

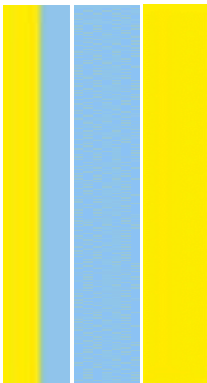
DOUTORAMENTO

BIOLOGIA BASICA E APLICADA (GABBA)

Gene Therapy: Finding Innovative Approaches to Treat Hemoglobinopathies

Sílvia Pires Lourenço

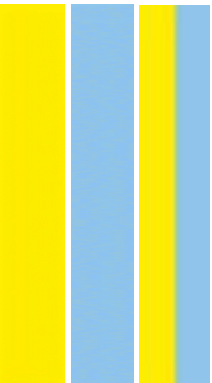
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2024



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2024



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GENE THERAPY: FINDING INNOVATIVE APPROACHES TO TREAT HEMOGLOBINOPATHIES

Tese de Candidatura ao grau de Doutor em
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ABSTRACT

Hemoglobinopathies are inherited monogenic recessive disorders caused by mutations in the globin genes. Sickle Cell Disease (SCD) and β -thalassemia are triggered by mutations in the β -globin locus, leading to lifelong clinical management without a definitive cure. Gene therapies offer the possibility of a one-time treatment that can be available to every patient. Here, we report the establishment of engineered cell lines that undergo erythroid differentiation and possess the gene and protein expression profiles required to be used as a platform to screen gene therapy-based drugs for β -globinopathies. Additionally, we used one of these cell lines (M#9) as well as CD34⁺ cells isolated from peripheral blood of SCD and β -thalassemia patients to evaluate novel lentiviral vectors. Our approach combined gene addition of functional copies of the β -globin gene with gene silencing of BCL11A, a repressor of the γ -globin gene, into a single lentiviral vector. We hypothesized that treatment with the dual vector would lead to a triple-fold effect of 1) production of transgenic β -globin, 2) reactivation of γ -globin, a β -like globin chain, and 3) decrease the production of endogenous mutant β -globin. The production of β -globin would lead to an increase in adult hemoglobin (HbA^{T87Q}), the reactivation of γ -globin would result in the induction of fetal hemoglobin (HbF), and the decrease of endogenous β -globin would be most evident in SCD samples with the decrease in hemoglobin S (HbS). We cloned a shRNA^{miR} targeting BCL11A (miR-E-BCL11A) in non-coding regions of the β -globin gene of the ALS10-T87Q lentiviral vector and screened for the cloning position that induced the highest levels of HbA^{T87Q}+HbF. The ATM1 dual lentiviral vector was generated by cloning the miR-E-BCL11A into intron 1 of the ALS10-T87Q vector and showed the most promising results but the levels of HbA^{T87Q}+HbF did not surpass those of ALS10-T87Q. We then modified the secondary structure of miR-E-BCL11A and evaluated four additional vectors. Of these, ATM1.1 and ATM1.4 outperformed ALS10-T87Q and yielded higher levels of HbA^{T87Q}+HbF than the HbA^{T87Q} produced by ALS10-T87Q, per copy of the integrated vectors. As ATM1.1 showed the highest potential, we transduced CD34⁺ hematopoietic progenitor cells isolated from the peripheral blood of SCD and β -thalassemia patients and confirmed that ATM1.1 surpassed ALS10-T87Q. Additionally, SCD and β -thalassemia cells treated with ATM1.1 were less prone to sickle and showed the lowest levels of α -globin aggregates than control or cells treated with ALS10-T87Q, respectively. In summary, we demonstrated that the cell lines and the double vectors generated during this PhD thesis are promising models and therapeutic alternatives that can further the research of gene therapies for hemoglobinopathies.

RESUMO

As hemoglobinopatias constituem um grupo de doenças hereditárias monogênicas recessivas causadas por mutações nos genes das globinas. A Drepanocitose (Doença Falciforme, DF) e a β -talassemia são devidas a mutações no locus da β -globina, e requerem uma gestão clínica ao longo da vida sem uma cura definitiva. As terapias genéticas oferecem a possibilidade de um tratamento único que pode ser disponibilizado a todos os doentes. Aqui, reportamos o estabelecimento de linhas celulares modificadas que sofrem diferenciação eritroide e possuem os perfis de expressão de genes e proteínas necessários para serem utilizadas como uma plataforma para rastrear medicamentos baseados em terapia genética para β -globinopatias. Além disso, utilizámos uma destas linhas celulares (M#9), bem como células CD34⁺ isoladas do sangue periférico de doentes com DF e β -talassemia, para avaliar novos vetores lentivirais. A nossa abordagem combinou a adição de cópias funcionais do gene da β -globina com o silenciamento do gene BCL11A, um repressor do gene da γ -globina, num único vetor lentiviral. A hipótese subjacente a esta abordagem foi que o tratamento com o vetor duplo conduziria a um efeito triplo de 1) produção de β -globina transgénica, 2) reativação da γ -globina, uma globina semelhante à β -globina, e 3) diminuição da produção de β -globina mutante endógena. A produção de β -globina levaria a um aumento da hemoglobina adulta (HbA^{T87Q}), a reativação da γ -globina resultaria na indução de hemoglobina fetal (HbF) e a diminuição da β -globina endógena seria mais evidente em células de DF com a diminuição da hemoglobina S (HbS). Clonámos um shRNAmiR, que tem como alvo o BCL11A (miR-E-BCL11A), em regiões não codificantes do gene da β -globina do vetor lentiviral ALS10-T87Q e procurámos a posição de clonagem que induziu os níveis mais elevados de HbA^{T87Q}+HbF. O vetor lentiviral duplo ATM1 foi gerado através da clonagem do miR-E-BCL11A no intrão 1 do vetor ALS10-T87Q e apresentou os resultados mais promissores, mas os níveis de HbA^{T87Q}+HbF não ultrapassaram os do ALS10-T87Q. Em seguida, modificámos a estrutura secundária do miR-E-BCL11A e avaliámos quatro vetores adicionais. Destes, o ATM1.1 e o ATM1.4 superaram o ALS10-T87Q e produziram níveis mais elevados de HbA^{T87Q}+HbF do que a HbA^{T87Q} produzida pelo ALS10-T87Q, por cópia dos vetores integrados. Como o ATM1.1 mostrou o potencial mais elevado, transduzimos células progenitoras hematopoiéticas CD34⁺ isoladas do sangue periférico de doentes com DF e β -talassemia e confirmámos que o ATM1.1 superou o ALS10-T87Q. Além disso, as células de doentes com DF ou β -talassemia tratadas com ATM1.1 foram menos propensas à falciformação, e com níveis mais baixos de agregados de α -globina do que o controlo ou as células tratadas com ALS10-T87Q, respectivamente. Em resumo, demonstrámos que

as linhas celulares e os vetores duplos gerados no âmbito desta tese de doutoramento são modelos promissores e alternativas terapêuticas com a capacidade de avançar o desenvolvimento de novas terapias genéticas para hemoglobinopatias.

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LIST OF ABBREVIATIONS

ABE	adenine base editors
ADC	antibody-drug conjugate
AML	acute myeloid leukemia
APC	allophycocyanin
BEL-A BTM	IVS1-5 (G.C) β -thalassemia mutation
BEL-A SCM	BEL-A immortalized erythroid progenitor cell line with the sickle cell mutation
BCL11A	B cell Leukemia/Lymphoma 11A
BM	bone marrow
bp	base pair
b-thal	β -thalassemia
BV421	brilliant violet 421
BV711	brilliant violet 711
CBE	cytidine base editors
CD71	transferrin receptor
CGT	cell and gene therapy
c-KIT	SCF receptor
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	sequence-specific CRISPR RNA
DEXA	dexamethasone
ddPCR	digital droplet polymerase chain reaction
DMSO	dimethyl sulfoxide
Doxy	doxycycline
DSB	double strand break
EMA	European Medicines Agency
EMCV	encephalomyocarditis virus
EPO	erythropoietin
FBS	fetal bovine serum
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
FOG1	friend of GATA1
GATA1	GATA-binding factor 1
G-CSF	granulocyte-colony stimulating factor
GPA	glycophorin A
Hb	hemoglobin

HbA	adult hemoglobin
<i>HBA1 and HBA 2</i>	α -globin genes
HBA	α -globin chains
<i>HBB</i>	β -globin gene
HBB	β -globin chains
<i>HBG1 and HBG2</i>	γ -globin genes
HBG	γ -globin chains
HbC	type C hemoglobin
HbF	fetal hemoglobin
HbS	sickle hemoglobin
HCV	hepatitis C virus
HDR	homology directed repair
hKO1	Kusabira-orange 1 ⁺
HPFH	hereditary persistence of fetal hemoglobin
HPLC	high performance liquid chromatography
HS	hypersensitive
hSCF	human stem cell factor
HSCT	hematopoietic stem cell transplantation
HSPC	hematopoietic stem and progenitor cell
HUDEP-2	Human Umbilical cord Derived Erythroid Progenitor –2
ICT	iron chelation therapy
IE	ineffective erythropoiesis
IMDM	Iscoe's modified Dulbecco's medium
IRES	internal ribosome entry sequence
IU	international units
LCR	locus control regions
MEL	murine erythroleukemia cells
MoAbs	monoclonal antibodies
MOI	multiplicity of infection
NHEJ	non-homologous end joining
NHP	non-human primate
NTDT	non-transfusion-dependent thalassemia
NuRD	nucleosome remodeling and deacylase
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	prime editor

pegRNA	prime editing guide RNA
P/S	penicillin/streptomycin
PTC	premature termination codon
PVDF	polyvinylidene fluoride
qPCR	quantitative polymerase chain reaction
RBC	red blood cell
RCE	red cell exchange
RIC	reduced-intensity conditioning
ROS	reactive oxygen species
RT-qPCR	real-time quantitative PCR
SAE	serious adverse effect
SCA	sickle cell anemia
SCD	sickle cell disease
SFEM	StemSpan serum-free expansion media
shRNA^{miR}	short hairpin RNA
SNP	single-nucleotide polymorphism
TALE	transcription activator-like effector
TDT	transfusion-dependent thalassemia
TF	transcription factor
tracrRNA	trans-activating crRNA
USA	United States of America
WB	Western Blot
WPRE	woodchuck post-regulatory element
WT	wild type
VOC	vaso-occlusive crises
VCN	vector copy number
ZFN	zinc finger nuclease

PUBLICATIONS

During the development of this thesis, two works were published in peer-reviewed journals.

1. Breda, L., Motta, I., **Lourenco, S.**, Gemmo, C., Deng, W., Rupon, J.W., Abdulmalik, O.Y., Manwani, D., Blobel, G.A., & Rivella, S., 2016, Forced chromatin looping raises fetal hemoglobin in adult sickle cells to higher levels than pharmacologic inducers, *Blood*, 128(8), 1139-1143.
2. **Pires Lourenco, S***, Jarocha, D*, Ghiaccio, V., Guerra, A., Abdulmalik, O., La, P., Zezulín, A., Smith-Whitley, K., Kwiatkowski, J.L., Guzikowski, V., Nakamura, Y., Raabe, T., Breda, L., & Rivella, S., 2021, Inclusion of a short hairpin RNA targeting *BCL11A* into a β -globin expressing vector allows concurrent synthesis of curative adult and fetal hemoglobin, *Haematologica*, 106(10), 2740-2745.

*SPL and DJ contributed equally as co-first authors.

1. GENERAL INTRODUCTION

1.1. Hemoglobin and the Globin gene loci

Hemoglobin (Hb), a tetrameric protein responsible for oxygen transportation from the lungs to all tissues, is composed of different combinations of β -like and α -like globin subunits, each carrying a heme prosthetic group containing an iron molecule [1, 2]. The expression of these subunits is tightly regulated to ensure balanced synthesis. The β -globin gene cluster includes the β -like globin genes: ϵ (*HBE*), $G\gamma$ (*HBG2*), $A\gamma$ (*HBG1*), δ (*HBD*), and β (*HBB*), located on the short arm of chromosome 11 (11p 15.15) and arranged in a 5' to 3' direction in order of their developmental expression (Figure 1) [3]. The α -globin cluster includes ζ (*HBZ*) and the duplicated *HBA2* and *HBA1* genes at the end of chromosome 16 (16p 13.3), also arranged in a 5' to 3' direction in order of their developmental expression (Figure 1). The tetrameric combinations generate various types of Hb expressed at distinct growth phases via hemoglobin switching from embryonic to fetal and to adult [4]. Embryonic Hbs are expressed while erythropoiesis occurs in the yolk sac and include Hb Gower-1 ($\zeta_2\epsilon_2$), Hb Gower-2 ($\alpha_2\epsilon_2$), and Hb Portland ($\zeta_2\gamma_2$), followed by erythropoiesis in the fetal liver and the transition to fetal hemoglobin (HbF, $\alpha_2\gamma_2$) (Figure 1). At birth, a second switch in hemoglobin production leads to the decline of HbF paired with the increase of adult hemoglobins (HbA, $\alpha_2\beta_2$ and HbA₂, $\alpha_2\delta_2$), HbA accounting for >90% of Hb expressed in bone marrow and immature (reticulocytes) and mature (erythrocytes) red blood cells (RBCs) (Figure 1) [1, 3, 4, 5, 6, 7].

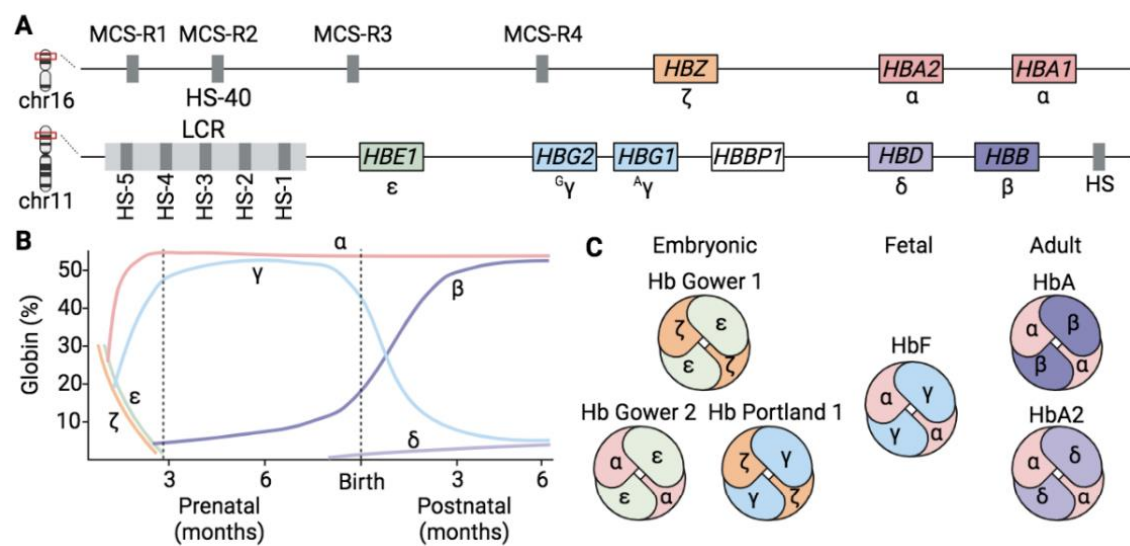


Figure 1 - Human α - and β -globin loci and hemoglobin switching.

(A) Schematic representation of the α - and β -globin loci on chromosomes 16 and 11, respectively. α -like (HBZ , $HBA2$ and $HBA1$) and β -like ($HBE1$, $HBG2$, $HBG1$, HBD and HBB) globin genes are depicted with colored boxes in 5' to 3' orientation. The globin encoded by each gene is reported below each box. $HBBP1$ pseudogene is depicted with a white box. The HSS are presented with grey boxes. (B) Diagrammatic representation of the human hemoglobin embryonic-to-fetal and fetal-to-adult switching, describing the expression pattern of α -like (α and ζ) and β -like (ϵ , γ , β and δ) globin chains, from conception to birth, and to adult life. (C) Composition of hemoglobin for different developmental stages (embryonic, fetal and adult Hb). Chromosome (Chr); multispecies conserved sequences (MCS); locus control region (LCR); hypersensitive site (HS); hemoglobin (Hb); fetal hemoglobin (HbF); adult hemoglobin (HbA); adult hemoglobin 2 (HbA2) [7].

The identification of specific promoters at the 5' end of each gene established that the expression of the genes is driven individually. The developmental stage-specific expression is allowed by distal regulation through the locus control regions (LCR) for α - and β -globin loci that loop with globin promoters via their DNase I hypersensitive sites (HS) that act as enhancers or insulators [7]. For the β -globin locus, the 5' hypersensitive region ranges from 6 kb to 40 kb upstream of the ϵ -globin gene (Figure 1) and has been further divided into five unique sites: HS1, HS2, HS3, HS4, and HS5 (Figure 1). At a proximal level, gene expression regulation is accomplished by numerous transcription factors (TFs) and co-factors that bind to the β -like globin gene promoters and allow their expression (Figure 2).

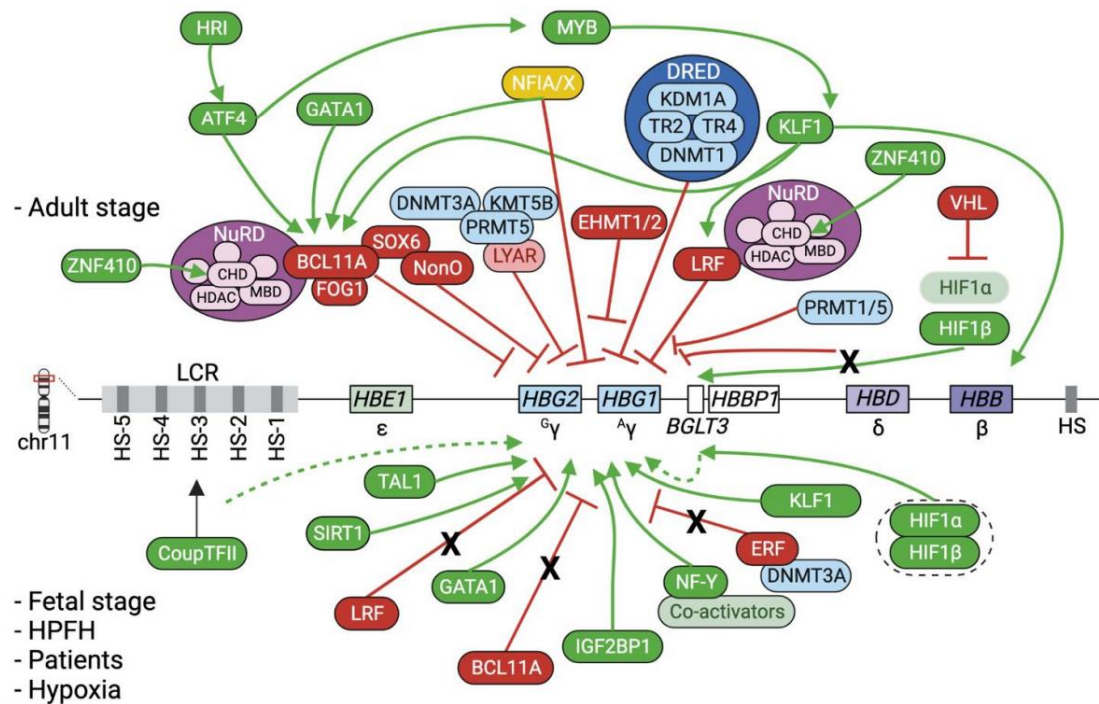


Figure 2 - Regulation of β -like globin gene expression.

Schematic model of β -like globin gene regulation in the adult (upper panel) and fetal stages, in individuals with hereditary persistence of fetal hemoglobin (HPFH) mutations, hemoglobinopathy patients, or low O_2 conditions (lower panel). Numerous transcription factors have been identified to regulate γ -globin expression in fetal/adult stages or HPFH conditions, either by directly silencing (NFIA/X [15], ERF [16]) or activating (Coup-TFII [17], IGF2BP1 [18], HIF1 α / β [19], NF-Y [20,21], GATA1 [20] and KLF1 [22]) γ -globin genes, or by co-acting with or indirectly regulating the expression of the two major γ -globin repressors BCL11A (KLF1 [23], NFIA/X [15], ATF4 [24,25], SOX6 [26]) and LRF (KLF1 [27]) or other key-players such as KLF1 (MYB [28]), ATF4 (HRI [24,25]), MYB (ATF4 [29]) and CHD4 (ZNF410 [30,31]). Ovals represent proteins with repressive (red), activating (green), repressive/activating (yellow) activity, or epigenetic modifying activity (blue and purple). Dotted green lines show indirect activation, and dotted black ovals indicate protein heterodimers [7].

1.1.1. The HbF to HbA switch and BCL11A

HbF expression is reduced to 1% in adult cells, except in cases of hereditary persistence of fetal hemoglobin (HPFH), a benign condition caused by mutations in the promoter of the γ -globin genes or the β -globin gene cluster [7]. The silencing of γ -globin genes and activation of the β -globin gene leads to the fetal-to-adult Hb switch through LCR-mediated looping formation and specific binding of TFs. The looping is mediated by a protein complex, the nucleosome remodeling and deacylase, (NuRD), FOG1, GATA1, and B Cell Leukemia/Lymphoma 11A (BCL11A) and facilitates the juxtaposition of the LCR to the γ - or β -globin gene promoters (Figure 3A). The identification of BCL11A as one of the major transcriptional repressors of the γ -globin gene was facilitated by genome-wide association studies which linked single-nucleotide polymorphisms (SNPs) with abnormally elevated HbF levels. BCL11A suppresses gene expression by binding to the γ -globin promoters and interacting and recruiting the other factors/complexes that act as co-repressors. Knockdown of BCL11A leads to an increase in γ -globin mRNA and HbF levels in human erythroid cells (Figure 3B). BCL11A contains several classical C2H2-type zinc finger domains and is not only expressed in erythroid cells, bone marrow (BM), HSCs, and B-cells but also in the brain and liver. Through alternative mRNA splicing, erythroid cells express stage-specific isoforms with adult cells expressing larger forms (XL and L), while fetal cells express shorter forms (S and XS). Only the longest isoform, BCL11A-XL, is implicated in fetal globin silencing [7].

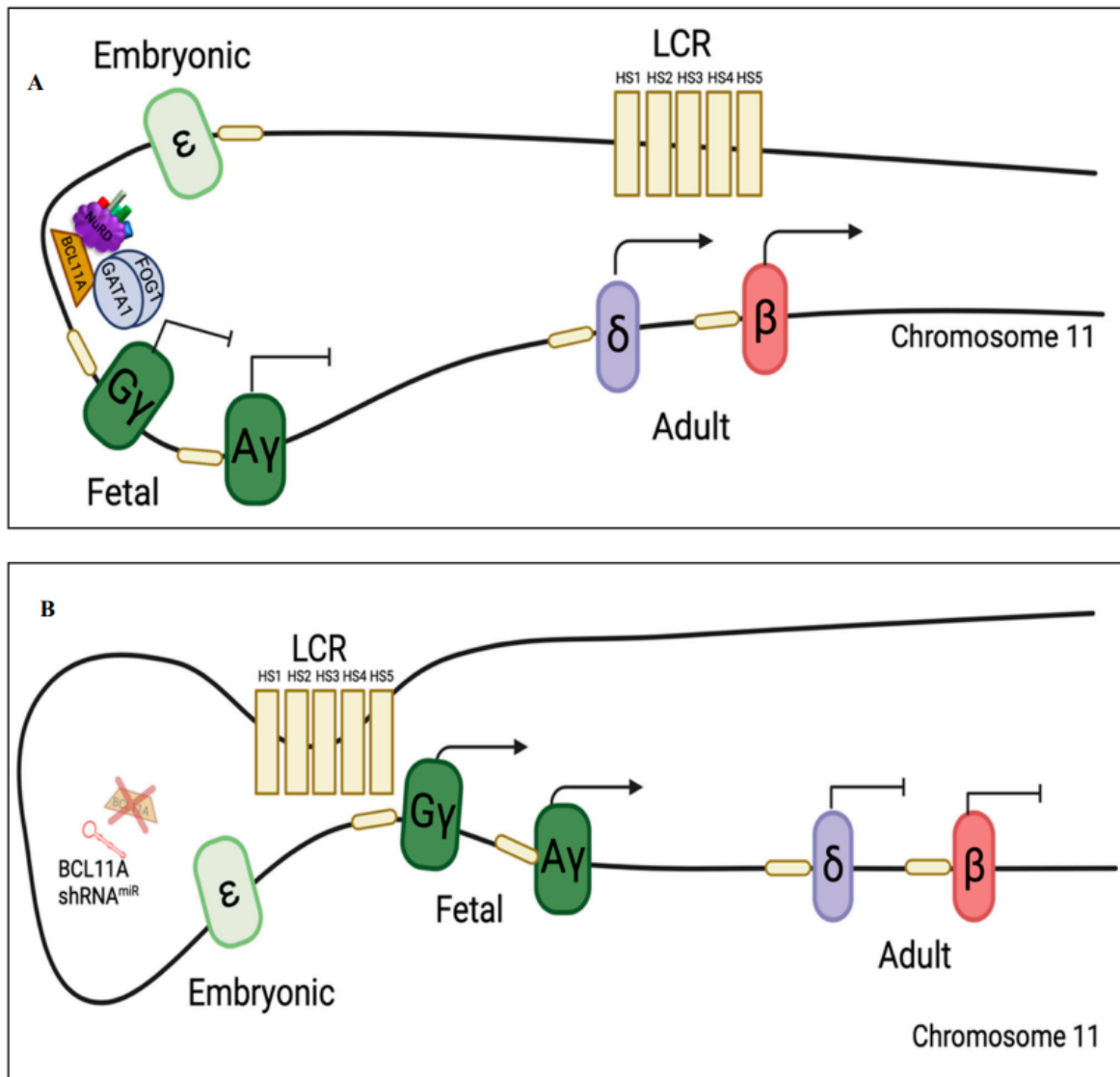


Figure 3 - Mechanism of HbF regulation.

(A) Endogenous HbF silencing. The human β -globin locus is comprised of the Locus Control Region (LCR) which regulates and enhances the expression of each of the globin genes through chromatin looping. When hemoglobin switching occurs from HbF to HbA expression, the nucleosome remodeling and deacetylase (NuRD) complex, FOG1, GATA1, and B Cell Lymphoma 11A (BCL11A) bind to the fetal globin promoters, leading to the repression of fetal globin expression and the activation of the adult globin genes. (B) Mechanism of BCL11A shRNA^{miR} silencing. The developed BCL11A shRNA^{miR} binds to BCL11A mRNA and targets it for breakdown in the cell, subsequently depleting BCL11A protein. Such a process prevents the BCL11A protein and protein complex binding to the fetal globin promoter binding site, thereby inducing γ -globin expression [3].

1.2. Hemoglobinopathies

Hemoglobinopathies is an umbrella term that defines a group of inherited diseases characterized by mutations in the globin genes. Beta(β)-Hemoglobinopathies (including Sickle Cell Disease (SCD) and β -thalassemia) are caused by deletions or point mutations in the gene encoding the hemoglobin (Hb) subunit β (HBB) [1, 4]. Another form of thalassemia is α -thalassemia, which arises from mutations in the α -globin genes (*HBA1* and *HBA2*) that result in deficit or qualitative change in the production of the Hb subunit α [8]. Although α - and β -thalassemia share conventional standard treatments, the difference in the molecular mechanism for each disease necessitates individual gene therapy and editing strategies. The work of this thesis will focus on the diseases caused by mutations in the β -globin chain, and throughout this document, the terms hemoglobinopathies and Beta(β)-Hemoglobinopathies may be used interchangeably.

Hemoglobinopathies are estimated to be the most common monogenic diseases worldwide [9], with SCD being the most prevalent single-gene disorder [10] and being recognized as a global health problem by the World Health Organization in 2006 [11]. Annually, over 300,000 and 40,000 infants are born with SCD and β -thalassemia, respectively [5, 12]. The protection against severe *P. falciparum* malaria in heterozygote carriers resulted in SCD being most prevalent in India, the Democratic Republic of Congo, and Nigeria [4, 5, 11]. Population movements have led to a much wider spread of the disease, which now affects 100,000 patients in the United States of America (USA). In 1975, the state of New York employed state-wide newborn screening, which opened the way to universal screening programs implemented by all states by the year 2007. β -thalassemia's highest prevalence is in the Mediterranean region, the Middle East, and Southeast Asia, remaining a rare disease in the USA with ~1200 patients identified. However, in the USA the prevalence of β -thalassemia has increased by approximately 7.5% over the last 50 years due to immigration from Asian countries and the adoption rate [12]. It has been estimated that approximately 1.5% of the world population are thalassemia heterozygotes or carriers [1, 13].

1.2.1. Sickle Cell Disease (SCD)

SCD was first reported in 1910 by James Herrick who described it as “peculiar elongated and sickle-shaped red blood corpuscles in a case of severe anemia” [14]. The molecular basis of SCD was established in 1949 by Pauling and colleagues who reported an abnormal form of hemoglobin [15]. However, it wasn't until 1957 that Ingram discovered that abnormal hemoglobin is caused by a change in a single amino acid [16].

SCD is characterized by RBCs expressing hemoglobin S (sickle hemoglobin, HbS) without additional HbA. A single nucleotide conversion of an adenine (A) to thymidine (T) at position 20 in the HBB gene (β^S allele) leads to the substitution of a glutamic acid for valine at codon 6 (GAG to GTG) in the β -globin chains of HbS [1, 4, 5]. Individuals who are heterozygous for the β^S allele carry the sickle cell trait (HbAS) but do not have SCD. The most severe and common form of SCD is Sickle Cell Anemia (SCA), in which individuals are homozygous for the β^S allele. Other relatively common forms of SCD include compound heterozygous conditions, such as HbSC disease and HbS β -thalassemia. Genotypically, HbSC disease has one β^S allele and one β^C allele. The β^C also results from another nucleotide conversion at codon 6 (GAG to AAG), leading to the substitution of the glutamic acid for lysine. HbS-thalassemia has one β^S allele with either a null (β^0) or hypomorphic (β^+ or β^{++}) β -thalassemia allele (Figure 4) [1, 4, 5].

Under hypoxic conditions, the mutant HbS polymerizes into numerous fibers of ~21 nm in diameter and up to several μ m in length. HbS has reduced oxygen affinity compared to HbA and this condition increases HbS polymerization, which in turn further reduces HbS oxygen affinity. As HbS bundles together in parallel, red blood cells are distorted, forming the characteristic “sickle” shape (SS-RBCs) [1, 4, 5, 10]. HbS polymerization correlates exponentially with the concentration of HbS within the erythrocyte, and the composition of other hemoglobins that participate in polymers. Hb tetramers with one HbS subunit can also polymerize, albeit not as efficiently as tetramers with two HbS subunits [4]. HbS polymerization changes the shape and physical properties of RBCs, impairing their rheology (how they flow) and survival [1, 5]. Sickle erythrocytes are highly unstable, with a lifespan that is reduced by >75%. Repeated episodes of HbS polymerization and erythroid sickling in low pO_2 and unsickling in high pO_2 can lead to severe membrane deformation and erythrocyte dehydration which eventually results in the formation of an irreversible sickle cell [4].

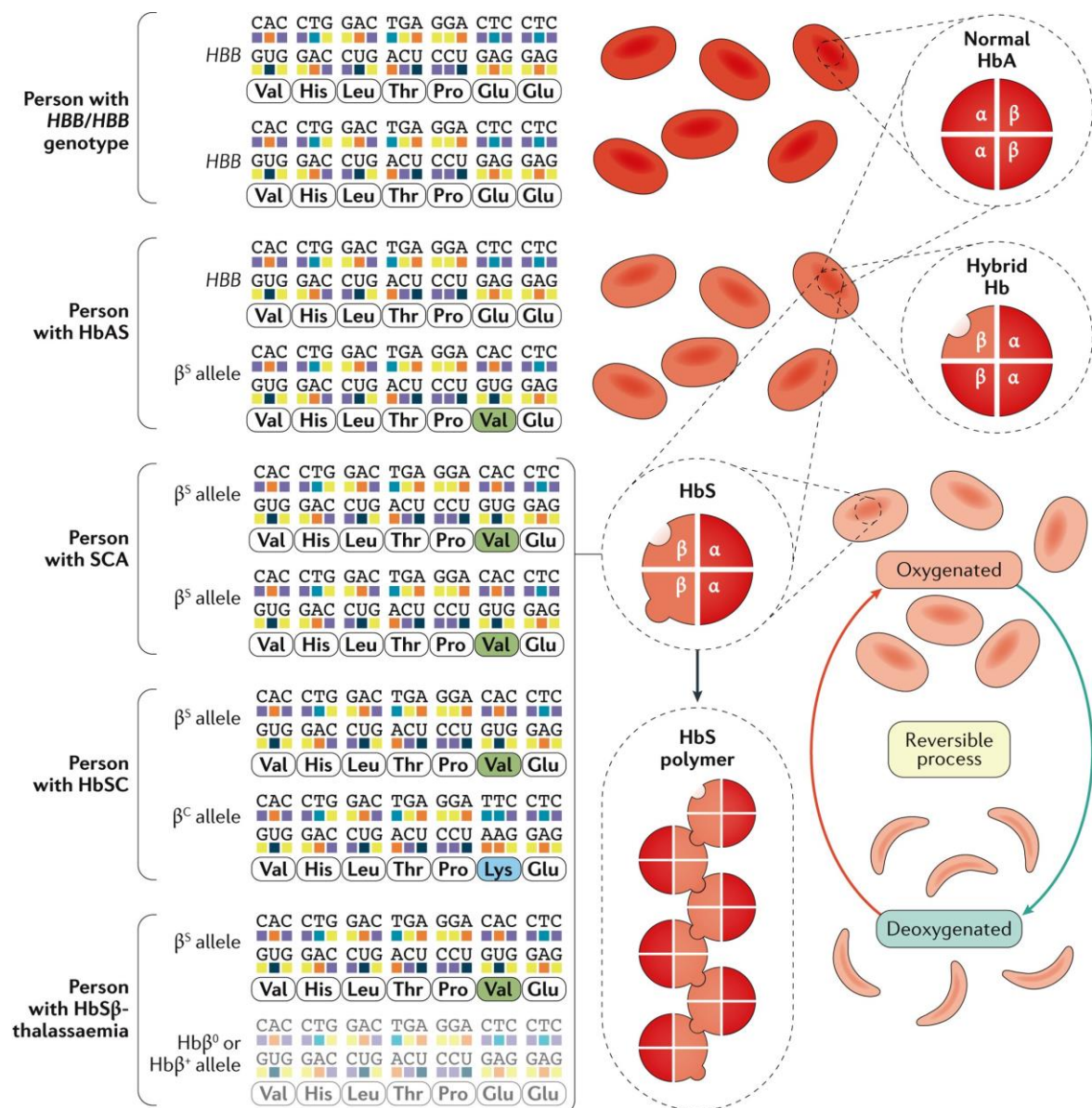


Figure 4 - Most common HBB genotypes and erythrocyte phenotypes in SCD.

