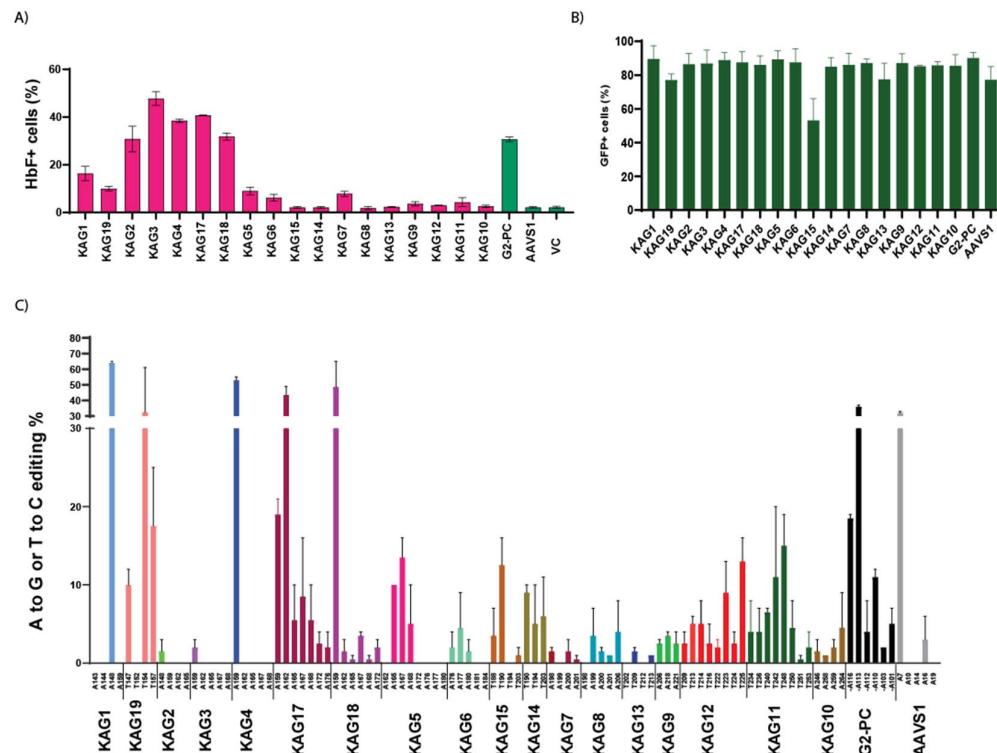


Figure 4.2.1 : Mutagenesis screening of gamma globin intron 1 using ABE 7.10. A) Evaluation of F⁺ cells after base editing in HUDEP2 cells. B) Transduction efficiency of gRNAs used in the study measured as GFP⁺ cells using flow cytometry. C) Evaluation of editing efficiency of the gRNAs used in the study by sanger sequencing.

We had previously demonstrated that the frequency of interstitial deletions in homologous regions decreases with the use of hyperactive base editor variant ABE8e. Hence, to rule out any effects mediated by 4.9kb deletion (in KAG2 and 3) and to

evaluate the effect of increased base conversion frequency, we selected the top 8 gRNAs in this region to edit with ABE8e which was shown to have higher editing efficiency and broader window. An arrayed screening via lentivirus mediated delivery of gRNAs into HUDEP2 cells stably expressing ABE8e was performed (Fig 4.2.2 A,B). Sequencing showed that all gRNAs were able to edit that target regions with ABE8e, but the editing frequency was lower in KAG2 and KAG3(~50%) compared to others(>90%), even though the transduction efficiency was similar (Fig 4.2.2 C). Upon evaluating the frequency of large deletion by qRT PCR, we found that no other guides except KAG2 and KAG3 caused large deletion(Fig 4.2.2 D).

The only property that distinguishes these two guides from others is the lack of adenosine in the preferred editing window for ABE. This might have caused the gRNA to bind repeatedly to the target site increasing the propensity for large deletion. All sgRNAs except KAG1 and KAG19 elevated the proportion of F positive cells to a significant level compared to the negative control and the levels were comparable to or better than the positive control. The F+ve cells in KAG19 were comparable to the negative control suggesting no role for these mutations in HbF elevation. KAG1 on the other hand had lesser F+ve cells probably because of the high editing in the exonic region which had hampered gamma globin expression.

Evaluation of globin chain synthesis corroborated with the previous observations(Fig 4.2.2 E,F). KAG2 and KAG3 had lower levels of G Gamma compared to A gamma while all the other guides maintained a higher G gamma level as expected confirming that large deletion would have occurred only in KAG2/3. Since four gRNAs in this region elevated HbF without any appreciable large deletion, it rules out the possibility of deletion mediated alterations in chromatin looping that

results in reactivated gamma globin expression. What was surprising is that there was only one adenosine editing that was common to all the 4 sgRNAs and the conversion at this site varied considerably but was not reflected in the HbF elevation. The number of F+ve cells were similar for these gRNAs even post differentiation.

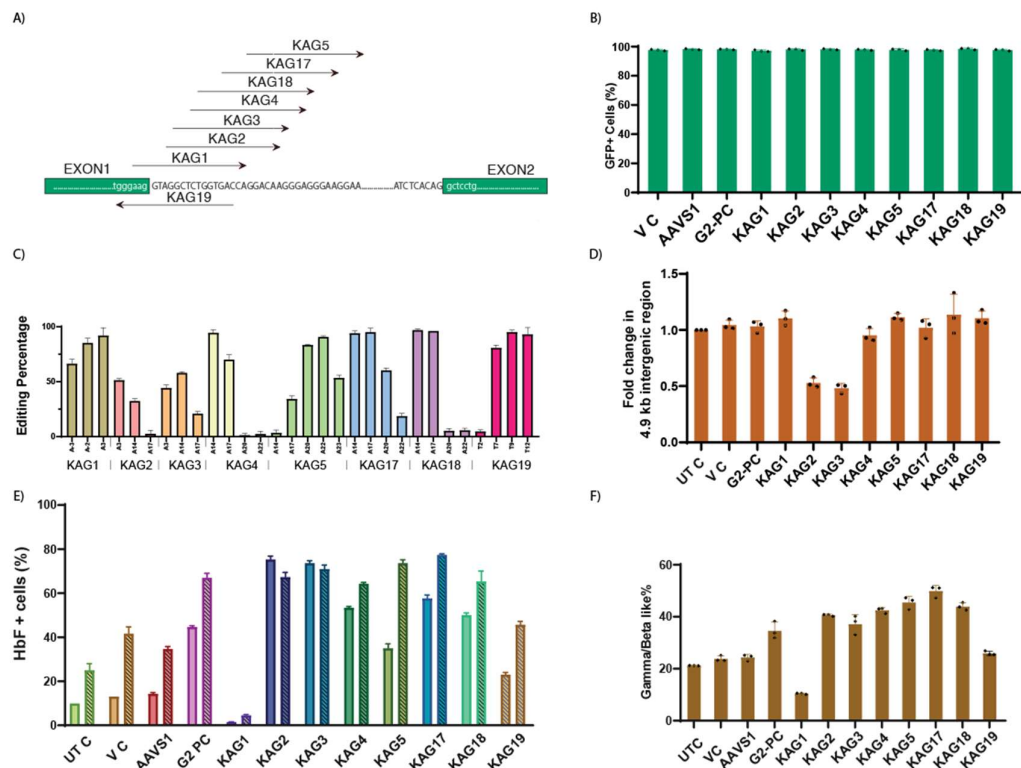


Figure 4.2.2: Evaluation of gRNAs in gamma globin intron 1 using ABE8e. A) Schematic representation of gRNAs used in the study. B) Transduction efficiency C) Editing efficiency D) Frequency of 4.9 Kb deletion E) percentage of F+ve cells and F) Globin chain production by HPLC in samples edited with ABE8e.

Since the editing pattern and HbF levels were not correlating and inconclusive, we tested these gRNAs for their HbF elevation potential using CBE. The base conversion frequency varied between the gRNAs and was dependent on the editing window even when the transduction efficiency was >95% for all. Even with CBE, there was no correlation between editing efficiency and HbF elevation. It was also interesting that the globin chain elevation was high compared to the negative control

and only slightly decreased compared to what was observed in ABE8e (Fig 4.2.3 A-C). However, the background HbF production (in negative controls) was also low in CBE.

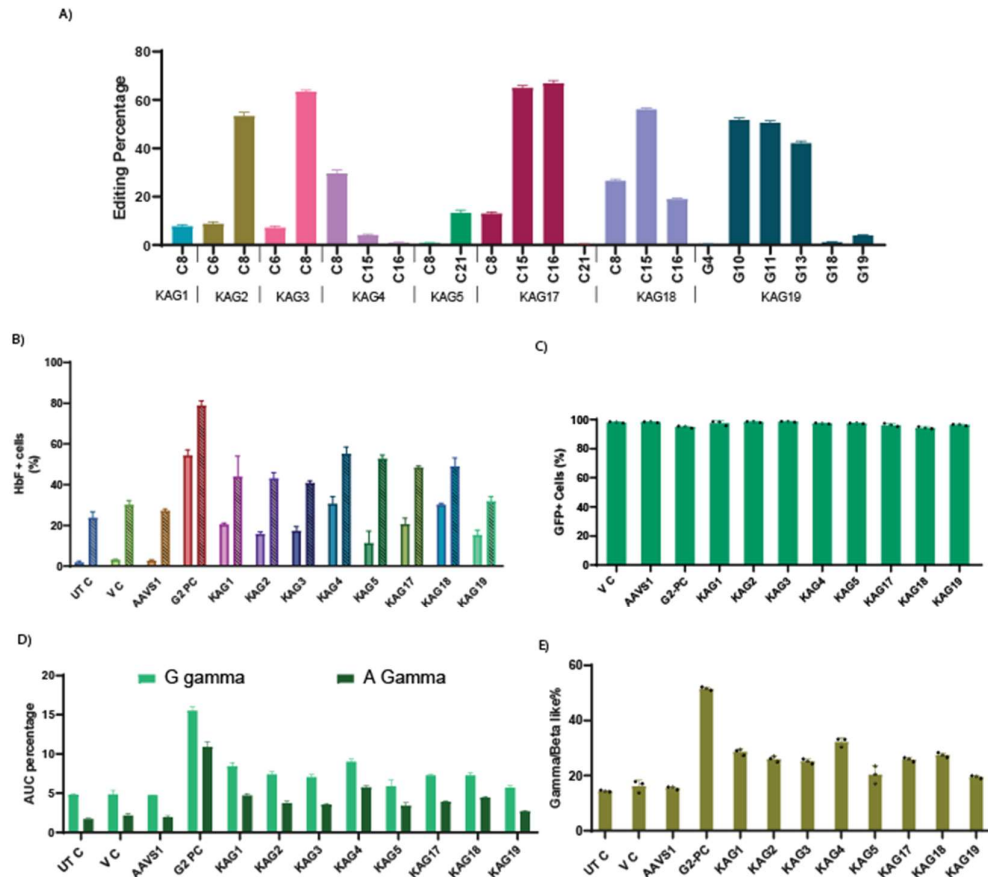


Figure 4.2.3: Evaluation of gRNAs in gamma globin intron 1 using CBE A) Editing efficiency B) percentage of F+ve cells C) Transduction efficiency and D-E) Globin chain production by HPLC in HUDEP2 cells edited with CBE.

There were no observable large deletions even when the editing efficiency was low as evidenced by the normal HPLC pattern with G gamma higher than A gamma (Fig 4.2.3 D-E). We had previously demonstrated that the frequency of large deletion varies with gRNA efficiency and since CBE showed good editing with KAG2 and KAG3, the chances for repeated gRNA binding and consequently large deletion might

have reduced.

Comparison of data from all three base editors suggests that a cluster of gRNAs in the first intron of gamma globin is capable of elevating HbF levels. However, why the gamma globin levels were not correlating with the editing efficiency during base editing in these gRNAs is not understood. The absence of large deletion in ABE8e and CBE samples exclude 4.9kb deletion mediated chromatin reorganization as the reason for HbF elevation but this needs further validation.

4.2.2 Nicking but not editing is responsible for elevated fetal hemoglobin while targeting gamma globin intron.

To understand the mechanism driving HbF elevation in edited cells, we decided to separately evaluate the two genomic effects of base editing – nicking and base conversion. We used the same gRNAs to target the genome either using a Cas9 D10A nickase (which would nick the DNA without base conversion) or a dCas9 ABE8e (which would not nick the DNA but still edit the base) via lentiviral delivery to the stable cell lines. Uniform transduction of >90% was maintained in all samples. As expected, there were base conversions in all the gRNAs treated with dCas9 ABE8e although the editing efficiency was slightly lower than when editing with ABE8e (Fig 4.2.4 A,C). To our surprise there was no appreciable increase in the HbF levels in any of the intronic targets even with high editing efficiency (Fig 4.2.4 B). The editing was higher than what was observed with ABE 7.10, but the HbF levels were unaffected compared to the control. On the other hand, the positive control gRNA targeting the gamma globin promoter elevated HbF even with dCas9 ABE8e proving that editing has crucial role in HbF elevation in the promoter site but not in the intronic region. KAG1 gRNA again showed a decrease in gamma globin levels similar to what has

been observed in ABE8e editing possibly due to the exonic base conversions.

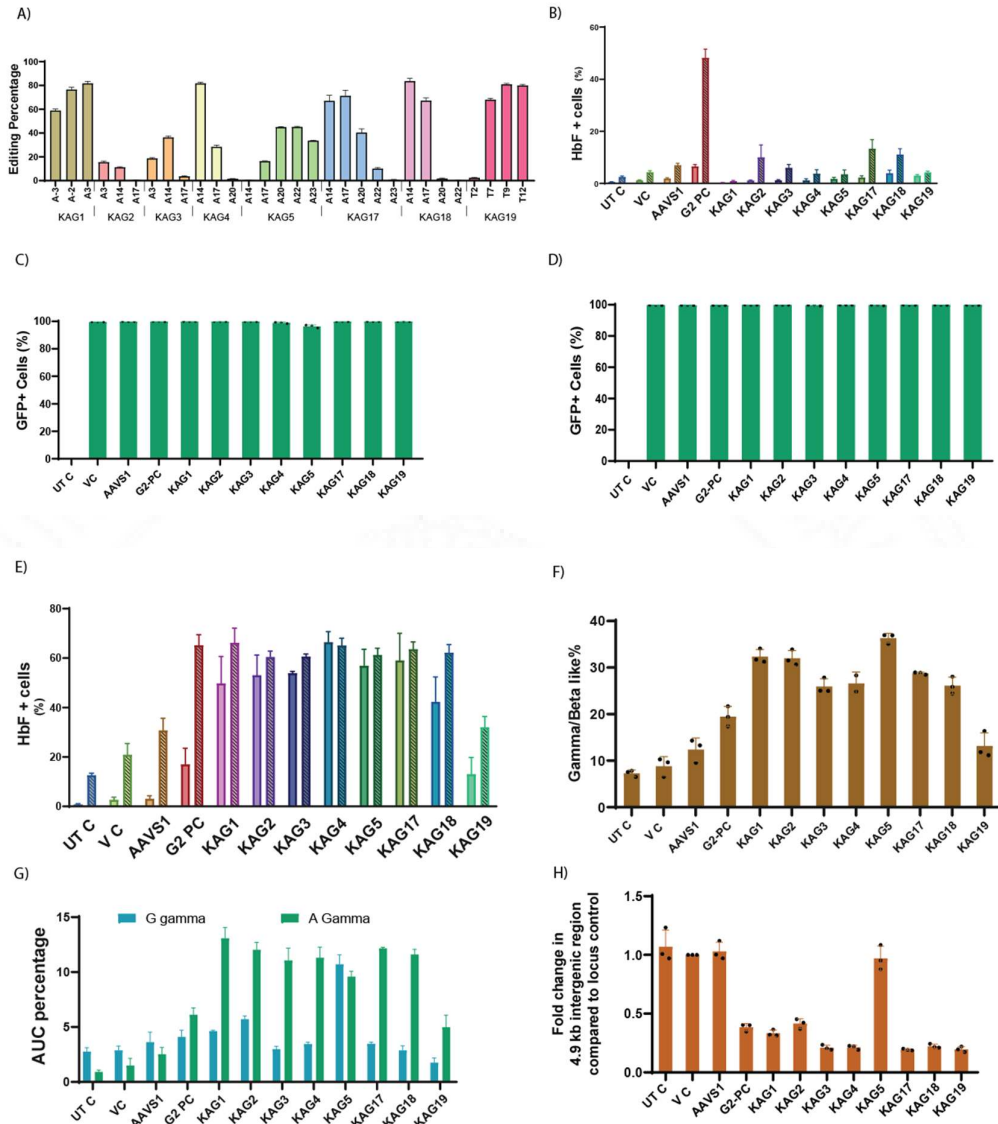


Figure 4.2.4: Evaluation of gRNAs in gamma globin intron 1 using dCas9 ABE8e and nCas9 A) Editing efficiency B) F+ve cell percentage and C) transduction efficiency of HUDEP 2 cells with the use of dCas9 ABE8e. D) Transduction efficiency, E) percentage of F+ve cells F-G) Globin chain production by HPLC and H) frequency of 4.9kb deletion in HUDEP2 cells edited with nCas9 (D10A).

What was more surprising was that with nCas9, all the gRNAs targeting the positive strand (7 out of 8) showed HbF⁺ cells including the positive control targeting the promoter. The elevation in gamma chains were not so significant for the

positive control gRNA compared to the intronic gRNAs (Fig 4.2.4 D-G). In line with the flow cytometry data, we also saw elevated globin chain production by HPLC in intronic gRNAs whereas the control gRNA targeting the promoter did not show appreciable globin chain elevation. We tested if this elevation in HbF is in any way related to 4.9kb deletion as the screening was lentivirus based allowing the gRNAs to bind repeatedly to the target site. All the gRNAs assessed except KAG5 showed large deletion with frequency of more than 70% which was reflected in the globin chain (G gamma /A gamma) ratio as well (Fig 4.2.4 H).

However, KAG19 which binds the negative strand did not alter globin gene expression even with 80% deletion suggesting that large deletion in this region has no role in HbF elevation observed with the use of DNA nickase. The promoter targeting gRNA also showed deletion which did not translate to elevated globin chain level. Moreover, KAG5 which elevated HbF without any editing or large deletion suggests a role for site specific DNA nicking in the elevation of fetal hemoglobin. Taken together the data suggest that the observed HbF elevation is not mediated by transcription factors, rather through some other mechanisms probably involving the DNA repair machinery.

We also considered the possibility of effects mediated by the off-target sites. The possible off-target sites for all the gRNAs were predicted using *in-silico* tools (Data not shown). Although there were multiple possible off-target sites for each gRNA there was no off-target site that was common between all the gRNAs. Only KAG4 and KAG18 shared a few off-target sites. This rules out the possibility that the HbF elevation seen is a result of off-target modifications.

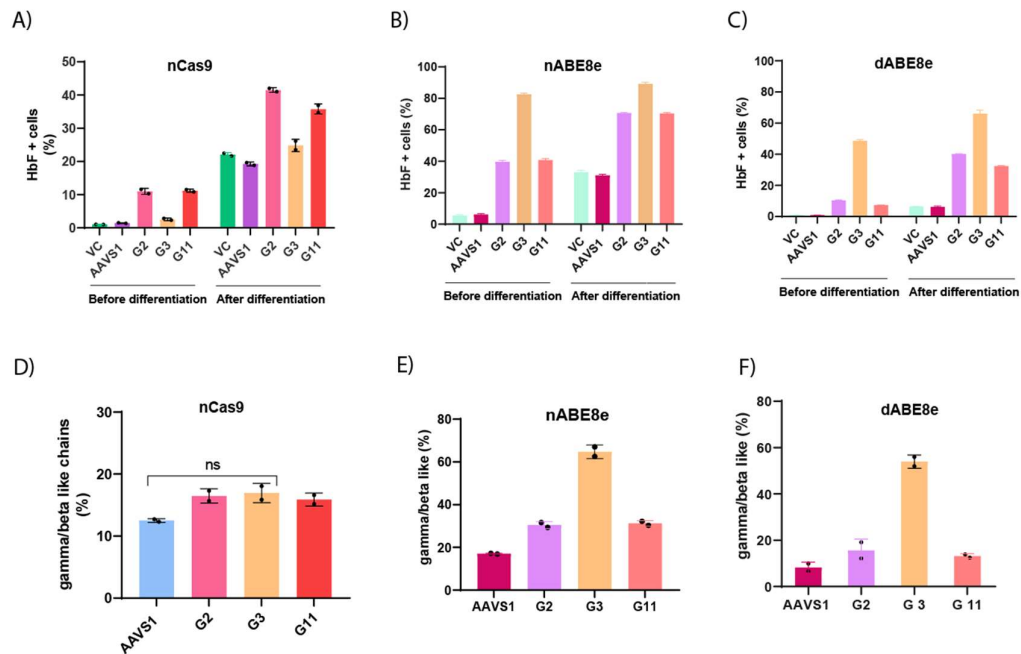


Figure 4.2.5: Evaluation of the effect of DNA nicking on HbF levels in gamma globin promoter A-C) Percentage of F+ve cells after editing with nCas9 (A), nABE8e(B) and dABE8e(C). D-F) Globin chain production after editing with nCas9 (D), nABE8e (E) and dABE8e (F).

4.2.3 The effect of DNA nicking is not evident in transcription factor binding sites at the promoter

We further evaluated if this nick mediated effect can be observed in any of the known HPFH target sites in the gamma globin promoter. We had previously demonstrated nick mediated large deletions happening with gRNAs targeting known HPFH sites. We evaluated the HbF induction in these cells. We noted an increase in F+ve cells in all samples. The elevation in HbF levels correlated well with the editing efficiency, with ABE8e giving the highest HbF elevation followed by dCas9ABE8e. However, the elevation in F+ve cells noted in nCas9 samples were probably induced by the stress of large deletion as there was no significant increase in globin chains compared to the control (Fig 4.2.5 A-F). It is interesting to note that the drop in globin chain levels with dCas9ABE8e is less pronounced for G3 compared to G2 or G11

suggesting that the TAL binding upon base conversion is solely responsible for HbF elevation in this site. There might be mechanisms other than disruption of repressor binding site or the creation of activator binding site responsible for HbF elevation in G2 and G11 which needs further investigation.

4.2.4 Transient nicking is sufficient for altering gene expression for a prolonged period.

The possibility of alteration of chromatin structure by sustained gRNA binding and the resultant HbF elevation was rejected since HbF elevation was not seen with dCas9ABE8e mediated editing. Further, to verify if the observation holds true even with transient editing, we delivered synthetic gRNAs by nucleofection to HUDEP cells. We nucleofected KAG17 along with positive (G2) and negative controls (AAVS1) in nickase Cas9 and nABE8e stables and evaluated the outcomes.

With nickase Cas9, as expected there was no base conversion, and we observed HbF levels of more than 50% before differentiation and it elevated to ~70% after erythroid differentiation. This was reflected in the mRNA level as well as the protein level. However, there was 4.9kb large deletion in KAG17 although to a very less extent (~40%) compared to lentiviral delivery (Fig 4.2.6 A-E). Even when compared to KAG17, the frequency of large deletion in G2 was minimal and the fetal globin elevation was also lesser although it was elevated compared to control.

The observations were also similar with respect to HbF elevation while editing with ABE8e. The editing efficiency reached almost 80% in KAG17, but the HbF elevation was similar to what was observed with nickase Cas9. There was no large deletion, and the fetal globin elevation was noted in the RNA as well as protein level. The major difference observed between nCas9 and ABE8e is the positive control, in

which HbF elevated significantly upon editing with ABE8e (Fig 4.2.6 F-K). Taken together these data suggest that nicking and not editing or large deletion is responsible for fetal hemoglobin elevation in the intronic site.

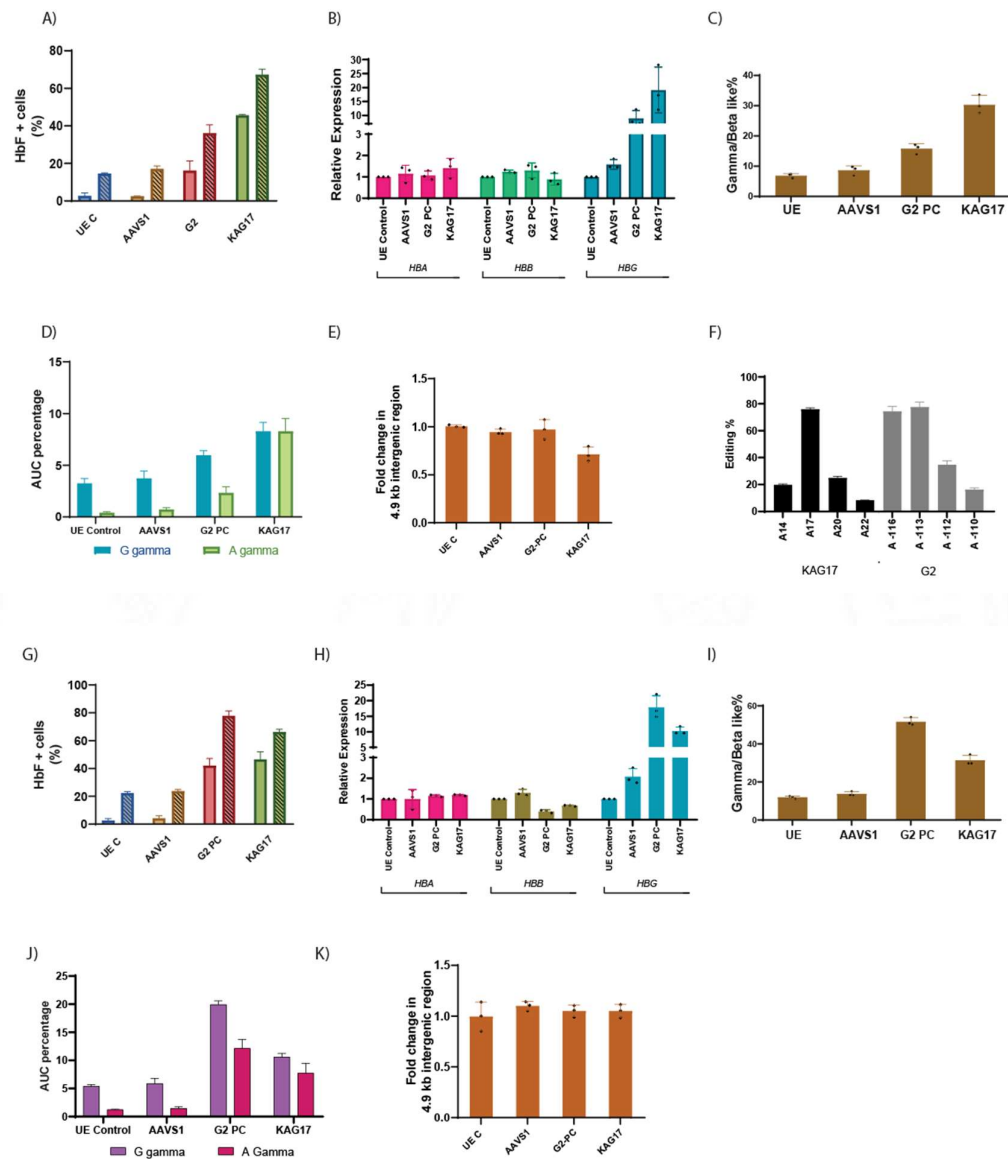


Figure 4.2.6: Evaluation of genotypic and phenotypic outcomes after transient editing in gamma globin intron. A-F) Using nABE8e A) Percentage of F+ve cells B) relative expression of globin genes measured by RT PCR C-D) total and individual globin chain production measured by HPLC E) frequency of 4.9kb deletion measured by qRT PCR and F) editing efficiency measured by NGS. G-K) Using nCas9 G) Percentage of F+ve

cells H) relative expression of globin genes measured by RT PCR I-J) total and individual globin chain production measured by HPLC and K) frequency of 4.9kb deletion measured by qRT PCR

4.2.5 D10A nickase but not H840A nickase elevates fetal hemoglobin

We further aimed to investigate if there is any strand bias in the HbF elevation observed during DNA nicking in the intron. KAG17 gRNA was nucleofected with D10A or H840A nickase (61 pmol nCas9: 100 pmol sgRNA) and the HbF elevation was evaluated at different time points over 20 days. While H840A nicked cells did not elevate HbF, D10A nicked cells showed HbF elevation persisting throughout the experiment (Fig 4.2.7.A). The data suggests two possibilities, HbF elevation driven by a strand specific effect or a nickase specific effect.

