COMPOSITIONS AND METHODS FOR THE TREATMENT OF HEMOGLOBINOPATHIES

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application is being filed on 22 February 2013, as a PCT International patent application, and claims priority to U.S. Provisional Patent Application No. 61/603,231, filed February 24, 2012, the disclosure of which is hereby incorporated by reference herein in its entirety.

SEQUENCE LISTING

[0002] The present application includes a Sequence Listing in electronic format as a txt file entitled "sequence listing 54428.0006WOU1_ST25" and created on February 22, 2013 and which has a size of 174 kilobytes (KB). The contents of the txt file "sequence listing 54428.0006WOU1_ST25" are incorporated by reference herein.

BACKGROUND OF THE DISCLOSURE

Technical Field of the Disclosure

[0003] The present disclosure relates, generally, to the treatment of genetic diseases. More specifically, the present disclosure provides endonuclease-based compositions and methods, including homing endonuclease- and Cas9 endonuclease-based compositions and methods, for altering the expression of globin genes, which compositions and methods are useful for the treatment of thalassemias, sickle cell disease, and other hemoglobinopathies.

Description of the Related Art

[0004] Hemoglobinopathies, such as thalassemias and sickle cell disease, are highly prevalent genetic red blood cell disorders that cause a significant health burden worldwide. Over 1,300,000 people with severe hemoglobin disorders are born each year. While 5% of people worldwide are carriers, the birth rates are 0.44 and 1.96 per thousand for clinically significant forms of thalassemia and sickle cell disease (SCD), respectively.

[0005] In the normal state, hemoglobins found in mammalian erythroid cells predominantly consist of heterotetramers of two α-like chains (polypeptides) and two β-like chains. The five genes of the β-globin locus reside in a cluster on chromosome 11. The genes are expressed in an erythroid, and developmentally stage specific manor; the ε, A_γ and G_γ, and δ and β genes being expressed primarily during the embryonic, fetal and post-natal periods respectively. At birth 95% of β-like chains are γ, with the rest being β. This ratio gradually inverts during the first year of life, explaining why phenotypes limited to the β-globin gene such as sickle cell and most β-thalassemias do not manifest until several months of age. Expression of the chromosome 16 based α-like genes differs; the embryonic ζ-gene parallels the expression of ε, but the twin α-genes are expressed from the fetal period onward. Thus α abnormalities manifest in utero, potentially with devastating consequences (e.g. hydrops fetalis). The resultant α-, β-heterotetramers are developmentally expressed; embryonic: Hb Gower1 (ζ_2,ε_2), Hb Gower2 (α_2,ε_2) and Hb Portland (ζ_2,γ_2); fetal: HbF (Fetal) (α_2,γ_2) and Adult: HbA2 (α_2,δ_2) and HbA (Adult) (α_2,β_2).

[0006] β-thalassemia is caused by an abnormality in the adult β-globin locus, which results in an abnormal stoichiometry of β-like globin chains to α-like chains, resulting in the precipitation of the unpaired α-like chains. The severity of thalassemia is directly related to the degree of this globin chain imbalance. The ensuing damage meditated through several pathways including oxidation of cellular and membrane proteins culminates in ineffective erythropoiesis, apoptosis, and decreased red cell survival. Over 200 mutations have been described that are responsible for β-thalassemia.

[0007] Sickle cell disease is caused by a single nucleotide substitution within the β-globin gene, which results in glutamic acid being substituted by valine at amino acid position 6 of the peptide resulting in β^S. Hemoglobin S (α_2,β^S_2) , which carries this mutation, is referred to as HbS, as opposed to the normal adult hemoglobin (HbA). Under conditions of low oxygen concentration HbS undergoes an allosteric change at which point it can polymerize. The deoxy- form of hemoglobin exposes a hydrophobic patch on the protein between the E and F helices. The hydrophobic valine at position 6 of the hemoglobin β-chain forms a

hydrophobic patch which can associate with the hydrophobic patch of other hemoglobin S molecules causing hemoglobin S molecules to aggregate and form fibrous precipitates which, in turn, cause the red blood cells to adopt a sickle-shape and leads to alterations in numerous pathways that result in tissue damage via vaso-occlusion and hemolyis.

[0008] Although β-thalassemia and sickle cell disease (SCD) are quantitative and qualitative disorders, respectively, of the β-globin locus, the expression of normal β-like globin genes can ameliorate both diseases. In thalassemia, any improvement in the globin chain imbalance provides a selective advantage for each cell and results in clinical benefit. In sickle cell disease the presence of normal or certain mutant β-like chains can ameliorate the clinical phenotype by competing for α-like chains more effectively than the mutant sickle cell chains thus reducing the amount of HbS, by forming hemoglobins that block the polymerization of Hbs (e.g. HbF) and increasing the amount of non-sickling hemoglobin per cell. For example, in sickle cell disease, fetal hemoglobin (HbF) levels of only 8% inhibit polymerization of HbS, which results in increased survival, while HbF levels of 20% provide nearly complete phenotypic correction. Critically, the progeny of donor erythroid cells containing normal HbA have a strong selective advantage following hematopoietic stem cell transplantation (HSCT) over endogenous derived cells containing HbS. A patient with 11% donor cells in the marrow had 35% donor BFUe and 73% donor erythrocytes, which resulted in transfusion independence. Thus, correction in a relatively small fraction of transplanted HSCs provides clinical benefit.