Sickle cell disease (SCD) occurs when both HBB alleles are mutated and at least one of them is the β^S allele. Deoxygenated (not bound to oxygen) HbS can polymerize, and HbS polymers can stiffen the erythrocyte. Individuals with one β^S allele have the sickle cell trait (HbAS) but not SCD; individuals with sickle cell anemia (SCA), have two β^S alleles (β^S/β^S). Individuals with the HbSC genotype have one β^S allele and one HBB allele with a different nucleotide substitution (β^C allele). The β^S allele combined with a null HBB allele (Hb β^0) that results in no protein translation causes HbS β^0 -thalassaemia. The β^S allele combined with a hypomorphic HBB allele (Hb β^+ ; with a decreased amount of normal β -globin protein) results in HbS β^+ -thalassaemia [4].

SCD reduces a patient's life expectancy by several decades. The landmark complication associated with SCD is pain crisis due to the vaso-occlusion of blood vessels to the chest, abdomen, and joints (VOCs, vaso-occlusive crises). VOCs are a complex phenomenon, triggered by multiple factors such as stress, inflammation, ischemia, hemolysis, activated

endothelial cells, recruitment of adherent leukocytes, activation of neutrophils, increased plasma viscosity, and diminished blood flow. SCD features a vicious circle between inflammation and abnormal RBC rheology, which modulates clinical severity in patients. In addition to VOCs, the clinical manifestations include asplenia, severe infection, neurological changes, priapism, stroke, acute chest syndrome, renal failure, systemic inflammation pulmonary hypertension [1, 4, 5, 10].

Management options for SCD include supportive care (blood transfusions and medications). SCD patients need single acute transfusions for immediate benefit, while chronic transfusions help in preventing long-term complications [1, 4, 10]. Many SCD patients require chronic red cell exchange (RCE), an automated procedure in which the patient's RBCs are removed while donor RBCs are concurrently transfused. RCE is performed in an outpatient setting and requires trained nursing and medical staff, specialized medical equipment, good patient compliance, and a compatible supply of transfusable RBCs. RCE is a risk for additional morbidities like extreme alloimmunization, in which patients form antibodies against foreign antigens in transfused RBCs. As the number of alloantibodies rises, compatible RBC products dwindle, complicating or even preventing further treatment with transfusions [17]. The US Food and Drug Administration (FDA) has approved four drugs for the treatment of SCD (hydroxyurea, L-glutamine, voxelotor, and crizanlizumab). Hydroxyurea was approved in 1998 by the FDA and in 2007 by the European Medicines Agency (EMA), being the only approved treatment for SCD until 2017. Hydroxyurea is a myelosuppressive agent that evidence suggests acts by inducing HbF and inhibiting intracellular HbS polymerization. It is prescribed to reduce VOC events and hospitalizations. L-glutamine raises nicotinamide adenine dinucleotide (NADH) levels and, as a result, increases cellular antioxidant activity, which reduces the frequency of acute complications. Voxelotor enhances the microvascular flow, reducing the duration and severity of acute pain crises. Crizanlizumab is a P-selectin inhibitor that reduces acute complication incidence [4, 18]. Apart from hydroxyurea, which can be used as a standalone therapy, these drugs are only effective in combination with transfusion support.

1.2.2. β -thalassemia

β -thalassemia was first described more than 90 years ago by Cooley and Lee [19]. Since the first characterization of the human β -globin gene cluster in 1980, more than 350 mutations have been identified. Most alleles identified result in a single nucleotide substitution within the β -globin gene or its flanking regions. Small deletions or insertions within the gene or upstream regulatory elements may also occur [1, 13, 20]. The quantitative reduction in β -globin production depends on the underlying mechanisms. HBB mutations either impair β -globin synthesis at a variable degree (β^+ - or β^{++} - thalassemia) or abolish its production (β^0 - thalassemia) [7, 21]. Structurally abnormal β -globin variants may also be quantitatively reduced with a phenotype of β -thalassemia. Hemoglobin E (β^E , HbE) results from the substitution of a lysine for glutamic acid at the codon 26 in the β -globin chains. This substitution creates an alternate splicing site, reducing the efficiency of the normal splicing pathway and leading to the non-functional β -globin chain production that gives rise to β -thalassemia [21]. The quantitative reduction of β -globin causes its hemoglobin tetramer partner α -globin to be in excess affecting the tightly coordinated equilibrium of adult hemoglobin chains [20, 21]. The excess of free α -globin is responsible for the pathophysiology of β -thalassemia with the degree of imbalance between α - and β -like chain synthesis influencing the severity of the phenotype [21]. The accumulation and precipitation of free α -globins form toxic aggregates (α -aggregates). Free Hb and heme catalyze the formation of reactive oxygen species (ROS), leading to oxidative stress, hemolysis of circulating RBCs, and premature apoptosis of erythroid precursors in the BM [1, 22].

Traditionally, β -thalassemia has been described based on the severity of the disease phenotype and underlying genotype as three main forms: thalassemia -major ($\beta^0\beta^0$ or $\beta^0\beta^+$), -intermedia ($\beta^+\beta^+$) and -minor (heterozygote carrier) [22]. However, more recently, patients have been divided based on transfusion therapy requirements into patients who rely on regular, lifelong RBC transfusions for survival indicated as transfusion-dependent thalassemia (TDT), or patients who are transfusion-independent or need limited transfusion support over a specific period of time, indicated as non-transfusion dependent thalassemia (NTDT) [1, 22]. TDT and NTDT classifications are used in clinical trials to determine eligibility criteria and define disease management guidelines [22].

Clinically, β -thalassemia major presents with severe anemia that requires RBC transfusions starting during the first 2 years of life. Chronic severe anemia stimulates the production of erythropoietin (EPO), leading to ineffective erythropoiesis (IE), altered BM homeostasis, and stress signals. If left untreated or if inadequately transfused, patients commonly experience growth retardation, pallor, jaundice, weak musculature, leg ulcers,

extramedullary hematopoiesis causing splenomegaly and hepatomegaly and skeletal abnormalities due to the intensive but ineffective expansion of the BM erythroid compartment [1, 21]. Data from registries shows that 64-89% of patients require regular transfusions at intervals between 2 and 4 weeks. While regular RBC transfusions are critical, chronic transfusions can cause transfusion-related iron overload. Iron loading overwhelms serum transferrin capacity, increasing the amount of unbound, reactive iron species that cause end-organ damage from ROS and lipid peroxidation. If not well managed, excess iron can cause severe and irreversible organic damage, such as cirrhosis, diabetes, heart disease, and hypogonadism [21, 22]. Patients with thalassemia intermedia present later in life with moderate anemia and are non-transfusion dependent. The main clinical features in these patients are hypertrophy of erythroid marrow with medullary complications, gallstones, leg ulcers, and predisposition to thrombosis [21]. Thalassemia minor is clinically asymptomatic, although some patients may present moderate anemia and related symptoms such as headache, fatigue, exercise intolerance, dizziness, and lethargy [21].

Chronic management for β -thalassemia depends on the severity of the phenotype. The first line of treatment is blood transfusions coupled with iron chelation therapy (ICT) such as deferoxamine, deferiprone, and deferasirox, which can lower systemic, hepatic, and myocardial iron levels. ICT should be started within a year of initiating regular blood transfusions and improves overall survival and decreases risks of complications related to iron overload. Still, chronic RBC transfusions and extended use of ICT are also associated with an increased risk of alloimmunization and infection. Early initiation of transfusions, avoidance of splenectomy, and extended matching for Kell (K), C, E, and Jkb antigens can improve the safety of this therapy by reducing the incidence of alloimmunization in patients with β -thalassemia major. The FDA has approved the use of hydroxyurea in NTDT for its role in increasing HbF and enhancing total Hb levels [4, 5, 22] and the use of luspatercept for the reduction of ineffective erythropoiesis, associated with sustained increases in hemoglobin levels in NTDT patients and reduction of transfusion requirements in TDT patients [22, 23].

1.2.3. Therapeutic options for Beta(β)-Hemoglobinopathies

1.2.3.1. Hematopoietic Stem Cell Transplantation

The potentially curative standard for both β -thalassemia and SCD patients is allogeneic hematopoietic stem cell transplantation (HSCT) from HLA-matched family donors (mostly siblings). Worldwide, it is estimated that ~2000 patients with SCD have undergone HSCT, with survival rates that exceed 90% in the United States and European countries. Higher mortality with increased age was observed in patients who underwent transplants; thus, the current recommendations offer HSCT to pediatric and adolescent patients (≤ 18 years), limiting the curative options for the adult population. Prior to HSCT, HSCT conditioning is required to improve the engraftment of donor stem cells. The conditioning can be divided into two categories: (1) nonmyeloablative regiment, in which adjusted doses of busulfan combined with cyclophosphamide open the HSC niche space in host bone marrow, providing conditioning for engraftment, and (2) immunosuppression to prevent immunological rejection of graft cells by the host [4, 5, 24].

The lack of matched family donors remains a key obstacle with only 10-20% of patients having a histo-compatible sibling donor. The use of expanded donor pools (haploidentical donors who share 50% of the HLA antigens with the recipient) and unrelated HLA-matched donors has shown promising results but with increased rates of graft rejection and graft-versus-host disease. Another drawback to the procedure is the need for long-term immunosuppression to prevent or treat immunological complications [2, 22, 24].

1.2.3.2. Cell and gene therapy (CGT) approaches

Over the last few decades, the limited availability and risks associated with allogeneic HSCT have led to the interest in developing new potentially curative approaches for β -globinopathies. Promising therapeutic alternatives have been developed through gene addition, gene editing, and gene silencing. These one-time cure therapies rely on autologous transplantation of genetically modified hematopoietic stem and progenitor cells (HSPCs). This eliminates the risks of alloreactive complications and the requirement for immunosuppressive drugs [1, 22]. HSPCs express CD34⁺ and can be harvested from either the bone marrow or from the peripheral blood. However, several BM aspirations must be performed to achieve a sufficient number of CD34⁺ cells for gene therapy, limiting the appeal of this approach. A less invasive procedure is the mobilization of bone marrow CD34⁺ HSPCs to peripheral blood circulation. β -thalassemia patients are treated with granulocyte-colony stimulating factor (G-CSF) in combination with Plerixafor, a CXCR4 antagonist while Plerixafor alone is used in SCD patients. The plerixafor/G-CSF combination optimally increases the CD34⁺ yield. However, G-CSF is contraindicated in SCD patients as it induces VOCs, multi-organ failure, and even death. Following leukapheresis, the CD34⁺ cell fraction is isolated by magnetic beads, genetically engineered ex vivo and re-administered to the preconditioned patient (Figure 5) [2, 10].

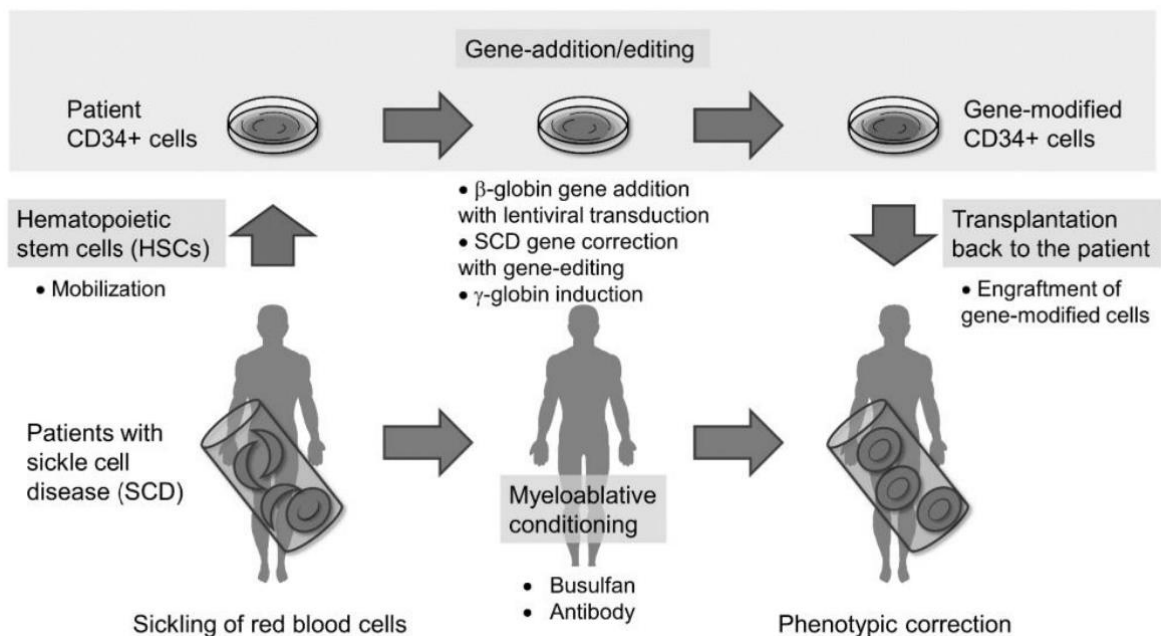


Figure 5 – Overview of hematopoietic stem cell-targeted gene therapy (addition/editing) in sickle cell disease [10].

Multiple strategies have been developed for the genetic manipulation of CD34⁺ cells. The following gene-addition/editing/silencing strategies will be covered here: (1) lentiviral vector (LV)-mediated insertion of a therapeutic β -globin or γ -globin gene and LV-mediated BCL11A silencing, (2) gene and base editing as an alternative to gene addition.

1.2.3.2.1. Lentiviral vectors (β -globin variants, anti-sickling γ -globin vectors, and BCL11A silencing)

Lentiviral vectors can introduce genes into non-dividing cells, which is useful for gene delivery to cells such as quiescent hematopoietic stem cells and enables shorter ex vivo culture time. LVs can carry large transgene sequences, which enables the inclusion of the complex β -globin gene cassettes identified as necessary for high-level, erythroid-specific expression (LCR HS elements, introns) [3]. Below, I will outline the scientific background, vector design, and clinical outcomes for keystone lentiviral vectors.

β -globin LV (TNS9)

In 2000, the first successful therapeutic use of β -globin LV, termed TNS9, was demonstrated by Sadelain and colleagues [25]. In 2012, this TNS9 LV was investigated in a clinical trial for severe β -thalassemia patients, the first trial in the United States for a severe inherited globin disorder (NCT01639690) (Table 1).

β^{A-T87Q} LVs (HPV569 and BB305)

Parallel studies revealed that β^S globin initiates HbS polymerization by binding threonine 87 (T87) of the β -globin chain of an adjacent HbA and that the different amino acid composition (of both γ - and δ -globin chains) is partly responsible for the anti-sickling effect. In 2001, Leboulch and colleagues designed the first anti-sickling β -globin gene variant, β^{A-T87Q} , replacing the T87 with a glutamine. This anti-sickling β -globin LV improved SCD phenotype in a murine SCD model by reducing sickled RBC and splenomegaly [26] and in 2006, the first gene therapy patient with TDT was treated with a β^{A-T87Q} lentiviral globin vector (LentiGlobin HPV569), in France. Although the trial was terminated due to vector insertion in the HMGA2 oncogene, a next generation of trials was built based on the insights gained from it, and in 2013, bluebird bio funded two studies that tested BB305, a β^{A-T87Q} lentiviral vector, in patients with SCD and TDT (NCT02151526 and NCT01745120) [3, 10] (Table 1). Following these initial studies, two Phase 3 trials were initiated in 2016 and 2017 to further assess the clinical benefits of BB305 in less severe β -thalassemia and to study the efficacy of G-CSF/plerixafor mobilization in both β -thalassemia major and minor

(NCT02906202 and NCT03207009) (Table 1). Although these trials were suspended in 2021 due to the serious adverse effect (SAE) of acute myeloid leukemia (AML), they were later restarted as it was concluded that the development of AML in the patient was unlikely due to vector integration. Following the successful outcomes of the trials with patients achieving transfusion independence and near-normal levels of hemoglobin, Zynteglo (Betibeglogene autotemcel, beti-cel) was approved by the FDA in August 2022, for the treatment of adults and pediatric patients with TDT. The same treatment had previously received conditional approval by the EMA in 2019 but was withdrawn in 2022 for commercial reasons by the marketing authorization holder [22]. In addition to the β -thalassemia trials, in 2014, bluebird bio initiated another phase 1/2 clinical trial solely for SCD patients and a phase 3 clinical trial (NCT02140554 and NCT04293185) (Table 1) [3, 10]. Results in 32 SCD patients supported initial safety and efficacy with 88% of the patients achieving complete resolution of VOCs during the study and in December 2023, FDA approved Lyfgenia (lovotibeglogene autotemcel, lovo-cel) for the treatment of SCD patients 12 years and older [27].

β^{AS3} LV (*Lenti- β^{AS3}*)

In 2003, another anti-sickling β -globin variant was developed by the Townes laboratory, by substituting three amino acids in the β -globin gene, β^{AS3} . In addition to the T87Q variant described above, β^{AS3} includes the substitution of the glutamic acid in codon 22 with alanine (E22A) and replaces the glycine at codon 16 with aspartic acid (G16D) [28]. A β^{AS3} -globin LV, referred to as *Lenti- β^{AS3}* , further established the efficiency of β -globin variant LVs and led to the use of β^{AS3} in a clinical trial for SCD in 2014 (NCT02247843) (Table 1).

γ -globin LVs (*ARU-1801 and GGHI-mB-3D*)

LVs carrying HBG1 or HBG2 were developed to increase exogenous expression of γ -globin chains as co-inheritance of HPFP with β -thalassemia or SCD ameliorates their clinical severity and reduces or abrogates blood transfusions or SCD-associated complications [29]. In severe β -thalassemia, the γ -globin chains bind to the unpaired α -globin chains to produce HbF and reduce toxicity of α -aggregates while in SCD, the Q87 amino acid variation impedes aggregation of γ -globin chains in HbS polymers. Malik and colleagues modified a previous β -globin LV to incorporate the γ -globin exons alongside a minimal β -globin promoter and elements of the LCR [30], to generate the ARU-1801 vector currently undergoing a Phase 1/2 clinical evaluation (NCT02186418) (Table 1). A new vector, GGHI-mB-3D γ -globin LV, is being tested for potential treatments for SCD and β -thalassemia through HbF induction by incorporating HPFP elements. This vector yielded high titers as

it is a β -globin LCR-free LV with γ -globin gene being flanked by regulatory elements from the γ -globin and α -globin loci [31, 32]. However, clinical efficacy is yet to be assessed.

shRNA^{miR} LV (BCH-BB694)

Another target for γ -globin induction involves knocking down BCL11A with shRNA^{miR} technology. These shRNA^{miRs} are modified shRNAs with flanking regions designed from miRNA scaffolds to allow the use of a polymerase II promoter that enables cell and lineage-specific targeting with low passenger strand activity [33]. In 2017 and 2022, this gene therapy strategy was brought to clinical trials for SCD (NCT03282656, NCT05353647) (Table 1) reporting 24-44% HbF and marked decreases in clinical symptoms [3].

Table 1 - A summary of lentiviral HSC gene-addition therapy trials in SCH and transfusion-dependent β -thalassemia (TDT).

Trial	Phase	Year	Study Drug	Target Gene	Sponsor
Sickle cell disease					
NCT04628585	N/A	2020	BB305	β^{T87Q_globin}	bluebird bio
NCT04293185	3	2020	BB305	β^{T87Q_globin} γ^{G16D_globin}	bluebird bio
NCT04091737	1	2019	CSL200	shRNA-HPRT	CSL Behring
NCT03964792	1/2	2019	GLOBE1	β^{AS3_globin}	APHP Boston
NCT03282656	1	2018	BCH-BB694	shmiR-BCL11A	Children's Hospital University of
NCT02247843	1/2	2014	β^{AS3} -FB	β^{AS3_globin}	California, Los Angeles
NCT02186418	1/2	2014	ARU-1801	γ -globin	Aruvant Sciences
NCT02140554	1/2	2014	BB305	β^{T87Q_globin}	bluebird bio
NCT02151526	1/2	2013	BB305	β^{T87Q_globin}	bluebird bio
β -Thalassemia					
NCT05015920	1	2021	BD211	β^{T87Q_globin}	Shanghai Bdgene Shenzhen Children's
NCT04592458	1	2020	LentiHBBT87Q	β -globin	Hospital, BGI- Research
NCT03207009	3	2017	BB305	β^{T87Q_globin}	bluebird bio
NCT02906202	3	2016	BB305	β^{T87Q_globin}	bluebird bio
NCT02453477	1/2	2015	GLOBE	β -globin	TIGET
NCT01745120	1/2	2013	BB305	β^{T87Q_globin}	bluebird bio
NCT02151526	1/2	2013	BB305	β^{T87Q_globin}	bluebird bio
NCT02633943	N/A	2013	BB305		bluebird bio Memorial
NCT01639690	1	2012	TNS9.3.55	β -globin	Solan Kettering
LG001	1/2	2007			bluebird bio

N/A: not applicable

1.2.3.2.2. Gene and base editing as alternative approaches to gene addition

Gene editing has emerged in the past decade as an alternative modality to gene addition due to its site-directed properties and theoretical reduction of genotoxicity risks compared to semi-random integration of viral vectors [2].

The different genome editing tools share the common feature of accurate introduction of double-strand breaks (DSBs) at the genome sequence of interest, leading to endogenous DNA repair mechanisms. The DSB could be repaired by non-homologous end joining (NHEJ) or homology directed repair (HDR). NHEJ occurs during any phase of the cell cycle and can introduce insertions and deletions (indels). These indels result in frameshift mutations and non-functioning gene products, generally used for knockouts such as the disruption of the binding sites for erythroid-specific TFs like BCL11A. HDR is less error-prone but requires a homologous DNA donor template for DSB correction. HDR is mainly active in the S and G2 phases of the cell cycle, leading to poor efficiency of gene correction in quiescent HSCs. High-fidelity HDR is usually used for homologous recombination such as the replacement of the SCD mutation on the β -globin gene [2, 10].

Several gene editing strategies have been studied for their usage in HSCs for the treatment of SCD and β -thalassemia, such as Zinc Finger Nucleases (ZFN), TALENs, and especially the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated Cas9 [3] (Figure 6A, B, C).

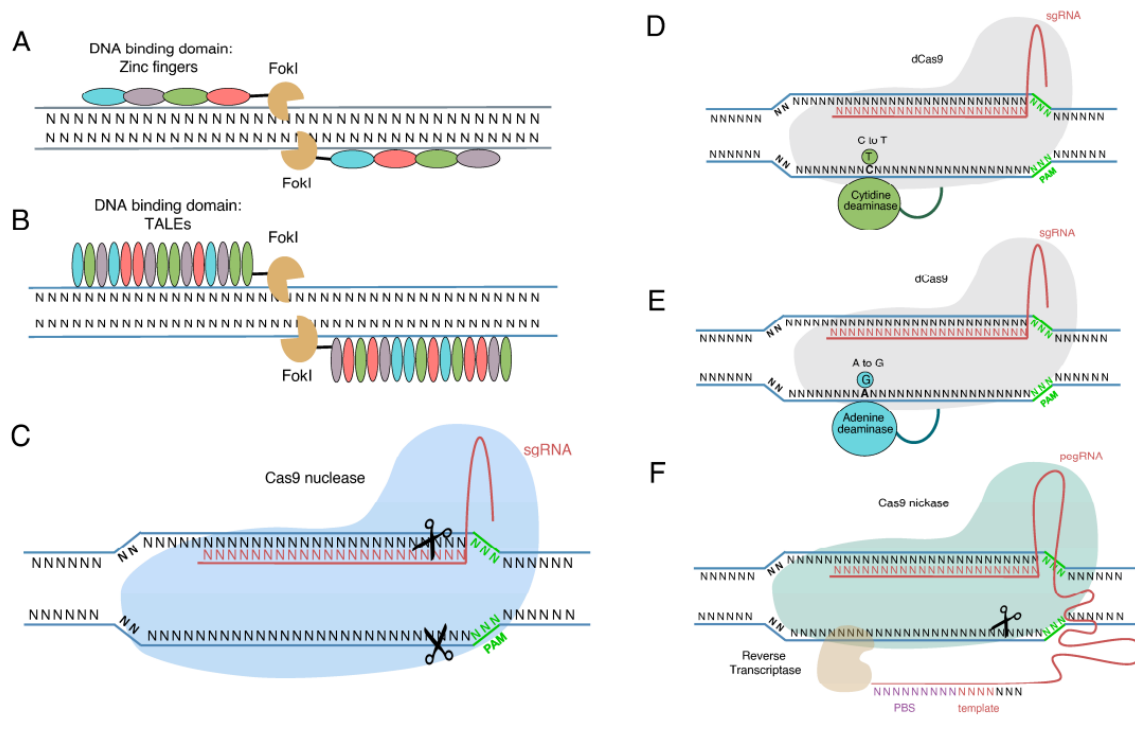


Figure 6 - Genome editing platforms.

(A) Zinc finger nucleases (ZFNs); (B) transcription activator-like nucleases (TALENs); (C) clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9); (D) cytidine base editor (CBE); (E) adenine base editor (ABE). (F) Prime editor (PE) sgRNA: single guide RNA, pegRNA: prime editor guide RNA, PAM: Palindromic Adjacent Motif, dCas9: dead-Cas9 nuclease, PBS: primer binding sequence [2].

ZFNs are artificial nucleases composed of two domains and developed by combining a bacterial endonuclease of Fok1 (a double-stranded DNA nickase; DNA cleavage domain) with DNA binding zinc finger domains to target and cut a particular 3-4 bp DNA sequence site of the genome (DNA recognition domain) [6]. In 2018, ZFN was the first genome editing tool used in a Phase I/II clinical trial for TDT by Sangamo Therapeutics and Sanofi (NCT03432364) which targeted the erythroid enhancer of BCL11A and led to increased HbF expression [34] (Table 2).

TALENs are based on the application of transcription activator-like effector (TALE) proteins found in *Xanthomonas* species. Similarly to ZFNs, TALENs consist of a nonspecific DNA recognition/cleavage domain fused to a customizable sequence-specific DNA-binding domain (Fok1 endonuclease). TALEN-mediated disruption of regulatory elements within HBG1 and HGB2 promoters resulted in elevated HbF levels in a humanized mouse model transplanted with edited human CD34⁺ cells [35].

The CRISPR-Cas9 system was first described as part of bacterial and archaeal adaptive immunity against foreign DNA. It includes two components, an endonuclease (Cas9), and a 100-nucleotide chimeric RNA (g or sgRNA), resulting from the fusion of a sequence-

specific CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA). CRISPR-Cas9 is the gene-editing technique most extensively used in β -thalassemia and SCD approaches such as the introduction of HPFP-like mutations [36], disruption of HbF regulatory binding sites proximal to HBG1 and HBG2 promoters [37], or correction of the β^S mutation [38].

Table 2 – A summary of HSC gene-editing therapy trials in SCD and TDT.

Trial	Phase	Year	Study Drug	Target Gene	Sponsor
Sickle cell disease					
NCT04774536	1/2	2021	CRISPR/Cas9: CRISPR_SCD001	β -globin	University of California, San Francisco
NCT04853576	1/2	2021	CRISPR/Cas12: EDIT-301	<i>BCL11A</i> ESE	Editas Medicine
NCT04819841	1/2	2021	CRISPR/Cas9: GPH101	β -globin	Graphite Bio
NCT05145062	N/A	2021	ZFN: BIVV003	<i>BCL11A</i> ESE	Bioverativ
NCT04443907	1/2	2020	CRISPR/Cas9: OTQ923/HIX763	<i>BCL11A</i> ESE	Novartis Pharmaceuticals
NCT03653247	1/2	2019	ZFN: BIVV003	<i>BCL11A</i> ESE	Bioverativ
NCT04208529	N/A	2019	CRISPR/Cas9: CTX001	<i>BCL11A</i> ESE	Vertex Pharmaceuticals
NCT03745287	2/3	2018	CRISPR/Cas9: CTX001	<i>BCL11A</i> ESE	Vertex Pharmaceuticals
β -Thalassemia					
NCT04205435	1/2	2021	CRISPR/Cas9	β -globin	Biorary Laboratories
NCT04208529	N/A	2021	CRISPR/Cas9: CTX001	<i>BCL11A</i> ESE	Vertex Pharmaceuticals
NCT04211480	1/2	2020	CRISPR/Cas9	<i>BCL11A</i> ESE	Biorary Laboratories
NCT03655678	2/3	2018	CRISPR/Cas9: CTX001	<i>BCL11A</i> ESE	Vertex Pharmaceuticals
NCT03432364	1/2	2018	ZFN: ST-400	<i>BCL11A</i> ESE	Sangamo Therapeutics

ESE: erythroid-specific enhancer, N/A: not applicable.

Several clinical trials involving CRISPR-Cas9 have been conducted on β -thalassemia and SCD. In 2018, Vertex Therapeutics, in collaboration with CRISPR-Therapeutics, funded two clinical trials for CTX001, based on CRISPR-Cas9 disruption of the GATA1 binding site at +58 in the *BCL11A* erythroid-specific enhancer in CD34⁺ cells derived from TDT and SCD patients (NCT03655678 and NCT03745287, respectively) (Table 2). These clinical trials were followed by a long follow-up study in patients who received CTX-001, initiated in 2019 [2, 22]. The efficacy and safety data of these ongoing studies were reported in 2023

from a pre-specified interim analysis. The primary efficacy outcome measure was freedom from severe VOC for at least 12 consecutive months during the 24-month follow-up period. Of the 31 patients with sufficient follow-up time, 29 achieved primary outcome and all 31 treated patients achieved successful cell engraftment with no cases of graft rejection or failure. Patients had early and sustained increases in total and fetal hemoglobin and the safety profile was consistent with myeloablative busulfan conditioning and autologous HSCT [22, 27]. On 16 November 2023, these results led to the approval of Casgevy (exagamglogene autotemcel, exa-cel) by the MHRA (UK Medicines and Healthcare products Regulatory Agency) for the treatment of patients ≥ 12 years of age with TDT for whom HSCT is appropriate but without an available HLA-matched donor, and for treatment of SCD patients aged ≥ 12 years with recurrent VOCs and for whom HSCT is appropriate but without an available HLA-matched donor. This first approval was followed by the approval by the FDA on 8 December 2023 and 16 January 2024, for the treatment of SCD in patients ≥ 12 years with recurrent VOC and TDT patients, respectively; and on 15 December 2023, the EMA approved Casgevy for both SCD and TDT [27].

Recently, a new technology known as base editing (BE) has been studied and seems to be a safer and equally effective genome editing approach. While gene editing relies on DSBs followed by HDR or NHEJ, base editing allows the targeted incorporation of a specific point mutation, independently of DSBs. Base editing is particularly interesting for diseases such as SCD or β -thalassemia when caused by well-documented point mutations [2, 10]. There are two categories of base editors: cytidine base editors (CBE) and adenine base editors (ABEs) (Figure 6D, E).

The first-generation CBE (CBE1) was created in 2016 by fusing the nuclease dead Cas9 (dCas9) with a cytosine deaminase. The CBE opens the DNA creating an R-loop that turns C to U after the hybridization of the spacer sequence with the gRNA. The endogenous DNA mismatch repair system recognizes uracil as a thymidine resulting in C-G to T-A transition. Since then, new and more effective generations of CBEs have led to higher editing and lower frequency of undesired byproducts [2, 10]. CBE technologies have been successfully used to edit the +58 BCL11A erythroid-specific enhancer in erythroid cells derived from β -thalassemia and SCD patient HSPCs and to edit the -28 (A>G) mutation [39], or a point mutation in the β -thalassemia promoter region in β -thalassemia HSPCs [40].

In 2017, the first ABEs were engineered by fusing a dCas9 with tRNA adenosine deaminase enzyme, TadA, leading to A-T to G-C conversion. Continuous optimization has been performed since then, and current 8th-generation ABEs provide cleaner editing compared to CBEs, without a significant percentage of indels or off-target effects. Although neither CBEs nor ABEs can create the transversion needed to correct the SCD mutation (A<T), ABEs can replace the A-T with a C-G resulting in the benign G-Makassar phenotype

[41]. ABEs have also been employed to target the γ -globin promoter regions to increase HbF [42].

A complementary technology to base editing that avoids double-strand breaks and allows for the incorporation of all 12 possible combinations of base substitutions is known as prime editors (PEs) (Figure 6F). PE can also install deletions of at least 80 nucleotides and insertions of at least 44 nucleotides with incredible precision and few off-target edits. PEs fuse a dCas9 to an engineered RNA-dependent DNA polymerase with a prime editing guide RNA (pegRNA), an RNA not only complementary to the target but also contains a template sequence [2, 10]. PEs have been shown to correct the SCD mutation in HEK293T cells [43].

1.2.3.3. Unmet needs for β -thalassemia and SCD

After decades of research, the recent advances in technologies manipulating the human genome have resulted in numerous clinical trials for SCD and β -thalassemia and, ultimately, the recent approval of Zynteglo, Lyfgenia and Casgevy. While Zynteglo is approved for both pediatric and adult TDT patients, Lyfgenia and Casgevy are only available to SCD patients ≥ 12 years of age who have recurrent VOCs. Additionally, we have yet to reach the 15-year follow-up requested by regulatory bodies to define the long-term safety and sustainability of the clinical benefit provided by the two approaches. Emerging techniques and continuous studies in globin research are indispensable to guarantee that all patients regardless of their age, phenotype, or medical history, have access to effective, safe, and long-lasting therapeutics.

2. GOALS

This Thesis presents the results obtained during the doctoral work divided into two parts.