To differentiate between the two, we chose an sgRNAs (KAG16) targeting the strand complementary to KAG17. The sgRNAs were delivered as RNP complex with either D10A or H840A nickase (20 pmol nCas9: 40 pmol sgRNA) by nucleofection to HUDEP2 cells. We observed that only D10A nickase but not H840A nickase was able to elevate HbF with both the sgRNAs suggesting a Cas9 nickase specific effect rather than a strand specific effect (Fig 4.2.7 B). While the elevation was very pronounced with KAG17 gRNA, it was less with KAG16, and this could possibly be due to the less efficiency of this gRNA. To rule out inactivity of H840A protein, we delivered KAG17 to previously validated prime editor stable cells which has an active H840A nickase. Even in these stables we could not observe any HbF elevation (data not shown).

Subsequently, we compared the effect of nickases vs Cas9 nuclease using KAG17 in HUDEP2 cells by RNP delivery (75pmol Cas9: 100 pmol sgRNA). While the nickases showed a similar pattern as before with D10A nickase showing high

elevation and H840A nickase not showing any elevation, Cas9 edited samples showed moderately high F+ve cells level.

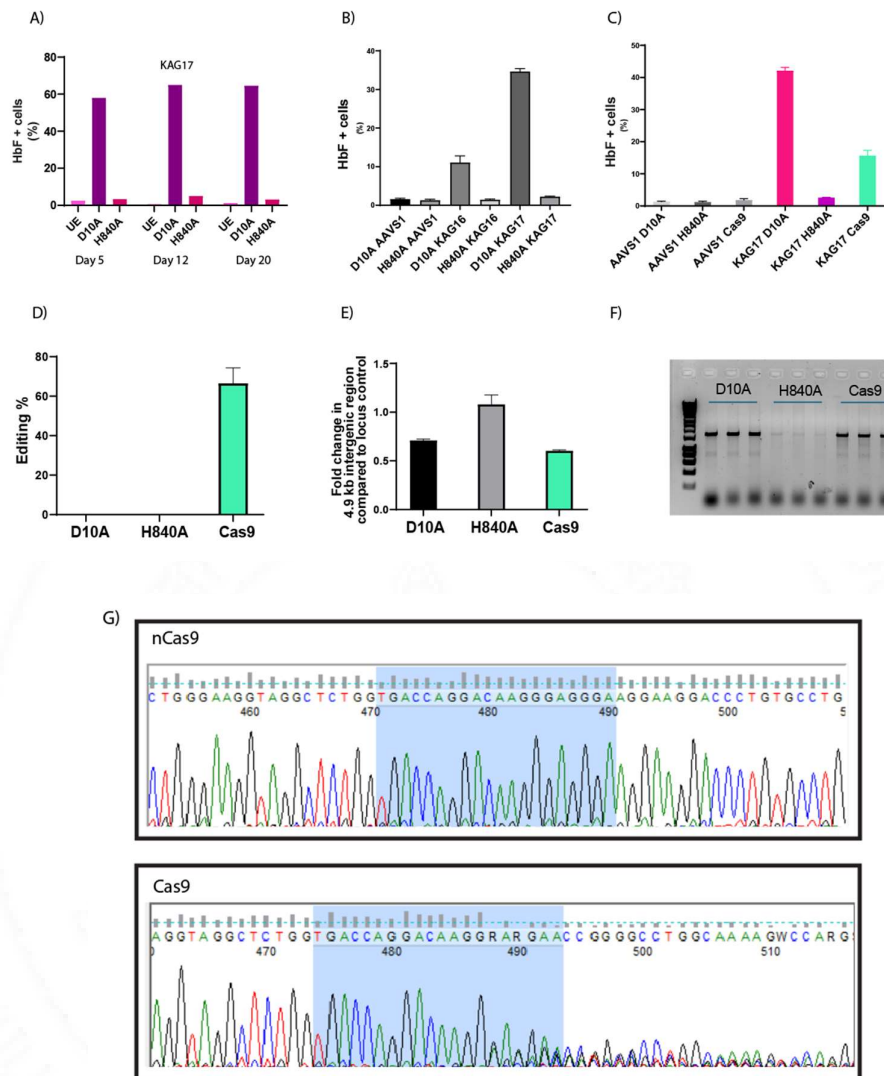


Figure 4.2.7: Comparison of different Cas9 proteins for the HbF regulation potential. A) Evaluation of F+ve cells over a period of 20 days using different nickases. B) Evaluation of F+ve cells using different nickases and gRNAs targeting complementary strands. C) Comparison of elevation in F+ve cells using Cas9 nickases and nuclease. D) Editing efficiency at the target site measures by sanger sequencing using different gene editors. E) Frequency of 4.9kb deletion measured by qRT PCR F) Formation of fusion gene after 4.9kb deletion detected by conventional PCR. G) representative chromatogram of the cut site sequencing of fusion gene formed by nCas9 and Cas9 nuclease.

A similar pattern was observed with KAG16 but at a lower level. We observed that only D10A nickase but not H840A nickase was able to elevate HbF with both the sgRNAs suggesting a Cas9 nickase specific effect rather than a strand specific effect (Fig 4.2.7 C). While the elevation was very pronounced with KAG17 gRNA, it was less with KAG16, and this could possibly be due to the less efficiency of this gRNA.

While no editing was seen with nickases, the indel frequency in Cas9 nuclease edited samples ranged from 60-70%. We observed that only D10A nickase but not H840A nickase was able to elevate HbF with both the sgRNAs suggesting a Cas9 nickase specific effect rather than a strand specific effect (Fig 4.2.7 D). While the elevation was very pronounced with KAG17 gRNA, it was less with KAG16, and this could possibly be due to the less efficiency of this gRNA. We also quantified the frequency of 4.9kb large deletion in these samples and found that while D10A nickase and Cas9 generated large deletions up to 50%, H840A nickase showed minimal deletion. Upon sequencing the fusion gene formed after deletion, we found that the product of nCas9 mediated deletion was largely InDel free unlike that formed by Cas9 (Fig 4.2.7 E-G).

4.2.6 Nick mediated HbF elevation is observed in primary HSPCs as well

To test if the observed phenomenon is limited to cell lines, we performed nicking as well as adenosine base editing using KAG17 in 3 different healthy donors derived HSPCs. Base editor was delivered as mRNA while nickase was given as protein via electroporation. ABE8e mediated editing of BCL11A binding site was used as a positive control to test if this approach can be used therapeutically.

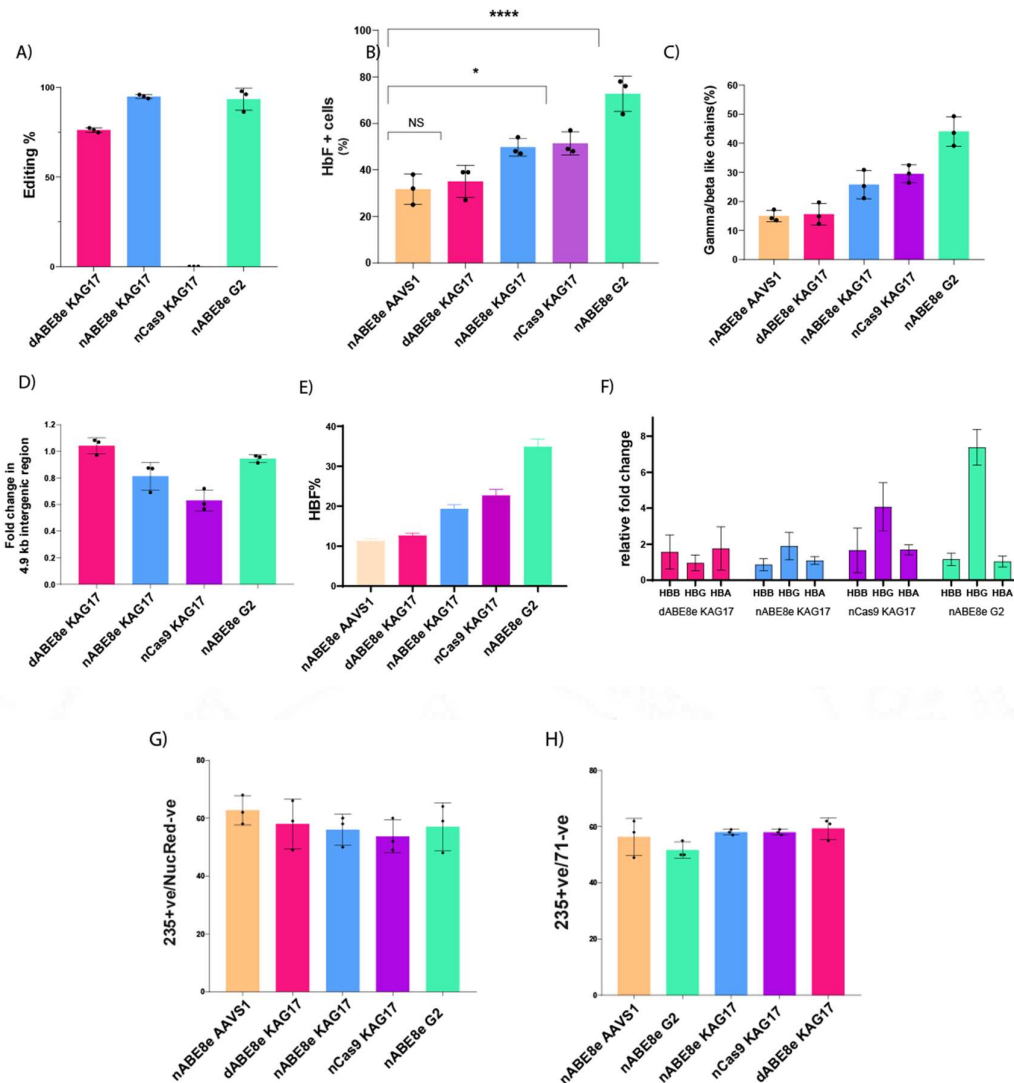


Figure 4.2.8: Evaluation of nick mediated HbF elevation in HSPCs. A) Editing efficiency at the target site measures by sanger sequencing using different gene editors . B) Evaluation of F+ve cells using different nickases. B) Evaluation of F+ve cells after erythroid differentiation. C) Globin chain production and E) total HbF production after erythroid differentiation of the edited samples. D) Frequency of 4.9kb large deletion measured by qRT-PCR. F)Relative fold change in expression of globin genes. G) Enucleation and H) Differentiation pattern of edited cells.

While both the editors elevated HbF to a similar level in all three donors, the levels were not as high as that of positive control(Fig 4.2.8 A-F). On the other hand, dABE8e editing did not give HbF elevation although the editing efficiency was very high. Moreover, we also observed large deletions, with ABE8e as well as nCas9 using

KAG17 however this did not affect erythroid differentiation or enucleation capacity of the cells (Fig 4.2.8 G-H, Fig 4.2.9). Taken together, the data suggest that intronic nick mediated HbF elevation is driven by some mechanism not just restricted to cell lines but also active in primary cells and could be tapped for therapeutic applications if the mechanism is unraveled.

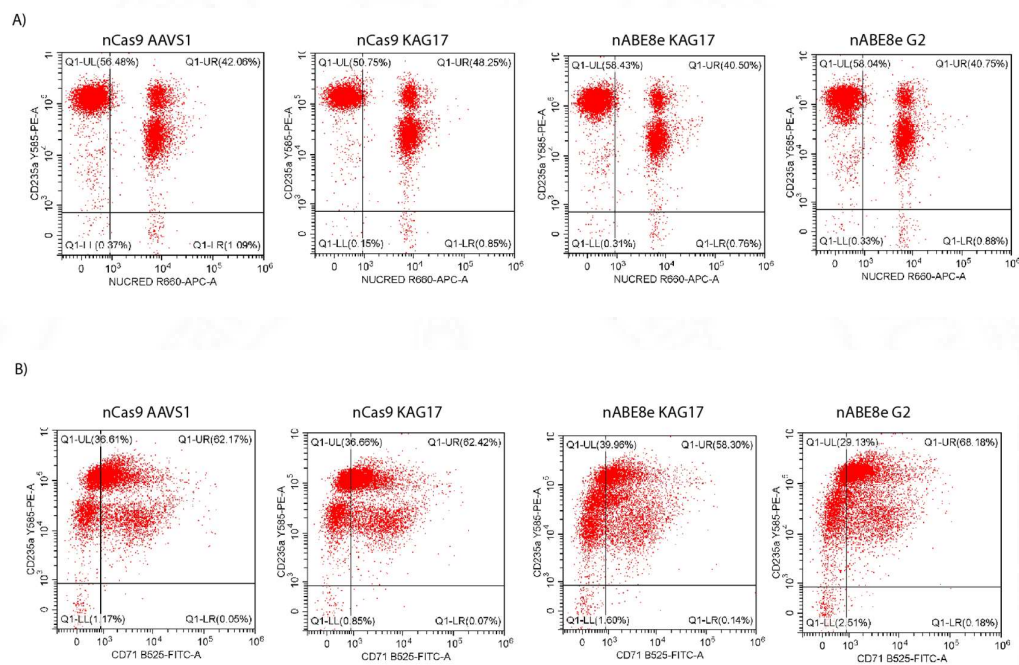


Figure 4.2.9: Representative flow cytometry data of edited HSPCs A) Enucleation pattern B) differentiation pattern

4.3 Correcting the causative point mutation of sickle cell disease in beta globin gene (E6V) using prime editing

Sickle cell anemia is caused by a single point mutation (GAG>GTG) that converts glutamine to valine in the beta globin coding region. This conversion from hydrophilic to hydrophobic amino acid is responsible for the aggregation of Hb and the sickling of RBCs under low oxygen saturation. Currently available base editors are not capable of converting Thymine to Adenine. Additionally, use of DSB mediated approaches in this region poses the risk of generating β -thalassemic mutations if the repair is not precise. Hence, we sought to use prime editing for correcting the mutation.

4.3.1 Generation of a modular lentiviral PE system

One of the major limitations of PE is its low efficiency, especially in specialized cell lines and in primary cells. This hinders the accurate evaluation of on and off-target effects of the editing. We sought to generate a lentiviral PE system which can be constitutively expressed and thus would give better editing efficiency. We developed a two-part system, as depicted in Figure 4.3.1 A. The PE2 is encoded in the first lentiviral vector driven by EF1 α promoter and includes a puromycin selection marker. This would enable the preparation of stable cell lines in which pegRNAs can be screened. Once the stable cell lines are prepared, pegRNAs can be delivered in multiple ways to evaluate the editing efficiency. Some methods include plasmid transfection, plasmid electroporation, synthetic pegRNA electroporation etc.

We also developed a lentiviral pegRNA acceptor cassette, to which the pegRNA can be assembled by golden gate assembly after digestion with *BsmBI* V2 enzyme. The pegRNA expression is driven by U6 promoter. This construct has a GFP

reporter so that the cells with pegRNA integration can be selected for. The design of the construct is in such a way that the pegRNA components with the overhangs suitable for cloning in Addgene #132777 vector can be used for cloning in this vector as well (Fig 4.3.1 B).

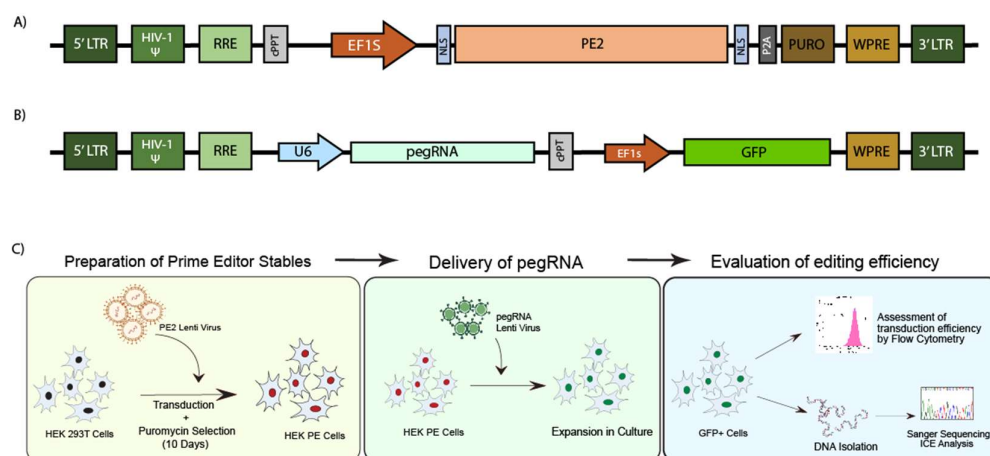


Figure 4.3.1: Development of modular lentiviral prime editing system. A) Schematic representation of PE lentiviral construct. B) Schematic representation of pegRNA lentiviral construct. C) Overall workflow for evaluation of lenti PE system.

4.3.2 Lentiviral delivery of PE2 enables efficient editing in HEK293T cells across multiple target sites.

We tested the efficiency of the lentiviral system using pegRNA targeting CTT insertion in HEK locus, which was tested in previous publications. The pegRNA was cloned into both acceptor vectors. PE2 stable cell lines were prepared by transduction of lentivirus followed by puromycin selection for 10 days (Fig 4.3.1 C). We compared 3 systems in parallel in HEK293T cells, transient transfection of both PE2 and pegRNA, transfection of pegRNA in PE2 stables and transduction of lentiviral pegRNA in PE2 stables. The genomic DNA was isolated after three and six days of

nucleofection and the editing efficiency was evaluated. There was a significant increase in editing efficiency in the dual lentiviral system. This was followed by transient delivery of pegRNA and transfection of both PE and pegRNA showed the least efficiency (Fig 4.3.2 A). Increase in editing efficiency with lentiviral delivery suggests that availability of editing components might be a limiting factor in prime editing. The development of modular lentiviral PE system however enables efficient screening of multiple pegRNAs parallelly.

We validated the utility of the modular lentiviral PE system by editing two other target sites in HEK293T cells. pegRNAs for RNF +1C>G conversion and for TATC insertion in HEXA locus was cloned in lentiviral pegRNA acceptor vector and editing efficiency was evaluated for 15 days and compared to that of CTT insertion in HEK3 locus. As observed previously, CTT insertion was the most efficient followed by RNF C>G conversion and the least efficient was TATC insertion (Fig 4.3.2 B). While CTT insertion reached close to 100% in 15 days, the maximum efficiency for RNF C>G conversion was 50% and that of TATC insertion was ~10%. The data suggests that even with unlimited supply of editors, the prime editing efficiency can be affected by locus and cell intrinsic factors thus requiring multiple screening and optimization steps to select the best pegRNA in any loci. This need underscores the importance of modular lentiviral system that can aid in efficient parallel screening of pegRNAs.

4.3.3 PE2 mediated editing in human erythroid cell lines

After testing the validity of PE2 lentiviral system in HEK293T cells, we further evaluated its ability to edit erythroid cell lines that are used widely for preclinical evaluations in hematological diseases. PE-2 expressing stable cell lines of K562 and

HUDEP 2 were prepared by lentiviral transduction followed by puromycin selection.

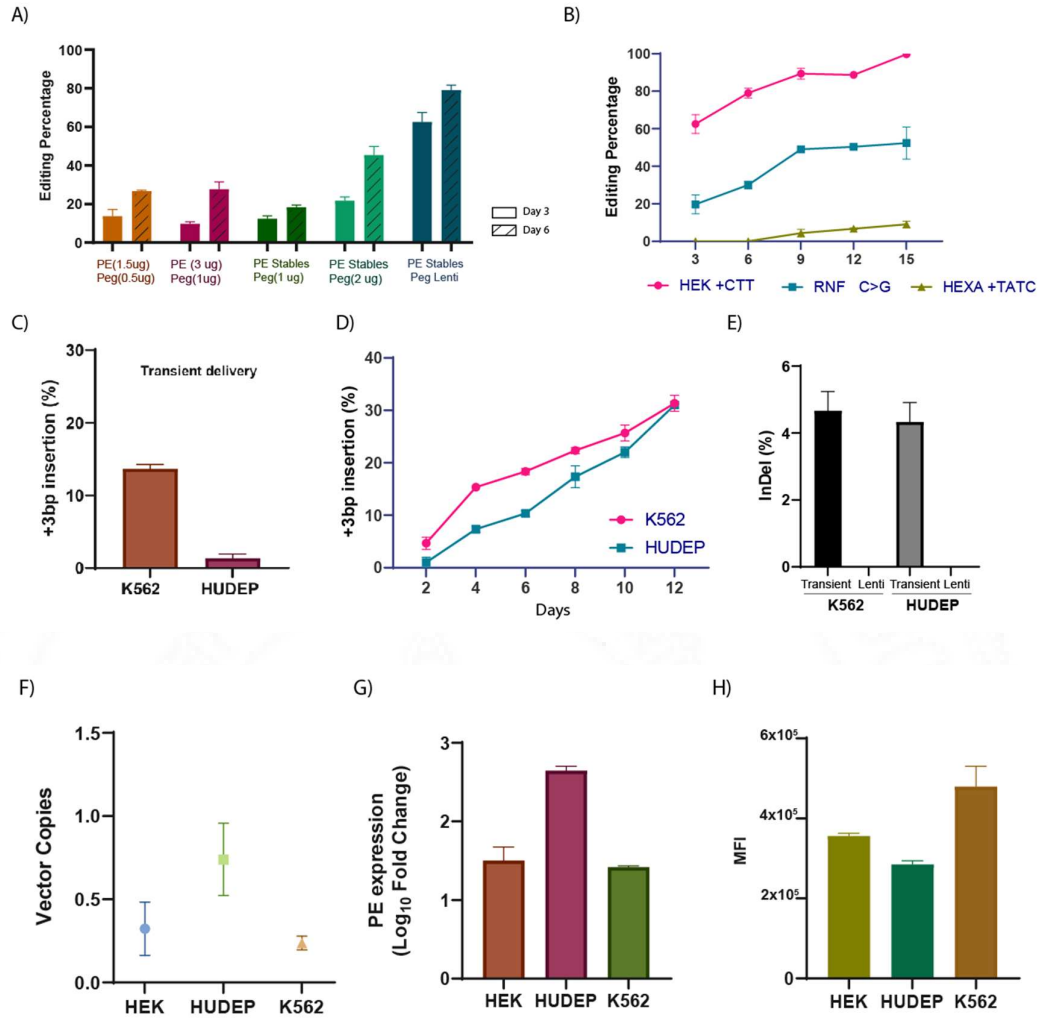


Figure 4.3.2: Evaluation of lentiviral prime editing system in cell lines. A) comparison of editing efficiency using various PE delivery approaches in HEK3 locus in HEK293T cells. B) Evaluation of editing efficiency at different target sites using lenti PE2 system in HEK293T cells. C) Efficiency of 3 bp insertion at HEK3 locus in K562 and HUDEP2 cells by transient delivery of pegRNA into PE2 stable cells. D) Efficiency of 3 bp insertion at HEK3 locus in K562 and HUDEP2 cells by lentiviral delivery of prime editing components. E) comparison of indel formation at target site using lentiviral and transient delivery of components. F) Vector copy number of PE construct integrated in respective cell lines. G) Expression of PE and H) pegRNA in the respective cell lines

We then electroporated the pegRNA for CTT insertion using Lonza electroporator in both the stable cell lines and evaluated the editing efficiency.

Although K562 cells showed moderate editing, the editing efficiency was very minimal in HUDEP 2 cells. As the efficiency was not as expected, we went on to do transduction of pegRNA in PE2 stable cells. With lentiviral delivery we were able to achieve better editing efficiency reaching close to 30% in both the cell lines (Fig 4.3.2 C-E). However, the editing was significantly lower compared to the editing attained in HEK293T cells.

Even with stable expression of PE2 and pegRNA, as we observed varying editing efficiencies, we went on to test the factors that might be responsible. We first tested the vector copies integrated into these stable cell lines and found no appreciable difference between HEK293T and K562 cells but HUDEP2 cells showed a higher VCN for PE2. This pattern was also reflected in the PE2 mRNA expression. The pegRNA expression, quantified by mean fluorescence intensity also did not corroborate with the editing efficiency (Fig 4.3.2 F-H). Taken together, the data leads us to speculate that even in saturating levels of editing components, other intrinsic factors might play a significant role in controlling the editing efficiency. This could be target specific factors such as chromatin accessibility or cell specific factors such as the availability of DNA repair proteins.

Once the lentiviral PE2 system was validated in erythroid cells, we went on to test the creation of sickle mutation in these stable cells. The pegRNA that was shown to give the highest editing efficiency reported in Anzalone et al. was taken forward for the installation of E6V mutation. After transduction, the cells were cultured for 12 days, and the editing efficiency was checked by sanger sequencing (Fig 4.3.3 A-B). We observed comparable editing efficiency in HEK293T cells and in HUDEP2 cells. Surprisingly, the creation of HBB E6V mutation was more efficient than CTT insertion

in HUDEP2 cells but the pattern was inverse in HEK293T cells. This is again suggestive of the role of intrinsic factors in determining prime editing efficiency. Unexpectedly, the editing efficiency in K562 cells was very minimal even with ~100% transduction of pegRNA. We hypothesize that the prime editing efficiency is higher in genes that are actively expressed and since beta globin is expressed in HUDEP2 while repressed in K562, the difference in editing efficiency can be explained. Taken together we show that using the lentiviral PE system, efficient screening of pegRNAs and editing can be performed in multiple cell types.

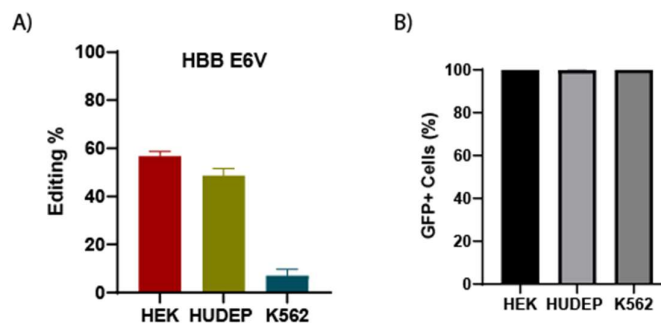


Figure 4.3.3: Installation of HBB E6V mutation using lentiviral prime editing system in cell lines. A) comparison of editing efficiency in different cells. B) Transduction efficiency of pegRNAs

4.3.4 Modelling of sickle cell anemia in HUDEP-2 cell using PE2

HUDEP2 cells were selected for the generation of sickle cell anemia model system because it mimics adult erythroid cells and predominantly expresses beta globin. HUDEP2 PE stables were transduced with pegRNA for insertion of sickle mutation (Fig 4.3.4). The dynamics of prime editing efficiency was evaluated for a period of 30 days. The editing efficiency increased from 16% on day 2 to ~70% by day 30. Even with very high editing efficiency there was no formation of unwanted InDels at the target site (Fig 4.3.5 A-C). We tested the off-target editing using *in-silico*

prediction tool Cas-offinder followed by deep sequencing. Only delta globin gene which shares perfect sequence homology with beta globin had base conversions (Fig 4.3.5.D).