[0009] Severe forms of thalassemia require chronic transfusions, resulting in iron overload. Survival directly correlates with the efficacy of chelation, though cost, side effects, and compliance severely limit efficacy. The only FDA approved drug for SCD is hydroxyurea, which can attenuate morbidity and mortality. This treatment, however, is under-prescribed, compliance is poor, and it does not adequately protect health.

[0010] Hematopoietic cell transplantation (HCT) is an important therapeutic option for thousands of patients each year with hematologic malignancies and

related disorders. According to the Center for International Blood and Marrow Transplant Research (CIBMTR), approximately 60,000 transplants were performed in 2009, an increase of over 15,000 transplants per year compared to a decade earlier. The effectiveness of transplantation is also increasing, with more recent outcomes demonstrating a significant reduction in the risk of relapse, non-relapse mortality, and overall mortality. Gooley *et al.*, *N. Engl. J. Med.* 363:2091-101 (2011).

[0011] Allogeneic hematopoietic cell transplantation (HCT) from HLA-matched sibling or unrelated donors offers a cure for patients with hemoglobinopathies, but is limited by the need for a suitably matched related or unrelated donor and is complicated by graft versus host disease (GVHD) and infections. In addition, a major barrier is a high rate of graft failures, which is higher than observed for HCT for malignancies. Alternative approaches include performing HCT with donor cord blood cells, as cord blood donors can be identified for nearly all patients. Additional experimental approaches are focused on using a patient's own hematopoietic stem cells (HSCs) and inducing expression of the endogenous globin genes, or adding an exogenous β-like globin gene.

[0012] For many patients who are unable to find a donor, particularly those of ethnic minority or mixed race background, umbilical cord blood (CB) transplantation may offer the best hope for cure. A source of donor stem cells (easily collected at the time of birth without risk to the mother or infant), CB also has the advantage of being readily available and safely used in an HLA-mismatched setting without increasing the risk of GVHD.

[0013] Unfortunately, several factors, including the low cell dose available in many cord blood units lead to slow engraftment and an increase in transplant related mortality in adults and larger children. Significantly delayed hematopoietic recovery of both neutrophils and platelets is a known risk factor for cord blood transplant (CBT) recipients and is associated with the low total nucleated cell (TNC) and $CD34^+$ cell doses provided in a single or double CB transplant. Similarly, these low cell numbers correlate with higher rates of graft failure, thus a particular concern in hemoglobinopathies where there is already high risk of graft failure. In

fact, a recent analysis of adult single CBT recipients demonstrated that infused $CD34^+$ cell dose is the most important predictor of myeloid engraftment.

[0014] Non-relapse mortality (NRM) is highest in double CBT (dCBT) recipients when compared to matched and mismatched unrelated donor recipients. Brunstein *et al.*, *Blood* 116:4693-9 (2010). The majority of the NRM occurs within the first 100 days post transplant with infection being the most common cause of death. Importantly, an analysis of the risk factors for NRM among dCBT recipients revealed a higher risk in patients with delayed myeloid recovery (time to absolute neutrophil count (ANC) >500/ml) if the recovery was ≥ 26 days, the median time to engraftment in dCBT recipients. When, however, the analysis of risk factors for NRM was restricted to include only those dCBT recipients engrafting before day 26, no difference was found between the donor sources, emphasizing the important contribution of delayed engraftment to increased risk of NRM.

[0015] Moreover, an ANC of >100 on any given day post stem cell transplant has been previously shown to be a critical threshold for a decreased risk of mortality before day 100 post transplant (Offner *et al.*, *Blood* 88:4058-62 (1996)). Thus, the significant delay in myeloid recovery that is observed in CBT recipients remains a critical barrier to successful outcomes in the CBT setting. The ability to increase not only the absolute number of CB progenitor cells available for transplantation, but also cells that can reliably result in more rapid myeloid recovery post-transplant, should improve overall survival for patients undergoing CBT. Strategies utilizing *ex vivo* expansion of cord blood stem/progenitor cells are being developed to overcome the low cell dose available in a cord blood graft with the goal of enhancing hematopoietic recovery and overall survival in CBT.