The main goals were to:

Main goal 1: Generate in vitro cell models to expedite the screening of potential new therapies for β -globinopathies.

Main goal 2: Develop and test double vectors with the potential to simultaneously produce 1) transgenic HbA, 2) reactivate endogenous HbF, and 3) decrease the production of endogenous mutant protein.

3. ESTABLISHMENT AND CHARACTERIZATION OF ENGINEERED CELL LINES

3.1. Introduction

The in vitro development of therapies to treat genetic human disorders such as SCD and β -thalassemia, relies mainly on the isolation of primary cells from patients. The Rivella lab has established the conditions to isolate and expand CD34⁺ cells from 30 mL of peripheral blood, achieving more than 6×10^7 cells. For isolation and expansion, the Rivella lab modified the first version of the two-phase liquid culture system originally described by Fibach [44]. Human stem cell factor in the first phase was added to promote the expansion of the CD34⁺ population starting from the beginning of the culture [45]. However, the use of primary cells is limited as they are restricted to the number of proliferation cycles before terminal differentiation. Additionally, access to these specimens strictly depends on establishing protocols with hospitals and the availability of volunteers. Alternatively, Kurita's group established the HUDEP-1, -2, and -3 immortalized cell lines, derived from human cord blood cells, by transducing a vector that expresses the HPV16-derived proteins HPV16-E6/E7 [46]. These cells can be used as a semi-primary adult hematopoietic cell model that can be propagated indefinitely in the presence of tetracycline or differentiated into red cells under appropriate culture conditions [46]. Gene expression profiles after differentiation of the three cell lines revealed that HUDEP-1 and -3 mainly expressed γ -globin, while HUDEP-2 expressed β -globin. In agreement with the γ - and β -globin patterns, HUDEP-2 showed high expression of BCL11A, while the expression of the same transcription factor in HUDEP-1 and -3 was negligible [46]. Moreover, we confirmed the gene expression results by chromatographic separation of hemoglobins in differentiated HUDEP-2 with HbA being the most predominant form of hemoglobin (Figure 7A and 7B) and nearly identical to that of erythroblasts isolated from the blood of healthy individuals. This profile provides HUDEP-2 with the potential for research use in BCL11A-targeted therapies, but the almost exclusive expression of HbA is still a limitation in approaches based on the addition of a WT β -globin gene. To overcome this limitation, the HUDEP-2 cell line could be used as the parental line for targeted mutagenesis of the β -globin gene to establish engineered cell lines.

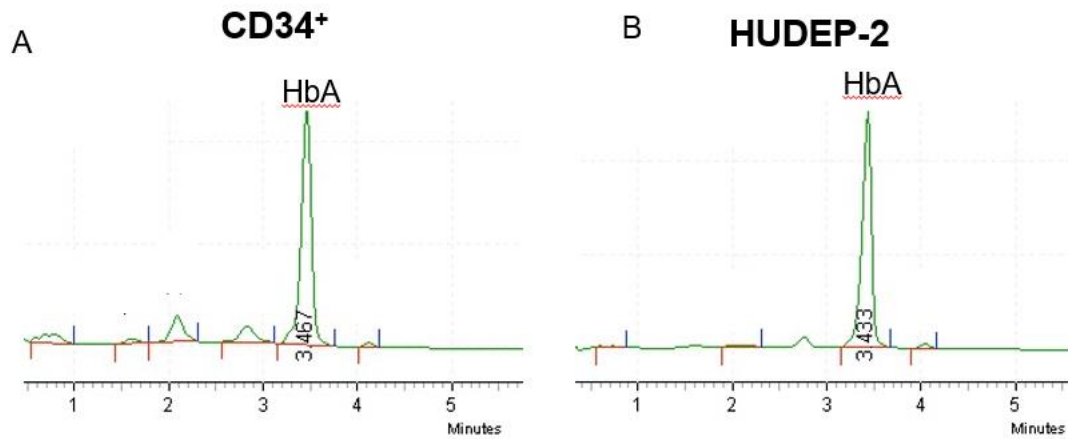


Figure 7 - Representative chromatographic separation of hemolysates.

(A) Representative chromatographic separation (high-performance liquid chromatography [HPLC]) of hemolysates from WT CD34⁺ cells. (B) Representative chromatographic separation (high-performance liquid chromatography [HPLC]) of hemolysates from HUDEP-2 cell line.

Alternatively, the successful establishment of immortalized erythroid progenitor cell lines derived from CD34⁺ cells from the umbilical cord blood raised the question of whether the same procedure could be used to immortalize CD34⁺ cells derived from SCD or β -thalassemia patient's whole blood.

The goal of this section was to generate in vitro cell models to expedite the screening of potential new therapies for β -globinopathies.

3.2. Methodology

3.2.1. Cell Culture of HUDEP-2 Cell Lines

HUDEP-2 parental cell line and established clones were cultured in a three-phase erythroid differentiation protocol. Cells were expanded (Phase I) in StemSpan serum-free expansion media (SFEM) (Stem Cell Technologies, Cambridge, Canada) supplemented with 10^{-6} M dexamethasone (DEXA) (Sigma, St Louis, MO, USA), 50 ng/mL human stem cell factor (hSCF) (Peprotech, Rocky Hill, NJ, USA), 2 international units (IU)/mL erythropoietin (EPO) (Amgen, Thousand Oaks, CA, USA), 1 µg/mL doxycycline (Doxy) (Clontech, Mountain View, CA, USA) and 1% penicillin/streptomycin (P/S) (Life Technologies, Carlsbad, CA, USA).

Cells were differentiated in Phases II and III. In Phase II, the cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Life Technologies, Carlsbad, CA, USA) supplemented with 200 µg/mL holo-transferrin (TF) (Athens Research & Technology, Athens, GA, USA), 10 µg/mL recombinant human insulin (Sigma, St Louis, MO, USA), 3% human serum type AB (Atlanta Biologicals, Flowery Branch, GA, USA), 2% human plasma (Stemcell Technologies, Vancouver, Canada), 10 ng/mL hSCF (Peprotech, Rocky Hill, NJ, USA), 2 IU/ml EPO (Amgen, Thousand Oaks, CA, USA), 1 µg/mL Doxy (Clontech, Mountain View, CA, USA) and 1% P/S (Life Technologies, Carlsbad, CA, USA). After 3 days in Phase II, the cells were transferred for 3 days to Phase III media containing IMDM (Life Technologies, Carlsbad, CA, USA) supplemented with 1000 µg/mL TF (Fisher Scientific, Waltham, MA, USA), 10 µg/mL recombinant human insulin (Sigma, St Louis, MO, USA), 3% human serum type AB (Atlanta Biologicals, Flowery Branch, GA, USA), 2% human plasma (Stemcell Technologies, Vancouver, Canada), 3 IU/ml EPO (Amgen, Thousand Oaks, CA, USA) and 1% P/S (Life Technologies, Carlsbad, CA, USA). Cells were collected on Day 6-7 of differentiation for all analyses and the level of differentiation was assessed by benzidine (Sigma, St Louis, MO, USA) staining.

3.2.2. Establishment of HUDEP-2 Engineered Cell Lines

The GR1 sequence (5'gtaacggcagacttctctc3') targeting the first exon of HBB at codon 6 was cloned into the pSpCas9(BB)-2A-GFP (PX458) plasmid (Addgene plasmid ID 48138) backbone using BbsI sites. The final construct coding for GR1, Cas9 protein, and EGFP was electroporated into the parental HUDEP-2 cell line. Cells efficiently transfected were GFP⁺ selected and single-cell sorted into 96-well plates.

After 21 days of clonal expansion, genomic DNA was extracted and the generated clones were screened for mutations in the HBB gene by DNA sequencing (outsourced to the Viral Molecular High-Density Sequencing Core at the Perelman School of Medicine, University of Pennsylvania).

3.2.3. Establishment of Patient-Derived Cell Lines

Non-mobilized CD34⁺ cells were isolated (Day 0) from whole blood samples by immunomagnetic separation using the CD34 MicroBead Kit (Miltenyi Biotec Inc., Auburn, CA, USA). CD34⁺ cells were expanded and kept at a cell density $<0.5 \times 10^6$ cells/mL in SFEM (Stemcell Technologies, Vancouver, Canada) supplemented with 10 μ L/mL CC100 (Stemcell Technologies, Vancouver, Canada), 2 U/mL EPO (Amgen, Thousand Oaks, CA, USA), 10^{-6} M DEXA (Sigma, St. Louis, MO, USA), and 1% P/S (Life Technologies, Carlsbad, CA, USA). Cells in the expansion phase (Day 3-5) were transduced with the CSIV-TRE-HPV16-E6/E7-UbC-KT (plasmid kindly provided by Kurita et al [46]) lentiviral vector at a low MOI (<10). On Day 10-12, cells were Kusabira-Orange 1⁺ (hKO1) selected and pooled. After selection, 1 μ g/mL Doxy (Clontech, Mountain View, CA, USA) was introduced to the expansion media. On Day 15-18, cells were transitioned to HUDEP-2 Phase I media. Cells were differentiated in Phases II and III as per section 3.2.1. The differentiation level was assessed by benzidine (Sigma, St Louis, MO, USA) staining, hemoglobin synthesis was assessed by HPLC, the degree of cell sickling in SCD-patient derived cells was evaluated by in vitro RBC sickling and morphological analysis, protein expression was assessed by Western Blot (WB) and the expression of cell surface molecules was evaluated by flow cytometry.

3.2.4. Tetrameric Analysis by HPLC

Red cell pellets were lysed with HPLC-grade water, and the resulting membrane-free hemolysates were loaded into a System Gold 126 Solvent Module instrument (Beckman Coulter, Fullerton, CA, USA). Hemoglobins were separated on a weak cation-exchange PolyCAT A column (PolyLC, Inc, Columbia, MD, USA), and detected at a wavelength of 415 nm. The hemoglobins were bound to the column with mobile phase A (20 mmol/L Bis-Tris, 2 mmol/L KCN, pH 6.96) and eluted with mobile phase B (20 mmol/L Bis-Tris, 2 mmol/L KCN, 200mmol/L NaCl, pH 6.55). Standards of adult hemoglobin (HbA), fetal hemoglobin (HbF), sickle cell disease hemoglobin (HbS), and type C hemoglobin (HbC) (Analytical Control Systems, Fishers, IN, USA) were used to determine various hemoglobin peak types. Single-chain quantification was assessed via reversed-phase HPLC. Hb

samples, in this case, were injected on a Hitachi D-7000 HSM Series apparatus (Hitachi Instruments, San Jose, CA, USA) using a Zorbax 5 μ m 300SB-C8 300 Å, LC 150 x 2.1 mm column (Agilent Technologies, Santa Clara, CA, USA) and a gradient from 20% to 60% acetonitrile in 0.1% trifluoroacetic acid in 25 minutes, with UV detection at 215 nm.

3.2.5. In Vitro RBC Sickling and Morphological Analysis

We assessed the degree of cell sickling in our experimental samples using previously reported methodology, with slight modifications [47, 48, 49, 50]. Briefly, 5 to 15 million cells were suspended in 150 μ L isotonic Hemox buffer (TCS Scientific Corp, Southampton, PA, USA), pH 7.4, supplemented with 10 mM glucose and 0.2% bovine serum albumin, in individual wells of a Costar polystyrene 96-well microplate (№ 9017; Corning, Corning, NY, USA). The microplate was then transferred to a Thermomixer R shaker-incubator (Eppendorf, Hamburg, Germany), and maintained under hypoxia (Nitrogen gas), with continuous agitation at 500 rpm, at 37°C for 2 hours. At conclusion, aliquots (~ 20 μ L) of each sample were collected in 2% glutaraldehyde solution for immediate fixation without exposure to air. Subsequently, fixed cell suspensions were introduced into specialized glass micro slides (Dawn Scientific, Inc., Newark, NJ, USA) for the acquisition of bright field images (at 40x magnification) of single-layer cells on an Olympus BX40 microscope fitted with an Infinity Lite B camera (Olympus) and the coupled Image Capture software.

3.2.6. Western Blot Analysis

Red cell pellets were lysed in RIPA buffer (Millipore, Burlington, MA, USA) combined with 1X proteinase and phosphatase inhibitors (Cell Signaling, Danvers, MA, USA). Protein lysates were collected and quantified by BCA analysis (Thermo Fisher Scientific, Waltham, MA, USA). Thirty micrograms of protein were used to detect BCL11A, HBG as well as GAPDH, and 3 μ g to probe HBB and HBA and loaded onto NuPAGE 4-12% Bis-Tris gel and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, MA, USA). Following blocking in TBS (Fisher Scientific, Waltham, MA, USA) with 0.1% Tween-20 and 5% BSA, the PVDF membrane was incubated with the corresponding antibodies: rabbit anti-HBB (Proteintech, Chicago, IL, USA), rabbit anti-HBA (Santa Cruz Biotechnology, Dallas, TX, USA), mouse anti-BCL11A (Abcam, Cambridge, MA, USA), rabbit anti-HBG (Santa Cruz Biotechnology, Dallas, TX, USA), and rabbit anti-GAPDH (Proteintech, Chicago, IL, USA) primary antibodies. Anti-mouse and anti-rabbit IgG HRP-linked secondary antibodies (cell signaling) were used for detection with the Amersham ECL western blotting analysis system (GE Healthcare, Malborough, MA, USA).

3.2.7. Flow Cytometry of In Vitro Culture

A sample of cells ($<1 \times 10^6$) in expansion was stained with monoclonal antibodies (MoAbs) in 100 μ l of phosphate-buffered saline (PBS) (Life Technologies, Carlsbad, CA, USA) containing 2% fetal bovine serum (FBS) (Hyclone, South Logan, UT, USA) for 30 minutes on ice. MoAb against CD223⁺ (glycophorin A) was conjugated with allophycocyanin (APC) (BD Biosciences, San Jose, CA, USA), MoAb against CD44⁺ (c-KIT) was conjugated with Brilliant Violet 421 (BV421) (BD Biosciences, San Jose, CA, USA), MoAb against CD36⁺ was conjugated with Fluorescein isothiocyanate (FITC) (Biolegend, San Diego, CA, USA) and MoAb against CD71⁺ was conjugated with Brilliant Violet 711 (BV711) (BD Biosciences, San Jose, CA, USA). Samples were analyzed on LSR-Fortessa flow cytometer (BD Biosciences, San Jose, CA, USA) and the results were analyzed using FlowJo (Treestar, Ashland, OR, USA) software.

3.3. Results

3.3.1. Establishment and Characterization of HUDEP-2 M#9 and M#13

To establish a screening system for lentiviral vectors engineered for the treatment of hemoglobinopathies, using the CRISPR/Cas9 approach, we modified the HUDEP-2 cells to synthesize a hemoglobin variant, which can be discriminated from that produced by the lentiviruses. We designed a gRNA (GR1) targeting the first exon of HBB (more specifically the triplet that translates to codon 6) and by electroporation, transiently expressed both the Cas9 protein and GR1 in the HUDEP-2 cells. We established various clones that differed from the parental cell line by HPLC profile. Two clones (M#9 and M#13) were selected for further characterization and sequencing analysis confirmed homozygous or compound heterozygous mutated HBB alleles in codon 6 (Table 3).

Table 3 – Sequences of the DNA Region Coding for Amino Acids 5-9 of the β -globin Gene and Length of the Respective Generated Proteins for the HUDEP-2, M#9 and M#13 Cell Lines

Cell Line	Sequence	Protein Size
HUDEP-2	5' gag gag aag tct gcc 3'	147aa protein
	5' gag gag aag tct gcc 3'	147aa protein
M#9	5' gag --- aag tct gcc 3'	146aa protein
	5' gag --- aag tct gcc 3'	146aa protein
M#13	5' gag --- aag tct gcc 3'	146aa protein
	5' gag – ag aag tct gcc 3'	PTC – 19aa protein

M#9 is homozygous for the deletion of codon 6 (Glu6) and was predicted to translate into a shorter variant of the β -globin protein made of 146aa instead of 147aa. M#13 carries the same deletion on one allele, and a single nucleotide deletion on the second allele, which by frameshift, causes a downstream premature termination codon (PTC), which was predicted to encode a 19aa long protein. At Day 7 of differentiation, tetrameric chromatographic separation of the hemolysates of clones M#9 and M#13 confirmed the presence of a new form of hemoglobin indicated as Hb-mutant, which is eluted at a different time and distinct from both HbA (Figure 8A and 8B) or HbS (not shown), while no HbA is present, as expected. Moreover, M#9 and M#13 showed comparable levels of HbA, HbB, HbG, and BCL11A to those of HUDEP-2 by WB analyses (Figure 8C).

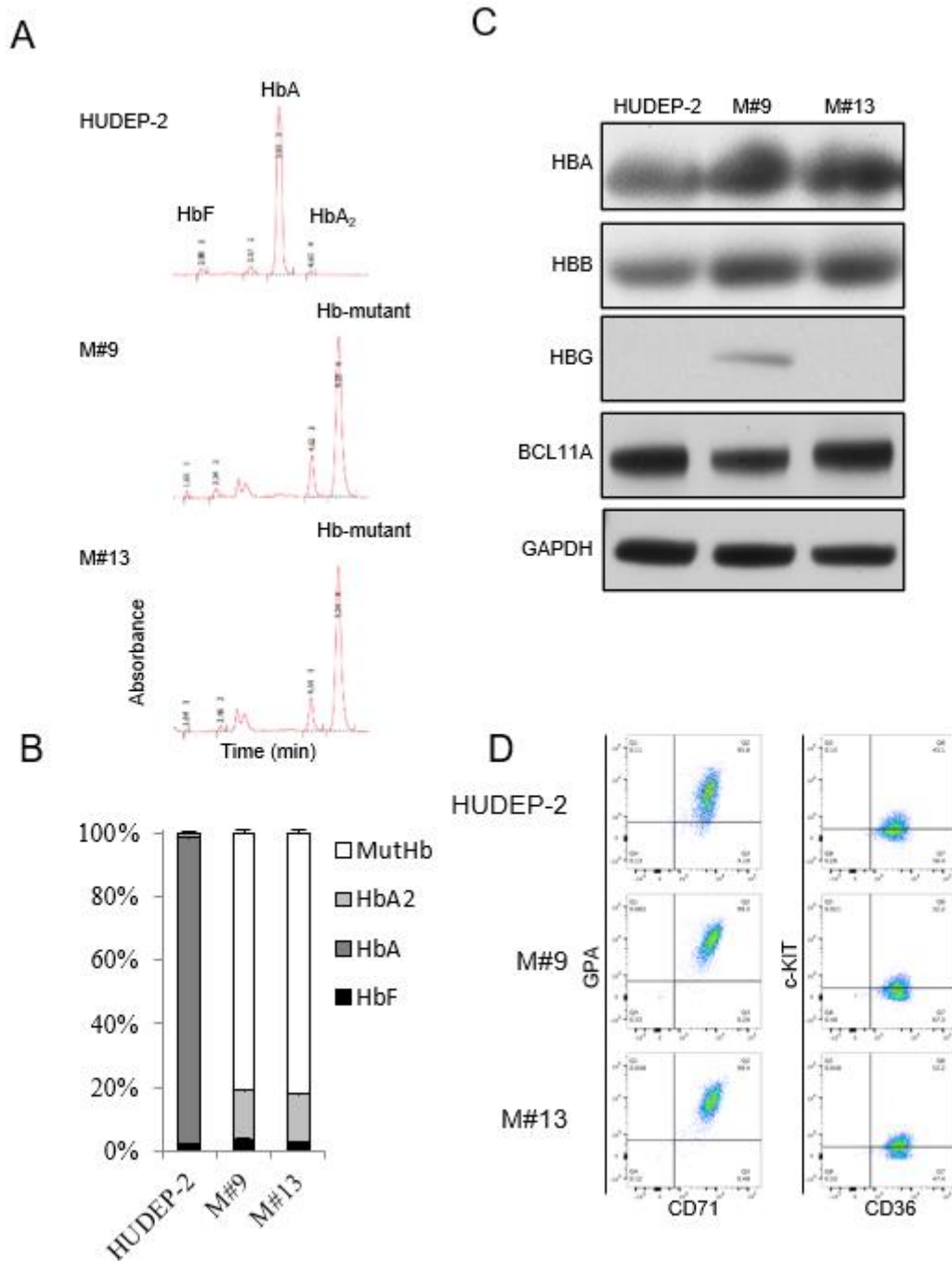


Figure 8 - Characterization of M#9 and M#13 cell lines

(A) Representative chromatographic separation [HPLC] of hemolysates from the HUDEP-2 (top), M#9 (middle), and M#13 (bottom) erythroblasts showing HbF, HbA, HbA₂ and Hb-mutant peaks at Day 7 of differentiation. (B) Relative proportion of HbF, HbA, HbA₂ and Hb-mutant in HUDEP-2, M#9, and M#13 quantified by HPLC at Day 7 of differentiation. (C) Representative WB showing HBA, HBB, HBG and BCL11A proteins in HUDEP-2, M#9, and M#13 at Day 7 of differentiation. (D) Representative flow cytometry plots of HUDEP-2, M#9 and M#13 stained for GPA, CD71, c-KIT and CD36 markers.

Flow cytometry analyses demonstrated that the genomic modifications in M#9 and M#13 did not disturb the expression of the erythroid-specific markers glycophorin A (GPA), transferrin receptor (CD71), SCF receptor (c-KIT) and a marker of immature erythroid cells (CD36) (Figure 8D).

From these properties, we predicted that M#9 and M#13 could be used as new cell models for β -globinopathies, by providing a tool to measure the levels of HbA or HbF generated after lentiviral treatment.

3.3.2. Establishment and Characterization of SCD13 Cell Line

In addition to the HUDEP M#9 and M#13 cell lines, an alternative screening system was established by immortalizing CD34⁺ hematopoietic progenitor cells isolated from peripheral blood of SCD patients. A combined protocol (Figure 9) was used in which CD34⁺ cells were isolated on Day 0 from a whole blood sample provided by patient SCD13. Following isolation and a 3–5-day expansion, cells were transduced with the CSIV-TRE-HPV16-E6/E7-UbC-KT lentiviral vector, which was produced using the plasmid kindly provided by the Kurita lab. After transduction, cells were expanded until enough cells were available for selection by mOrange⁺ (Day 10-12). After selection, mOrange⁺ cells were pooled and Doxy was added to the expansion media as a selective agent. Cells were banked after 3-8 days in the presence of the selection marker. After Day 18, cells were also kept in culture in HUDEP-2 Phase I media for over 2 months to demonstrate immortalization. To establish SCD#13 as a screening system, the cells were differentiated in parallel with M#9 and M#13 cells. HPLC analysis (Figure 10A and 10B) revealed HbS as the most predominant hemoglobin expressed by the cell line (89.0%), followed by HbA (9.30%) and HbF (1.70%). Flow cytometry analyses demonstrated that the immortalized SCD#13 express the erythroid-specific markers GPA, CD71, c-KIT, and CD36 at the same levels as HUDEP-2, M#9, and M#13 (Figure 11). Moreover, when exposed to hypoxic conditions, differentiated SCD#13 cells presented similar features to those observed in differentiated cells from the primary culture of CD34⁺ SCD patients (Figure 12).

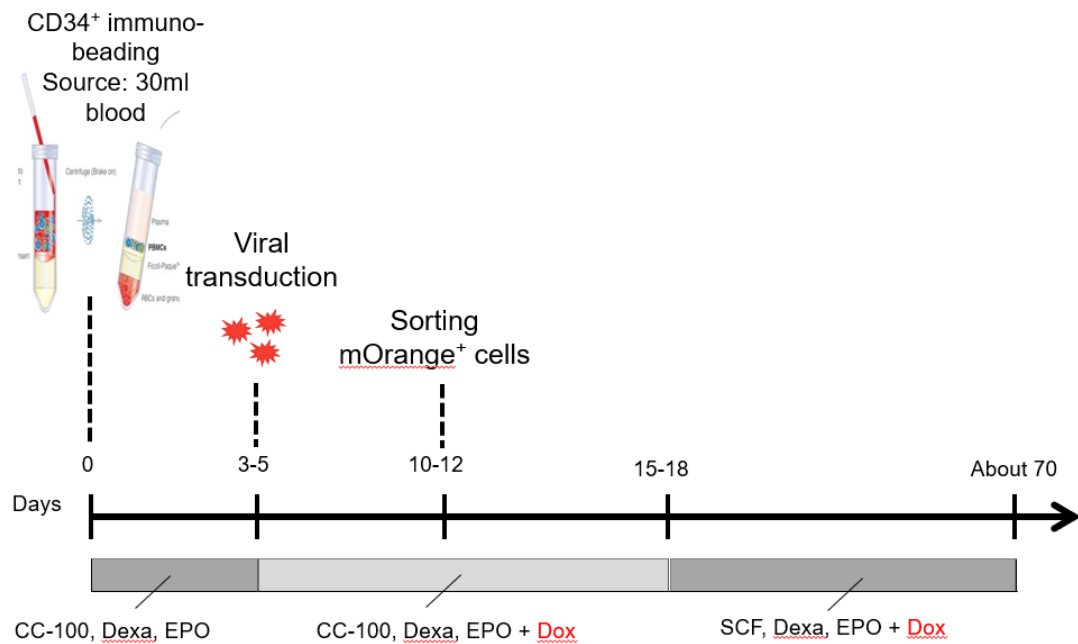


Figure 9 - Schematic outline of the procedures for establishing immortalized human erythroid progenitor cell line from CD34⁺ hematopoietic progenitor cells isolated from peripheral blood of patient SCD13.

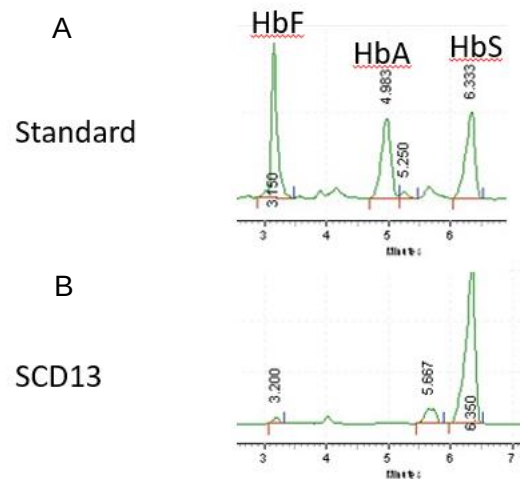


Figure 10 - (A) Representative chromatographic separation (HPLC) of analytical control standard of HbF, HbA, and HbS. (B) Representative chromatographic separation (HPLC) of hemolysates from SCD13 cell line.

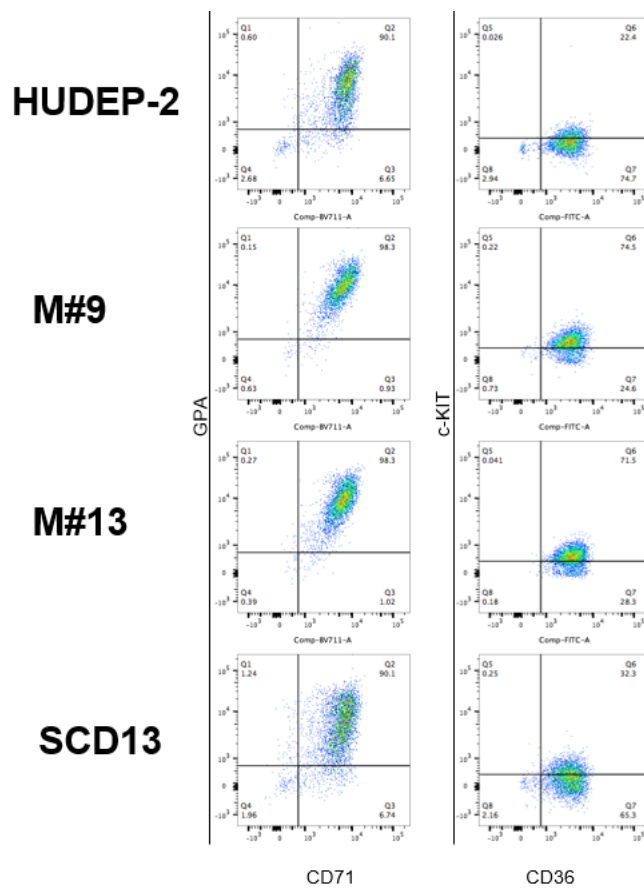


Figure 11 - Representative flow cytometry plots of HUDEP-2, M#9, M#13, and SCD13 stained for GPA, CD71, c-KIT and CD36 markers.

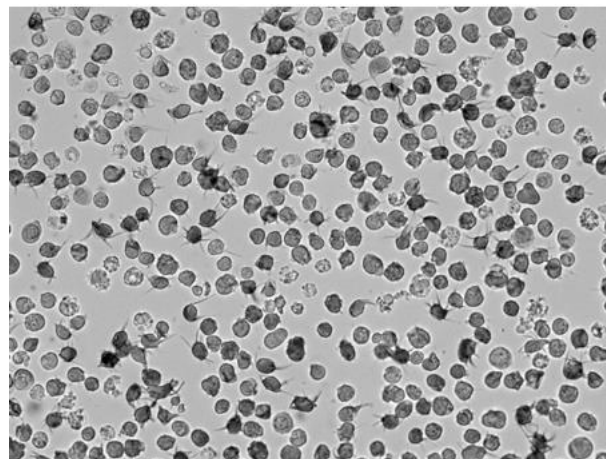


Figure 12 - Representative image of SCD13 cell cultures under hypoxia.

3.4. Discussion

We established engineered cell lines from the HUDEP-2 parental cell line [46]. To our knowledge, this was the first report describing erythroid progenitor-engineered cell lines able to differentiate into RBCs in vitro, which do not produce HbA. Although the initial goal to knock in the SCD mutation into codon 6 of the β -globin gene was not achieved, the first attempt at mutagenizing the parental cell line resulted in the establishment of two clones, M#9 and M#13, which produce a Hb-mutant distinguishable from HbF, HbA, and HbS by tetrameric HPLC analysis. Further characterization by WB and flow cytometry, confirmed that the CRISPR-Cas9 gene editing did not affect the expression of erythroid-specific proteins (HBA, HBB, HBG, and BCL11A) and markers (CD223, CD71, CD36, and CD44). Our results showed that M#9 and M#13 maintained the advantages of the parental cell line while possessing unique characteristics that predicted their value in being used as a platform to test both HbF and HbA-inducing therapies for treating β -globinopathies.

To establish a SCD cell model, we tested the protocol developed by Kurita et al. [46], in an alternative source of CD34⁺ cells. While the original protocol established the HUDEP cell lines from CD34⁺ derived from the umbilical cord blood of healthy donors, our modified protocol used 30 mL of peripheral blood of SCD patients as the source of CD34⁺ cells. Additional changes to the original protocol were required to accommodate the change in source. Due to the low yield of CD34⁺ cells isolated from 30 mL of blood, the cells stayed in expansion media for extra days before viral transduction and we based the initial expansion media on the protocol established by the Rivella lab to expand and bank primary cells derived from SCD and β -thalassemia patients. To enrich the population with cells transduced with the HPV-16-E6/E7 system, we introduced a selection step in which the cells were sorted for the presence of the mOrange⁺ selection marker. To our knowledge, these changes to the original protocol enabled the establishment of the first SCD-immortalized cell line. Since then, Gupta et al. [51] used a CRISPR-Cas9 system to engineer the BEL-A immortalized erythroid progenitor cell line with the sickle cell mutation (BEL-A SCM), or with the IVS1-5 (G.C) β -thalassemia mutation (BEL-A BTM).

In conclusion, establishing M#9, M#13, and SCD13 cell lines was another step toward building versatile screening systems for the various emerging therapeutic approaches for β -globinopathies.

4. GENERATION OF DOUBLE-PRONGED LENTIVIRAL VECTORS THAT INDUCE HBA AND REACTIVATE HBF

4.1. Introduction

The Rivella lab further improved the vector TNS9, utilized in the clinical trial led by Dr. Sadelain, and generated a new lentiviral vector, named AnkT9W (Figure 13). AnkT9W incorporated both the human β -globin gene and the erythroid-specific ankyrin 5' hypersensitive (HS) barrier insulator. Insulator elements generally have two functions: (1) enhancer-blocking activity, when placed between an enhancing element and a promoter and (2) preventing the spread of heterochromatin into the integrated transgenic cassette from a nearby heterochromatinized region [52]. In AnkT9W, the 5' HS ankyrin insulator exhibits only the latter activity, and significantly increased expression of the percentage of corresponding aggregates with increasing VCN, in patient-derived β^0/β^0 thalassemic erythroid cells untreated or treated with AnkT9W. Statistical significance was assessed by computing means with a one-way Anova test followed by a Tukey test to compare every mean with every other mean. β -globin was compared to vectors without this insulator. The Rivella lab then compared AnkT9W to TNS9 (the vector without the ankyrin element) in vivo, engrafting mice affected by β -thalassemia ($Hbb^{th3/+}$) with BM cells from $Hbb^{th3/+}$ mice infected by either TNS9 or AnkT9W (N=5 and 6 per vector, respectively). Compared with β -thalassemic mice transplanted with BM cells transduced with TNS9, animals treated with AnkT9W showed a 30% improvement in Hb synthesis and hematologic parameters [45]. Next, AnkT9W was tested in human $CD34^+$ peripheral blood stem cells, using optimal conditions for infecting the cells to achieve a clinically relevant vector copy number (VCN) between 0.1 and 2. Following in vitro β -globin gene transfer with AnkT9W and erythroid differentiation, the correlation between the VCN, the number of β -globin mRNA molecules, and the level of Hb synthesis were evaluated. AnkT9W increased the synthesis of β -globin protein in many patient specimens to levels comparable to those observed in carriers and control samples [45].