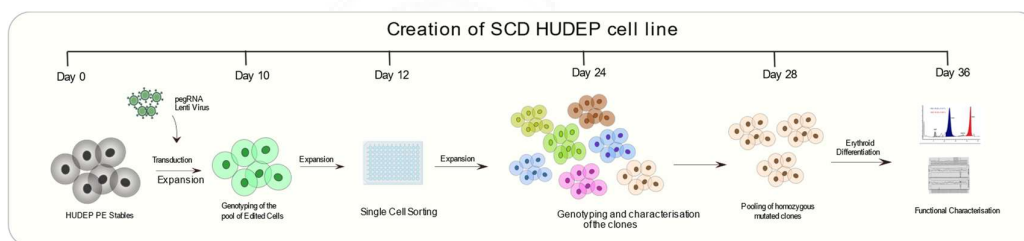


Figure 4.3.4: Schematic representation of the workflow for creation of sickle mutation in HUDEP2 cell line

However, the base conversion was significantly lower than that was seen in the beta globin, although the off-target editing also increased overtime. Off-target editing at delta globin would not be a concern during editing of sickle mutation as patient wild type sequence would be present at delta globin region inherently.

To obtain cells that are homozygous for sickle mutation we performed single cell sorting of GFP positive cells. Of the 21 clones that were evaluated, 8 clones were homozygous for sickle mutation and the remaining were heterozygous. Clones with homozygous editing were chosen for further studies (Fig 4.3.6 A) . The overall editing efficiency on the day of sorting was around 55%.

First, we differentiated the cells and looked at the hemoglobin expression. We found that 6 out of 8 clones expressed high HbF levels. When we checked the genotype of these cells, we found that they harbored the deletion of intervening region spanning the region from nick site at delta globin to the nick site at beta globin. This was in line with our observation that DNA nicking causes large deletions involving the intervening regions in homologous sites (fig 4.3.6 B-C).

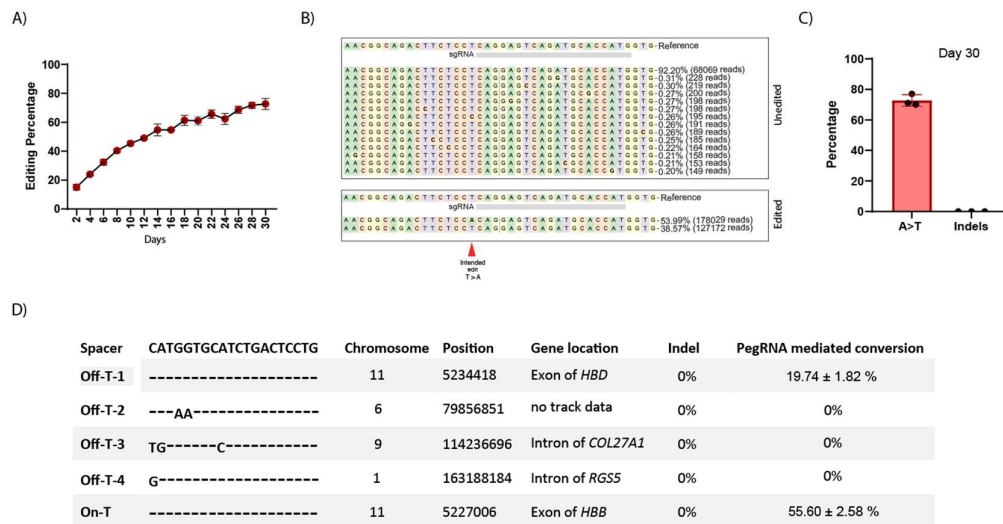


Figure 4.3.5 : Creation of sickle mutation in HUDEP2 cells: A) editing efficiency of pegRNA in HUDEP2 PE stables over a period of 30 days B) installation efficiency of sickle mutation in bulk edited population on the day of sorting. C) Total editing efficiency and indels on day 30 in bulk edited population D) Analysis of off-target editing at sites predicted by Cas-offinder

The two clones that did not have any deletions were chosen for further validation. MLPA showed that there were no genomic alterations except the introduction of sickle mutation (Fig 4.3.6 D-E). HPLC analysis of differentiated samples also showed that HbS was the predominant hemoglobin expressed in these cells (Fig 4.3.6 F). Thus, we developed HUDEP sickle mutant cell lines that can be used for testing the pegRNAs for correction of sickle mutation or other therapeutic approaches.

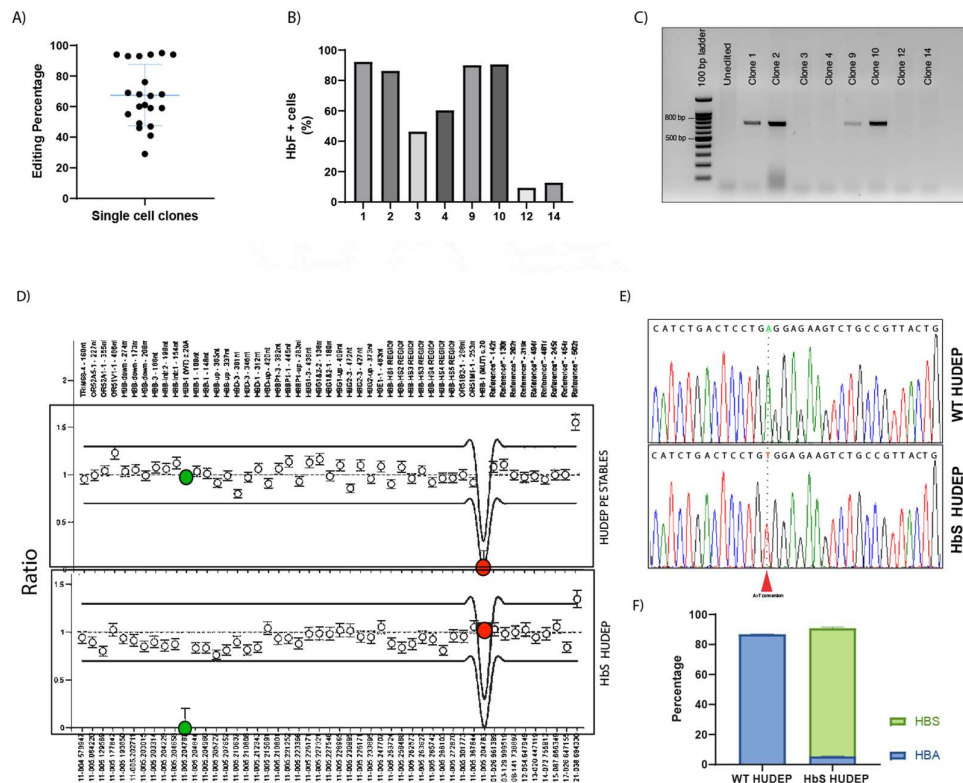


Figure 4.3.6 : Characterization of HUDEP2 sickle cell disease model : A) Editing pattern of single cell clones. B) Percentage of F+ve cells in clones having homozygous sickle mutation C) Detection of large deletion in single cell clones using conventional PCR. D) Characterization of mutations in globin locus using MPLA in the HUDEP sickle model. E) Sanger sequencing chromatogram of the HUDEP sickle model. F) HPLC showing expression of sickle hemoglobin in the HUDEP sickle model.

4.3.5 Correction of sickle mutation and reconstitution of adult hemoglobin using in HUDEP2 cells using PE2

We performed the proof-of-concept evaluation of sickle mutation correction using the SCD model that we generated. pegRNA for sickle correction was cloned in the pegRNA acceptor construct with RFP as the marker and delivered to SCD model using lentiviral transduction (Fig 4.3.7 A). The pegRNA for correction of sickle mutation contained an additional PAM site mutation to prevent the conversion back to sickle mutation by the pegRNA for creation of SCD mutation which was already integrated

in these cells.

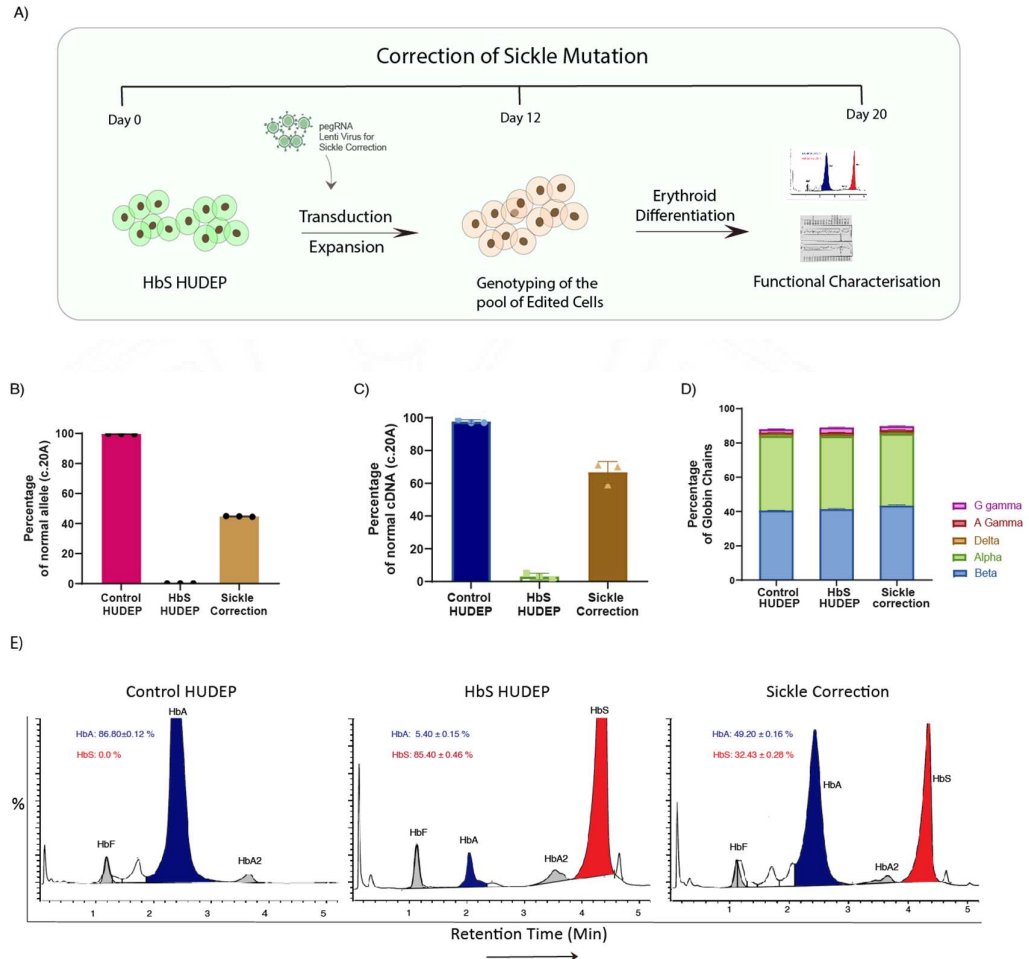


Figure 4.3.7 : Correction of sickle mutation using Prime Editing A) Schematic representation of the approach for correction of sickle mutation B) Evaluation of overall editing efficiency after correction of mutation C) Evaluation of editing efficiency at the cDNA level D) Globin chain analysis of edited and unedited cells. E) Hemoglobin variant analysis of edited and unedited samples. Sickle hemoglobin is represented in red color.

The editing efficiency was evaluated on Day 12 by NGS and showed that correction efficiency was comparable to that of installation (~50%). The editing at cDNA level was also comparable and there was no formation of InDels in these cells (Fig 4.3.7 B-C). Erythroid differentiation followed by HPLC showed that there was an increase in production of HbA and decrease in the production of HbS corresponding

to the editing efficiency (Fig 4.3.7 D-E). As the correction is without any undesired mutations, we expect that the corrected alleles would not show sickling. The frequency of installation of PAM mutation was evaluated in HBD region and was found to be present.

4.3.6 pegRNA mediated large deletion can be overcome by careful selection of pegRNAs

As we observed large interstitial deletions between HBD and HBB genes due to simultaneous binding of pegRNA and nicking by nCas9, we sought to evaluate approaches to overcome this. As the nickase activity cannot be removed in prime editing we chose a spacer that does not have sequence homology with delta globin and compared it with the validated pegRNA.

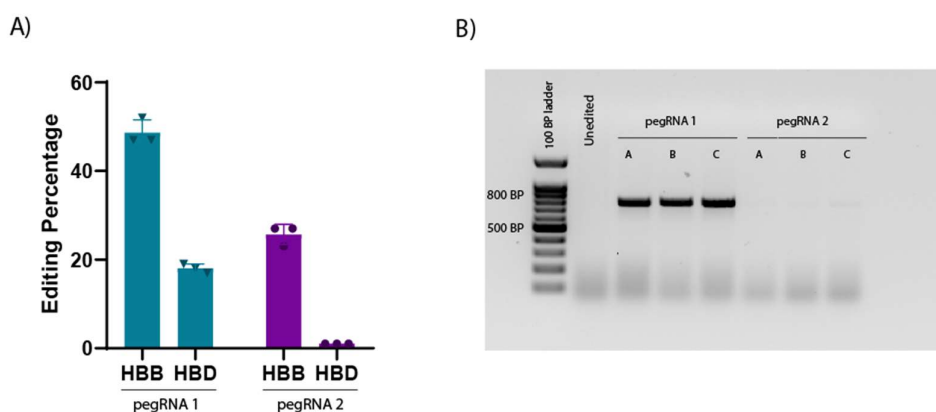


Figure 4.3.8: Evaluation of alternative pegRNAs for correction of sickle mutation A) editing efficiency at HBB and HBD genes using different pegRNAs. B) Detection of large deletions in cells edited with different pegRNAs by conventional PCR.

Although we were able to overcome large deletion, the editing efficiency dropped significantly using this pegRNA (Fig 4.3.8 A-B). Thus, even when multiple pegRNAs are available to install any mutation, careful consideration should be given to balance efficiency with undesired outcomes.

4.3.7 Evaluation of mRNA mediated delivery of prime editor

In an attempt to edit HSPCs for therapeutic applications, we attempted mRNA mediated delivery of PE max system along with synthetic pegRNAs. All the three mRNAs with different caps gave almost the same editing percentage. The editing was without any bystander edits or indels (Fig 4.3.9 A-B). Although we were able to achieve editing in HUDEP2 cells, there was no editing in HSPCs (data not shown). Further modifications and optimizations of mRNA synthesis and delivery would be required to achieve therapeutically relevant editing in HSPCs.

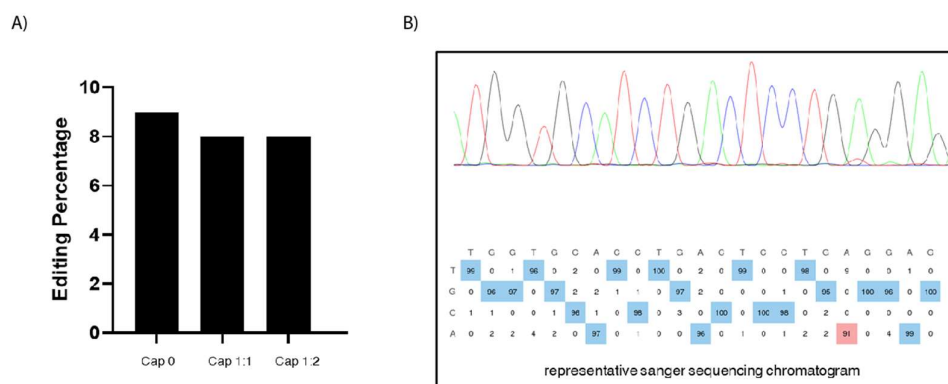


Figure 4.3.9: Evaluation of PE2 mRNA in HUDEP cells A) Overall editing efficiency with different Cap modifications B) Representative chromatogram of cells after prime editing with mRNA

DISCUSSION

5.1 Improving the precision of base editing in globin locus using non-nicking approaches

As base editors are entering into clinical trials for treatment of various disorders, there is an increasing interest in improving the precision of genome editing while minimizing undesired genomic alterations. With precision genome engineering there is often a very limited number of available gRNAs to generate the target mutation and hence the base editors must be precise with minimal undesired outcomes. It has been reported previously that base editors generate undesired genotoxic effects due to DNA nicking and that single strand breaks can have detrimental effects on cell fitness (Amendola et al., 2022; Park et al., 2022). In this study we show that large deletions are generated in homologous regions during base editing in a gRNA dependent manner.

Repeated binding of the gRNA might provide more chance for the nicks to occur simultaneously with DNA replication thereby resulting in large deletions. The frequency of deletion reduced significantly with efficient base editing and with transient gRNA delivery as both reduces the chances of gRNA binding to DNA. Although large deletions occurring during base editing in globin locus has been reported by us and others, this study offers more insights to understand the mechanism (Prasad et al., 2024; Ravi et al., 2022). Even with constitutive expression, gRNA3 in promoter does not cause any appreciable deletion even with Cas9 nickase. The gRNA features that result in this difference can be explored to develop safer therapeutic approaches. It is also interesting that CBE has less deletion frequency compared to ABE7.10 even though editing efficiency is low for both. There are several

homologous regions that are targets for gene therapy beyond the globin cluster and developing approaches that effect gene editing without deletion of intervening sequences will go a long way in improving the safety of therapeutic gene editing.

These deletions can be overcome using nickase deficient dABE8e that can edit efficiently even in CD34⁺ve HSPCs. The editing occurs even in CD90⁺ve HSPCs and the edited cells show no lineage bias suggesting the potential for long term engraftment and repopulation. The editing efficiency by dABE8e is however lower by 20-30% than ABE8e in all target sites tested. Hence in non-homologous regions or sites that do not cause formation of InDels, ABE8e can be used as it would provide better editing efficiency. While here we demonstrate only the use of ABE8e, it is similarly possible to develop nickase deficient CBEs using hyperactive variants of CBEs. A recent study has shown the proof of concept for overcoming InDels using nickase deficient CBEs (Yoon et al., 2023). In addition to targeting the homologous regions, dABE8e can also be used for multiplexed editing in a single locus or simultaneous editing in complementary strands without risking the generation of large deletions or InDels.

There are, however, certain limitations to the study. First, the approach was tested in two sites in the globin locus (*HBG* and *HBB* genes) to evaluate the abolishment of large deletions. There are other homologous loci such as *CCR5*, *CD33* etc which can be tested for the same. Additionally, cell cycle dependence of dABE8e mediated editing was not directly tested and only an engraftment study would show the long-term repopulation potential of the cells edited with dABE8e. Considering the in vitro data, we believe that nABE8e is still a better option in non-homologous sites as the editing efficiency is evidently higher compared to dABE8e. However, dABE8e would be a suitable approach, in terms of purity of outcomes while base editing in

situations where two or more nicks are introduced in the same locus as in homologous regions or during multiplexed editing.

5.2 Identifying regulatory elements in the gamma globin gene amenable to genome engineering for elevation of fetal hemoglobin

Reactivation of developmentally silenced fetal hemoglobin is a very promising approach for the treatment of hemoglobinopathies. Most of the current strategies aimed at elevating fetal hemoglobin target known regulatory elements such as gamma globin promoter or the associated transcription factors. Some approaches also rely on rewiring the chromatin looping (Bauer et al., 2012; Li et al., 2021). Although well studied, it is accepted that fetal hemoglobin regulation involves a complex interplay of various elements which is not completely understood. Therefore, it is important to uncover regulatory elements involved in globin switching both for understanding complex biology as well as for translational applications. In an attempt to discover regulatory elements, we found that the first intron of gamma globin gene possesses elements that control gene expression and can be manipulated by gene modifying tools.

The knowledge that intron contains regulatory elements is not new. Several studies have shown the essentiality of introns both in animals and plants with various mechanisms. Even in the globin cluster Beta globin second intron is found to have gene regulatory regions and this has been utilized in the development of gene therapy vectors (Buzina et al., 2008; Rose, 2019). However there have been no studies so far on introns of gamma globin probably because there are no reported naturally occurring HPFH mutations in the intron. Here we show that the first intron of gamma globin indeed has regulatory elements but what is fascinating is that DNA nicking (not base conversion) in this region is sufficient to reactivate gamma globin. The exact

mechanism of how this upregulation is happening is unknown. More studies are needed to understand the exact mechanism.

It is also important to note that the effects of nicking are different in the gamma globin promoter. Editing is required for the alteration of transcription factor binding sites and nicking has very minimal effect. gRNA 3 which creates a binding site for TAL 1 shows the most significant correlation between editing and HbF induction. This suggests that no other mechanisms might be interfering during the activator binding site creation. This is not exactly the case with gRNA2 or gRNA11 where other mechanisms beyond TF based regulation might be involved. Further studies are required to explore this pattern of HbF induction.

The data suggest that HbF elevation could be driven by the chromatin changes induced by D10A nickase. Recent studies have shown that there is difference in the processing of D10A and H840A nickase, with the latter being rapidly removed from the cut site due to the presence of exposed 3' end(Pavani et al., 2024; Reginato et al., 2024). This could also explain why Cas9, which has both the activities, is not able to elevate HbF to the same levels as D10A nickase although both causes DNA strand breaks. However, DNA nick is essential for HbF elevation as dABE8e is not able to elevate HbF levels. This phenomenon is also evident in primary CD34+ve HSPCs suggesting that it is a universal phenomenon and not driven by the altered DNA repair pathways in immortalized cell lines.

Deeper investigations into the dynamics of Cas9 clearance, epigenetic and chromatin changes induced by DNA nicking and differences in the DNA repair pathways with the use of different genome editors might provide insights into the mechanism driving this phenomenon. Although it does not seem an ideal therapeutic

target using gene editing, understanding the mechanism of HbF elevation would help in developing new therapeutic strategies beyond gene editing. One of the major limitations of this work is that beyond the observation in gamma globin intron, other genes were not tested to evaluate the occurrence of a similar phenomenon. Uncovering the mechanism driving this enhanced gene expression might help us narrow down other target genes that could be regulated by a similar phenomenon.

5.3 Correcting the causative point mutation of sickle cell disease in beta globin gene (E6V) using prime editing

Sickle cell Disease (SCD) being a monogenic disorder and owing to the lack of other efficient curative approaches is an attractive target for gene therapy. Homology directed repair and base editing has been used in the past to demonstrate correction of SCA, but both the approaches have dire inherent limitations. HDR, mediated by DSB could be deleterious in the correction of SCA if the proportion of indels are high due the risk of creating beta-thalassemic mutations. Base editing on the other hand, cannot be used to install T>A transition mutation and has relied on the creation of Hb-G Makassar, a non-pathogenic Hb variant to overcome the SCA mutation. The functional efficiency of Hb-G Makassar variant has not been evaluated extensively(DeWitt et al., 2016; Newby et al., 2021). Prime editing overcomes both these issues by avoiding DSB and the ability to install precise T>A mutation.

Here, we sought to evaluate prime editing for correction of HbS mutation in HUDEP2 cells using PE2 system that would install the mutation without any bystander edits or indels. Most of the PE studies so far have relied on the use of PE3 system, which requires a gRNA that nicks the opposite strand, to achieve better editing efficiency but carries the risk of generation small insertions and deletions at the target

site. Undesired edits and indels can interfere with the evaluation of functional effects of the mutation especially in the coding regions.

Delivery approaches affect prime editing efficiency to a great extent as the availability of the prime editor and pegRNA for a longer period has been shown to ensure efficient editing at the target site. Lentiviral vectors offer an attractive platform for delivery of PE as they would integrate into the genome and constitutively express the components. Previous studies have attempted to deliver pegRNA and prime editor as an all-in-one lentiviral vector which necessitated either the development of truncated version of PE or the delivery as split-intein based system, owing to the decrease in transduction efficiency with increased size of the vector(Gao et al., 2022). Both approaches, however, limited the editing efficiency considerably and a single full sized lentiviral vector had failed to produce any editing. We aimed at overcoming this problem by uncoupling PE and pegRNA delivery and developed two different constructs that can separately deliver the components. The potential to prepare the cell lines stably expressing PE which can then be transduced with pegRNAs enable efficient arrayed screening of multiple pegRNAs for initial optimization. We also show that sequential delivery of two pegRNAs does not affect the expression of either making it possible to use the lentiviral system for studies that require dual pegRNA delivery.

Using the optimized modular lentivirus based approach, we were able to modify difficult to manipulate HUDEP2 cells with editing efficiency reaching up to 75% overtime using prime editor 2 system. HUDEP2 cells are important cellular model for preclinical studies involving beta-haemoglobinopathies as they can easily be differentiated into erythroid lineage and express adult haemoglobin to a quantifiable

level. In addition to evaluating prime editing as a tool for correction of mutations, this approach facilitates the use of prime editing in understanding globin biology and gene switching with its ability to introduce precise mutations with higher efficiency. Higher editing efficiency mediated by lentiviral PE system also overcomes the need for single cell sorting for most applications avoiding the biases associated with the clonal selection when evaluating the gene expression. We hope the lentiviral system can be used in other cell types as well relevant to researchers in different domains.

PE2 system would be a better choice in studies involving coding regions or regions where sequence integrity needs to be preserved as the phenotypic outcomes would be an accurate reflection of the installed edit and not influenced by the by-products even to a minimal degree. The model system that we developed produced sickle haemoglobin exclusively without compromising the expression of any other globin chains. The major drawback of developed model system is the constitutive expression of PE and pegRNA that limit its use for prime editing studies not affected by the expression of the initially transduced pegRNA. Transient delivery of prime editing components could overcome the limitation although with reduced efficiency and would require delivery as mRNA or RNP complex and the screening of a large number of clones to obtain the desired homozygous edited clones.

The system also provides a platform for efficient screening of pegRNAs for the installation of mutation, best of which could be taken for further evaluation in the patient CD34⁺ HSPCs thereby lowering the number of primary cells required for the genetic manipulation as well as cutting down the expenditure. However, before this statement can be generalized, comparison of prime editing efficiency at the specific locus in different cell lines and CD34⁺ HSPCs need to be performed as we have

observed a cell type dependent variation in editing efficiency for the same pegRNA even when the level of expression of PE and pegRNA was consistent. The resulting variation might be due to cell intrinsic factors or gene expressions profiles that need thorough evaluation for the development of improved versions of PE specific for the cell type in question. In addition, we and others have noticed a consistent increase in prime editing efficiency with constitutive expression overtime hinting the cell division dependency of prime editing which might be a bottleneck when editing primary cells.

Delivery of base editors as mRNA with synthetic gRNA has been shown to edit efficiently in HSPCs. A recent study compared different delivery modalities of prime editor in Human induced pluripotent stem cells and found that PE mRNA combined with synthetic pegRNA gave the highest editing efficiency(Li et al., 2022). A similar approach would be most suitable for prime editing in HSPCs as well and the recently described modifications in the prime editor (PE max) and pegRNA (epgRNA) could improve the overall efficiency. Recently , prime editing with improved constructs and pegRNA modifications has been used to correct sickle mutation in HSPCs with good editing efficiency(Everette et al., 2023). But here also there was presence of InDels and large deletions in the edited cells in addition to the desired effect. This underscores the importance of developing approaches that cause minimal undesired effects in the genome while maintaining editing efficiency. The delivery of PE as Integration deficient lentivirus (IDLV) could be another viable approach for editing HSPCS. Prime editing has been shown to generate minimal chromosomal aberrations compared to DSB mediated approaches and hence the edited cells might have better engraftment potential improving the treatment outcome.