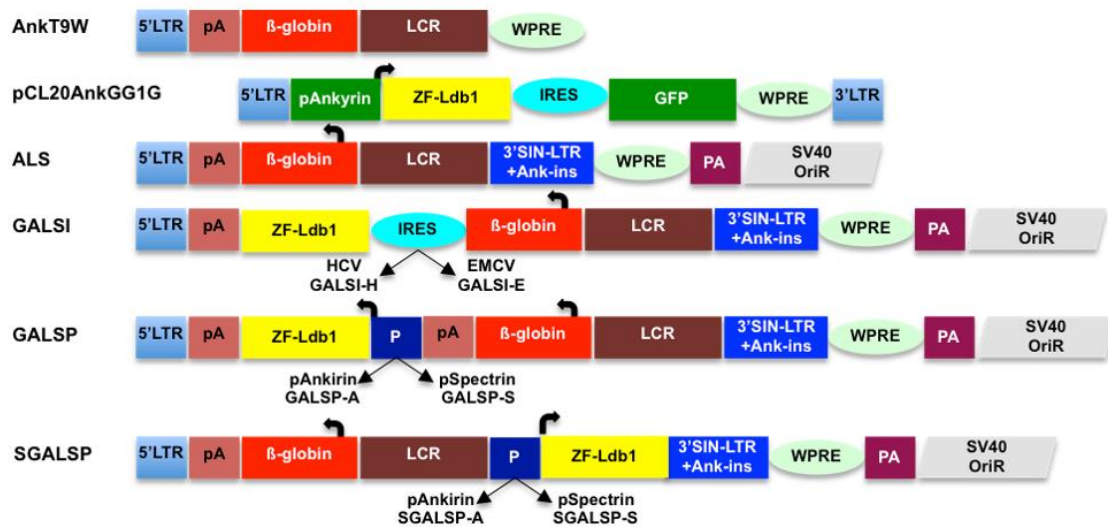


Figure 13 - Maps of AnkT9W, pCL20AnkGG1G, ALS, GALSI (-H and -E), GALSP (-A and -S), and SGALSP (-A and -S).

Viral features: WPRE - Woodchuck hepatitis virus posttranscriptional regulatory element; LCR - Locus Control Region; LTR - Long Terminal Repeat; SIN - self inactivating LTR; pA - polyadenylation sequence; β-globin - β-globin gene, including promoter and introns 1 and 2; Ank-ins - Ankyrin insulator; IRES - internal ribosomal entry site; P - promoter (Ankyrin or Spectrin); ZF-Ldb1 - zinc-finger-Ldb1 transcription factor fusion protein; HCV - hepatitis C virus; EMCV - encephalomyocarditis virus; SSV-OriR - origin of replication. Not in scale.

The Rivella lab made additional changes to the AnkT9W vector to increase its expression and safety. First, to mimic the endogenous β-globin sequence and preserve its regulation by endogenous elements, the vector was modified to include a complete intron 2. Previous vectors used a truncated version of intron 2 since this element was shown to decrease titer in oncoretroviral vectors. Second, the Woodchuck Post-Regulatory Element, or WPRE, was eliminated from the integrating sequence to increase the safety of the vector. The WPRE was originally part of the integrated portion of the vector since it was placed between the LCR and the 3' LTR (Figure 13); its original purpose was to increase the titer of the lentivirus, but based on Rivella's unpublished data, it seemed that it underwent chromosomal rearrangement upon integration. To preserve the ability of WPRE to increase viral titers without having this viral element in the integrating sequence, the Rivella lab removed the WPRE from the integrating portion and added it back after the 3'LTR. In addition, a strong bovine growth hormone polyA tail was cloned after the WPRE region. These modifications led to the establishment of ALS vectors.

Additionally, in collaboration with Dr. Blobel at UPENN-CHOP, the Rivella lab used a lentivirus that encodes the ZF-Ldb1 cassette (pCL20AnkGG1G, Figure 13) [53]. This vector remarkably and significantly increased the synthesis of HbF in erythroid cells derived from SCA patients' HSCs. As HbF increased, the synthesis of HbS decreased. Noticeably, and

in line with the role of ZF-Ldb1, a decrease of HbA₂ ($\alpha_2\delta_2$) was also observed. The number of HbF-positive cells measured by flow cytometry increased proportionally to the VCN. Accordingly, the γ/α -globin mRNA ratio increased while β/α -globin mRNA ratio decreased in the samples treated with the ZF-Ldb1 vector, supporting the model in which the ZF-Ldb1 redirects the LCR activating effect from the β - and δ -globin promoters to the γ -globin promoter [54].

The first aim reported in the work plan for this thesis was to develop a novel therapeutic vector that carried both the transgenic β -globin gene and ZF-Ldb1, leading to the production of both HbF and HbA. The approach was to transcribe β -globin and the ZF-Ldb1 genes into a single mRNA molecule. In these constructs, the expression of the β -globin gene was driven by its promoter and regulatory elements while the expression of ZF-Ldb1 was supported by an internal ribosome entry sequence (IRES). Depending on the IRES element used, the constructs were named GALSI-H (using hepatitis C virus (HCV)) and GALSI-E (using encephalomyocarditis virus (EMCV)) (Figure 13). A second strategy had the ZF-Ldb1 gene transcribed by an alternative erythroid-specific promoter (Ankyrin or Spectrin), and those constructs were designated GALSP-A and GALSP-S, respectively (Figure 13). Both strategies relied on the unidirectional expression of both genes. A third approach was based on the bi-directional expression of the β -globin gene and ZF-Ldb1, and those constructs were denominated SGALSP-A and SGALSP-S (Figure 13). The success of the dual approach was motivated by the proof-of-concept data obtained by *in vitro* co-transduction of SCD CD34⁺ cells isolated from patient peripheral blood mononuclear cells (PBMCs), which resulted in a simultaneous increase of HbA and HbF, as well as the highest reduction in SCD variant HbS (data not shown). Preliminary data (Figure 14) showed that when treated with GALSP-S, even at a VCN of 2.64 copies/cell, the HbA and HbF increase was minimal (2.3 and 7.3%, respectively) (Figure 14B). When transduced at a similar MOI, SGALSP-S resulted in an average VCN of only 0.24 copies/cell with HbA and HbF increases of 6.8 and 2.8%, respectively (Figure 14C). Additionally, and highly likely due to the large size of the β -globin+ZF-Ldb1 vectors, we observed much lower titers for the dual vectors when compared to the ALS and pCL20AnkGG1G single vectors. These results led us to research additional approaches to establish vectors with the ability to express HbA and concurrently reactivate HbF (dual vectors).

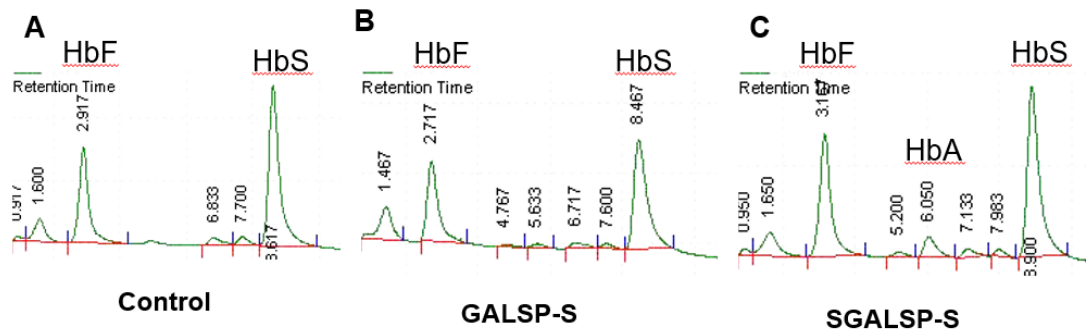


Figure 14 - Representative chromatographic separation (HPLC) of hemolysates from the SCD patients' cells showing HbF, HbA, and HbS.

(A) Control/untreated. (B) transduced with GALSP-S at VCN of 2.64 copies/cells. (C) transduced with SGALSP-S at VCN of 0.24 copies/cell.

The transcription factor BCL11A is a known alternative player involved in the repression of the γ -globin gene. Because of its smaller size, the use of a microRNA-adapted short hairpin RNA (shRNA^{miR}) to target BCL11A was chosen as the next approach to developing a dual lentiviral vector.

This section aimed to develop and test double vectors with the potential to simultaneously produce 1) transgenic HbA, 2) reactivate endogenous HbF, and 3) decrease the production of endogenous mutant protein.

4.2. Methodology

4.2.1. Construction of Lentiviral Vector Plasmids for Expression of β -globin Human and miRNA Targeting BCL11A

To generate ALS10-T87Q, we modified the AnkT9W vector [45] by cloning the full sequence of the intron 2 of the β -globin gene, and re-position of WPRE downstream of the 3'LTR. Moreover, we mutagenized the coding sequence to encode the T87Q version of the β -globin gene.

The miR-E-BCL11A sequences were synthesized by GenScript Biotech (Piscataway, NJ, USA) by combining the passenger and guide sequences of shRNA^{miR5} [33] with flanking miR-E backbone sequences [55]. The combined vectors were constructed by cloning the miR-E-BCL11A sequence into the β -globin gene intron 1 position c.79+36 of ALS10-T87Q (ATM1) and the intron 2 positions c.303-163 and c.303-172 (ATM2.1 and ATM2.2, respectively). The ATM1.1, ATM1.2, ATM1.3, and ATM1.4 vectors were generated by modifying the miR-E-BCL11A sequences of ATM1, also synthesized by GenScript Biotech

(Piscataway, NJ, USA). ALS10-T87Q-silent and ATM1.1-silent were generated by introducing variant sequences S1 and S2 to the β -globin gene exons 1 and 2, respectively.

4.2.2. Virus Production and Titration

Viral stocks were generated by co-transfection of the gene transfer plasmid (i.e. ALS10-T87Q, ATM1, ATM2.1, ATM2.2, ATM1.1, ATM1.2, ATM1.3, ATM1.4, ALS10-T87Q-silent, and ATM1.1-silent) together with the envelope plasmid (VSV-G) (Addgene plasmid ID #12259), the packaging plasmid (pMDLg/p RRE, Addgene plasmid ID #12251), and the pRSV-REV vector into HEK293T (ATCC) cells using CalFectin mammalian cell transfection reagent (SigmaGen Laboratories, Rockville, MD, USA). Viral supernatants were collected 48 hours after transfection, and filtered through a 0.22 μ m PES filter (USA Scientific, Orlando, FL, USA). Following ultraconcentration by ultracentrifugation, serial dilutions of concentrated virus (4, 0.4, and 0.04 μ L, respectively) were used to infect 1×10^5 NIH 3T3 cells (ATCC) in the presence of 8 μ g/mL polybrene (Millipore, Burlington, MA, USA). After 3 days, genomic DNA was extracted, precipitated with phenol, chloroform, and isoamyl alcohol (25:24:1), saturated with 10 mM Tris (pH 8.0) and 1 mM EDTA (Sigma, St. Louis, MO, USA), and the number of integrations (VCN) was quantified by qPCR using 100 ng of mouse or human gDNA in duplicate and oligos (FW: 5'-GTGCGAGAGCGTCAGTATTAAG-3'; Rev: 5'-TCCCTGCTTGCCCATACTA-3') for the specific sequence present in the vector (Psi) and compared to the PCBP2 endogenous control present in two copies within the genome (FW: 5'-TTGTGTCTCCAGTCTGCTTG-3'; Rev: 5'-AGGTGGTGGTGGTGGTA-3'). The multiplicity of infection (MOI) was calculated using the following formula: number of cells (1×10^5) * dilution factor (1 mL/ μ L viral preparation) * VCN. VCN of transduced cells was calculated by relating the difference of their Psi vs. PCBP2 signal to the value of copy controls signals using a dependency curve.

4.2.3. MEL Cell Culture and Differentiation

Murine erythroleukemia cells (MEL) were grown in RPMI (Life Technologies, Carlsbad, CA, USA), supplemented with 10% FBS (Hyclone, South Logan, UT, USA) and 1% P/S (Life Technologies, Carlsbad, CA, USA). Erythroid differentiation was achieved by seeding 2×10^6 cells on Day 1, in log phase, into 3 mL of fresh culture media containing HMBA, at a final concentration of 1 mg/mL. Spent media was replaced on Day 3 and Day 5 and cells were collected for analysis on Day 7.

4.2.4. Cell Culture and Transduction of HUDEP Cell Lines

HUDEP-2 parental cell line and established clones were cultured as per section 3.2.1. HUDEP M#9 cells in expansion were transduced with lentiviral vectors expressing β -globin gene and/or miRNA targeting BCL11A in the presence of 8 $\mu\text{g/mL}$ polybrene (Millipore, Burlington, MA, USA) for 6 hours at 37°C and 5% CO_2 , at a low MOI (<10) to reach a low integration rate ($\leq 3\text{--}4$ copies). After transduction, cells were seeded into fresh expansion medium and cultured for 2 more days, then cells were induced to terminally differentiate over 6-7 days in Phases II and III as per section 3.2.1. Cells were collected on Day 6-7, the differentiation level was assessed by benzidine (Sigma, St Louis, MO, USA) staining, and hemoglobin synthesis, VCN, and gene and protein expression were assessed by HPLC, ddPCR, qPCR, and WB, respectively.

4.2.5. Cell Culture and Transduction of Patient Derived CD34⁺ Cells

Non-mobilized CD34⁺ cells were isolated from whole blood samples by immunomagnetic-separation using the CD34 MicroBead Kit (Miltenyi Biotec Inc., Auburn, CA, USA). CD34⁺ cells were kept undifferentiated with bi or tri-weekly media changes at a cell density $<0.5 \times 10^6$ cells/mL in SFEM (Stemcell Technologies, Vancouver, Canada) supplemented with 10 $\mu\text{L/mL}$ CC100 (Stemcell Technologies, Vancouver, Canada), 2 U/mL EPO (Amgen, Thousand Oaks, CA, USA), 10^{-6} M DEXA (Sigma, St. Louis, MO, USA), and 1% P/S (Life Technologies, Carlsbad, CA, USA). Cells in the expansion phase were frozen after 6–10 days in freezing media containing 50% characterized FBS (Hyclone, Logan, UT, USA), 10% dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA), and 40% IMDM (Life Technologies, Carlsbad, CA, USA). Once thawed in thawing medium (IMDM (Life Technologies, Carlsbad, CA, USA) supplemented with 5% characterized FBS (Hyclone, Logan, UT, USA), cells within the expansion phase were transduced with lentiviral vectors at a low MOI (<10) to reach a low integration rate ($\leq 3\text{--}4$ copies). After transduction, cells were seeded into fresh expansion medium and cultured for 2 more days, then they were induced to terminally differentiate over 7 days. Differentiation medium was prepared using IMDM (Life Technologies, Carlsbad, CA, USA) supplemented with 3% human AB serum (Atlanta Biologicals, Flowery Branch, GA, USA), 2% human plasma (Stemcell Technologies, Vancouver, Canada), 10 $\mu\text{g/mL}$ insulin (Sigma, St. Louis, MO, USA), 200 $\mu\text{g/mL}$ TF (Athens Research & Technology, Athens, GA, USA), 10 ng/mL hSCF (Peprotech, Rocky Hill, NJ, USA), 3 U/mL EPO (Amgen, Thousand Oaks, CA, USA), and 1% P/S (Life Technologies, Carlsbad, CA, USA).

The differentiation level was assessed by benzidine (Sigma, St Louis, MO, USA) staining, and hemoglobin synthesis, VCN, and gene expression were assessed by HPLC, ddPCR, and qPCR, respectively.

4.2.6. Single Chain Analysis by HPLC

Single-chain quantification was assessed via reversed-phase HPLC. Hb samples were injected on a Hitachi D-7000 HSM Series apparatus (Hitachi Instruments, San Jose, CA, USA) using a Zorbax 5 μ m 300SB-C8 300 Å, LC 150 x 2.1 mm column (Agilent Technologies, Santa Clara, CA, USA) and a gradient from 20% to 60% acetonitrile in 0.1% trifluoroacetic acid in 25 minutes, with UV detection at 215 nm.

4.2.7. Analysis by RT-PCR, qPCR and ddPCR

Genomic DNA was precipitated with phenol, chloroform, and isoamyl alcohol (25:24:1) saturated with 10 mM Tris (pH 8.0) and 1 mM EDTA (Sigma, St. Louis, MO, USA), and it was then analyzed by qPCR and ddPCR for VCN and by real-time PCR (RT-PCR) for the transgene mutation.

RNA was isolated using Trizol Reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and cDNA was prepared using one step qScript™ XLT cDNA SuperMix kit (Quantabio, Beverly, MA, USA). The procedure followed the manufacturer's recommendations. The resulting cDNA was used in subsequent PCR, qPCR, and ddPCR reactions. qPCR was performed using qPCR reactions prepared with Power SYBR Green (ThermoFisher Scientific, Waltham, MA, USA) and specific primers and were run on a ViiA7 Real-Time qPCR instrument (Thermo Fisher Scientific, Waltham, MA, USA). The sequences of the oligos used in RT-PCR and qPCR are summarized in Table 4.

Table 4-- Sequences of primers used in RT-PCR and qPCR to amplify β -globin, Sil β -globin, γ -globin and β -actin.

Target	Forward Primer 5' – 3'	Reverse Primer 5' – 3'
Silβ-globin	GTGGATGAAGTCGGCGGC	GGAGAAGGCACCGAGCAC
WTβ-globin	GTGGATGAAGTTGGTGGT	ACTAAAGGCACCGAGCAC
γ-globin	TGGCCTGTGGAGTAAGGTCAA	GAAGCAGAGGACAAGTTCCCA
β-actin	GTTTGAGACCTTCAACCCAGCC	TACATGGGCACAATGTGGGTGA

ddPCR reaction used specific, exon-spanning primers and fluorescently labeled probes (Biorad) allowing for specific detection of vector-derived, T87Q, Sickie, or β -globin expression using Biorad ddPCR system. The sequences of the oligos used in ddPCR targeted specific sequence in human, vector-derived, T87Q β -globin (FW: 5'-CTGGCTCACCTGGACAA-3'; Rev: 5'-CAGGAGCCTGAAGTTCTCA-3'; Probe: 5'-CACCTTTGCCCAGCTGAGTGAG-3'), human SCD β -globin (FW: 5'-CCAGCAGCCTGCCCAG -3'; Rev: 5'-ATCTATTGCTTACATTTGCTTCTGACA-3'; Probe: 5'-AGACTTCTCCACAGGAGT-3') and human γ -globin (FW: 5'-AGGAGGACAAGGCTACTATCACAAG -3'; Rev: 5'-AGGAGCCTTCCCAGGGTTT -3'; Probe: 5'-CAAGGTGAATGTGGAAGAT-3') referred to human α -globin as an endogenous control (FW: 5'-GCCCTGGAGAGGATGTTTCCT-3'; Rev: 5'-TCGAAGTGCGGGAAGTAGGT -3'); Probe: 5'-TCCTTCCCCACCAACAA-3').

4.2.8. In Vitro RBC Sickling and Morphological Analysis

The degree of cell sickling in the experimental samples was accessed as per section 3.2.5.

4.2.9. Statistical Analyses

Statistical significance was assessed by computing means with one-way Anova test followed by a Tukey test to compare every mean with every other mean.

All tests were done using GraphPad Prism software, version 7.0. The software package was used for statistical analysis.

Table 5 - Sequences of BCL11A miRNAs in ATM1, ATM1.1, ATM1.2, ATM1.3 and ATM1.4 constructs.

ATM1	...TTGCTGTTGACAGTGAGCG- - - - gatcgagtggtgaataatgataGGGTGAAGCCACAGATGCCatcattattcaacactcgatc- - - -TGCCTACTGC..
ATM1.1	...TTGCTGTTGACAGTGAGCG- gcgc gatcgagtggtgaataa- - - -GGGTGAAGCCACAGATGCC- - - ttattcaacactcgatc gcgc -TGCCTACTGC...
ATM1.2	...TTGCTGTTGACAGTGAGCG- - - ag atcgagtggtgaataatgat -GGGTGAAGCCACAGATGCC- atcattattcaacactcgatc c - - -TGCCTACTGC...
ATM1.3	...TTGCTGTTGACAGTGAGCG- - - - gatcgagtggtgaataatgata T AGTGAAGCCACAGATG T atcattattcaacactcgatc- - - -TGCCTACTGC...
ATM1.4	...TTGCTGTTGACAGTGAGCG agcgc gatcgagtggtgaataa - - - T AGTGAAGCCACAGATG TA - - - ttattcaacactcgatc gcgc c TGCCTACTGC...

To generate proof-of-concept data and evaluate the ability to function as a screening system for therapeutic approaches, we transduced M#9 and M#13 with ALS10-T87Q and one of the ATM vectors. The HbA^{T87Q} generated by both the single and double vectors and the HbF induced by the ATM vector were distinguishable from the Hb-mutant produced by M#9 and M#13 by tetrameric HPLC (Figure 16). Single-chain HPLC cannot distinguish HbA^{T87Q} from Hb-mutant, but it confirms the increase in γ -globin chains in samples treated with the ATM vector (Figure 17). Additionally, the single-chain data shows that the ratio between α -globin and β -globin like chains (β - and γ -globin) is maintained when cells are treated with either the single or the double vector (Figure 17). However, when assessing the relative production of HbA^{T87Q} and HbF in the ATM vector, M#9 showed a better dose/response correlation to the VCN than M#13 (Figure 18). For this reason, we chose M#9 as the system to screen cloning positions for the miR-E-BCL11A. M#13 was the system chosen to screen improved β -globin expressing lentiviral vectors, and those results were published by Breda et al. in 2021 [55].

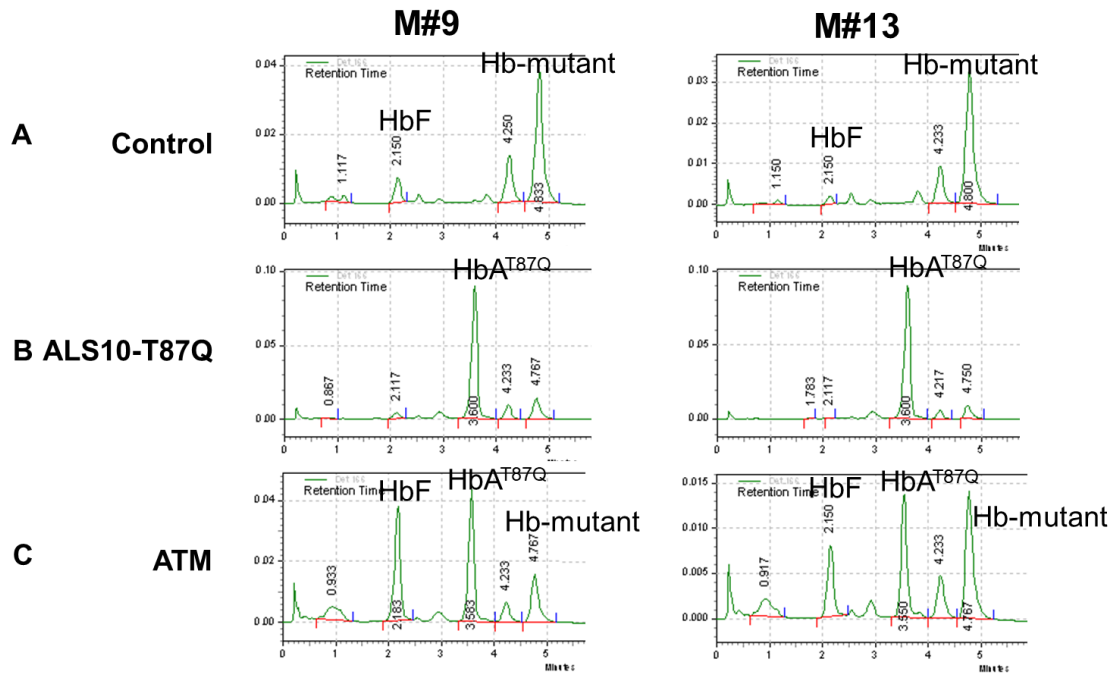


Figure 16 - Representative chromatographic separation (HPLC) of hemolysates

from (A) control/untreated M#9 (left) and M#13 (right) erythroblasts showing HbF, HbA² and Hb-mutant peaks at Day 7 of differentiation (top). (B) Representative chromatographic separation of M#9 and M#13 cell lines after transduction with ALS10-T87Q which produced HbA^{T87Q}. (C) Representative chromatographic separation of M#9 and M#13 cell lines after transduction with one of the vectors that simultaneously produced HbA^{T87Q} and induced HbF expression.

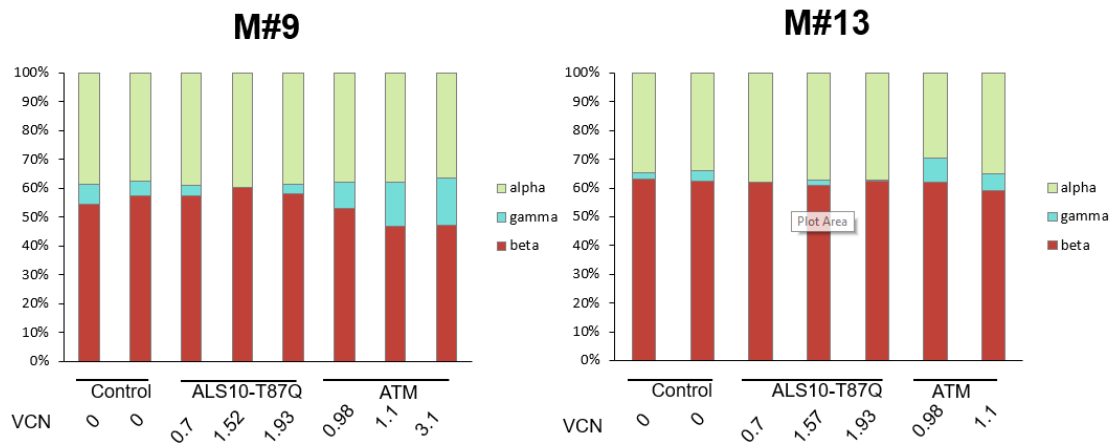


Figure 17 - Relative proportion of α , β , and γ chains in M#9, and M#13:

control/untreated, transduced with ALS10-T87Q, or transduced with ATM, with increasing VCN, quantified by single-chain HPLC at Day 7 of differentiation.

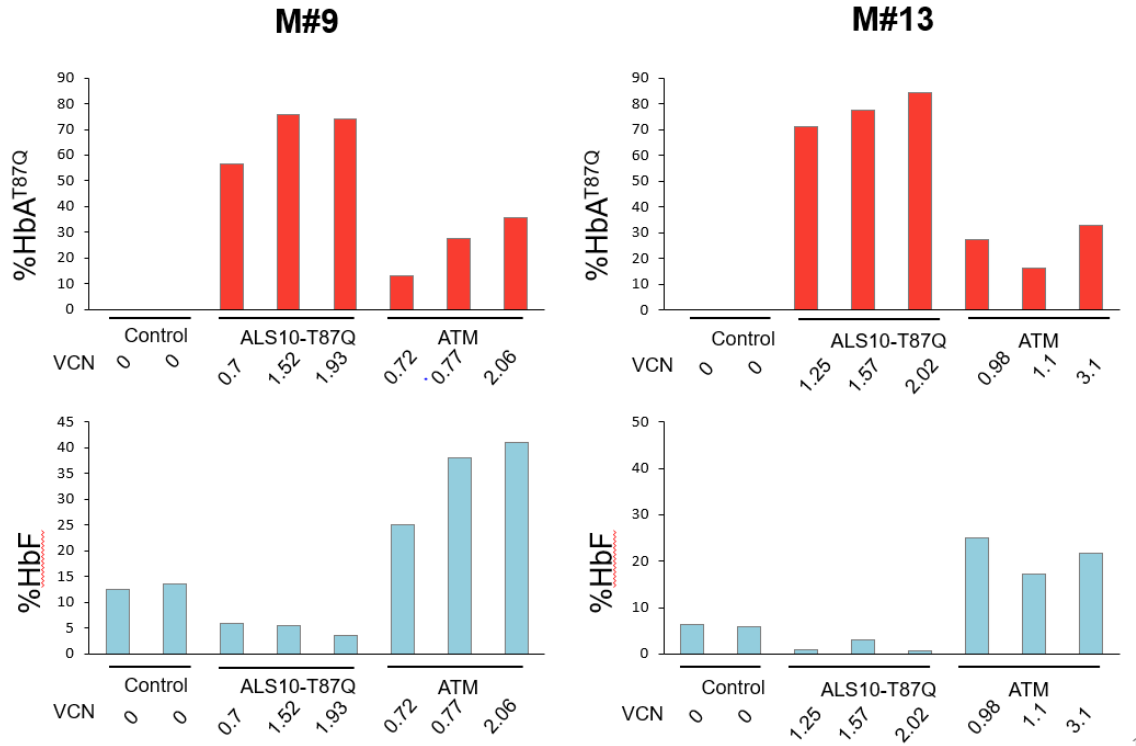


Figure 18 - Relative proportion of HbA^{T87Q} and HbF, in M#9 and M#13, with increasing VCN, quantified by HPLC at Day 7 of differentiation.

Upon transduction of M#9, we observed a dose-dependent response in ALS10-T87Q and the double vectors ATM2.1, ATM2.2 and ATM1 (Figure 19). Linear regression analysis of the data points for ALS10-T87Q showed that HbF+HbA^{T87Q} production was 30% per 1 integrated copy (Table 6). Comparatively, the double vectors induced 21.1%, 18.2% and 26.7% HbA^{T87Q}+HbF, respectively (Table 6). Overall, the percentage of curative hemoglobins produced by the double vectors was lower than that observed for the single vector ALS10-T87Q. Analysis of single data points showed that the production of HbA^{T87Q} was severely impaired by adding the miR-E-BCL11A (Figure 20). Additionally, the low levels of HbF induced by ATM2.1, ATM2.2 and ATM1 (Figure 20) led to a total %HbA^{T87Q}+HbF induction lower or equivalent to the total HbA^{T87Q} produced by ALS10-T87Q (Figure 20). Moreover, cloning the miR-E-BCL11A on the 3'UTR of the beta-globin gene was detrimental to the expression of β -globin with no HbA^{T87Q} produced (data not shown).

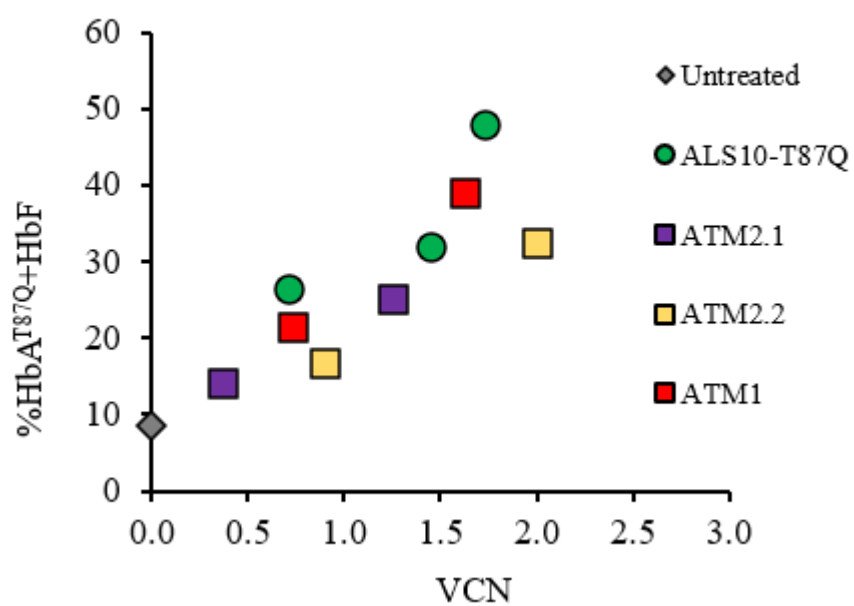


Figure 19 - Percentage of total curative hemoglobins (HbA^{T87Q}+HbF) over a range of viral integrations after transducing M#9 cells.

Table 6 - Results of mixed-effects linear regression models to identify the percentage of curative HbA^{T87Q}+HbF produced by ALS10-T87Q, ATM2.1, ATM2.2 and ATM1 at VCN=1.

	Equation	%HbA ^{T87Q} +%HbF VCN = 1	Compared to ALS10 – T87Q [(HbA ^{T87Q} +HbF)/HbA ^{T87Q}]%
ALS10-T87Q	$Y = 18.32 \cdot X + 11.66$	30.0%	100%
ATM2.1	$Y = 14.63 \cdot X + 6.517$	21.1%	70.3%
ATM2.2	$Y = 14.06 \cdot X + 4.123$	18.2%	60.7%
ATM1	$Y = 19.43 \cdot X + 7.254$	26.7%	89.0%

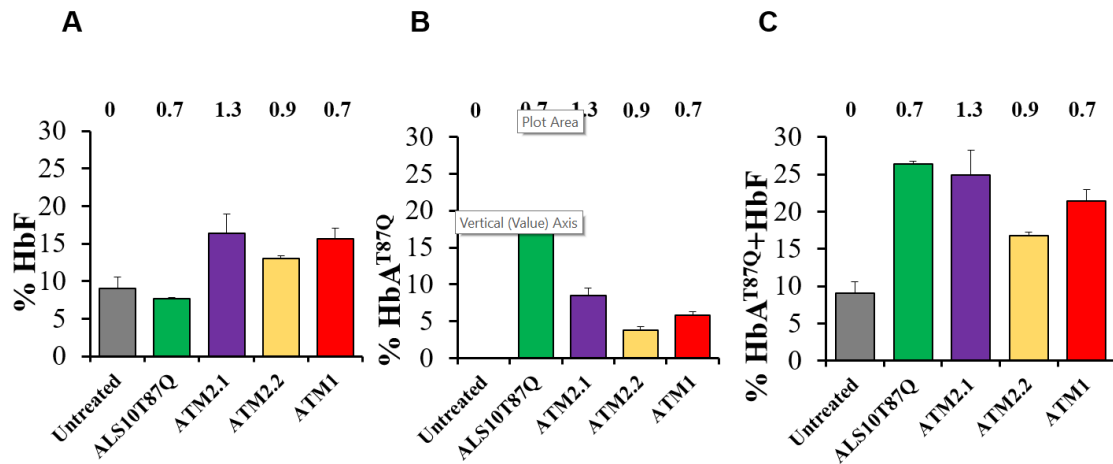


Figure 20 - Screening of positions for miR-E-BCL11A within the ALS10-T87Q vector.

(A) Percentage of HbA^{T87Q} (B) HbF and (C) HbA^{T87Q}+HbF levels quantified by HPLC over the number of viral integrations per cell after transduction of M#9 with ALS10-T87Q, ATM2.1, ATM2.2 or ATM1. Data are indicated as the mean ± SD, n=3.

WB analyses showed a clear increase in the levels of γ -globin in the samples treated with ATM2.1, ATM2.2, and ATM1 (Figure 21). However, the modest knockdown of BCL11A observed explains the overall low HbF levels observed in the samples treated with ATM2.1, ATM2.2, and ATM1 (Figure 21). This data suggests that including the miR-E-BCL11A in intron 1 and 2 was suboptimal, as it lessened the production of HbA^{T87Q} and the miRNA processing efficiency.

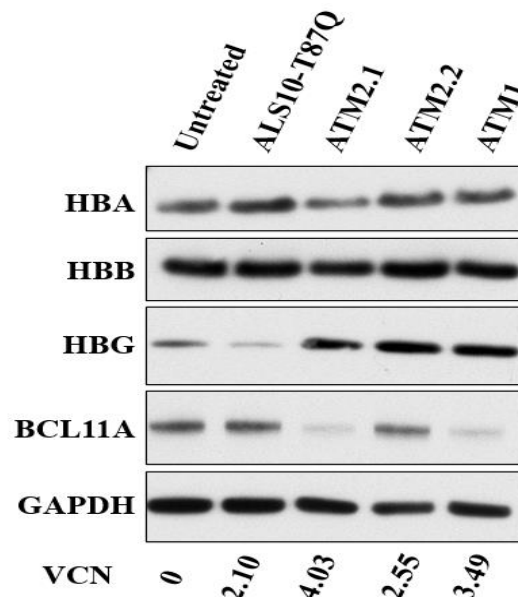


Figure 21 - Representative WB showing HBA, HBB, HBG and BCL11A proteins in differentiated M#9 cells at Day 7 of differentiation.

4.3.2. Generation of Improved Double-pronged Lentiviral Vectors with Modified Secondary Structures of miR-E-BCL11A

ATM1 showed the most promising results inducing 89% of the %HbA^{T87Q}+HbF produced by ALS10-T87Q, while ATM2.1 and ATM2.2 only produced 70.3% and 60.7% (Table 6), respectively. To overcome the limitations previously shown by the dual vectors, we further modified the secondary structure of miR-E-BCL11A in ATM1 (Figure 22 and Table 7). We tested four different modifications of ATM1: 1) ATM1.1 differed from ATM1 in the last four bases of the guide to achieve higher 3'-end stability and adapt from shRNA to a miR-based design; 2) ATM1.2 included an additional mismatch at the base of the guide that creates a "bulge" in the design to enhance pri-miRNA recognition by Drosha [54]; 3) ATM1.3 included a 2bp modification of the loop of the miR-30 scaffold itself to promote more precise strand selection by Dicer and improve performance; and 4) ATM1.4 included the 3 changes introduced in ATM1.1-3 into a single vector.

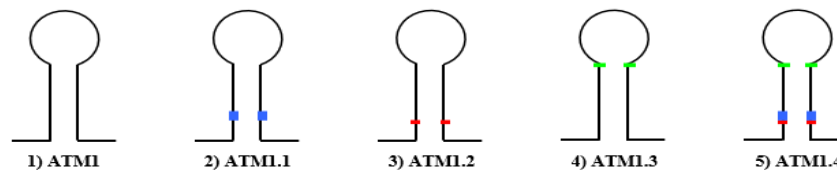


Figure 22 - Optimization of BCL11A miRNA design.

Illustration of changes introduced into BCL11A miRNA sequence of ATM1 vector. ATM1.1 differs from ATM1 in the last 4 bases of the guide and passenger strands. ATM1.2 contains 1 basepair (bp) mismatch between the guide and the passenger creating a "bulge" in the design. ATM1.3 presents a 2 bp modification of the published miR-30 loop. The modifications tested in ATM1.1, -1.2 and -1.3 were combined in the final design of ATM1.4.

Table 7 - Results of mixed-effects linear regression models of % of curative HbA^{T87Q}+HbF obtained with ALS10-T87Q, ATM1, ATM1.1, ATM1.2, ATM1.3 or ATM1.4 in M#9 treated cells.

	Equation	R ²	%HbF+%HbA ^{T87Q} VCN = 1	%HbF+%HbA VCN = 2	Compared to ALS10-T87Q [(HbA ^{T87Q} +HbF)/HbA ^{T87Q}]% VCN = 2
ALS10-T87Q	Y = 16.18*X + 15.81	0.9919	32.0%	48.2	100%
ATM1	Y = 16.48*X + 9.581	0.9985	26.1%	42.5	81.6%
ATM1.1	Y = 21.85*X + 20.94	0.9996	42.8%	64.6	133.8%
ATM1.2	Y = 16.51*X + 14.2	0.9426	30.7%	47.2	96.0%
ATM1.3	Y = 16.12*X + 14.01	0.9927	30.1%	46.3	94.1%
ATM1.4	Y = 14.06*X + 24.34	0.9972	38.4%	52.5	120%

4.3.3. Improved Dual Vectors Induce HbA^{T87Q}+HbF Levels Higher than the Total HbA^{T87Q} Produced by ALS10-T87Q

The transduction of M#9 cells with the modified lentiviral vectors showed ATM1.1 to be the most potent of the ATM1 vectors. WB analyses confirmed functional repression of BCL11A by the miR-E-BCL11A shRNA^{miR}, leading to a concurrent increase of γ -globin protein levels in samples treated with the ATM1 vectors but not in untreated or cells treated with ALS10-T87Q (Figure 23). Tetrameric HPLC analysis of lysates from M#9 cells treated with ATM1.1, ATM1.2, and ATM1.4, but not ATM1.3, showed increased levels of %HbA^{T87Q} in comparison to ATM1 both at low (0.6 ± 0.1) and high (2 ± 0.2) VCN. Moreover, the induction of HbF was the highest for ATM1.1 compared to the remaining ATM vectors (Figure 24). These results translated into ATM1.1 total HbA^{T87Q}+HbF being significantly higher than the HbA^{T87Q} induced by ALS10-T87Q (Figure 24). Linear regression analysis of the ratio of HbA^{T87Q}+HbF to VCN in a range from 0 to 5 (Figure 25) showed that ATM1.1 and ATM1.4 yield 133.8% and 120% more HbA^{T87Q}+HbF than ALS10-T87Q (Table 7).

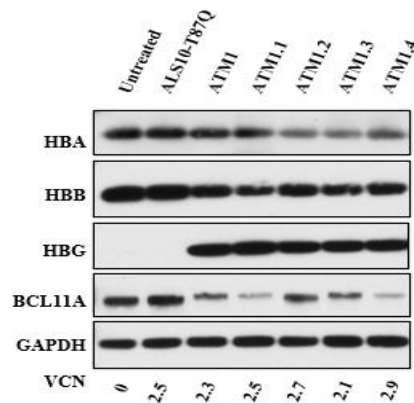


Figure 23 - Representative WB

showing side by side the HBA, HBB, HBG, and BCL11A expression in control and different vectors transduced M#9 cells at Day 7 of differentiation.

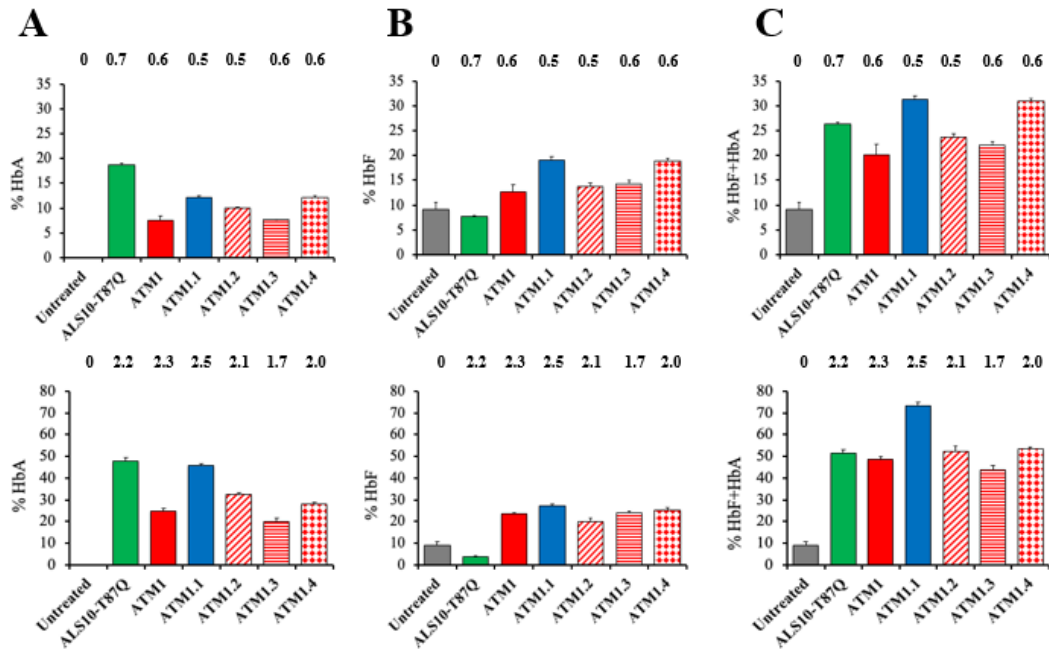


Figure 24 - Screening of vectors with optimized miR-E-BCL11A design.

(A) HbA^{T87Q} (B) HbF and (C) HbA^{T87Q}+HbF levels at low (0.6 ± 0.1 , top panels) or high (2.0 ± 0.3 , bottom panels) integrations per cell after transduction of M#9 with ALS10-T87Q, ATM1, and its variants ATM1.1, ATM1.2, ATM1.3, or ATM1.4. Data are the mean $\pm$ standard deviation, $n=3$.

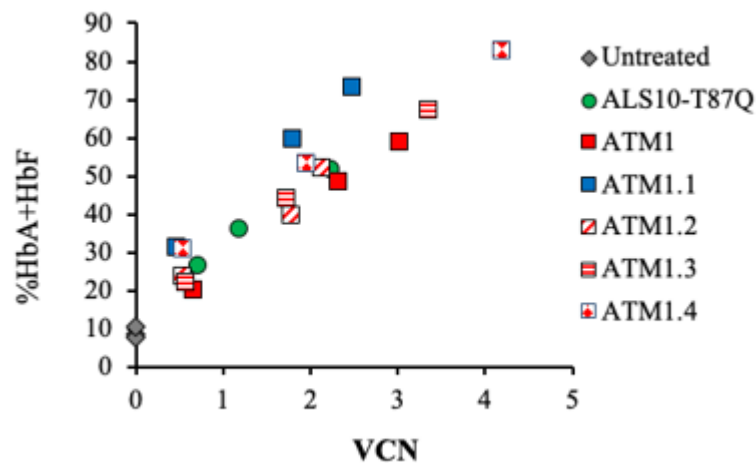


Figure 25 - Graphic representation of the proportion of curative HbA^{T87Q}+HbF hemoglobin

over a range VCN after transduction of M#9 with ALS10-T87Q, ATM1, ATM1.1, ATM1.2, ATM1.3, or ATM1.4. Data are represented as mean $\pm$ standard deviation, $n=3$

To further characterize the effects of the inclusion of miR-E-BCL11A in the ATM1.1, we introduced silent mutations in exon 1 (S1) and exon 2 (S2) (Figure 26) of the β -globin gene (Sil β -globin gene) of ALS10-T87Q and ATM1.1, allowing for detection of splicing variants and specific amplification of the endogenous and transgenic mRNA and DNA. We designed

oligonucleotides (Table 4) to target the variant sequences and confirmed the specificity of amplification by PCR analysis of the plasmids containing the ALS10-T87Q, ALS10-T87Q-silent, ATM1.1 and ATM1.1-silent sequences (Figure 27). MEL cells transduced with ALS10-T87Q, ALS10-T87Q-silent, ATM1.1 and ATM1.1-silent in VCN range of 0.5-7 copies/cell showed no difference between the WT and the silent vectors in dose/response relationship between VCN, the HbA tetramers (mouse α -globin/human β -globin-T87Q) (Figure 28), mHBA, hHBB and mBCL11A (Figure 29) and human β -globin (Figure 30).

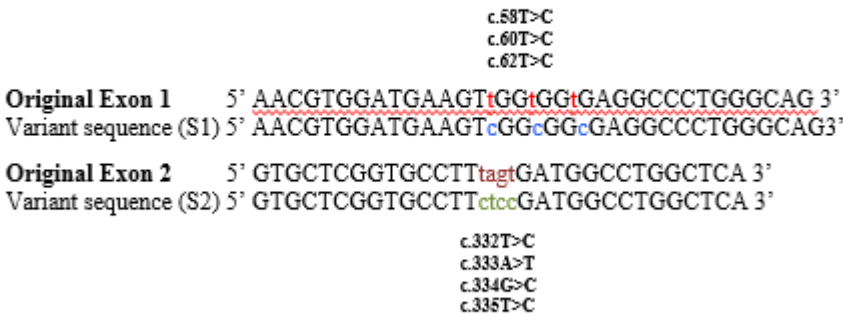


Figure 26 - Modifications of the sequences in exon 1 (top) and exon 2 (bottom) of the β -globin gene to generate the silent version of the β -globin gene.

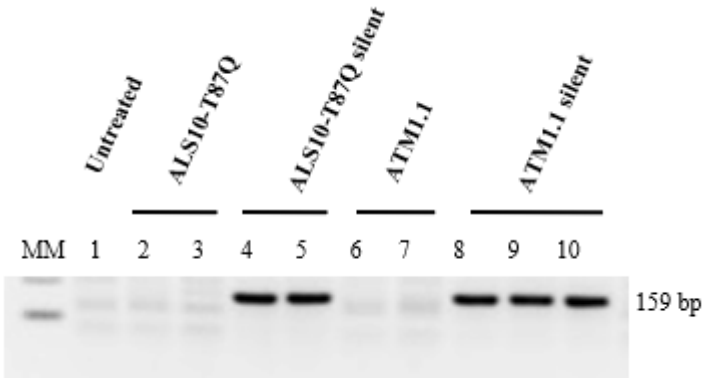


Figure 27 - Amplification of β -globin sequences carrying silent mutations on plasmids that contain the sequence of ALS10-T87Q, ALS10-T87Q-silent, ATM1.1 or ATM1.1-silent.

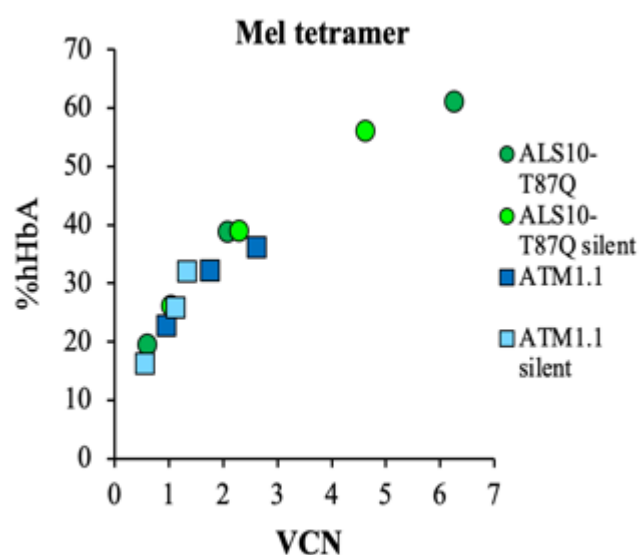


Figure 28 - Chimeric HbA (human β -globin/mouse α -globin tetramer) production following transduction with ALS10-T87Q, ALS10-T87Q-silent, ATM1.1 or ATM1.1-silent vectors in MEL cells, over range of viral integrations (VCN up to 7 copy/genome).

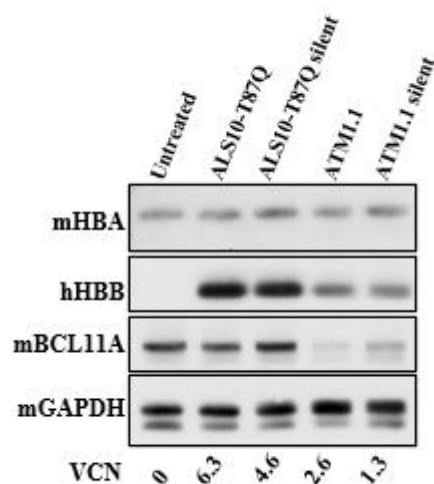


Figure 29 – Representative WB showing mouse HBA (mHBA), human HBB (hHBB) and mouse BCL11A (mBCL11A) protein levels in differentiated MEL cells treated with ALS10-T87Q, ALS10-T87Q-silent, ATM1.1 or ATM1.1-silent vectors.

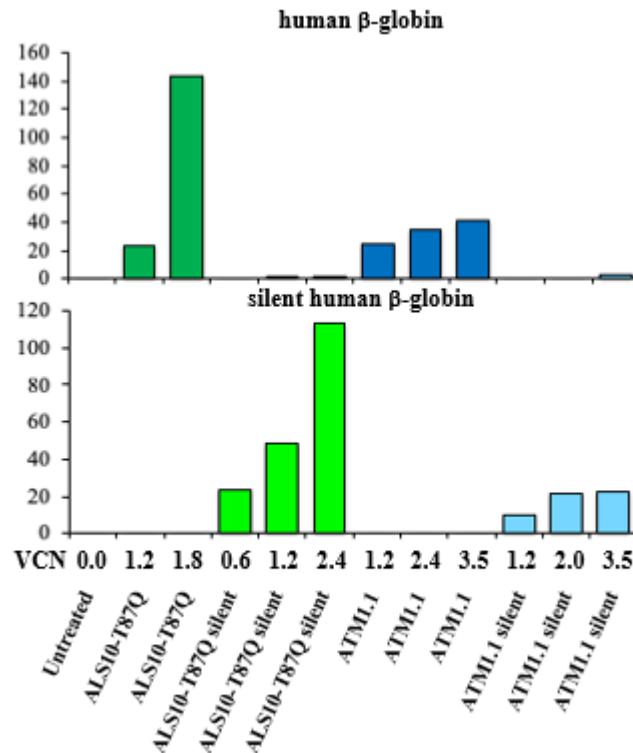


Figure 30 – Evaluation of gene expression of WT, β-globin, and silent-β-globin in untreated and transduced MEL cells

compared to the endogenous control mouse β-actin mRNA at different VCN.

To validate that the inclusion of the miR-E-BCL11A did not lead to recombinatory events in the mRNA or DNA of the ATM1.1, an additional proof-of-concept study was performed in SCD patient CD34⁺ cells treated with ALS10-T87Q, ALS10-T87Q-silent, ATM1.1 and ATM1.1-silent. In this study, we were able to selectively amplify SCD and β-globin-T87Q mRNA from the Siβ-globin mRNA encoded by the silent vectors (Figures 31 and 32), indicating that the presence of the miR-E-BCL11A in ATM1.1 does not affect the splicing nor lessen the production of the β-globin mRNA.

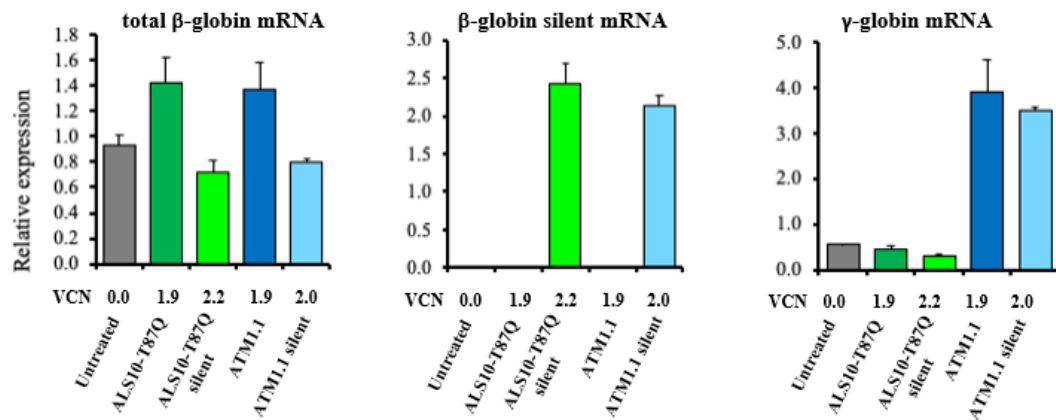


Figure 31 - Quantitative RT-PCR analysis of a SCD patient specimen.

The analysis includes quantification of the total β-globin (left panel: SCD β-globin mRNA, β-globin^{T87Q} or Silβ-globin transcribed by the vectors), the Silβ-globin (middle panel), and induced γ-globin mRNAs (right panel) and normalized to endogenous GAPDH mRNA.

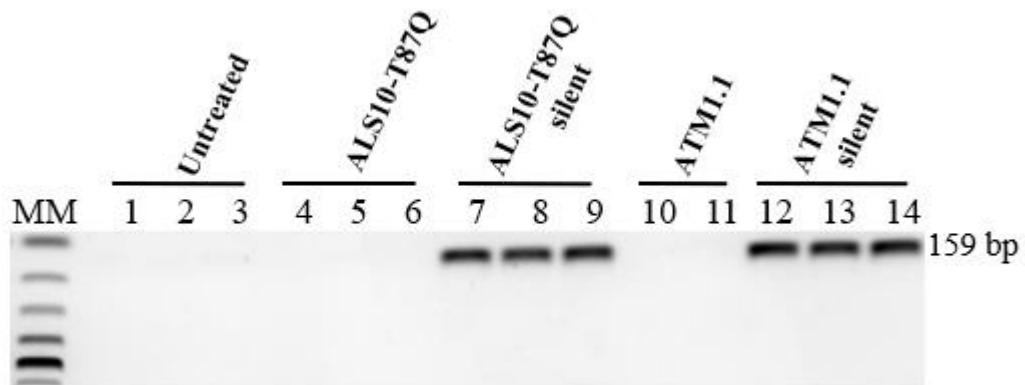


Figure 32 - Amplification of the β-globin region encompassing exon 1 (S1) and 2 (S2).

4.3.4. The Dual Vector ATM1.1 Outperforms the β -globin Vector in SCD and β^0/β^0 Primary Cells

To evaluate the potential use of these vectors in gene therapy for SCD and β -thalassemia, we transduced CD34⁺ hematopoietic progenitor cells isolated from peripheral blood of SCD and β^0/β^0 patients.

Consistent with the results obtained in M#9 cells, transduction of SCD erythroblasts with ALS10-T87Q, ATM1.1 and the corresponding silent vectors, resulted in a dose-dependent induction of HbA^{T87Q} and HbF (Figure 33A and 33B). As expected, treatment with the ALS10-T87Q vectors resulted in expression of HbA^{T87Q} but not HbF, while the ATM1.1 vectors led to the expression of both HbA^{T87Q} and HbF (Figure 33A and 33B). Overall, the concomitant induction of HbA^{T87Q}+HbF by the dual vectors ATM1.1 and ATM1.1-silent surpassed the single induction of HbA^{T87Q} by ALS10-T87Q and ALS10-T87Q-silent (Figure 33C). Linear regression analysis of the ratio of HbA^{T87Q}, HbF and HbA^{T87Q}+HbF (Tables 8, 9, 10) to VCN in a range from 0 to 3 showed an unexpected trend with the silent vectors outperforming the WT vectors. However, this tendency is likely due to the lower number of data points and, consequently, the lower statistical power of the data generated for the silent vectors. Nevertheless, direct comparison of the %HbA^{T87Q}+HbF at VCN=1 confirmed that the double vectors outperformed the single vectors with total yields of 100, 115, 115.4, and 138% for ALS10-T87Q, ALS10-T87Q-silent, ATM1.1 and with ATM1.1-silent.

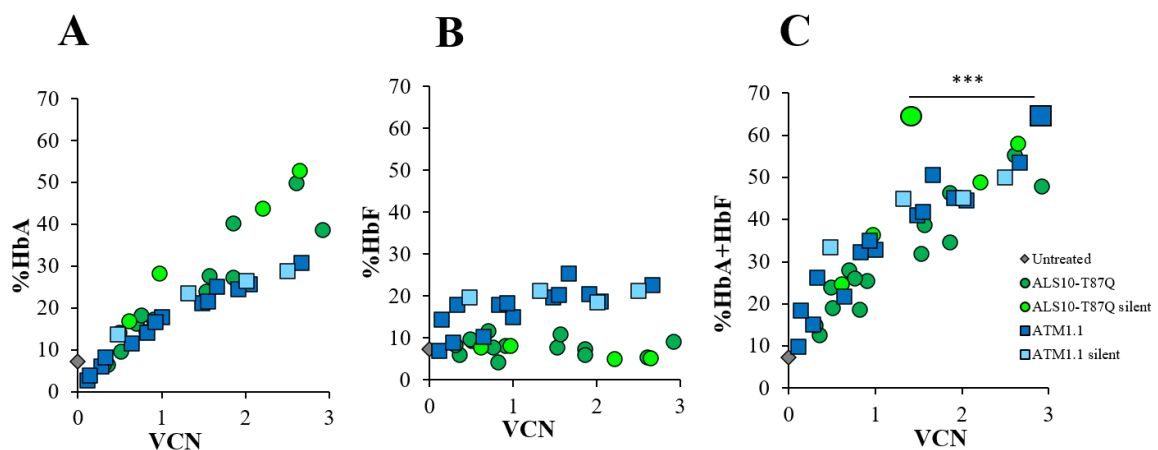


Figure 33 - Efficiency of ATM1.1 construct in sickle cell disease patients' cells.

Dose/response analyses obtained by plotting integration levels VCN against the percentage values of (A) HbA^{T87Q}, (B) HbF and (C) HbA^{T87Q}+HbF, respectively, in SCD erythroid cells untreated or treated with ALS10-T87Q, ALS10-T87Q-silent, ATM1.1, or ATM1.1-silent. Data are represented as mean $\pm$ standard deviation (SD), n=3.

Table 8 – Results of mixed-effects linear regression models of % of curative HbA^{T87Q} obtained in SCD cells treated with ALS10-T87Q, ALS10-T87Q-silent, ATM1.1 or ATM1.1-silent

HbA ^{T87Q}	Equation	R ²	%HbA ^{T87Q} VCN = 1	Compared to ALS10-T87Q
ALS10-T87Q	Y = 14.76*X + 4.082	0.8823	18.4%	100%
ALS10-T87Q silent	Y = 16.22*X + 9.314	0.9759	25.5%	139%
ATM1.1	Y = 10.99*X + 4.262	0.9592	15.3%	82%
ATM1.1 silent	Y = 7.324*X + 11.61	0.9312	18.9%	103%

Table 9 - Results of mixed-effects linear regression models of % of curative HbF obtained in SCD cells treated with ALS10-T87Q, ALS10-T87Q-silent, ATM1.1 or ATM1.1-silent.

HbF	Equation	R ²	%HbF VCN = 1	Compared to ALS10-T87Q
ALS10-T87Q	Y = -0.357*X + 8.39	0.02041	8.0%	100%
ALS10-T87Q silent	Y = -1.604*X + 9.08	0.9111	7.5%	109.2%
ATM1.1	Y = 5.101*X + 11.25	0.5878	16.4%	114.1%
ATM1.1 silent	Y = 0.211*X + 19.85	0.0204	20.1%	140.8%

Table 10 - Results of mixed-effects linear regression models of % of curative HbA^{T87Q}+HbF obtained in SCD cells treated with ALS10-T87Q, ALS10-T87Q-silent, ATM1.1 or ATM1.1-silent

HbF + HbA ^{T87Q}	Equation	R ²	%HbA ^{T87Q} +%HbF VCN = 1	Compared to ALS10-T87Q [(HbA ^{T87Q} +HbF)/HbA ^{T87Q}]%
ALS10-T87Q	Y = 14.45*X + 14.44	0.7692	28.89%	100%
ALS10-T87Q silent	Y = 15.76*X + 17.57	0.9101	33.33%	115%
ATM1.1	Y = 17.39*X + 15.96	0.7865	33.35%	115.4%
ATM1.1 silent	Y = 8.112*X + 31.75	0.7826	39.86%	138.0%

These results were corroborated by the relative expression data (Figure 34) which showed no significant difference of T87Q or SCD β -globin mRNA expression between the single and the double vectors (Figure 34A and 34B) while γ -globin mRNA expression was significantly higher in the double vectors when compared to untreated or treated with single vector samples (Figure 34C) but not significantly different between the two dual vectors. Together with the data generated in M#9 cells, this data provides further evidence that the introduction of the silent mutations does not affect the production of transgenic β -globin mRNA. WB analyses confirmed the induction of γ -globin chains and an incomplete suppression of BCL11A in SCD cells treated with ATM1.1 (Figure 35). The partial knockdown of BCL11A observed in cells treated with ATM1.1 could be advantageous considering reported evidence suggesting that disruption of BCL11A leads to an erythroid

differentiation defect or survival disadvantage [56, 65]. Morphological analysis of the SCD erythroblasts treated with ALS10-T87Q and ATM1.1 showed that when exposed to low oxygen tension, cells transduced with ATM1.1 were less prone to sickle than control cells (Figure 36). ATM1.1 treated cells showed the lowest percentage of sickle-like morphology (58.1%) when compared to cells transduced with ALS10-T87Q (81.9%) (Figure 37).

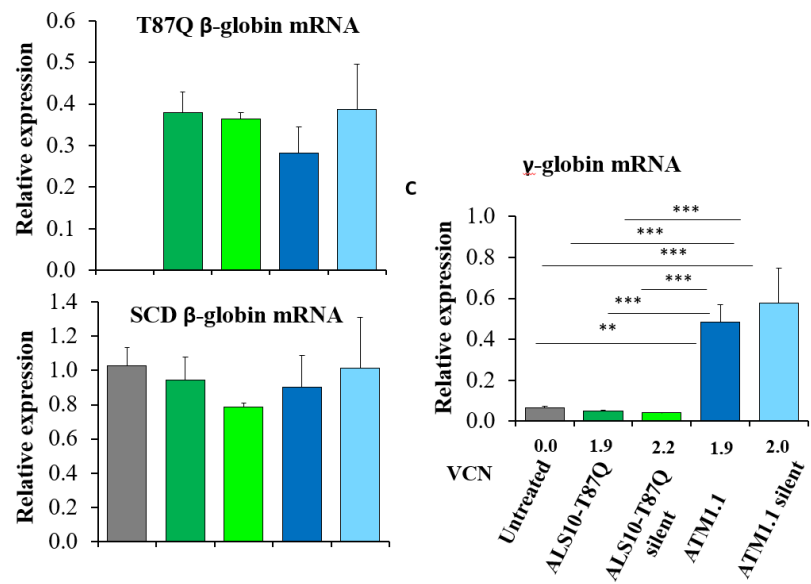


Figure 34 - Quantification by ddPCR of vector-derived

(A) T87Q β-globin, (B) sickle β-globin and (C) γ-globin mRNAs and normalized to endogenous α-globin mRNA

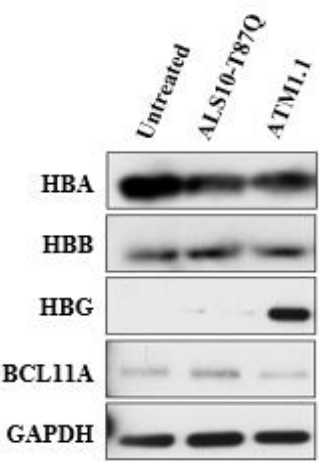


Figure 35 - Representative WB showing the HBA, HBB, HBG and BCL11A protein content

in differentiated SCD cells untreated or treated with ALS10-T87Q or ATM1.1.

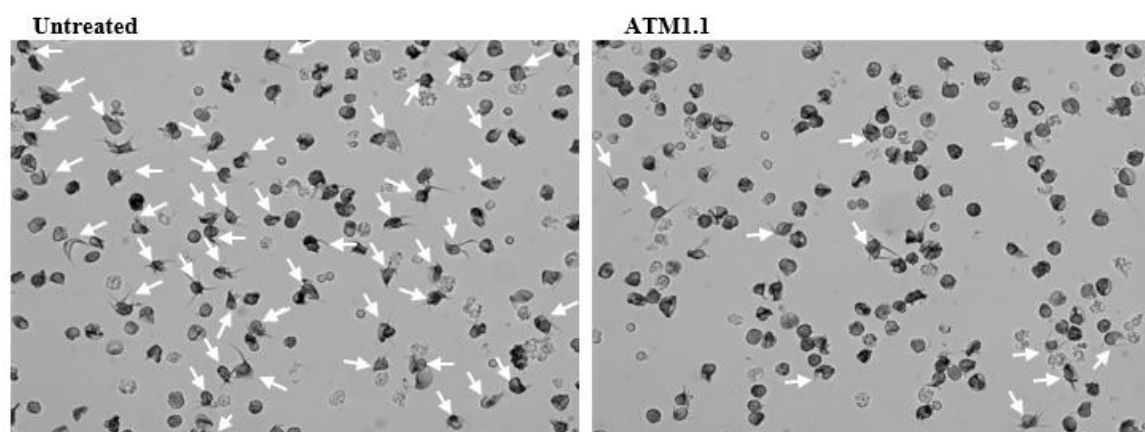


Figure 36 - Representative image of patient derived SCD erythroid cultures under hypoxia.
Arrows mark cells with sickle-like morphology.

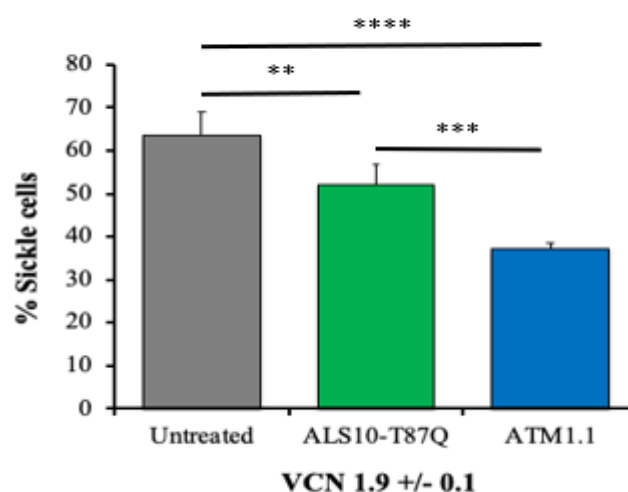


Figure 37 - Percentage of blind counts of sickle-shaped cells exposed to hypoxia in four fields
from untransduced SCD erythroid cells or after treatment with ALS10- T87Q or ATM1.1 at VCN 1.9±0.1. Averages and SD are indicated.