SUMMARY AND CONCLUSION

This work was aimed at developing CRISPR/Cas9 based genome editing approaches for the treatment of beta globin disorders specifically beta thalassemia and sickle cell anemia. Within the overall aim, the study addressed three major aspects.

The first objective was aimed at improving the precision of genome engineering at the globin locus. Globin locus is peculiar in that there are multiple genes that are highly homologous and strand mediated approaches relying on RNA guided endonucleases often bind to and cut multiple sites in the locus resulting in InDels and large deletions, including chromosomal loss. In this work we specifically addressed the problem of large interstitial deletions formed during base editing targeting either the gamma globin or beta globin locus. We demonstrated that DNA nicking by itself (without base conversion) can cause large deletions. Based on this observation we hypothesized that a nickase deficient version of hyperactive adenosine base editor (ABE8e) could be developed, that would overcome large deletions. The construct was developed and validated in cell lines as well as in primary human CD34⁺ve HSPCs. Although the editing efficiency was slightly lower than the nickase counterpart, it was able to completely abolish the formation of large deletion. This resulted in less activation of DNA damage response pathways as well. We further extrapolated the application of this nickase deficient variant for multiplexed base editing on complementary strands. As expected, the formation of InDels during complementary strand editing due to nicks were totally absent in dABE8e edited samples resulting in higher base conversion efficiency. Overall, this approach improves the precision of preexisting gene editing approaches for the treatment of beta hemoglobinopathies such

as promoter editing at gamma globin and direct correction of thalassemia mutations.

The second objective was aimed at identifying novel regulatory regions in gamma globin gene that can be tapped for therapeutic applications. A mutagenesis screening in the first intron of gamma globin identified an ~30 bp region that can be targeted for elevated gene expression by Cas9 based DNA nickase. As only D10A nickase elevates gene expression while targeting this region, we believe this would be driven by some epigenetic changes involving the DNA repair pathways. Although the exact mechanism was not identified, we show that the phenomenon occurs in human CD34+ve HSPCs as well, suggesting the possibility for development of therapeutic applications. If the mechanism is identified and could be manipulated by pharmacological agents, this would provide an alternative cheaper approach for treatment of beta hemoglobinopathies.

In the third objective we developed a modular lentiviral PE system that works efficiently across cell lines and in different target sites. This approach enabled several fold higher editing of targets compared to previously reported approaches. Using this system, we showed the development of a cellular disease model of sickle cell anemia in HUDEP-2 cells. This model was further used for pre-clinical validation of the utility of prime editing in correcting sickle mutation. Additionally, we show that a careful designing of pegRNAs is needed while editing in homologous regions with the risk of formation of interstitial deletions as nickase deficient approach would not be feasible with prime editing. Overall, the modular lentiviral system is a cheap, versatile and efficient approach for Prime editor and pegRNA screening and validation which is often the bottleneck in prime editing experiments.

FUTURE DIRECTIONS

Objective 1

1. Validation of the engraftment and multilineage repopulation potential of hematopoietic stem cells edited with dCas9 ABE8e
2. Development of other base editors with nickase deficient Cas9

Objective 2

1. Identification of the mechanism by which HbF expression is elevated by DNA nicking in gamma globin intron 1
2. Studying the persistence of HbF elevation in edited cells by engraftment studies in mice models.

Objective 3

1. Development of efficient delivery approaches for prime editing in HSPCs without causing large deletions or formation of InDels at the target site.

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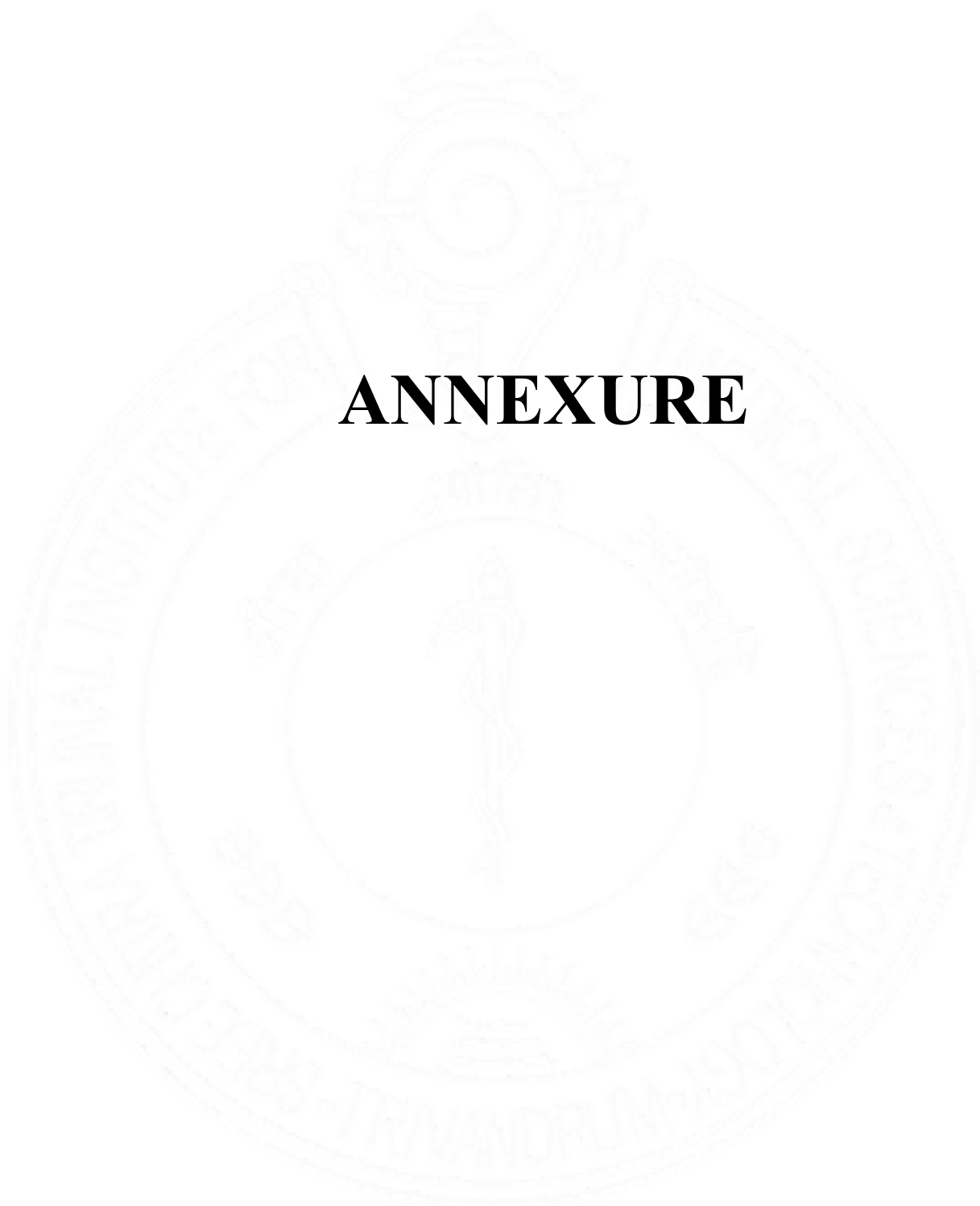
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ANNEXURE



LIST OF PUBLICATIONS

Research Articles:

1. George A, Ravi NS, Vaishnavi B, Marepally S, Thangavel S, Velayudhan SR, et al. Editing of homologous globin genes by nickase deficient base editor mitigates large intergenic deletions in HSPCs. *Molecular Therapy -Nucleic acids* 2024 Sep 30;
2. George A, Ravi NS, Prasad K, Panigrahi L, Koikkara S, Rajendiran V, et al. Efficient and error-free correction of sickle mutation in human erythroid cells using prime editor-2. *Front Genome Ed.* 2022 Dec 20;4:1085111.

Protocol:

1. Ravi NS, George A, Mohankumar KM. Protocol for arrayed gRNA screening by base editors in mammalian cell lines using lentiviral system. *STAR Protocols.* 2023 Dec 15;4(4):102668.

Review article:

1. Prasad K, George A, Ravi NS, Mohankumar KM. CRISPR/Cas based gene editing: marking a new era in medical science. *Mol Biol Rep.* 2021 May;48(5):4879–95.

Anila George

Centre for Stem Cell Research, Vellore, India

Email: anilageorge2011@gmail.com

Phone: +91-8893962525

EDUCATION

PhD: 2019- Till date

Centre for Stem Cell Research (a unit of InStem, Bengaluru), Christian Medical College Campus, Bagayam, Vellore, Tamil Nadu, India

MSc Medical Biochemistry: 2016-2019

Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry, India
University Gold Medallist.

BSc Medical Biochemistry: 2011- 2015

Centre for Health Sciences, University of Calicut, Kerala, India
University Topper. Awarded First Class with Distinction.

RESEARCH EXPERIENCE

1. Centre For Stem Cell Research (2019 – 2024): CSIR Research Fellow/ PhD Student

Project: Genome editing strategies for treatment of beta hemoglobinopathies

Supervisor: Dr Mohankumar KM

- Experienced in preclinical CRISPR based genome engineering (including base and prime editing).
- Capable of independently designing, planning, and executing all steps of in vitro gene editing.

Skills learned:

- **Molecular Biology:** Nucleic acid isolation, PCR (conventional PCR, ddPCR, qRT PCR), sequencing, IVT, cloning, protein purification etc
- **Cell Culture:** Handling of cell lines as well as primary cells (HSPCs), transfection, lentivirus production, and transduction, Nucleofection (Maxcyte, Lonza, Neon).
- **Others:** Handling of laboratory mice, Flow cytometry, Basic microscopy, Softwares – GraphPad Prism, Adobe Illustrator, FlowJo.

2. Department of Biochemistry, JIPMER (2017-2019): Master's Project

Project: Assessment of systemic inflammation and its associated biochemical derangements in different stages of breast cancer

Supervisor: Dr Zachariah Bobby

Skills learned: ELISA, CLIA, clinical Biochemistry (basic parameters), phlebotomy, research proposal writing, basic statistics.

PUBLICATIONS

1. **George A**, Ravi NS, Vaishnavi B, Marepally S, Thangavel S, Velayudhan SR, et al. Editing of homologous globin genes by nickase deficient base editor mitigates large intergenic deletions in HSPCs. *Molecular Therapy -Nucleic acids* 2024 Sep 30;
2. Prasad K, Devaraju N, **George A**, Ravi NS, Paul J, Mahalingam G, et al. Precise correction of a spectrum of β -thalassemia mutations in coding and non-coding regions by base editors. *Molecular Therapy - Nucleic Acids*. 2024 Jun 11;35(2):102205.
3. Rajendiran V, Devaraju N, Haddad M, Ravi NS, Panigrahi L, Paul J, et al. Base editing of key residues in the BCL11A-XL-specific zinc finger domains derepresses fetal globin expression. *Molecular Therapy*. 2024 Mar 6;32(3):663–77.
4. Nandy K, Babu D, Rani S, Joshi G, Ijee S, **George A**, et al. Efficient gene editing in induced pluripotent stem cells enabled by an inducible adenine base editor with tunable expression. *Sci Rep*. 2023 Dec 11;13(1):21953.
5. Ravi NS, **George A**, Mohankumar KM. Protocol for arrayed gRNA screening by base editors in mammalian cell lines using lentiviral system. *STAR Protocols*. 2023 Dec 15;4(4):102668.
6. **George A**, Ravi NS, Prasad K, Panigrahi L, Koikkara S, Rajendiran V, et al. Efficient and error-free correction of sickle mutation in human erythroid cells using prime editor-2. *Front Genome Ed*. 2022 Dec 20;4:1085111.
7. Ravi NS, Wienert B, Wyman SK, Bell HW, **George A**, Mahalingam G, et al. Identification of novel HPFH-like mutations by CRISPR base editing that elevate the expression of fetal hemoglobin. *eLife*. 2022 Feb 11;11:e65421.
8. **George A**, Bobby Z, Dubashi B. Utility of ferritin and inflammatory biomarkers in the diagnosis of different stages of breast cancer. *Saudi Med J*. 2021 Aug;42(8):825–31.
9. Prasad K, **George A**, Ravi NS, Mohankumar KM. CRISPR/Cas based gene editing: marking a new era in medical science. *Mol Biol Rep*. 2021 May;48(5):4879–95.

AWARDS AND HONOURS

- Junior Research Fellowship (2018): Awarded by the Council for Scientific and Industrial Research, Government of India.

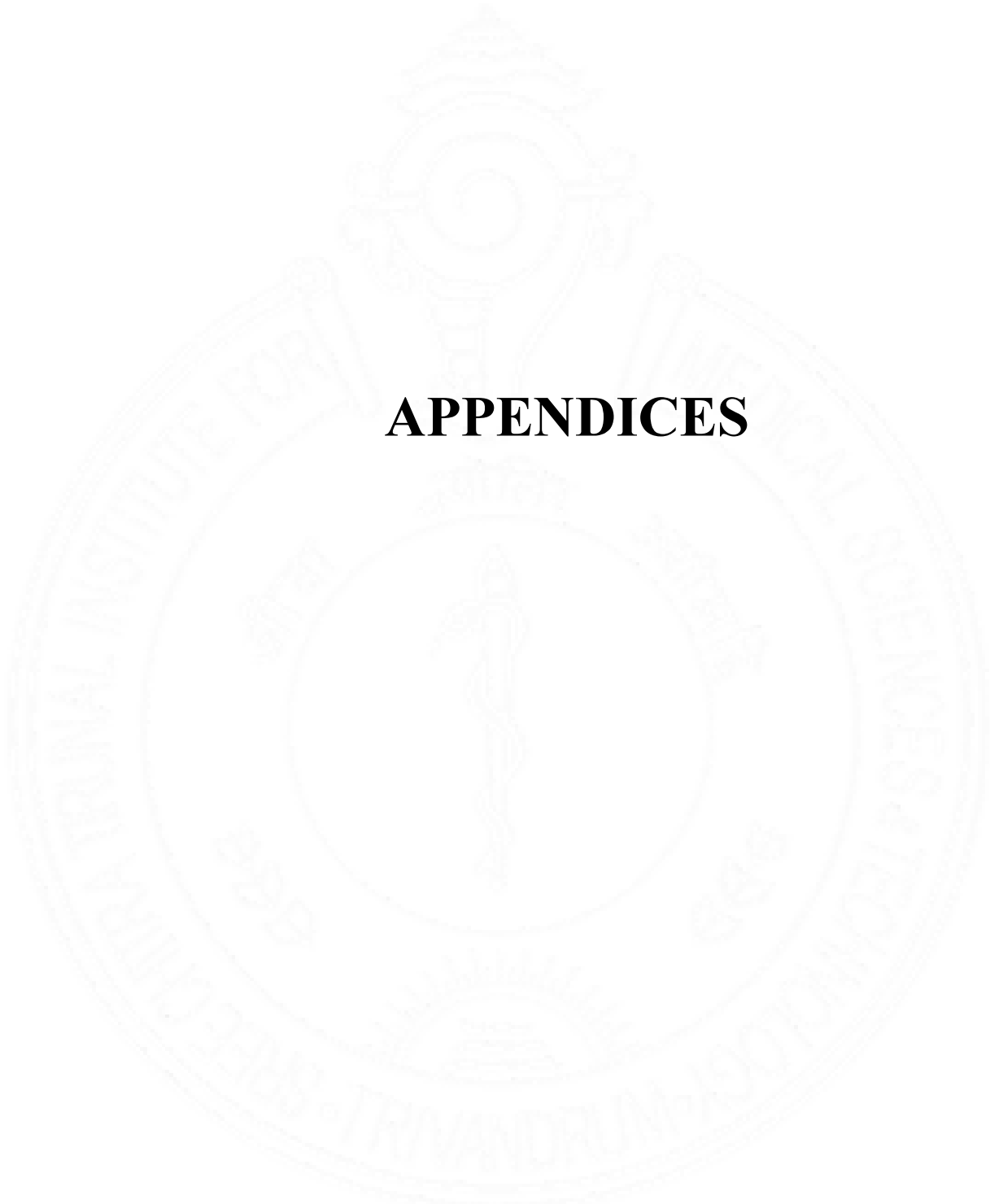
- Travel Grant from International Max Planck Research School for attending “*Horizons in Molecular Biology 2023 symposium*”.
- NCATS scholarship awardee for attending “*Keystone Symposia: Precision Genome Engineering 2023*”.
- One among 6 students invited to represent India in “*JENESYS 2018 (Theme - Health): Student and Youth exchange programme*” conducted by the Government of Japan.
- Winner of genome engineering quiz at FGE conference, 2023.
- Poster Award in PhD category at Annual Research Day, 2022, CMC, Vellore, India

POSTER PRESENTATIONS

- Frontiers in Genome Engineering 2023, Goa, India:
“Nickase deficient ABE8e efficiently edits paralogous HBG genes without deletion of intervening 4.9kb region”
- ESGCT Annual Conference, Brussels, 2023 (Virtual):
“Nickase deficient ABE8e efficiently edits paralogous HBG genes without deletion of intervening 4.9kb region”
- Horizons in Molecular Biology 2023, IMPRS, Gottingen, Germany:
“DNA nicks in intron of human gamma globin gene activate gene expression during base editing independent of base conversions”
- InStem Annual Review of Research 2023, Bengaluru, India
“Precise modelling and correction of sickle cell mutation in Human erythroid cells using Prime Editing”

OTHER PROFESSIONAL CONTRIBUTIONS

- Mentor for several undergraduate and master’s students at the Centre for Stem Cell Research over the last five years.
- Reviewer for Clinica chimica acta – Medical biochemistry
- Member of the organising committee at the Centre for Stem Cell Research for the yearly Cell and Gene Therapy Symposium as well as the community outreach programmes over the years.



APPENDICES



**OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA**

Ethics Committee Registration No: ECR/326/INST/TN/2013 Re Reg-2016 Issued under Rule 122D of the Drugs & Cosmetics Rules 1945, Govt. of India

Dr. George Thomas, D. Ortho., Ph.D.,
Chairperson, Ethics Committee

Dr. Antonisamy, Ph.D., FSMS, FRSS.,
Secretary, Research Committee

Prof. Keith Gomez, MA (S.W), M.Phil.,
Deputy Chairperson, Ethics Committee
January 31, 2020.

Dr. Anna Benjamin Pulimood, MD., Ph.D.,
Chairperson, Research Committee & Principal

Dr. Biju George, MD., DM.,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Dr. Mohankumar K,
Assistant Investigator,
Department of CSCR,
Christian Medical College,
Vellore – 632 004

Sub: **External Research Grant: New Proposal: DBT**

Recreating HPFH-associated point mutation to elevate the fetal haemoglobin levels for the treatment of beta-hemoglobinopathies using CRISPR based tools.

Dr. Mohankumar K. Murugesan, Assistant Investigator, CSCR, Dr. Srujan Marepally,
Dr. Alok Srivastava, CSCR & Haematology

Ref: IRB Min. No. 12309 (OTHER) dated 30.10.2019

Dear Dr. Mohankumar K,

The Institutional Review Board (**Silver**, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Recreating HPFH-associated point mutation to elevate the fetal haemoglobin levels for the treatment of beta-hemoglobinopathies using CRISPR based tools" on October 30, 2019. I am quoting below the minutes of the meeting.

The Committee reviewed the following documents:

1. IRB Application Format
2. Cvs of Drs. Alok S, Mohankumar, Srujan.
3. No. of Documents 1- 2

The following Institutional Review Board (**Silver**, Research & Ethics Committee) members were present at the meeting held on October 30, 2019 at 9.45 am in the New IRB Room, Christian Medical College, Bagayam, Vellore -632002.



OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

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Secretary, Research Committee

Dr. Biju George, MD., DM.,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Prof. Keith Gomez, MA (S.W), M.Phil.,
Deputy Chairperson, Ethics Committee

Name	Qualification	Designation	Affiliation
Dr. George Thomas	D Ortho, PhD	Orthopaedic Surgeon, St. Isabella Hospital, Chennai, Chairperson, Ethics Committee, IRB, Chennai	External, Clinician
Prof. Keith Gomez	BSc, MA (S.W), M. Phil (Psychiatry Social Work)	Student counselor, Loyola College, Chennai, Deputy Chairperson, Ethics Committee, IRB	External, Lay Person & Social Scientist
Dr. Biju George	MBBS, MD, DM	Professor, Haematology, Additional Vice Principal (Research), Deputy Chairperson (Research Committee), Member Secretary (Ethics Committee), IRB, CMC, Vellore.	Internal, Clinician
Dr. P. Zachariah	MBBS, PhD	Retired Professor, Vellore	External, Clinician
Dr. Sathya Subramani	MD, PhD	Professor, Physiology, CMC, Vellore	Internal, Clinician
Dr. Prasanna Samuel	M. Sc, PhD	Lecturer, Biostatistics, CMC, Vellore	Internal, Statistician
Dr. Jayaprakash Muliylil	MBBS, MD, MPH, Dr PH (Epid), DMHC	Retired Professor, Vellore	External, Scientist & Epidemiologist
Rev. Dr. T. Arul Dhas	MSc. BD, DPC, PhD(Edin)	Chaplaincy Department, CMC, Vellore	Internal, Social Scientist
Dr. Blessed Winston	MBBS., MD	Associate Professor, Clinical Pharmacology, CMC, Vellore	Internal, Pharmacologist
Dr. Suresh Devasahayam	BE, MS, PhD	Professor of Bio-Engineering, CMC, Vellore	Internal, Basic Medical Scientist
Dr. P. Zachariah	MBBS, PhD	Retired Professor, Vellore	External, Clinician
Dr. Ajith Sivadasan	MD, DM	Professor, Neurological Sciences, CMC, Vellore	Internal, Clinician
Mr. Samuel Abraham	MA, PGDBA, PGDPM, M. Phil, BL.	Sr. Legal Officer, Vellore	External Legal Expert
Mr. C. Sampath	BSc, BL	Advocate, Vellore	External, Legal Expert
Mrs. Ilavarasi Jesudoss	M Sc (Nursing)	Deputy Nursing Superintendent, College of Nursing, CMC, Vellore	Internal, Nurse
Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay person

IRB Min. No. 12309 (OTHER) dated 30.10.2019

2 of 3



OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Ethics Committee Registration No: ECR/326/INST/TN/2013 Re Reg-2016 Issued under Rule 122D of the Drugs & Cosmetics Rules 1945, Govt. of India

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Dr. Antonisamy, Ph.D., FSMS, FRSS.,
Secretary, Research Committee

Dr. Biju George, MD., DM.,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Prof. Keith Gomez, MA (S.W.), M.Phil.,
Deputy Chairperson, Ethics Committee

Dr. Abhay Gahukamble	MS, D Ortho, DNB(Ortho)	Associate Professor, Paediatric Orthopaedics, CMC, Vellore	Internal, Clinician
Dr. Suceena Alexander	MBBS, MD, DM	Associate Professor, Nephrology, CMC, Vellore	Internal, Clinician
Dr. Sujith J Chandy	MD., Ph.D., FRCP (E)	Professor, Clinical Pharmacology, CMC, Vellore	Internal, Pharmacologist
Dr. Shirley David	MSc, PhD	Professor, Head of Fundamentals Nursing Department, College of Nursing, CMC, Vellore	Internal, Nurse

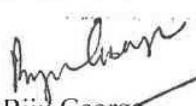
We approve the project to be conducted as presented.

Kindly provide the total number of patients enrolled in your study and the total number of Withdrawals for the study entitled: "Recreating HPFH-associated point mutation to elevate the fetal haemoglobin levels for the treatment of beta-hemoglobinopathies using CRISPR based tools" on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in).

The Institutional Ethics Committee expects to be informed about the progress of the project, any **adverse events** occurring in the course of the project, any **amendments in the protocol and the patient information / informed consent**. On completion of the study you are expected to submit a copy of the **final report**. Respective forms can be downloaded from the following link: http://172.16.11.136/Research/IRB_Policies.html in the CMC Intranet and in the CMC website link address: <http://www.cmch-vellore.edu/static/research/Index.html>.

Administrative Committee's approval is to be obtained for opening the account-head, employing any personnel or purchasing any equipment. The investigator also needs to present to Administrative Committee, the terms and condition of the Funding agency for approval

Yours sincerely,


Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board.

Dr. BIJU GEORGE
MBBS., MD., DM.
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

IRB Min. No. 12309 (OTHER) dated 30.10.2019

3 of 3



OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

CDSCO - Ethics Committee Registration No: ECR/326/INST/TN/2013/RR-2019, DHR Provisional Registration No: EC/NEW/INST/2020/818

Dr. George Thomas, D. Ortho., Ph.D.,
Chairperson, Ethics Committee

Dr. Prasanna Samuel, M.Sc., Ph.D.,
Secretary, Research Committee

Prof. Keith Gomez, MA (S.W), M.Phil.,
Deputy Chairperson, Ethics Committee

Dr. Biju George, MD., DM.,
Chairperson, Research Committee

Dr. Suceena Alexander, MD., DM., FASN.,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

September 23, 2022.

Dr. Mohankumar K. Murugesan,
Scientist,
Centre for Stem Cell Research,
Christian Medical College,
Vellore – 632 002.

Sub: External Research Grant: New Proposal: DBT:
Targeting the gamma globin regulatory elements using base editors for clinical application in patients with β -hemoglobinopathies.
Dr. Mohankumar K. Murugesan, Employment Number: 27118 , Scientist-E Centre for Stem Cell Research, Dr. Alok Srivastava, Haematology and Centre for Stem Cell Research (CSCR), Dr. R. V. Shaji, Haematology, Adjunct Scientist- Centre for Stem Cell Research (CSCR), Dr. Saravanabhavan Thangavel, Centre for Stem Cell Research (CSCR).

Ref: IRB Min. No. 14707 [OBSERVE] dated 29.06.2022

Dear Dr. Mohankumar K. Murugesan,

The Institutional Review Board (Silver, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled “Targeting the gamma globin regulatory elements using base editors for clinical application in patients with β -hemoglobinopathies” on June 29, 2022.

The Committee reviewed the following documents:

- 1) IRB Application Format
- 2) Patient information sheet and Informed consent forms (English, Odiya and Hindi)
- 3) Cvs. of Dr. Mohankumar K. Murugesan, Dr. Alok Srivastava, Dr. R. V. Shaji, Dr. Saravanabhavan Thangavel.
- 4) No. of Documents 1 – 3.