In addition to SCD erythroblasts, we tested ALS10-T87Q and ATM1.1 in $\beta 0/\beta 0$ thalassemic patients' cells. Due to the lack of β -globin being expressed in $\beta 0/\beta 0$ patients, untransduced cells expressed >90% HbF, with the remaining tetramers being attributed to either α -aggregates [45] or HbA₂ (Figure 38). For this reason, transduction of $\beta 0/\beta 0$ cells with ALS10-T87Q and ATM1.1 is uninformative when comparing the potency of the single versus dual vectors. In both cases, the total HbA^{T87Q}+HbF was close to 100% with ALS10-T87Q yielding higher levels of HbA^{T87Q} and HbF being highest expressed in ATM1.1 treated

cells (Figure 39). The most enlightening piece of data generated was the change observed in α -globin aggregates (Figure 40). Transduction with ALS10-T87Q and ATM1.1 resulted in a decrease of the % α -globin aggregates, with ATM1.1-transduced cells showing the lowest levels. Such an observation suggests that treatment with ATM1.1 produces higher absolute values of curative β -like chains (β^{T87Q} - and γ -globins).

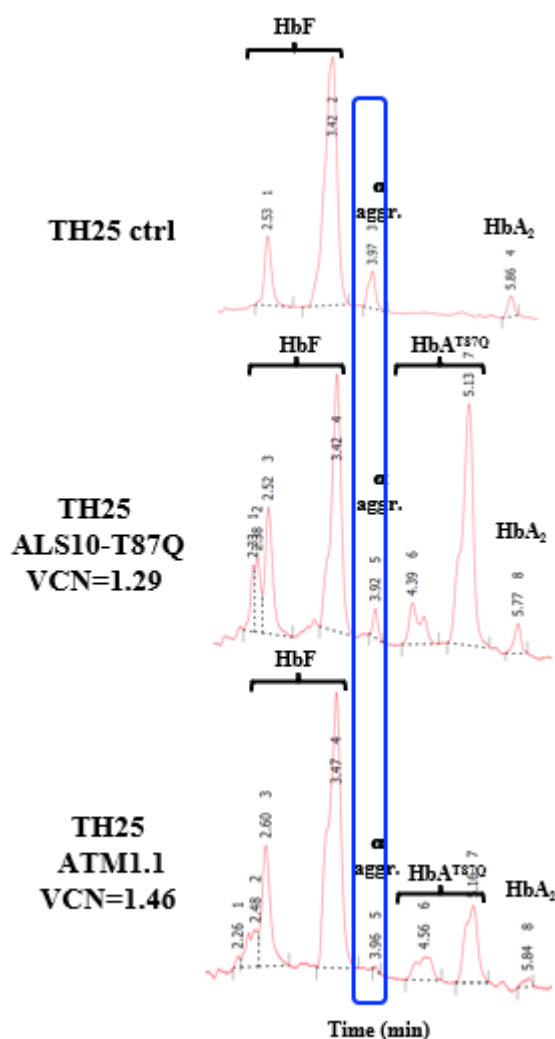


Figure 38 - Representative chromatographic separation (HPLC) of hemolysates from β^0/β^0 TH25 patient' cells. Control, untransduced (top), ALS10-T87Q (middle) and ATM1.1 (bottom) vector transduced erythroblasts show: HbF, (acetylated and main form), α -aggregates, HbA (acetylated and main form) and HbA₂ peaks at Day 7 of differentiation.

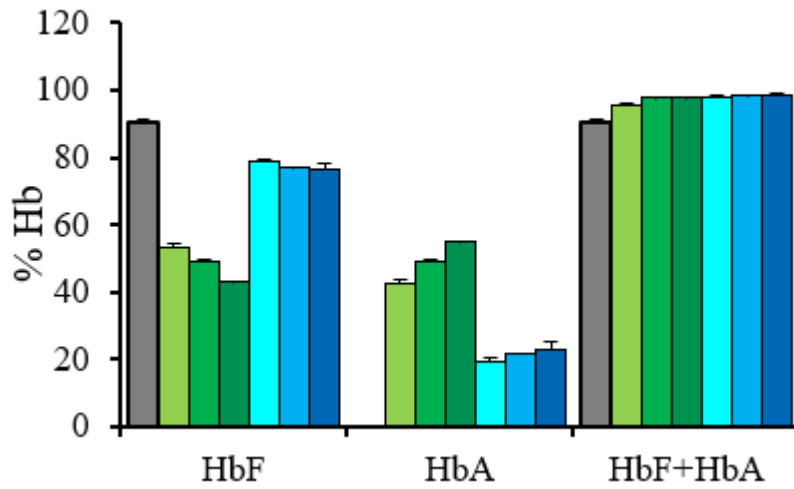


Figure 39 - Dose/response analyses obtained for HbF, HbA^{T87Q}, and HbA^{T87Q}+HbF, with increasing VCN, in patient-derived $\beta 0/\beta 0$ thalassemic erythroid cells untreated or treated with ALS10-T87Q, ATM1.1.

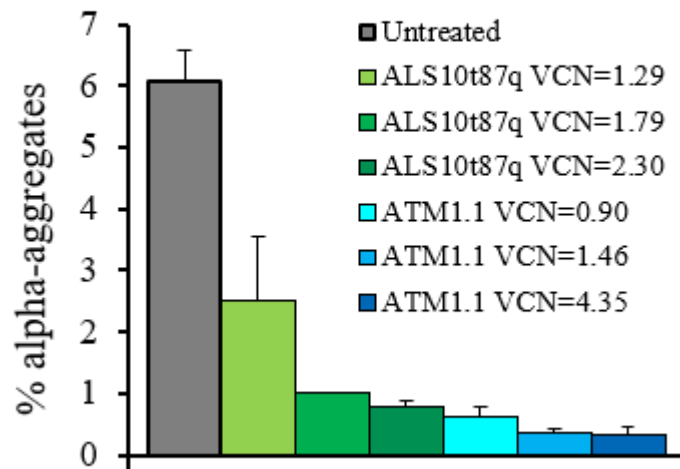


Figure 40 - Percentage of corresponding aggregates with increasing VCN, in patient-derived $\beta 0/\beta 0$ thalassemic erythroid cells untreated or treated with ALS10-T87Q, ATM1.1. Statistical significance was assessed by computing means with one-way Anova test followed by a Tukey test to compare every mean with every other mean.

4.4. Discussion

The development of dual-pronged vectors aimed to establish a “threefold” approach consisting in the production of curative HbA and HbF while also reducing the production of mutant β -globin RNA and protein for the cure of SCD and β -thalassemia. The initial strategy consisted of combining in a single lentiviral vector, a transgenic β -globin gene and the ZF-Ldb1 transcription factor fusion protein. Both approaches had proven successful when used independently [45, 53] and combining them emerged as a response to the need for more powerful and versatile vectors to achieve therapeutic levels in the most severe genotypes, with minimal VCN per cell. After testing multiple iterations of ALS10-T87Q combined with pCL20AnkGG1G without success, it became imperative to find options that could still satisfy the threefold approach. The use of shRNA^{miR} sequences, occurred as an innovative approach that fulfilled our requirement to reduce the overall size of the double vectors and BCL11A appeared the most obvious option being one of the dominant elements involved in the repression of γ -globin [15, 16, 17]. For the construction of the next generation of double vectors, we incorporated in ALS10-T87Q, a modification of the shRNA^{miR} sequences targeting BCL11A [33,58,59] developed by Guda et al. and further characterized in Bredel et al., by replacing the flanking regions with an optimized backbone termed “miR-E” [55]. Our strategy and vector configuration underwent several stages of development to address a variety of limitations. As a first step, we screened for the optimal position for the miR-E-BCL11A within the ALS10-T87Q. Although not optimal, cloning miR-E-BCL11A in Intron 1 (ATM1) of the β -globin gene produced the highest levels of curative hemoglobins. Then, we optimized ATM1 by introducing several modifications to the miR-E-BCL11A. With this stepwise approach, we identified a double vector which expressed HbA^{T87Q}+HbF at higher levels than the HbA levels produced by ALS10-T87Q.

Our results are proof-of-concept that combined strategies are feasible and can outperform the current single-pronged methods used in clinical trials. With ATM1.1, we showed that HbA and HbF can be elevated simultaneously using a single lentiviral construct surpassing the HbA production of the corresponding single vector ALS10-T87Q. With this approach, we expect to address the unmet need for more powerful and versatile vectors. Such vectors are expected to maximize efficiency and decrease the risk of genotoxicity associated with increasing VCN by successfully treating the most severe genotypes with minimal integrations per genome. Moreover, we expect that in addition to β -globinopathies, many other genetic disorders can benefit from the combination of gene transfer with RNA interference as one single therapeutic approach.

The idea of combining LV-driven HbA production with HbF inducers has been under consideration for over a decade [64], while recently, the combination of HbF inducers and

gene editing of a β -thalassemia mutation has been reported [67]. However, such combined strategies require co-treatment protocols of autologous HSCT and pharmacologic induction of HbF. Our bifunctional vector provides an identical therapeutic effect while bypassing the need for life-long treatment with HbF inducers.

Since the publication of our study, the field has been progressing, and several multiplex approaches have been reported. In parallel to our study, Nualkaew et al., reported the inclusion of a miR30-shRNA targeting the α 2-globin mRNA in intro 2 of BB305, the LV vector of Zynteglo and Lyfgenia [68]. While the LV β -sh α 2 vector improved α/β ratios, it does not fully address the need for increased Hb absolute values in the more severe phenotypes. By generating a vector that leads to higher levels of β -like chains, we also showed an indirect decrease of α -aggregates without targeting the α -globin mRNA. Brusson et al. [61], inserted a shRNA^{miR} targeting the exon 2 of the β -globin gene (miRHBB) into the β^{AS3} LV vector. To specifically downregulate the endogenous β^S expression, the β^{AS3} LV vector was modified at the position recognized by the miRHBB. This strategy showed increased incorporation of β^{AS3} in Hb tetramers and better correction of the sickling phenotype compared with control cells. A similar strategy was used by Koniali et al. [62], who cloned a shRNA^{miR30} targetting the HBB^{IVSI-110(G>A)} splicing mutation into the intro 2 of the β^{AS3} LV vector. This strategy resulted in concomitant stable expression of β^{AS3} and the reduction of the aberrant HBB^{IVSI-110(G>A)} mRNA. Another example of a bifunctional lentiviral vector was reported by Liu et al. [60], which, unlike our ATM1.1, did not combine gene addition with gene silencing. Instead, it expressed two shRNA^{miRs} targeting the γ -globin repressors ZNF410 and BCL11A in a single lentiviral vector. The double shmiR (DS) LV showed a robust reduction of both ZNF410 and BCL11A proteins, resulting in a 10% increase of HbF compared to the vector targeting BCL11A only. While promising, this approach is limited by the maximum amount of HbF that can be induced in a cell. The advantage of using a vector that expresses exogenous β -globin such as ATM1.1, is that its expression and regulation are independent of the sequences of the endogenous β -globin locus. Alternatively, two therapeutic approaches combining LV-based gene addition and CRISPR-Cas9 gene editing were described by Ramadier et al. [69]. The LV vectors combined the *de novo* expression of β^{AS3} with the expression of a sgRNA that either introduced frameshift mutations in the HBB gene or disrupted the BCL11A binding site in the γ -globin promoters. However, the Cas9-GFP protein had to be transfected into the cells separately to induce gene editing.

Multiplex approaches have also been adopted for gene editing procedures. Han et al. [70], described the combination of two single gene editing strategies via CRISPR-Cas9 technology. Simultaneous gene editing of the BCL11A erythroid-specific enhancer and the BCL11A binding site on the γ -globin gene promoters resulted in higher γ -globin expression

without a major increase in off-target effects. However, other studies found that multiplex CRISPR-Cas9 generated chromosomal translocations in hematopoietic stem cells [71]. The genomic instability and genotoxicity of multiplex CRISPR-Cas9 is still an unresolved concern that warrants further research. Base editing is of great interest for multiplex strategies due to its lower level of genotoxicity. Zeng et al. [39], simultaneously targeted the BCL11A erythroid-specific enhancer and corrected the HBB-28A>G promoter mutation by A3A(N57Q)-BE3.

The possible combination of gene addition, silencing, and/or editing deserves further studies to determine the real impact on the management of SCD and β -thalassemia patients.

5. GENERAL DISCUSSION

Although three gene addition/editing therapeutic drugs have recently been approved for SCD and β -thalassemia, these therapies still face many challenges.

Autologous HSCT relies on ex vivo cell culture and efficient engraftment for maximum therapeutic effect. Minimal cytokine stimulation and short-term culture have shown the best results in maintaining the balance between efficient gene addition and robust engraftment of the gene-modified cells [63]. Another challenge is the short- and long-term toxicity in hematopoietic and non-hematopoietic tissues which can be alleviated by adjusting the dosage based on therapeutic levels required for disease conditioning or reduced-intensity conditioning (RIC). However, this risk mitigation may lead to lower levels of therapeutic effect and inefficient engraftment due to partial myeloablation. Since such an approach isn't feasible for patients with more severe phenotypes, alternatives are being explored that reduce toxicity without affecting engraftment levels. The use of Briquilimab (also known as JSP191) a monoclonal antibody targeting the cell surface receptor CD117 (c-Kit) is currently being evaluated in a phase I/II clinical trial (NCT05357482) with preliminary successful and safety outcomes as a nonmyeloablative agent in hematopoietic cell transplantation for SCD and β -thalassemia. As CD117 targets HSCs and hematopoietic progenitors but not lymphocytes, it serves as an optimal target for myeloablative conditioning without immunosuppression.

Another hurdle of ex vivo gene therapies is the high price which makes it inaccessible in developing countries where the vast majority of SCD and β -thalassemia patients reside. Even in developed countries, the exorbitant costs of commercialized gene therapy products preclude access to treatment for those in need. In Europe, Zynteglo was priced at 1.5 million euros which led to reimbursement negotiation failure and its commercialization arrest. Yet, after its approval by the FDA, it was then priced at \$2.8 million becoming the most expensive medicine in history.

In vivo gene therapy emerges as a platform with potential to circumvent some of the challenges described above. In vivo injection of integrating HDAd5/35⁺⁺ vectors resulted in stable γ -globin expression in a mouse model of thalassemia intermedia and non-human primates [72, 73, 74]. This platform avoids leukapheresis and its low yields, ex vivo cell manipulation, and its potential for reducing engraftment, myeloablation, and its cytotoxicity, as well as transplantation. This approach would not only simplify the current methodology but should also decrease the cost associated with clinical support required for HSCT.

The advancement of gene therapy/editing has promised a potential cure for SCD and β -thalassemia genetically. Nonetheless, despite the significant progress gene therapy/editing still has limitations. Continuous research is key and necessary to support the development

of newer and improved curative treatments. Epigenome editing, viral vs non-viral delivery systems, miRNA-based targeted therapeutics, antisense oligonucleotides, and engineering of the endogenous HBD promoter, are additional avenues of scientific innovation being explored with the final goal in mind of making gene therapy available for all patients with SCD and β -thalassemia.

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6. PEER-REVIEWED PUBLICATIONS

RED CELLS, IRON, AND ERYTHROPOIESIS

Forced chromatin looping raises fetal hemoglobin in adult sickle cells to higher levels than pharmacologic inducers

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Key Points

- Ldb1 transcription factor self-association domain fused to γ -globin promoter-specific ZF protein increases HbF, reduces HbS in hSCD cells.
- In vitro reactivation of HbF mediated by ZF-Ldb1 exceeds pharmacologic treatment in adult hSCD cells.

Overcoming the silencing of the fetal γ -globin gene has been a long-standing goal in the treatment of sickle cell disease (SCD). The major transcriptional enhancer of the β -globin locus, called the locus control region (LCR), dynamically interacts with the developmental stage-appropriate β -type globin genes via chromatin looping, a process requiring the protein Ldb1. In adult erythroid cells, the LCR can be redirected from the adult β - to the fetal γ -globin promoter by tethering Ldb1 to the human γ -globin promoter with custom-designed zinc finger (ZF) proteins (ZF-Ldb1), leading to reactivation of γ -globin gene expression. To compare this approach to pharmacologic reactivation of fetal hemoglobin (HbF), hematopoietic cells from patients with SCD were treated with a lentivirus expressing the ZF-Ldb1 or with chemical HbF inducers. The HbF increase in cells treated with ZF-Ldb1 was more than double that observed with decitabine and pomalidomide; butyrate had an intermediate effect whereas tranylcypromine and hydroxyurea showed relatively low HbF reactivation. ZF-Ldb1 showed comparatively little toxicity, and reduced sickle hemoglobin (HbS) synthesis as well as sickling of SCD erythroid cells under hypoxic conditions. The efficacy and low cytotoxicity of lentiviral-mediated ZF-Ldb1 gene transfer compared with the drug regimens support its therapeutic potential for the treatment of SCD. (*Blood*. 2016;128(8):1139-1143)

hemoglobin (HbS) synthesis as well as sickling of SCD erythroid cells under hypoxic conditions. The efficacy and low cytotoxicity of lentiviral-mediated ZF-Ldb1 gene transfer compared with the drug regimens support its therapeutic potential for the treatment of SCD. (*Blood*. 2016;128(8):1139-1143)

Introduction

Sickle cell disease (SCD) represents a major challenge and a growing health problem in Western countries, with ~300 000 births every year.¹ Altered deformability of sickle cells is caused by the polymerization of deoxygenated sickle hemoglobin (HbS), whose rate and extent are proportional to the extent and duration of hemoglobin (Hb) deoxygenation and the levels of fetal hemoglobin (HbF) in the erythrocyte.² As a consequence, patients experience vaso-occlusive crises and red cell destruction in the peripheral circulation.³ Since the discovery that patients with hereditary persistence of HbF have a more benign clinical course,⁴ considerable research has been devoted toward approaches to augment HbF levels in adult red blood cells. Hydroxyurea (HU) is the only approved drug to induce HbF.⁵ Although its use is associated with marked clinical improvement, patients' compliance and concerns about long-term effects hinder widespread utilization.⁶

Despite the improvement in the management of SCD, allogeneic hematopoietic stem cell transplantation remains the only curative option.⁷ New approaches from alternative donors showed promising results, however, they are limited to experimental protocols.⁸

Gene transfer using autologous bone marrow is a suitable option to cure SCD, and could resolve the issue of a matched donor and eliminate the risks of graft-versus-host disease.⁹ In recent clinical trials, the successful use of lentiviral vectors carrying the β - or γ -globin genes^{9,10} has opened the doors to other approaches, based on knockdown of γ -globin repressors and genome editing.¹¹⁻¹³

Study design

We have developed another approach in which long-range enhancer-promoter contacts can be established by zinc finger (ZF)-mediated tethering of the "looping factor" Ldb1, leading to transcriptional activation.¹⁴ In murine and human adult erythroid cells from healthy individuals, this approach was successful in activating embryonic and fetal globin genes, respectively, thus partially reversing the developmental gene switch.¹⁵

The aim of this study was to compare the forced chromatin-looping strategy to known pharmacologic HbF inducers in adult erythroid cells from patients with SCD.

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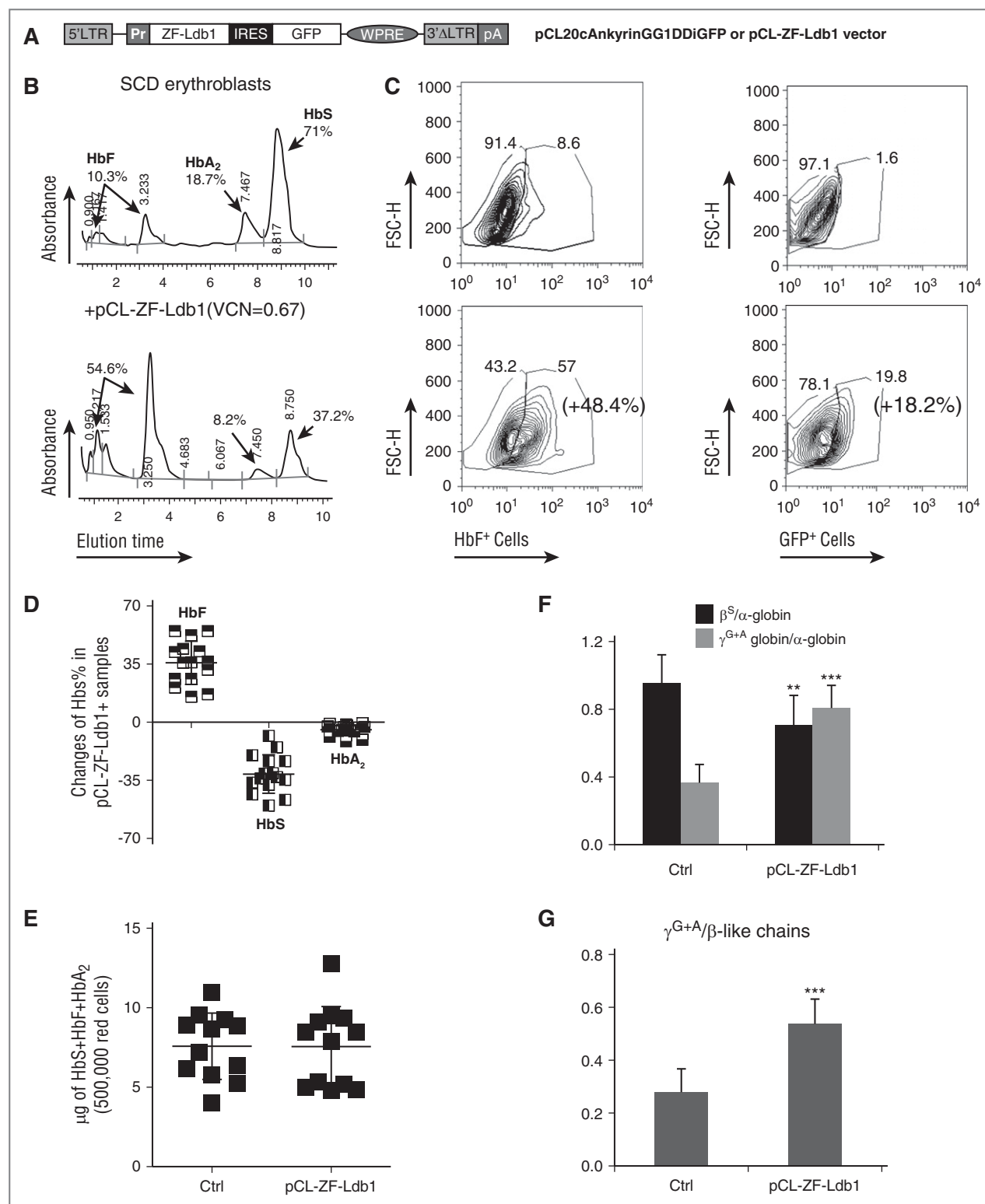


Figure 1. Increased HbF and reduced HbS synthesis after treatment with pCL-ZF-Ldb1. (A) Lentiviral construct carrying ZF-Ldb1 and the GFP genes expressed under the ankyrin promoter (Pr) via an internal ribosomal entry site (IRES). (B) Percentage of HbF, HbS, and HbA₂ measured by high-performance liquid chromatography (HPLC) in representative erythroid cells untreated (top) or treated with ZF-Ldb1 (bottom). The transduced cells have, on average, 0.7 copies of viral molecules integrated per genome. (C) Changes in percentage of cells expressing HbF (left panels) and GFP (right panels), measured by flow cytometry. (D) Overall percentages of HbF, HbS, and HbA₂ changes in SCD samples treated with pCL-ZF-Ldb1 (n = 10, 5 samples were duplicated; average VCN = 1.2). (E) Hb (F + S + A₂) content in erythroid cells with or without pCL-ZF-Ldb1. (F) Ratio of β- or γ-globin chains over α-globin chains. The quantity of the globin chains (in μg) is measured via liquid chromatography under denaturing conditions (**P < .05). (G) Ratio of γ/β-like chains measured in 250 000 erythroid sickle cells untreated or treated with pCL-ZF-Ldb1 (n = 5, VCN = 1.6; ***P < .005 and **P < .05). FSC-H, forward scatter height; LTR, long terminal repeat; pA, polyadenylation signal; pCL, vector backbone; VCN, vector copy number; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

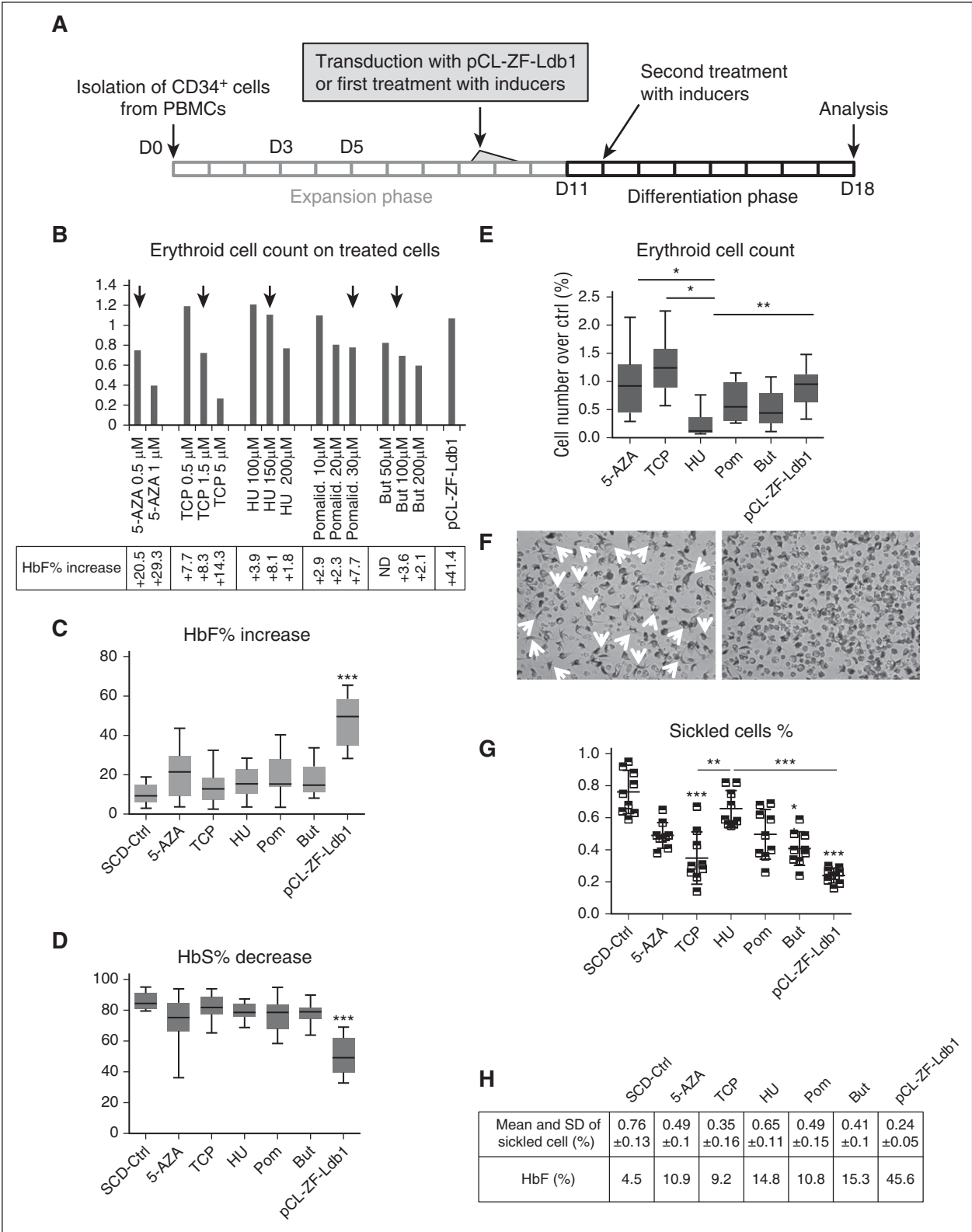


Figure 2. Variation of HbF and HbS levels in CD34⁺-derived SCD erythroid cells treated with pCL-ZF-Ldb1 LV or HbF inducers in vitro. (A) Schematic representation of the experimental protocol of human SCD CD34⁺ cells treated with the inducer drugs or pCL-ZF-Ldb1. (B) Erythroid cell number (by benzidine staining; N = 2) using the indicated doses of drugs and compared with pCL-ZF-Ldb1 (with VCN ~1 copy per cell). Black arrows indicate the dose of each drug chosen for the subsequent experiments. (C) Net increase of HbF percentage and (D) net decrease of HbS percentage in SCD erythroblasts (N = 7, 2 of which were replicated once or twice, respectively) treated with the HbF inducers or pCL-ZF-Ldb1 (***P < .005). (E) Erythroid cell count for each treatment normalized to count of untreated samples (*P = .05, **P < .05). (F) Morphological assessment of untreated (ctrl, left) vs pCL-ZF-Ldb1-treated cells (right) in hypoxic conditions. (G) Proportion of sickled cells/total cell count and (H) relative means and SD, measured on 3 replicas on 3 sets of samples treated with the various inducers or pCL-ZF-Ldb1 (**P < .05, ***P < .005) and exposed to hypoxic conditions. P values under and above bars refer to differences between treated and untreated samples and between treated samples, respectively. 5-AZA, 5-aza-2'-deoxy-cytidine; But, butyrate; ND, not determined; PBMC, peripheral blood mononuclear cell; Pom/Pomalid., pomalidomide; SD, standard deviation; TCP, tranyl-cypromine.

Results and discussion

We aimed to improve HbF synthesis and the quality of erythroid cells derived from patients with SCD, using the lentiviral vector (pCL20cAnkyrinGG1DDiGFP), henceforth called pCL-ZF-Ldb1 (Figure 1A), which enabled Hb switch in healthy erythroblasts.¹⁵ As expected, sickle CD34⁺ cells produced predominantly HbS ($\alpha_2\beta^S_2$) once differentiated into erythroblasts in vitro (Figure 1B top). Other Hb, such as HbF ($\alpha_2\gamma_2$) and HbA₂ ($\alpha_2\delta_2$), were produced to a lower degree (Figure 1B top). Following infection with pCL-ZF-Ldb1 vector, the same SCD-derived erythroid cells increased the proportion of HbF and green fluorescent protein (GFP)-expressing erythroblasts (Figure 1C; supplemental Figure 1, available on the *Blood* Web site) and HbF from 10.3% (Figure 1B top) to 54.6% (Figure 1B bottom).

We analyzed the messenger RNA and Hb protein content of erythroblasts, untreated or after transduction with pCL-ZF-Ldb1, derived from 10 SCD subjects. On average, cells treated with pCL-ZF-Ldb1 produced nearly 40% more HbF compared with untreated cells (from 27.26% to 65.52%) and lowered production of HbS (−31.14%; from 62.83% to 31.69%) and HbA₂ (−4.5%; from 9.5% to 5%) (Figure 1D). Messenger RNA content of γ -, β -, δ -globins and transgenic Ldb1 changed accordingly (supplemental Figure 2).

To assess the effect of ZF-Ldb1 treatment on the total amount of cellular Hb, we measured absolute Hb content in differentiated cells. Total Hb synthesis (HbF + HbS + HbA₂) remained essentially unaltered (Figure 1E), regardless of significant shifts in the ratio of fetal to adult globin. Reversed-phase liquid chromatography, which allows for the quantification of single globin chains rather than tetrameric hemoglobin molecules, revealed that β^S chains were diminished whereas both γ^A + γ^G chains were increased in specimens treated with ZF-Ldb1 (Figure 1F-G; supplemental Figure 3).

The experimental outline to compare pCL-ZF-Ldb1 to pharmacologic inducers of HbF is illustrated in Figure 2A. Briefly, SCD erythroid progenitor cells were infected with pCL-ZF-Ldb1 or treated with 5-aza-2'-deoxy-cytidine (0.5 μ M), tranilcypromine (1.5 μ M), HU (150 μ M), pomalidomide (30 μ M), or butyrate (100 μ M). Concentrations of inducers were determined through evaluation of efficacy (net increase of HbF) vs toxicity (cell death); the original scaling dosage (Figure 2B) was extrapolated from the most recent literature.¹⁶⁻¹⁸

Studies were performed at the end of phase 3 (day 18 from initial cell isolation), at the orthochromatophilic stage when Hb levels peaked. PCL-ZF-Ldb1-treated cells showed the most robust increase of HbF (Figure 2C) and decrease in HbS (Figure 2D). On average, the HbF in control (ctrl) SCD erythroblasts was 10.36% vs 21.05%, 14.16%, 15.98%, 18.91%, 17.34%, and 45.12% after treatment, respectively, with 5-aza-2'-deoxy-cytidine, tranilcypromine, HU, pomalidomide, butyrate, and ZF-Ldb1. In the same samples, on average, HbS decreased from 85.6%, in untreated SCD erythroblasts, to 74.8%, 81.7%, 79.1%, 76.9%, 78.1%, and 50.3% after treatments. ZF-Ldb1-expressing cells presented significantly higher variation in HbF and HbS than untreated and all other treated cells. Along with greater improvement of HbF levels, cells expressing ZF-Ldb1 did not show significant changes in viability compared with untreated samples, whereas cells treated with pomalidomide, butyrate, and HU showed reduced viability (Figure 2E). Moreover, the number of sickled cells in 3 independent samples treated with pCL-ZF-Ldb1 and exposed to hypoxic conditions was significantly reduced, also showing the least variability, when compared with controls or pharmacologically treated cells (Figure 2F-G). Taken together, pCL-ZF-Ldb1 was superior to all tested compounds in augmenting HbF and F-cell levels and importantly was associated with minimal toxicity.

Clinical observations indicate that the increase of HbF can ameliorate the severity of SCD. Although administration of HU in patients is associated with increased HbF levels, its therapeutic effects in children and adults are extremely variable, with increase of HbF ranging between 10% and 30%.^{6,19}

Here, we investigated to which extent manipulation of the chromatin organization of the β -globin gene locus, via targeted tethering of the Ldb1 self-association domain to the β -like γ^G - and γ^A -globin promoters, can reverse the developmental gene switch in erythroblasts derived from SCD patients. We found that sickle erythroblasts treated with pCL-ZF-Ldb1 can yield up to a 40% increase of HbF synthesis and concurrently reduce HbS synthesis. Expression of ZF-Ldb1 in SCD led to 2 major beneficial outcomes, that is, increased HbF and reduced HbS content. Although the HbF content was 2.3-fold higher in treated erythroblasts, the overall Hb concentration, comprising HbF, HbS, and HbA₂, remained unchanged, consistent with unimpeded erythroid maturation.