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Chairperson, Ethics Committee

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Secretary, Research Committee

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Dr. Biju George, MD., DM.,
Chairperson, Research Committee

Dr. Suceena Alexander, MD., DM., FASN.,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

ETHICS COMMITTEE MEMBERS			
Name	Qualification	Designation	Affiliation
Dr. George Thomas	D. Ortho, PhD	Orthopaedic Surgeon, St. Isabella Hospital, Chennai, Chairperson, Ethics Committee, IRB, CMC. Vellore	External, Clinician
Dr. Suceena Alexander	MD, DM, FASN.	Professor, Nephrology, Additional Vice-Principal (Research), Member Secretary (Ethics Committee), IRB, CMC, Vellore.	Internal, Clinician
Rev. Joseph Devaraj	BSc, BD	Chaplaincy Department, CMC, Vellore	Internal, Social Scientist
Prof. Keith Gomez	MA (S.W), M. Phil (Psychiatry Social Work)	Deputy Chairperson, Ethics Committee, IRB Student counselor, Loyola College, Chennai.	External, Lay Person & Social Scientist
Mr. C. Sampath	BSc, BL	Advocate, Vellore	External, Legal Expert
Mr. Samuel Abraham	MA, PGDBA, PGDPM, M. Phil, BL.	Sr. Legal Officer, Vellore	External Legal Expert
Mrs. Ilavarasi Jesudoss	M Sc (Nursing)	Deputy Nursing Superintendent, College of Nursing, CMC, Vellore	Internal, Nurse
Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay Person
Dr. Jayaprakash Muliyil	MD, MPH, Dr PH (Epid), DMHC	Retired Professor, CMC, Vellore	External, Scientist & Epidemiologist
Dr. Manoranjitham Sathyaseelan	MSc., PhD	Professor and Head of Psychiatric Nursing, CMC, Vellore.	Internal Nurse

IRB Min. No. 14707 [OBSERVE] dated 29.06.2022

2 of 4



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Chairperson, Research Committee

Dr. Suceena Alexander, MD., DM., FASN.,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Mrs. Shandрила Immanuel	M Sc (Nursing)	Deputy Dean, College of Nursing, CMC, Vellore	Internal, Nurse
Dr. Premila M Wilfred	MBBS, MD, Pharmacology.	Assistant Professor, Pharmacology, CMC Vellore.	Pharmacologist
Dr. Anuradha Rose	MBBS, MD, MHSC (Bioethics)	Professor, Community Health, CMC, Vellore.	Internal, Clinician
RESEARCH COMMITTEE MEMBERS			
Dr. Biju George	MD, DM	Chairperson (Research Committee), Professor, Haematology, CMC, Vellore.	Internal, Clinician
Dr. RV. Shaji	M.Sc, PhD	Professor, Haematology, CMC, Vellore	Internal, Basic Medical Scientist
Dr. Prasanna Samuel	M. Sc, PhD	Secretary (Research Committee) Associate Professor, Biostatistics, CMC, Vellore	Internal, Statistician
Dr. Jacob John	MD, MPH	Professor, Community Medicine, CMC, Vellore	Internal, Clinician
Dr. Sivakumar Balasubramanian	M. Tech, PhD	Professor of Bio-Engineering, CMC, Vellore	Internal, Basic Medical Scientist
Dr. Abhay Gahukamble	MS, D Ortho, DNB (Ortho)	Professor, Paediatric Orthopaedics, CMC, Vellore	Internal, Clinician
Dr. Elizabeth Vinod	MBBS, MD,	Associate Professor, Department of Physiology, CMC Vellore	Internal Clinician
Dr. Joe Varughese	MBBS, MD	Professor, Biochemistry, CMC	Internal, Clinician
Dr. Christhunesa S. Christudass	M.Sc., P.hd.,	Professor, Neurochemistry, CMC	Internal, Basic Medical Scientist



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Dr. Biju George, MD., DM.,
Chairperson, Research Committee

Dr. Suceena Alexander, MD., DM., FASN.,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Dr. Sridhar Gibikote	DMRD, DNB	Professor, Radiology, CMC, Vellore	Internal, Clinician
Dr. Winsely Rose	MBBS, MD (Paed)	Professor, Paediatrics, CMC Vellore	Internal, Clinician

We approve the project to be conducted as presented for the duration 03 Years.

Kindly provide the total number of patients enrolled in your study and the total number of Withdrawals for the study entitled: "Targeting the gamma globin regulatory elements using base editors for clinical application in patients with β -hemoglobinopathies" on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in).

The Institutional Ethics Committee expects to be informed about the progress of the project, Any adverse events occurring in the course of the project, any amendments in the protocol and the patient information / informed consent. On completion of the study, you are expected to submit a copy of the final report. Respective forms can be downloaded from the following link: http://172.16.11.136/Research/IRB_Policies.html in the CMC Intranet and in the CMC website link address: <http://www.cmch-vellore.edu/static/research/Index.html>.

Administrative Committee's approval is to be obtained for opening the account-head, employing any personnel or purchasing any equipment. The investigator also needs to present to Administrative Committee, the terms and condition of the Funding agency for approval.

Yours sincerely,


Dr. Suceena Alexander

Secretary (Ethics Committee)
Institutional Review Board.

Dr. SUCEENA ALEXANDER
MD, DM(Nephrology), FRCP(Lon), FASN, PhD.
Secretary - (Ethics Committee),
Institutional Review Board, Christian Medical College,
Vellore - 632 002, Tamil Nadu, India.

IRB Min. No. 14707 [OBSERVE] dated 29.06.2022

4 of 4



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Prof. Keith Gomez, MA (S.W), M.Phil.,
Deputy Chairperson, Ethics Committee.

Dr. Jacob John, MD., Ph.D.,
Chairperson, Research Committee

Dr. Suceena Alexander, MD, DM (Nephrology),
FRCP (Lon), FASN., Ph.D.,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Ref: IRB – A – 20.09.2023

January 20, 2024,

Dr. Mohankumar K. Murugesan,
Department of CSCR,
Christian Medical College,
Vellore.

Ref: IRB Min. No: 14707 dated 29.06.2022.

Dear Dr. Mohankumar K. Murugesan,

The Institutional Review Board (Silver, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed for the study titled "Targeting the gamma globin regulatory elements using base editors for clinical application in patients with β -hemoglobinopathies" on September 20, 2023.

a) Methodology: (page number: 15,16 and 17)

We would like to include a mutagenesis screening in Gamma Globin introns using Cas9 based gene editors in HUDEP-2 cell line. If any targets are identified we would include that in the remaining experiments similar to the gRNAs targeting promoter and BCLIIA enhancer.

a. References:

1Frontiers I Introns as Gene Regulators: A Brick on the Accelerator
[<https://www.frontiersin.org/articles/10.3389/fgene.2018.00672/full>]

2Anzalone AV, Koblan LW, Liu DR. Genome editing with CRISPR-Cas nucleases, base editors, transposases and prime editors. Nat Biotechnol. 2020ul;38(7):824-44.

3Venkatesan V, Christopher AC, Rhiel M, Azhagiri MKK, Babu P, Walavalkar K, et al. Editing the core region in HPFH deletions alters fetal and adult globin expression for treatment of β hemoglobinopathies. Molecular Therapy - Nucleic Acids. 2023 Jun 13;32:671-88.



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Secretary, Research Committee

Prof. Keith Gomez, MA (S.W), M.Phil.,
Deputy Chairperson, Ethics Committee.

Dr. Jacob John, MD., Ph.D.,
Chairperson, Research Committee

Dr. Suceena Alexander, MD, DM (Nephrology),
FRCP (Lon), FASN., Ph.D.
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

The following Institutional Review Board (Silver, Research & Ethics Committee) members were present at the meeting held on September 20, 2023 at 09.45 am in the New IRB Room, Christian Medical College, Bagayam, Vellore-632002.

ETHICS COMMITTEE MEMBERS			
Name	Qualification	Designation	Affiliation
Dr. J. Amalorpavanathan	M.S (Gen. Surg), Dip. NBE (Gen. Surg), M. Ch (Vascular Surgery)	Chairperson, Ethics Committee, IRB, CMC Vellore, Vascular Surgeon, Retired Faculty, Chennai.	External, Clinician
Dr. Suceena Alexander	M.D, D.M (Nephro), FRCP (Lon.), FASN, Ph.D.	Secretary – (Ethics Committee), IRB, Addl. Vice Principal (Research), Professor of Nephrology, CMC, Vellore	Internal, Clinician
Prof. Keith Gomez	M.A (S.W), M. Phil (Psychiatry Social Work)	Deputy Chairperson, Ethics Committee, IRB, Student counselor, Loyola College, Chennai.	External, Social Scientist
Dr. George Thomas	D. Ortho, Ph.D.	Vice Chairperson, Ethics Committee, IRB, Orthopedic Surgeon, CMC, Ranipet Campus,	Internal, Clinician
Rev. Joseph Devaraj	BSc, BD	Theology	Internal Social Scientist
Mrs. Mary Anbarasi Johnsons	M. Sc., Ph.D	Professor & Head, Paediatric Nursing College of Nursing, CMC, Vellore	Internal, Nurse
Mrs. Shandril Immanuel	M. Sc (Nursing)	Deputy Dean, College of Nursing, CMC, Vellore	Internal, Nurse
Dr. Blessed Winston	M.D Pharmacology	Associate Professor, Clinical Pharmacology, CMC, Vellore	Internal, Pharmacologist



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Dr. Prasanna Samuel, M.Sc., Ph.D.,
Secretary, Research Committee

Prof. Keith Gomez, MA (S.W.), M.Phil.,
Deputy Chairperson, Ethics Committee.

Dr. Jacob John, MD., Ph.D.,
Chairperson, Research Committee

Dr. Suceena Alexander, MD, DM (Nephrology),
FRCP (Lon), FASN., Ph.D.,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Mrs. Anne Jarone	M. Sc (Nursing)	Professor, Associate Nursing Superintendent, CMC, Vellore	Internal, Nurse
Mr. Samuel Abraham	MA, PGDBA, PGDPM, M. Phil, BL.	Sr. Legal Officer, CMC, Vellore	External, Legal Expert
Mr. C. Sampath	B.Sc, BL	Sr. Legal Officer, Vellore	External, Legal Expert
Mrs. B. Scholastica Mary Vithiya	M. Phil, Ph. D.	Assistant Professor, Auxilium College, Vellore	External, Layperson
RESEARCH COMMITTEE MEMBERS			
Dr. Jacob John	MD, MPH	Chairperson, Research Committee, Professor, Community Medicine, CMC, Vellore	Internal, Clinician
Dr. Prasanna Samuel	M. Sc, Ph.D.	Secretary, Research Committee, Associate Professor of Biostatistics, CMC, Vellore	Internal, Statistician
Dr. Rajdeep Ojha	M. Tech, PhD	Associate Professor of Physical Medicine and Rehabilitation, CMC, Vellore	Internal, Basic Medical Scientist
Dr. Nihal Thomas	MD MNAMS DNB (Endo) FRACP (Endo) FRCP (Edin) FRCP (Glas) FRCP (London) FACP Ph.D. (Copenhagen)	Professor & Head Department of Endocrinology, Diabetes, and Metabolism	Internal, Clinician
Dr. D. J. Christopher	DTCD DNB, FRCP(Glasg), FCCP(USA)	Professor, Pulmonary Medicine, CMC, Vellore	Internal, Clinician
Dr. Joe Varghese	MBBS, MD	Professor, Biochemistry, CMC	Internal, Basic Medical Scientist



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Dr. J. Amalorpavanathan, M.S. Dip. NBE, M. Ch.
Chairperson, Ethics Committee

Dr. Prasanna Samuel, M.Sc., Ph.D.,
Secretary, Research Committee

Prof. Keith Gomez, MA (S.W), M.Phil.,
Deputy Chairperson, Ethics Committee.

Dr. Jacob John, MD., Ph D.,
Chairperson, Research Committee

Dr. Suceena Alexander, MD, DM (Nephrology),
FRCP (Lon), FASN., Ph.D.
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

We approve the above amendment as presented.

Yours sincerely,

Dr. Suceena Alexander
Secretary (Ethics Committee)
Institutional Review Board.

Dr. Suceena Alexander, MD.,DM.,FASN.
Secretary - (Ethics Committee)
Institutional Review Board
Christian Medical College,
Vellore - 632 002, Tamil Nadu, India.





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Chairperson, Ethics Committee

Dr. Prasanna Samuel, M.Sc., Ph.D.,
Secretary, Research Committee

Prof. Keith Gomez, MA (S.W), M.Phil.,
Deputy Chairperson, Ethics Committee

Dr. Jacob John, MD., Ph D.,
Chairperson, Research Committee

Dr. Succena Alexander, MD, DM (Nephrology),
FRCP (Lon), FASN., Ph.D.

Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

May 12, 2023

Dr. Mohankumar K. Murugesan,
Scientist E,
Department of CSCR,
Christian Medical College,
Vellore – 632 002.

Sub: External Research Grant: New Proposal: DBT:
Prime Editor mediated gene editing for the treatment of β -hemoglobinopathies.
Dr. Mohankumar K. Murugesan (Emp. No. 81774), Scientist E, CSCR, Dr.
Saravanabhavan Thangavel, Dr. Srujan Marepalley, Dr. RV Shaji, Dr. Alok Srivastava,
Haematology, CSCR.

Ref: IRB Min. No. 14854 [OTHER] dated 28.09.2022

Dear Dr. Mohankumar K. Murugesan,

The Institutional Review Board (Silver, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Prime Editor mediated gene editing for the treatment of β -hemoglobinopathies" on September 28, 2022.

The Committee reviewed the following documents:

- 1) IRB application
- 2) Patient information sheet and consent forms (Tamil, English, Telugu, Kannada, Bengali, Malayalam)
- 3) Signature page
- 4) CVs of Dr. Mohankumar K. Murugesan, Dr. Saravanabhavan Thangavel, Dr. Srujan Marepalley, Dr. RV Shaji, Dr. Alok Srivastava.
- 5) No of documents 1-4.

The following Institutional Review Board (Silver, Research & Ethics Committee) members were present at the meeting held on September 28, 2022 at 9.45 am in BIostatistics Resource and Training Centre, Bagayam Campus, Vellore -632002.

1 of 4



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Dr. Jacob John, MD., Ph D.,
Chairperson, Research Committee

Dr. Suceena Alexander, MD, DM (Nephrology),
FRCP (Lon), FASN., Ph.D.

Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

ETHICS COMMITTEE MEMBERS			
Name	Qualification	Designation	Affiliation
Dr. J. Amalorpavanathan	M.S (Gen. Surg), Dip. NBE (Gen. Surg), M.Ch (Vascular Surgery)	Chairperson, Ethics Committee, IRB, CMC Vellore, Vascular Surgeon, Retired Faculty, Chennai.	External Clinician
Dr. George Thomas	D. Ortho, PhD	Vice Chairperson, Ethics Committee, IRB, Orthopedic Surgeon, CMC, Ranipet Campus.	Internal, Clinician
Dr. Suceena Alexander	M.D, D.M (Nephro), FRCP (Lon.), FASN, Ph.D.	Secretary – (Ethics Committee), IRB, Addl. Vice Principal (Research), Professor, Department of Nephrology, CMC, Vellore	Internal, Clinician
Prof. Keith Gomez	M.A (S.W), M. Phil (Psychiatry Social Work)	Deputy Chairperson, Ethics Committee, IRB, Student counsellor, Loyola College, Chennai.	External, Lay Person & Social Scientist
Mr. C. Sampath	B.Sc, BL	Sr. Legal Officer, Directorate office, CMC, Vellore, Advocate, Vellore	External, Legal Expert
Mr. Samuel Abraham	MA, PGDBA, PGDPM, M. Phil, BL.	Sr. Legal Officer, Vellore	External Legal Expert
Mrs. Ilavarasi Jesudoss	M Sc (Nursing)	Deputy Nursing Superintendent, College of Nursing, CMC, Vellore	Internal, Nurse
Dr. Jayaprakash Muliyl	M.D, MPH, Dr. PH (Epid), DMHC	Retired Professor, Department of Community Medicine, CMC, Vellore	External, Scientist & Epidemiologist
Dr. Premila M Wilfred	MD, Pharmacology.	Assistant Professor, Pharmacology, CMC Vellore.	Internal, Pharmacologist

IRB Min. No. 14854 [OTHER] dated 28.09.2022

2 of 4



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Dr. Suceena Alexander, MD, DM (Nephrology),
FRCP (Lon), FASN., Ph.D.

Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Dr. Anuradha Rose	M.D, MHSC (Bioethics)	Professor, Department of Community Health, CMC, Vellore	Internal, Clinician
RESEARCH COMMITTEE MEMBERS			
Dr. Jacob John	MD, Ph D.	Chairperson, Research Committee, Professor, Department of Community Health, CMC, Vellore	Internal, Clinician
Dr. Prasanna Samuel	MSc, Ph D.	Secretary, Research Committee, Associate Professor, Department of Biostatistics, CMC, Vellore	Internal, Statistician
Dr. D. J. Christopher	DTCD DNB, FRCP(Glasg), FCCP(USA)	Professor, Department of Pulmonary Medicine, CMC, Vellore	Internal, Clinician
Dr. Rajdeep Ojha	M. Tech, Ph.D.	Associate Professor, Department of Physical Medicine and Rehabilitation, CMC, Vellore	Internal, Basic Medical Scientist
Dr. Sridhar Gibikote	DMRD, DNB	Professor, Department of Radiology, CMC, Vellore	Internal, Clinician
Dr. Santhanam Sridhar	DCH, DNB	Professor, Department of Neonatology, CMC, Vellore	Internal, Clinician
Dr. Elizabeth Vinod	MBBS, MD,	Associate Professor, Department of Physiology, CMC Vellore	Internal. Clinician
Dr. Joe Varughese	M.D., Ph.D.	Professor, Department of Biochemistry, CMC	Internal, Clinician
Dr. Christhunesa S. Christudass	M.Sc., Ph.D.	Professor, Department of Neurochemistry, Department of Neurological Sciences	Internal, Basic Medical Scientist
Dr. Winsely Rose	MD (Paed)	Professor, Department of Paediatrics, CMC Vellore	Internal, Clinician

IRB Min. No. 14854 [OTHER] dated 28.09.2022

3 of 4



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Chairperson, Research Committee

Dr. Suceena Alexander, MD, DM (Nephrology),
FRCP (Lon), FASN., Ph.D.
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

We approve the project to be conducted as presented for the duration 3 Years.

Kindly provide the total number of patients enrolled in your study and the total number of Withdrawals for the study entitled: "Prime Editor mediated gene editing for the treatment of β -hemoglobinopathies" on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in).

The Institutional Ethics Committee expects to be informed about the progress of the project, Any adverse events occurring in the course of the project, any amendments in the protocol and the patient information / informed consent. On completion of the study, you are expected to submit a copy of the final report. Respective forms can be downloaded from the following link: http://172.16.11.136/Research/IRB_Policies.html in the CMC Intranet and in the CMC website link address: <http://www.cmch-vellore.edu/static/research/Index.html>.

Yours sincerely,

Dr. Prasanna Samuel
Secretary (Research Committee)
Institutional Review Board

SECRETARY
Institutional Review Board
(Research Committee)
Christian Medical College,
Vellore - 632 002, Tamil Nadu, India.
VELLORE
INDIA

IRB Min. No. 14854 [OTHER] dated 28.09.2022

4 of 4



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EDITED BY
Ciaran Michael Lee,
University College Cork, Ireland

REVIEWED BY
Giulia Pavani,
Children's Hospital of Philadelphia,
United States
Dan Wang,
University of Massachusetts Medical
School, United States

*CORRESPONDENCE
Kumarasampet M. Mohankumar,
✉ mohankumarm@cmcvellore.ac.in

SPECIALTY SECTION
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Editing in Blood Disorders,
a section of the journal
Frontiers in Genome Editing

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Balasubramanian P, Thangavel S,
Marepally S, Velayudhan SR, Srivastava A
and Mohankumar KM (2022), Efficient
and error-free correction of sickle
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Efficient and error-free correction of sickle mutation in human erythroid cells using prime editor-2

Anila George^{1,2}, Nithin Sam Ravi^{1,2}, Kirti Prasad^{1,3},
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Sickle cell anaemia (SCA) is one of the common autosomal recessive monogenic disorders, caused by a transverse point mutation (GAG > GTG) at the sixth codon of the beta-globin gene, which results in haemolytic anaemia due to the fragile RBCs. Recent progress in genome editing has gained attention for the therapeutic cure for SCA. Direct correction of SCA mutation by homology-directed repair relies on a double-strand break (DSB) at the target site and carries the risk of generating beta-thalassaemic mutations if the editing is not error-free. On the other hand, base editors cannot correct the pathogenic SCA mutation resulting from A > T base transversion. Prime editor (PE), the recently described CRISPR/Cas 9 based gene editing tool that enables precise gene manipulations without DSB and unintended nucleotide changes, is a viable approach for the treatment of SCA. However, the major limitation with the use of prime editing is the lower efficiency especially in human erythroid cell lines and primary cells. To overcome these limitations, we developed a modular lenti-viral based prime editor system and demonstrated its use for the precise modelling of SCA mutation and its subsequent correction in human erythroid cell lines. We achieved highly efficient installation of SCA mutation (up to 72%) and its subsequent correction in human erythroid cells. For the first time, we demonstrated the functional restoration of adult haemoglobin without any unintended nucleotide changes or indel formations using the PE2 system. We also validated that the off-target effects mediated by the PE2 system is very minimal even with very efficient on-target conversion, making it a safe therapeutic option. Taken together, the modular lenti-viral prime editor system developed in this study not only expands the range of cell lines targetable by prime editor but also improves the efficiency considerably,

enabling the use of prime editor for myriad molecular, genetic, and translational studies.

KEYWORDS

prime editing, sickle cell anaemia, CRISPR, haemoglobinopathies, beta-globin

Introduction

Sickle cell Anaemia (SCA) is one of the most prevalent monogenic disorders worldwide, caused by a single transverse point mutation (GAG>GTG) in the sixth codon of the beta-globin (*HBB*) gene (Brittenham et al., 1985). This mutation results in the replacement of a polar amino acid residue (glutamate) with a non-polar residue (valine) causing adult haemoglobin to polymerise under conditions of low oxygen saturation and RBCs acquire the characteristic sickle shape with increased susceptibility to haemolysis. SCA patients present with haemolytic anaemia as well as other acute and chronic complications, such as recurrent episodes of pain and organ damage due to the obstruction of blood vessels by the distorted RBCs (Kavanagh et al., 2022). Being an autosomal recessive disorder, HbS mutation can be present in heterozygous (Sickle Cell Trait), homozygous (Sickle cell Anaemia), or compound heterozygous state (in combination with other structural or functional beta-globin variants) and this spectrum of diseases is commonly referred to as Sickle Cell Disease (SCD). Sickle Cell trait is a less adverse condition and patients do not present with clinical complications unless exposed to extreme conditions that can induce sickling of RBCs but SCA is a severe condition with reduced life expectancy and vast healthcare burden (Kato et al., 2018). The treatment approaches currently in practice are the administration of hydroxyurea that can elevate non-sickling fetal haemoglobin or blood transfusions with iron chelating agents that can improve the number of non-sickling RBCs (Platt et al., 1984; Sheth et al., 2013). The only curative approach approved for SCA is allogeneic stem cell transplant but is limited by the lack of availability of HLA matched donors and the risk of graft rejection which has prompted the research for other curative approaches (Salinas Cisneros and Thein, 2020).

Gene therapy and *ex vivo* genome editing approaches have proven successful in preclinical studies and are now being tested in ongoing clinical trials (Morgan et al., 2017; Magrin et al., 2019). Lenti-viral based approaches to complement the defective adult haemoglobin with non-sickling fetal or adult haemoglobin are currently the preferred strategies, but the long-term safety issues due to insertional mutagenesis are unknown (Pawliuk et al., 2001; Ribeil et al., 2017; Kanter et al., 2022). CRISPR/Cas based approaches are now gaining recognition as *ex vivo* gene editing tools owing to the efficiency and versatility (Barbarani et al., 2020; Prasad et al., 2021).

Previous studies have attempted the correction of HbS mutation using Homology Directed Repair (HDR) with Cas9 but suffered from low efficiency and engraftment potential (DeWitt et al., 2016). Furthermore, unrepaired indels from DSB at the target site would result in a more severe beta-thalassemia phenotype rather than correcting SCD. Over the years attempts at improving the HDR efficiency and reducing indel formation at the target site have been successful but still do not overcome the general risks posed by DSB such as large deletions, translocations, chromothripsis and p53 activation (Leibowitz et al., 2021; Papathanasiou et al., 2021; Amendola et al., 2022).

Base editing for the substitution of key nucleotides at the gamma-globin regulatory elements to mimic the naturally occurring HPFH conditions thereby elevating fetal haemoglobin is a potential alternative strategy for the treatment of SCD (Psatha et al., 2018, 11; Esrick et al., 2021, 11; Ravi et al., 2022). The use of single-strand nick at the target site keeps the generation of indels to a minimum and base editors were shown to be highly efficient in installing transition mutations (by deaminating the purine or pyrimidine to the respective bases) in CD34+ HSPCs without affecting the engraftment potential (Zeng et al., 2020). However, correction of transverse point mutation (T>A) is not possible with base editors described so far which poses a challenge in the treatment of SCD (Anzalone et al., 2020). Recently, the conversion of sickle beta-globin to a non-sickling haemoglobin variant HbG Makassar, using adenosine base editor (GTG>GCG, Val > Lys) has proven to be successful *in vitro* and in SCD mouse models. However, this approach uses a more common NRCH PAM which increases the number of potential off-target sites. Further, the functional utility of this approach needs to be extensively characterised for therapeutic applications (Newby et al., 2021).

Prime editing is a CRISPR-based novel gene editing tool that allows the installation of precise mutations in the target locus without causing double-strand break (DSB) and limited PAM constraints. PE relies on a reverse transcriptase fused to a Cas9 nickase to incorporate the mutations encoded by the prime editing guide RNA (pegRNA) into the target DNA (Anzalone et al., 2019). This approach offers several advantages over conventional DSB based approaches as well as base editing in therapeutic gene editing. PE enables precise introduction of point mutations including transition mutations, insertions, or deletions in the genomic region of

interest while overcoming the limitations associated with DSB, thus expanding the scope of gene therapy (Anzalone et al., 2020). Prime editing has been successfully harnessed in proof-of-concept studies for correcting genetic disorders as well as developing cellular and animal models for genetic disorders (Schene et al., 2020; Petri et al., 2021; Doman et al., 2022; Lu et al., 2022). However, the efficiency of prime editing is low compared to other approaches and the cells that can be edited are limited. One approach to increase the editing efficiency was the PE3 system that uses an additional gRNA that nicks the unedited strand in the PE3 system along with the targeting pegRNA and prime editor (PE2) but was reported to generate a higher proportion of InDels. Till date prime editing has not been demonstrated for the correction of HbS mutation in human erythroid cells and the functional evaluation and safety profiling have not been carried out, which limits its clinical application although Anzalone et al. had described the proof of concept for correction of SCA using HEK293T cells.

In this study, we describe the use of prime editing for the correction of HbS mutation in human erythroid cell line (HUDEP2) and demonstrate the reconstitution of functional adult haemoglobin in the SCA model with high efficiency and precision. As the expression of PE components for a longer duration of time might increase the editing efficiency, we developed a selectable dual lenti-viral system for delivery of PE and pegRNA that showed highly efficient editing than previously reported approaches in human erythroid and other cell lines. We hope this study would pave the foundation for therapeutic *ex vivo* gene editing of HSPCs for the treatment of SCD and other beta-haemoglobinopathies. Further, the modular lenti-viral PE delivery system that we developed would expand the type of cells targetable by prime editing, enabling efficient screening and functional evaluation of prime editing for various genetic diseases.

Materials and methods

Plasmids constructs

Plasmids pCMV-PE2 (Addgene #132775), pU6-pegRNA-GG-acceptor (Addgene #132777) and pU6-Sp-pegRNA-HEK3_CTT_ins (Addgene #132778) were a gift from David Liu. pMD2.G and psPAX2 (second-generation lenti-viral packaging construct, Addgene #12259, #12260) were a gift from Didier Trono. pLenti_PE2_P2A_Puro vector was constructed by assembling amplified PE2 (from Addgene #132775; primers listed in Table) into BamH1 and Nhe1 digested pLenti-ABERA-P2A-Puro vector (Addgene#112675; a gift from Lucas Dow) using NEBuilder HiFi DNA Assembly Master Mix as per the manufacturer's instructions. Lenti_pegRNA_GFP/RFP vector was constructed by removing the

gRNA scaffold from pLKO5.sgRNA.EFS.GFP/RFP (Addgene #57822/57823; Gift from Benjamin Ebert).