ZF-Ldb1 is a relatively small gene construct driven by a short promoter, which requires relatively low expression levels to activate the γ -globin genes. As small vectors significantly simplify production of lentiviral particles, this may also represent a considerable advantage over lentiviral vectors based on a classic additive gene-therapy approach, like those carrying curative β/γ -globin genes, which require additional large genomic elements to achieve therapeutic expression levels.²⁰⁻²³ Compared with gene editing and gene disruption, gene transfer for the cure of β -globinopathies has been shown to be safe in at least 10 patients.^{24,25} The therapeutic implication of our strategy for SCD patients has potential clinical application, as it not only sustains high levels of endogenous functional hemoglobin, but also reduces the quantity of sickled cells in hypoxic conditions.

In conclusion, a lentiviral vector carrying the self-association Ldb1 domain linked to a ZF protein, which selectively binds the γ -globin promoters, significantly increased HbF synthesis and exceeded the effects of previously described pharmacologic inducers in human cultured cells. Further lines of investigation will provide additional insight on the impact of this approach in vivo and on its safety and applicability for clinical purposes.

Acknowledgments

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Authorship

Contribution: L.B., S.R., I.M. and G.A.B. designed research, analyzed and interpreted data, and wrote the paper; L.B., I.M., C.G., S.L., and

O.Y.A. performed experiments and collected data; and D.M., W.D., and J.W.R. contributed vital new reagents and contributed to the editing of the paper.

Conflict-of-interest disclosure: S.R. is a consultant for Novartis, Ionis and Keryx Pharmaceuticals, Nektar and Rana Therapeutics, and Merganser Biotech. In addition, S.R. is a co-inventor for the patents US8058061 B2 C12N 20111115 and US7541179 B2 C12N 20090602. The consulting work and intellectual property of S.R. did

not affect in any way the design, conduct, or reporting of this research. The remaining authors declare no competing financial interests.

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MATERIALS AND METHODS

Human and Animal Ethics

Peripheral blood samples from SCD patients were obtained during automated red cell exchange as part of their routine clinical care at Montefiore Medical Center. Since the samples were unlinked and de-identified medical waste, the Montefiore Medical Center Institutional Review Board deemed them to be IRB exempt.

Vector production

Viral stocks were generated by co-transfection of the gene transfer plasmid (pCL20cAnkyrinGG1DDiGFP or pCL-ZF-Ldb1, ^{1,2}) together with the envelope plasmid (VSV-G), the packaging plasmid (pMDLg/p RRE), and the pRSV-REV plasmid into 293T cells, using a modified protocol already described ³. An aliquot (5×10^6) of 293T cells was seeded into cell culture dishes (10 cm) 24 hours prior to transfection in Dulbecco's Modified Eagle's medium (DMEM, Cellgro, Manassas, VA) with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin, at 37°C under 5% CO₂. The culture medium was changed 30' to an hour prior to transfection. The precipitate was formed by adding drop-wise 45 uL of Calfectin reagent (Signagen, Ljamsville, MD) in DMEM to 500 uL of the plasmids in DMEM, and added to cells as per manufacturer's instructions. Viral supernatant, collected after 48 hours was concentrated 1300 times by ultracentrifugation.

Real Time (RT)-PCR

Retrotranscription of total mRNA was done using the SuperScript™ II First Strand Kit (Invitrogen, Carlsbad, CA). Q-PCR reactions were performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), with either TaqMan (TaqMan PCR 2× Master mix; Applied Biosystems) or SYBR Green (iTaq™ SYBR® Green Supermix, Bio-Rad Laboratories, Hercules, CA) chemistry. Quantitative real-time PCR assays of globin transcripts were

carried out using gene-specific double fluorescently labeled probes. The following primer and probe sequences were used (forward, reverse and probe, when used, of each gene, respectively): β -globin: Fw: 5'-CAAGAAAGTGCTCGGTGCCT-3'; Rev: 5'-GCAAAGGTGCCCTTGAGGT-3'; 5'-FAM-TAGTGATGGCCTGGCTCACCTGGAC-TAMRA-3'; α -globin: Fw: 5'-TCCCCACCACCAAGACCTAC-3'; Rev: 5'-CCTTAACCTGGGCAGAGCC-3'; 5'-FAM-TCCCGCACTTCGACCTGAGCCA-TAMRA-3'; γ -globin: Fw: 5'-TGGCAAGAAGGTGCTGACTTC-3'; Rev: 5'-TCACTCAGCTGGGCAAAGG; 5'-FAM-TGG GAGATGCCATAAAGCACCTGG-TAMRA-3'; Ldb1: Fw: 5'-AGACACCAAAGAACCCATACC-3'; Rev: 5'-CGGGACATTTGTAAGGCTTTT-3'. As for the reference gene, the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), we used a kit, in which the probe is fluorescently labeled with VIC (Applied Biosystems). The number of transgene integrations (VCN) was quantified using oligos (Fw: 5'-TGAAAGCGAAAGGGAAACCA -3'; Rev: 5'-CCGTGCGCGCTTCAG -3') and a probe (5'-FAM-AGCTCTCTCGACGCAGGACTCGG -TAMRA-3') for the viral encapsidation site sequence (Psi) and compared it to an endogenous control present in two copies within the genome (ID-1 Fw: 5'-AAGGTGAGCAAGGTGGAGATTC-3'; Rev: 5'-TTCCGAGTTCAGCTCCAACTG-3').

Two and three-phase liquid cultures, transduction and treatment with HbF inducers

CD34⁺ cells selection from blood samples was performed by immunomagnetic separation, using the CD34 microbeads kit (Miltenyi Biotec Inc., Auburn, CA). Cells were then expanded and differentiated following a 2-phase protocol previously developed ⁴. To minimize basal HbF production, intrinsic to culture conditions, the experiments in which we compared various compounds to pCL-ZF-Ldb1 treatment were carried out using a 3-phase cell culture system. After the initial expansion in serum-free StemSpan media with StemSpan CC-100 cytokine cocktail (both from Stemcell Technologies, Vancouver, BA, Canada), 2 U/mL Erythropoietin (Amgen, Thousand Oaks, CA), 10⁻⁶ M dexamethasone (Sigma) and 1% penicillin streptomycin, cell media was replaced twice, according to

phase II and III, as previously reported ⁵. Cells were infected with 3-5MOI/ cell of the virus or treated with inducers within days 9-11 in expansion phase. Low MOI was necessary to keep the number of viral molecules inserted below 2-3/cell and reduce the risk of genome toxicity due to random integration. Treatment with inducers was repeated few days later, according to the timeline shown on Fig 2A. Cells were collected on day 18 of phase II (for samples shown Figs. 1 and S1-2) or phase III (for samples shown in Fig. 2) for analyses. Erythroid differentiation in the cultures was assessed by benzidine staining ⁴. At this stage, cells were analyzed by flow cytometry for GFP and HbF expression. The inducers: 5-aza-2'-deoxy-cytidine (5-AZA, 0.5mM), tranyl-cypromine (TCP, 1.5mM), hydroxyurea (HU, 150mM), pomalidomide (Pom, 30mM) and sodium butyrate (But, 100mM) were used at the final concentration indicated in parentheses. For our experiments final doses were determined via titration of known doses extrapolated from the most recent literature ⁶⁻¹⁰ and relative cytotoxicity and HbF increase, assessed by erythroid cell count and HPLC, respectively (Figure 2B).

Tetrameric and single chain analysis by high performance liquid chromatography (HPLC).

Red cell pellets were lysed with HPLC-grade water, and the resulting membrane-free hemolysates loaded into a System Gold 126 Solvent Module instrument (Beckman Coulter, Fullerton, CA). Hemoglobins were separated on a weak cation-exchange PolyCAT A column (PolyLC, Inc, Columbia, MD), and detected at a wavelength of 415 nm. The Hbs were bound to the column with mobile phase A (20 mmol/L Bis-Tris, 2 mmol/L KCN, pH 6.96) and eluted with mobile phase B (20 mmol/L Bis-Tris, 2 mmol/L KCN, 200mmol/L NaCl, pH 6.55). Single chain quantification was assessed via reversed-phase HPLC. Hb samples in this case were injected on a Hitachi D-7000 HSM Series apparatus (Hitachi Instruments, San Jose, CA) using a Zorbax 5 µm 300SB-C8 300 Å, LC 150 x 2.1 mm column (Agilent Technologies, Santa Clara, CA) and a gradient from 20% to 60% acetonitrile in 0.1% trifluoroacetic acid in 25 minutes, with UV detection at 215 nm. Serial dilutions of a solution with known concentrations of Hbs A-F-S-C,

(Helena Laboratories, Beaumont, TX) were used to generate a calibration curve, where the peak areas were plotted against the concentration values. Types and relative quantity of Hbs in samples were assessed by comparison to standard hemoglobin controls.

In vitro RBC sickling and morphological analysis.

We assessed the degree of cell sickling in our experimental samples using previously reported methodology, with slight modifications^{11 12-14}. Briefly, 5 to 15 million cells were suspended in 150 μ L in isotonic Hemox buffer (TCS Scientific Corp, Southampton, PA), pH 7.4, supplemented with 10 mM glucose and 0.2% bovine serum albumin, in individual wells of a Costar polystyrene 96-well microplate (№ 9017; Corning, Corning, NY). The microplate was then transferred to a Thermomixer R shaker-incubator (Eppendorf), and maintained under hypoxia (Nitrogen gas), with continuous agitation at 500 rpm, at 37° C for 2 hours. At conclusion, aliquots (~ 20 μ L) of each sample were collected in 2% glutaraldehyde solution for immediate fixation without exposure to air. Subsequently, fixed cell suspensions were introduced into specialized glass microslides (Dawn Scientific, Inc., Newark, NJ)¹⁵ for acquisition of bright field images (at 40x magnification) of single layer cells on an Olympus BX40 microscope fitted with an Infinity Lite B camera (Olympus) and the coupled Image Capture software.

Statistics

For two-group comparisons we used either paired or unpaired t-tests or Mann Whitney and Wilcoxon tests. For three or more groups, we compared means with a one-way Anova test and Bonferroni's Multiple Comparison test. Dose/response fit was assessed by linear regression. All tests were done using GraphPad Prism software, version 5.0a.

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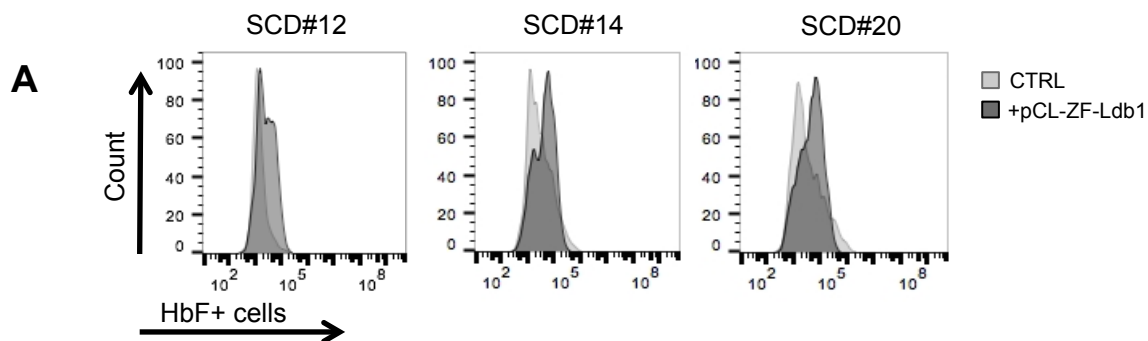
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Figure legends

Figure S1. pCL-ZF-Ldb1 increases number of HbF⁺ cells and HbF synthesis in SCD erythroblasts. (A) The shift in number of HbF⁺ cells in erythroid culture from 3 independent SCD specimens (#12-14-20) untreated and treated with pCL-ZF-Ldb1, assessed by flow cytometry. (B) Number of HbF⁺ cells, measured by flow cytometry (FC), and relative HbF% levels in the same specimens, measured by HPLC.

Figure S2. pCL-ZF-Ldb1 supports high levels of γ -globin/HbF induction and concurrently reduces HbS levels in SCD erythroblasts. (A) β -like globin and transgenic Ldb1 mRNA content (γ , left, and β^S , middle) was measured by Q-PCR and normalized by endogenous GAPDH expression. β -like globin expression was further normalized by α -globin expression (which is a measure of erythroid maturation across samples) $**=P<0.05$. Right: transgenic ZF-Ldb1 mRNA expression in treated samples is plotted against integration of pCL-ZF-Ldb1, and tested for linear regression, $P<0.0001$. (B) HbF (left), HbS (middle), and HbA₂ (right) changes in all SCD samples untreated and treated with pCL-ZF-Ldb1 LV. For all experiments ($n=10$, $***=P<0.005$).

Figure S3. Differential single globin chain synthesis in cells treated with pCL-ZF-Ldb1. (A) Chromatographic profile of a representative sickle cell's hemolysate untreated or treated with pCL-ZF-Ldb1. (B) Calibration curve obtained using serial dilution of a commercially available solution (Hbs A-F-S-C) with known Hb concentration. (C) Areas under peak of single globin chains extrapolated from the calibration curve (from B) in samples untreated or after treatment with pCL-ZF-Ldb1 ($n=5$, VCN=1.6).



B

	HbF+ cells% (FC) untreated	HbF+ cells% (FC) +pCL-ZF-Ldb1	HbF% (HPLC) untreated	HbF% (HPLC) +pCL-ZF-Ldb1
SCD#12	20.1	56.6	3.47	28.32
SCD#14	36.1	56.1	2.99	56.72
SCD#20	56.1	69.1	7.12	51.66

Figure S1

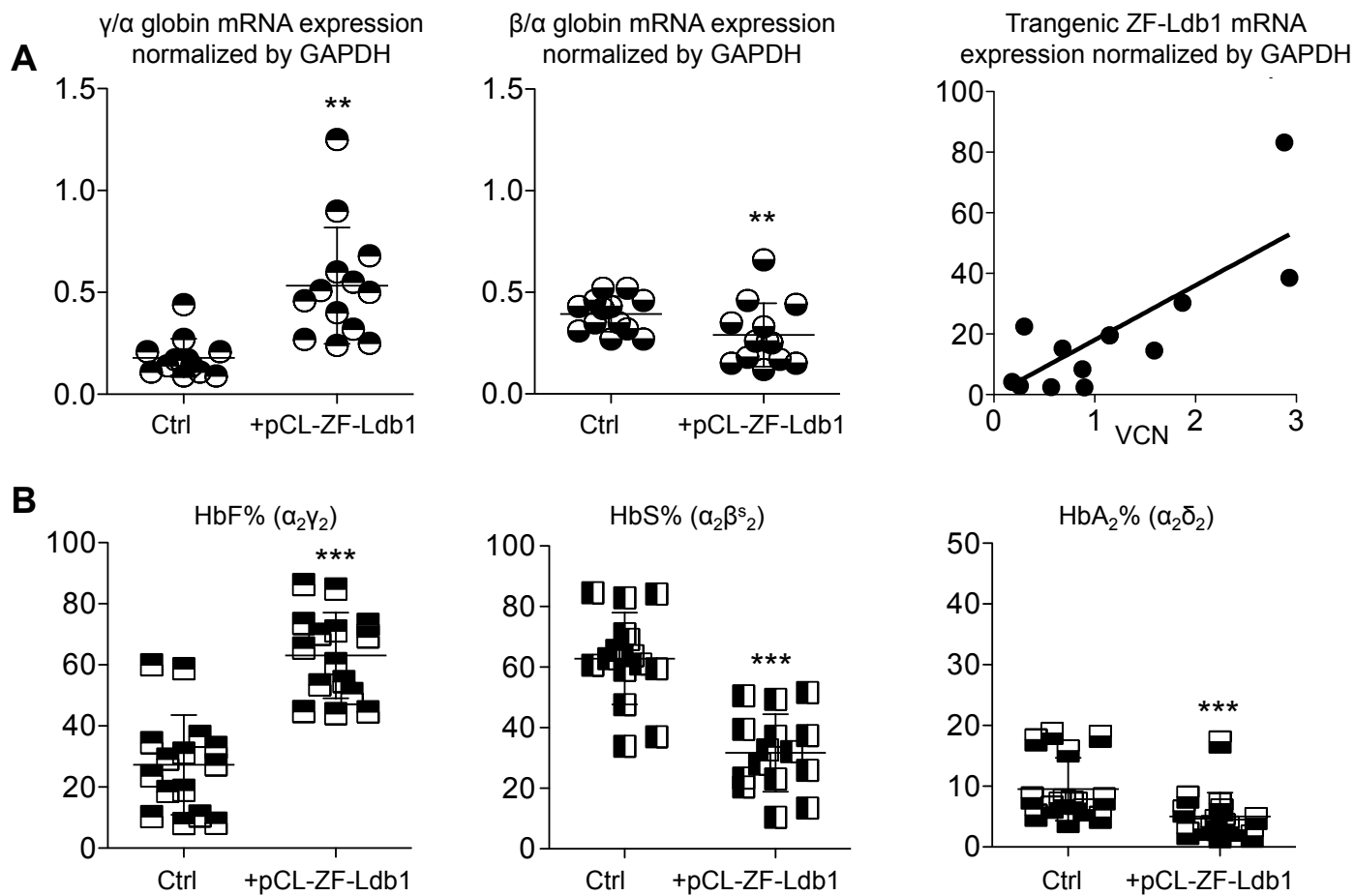


Figure S2

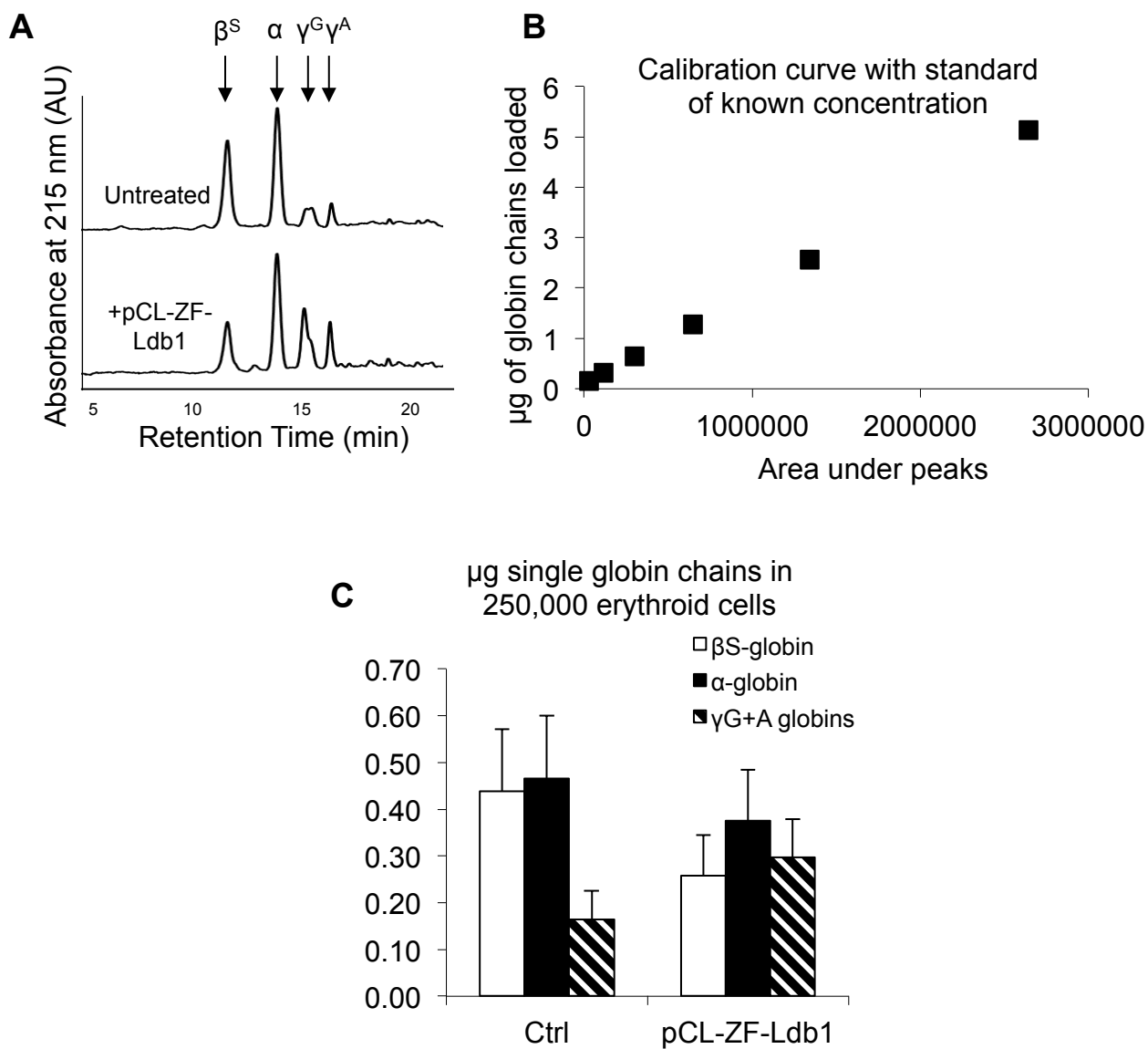


Figure S3

Inclusion of a short hairpin RNA targeting *BCL11A* into a β -globin expressing vector allows concurrent synthesis of curative adult and fetal hemoglobin

Addition of a functional copy of the β -globin gene and reactivation of fetal hemoglobin (HbF) are promising therapeutic approaches for β -globinopathies such as sickle cell disease (SCD) and β -thalassemia. Results from ongoing clinical trials for β -globinopathies indicate that a

successful outcome is genotype-dependent. In studies that included patients with the β^0/β^0 genotype or SCD, vectors expressing a single curative gene have failed or have required several integrations per genome to produce enough adult hemoglobin (HbA) levels to correct the phenotype of the patients.¹⁻⁶ Therefore, to achieve HbA synthesis at therapeutic levels in the most severe of genotypes, and with minimal vector copy number (VCN) per cell, more powerful and versatile vectors are required.

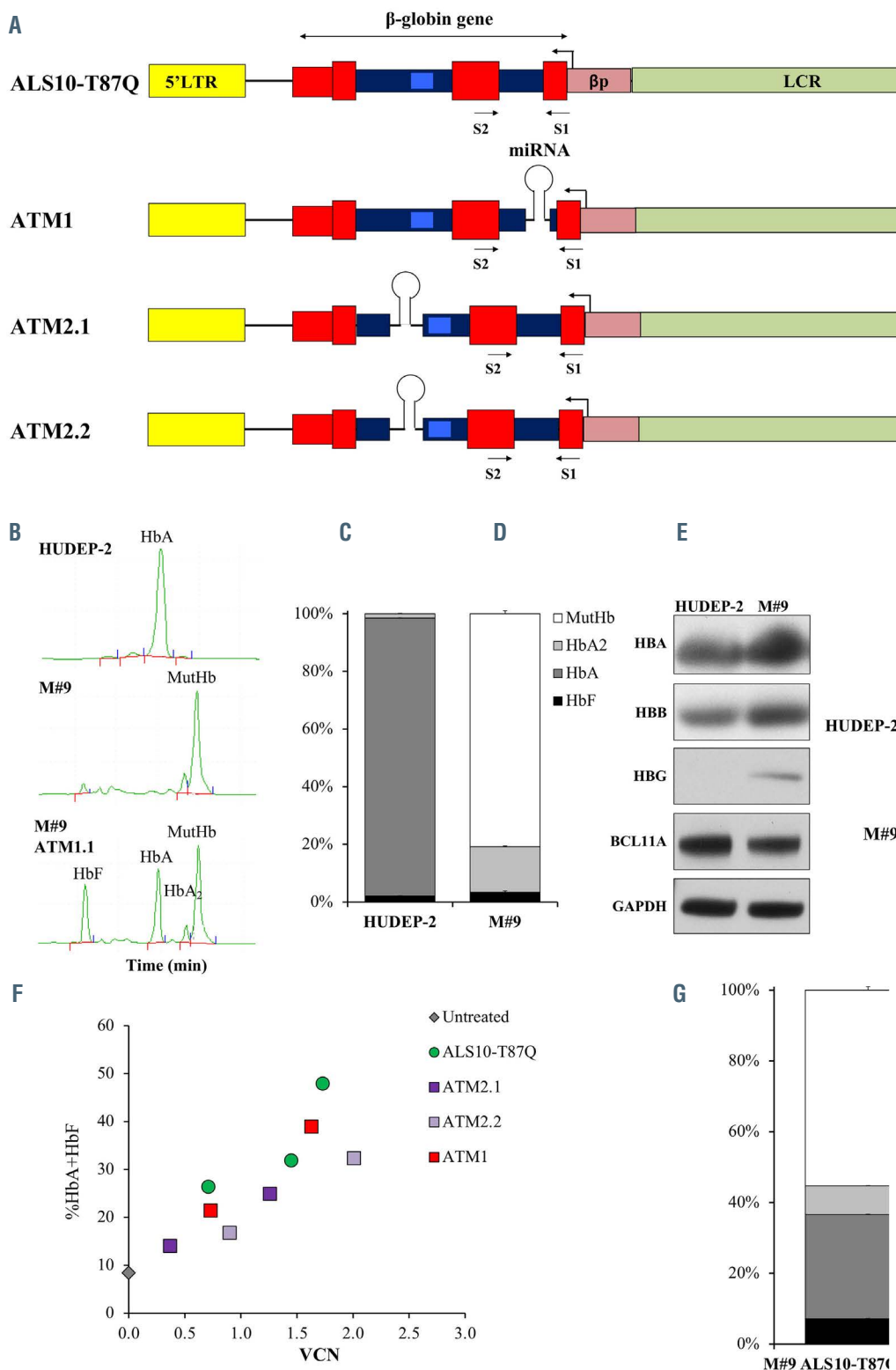


Figure 1. Legend on following page.

Figure 1. Initial constructs design, Hudep9 (M#9) line development and characterization of constructs efficiency. (A) Initial constructs used in the study: ALS10-T87Q, ATM1.1, ATM2.1, ATM2.2. The miR-E-BCL11A5 sequences were synthesized by Genscript USA (NJ) by combining the passenger and guide sequences of short hairpin RNA (shRNA^{mir5}) with the flanking miR-E backbone sequences.^{3,4} The combined miR-E-BCL11A5 sequences were cloned in intron 1 position c.79+36 of ALS10-T87Q (ATM1) and intron 2 positions c.303-163 and c.303-172 (ATM2.1 and ATM2.2, respectively) of the β -globin transgene. Viral production and titration were performed according to methods previously reported.¹⁵ All vectors include the human β -globin gene and regulatory elements (β -globin promoter (β p) and portions of the locus control region (LCR) as in our previously described vector AnkT9).⁸ Some of these vectors present a miRNA as described in the main manuscript. Erythroid specific expression of the β -globin gene and shRNAmiR is achieved by using the β -globin promoter and its locus control region (LCR), in a pol II-promoter driven system. Transgene expression under these regulatory elements will be limited to the latest stages of erythroid development,⁸ preventing unwanted effects as previously seen in B lymphocytes and hematopoietic stem cells.¹⁰ (B) Representative chromatographic separation (high-performance liquid chromatography [HPLC])² of hemolysates from the HUDEP-2 (top) and M#9 (middle) erythroblasts (developed using methodology previously described),¹⁵ showing fetal hemoglobin (HbF), adult hemoglobin (HbA), HbA₂ and mutant HbA (MutHbA) peaks at day 7 of differentiation as described.^{1,15} At the bottom, a representative chromatographic separation¹⁵ of the M#9 cell line after transduction with one of the vectors that simultaneously produced HbA^{T87Q} and induced HbF expression. (C) Relative proportion of HbF, HbA, HbA₂ and MutHb in HUDEP-2 and M#9 quantified by HPLC at day 7 of differentiation. (D) Representative western blot showing HbA, HbB, HbG and BCL11A proteins in HUDEP-2 and M#9 at day 7 of differentiation. The evaluation was performed using rabbit anti-HbB (Proteintech, Chicago, IL), rabbit anti-HbA (Santa Cruz Biotechnology), mouse anti-BCL11A (Abcam, Cambridge, MA, USA) rabbit anti-HbG (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-GAPDH (Proteintech) primary antibodies. Secondary, anti-mouse and anti-rabbit IgG HRP-linked antibodies (Cell Signaling) were used for detection with the Amersham enhanced chemiluminescence (ECL) western blotting analysis system (GE Healthcare, Marlborough, MA, USA). (E) Representative fluorescence-activated cell sorting (FACS) plots of HUDEP-2 and M#9 stained for GPA, CD71, c-KIT and CD36 markers adapted from and analyzed as previously described.⁸ (F) Percentage of total curative hemoglobins (HbA^{T87Q}+HbF) over a range of viral integrations after transducing M#9 cells. (G) Proportion of the different hemoglobins produced in M#9 cells transduced with ALS10-T87Q with vector copy number (VCN)= 2.

Using a previous vector developed by our group indicated as AnkT9W, we generated ALS10-T87Q (Figure 1A), a vector that produces a functional β -globin gene that carries a modification (indicated as T87Q) with improved anti-sickling activity.⁷⁻⁸ Additionally, ALS10-T87Q vector includes the full sequence of the intron 2 of the β -globin gene (Figure 1A). We incorporated into the β -globin coding sequence a short hairpin RNA (shRNA^{mir}) targeting the transcription factor BCL11A, a known repressor of γ -globin.

The shRNA^{mir} sequences targeting *BCL11A*⁹⁻¹⁰ were flanked by an optimized backbone termed “miR-E” (11) to increase HbF levels through downregulation of *BCL11A* (Figure 1A). Our goal is to overcome some of the limitations of the vectors presently in clinical trials by simultaneous i) production of transgenic HbA; ii) reactivation of endogenous HbF; and iii) decrease in production of endogenous mutant β -globin mRNA and/or protein.

The erythroid HUDEP-2 immortalized human cord blood cell line was generated by inducible expression of the encoding genes HPV16-E6/E7 derived from the human Papilloma virus 16.¹² These cells can be used as a semi-primary adult hematopoietic cell model that can be propagated indefinitely in the presence of tetracycline or differentiated into red cells under appropriate culture conditions. In order to assess the level of HbA^{T87Q} and HbF produced by the new vectors, we mutagenized the first exon of the human β -globin gene (*HBB*) in erythroid HUDEP-2 cells using the CRISPR/Cas9 system. We established a clonal cell line, named M#9, carrying a homozygous deletion of codon 6, thus generating a mutant *HBB* gene (Hb-mutant). This line produced a predominant mutant adult HbA (Hb-mutant) distinguishable from the endogenous wild-type (WT) HbA by high-performance liquid chromatography (HPLC) (Figure 1A and B, top and middle panel, and Figure 1C), which was also visible by western blot analysis (Figure 1D). The change did not alter erythroid maturation (Figure 1E). The HbA^{T87Q} generated by ALS10-T87Q and HbF induced by the shRNA^{mir} were also both distinguishable from the Hb-mutant produced by M#9 via chromatographic separation (Figure 1B, bottom panel). We were able to assess the relative production of functional HbA^{T87Q}+HbF after gene transfer and correlate these values to VCN in a dose/effect relationship. Upon transduction in the M#9 cell line, ALS10-T87Q generated 18%, 23% and 44% of HbA^{T87Q} with VCN=0.6, 1.0 and 2.0, respectively (Figure 1F and G). We cloned the miR-E-BCL11A sequence either in the

β -globin intron 1 (ATM1), or in two different regions of intron 2 (ATM2.1 and ATM2.2) (Figure 1A). Upon transduction of M#9, all ATM vectors reduced BCL11A level and induced HbF production by western blot (Online Supplementary Figure S1A), confirming functional repression from the miR-E-BCL11A sequence. ATM1, the most effective among the ATM vectors, showed the highest HbF induction (Online Supplementary Figure S1B) and an HbA production equivalent to ~90% of that made by ALS10-T87Q at VCN=1 (Online Supplementary Figure S1C). However, the total production of HbA^{T87Q}+HbF by ATM1 vector was suboptimal, as the combination of the miR-E-BCL11A with the β -globin gene did not lead to an overall increase of therapeutic hemoglobins (HbA^{T87Q}+HbF) compared to ALS10-T87Q (Figure 1F; Online Supplementary Figure S1D to E).

In order to overcome this limitation, we modified the sequence of miR-E-BCL11A within intron 1, generating additional vectors named ATM1.1, ATM1.2, ATM1.3 and ATM1.4 (Figure 2A; Online Supplementary Table S1A). We tested two different modifications of the guide and one modification of the loop of the miR scaffold itself. ATM1.1 differs from ATM1 in the last four bases of the guide to adapt the shift from shRNA to miR-based design, as indicated in the legend of Figure 2A. These changes ensure higher 5' passenger stability for efficient guide recognition and incorporation into RISC complex as previously shown.⁹

Among these constructs, ATM1.1 produced the highest HbAT87Q and HbF expression levels (Figure 2B and C, top and bottom panels). ATM1.1 produced between ~25% and ~47% more therapeutic hemoglobins (HbA^{T87Q}+HbF) compared to the HbA^{T87Q} produced by ALS10-T87Q, at low and high VCN, respectively (Figure 2D, top and bottom panels). Using a mixed-effects linear regression model, the increase was quantified in ~33% more HbA^{T87Q}+HbF in ATM1.1 compared to the HbA^{T87Q} made by ALS10-T87Q (looking at similar levels of VCN; Figure 2E; Online Supplementary Table S1B). Western blot analysis confirmed the simultaneous reduction of BCL11A and the increase of γ -globin protein levels in M#9 samples treated with ATM1.1 (Figure 2F).