Design and cloning of pegRNA

PegRNA sequences were selected from previous publication (Details in [Supplementary Table](#)). pU6-Sp-pegRNA-HEK3_CTT_ins was obtained from Addgene (#132778); all other pegRNAs used in the study were cloned in Lenti_pegRNA_GFP/RFP vector. Spacer, extension template and scaffold sequences were obtained as oligonucleotides with respective overhangs in the top and bottom strands to facilitate Goldengate assembly ([Supplementary Figure S1A](#)). Oligo annealing was carried out for respective sets as per the previously published protocol (Ravi et al., 2022). Briefly, 1 μ L each of forward and reverse oligonucleotides (100p.m.), 0.5 μ L of T4 PNK(NEB), 1 μ L of T4 DNA ligase buffer (NEB) and 6.5 μ L of H₂O was mixed and oligo annealed. After annealing the top and bottom strands of the respective components, golden gate assembly was carried out using BsmB1 V2(NEB) digested Lenti_pegRNA_GFP/RFP vector as the backbone. In brief, 1 μ L each of 1:10 diluted annealed oligos (total 3 μ L), 50 ng of digested vector, 0.5 μ L of T4 DNA ligase, 0.25 μ L of BsmB1 V2 enzyme and 1 μ L of T4 DNA ligase was mixed and volume was made up to 10 μ L using nuclease-free water. The golden gate assembly reaction was carried out as follows: (42°C, 3 min $\rightarrow$ 16°C, 3 min) x 30 cycles, 60°C, 5 min $\rightarrow$ 4°C. The reaction product was then transformed into DH10B competent cells and plated in LB agar containing 100 μ g/ml of ampicillin for selection. The selected colonies were verified using colony PCR followed by sanger sequencing as per published protocol (Ravi et al., 2022). The positive clones were inoculated further in LB broth and cultured overnight. Plasmids were isolated using NucleoBond Xtra Midi EF (Macherey-Nagel) kit according to the manufacturer's instruction.

Cell culture

HEK293T cells were cultured in DMEM (HyClone) supplemented with 10% FBS and 1x Penicillin Streptomycin. K562 cells were maintained in RPMI supplemented with 10% FBS and 1x Penicillin Streptomycin. HUDEP2 cells were cultured in StemSpan™ SFEM II (STEMCELL Technologies) supplemented with 50 ng/ml SCF (ImmunoTools), 3 U/ml EPO (Zyrop 4000 IU Injection), 1x Pen-Strep, 1 μ M dexamethasone (Alfa Aesar), 1 μ g/ml doxycycline (Sigma-Aldrich) and 1x L-Glutamine 200 mM (Gibco™) (Kurita et al., 2013). Erythroid differentiation of HUDEP2 cells was carried out using

two-phase erythroid differentiation media. Phase I media consisted of IMDM glutamax (Gibco), 3% AB serum (MP Biomedicals), 2% FBS, 0.1% insulin solution human (Sigma-Aldrich), 3 U/ml Heparin sodium salt (MP Biomedicals), 200 µg/ml Holo Transferrin (BBI Solutions), 3 U/ml EPO, 10 µg/ml SCF, 1 ng/ml IL3 (Immuno Tools), 1x Pen-Strep, and 1 µg/ml doxycycline. Phase II media is similar in composition except that it is devoid of doxycycline and contains 500 µg/ml of holotransferrin. Around one million cells were used to set up differentiation. Media change was done on day-3 and cells were shifted to phase II media on day-6. The culture was terminated on day-9.

Transfection

2 × 10⁵ HEK293T cells were seeded in a 12-well cell culture dish. Upon reaching 80% confluency, the cells were transfected with the appropriate plasmids using FuGENE-HD (Promega) as per the manufacturer's protocol. Briefly, 100 µL OptiMem, 1,000 ng of plasmids and 3 µL of FuGENE-HD (1:3 ratio; scaled up when required) was mixed well, incubated for 15 min, and added to the cells. Media was changed 24 h later and cells were dissociated and taken for analysis from 72 h onwards. Prime Editor to pegRNA ratio was maintained at 3:1.

Electroporation

pegRNA plasmid was electroporated into K562/HUDEP2 PE stables using Lonza 4D electroporator using the program EN138. 2 µg of plasmid was nucleofected to one million cells as per the manufacturer's protocol.

Lenti-virus production and transduction

About 2 × 10⁶ HEK293T cells were seeded in a 10 cm cell culture dish with DMEM (HyClone) containing 10% FBS and 1x Pen-Strep. At 80% confluency, 2.5 µg of pMD2.G, 3.5 µg of psPAX2 and 4 µg of lenti-viral vector (PE2 or pegRNA constructs) were transfected using FuGENE-HD as per the manufacturer's protocol. Viral supernatant was then collected after 48 h and concentrated using Lenti-X Concentrator (Takara). The concentrated pellet was resuspended in 500 µL 1xPBS and stored at -80°C until use. For transduction, 0.2 million cells were taken in 12 well plate with 10 µL HEPES 1M buffer (Gibco) and 1 µL of polybrene (Sigma Aldrich; 6 mg/ml concentration). Virus was thawed, 100 µL added to each well and spininfected at 800 g for 30 min at room temperature. After 48 h s media was changed and 1 µg/ml puromycin (Gibco) was added for stable preparation. The transduction efficiency of

pegRNA was analysed using GFP/RFP expression by flow cytometry (BD celesta or BC Cytotflex).

DNA isolation, sequencing, and analysis of editing efficiency

To determine the prime editing efficiency, genomic DNA from the edited cells was isolated using DNA isolation kit (Qiagen). For experiments where editing was tested sequentially over time, DNA was isolated from about 0.2 million cells using quick DNA extract (epicentre). The target region was amplified using the respective primers ([Supplementary Tables](#)), and the amplified product was Sanger sequenced. The sequencing data were analysed either using Synthego ice (for insertions/deletions) ([Conant et al., 2022](#)) or EditR (for substitutions) ([Kluesner et al., 2018](#)). For next-generation sequencing, primer sets with ~250 bp amplicon size and appropriate adapters were used (Listed in Table). NGS data was analysed using Crispresso-2 ([Clement et al., 2019](#)).

cDNA sequencing

For checking the edits at RNA level, total RNA from the cells was isolated using NucleoSpin RNA kit (Macherey-Nagel) and reverse transcribed to cDNA using iScript cDNA synthesis kit (BioRad) as per the manufacturer's protocol. cDNA was PCR amplified using the primers listed in Table and Sanger sequenced. Editing efficiency was analysed using EditR.

MLPA

MLPA was performed using SALSA MLPA probe mix P102 HBB with 100 ng/µL DNA as per the manufacturer's protocol. The data was analysed and visualised using Coffalyser software.

HPLC

HPLC was used to quantify haemoglobin variants and individual globin chains in differentiated HUDEP2 cells. Cells were collected, washed with 1x PBS, and resuspended in double distilled water. The cells were then subjected to sonication following which the suspension was centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was collected and stored at -80 until analysis. The samples were analysed using VARIANT II Haemoglobin Testing System (Bio-Rad) for haemoglobin variants and data was quantified by the Bio-Rad's Clinical Data Management (CDMTM) Software. Individual globin

chains were quantified using Reverse phase HPLC (Shimadzu Corporation-Phenomenex).

VCN analysis by real-time PCR

Vector copy number was analysed in genomic DNA isolated from the stable cell lines. Primers targeting the Cas9 gene were used to detect the integration of prime editor. A primer set targeting Factor IX gene was used as an internal reference. pCMV-PE2 (Addgene #132775) and an in-house plasmid construct with Factor IX CDS were used as standards.

Prime editor expression analysis by real-time PCR

RNA was isolated from the cells using NucleoSpin RNA kit (Macherey-Nagel) and reverse transcribed to cDNA using iScript cDNA synthesis kit (BioRad). The relative expression of prime editor in the stables compared to the respective wild-type cells was quantified using primers targeting Cas9. GAPDH was used as the normalising internal control. The PCR reaction was carried out using SsoFast EvaGreen Supermixes (Bio-Rad) in QuantStudio six Flex Real-Time PCR System (Applied Biosystems) after three fold dilution of cDNA samples as reported previously (Ravi et al., 2022).

Single cell sorting

Single-cell sorting to obtain isogenic clones were done using BD FACS Aria cell sorter in 96-well format. After Sanger sequencing, desired clones were expanded and used for further analysis.

HbF intracellular staining

To evaluate the frequency of HbF-positive cells, the cells were fixed, permeabilized, and intracellular staining was performed using Fetal Haemoglobin Monoclonal Antibody (HBF-1), APC (Invitrogen) as previously described (Canver et al., 2015). The stained cells were analysed by Flow cytometry (CytoFLEX LX Flow Cytometer—BC) to measure the number of HbF-positive cells.

Off-target analysis

Cas-OFFinder/Cosmid was used to predict the gRNA-dependent off-target regions, up to three mismatches were allowed in selecting targets. The top four targets were

amplified and sequenced using Illumina MiSeq platform (Supplementary Table). CRISPResso2 was used for data analysis and visualisation.

Statistics

All analyses were performed using GraphPad Prism V8.1 and p -value < 0.05 was considered statistically significant.

Results

Prime editing has tremendously expanded the scope of therapeutic genome editing with its ability to correct various disease-causing mutations resulting from substitutions, small insertions, and deletions (Indels) without generating undesired editing outcomes. One of the major limitations in taking this approach forward for clinical translation is that the efficiency of prime editing decreases as we move from workhorse cell lines to more specialised cell lines and even further when taken to primary cells (Jin et al., 2021). This decline in efficiency also hinders the appropriate evaluation of on- and off-target effects mediated by the prime editor. Therefore prior to evaluating prime editing in erythroid cell lines for correction of SCA mutation, we aimed at developing a selectable lenti-viral PE system that could not only be used with ease in multiple cell lines but also yield better editing efficiency than the conventional delivery approaches.

Lenti-viral delivery of prime editor enables efficient editing across multiple target sites in HEK293T cells

We sought to develop a lenti-viral delivery system for prime editing that would ensure constitutive availability of components allowing for maximum editing efficiency. The prospect of cloning prime editor in a single lenti-viral vector has been dismissed previously owing to its large size (Gao et al., 2022). Limited number of studies have used lenti-viral delivery of PE and have relied on the split-intein approach which requires simultaneous delivery of two different constructs and has not shown significant elevation in editing efficiency (Anzalone et al., 2019). Nevertheless, with our past success in cloning base editor into a lenti-viral based vector (Ravi et al., 2022), we considered developing a similar prime editing system, with the PE delivered in one vector and pegRNA in another. We limited the study to PE2 system since multiple publications have shown an increase in indels with the use of additional nicking gRNA in the PE3 system.

Prime editor (PE2) sequence (Anzalone et al., 2019) was cloned into a lenti-viral backbone driven by EF1 alpha (short)

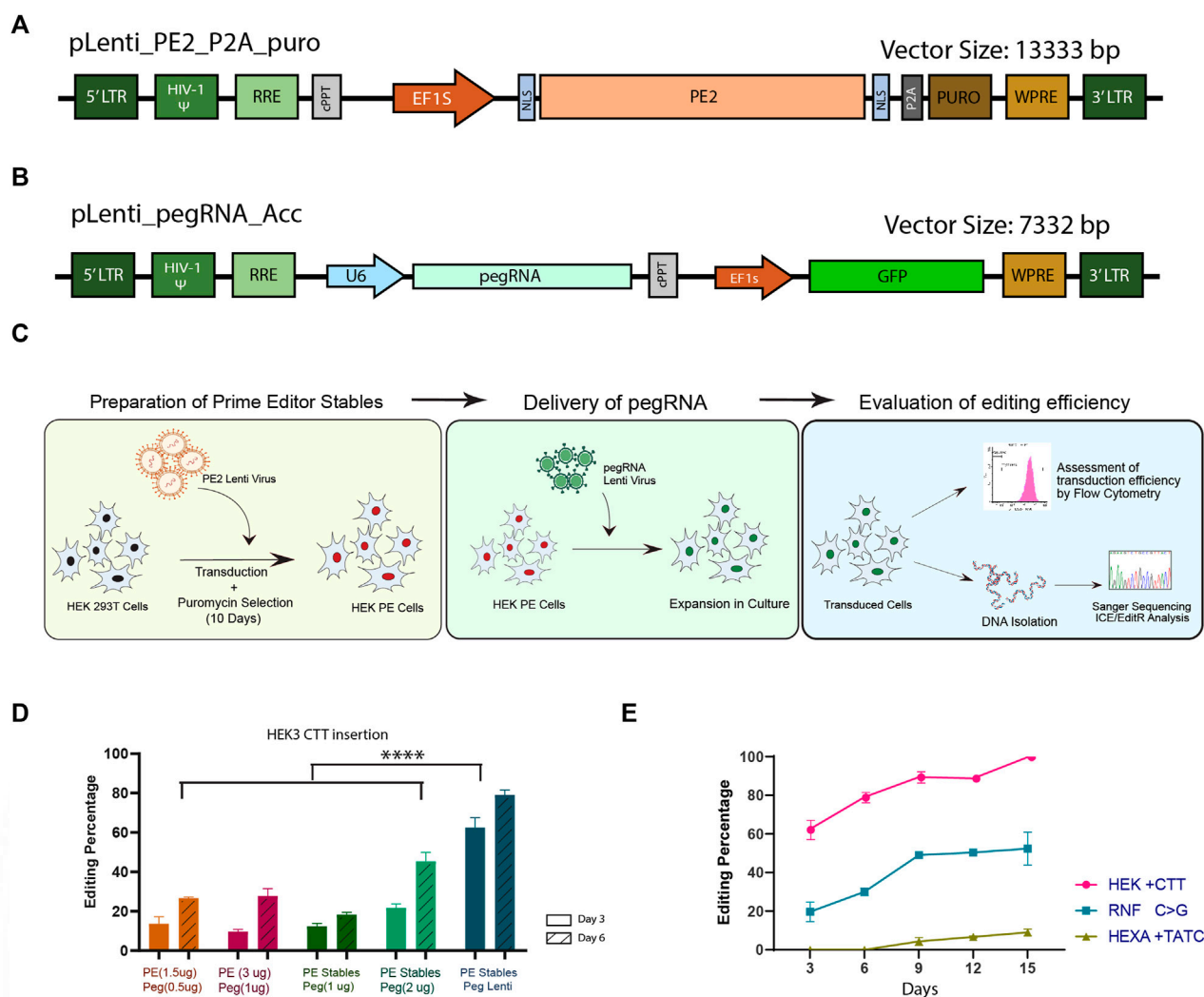


FIGURE 1

Generation and characterization of a modular lenti-viral prime editor system in HEK293T cells. Schematic representations of the prime editor (A) and pegRNA (B) lenti-viral constructs developed in this study. (C) Overview of the general workflow for prime editing by the dual lenti-viral system. (D) Evaluation of prime editing efficiency for CTT insertion in HEK3 locus; The efficiency of dual lenti-viral delivery of the components (blue) was compared against transfection of pegRNA plasmid in HEK293T PE stables (dark and light green) and dual transfection of PE2 and pegRNA (orange and magenta) by sanger sequencing at two different time points. (E) Prime editing efficiency by dual lenti-viral delivery at 3 different genomic loci evaluated by sanger sequencing over a period of 15 days. Results from three replicates are plotted as mean $\pm$ SD; Asterisks indicate the level of statistical significance; One way ANOVA with Tukey's test was used for comparison between the groups. (**** $p < 0.0001$).

promoter which is known to give better transgene expression and stability across various cell lines (Zafra et al., 2018). The PE sequence was linked *via* self-cleaving peptide (P2A) to puromycin resistance gene that would allow the selection of cells with stable expression of prime editor (Figure 1A). Subsequently, the pegRNA was cloned into a lenti-viral backbone driven by U6 promoter which co-express GFP, enabling positive selection and validation of transduction efficiency (Figure 1B; Supplementary Figure S1A). We presumed that the relatively low efficiency of PE compared to the other genome editing strategies could be overcome by

prolonged expression of PE through the dual lenti-viral system which will ensure the availability of PE components for multiple rounds of cell division till the desired conversion is achieved at the target site. Further, this system would enable the generation of cell lines stably expressing prime editor, which can be used for simultaneous arrayed screening of multiple pegRNAs overcoming the inconsistencies associated with transfection of large PE plasmid.

We first validated the efficiency of the lenti-viral PE system in HEK293T cells by inserting a CTT sequence in HEK3 locus using previously validated pegRNA (Anzalone et al., 2019). HEK293T

PE stables were generated by lenti-viral transduction of PE2 construct followed by puromycin selection for 10 days (Figure 1C). The HEK293T PE stables were either transduced with lenti-virus of pegRNA for inserting the CTT sequence or transiently transfected with pegRNA construct described by Anzalone et al. This approach was then compared against transient transfection of both PE2 and pegRNA in HEK293T cells. Three and six days after delivery of the components, cells were collected, genomic DNA isolated and subjected to sanger sequencing to assess the editing efficiency. We noted a significant elevation in editing efficiency in samples transduced with lenti-virus of pegRNA compared to the pegRNA transfected samples (Figure 1D). This could be accounted for by the perpetual expression of PE components in the transduced samples, as lenti-virus integrates into the host genome. By day-6, HEK293T PE cells transfected with pegRNA also showed a higher editing efficiency than dual transfection of PE2 and pegRNA which might be due to the increase in the delivery dosage of pegRNA alone when compared to the dual transfection. Higher editing efficiency noted with the increase in the concentration of components delivered hints that the availability of components might be a limiting factor in determining the editing efficiency. However, we did not find any discernible difference in the editing efficiency when the concentration of PE2 and pegRNA was increased in the dual transfection, probably because of the limitations in transfecting the large sized PE2 constructs. Though the editing efficiency of pegRNA alone transfected in PE stable might be lower than what would be expected with lenti-viral delivery, transfection at higher doses enables screening of multiple pegRNAs without the need for producing lenti-virus for each target.

We further tested the system by creating mutations at two additional genomic loci with previously described pegRNAs for *RNF* +1C>G conversion and a TATC insertion in *HEXA* locus (Anzalone et al., 2019). Lenti-viral pegRNAs were transduced into HEK293T PE cells and the editing efficiency was evaluated by sanger sequencing over a period of 15 days. Although the transduction efficiency was similar across all three target sites (Supplementary Figure S1B), editing efficiency varied considerably between the targets (Figure 1E). As has been previously observed (Wolff et al., 2021), the pegRNA for insertion of CTT sequence in *HEK3* locus resulted in the highest editing efficiency starting with ~60% insertion on day-3 and reaching ~100% by day-15 (Figure 1E). *RNF* C>G conversion showed modest editing of ~20% on day-3 and increased up to ~50% on day-15. On the other hand, *HEXA* TATC insertion did not show any appreciable editing till day-9 and the maximum efficiency obtained was around 10% on day-15. These data suggest that even though the availability of pegRNA and prime editor is not limited, the editing efficiency and the installation of the desired mutation might vary from one locus to the other, necessitating multiple steps of optimisation at each of the desired loci. Taken together, the modular prime

editing approach that we have described is versatile for studies requiring extensive screening and validation of pegRNAs.

Efficient prime editing in the human erythroid cell lines mediated through lenti-viral delivery system

Encouraged by the efficiency and versatility of the PE lenti system in HEK293T cells, we evaluated the prime editing efficiency in two commonly used human erythroid progenitor cell lines (K562 and HUDEP2) that are widely used as preclinical models in hematological studies. Although prime editing has been tested in K562 cells by different approaches, the editing efficiency was lower compared to HEK293T cells (Wolff et al., 2021) and the prime editing efficiency reported in HUDEP2 cells was very minimal (Zhang et al., 2022). We hypothesised that the lower efficiency in these cell lines could be overcome by a constitutive expression of prime editing components. We first tested whether a transient delivery of pegRNA into K562 and HUDEP2 cell lines stably expressing PE could edit efficiently as was seen in HEK293T PE cells and compared the efficiency against dual lenti-viral delivery in human erythroid cell lines. The efficiency of prime editing in both the erythroid cell lines was validated using the pegRNA for CTT insertion in *HEK3* locus, which showed the highest editing in the HEK293T cells. Upon nucleofection of pegRNA construct, we observed moderate editing efficiency (13.6%) in K562 cells and very low frequency of 3bp insertion (1.33%) in HUDEP2 cells by day-3 (Figure 2A; Supplementary Figure S2A). On the other hand, by lenti-viral mediated transduction of pegRNA, we achieved efficient insertion of the CTT sequence in K562 cells which was either comparable to or better than previously described prime editing delivery approaches (Wolff et al., 2021) (Figure 2B). We observed almost comparable editing efficiency for transient (day-3) vs. lenti-viral delivery (day-4) in K562 cells (13.6% vs. 15.33%). However, we noticed an increased editing efficiency by lenti-viral mediated delivery when compared to transient transfection in HUDEP2 cells (1.33% vs 7.33%). The editing efficiency in K562 cells was higher than HUDEP2 cells in the initial days but over time reached similar efficiency. The maximum efficiency that could be achieved by day-12 was around 30% in both cell lines, although the overall efficiency was lower than what was observed in HEK293T cells (~30% vs. 89%) (Figure 2A; Figure 1E). Surprisingly, we also observed indels in both the cell lines with transient delivery which was not seen with lenti-viral delivery possibly because of the continued availability of pegRNA in the lenti PE2 system for editing until error-free conversion is achieved at the target site (Supplementary Figure S2B).

As all 3 cell lines were shown to have been transduced with both prime editor as well as pegRNA constructs (evidenced by survival in puromycin selection and GFP expression)

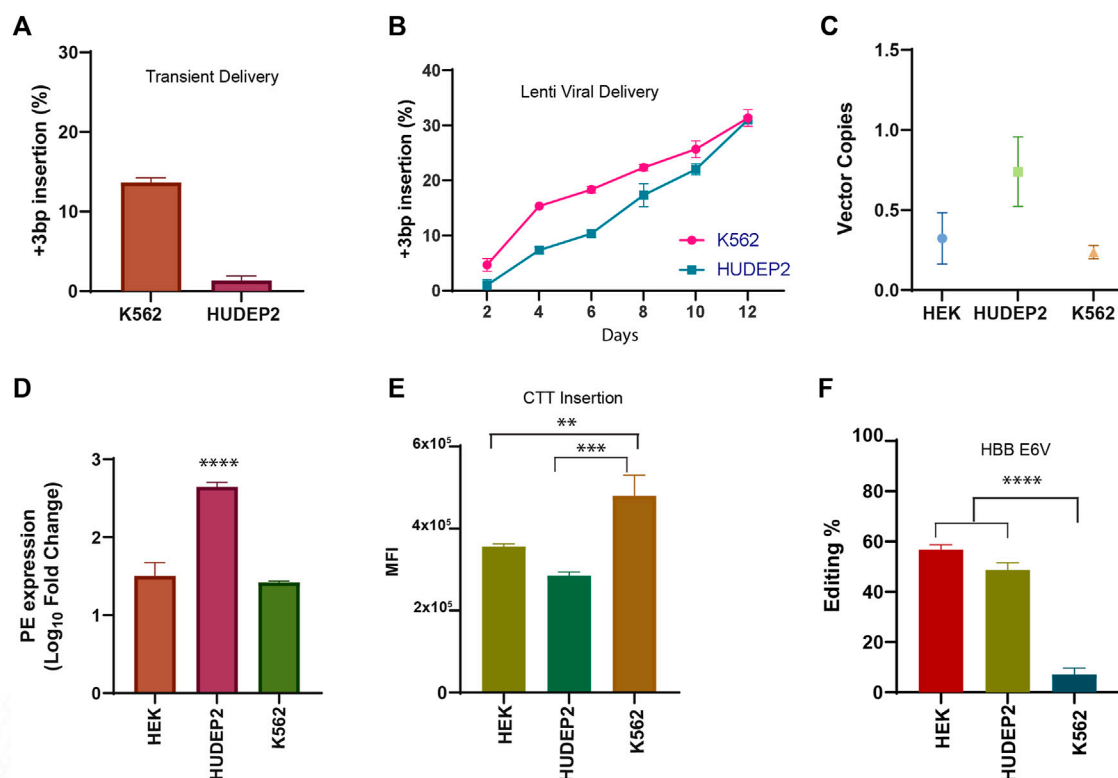


FIGURE 2

Validation of prime editing in human erythroid cell lines (A) Efficiency of CTT insertion in HEK3 locus in K562 and HUDEP2 PE cells upon nucleofection of pegRNA plasmid. (B) Dynamics of CTT insertion efficiency in HEK3 locus by lenti-viral delivery in K562 and HUDEP2 cells evaluated by sanger sequencing (C) The number of prime editor vector copies integrated in the genome of HEK293T, HUDEP2, and K562 PE cells assessed by RT-PCR. (D) Expression of prime editor in PE stable cell lines normalised to respective wild-type cells. (E) Mean Fluorescence Intensity of GFP expression in the PE stable cells transduced with pegRNA for CTT insertion. (F) Efficiency of installation of Sickle Mutation (A>T conversion) in the beta-globin gene in the three different cell lines by lenti-viral delivery measured by sanger sequencing 12 days after transduction. Results from three replicates are plotted as mean $\pm$ SD; asterisks indicate the level of statistical significance; One way ANOVA with Tukey's test was used for comparison between the groups. (** $p < 0.01$, *** $p < 0.001$, p**** < 0.0001).

(Supplementary Figure S2C), we compared whether there is any difference in expression of PE or pegRNA between these cell lines which results in varying editing efficiency at the same target site. The number of PE2 vector copies integrated into the genome for each of the stable cell lines was quantified by RT-PCR. There was no appreciable difference between HEK293T and K562 cell lines while HUDEP2 cells showed a higher number of vector copy integration (Figure 2C). This pattern was also reflected in the expression levels of PE2 mRNA in all these cell lines (Figure 2D). These results from integration and expression pattern of PE2 components did not correlate with the editing efficiency which was higher in HEK293T cells suggesting that prime editor availability might not be the only deciding factor for varying editing outcomes. The mean fluorescence intensity of GFP expression from the pegRNA was also compared in all 3 cell lines and the patterns did not corroborate the editing in the respective cell line (Figure 2E). The difference in editing efficiency might be due to cell-intrinsic factors affecting the

editing outcome such as the chromatin accessibility or the presence of mismatch repair proteins (Chen et al., 2021; Ferreira da Silva et al., 2022).

Upon ensuring that the prime editor system works efficiently in both the erythroid cell lines, we went ahead with creation of the HbS mutation (*HBB* E6V) in K562 and HUDEP2 cell lines along with HEK293T cells. We selected the pegRNA that was shown to produce the highest editing efficiency for *HBB* E6V installation in HEK293T cells by Anzalone et al. K562, HUDEP2 and HEK293T cell lines stably expressing PE2 were transduced with pegRNA and were cultured for 12 days before editing efficiency was evaluated by sanger sequencing. Although HEK293T cells showed the highest editing efficiency for HbS creation, the difference was not as marked as observed for CTT insertion in HEK3 locus (Figure 2F). Interestingly, we observed a higher editing frequency for the installation of HbS mutation compared to CTT insertion in HUDEP2 cells suggesting

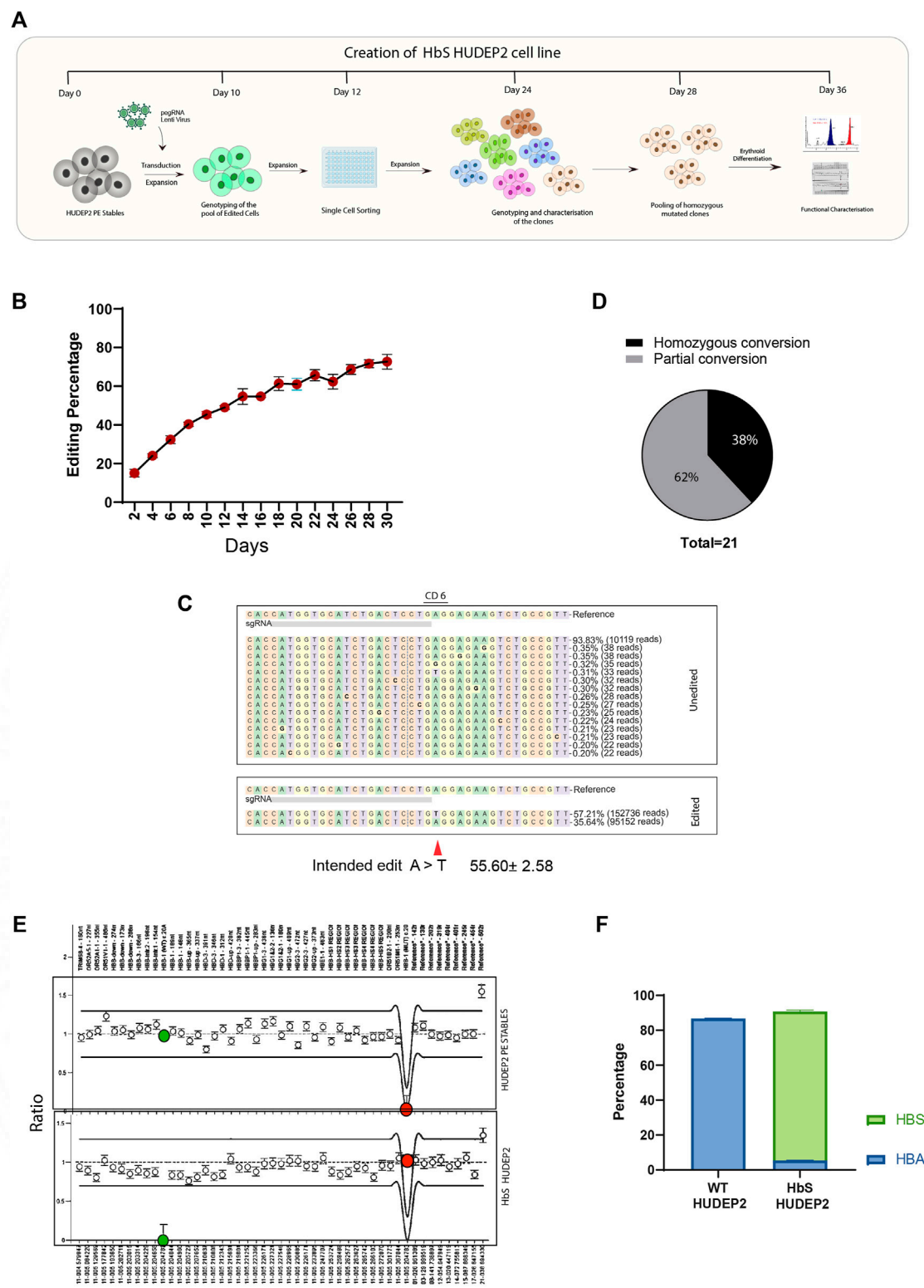


FIGURE 3 Modelling of Sickle Cell Mutation in human erythroid cells using prime editor (A) Schematic representation of the methodology for the creation of sickle mutant cell line using prime editor (B) Change in prime editing efficiency for 30 days in HUDEP2 PE cells transduced with pegRNA for installation of HBB E6V mutation measured by sanger sequencing. (C) Representative NGS data of overall editing efficiency on the day of single cell sorting (D) pie chart showing the proportion of the single cell clones obtained classified according to the genotype at the beta-globin gene (E) MLPA analysis chart with the WT probe (Green) and Sickle Mutant probe (Red) highlighted. WT probe amplified exclusively in the unedited cells while the Hbs HUDEP2 cells showed amplification with the Sickle probe. (F) Percentage of haemoglobin variants after erythroid differentiation of HUDEP2 cells assessed by HPLC. Results from three replicates are plotted as mean ± SD.

that the prime editing efficiency is dependent on the locus and varies from cell to cell. To our surprise, we did not observe any significant editing in K562 cells although the transduction efficiency was 100% (Supplementary Figure S2D). The result was similar to what was observed by Wolff et al. on day-10 of prime editing for the creation of HbS mutation in K562 cells by piggy-bac transposons. The editing efficiency in these cells might increase over time by prolonging the culture of cells (Wolff et al., 2021). Further experiments need to be performed to evaluate if this minimal editing is in any way associated with the gene expression pattern that varies between K562 and HUDEP2 cells or other epigenetic factors intrinsic to the cell line. In summary, we show efficient prime editing without any indels or bystander edits using lenti-viral system in two different human erythroid cell lines. Lenti-viral mediated delivery of prime editor enables the installation of mutations in cell lines that are not easily transfected, expanding the range of cell lines that can be prime edited. Therefore, with the expanded repertoire of targetable cells, it would be possible to utilise prime editing with its full potential in addressing a wide range of research questions in varied research areas.

Modelling of sickle cell anaemia in human erythroid cell line by prime editor yields a higher proportion of biallelic HbS mutation

Although other groups have previously demonstrated the modelling of SCA in HUDEP2 cells using CRISPR/Cas-9 mediated HDR (Demirci et al., 2020), we evaluated the efficiency of PE utilising the lenti-viral delivery approach to generate a cellular model of SCA in HUDEP2 cells. (Figure 3A). We selected HUDEP2 cells for modelling and correction of HbS mutation as it mimics the adult human erythroid cells and predominantly express beta-globin. We first characterised the dynamics of prime editing for HbS mutation creation by sanger sequencing over a period of 30 days in HUDEP2 PE stables as the PE components were transduced by the selectable lenti-viral vector shown to express for a longer duration of time. Most of the cells were completely transduced with pegRNA as seen by the GFP expression (Supplementary Figure S3A). On day-2 after transduction, the editing efficiency for installing HbS mutation was around 16%, reaching a maximum of ~70% by day-30 (Figure 3B). There was a consistent increase in editing efficiency during the experimental period which plateaued around day-26. We also evaluated the formation of indels at the nick site and as expected with PE2 system there was no indel formation even at day-30 when the overall editing efficiency had reached 73% (Supplementary Figure S3B). These data provide confidence that prime editor could be

used for precise modelling of SCA with the possibility to obtain a greater number of individual clones with the desired mutation compared to Cas9/HDR based approaches.

SCA phenotype is evident when expressed in the homozygous condition because it is inherited in an autosomal recessive manner. Therefore, we performed the single-cell sorting of edited cells on day-12 to obtain homozygous clones of HbS mutation for further phenotypic characterisation. Almost half of the cells were edited on the day of sorting with an overall editing efficiency of ~55% and no trace of undesired mutations or indels (Figure 3C). Of the 21 clones that proliferated, 38% were homozygous for HbS mutation (8 clones), and the average editing efficiency was 72% (Figure 3D, Supplementary Figure S3C). Further, all but one of the sequenced clones showed at least 50% desired mutation suggesting that all the cells in the pool of edited cells were harbouring the SCA mutation and given more time these cells also might achieve homozygous conversion. We selected the eight clones that showed homozygous conversion of HbS for further experiments. The clones were evaluated for fetal haemoglobin levels to eliminate any variation due to clonality which can interfere with the precise evaluation of sickle or adult haemoglobin. We found six out of eight clones with elevated HbF levels (Supplementary Figure S3D). We pooled the clones that showed lower HbF and expanded further before proceeding with functional characterisation.

Even after multiple passages, the homozygosity of the edited cells was maintained without any undesired edits or indel formation (Supplementary Figure S3E). We performed Multiplexed Ligation Dependent Probe Amplification (MLPA) in the beta-globin locus using the genomic DNA and reconfirmed the installation of HbS mutation, absence of normal genotype (HBB E6), and the intactness of the other regions in the globin locus (Figure 3E). We further differentiated the clones in the erythroid medium for 8 days and the haemoglobin variants produced were analysed by HPLC. The pool of homozygous clones showed almost complete presence of sickle haemoglobin in contrast to the unedited control (Figure 3F). There was no significant alteration in either HbF or HbD which suggests the successful creation of sickle cell mutation in HUDEP2 cells that mimic the human disease both phenotypically as well as genotypically. This pool of homozygous clones (Further referred to as HbS HUDEP2 Cells) was used for the proof of concept for correcting the HbS mutation using prime editor.

Our study reiterates the fact that prime editing could be utilised for efficient modelling of diseases in human erythroid cell lines. However, constitutive expression over a longer period might be required to achieve bi-allelic mutation at the target site.

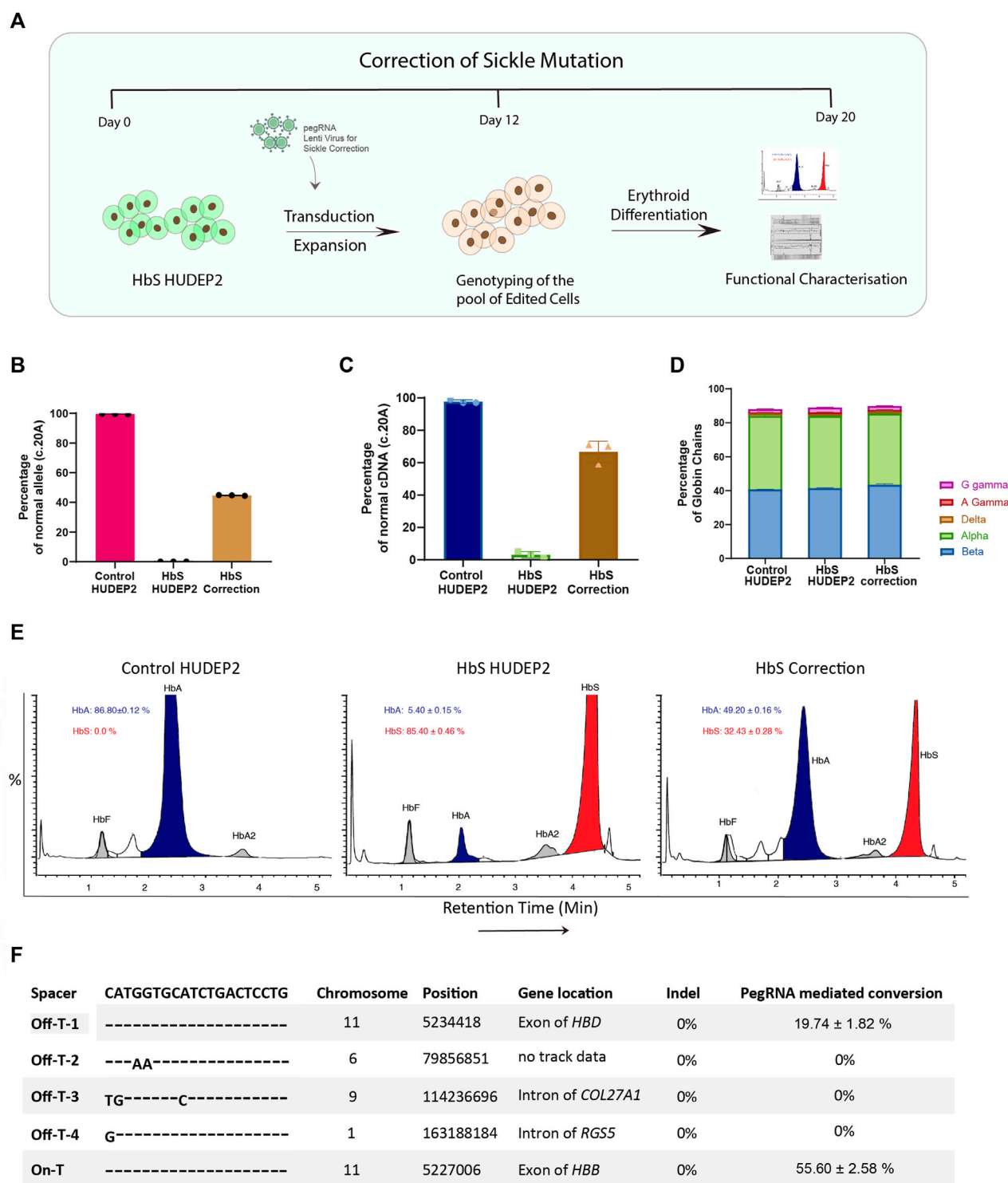


FIGURE 4

Correction of sickle cell mutation in human erythroid cells using prime editor (A) Schematic representation of the workflow for correction of sickle cell mutation in the HbS HUDEP2 cells. (B) Editing efficiency for correction of HbS mutation at Day 12 after transduction evaluated by next-generation sequencing (C) Correction of sickle mutation at the cDNA level evaluated by sanger sequencing (D) RP-HPLC analysis representing the percentage of individual globin chains upon erythroid differentiation after prime editing (E) Haemoglobin variant analysis by HPLC after erythroid differentiation of the edited cells. (F) Analysis of editing at the top 4 *in silico* predicted off-target sites using NGS compared with on-target editing for the installation of HbS mutation in the *HBB* gene. Results from three replicates are plotted as mean ± SD.

Seamless correction of sickle cell mutation and reconstitution of adult haemoglobin using prime editor

Using the created cellular model of sickle cell mutation in human erythroid cells (HbS HUDEP2 Cells), we performed the proof-of-concept evaluation for the correction of HbS mutation and restoration of adult haemoglobin production (Figure 4A). The major limitation of the generated cellular model was the constitutive expression of the initially transduced pegRNA for installation of HbS mutation even after long-term culturing owing to the lenti-viral delivery. To prevent the reconversion of the corrected alleles back to HbS mutation by the initially transduced pegRNA, we selected the pegRNA for the correction of mutation that would create a silent PAM mutation in addition to the desired T > A conversion. The pegRNA extension was 25 bp long with 12 nt Primer Binding Site (PBS) and 13 nt Reverse Transcriptase (RT) template and was similar to the one for installation of HbS mutation in all respects except at the SCA mutation and the installation of silent PAM mutation (GTG>GAA). Further, to assess the transduction efficiency of the subsequently transduced pegRNA for the correction of HbS mutation without interference from the other pegRNA, a lenti-viral vector that co-expresses RFP instead of GFP was used.

HbS HUDEP2 cells were transduced with the pegRNA for the correction of HbS mutation and the transduction efficiency was estimated using RFP expression. We used the unmodified vector as a negative control to account for any variations while performing a second round of transduction in cells with a previously integrated pegRNA. We obtained ~100% transduction efficiency in both the experimental and vector control transduced cells, similar to what has been observed before (Supplementary Figure S4A). This data gives confidence that a second pegRNA can be delivered effectively and would express efficiently even when another pegRNA is being constitutively expressed. The transduced HbS HUDEP2 cells were maintained in culture for 12 days and the editing efficiency was evaluated by next-generation sequencing. We found that the editing efficiency of correction is comparable to the installation of HbS mutation (47%) (Figure 4B). A similar editing efficiency was observed at the cDNA level as well suggesting that the correction of HbS mutation did not affect the transcription of the *HBB* gene (Figure 4C). There was no indel production at the target site even with higher efficiency of on-target conversion (Supplementary Figure S4B). These results highlight the advantage of using the PE2 system for editing in the coding region where the indel generation will lead to disease phenotype. Further analysis with MLPA also showed the reduction of SCD mutant alleles in the edited cells (Supplementary Figure S4C).

To extensively characterise the functional effect of HbS mutation correction by prime editor, the edited cells were

cultured under the erythroid differentiation conditions and analysed for the haemoglobin production. Reverse Phase HPLC analysis showed no marked difference in the production of individual globin chain levels in the edited cells compared to the control suggesting that prime editing at the target locus did not affect the translation efficiency (Figure 4D). We observed on haemoglobin variant analysis that the edited cells produce substantial amounts of wild-type adult haemoglobin (HbA: $49.20 \pm 0.16\%$ in edited cells vs. $5.40 \pm 0.15\%$ in unedited cells), with a concomitant decrease in sickle haemoglobin (HbS: $32.43 \pm 0.28\%$ in edited cells vs. $85.40 \pm 0.46\%$ in unedited cells), which corroborates with our observed on-target editing at the DNA and RNA levels (Figure 4E). There is a slight elevation in the number of HbF + cells in both the HbS mutation corrected as well as mock transduced HbS HUDEP2 cells when compared to the untransduced parental cells (Supplementary Figure S4D). As the number of HbF + levels were comparable between the two pegRNA transduced HbS HUDEP2 cells, the elevation might be due to transduction stress and might not be of importance since the levels of sickle haemoglobin and gamma globin chains detected by HPLC- analysis were not significantly altered. Since the correction is error-free and devoid of any other point mutations, we expect that the adult beta-globin produced would not sickle under hypoxic conditions.

After successfully confirming the use of prime editing system for the correction of HbS mutation and amelioration of SCA phenotype, we went on to characterise the safety profile of the pegRNA. Prime editor is considered safer than conventional gene editing tools as multiple hybridisation steps are required between spacer, PBS and RT template with their respective binding regions in DNA for successful prime editing to occur (Anzalone et al., 2019). Several studies have reported minimal off-target effects for prime editing by whole genome sequencing and targeted amplification (Kim et al., 2020; Jin et al., 2021). Nevertheless, it is important to evaluate the off-target effects for each pegRNA during preclinical validations.

To investigate the possible off-target effects, we used the unsorted edited cells that were transduced with pegRNA for installation of HbS mutation rather than the individual clones or the cell corrected of sickle mutation thereof. We reasoned that the use of unsorted edited cells would provide an unbiased view of off-target effects mediated by the pegRNA. As the spacer sequence is common between the pegRNA that is used for the creation and correction of HbS mutation, we presumed that the off-target effects would also be occurring in a similar frequency. We performed the targeted deep sequencing at the top four off-target sites predicted by the *in silico* tools. We did not observe any indels at any of the off-target sites or the on-target region. Further, we did not observe any pegRNA driven mutations at three out of four off-target sites compared to the control (Figure 4F).

The only off-target site that showed editing was the highly homologous *HBD* region almost 8 kb upstream of the *HBB* gene where the pegRNA had exact sequence homology. As expected, we noticed a considerable amount of editing at the *HBD* region (~19%) but this frequency of conversion was significantly lower than the editing observed at the on-target *HBB* region (Figure 4F). The editing efficiency at the off target site also increased with time as has been observed at the on target site (Supplementary Figure S4E). The off-target editing will not be a concern during the correction of HbS mutation in patient samples as the WT sequence would be present at the *HBD* region. The installation of silent PAM mutation could still occur with similar efficiency at *HBD* off-target site as seen by sanger sequencing of WT cells treated with pegRNA for the correction of HbS mutation (Supplementary Figure S4F).

Previous studies with programmable nucleases have suggested that 20–30% correction of HbS mutation in the patient CD34+ HSPCs would be sufficient for the treatment of SCD. Almost fifty percent of correction achieved by prime editing predicts a higher possibility of edited cells harbouring heterozygous HbS mutation. Based on the allelic frequency obtained from the individual clones during the HbS mutation creation and considering the normal phenotype of individuals with heterozygous HbS mutation, we expect that the level of error-free conversion achieved by the prime editor would be sufficient for the therapeutic correction of SCD mutation in patients.

Discussion

Sickle cell Disease (SCD) being a monogenic disorder and owing to the lack of other efficient curative approaches is an attractive target for gene therapy (Kato et al., 2018). Homology-directed repair and base editing have been used in the past to demonstrate the correction of SCA but both approaches have dire inherent limitations. HDR, mediated by DSB could be deleterious in the correction of SCA if the proportion of indels is high due to the risk of creating beta-thalassaemic mutations (DeWitt et al., 2016). Base editing on the other hand, cannot be used to install T>A transition mutation and has relied on the creation of Hb-G Makassar, a non-pathogenic Hb variant to overcome the SCA mutation (Newby et al., 2021). The functional efficiency of Hb-G Makassar variant has not been evaluated extensively. Prime editing overcomes both these issues by avoiding DSB and the ability to install precise T>A mutation. The proof of concept of correcting SCA using prime editing has been shown by Anzalone et al. in HEK293T cells; however, no studies have demonstrated a functional correction of HbS mutation and evaluated the safety of the use of prime editing in human erythroid cells.

Here, we sought to evaluate prime editing for the correction of HbS mutation in HUDEP2 cells using PE2 system that would

install the mutation without any bystander edits or indels. So far, most of the PE studies have relied on the PE3 system, which requires a gRNA that nicks the opposite strand to achieve better editing efficiency but carries the risk of generating small insertions and deletions at the target site. Undesired edits and indels can interfere with the evaluation of the functional effects of the mutation especially in the coding regions. To improve the PE2 system in an efficient and versatile manner, we first developed a lenti-viral based approach that can edit different target sites across various human cell lines. This approach was utilised for efficiently modelling the HbS mutation in HUDEP2 cells which was further used for demonstrating the utility of prime editing in the correction of HbS mutation. Further, we comprehensively evaluated the reconstitution of functional adult-haemoglobin in the created SCA cellular model and assessed the on- and off-target safety profile of the use of prime editor in the beta-globin locus.

Delivery approaches affect prime editing efficiency to a great extent as the availability of the prime editor and pegRNA for a longer period has been shown to ensure efficient editing at the target site (Eggenchwiler et al., 2021; Wolff et al., 2021). Lenti-viral vectors offer an attractive platform for the delivery of PE as they would integrate into the genome and constitutively express the components. Previous studies have attempted to deliver pegRNA and prime editor as an all-in-one lenti-viral vector which necessitated either the development of a truncated version of PE or the delivery as split-intein based system, owing to the decrease in transduction efficiency with increased size of the vector (Anzalone et al., 2019; Gao et al., 2022). Both approaches however limited the editing efficiency considerably and a single full sized lenti-viral vector had failed to produce any editing. We aimed to overcome this problem by uncoupling PE and pegRNA delivery and developed two different constructs that can separately deliver the components. We have shown that this system is highly efficient compared to other viral and non-viral delivery approaches and is able to achieve even 100% editing at certain target sites. In addition, the dual lenti-viral based system can be used efficiently in cell lines other than HEK293T as well, expanding the targeting scope of prime editing. The potential to prepare the cell lines stably expressing PE which can then be transduced with pegRNAs enable efficient arrayed screening of multiple pegRNAs for initial optimisations. We also show that the sequential delivery of two pegRNAs does not affect the expression of either making it possible to use the lenti-viral system for studies that require dual pegRNA delivery. The developed dual lenti-viral system greatly increases the targeting scope of prime editing for preclinical studies and allows efficient comparison of pegRNAs.

Using the optimised modular lenti-viral based approach, we were able to edit difficult-to-manipulate HUDEP2 cells with editing efficiency reaching up to 75% over time using prime editor 2 system. HUDEP2 cells are important cellular model for preclinical studies involving beta-haemoglobinopathies as they

can easily be differentiated into erythroid lineage and express adult haemoglobin to a quantifiable level. In addition to evaluating prime editing as a tool for the correction of mutations, this approach facilitates the use of prime editing in understanding globin biology and gene switching with its ability to introduce precise mutations with higher efficiency. Higher editing efficiency mediated by lenti-viral PE system also overcomes the need for single-cell sorting for most applications avoiding the biases associated with the clonal selection when evaluating the gene expression. We hope that the lenti-viral system can be used in other cell types as well, relevant to facilitate research in different domains.

Modelling of beta-thalassaemic mutations in HUDEP2 cells has been attempted by another research group but showed very poor efficiency (Zhang et al., 2022). We were able to install the HbS mutation with very high efficiency and obtained a high proportion of homozygous clones with SCA mutation. We also did not observe any small insertions or deletions in the nick site even when the editing efficiency had reached more than 70%. This can be attributed to the use of PE2 system, which does not generate any undesired mutations (Ferreira da Silva et al., 2022). PE2 system would be a better choice in studies involving coding regions or regions where sequence integrity needs to be preserved as the phenotypic outcomes would be an accurate reflection of the installed edit and not influenced by the by-products even to a minimal degree. The model system that we developed produced sickle haemoglobin exclusively without compromising the expression of any other globin chains. The major drawback of the developed model system is the constitutive expression of PE and pegRNA which limit its use for prime editing studies not affected by the expression of the initially transduced pegRNA. Transient delivery of prime editing components could overcome the limitation although with reduced efficiency and would require delivery as mRNA or RNP complex and the screening of a large number of clones to obtain the desired homozygous edited clones.

Correction of SCA mutation was evaluated using the developed model cell line using a pegRNA that installs a silent PAM mutation in addition to correcting the HbS mutation. The installation of the silent PAM mutation would overcome the limitation associated with the expression of the initially transduced pegRNA, which was used for the creation of HbS mutation. We demonstrated high efficiency in the correction of HbS mutation as well as validated the functional production of normal adult haemoglobin concomitant with the editing efficiency. This demonstrates the utility of prime editing for the correction of HbS mutation which could now be evaluated in SCD patient CD34+ HSPCs by optimising the delivery. During the assessment of editing at the off-target sites, although other off-target sites were not edited, we noticed a higher frequency of editing at the delta-globin gene which is highly homologous to the beta-globin. This might not have any consequence

during the correction of the SCD mutation in patient's CD34+ HSPCs as the corresponding sequence in the delta-globin would be intact. The system also provides a platform for efficient screening of pegRNAs for the installation of mutation, the best of which could be taken for further evaluation in the patient CD34+ HSPCs thereby lowering the number of primary cells required for the genetic manipulation as well as cutting down the expenditure. However, before this statement can be generalised, a comparison of prime editing efficiency at the specific locus in different cell lines and CD34+ HSPCs needs to be performed as we have observed a cell type dependent variation in editing efficiency for the same pegRNA even when the level of expression of PE and pegRNA was consistent. The resulting variation might be due to cell intrinsic factors or gene expression profiles that need a thorough evaluation to develop improved versions of PE specific to the cell type in question. In addition, we and others have noticed a consistent increase in prime editing efficiency with constitutive expression over time, hinting at the cell division dependency of prime editing which might be a bottleneck when editing primary cells (Wolff et al., 2021).

Delivery of base editors as mRNA with synthetic gRNA has been shown to edit efficiently in HSPCs. Hanqin et al. compared different delivery modalities of prime editor in Human induced pluripotent stem cells and found that PE mRNA combined with synthetic pegRNA gave the highest editing efficiency (Li et al., 2022). A similar approach would be most suitable for prime editing in HSPCs as well and the recently described modifications in the prime editor (PE max, PE4 and PE5) and pegRNA (epegRNA) could improve the overall efficiency (Chen et al., 2021; Nelson et al., 2022). The delivery of PE as Integrase Deficient Lenti Virus (IDLV) could be another viable approach for editing patient derived CD34+ HSPCs (Ortinski et al., 2017). Prime editing has been shown to generate minimal chromosomal aberrations compared to DSB mediated approaches and hence the edited cells might have better engraftment potential improving the treatment outcome.

To conclude, the current study demonstrates the development of a modular lenti-viral PE delivery system that can edit with high efficiency and precision across multiple cell lines and different genomic loci. This approach was used to precisely model and correct the HbS mutation (*HBB* E6V) without any undesirable conversion in HUDEP2 cells where functional reconstitution of beta-globin and safety of prime editing was characterised extensively for the first time. Overall, our study not only expands the application and scope of prime editing for the difficult-to-transfect cells but also provides a thorough preclinical evaluation of pegRNA for the correction of SCD. The results provide a framework for the preclinical evaluation of prime editing in CD34+ HSPCs for the correction of hematological disorders not just limited to SCD.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/>, PRJNA905550.