In order to verify that the mRNA or DNA of the ATM1.1 vector did not recombine due to the inclusion of the miR-E-BCL11A, both during integration and expression of the transcript, we introduced silent mutations in exon1 and exon2 of the β -globin gene (Sil β -globin gene) to distinguish, in human cells, DNA and mRNA of the endogenous and the transgenic Sil β -globin sequences

(Figure 3G). We confirmed that the oligonucleotides (Online Supplementary Table S1D) designed to amplify the Sil β -globin gene amplified the transgenic Sil β -globin gene exclusively (Online Supplementary Figure S1F). Using a murine erythroleukemia cell line (MEL), we confirmed a linear relationship between VCN, chimeric HbA (mouse α -globin/human β -globin-T87Q tetramer; Online

Supplementary Figure S1G), protein levels (Online Supplementary Figure S1H), and human β -globin RNA (Online Supplementary Figure S1I) produced upon integration of ALS10-T87Q, ALS10-T87Q-silent, ATM1.1 and ATM1.1-silent. Furthermore, in a pilot experiment on CD34⁺-derived SCD erythroblasts treated with ALS10-T87Q, ALS10-T87Q-silent, ATM1.1 and

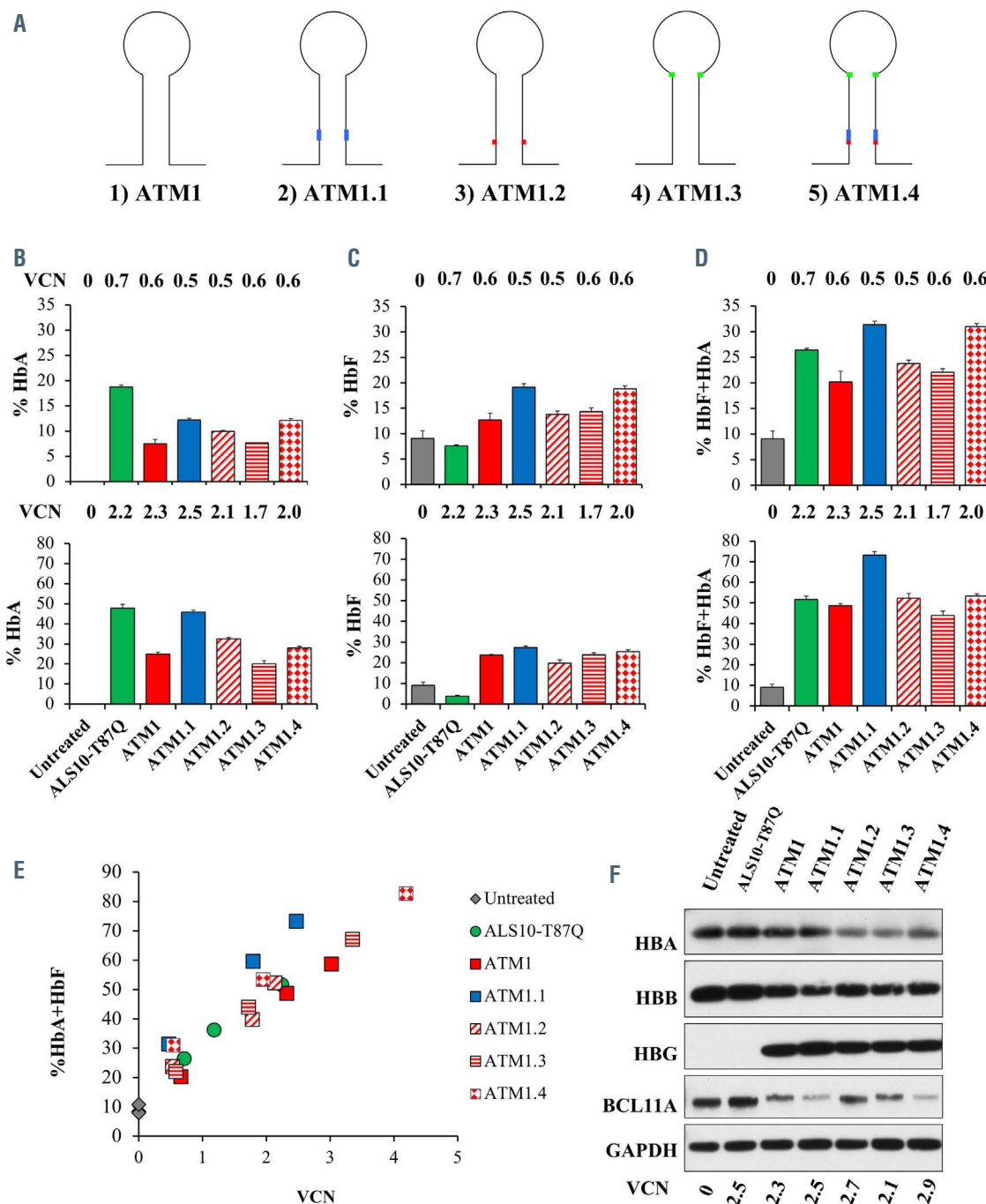


Figure 2. Optimization of *BCL11A* miRNA design. (A) Illustration of changes introduced into *BCL11A* miRNA sequence of ATM1.1 vector. ATM1.2 contains 1 basepair (bp) mismatch between the guide and the passenger creating a "bulge" in our design, based on evidence from Fellman's group, which demonstrated that inclusion of such an element can boost the potency of the miRNA.¹¹ ATM1.3 presents a 2 bp modification of the published miR-30 loop to improve performance.¹¹ The modifications tested in ATM1.1, -1.2 and -1.3 were combined in the final design of ATM1.4. (B) Adult hemoglobin (HbA^{T87Q}) (C) fetal hemoglobin (HbF) and (D) HbA^{T87Q}+HbF levels at low (0.6+/-0.1, top panels) or high (2.0+/-0.3, bottom panels) integrations per cell after transduction of M#9 with ALS10-T87Q, ATM1, and its variants ATM1.1, ATM1.2, ATM1.3, or ATM1.4. (E) Graphic representation of the proportion of curative HbA^{T87Q}+HbF hemoglobin over a range vector copy number (VCN) after transduction of M#9 with ALS10-T87Q, ATM1, ATM1.1, ATM1.2, ATM1.3, or ATM1.4. Data are represented as mean $\pm$ standard deviation, n=3. (F) Representative western blot showing side by side the HBA, HBB, HBG and BCL11A expression in control and different vectors transduced M#9 cells at day 7 of differentiation.

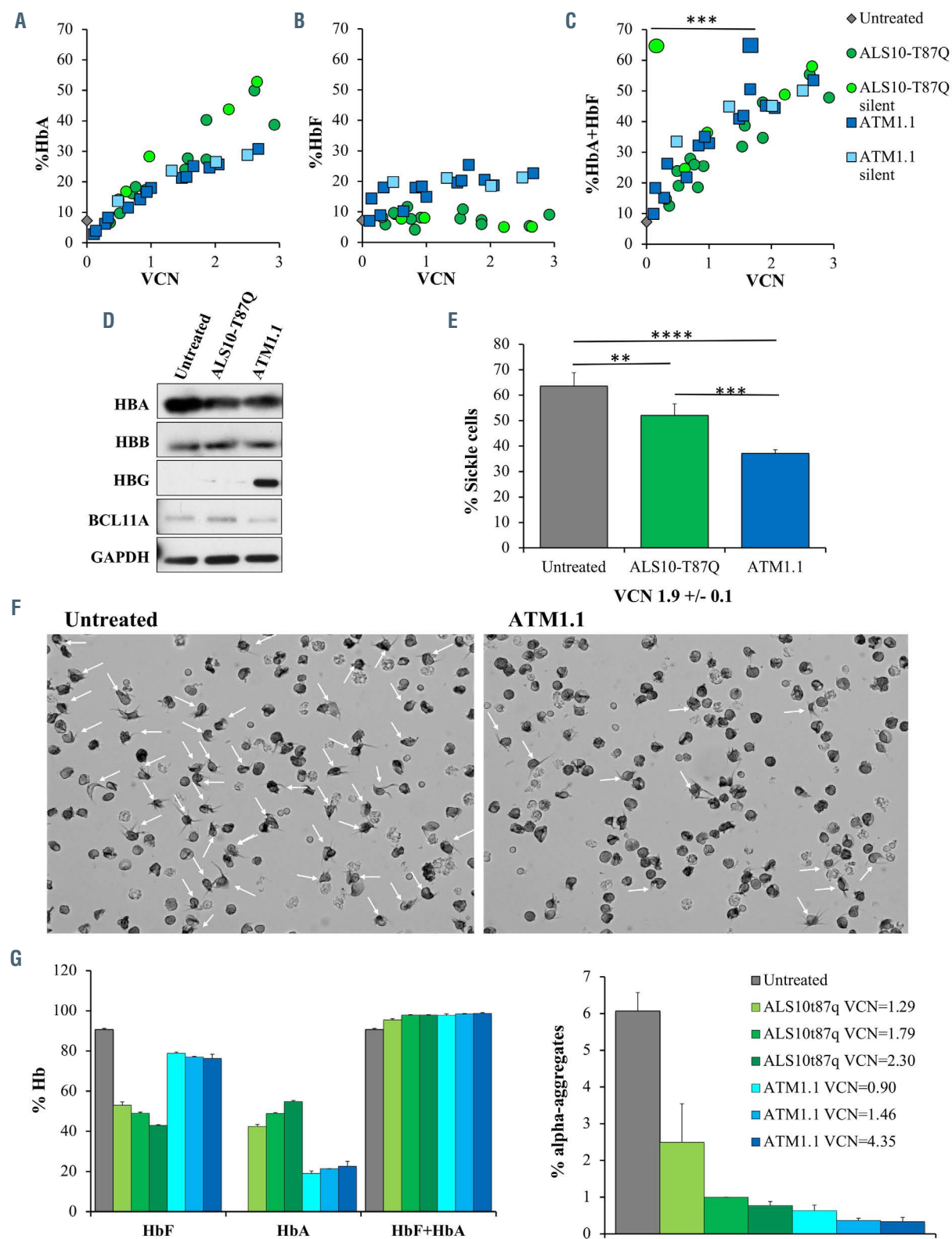


Figure 3. Efficiency of ATM1.1 construct in sickle cell disease and β^0/β^0 patients' cells. Dose/response analyses obtained by plotting integration levels (vector copy number [VCN]) against the percentage values of (A) adult hemoglobin (HbA^{T87Q}), (B) fetal hemoglobin (HbF) and (C) HbA^{T87Q}+HbF, respectively, in sickle cell disease (SCD) erythroid cells untreated or treated with ALS10-T87Q, ALS10-T87Q-silent, ATM1.1, or ATM1.1-silent. Data are represented as mean $\pm$ standard deviation (SD), n=3. (D) Representative western blot showing the HBA, HBB, HBG and BCL11A protein content in differentiated SCD cells. (E) Percentage of blind counts of sickle-shaped cells exposed to hypoxia, as previously described,⁹ in four fields from untransduced SCD erythroid cells or after treatment with ALS10-T87Q or ATM1.1. Averages and SD are indicated. (F) Representative image of patient-derived SCD erythroid cultures under hypoxia. Arrows mark cells with sickle-like morphology. (G) Dose/response analyses obtained for HbF, HbA^{T87Q}, and HbA^{T87Q}+HbF, with increasing VCN, in patient-derived β^0/β^0 thalassemic erythroid cells untreated or treated with ALS10-T87Q, ATM1.1., left panel. Percentage of corresponding alpha aggregates, right panel. Statistical significance was assessed by computing means with one-way Anova test followed by a Tukey test to compare every mean with every other mean. All tests were done using GraphPad Prism software, version 7.0. software package was used for statistical analysis.

ATM1.1-silent vectors, we were able to discriminate between the SCD and β -globin-T87Q mRNA and that encoded by the silent vectors (Online Supplementary Figure S2A). Amplification of the region encompassing exon 1 and 2 (Figure 1A), performed using oligonucleotides (Online Supplementary Table S1D) that selectively anneal to the silent β -globin mRNA, suggested that the presence of the miR-E-BCL11A in intron 1 did not affect the length and splicing of the portion of the β -globin mRNA amplified in the double vector (Online Supplementary Figure S2B). This indicated no apparent interference between the expression of the transgenic β -globin and the miR-E-BCL11A components.

In order to evaluate the potential use of this vector in gene therapy for SCD, we transduced CD34⁺-derived hematopoietic progenitor cells isolated from peripheral blood of three patients with SCD. We compared the production of HbA^{T87Q} and HbF by ATM1.1 with its counterpart ALS10-T87Q. We observed a dose-dependent induction of HbF and HbA^{T87Q} in SCD erythroblasts treated with ATM1.1 (Figure 3A and B). Moreover, our results showed that ATM1.1 outperformed ALS10-T87Q, inducing the highest levels of curative hemoglobins (HbA^{T87Q}+HbF) (Figure 3C; Online Supplementary Table S2C). Western blot analysis confirmed the induction of γ -globin chains in cells transduced with ATM1.1 (Figure 3D). While not completely suppressed, the partial knock-down of BCL11A observed in cells treated with ATM1.1 (Figure 3D) could be advantageous. Reported evidence suggests that disruption of BCL11A leads to an erythroid differentiation defect or survival disadvantage.¹³⁻¹⁴ Gene expression analysis of SCD cells showed comparable expression of transgenic β -globin, irrespectively of whether they carried the silent- β -globin mRNA or not (Online Supplementary Figure S2C). This also indicated that introduction of the silent mutation did not affect the ability of the vectors to produce the β -globin mRNA (Online Supplementary Figure S2C, top panel). Both ATM1.1 and its silent variant presented a significantly increased level of γ -globin mRNA, tightly correlating with the protein yield (Online Supplementary Figure S2A to C). Moreover, the SCD erythroblasts treated with ATM1.1 and exposed to hypoxic conditions showed lower percentage of sickle-like morphology (58.1%) by comparison to parallel specimens transduced with ALS10-T87Q (81.9%) (Figure 3E to F). We analyzed β 0/ β 0 thalassemic patients' cells and compared the percentage of total curative hemoglobin produced by ALS10-T87Q and ATM1.1. Untransduced β 0/ β 0 cells showed over 90% of baseline HbF (Figure 3G, left panel). The high level of HbF is in part due to intrinsic features of the culture conditions and lack of β -globin expression in these cells. Upon transduction, we observed that the total content of HbF+HbA was close to 100%, although in different proportions (Figure 3G, left panel). The most informative change was the abundance of a HPLC peak corresponding to α -globin aggregates, which we previously characterized in β 0/ β 0 CD34⁺ derived erythroid cells.⁸ ATM1.1-transduced cells showed lower levels of these aggregates when compared to the same cells transduced with ALS10-T87Q at similar VCN (Figure 3G, right panel; Online Supplementary Figure S2D). The higher suppression of free α -chains suggests that, overall, ATM1.1 was able to produce more curative chains (β + γ).

In conclusion, our results show that HbA and HbF can be elevated simultaneously using a single lentiviral construct, as our ATM1.1 vector. The ability of ATM1.1 to induce functional hemoglobin production (HbF+HbA) surpasses that of a vector expressing β -globin alone.

While the results *in vitro* demonstrate proof-of-principle potency of the vectors used, the level of functional hemoglobin produced *in vivo* may vary. Future studies will evaluate this approach analyzing erythroid cells derived from transduced SCD or thalassemic HSC *in vivo*. These studies will be performed after cloning the best miR-E-BCL11A into an enhanced vector recently developed in our lab.¹⁵

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Contributions: SPL designed the vector and experiments, conducted the experiments, analyzed the data and prepared the manuscript; DJ analyzed the data and prepared the manuscript; GV provided experimental support and analysis of the data; AG designed the vectors and analyzed the data; OA and LP provided experimental support; S-WK, KJ and GV prepared clinical specimens and analyzed the data; NY provided the Hudep2 cell line; RT helped with M#9 cell line development; BL designed the vectors and analyzed the data; RS designed the vectors and experiments, analyzed data and revised the final manuscript.

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Inclusion of a short hairpin RNA targeting *BCL11A* into a β -globin expressing vector allows concurrent synthesis of curative adult and fetal hemoglobin

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Supplementary appendix

Figure legends

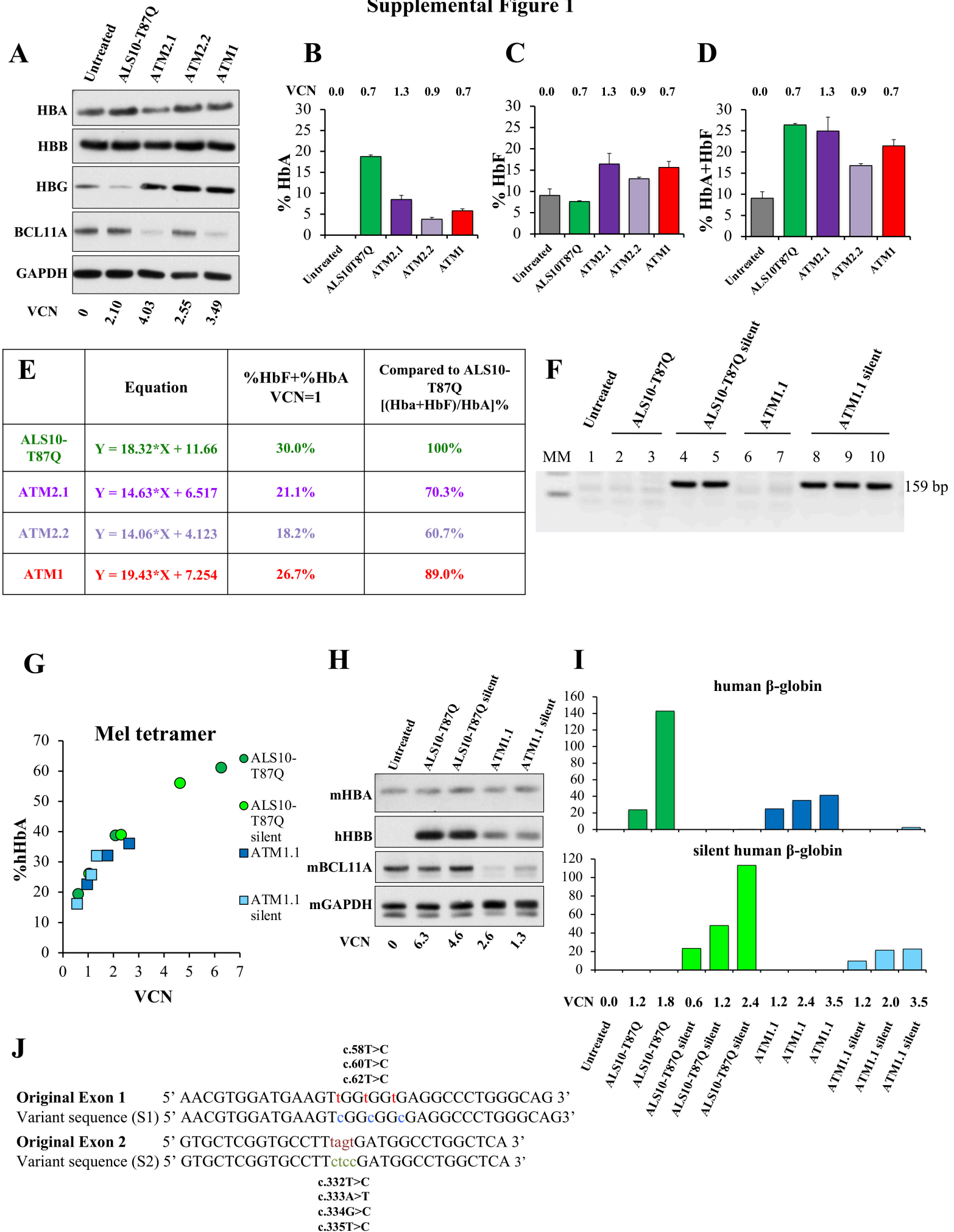
Supplemental Figure 1 – (A) Representative Western blot showing HBA, HBB, HBG and BCL11A proteins in differentiated M#9 cells. (B) Percentage of HbA^{T87Q} (C) HbF and (D) HbA^{T87Q}+HbF levels quantified by HPLC over the number of viral integrations per cell after transduction of M#9 with ALS10-T87Q, ATM2.1, ATM2.2 or ATM1. Data are indicated as the mean $\pm$ SD, n=3. (E) Results of mixed-effects linear regression models to identify the percentage of curative HbA^{T87Q}+HbF produced by ALS10-T87Q, ATM2.1, ATM2.2 and ATM1 at VCN=1. (F) Amplification of β -globin sequences carrying silent mutations (as indicated in **Fig.1A**) on plasmids that contain the sequence of ALS10-T87Q, ALS10-T87Q-silent, ATM1.1 or ATM1.1-silent. (G) Chimeric HbA (human β -globin/mouse α -globin tetramer) production following transduction with ALS10-T87Q, ALS10-T87Q-silent, ATM1.1 or ATM1.1-silent vector in MEL cells, over range of viral integrations (VCN up to 7 copy/genome). (H) Representative Western blot showing mouse HBA (mHBA), human HBB (hHBB) and mouse BCL11A (mBCL11A) protein levels in differentiated MEL cells treated with WT vectors and their counter silent variants. (I) Evaluation of gene expression of WT, β -globin, and silent- β -globin in untreated and transduced MEL cells compared to the endogenous control mouse β -actin mRNA at different VCN. (J) Modifications of the sequences in exon 1 (top) and exon 2 (bottom) of the β -globin gene to generate the silent version of the β -globin gene.

Supplemental Table 1 – (A) Sequences of BCL11A miRNAs in ATM1, ATM1.1, ATM1.2, ATM1.3 and ATM1.4 constructs. (B) Results of mixed-effects linear regression models of % of curative HbA^{T87Q}+HbF obtained with ALS10-T87Q, ATM1, ATM1.1, ATM1.2, ATM1.3 or ATM1.4 in M#9 treated cells. (C) Results of mixed-effects linear regression models of % of curative HbA^{T87Q}+HbF, HbA^{T87Q} and HbF obtained in SCD cells treated with ALS10-T87Q, ALS10-T87Q-silent, ATM1.1 or ATM1.1-silent. (D) Sequences of primers used in RT-PCR and qPCR to amplify β -globin, Sil β -globin, γ -globin and β -actin.

Supplemental Figure 2 – (A) Quantitative RT-PCR analysis of a SCD patient specimen. The analysis includes quantification of the SCD β -globin mRNA, β -globin transcribed by the vectors, including the Sil β -globin, and induced γ -globin mRNAs and normalized to endogenous GAPDH mRNA adapted from Breda L, et al. Lentiviral vector ALS20 yields high hemoglobin levels with low genomic integrations for treatment of beta-globinopathies Mol Ther. 2021. (B) Amplification of the β -globin region encompassing exon 1 and 2, from **Fig.1A**. (C) Quantification by ddPCR of vector-derived T87Q β -globin, sickle β -globin and γ -globin mRNAs and normalized to endogenous α -globin mRNA. Bio-Rad ddPCR system was used to perform the analysis using specific primer/probe custom sets detecting vector-derived, T87Q, Sickle or γ -globin expression. The sequences of the oligos used: vector-derived, T87Q β -globin (FW: 5'- CTGGCTCACCTGGACAA-3'; Rev: 5'- CAGGAGCCTGAAGTTCTCA-3'; Probe: 5'- CACCTTTGCCCAGCTGAGTGAG-3'), human SCD β -globin (FW: 5'- CCAGCAGCCTGCCAG -3'; Rev: 5'- ATCTATTGCTTACATTTGCTTCTGACA-3'; Probe: 5'-

AGACTTCTCCACAGGAGT-3') and human γ -globin (FW: 5'-AGGAGGACAAGGCTACTATCACAAG-3'; Rev: 5'-AGGAGCCTTCCCAGGGTTT-3'; Probe: 5'-CAAGGTGAATGTGGAAGAT-3') referred to human α -globin as an endogenous control (FW: 5'-GCCCTGGAGAGGATGTTTCCT-3'; Rev: 5'-TCGAAGTGCGGGAAGTAGGT-3'); Probe: 5'-TCCTTCCCCACCACCAA-3'). **(D)** Representative chromatographic separation (HPLC) of hemolysates from β^0/β^0 TH25 patient' cells. Control, untransduced (top), ALS10-T87Q (middle) and ATM1.1 (bottom) vector transduced erythroblasts show: HbF, (acetylated and main form), α -aggregates, HbA (acetylated and main form) and HbA₂ peaks at day 7 of differentiation. Dual vector transduced cells demonstrated highest reduction of α -aggregates peak.

Supplemental Figure 1



Supplemental Table 1

A

ATM1	...TTGCTGTTGACAGTGAGCG- - - -gatcgagtggtgaataatgataGGGTGAAGCCACAGATGCCatcattattcaacactcgatc- - - -TGCCTACTGC..
ATM1.1	...TTGCTGTTGACAGTGAGCG- g cgcgatcgagtggtgaataa- - - -GGGTGAAGCCACAGATGCC- - - -ttattcaacactcgatc g cgcg-TGCCTACTGC...
ATM1.2	...TTGCTGTTGACAGTGAGCG- - - - a gatcgagtggtgaataatgat -GGGTGAAGCCACAGATGCC-atcattattcaacactcgatc c - - - -TGCCTACTGC...
ATM1.3	...TTGCTGTTGACAGTGAGCG- - - -gatcgagtggtgaataatgata T AGTGAAGCCACAGATG T Aatcattattcaacactcgatc- - - -TGCCTACTGC...
ATM1.4	...TTGCTGTTGACAGTGAGCG a g g cgcgatcgagtggtgaataa - - - - T AGTGAAGCCACAGATG T A- - - -ttattcaacactcgatc g cg c T GCCCTACTGC...

B

	Equation	R ²	%HbF+%HbA VCN=1	%HbF+%HbA VCN=2	Compared to ALS10-T87Q [(Hba+HbF)/HbA]%
ALS10-T87Q	Y = 16.18*X + 15.81	0.9919	32.0%	48.2	100%
ATM1	Y = 16.48*X + 9.581	0.9985	26.1%	42.5	81.6%
ATM1.1	Y = 21.85*X + 20.94	0.9996	42.8%	64.6	133.8%
ATM1.2	Y = 16.51*X + 14.2	0.9426	30.7%	47.2	96.0%
ATM1.3	Y = 16.12*X + 14.01	0.9927	30.1%	46.3	94.1%
ATM1.4	Y = 14.06*X + 24.34	0.9972	38.4%	52.5	120.%

C

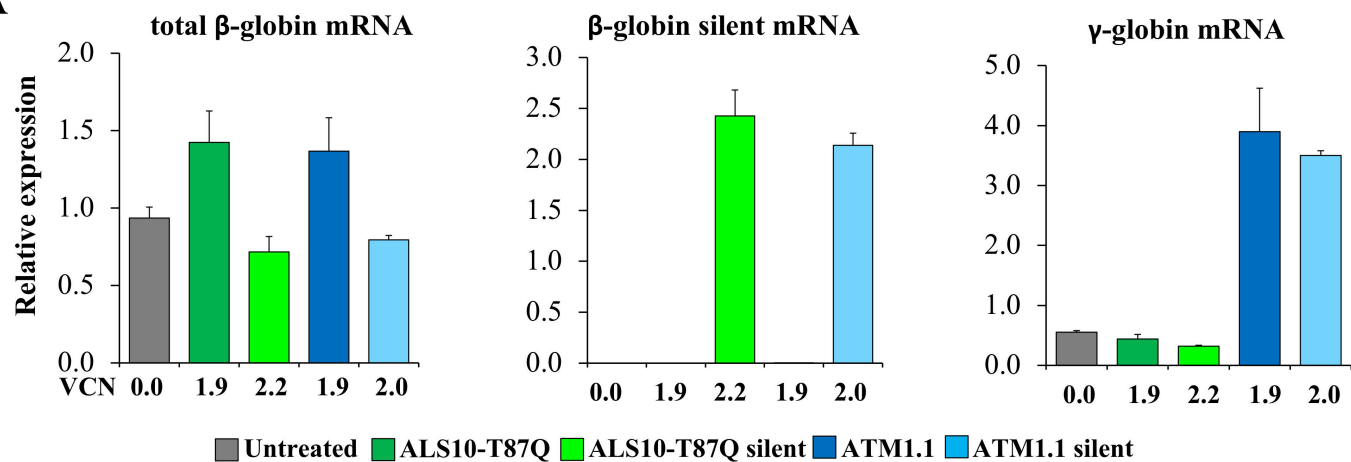
HbA	Equation	R ²	%HbA VCN=1	Compared to ALS10-T87Q	HbF	Equation	R ²	%HbF VCN=1	Compared to ALS10-T87Q
ALS10-T87Q	Y = 14.76*X + 4.082	0.8823	18.4%	100%	ALS10-T87Q	Y = -0.357*X + 8.39	0.0204	8.0%	100%
ALS10-T87Q silent	Y = 16.22*X + 9.314	0.9759	25.5%	139%	ALS10-T87Q silent	Y = -1.604*X + 9.08	0.9111	7.5%	109.2%
ATM1.1	Y = 10.99*X + 4.262	0.9592	15.3%	82%	ATM1.1	Y = 5.101*X + 11.25	0.5878	16.4%	114.1%
ATM1.1 silent	Y = 7.324*X + 11.61	0.9312	18.9%	103%	ATM1.1 silent	Y = 0.211*X + 19.85	0.0204	20.1%	140.8%
HbF + HbA		Equation		R ²	%HbF+%HbA VCN=1		Compared to ALS10-T87Q [(Hba+HbF)/HbA]%		
ALS10-T87Q		Y = 14.45*X + 14.44		0.7692	28.89%		100%		
ALS10-T87Q silent		Y = 15.76*X + 17.57		0.9101	33.33%		115%		
ATM1.1		Y = 17.39*X + 15.96		0.7865	33.35%		115.4%		
ATM1.1 silent		Y = 8.112*X + 31.75		0.7826	39.86%		138.0%		

D

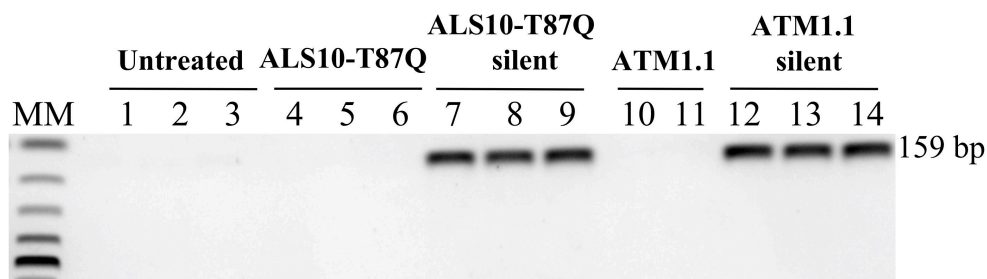
Target	Forward Primer	Reverse Primer
Silβ-globin	GTGGATGAAGTCGGCGGC	GGAGAAGGCACCGAGCAC
WTβ-globin	GTGGATGAAGTTGGTGGT	ACTAAAGGCACCGAGCAC
γ-globin	TGGCCTGTGGAGTAAGGTCAA	GAAGCAGAGGACAAGTTCCCA
β-actin	GTTTGAGACCTTCAACCCCAGCC	TAGATGGGCACAATGTGGGTGA

Supplemental Figure 2.

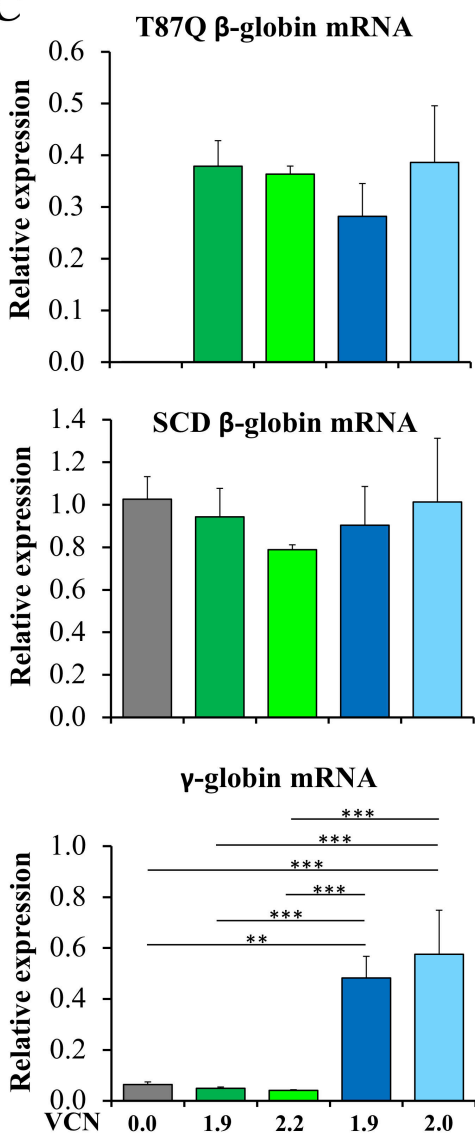
A



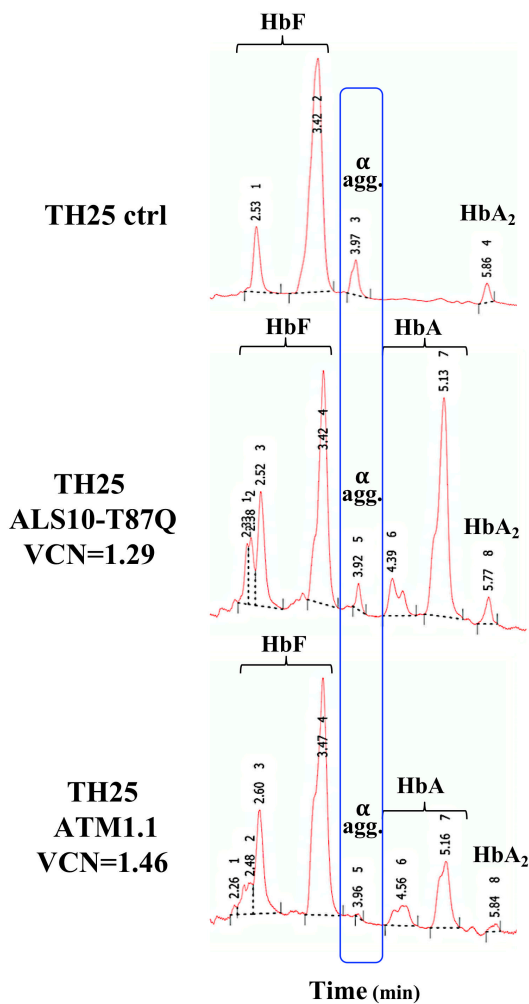
B



C



D



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