Author contributions

AG—Conceptualization, Methodology, Investigation, formal analysis, visualisation, data curation, writing-original draft; NR, KP, LP, SK, VR, ND, JP—methodology, writing-review and editing; —AP, PB—methodology and resources; YN, RK—resources; ST, SM—writing-review and editing; SRV—methodology, resources, writing-review and editing; AS—Project administration, writing-review and editing; KMM—Conceptualization, Supervision, Formal analysis, data curation, Funding acquisition, Project Administration, Resources, Writing-original draft, review and editing. All authors read and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgeed.2022.1085111/full#supplementary-material>

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Editing of homologous globin genes by nickase-deficient base editor mitigates large intergenic deletions in HSPCs

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Recent studies have shown that base editing, even with single-strand breaks, could result in large deletions of the interstitial regions while targeting homologous regions. Several therapeutically relevant genes such as *HBG*, *HBB*, *CCR5*, and *CD33* have homologous sites and are prone for large deletion with base editing. Although the deletion frequency and indels observed are lesser than what is obtained with Cas9, they could still diminish therapeutic efficacy. We sought to evaluate whether these deletions could be overcome while maintaining editing efficiency by using dCas9 fusion of ABE8e in the place of nickaseCas9. Using guide RNAs (gRNAs) targeting the γ -globin promoter and the β -globin exon, we evaluated the editing outcome and frequency of large deletion using nABE8e and dABE8e in human HSPCs. We show that dABE8e can edit efficiently while abolishing the formation of large interstitial deletions. Furthermore, this approach enabled efficient multiplexed base editing on complementary strands without generating insertions and deletions. Removal of nickase activity improves the precision of base editing, thus making it a safer approach for therapeutic genome editing.

INTRODUCTION

Base editing in the γ -globin promoter for the reactivation of fetal hemoglobin (HbF) or in the β -globin gene for direct correction of mutations is a promising approach for the treatment of β -hemoglobinopathies.^{1–5} Unlike other loci, the globin locus is highly homologous with the probability of guide RNA (gRNA) binding simultaneously at two or more sites in the locus. Consequently, while using double-strand break (DSB)-mediated approaches, large deletions involving the intervening regions occur in addition to the intended edits.^{6,7} Although base editing was expected not to cause large deletions due to the absence of DSBs, we and others have observed the occurrence of unintended large deletions in both gamma- and beta-globin genes, possibly because of simultaneous nicking at homologous sites.^{6,8,9} Recent work also showed that even with a 15% large deletion in input cells, upon long-term engraftment, 50% of the mice harbored large deletions of one of the globin genes, which would mean less hemoglobin production per cell.¹⁰ Addi-

tionally, the indirect consequences of large deletions such as chromothripsis, translocations, and p53 activation have not been investigated extensively.^{11,12} Hence, it is important to develop genome editing strategies that generate minimal changes in the genome while achieving therapeutic benefits. Here, we sought to evaluate whether fusing dCas9 (dead Cas9) to ABE8e could overcome deletions generated because of DNA nicks. We show that it not only overcomes large deletions while editing homologous regions but also prevents the formation of insertions or deletions (indels) while base editing in complementary strands.

RESULTS

Frequency of 4.9-kb deletion in the γ -globin locus varies with gRNAs

Base editors were designed to introduce point mutations in the target region while avoiding the DSBs caused by Cas9 nucleases. The initial design of base editors using deaminase fused to a dCas9, however, was less efficient.^{13,14} The use of D10A nCas9 (nickase Cas9) allowed the nicking of non-edited strands, thereby facilitating the effective installation of edits that resulted in significantly higher editing efficiency. Base editors were subsequently evolved to improve the activity, and the recently described hyperactive variant ABE8e was shown to edit, with conversion reaching $\sim 100\%$ in many target sites.¹⁵ However, the use of nCas9 base editors in highly homologous regions resulted in the deletion of intervening regions.^{8,9} We hypothesized that with its high processivity, ABE8e would be able to install mutations even when fused to catalytically dCas9 and thus can be used for base editing in homologous regions without risking deletion of the intervening region.

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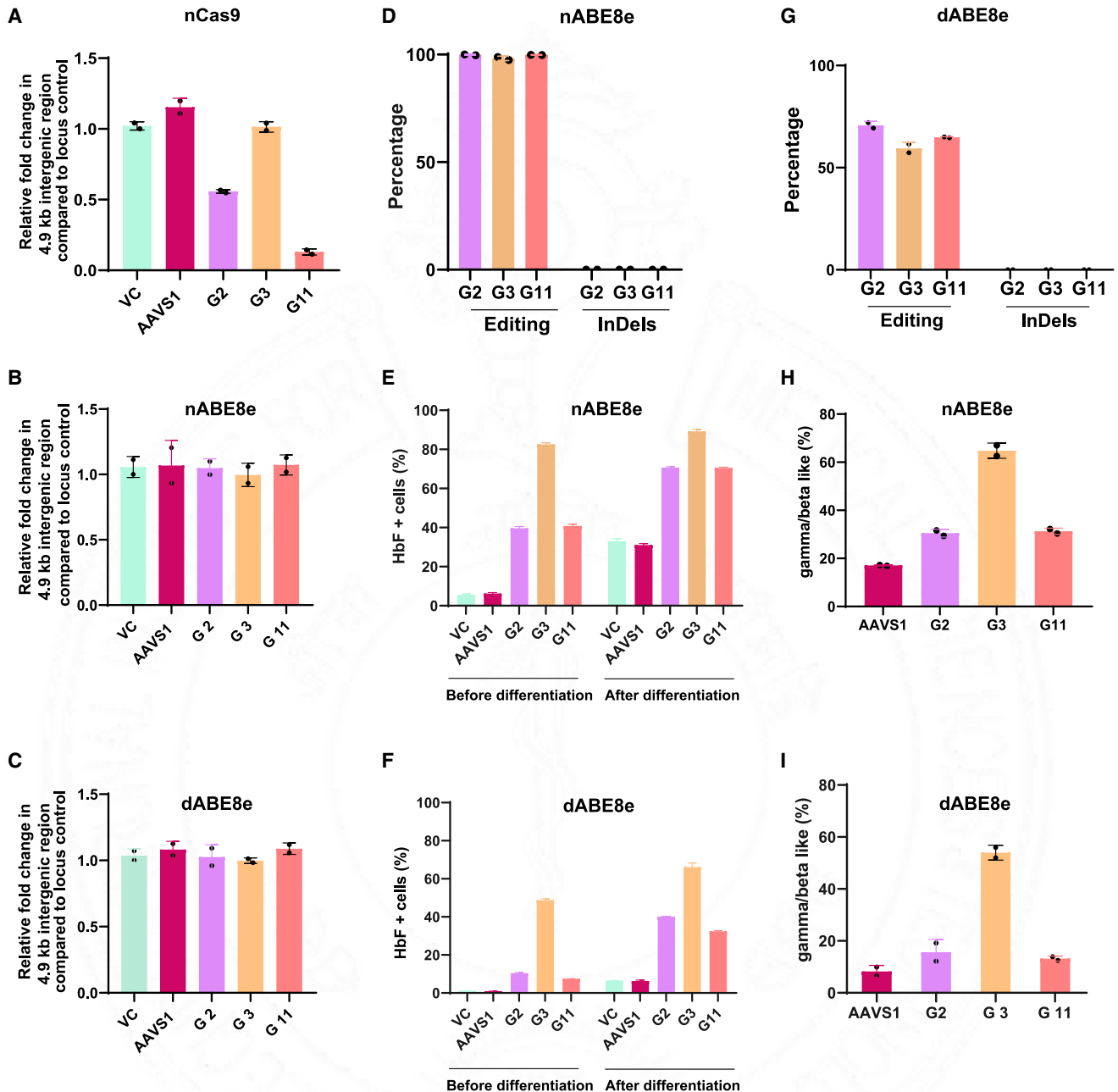
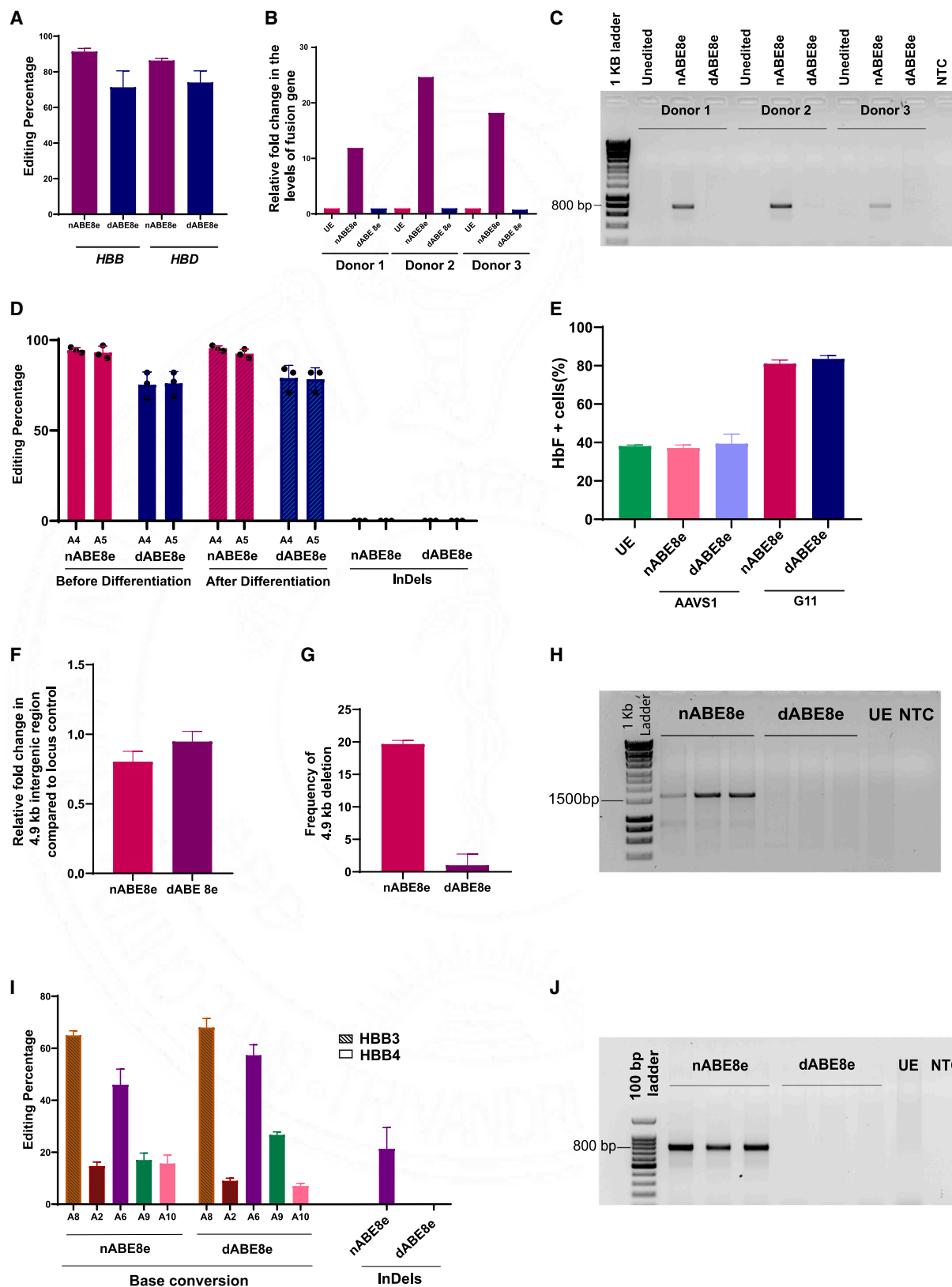


Figure 1. DNA nickases can introduce large interstitial deletions in homologous regions

(A–C) Quantification of changes in the 4.9-kb intergenic region upon targeting the γ -globin promoter by nCas9 (A), nABE8e (B), and dABE8e (C) in HUDEP 2 cells (by lentiviral delivery), measured as relative fold change compared to locus control by quantitative real-time PCR. (D and G) Editing efficiency and indels generated by nABE8e (D) and dABE8e (G) using gRNAs targeting γ -globin promoter evaluated by next-generation sequencing. (E and F) Percentage of HbF⁺ cells evaluated by intracellular staining, followed by flow cytometry upon base editing with nABE8e (E) and dABE8e (F) measured before and after erythroid differentiation. (H and I) Measurement of globin chains after base editing in the γ -globin promoter using RP-HPLC in nABE8e (H) and dABE8e (I). All experiments were performed as biological duplicates. Data represented as mean $\pm$ SD. VC, vector control.

Using previously validated gRNAs⁸ targeting the γ -globin locus (G2, G3, G11) with the potential for therapeutic applications, we sought to evaluate whether the frequency of 4.9-kb deletion varies between the

gRNAs, irrespective of the base editor used (Figure S1). These gRNAs were delivered as lentivirus to D10A nickase HUDEP-2 stables, and the genomic alterations were evaluated. There was no appreciable



(legend on next page)

base conversion or indels except in G11, which showed a very small percentage of indels (Figures S2A and S2D). However, by quantitative real-time PCR we detected large deletions occurring with the use of G2 and G11 but not with G3 (Figure 1A). We believe that gRNA efficiency might be a driving factor in determining the deletion frequency (Figures S2E and S2F; Note S1). While this level of deletion might not be reflected during base editing, it shows the potential for large deletion when the base editing components (gRNA and Base Editor mRNA) are available in excess as in therapeutic *ex vivo* editing of hematopoietic stem and progenitor cells (HSPCs).

Nickase-deficient ABE8e can edit efficiently in human erythroid cells

We introduced H840A mutation in nABE8e and tested whether it can edit efficiently to achieve therapeutic benefits. We tested this in HUDEP-2 cells stably expressing nABE8e or dABE8e using the same gRNAs. As expected, all three gRNAs resulted in >90% editing with nABE8e, while the efficiency was ~60%–70% with dABE8e (Figures 1D, 1G, S2B, and S2C). No indels were detected with either construct, and there was no alteration in the editing window (Figure S2G). We did not observe any significant 4.9-kb deletion in any of the samples by quantitative real-time PCR (Figures 1B and 1C). This suggests that even with nickase activity in nABE8e, rapid kinetics of deaminase resulting in accelerated editing in HUDEP-2 cells likely reduced the deletion frequency, which was not picked up in the quantitative real-time PCR. Corresponding to the editing efficiency, we also observed a drop in HbF levels while editing with dABE8e, but the levels would be sufficient for therapeutic applications in β -hemoglobinopathies (Figures 1E, 1F, 1H, and 1I).

dABE8e can efficiently edit in human CD34⁺ HSPCs and prevent the formation of interstitial deletions

As nicking by itself can generate large deletions in homologous regions, we evaluated whether the formation of large deletions during base editing in HSPCs can be overcome using dABE8e. We first tested base editing efficiency and the resulting large deletions in CD34⁺ HSPCs in the therapeutically relevant *HBB* gene using gRNAs targeting exon-1 with (HBB1) and without (HBB2) homology to *HBD* gene (Figure S3A). Cells were nucleofected with nABE8e/dABE8e mRNA and the respective single-guide RNA, and as expected, the editing efficiency was slightly reduced while targeting with dABE8e (Figure 2A).

Base conversion in *HBD* and resulting large deletion with nABE8e was observed only in HBB1 gRNA, which had binding sites at both genes and not with HBB2, which had binding sites only in *HBB* (Figure S3B). However, with dABE8e, large deletion was absent even when very high levels of editing were observed at both genes using HBB1 (Figure 2C). Quantitative real-time PCR with primers specific to large deletion between *HBD* and *HBB* showed that nABE8e had a 10-fold increase in amplicons with large deletion compared to unedited control and dABE8e edited samples (Figure 2B).

We further compared both of the editors in the *HBG* promoter. G11 was used along with AAVS1 targeting gRNA as the negative control. While the editing efficiency reached >90% in nABE8e, it was slightly lower in dABE8e (Figures 2D and S3C). As expected, we observed the 4.9-kb deletion with nABE8e but not in dABE8e (Figures 2F–2H). The edited cells upon differentiation showed similar elevation in F⁺ cells reaching close to 80% in both nABE8e and dABE8e, suggesting that editing by dABE8e would be sufficient for therapeutically relevant HbF elevation (Figure 2E). We also tested two other gRNAs in the *HBG* promoter that were shown to elevate HbF to therapeutic levels and found that while editing efficiency with dABE8e was slightly lower than what was observed with nABE8e, it abolished the creation of the 4.9-kb deletion (Figures S3D–S3G).

Additionally, we tested the utility of dABE8e in preventing indel formation during multiplexed editing in complementary strands using two sgRNAs targeting the *HBB* gene (HBB3 and HBB4) (Figure S4A). While nABE8e resulted in indel formation due to nicking on opposite strands, dABE8e resulted in pure base conversion without any indels/large deletions (Figures 2I, 2J, and S4B). These data suggest that simultaneous nicking in the homologous site during base editing with nABE8e is responsible for large deletions and can be overcome using dABE8e.

Characterization of dABE8e activity in CD34⁺ HSPCs

As dABE8e showed slightly reduced editing compared to nABE8e at all the sites, we evaluated whether this was due to the limited editing efficiency of dABE8e in the more primitive quiescent cells. The editing efficiency of nABE8e and dABE8e was compared in CD90⁺ primitive HSPCs with that of bulk edited cells, and no significant difference was observed (Figures 3A and S5). We also tested whether increasing

Figure 2. dABE8e can edit efficiently in HSPCs without generating large deletions

(A) Base editing efficiency of HBB1 gRNA in *HBB* and *HBD* genes using ABE8e and dABE8e (by mRNA delivery) measured by Sanger sequencing ($n = 3$ individual donors). (B) Relative increase in HBD/HBB fusion gene generated by large deletions upon editing with nABE8e and dABE8e compared to unedited control measured by quantitative real-time PCR (performed in three individual donors). (C) Formation of fusion gene (HBD-HBB) due to large deletion upon base editing detected by agarose gel electrophoresis after PCR. A band at ~800 bp indicates large deletion. (D) Editing efficiency of nABE8e and dABE8e using G11 in γ -globin promoter measured by Sanger sequencing before and after erythroid differentiation. (E) Percentage of HbF⁺ cells evaluated by flow cytometry after intracellular staining of samples edited with G11 using nABE8e and dABE8e after erythroid differentiation. (F) Reduction in 4.9-kb intergenic region caused by interstitial deletions while targeting the γ -globin promoter using G11 measured as relative fold change compared to locus control by quantitative real-time PCR and normalized using unedited control. (G) Frequency of 4.9-kb deletion created by base editors while targeting the γ -globin promoter by G11 measured by probe-based droplet digital (ddPCR) and compared to unedited control. (H) Formation of fusion gene (HBG2-HBG1) due to large deletion on base editing detected by agarose gel electrophoresis following PCR. A band at ~1,700 bp indicates large deletion. (I) Editing efficiency and indel formation upon multiplex editing with two sgRNAs targeting the complementary strands in β -globin exon 1 (HBB3/HBB4) measured by Sanger sequencing. (J) Formation of fusion gene (HBD-HBB) due to large deletion after multiplexed base editing detected by agarose gel electrophoresis after PCR. A band at ~800 bp indicates large deletion. ($n = 3$ for all experiments. Data represented as mean $\pm$ SD. NTC, non-templated control; UE, unedited control).

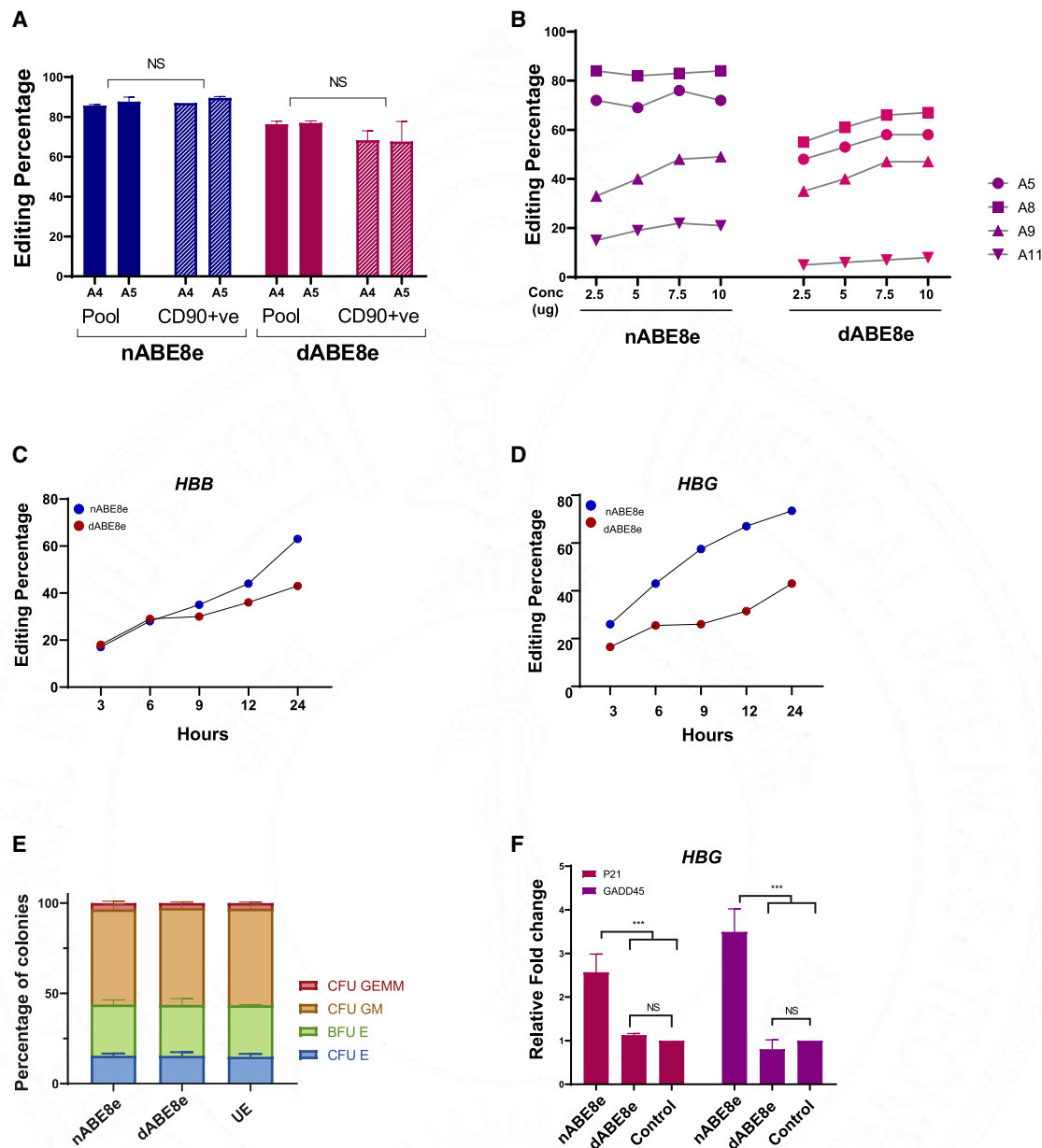


Figure 3. Characterization of nABE8e and dABE8e activity in HSPCs

(A) Base editing efficiency of nABE8e and dABE8e (by mRNA delivery) in a pool of HSPCs and in a primitive (CD90⁺) population, with G11 targeting γ -globin promoter measured by Sanger sequencing ($n = 3$). Student t test was used for comparison between the groups. (B) Comparison of base editing efficiency with escalating doses of base editors in HSPCs using G2 targeting γ -globin promoter after 48 h of electroporation measured by Sanger sequencing ($n = 1$). (C and D) Evaluation of base editing efficiency over time after electroporation in HSPCs using HBB1 (C) and HBG G11 (D) gRNAs measured by Sanger sequencing ($n = 1$). (E) Percentage of colonies formed from base edited HSPCs compared to unedited (mock electroporated) control after 14 days of seeding in MethoCult medium ($n = 3$). (F) Relative fold change in mRNA expression of P21 and GADD45 genes after 48 h of electroporation (mRNA) by HBG G4 measured by quantitative real-time PCR. Data are normalized to mock electroporated control (G4 gRNA, $n = 3$). (ANOVA with multiple comparisons was used for evaluating statistical significance.) Data represented as mean $\pm$ SD. *** $p < 0.001$. NS, nonsignificant; UE, unedited control.

the base editor cargo would improve the editing efficiency in target sites that showed moderate editing efficiency, but we did not observe any appreciable improvement in editing, even with a 4-fold increase in the cargo, suggesting that reagent availability is not the limiting fac-

tor for editing at these sites (Figures 3B and S3H). It was also noted that both nABE8e and dABE8e started editing within 3 h of nucleofection, but the editing kinetics is much faster in nABE8e (Figures 3C and 3D). The edited cells were also subjected to clonogenic assay, and

we observed no difference in colony-forming potential between the samples (Figure 3E). Finally, we tested the levels of *P21* and *GADD45*, both markers of DNA damage response and observed that while nABE8e showed a slight elevation in RNA levels, dABE8e and the mock electroporated sample showed similar levels (Figure 3F). Thus, dABE8e would be a better approach, in terms of purity of outcomes, while editing regions that are highly homologous and during multiplexed editing for therapeutic applications.

DISCUSSION

As base editors are entering into clinical trials for the treatment of various disorders, there is an increasing interest in improving the precision of genome editing while minimizing undesired genomic alterations.^{16,17} With precision genome engineering, there is often a very limited number of available gRNAs to generate the desired mutation, and hence the base editors must be precise, with minimal undesired outcomes.^{3,18–20} It has been reported that base editors generate undesired genotoxic effects due to DNA nicking¹¹ and that single-strand breaks can have detrimental effects on cell fitness.²¹ In this study, we show that large deletions are generated in homologous regions during base editing in a gRNA-dependent manner. These deletions can be overcome using nickase-deficient dABE8e that can edit efficiently even in CD34⁺ HSPCs. The editing occurs even in CD90⁺ HSPCs, and the edited cells show no lineage bias, suggesting the potential for long-term engraftment and repopulation. The editing efficiency by dABE8e is, however, lower than nABE8e by 20%–30% in all target sites tested. Hence, in non-homologous regions or sites that do not cause the formation of indels, nABE8e can be used because it would provide better editing efficiency. While here we demonstrate only the use of dABE8e, it is similarly possible to develop nickase-deficient cytosine base editors (CBEs) using hyperactive variants of CBEs.²² A recent study has shown the proof of concept for overcoming indels using nickase-deficient CBEs.²³ In addition to targeting the homologous regions, dABE8e can be used for multiplexed editing in a single locus or simultaneous editing in complementary strands without risking the generation of large deletions or indels.

There are, however, certain limitations to our study. First, the approach was tested in two sites in the globin locus (*HBB* and *HBB* genes) to evaluate the abolishment of large deletions. There are other homologous loci such as *CCR5* and *CD33* that can be tested for the same. Additionally, cell-cycle dependence of dABE8e-mediated editing was not directly tested, and only an engraftment study would show the long-term repopulation potential of the cells edited with dABE8e. Considering the *in vitro* data, we believe that nABE8e is still a better option in non-homologous sites as the editing efficiency is evidently higher compared to dABE8e. However, dABE8e would be a suitable approach in terms of purity of outcomes while base editing in situations where two or more nicks are introduced in the same locus as in homologous regions or during multiplexed editing.

MATERIALS AND METHODS

Details of materials and methods used in the study can be found in the [supplemental information](#).

DATA AND CODE AVAILABILITY

All data generated from this study have been made available in this paper.

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AUTHOR CONTRIBUTIONS

A.G.: conceptualization, methodology, investigation, formal analysis, visualization, data curation, and writing – original draft. P.S., N.S.R., and B.V.: methodology. S.M., S.T., and S.R.V.: resources. A.S.: resources and project administration. M.K.M.M.: conceptualization, supervision, formal analysis, funding acquisition, project administration, resources, and writing – review & editing. All authors read and approved the submitted version of the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omtn.2024.102347>.

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CERTIFICATE

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