[0016] With the goal of overcoming the significant delay in neutrophil recovery that occurs following transplantation with umbilical cord blood (CB), the role of the Notch signaling pathway in regulating *ex vivo* expansion of hematopoietic stem/progenitor cells has been investigated to generate increased numbers of progenitor cells capable of rapid repopulation *in vivo*. A clinically feasible methodology utilizing an engineered Notch ligand (Delta1) has been

developed, which results in a multi-log increase in the absolute numbers of $CD34^+$ cells and a cellular therapy capable of rapid repopulation *in vivo*.

[0017] Infusion of expanded, partially HLA-matched cells results in a significant reduction in the median time to achieve an initial absolute neutrophil count (ANC) of 500/ml to just 11 days as compared to a median time of 25 days ($p<0.0001$) in a concurrent cohort of 29 patients undergoing identical treatment but with two non-manipulated CB units. Although the number of patients treated was small (*i.e.* n=14), a significant effect on time to myeloid recovery was demonstrated, as was the safety and clinical feasibility of this approach.

[0018] Despite tremendous investment of resources by many laboratories for over 30 years, there has been little progress in the development of therapeutic regimens for hemoglobinopathies, in large part due to the lack of identified drugable targets and the requirement for gene therapy vectors to persistently express at extremely high levels, while not leading to insertional mutagenesis. While increased expression of fetal hemoglobin (HbF) ameliorates both hemoglobinopathies, extensive research has not yielded viable new agents based on that observation. Hematopoietic stem cell (HSC) gene therapy with integrating lentiviral vectors is being pursued by several investigators. HSC gene therapy, however, requires high-level persistent expression and carries a substantial risk of insertional mutagenesis and leukemia.

[0019] What is critically needed in the art are compositions and methods, which exhibit improved efficacy for the treatment of hemoglobinopathies, including thalassemias and sickle cell disease while overcoming the safety concerns of existing therapeutic modalities.

SUMMARY OF THE DISCLOSURE

[0020] The present disclosure addresses these and other related needs in the art by providing, *inter alia*, compositions and methods for the treatment of hemoglobinopathies. Compositions and methods disclosed herein employ one or more polynucleotide that encodes one or more endonuclease(s) or endonuclease fusion protein(s), including one or more homing endonuclease(s) and/or homing endonuclease fusion protein(s) and/or one or more CRISPR endonucleases (*i.e.* Cas9 endonucleases in combination with one or more RNA guide strands) and/or CRISPR endonuclease fusion protein(s) (*i.e.* Cas9 endonuclease fusion protein(s) in combination with one or more RNA guide strands): (a) to disrupt a Bcl11a coding region or a Bcl11a gene regulatory region; (b) to disrupt a HbF silencing DNA regulatory element or pathway, such as a Bcl11a-regulated HbF silencing region; (c) to mutate one or more γ-globin gene promoter(s) to achieve increased expression of a γ-globin gene; (d) to mutate one or more δ-globin gene promoter(s) to achieve increased expression of a δ-globin gene; and/or (e) to correct one or more β-globin gene mutation(s).

[0021] Within a first embodiment, the present disclosure provides compositions and methods that comprise a polynucleotide that encodes one or more endonuclease(s), such as a homing endonuclease (HE) and/or a CRISPR endonucleases (*i.e.* Cas9 endonucleases in combination with one or more RNA guide strands) to achieve the targeted disruption of a sequence within a Bcl11a coding region or a Bcl11a gene regulatory region, thereby increasing to therapeutic levels the expression of an endogenous gene such as a γ- or a ε- globin gene. Within related aspects, the compositions of these embodiments comprise a polynucleotide that encodes one or more TALEN, one or more TALE-HE fusion protein, and/or one or more TREX2 protein.

[0022] Within a second embodiment, the present disclosure provides compositions and methods that comprise a polynucleotide that encodes one or more endonuclease(s), such as a homing endonuclease (HE) or a CRISPR endonucleases (*i.e.* Cas9 endonucleases in combination with one or more RNA guide strands) to achieve the targeted disruption of a key regulatory sequence within a β-globin gene

locus, thereby increasing to therapeutic levels the expression of an endogenous gene such as a γ- or δ- globin gene. Within related aspects, the compositions of these embodiments comprise a polynucleotide that encodes one or more TALEN, one or more TALE-HE fusion protein, and/or one or more TREX2 protein.

[0023] Within certain aspects of this embodiment are provided HEs and CRISPR endonucleases that target a 3.6 kb region (SEQ ID NO: 1) within a β-globin gene locus (chr11:5212342-5215944 in HG18) that contains a binding site for the regulatory protein Bcl11a.

[0024] The homing endonucleases and CRISPR endonucleases described herein exhibit unique advantages over conventional gene targeting nucleases. Because they are broadly efficacious regardless of genotype, the homing and Cas9 endonucleases in combination with one or more RNA guide strands described herein are not patient specific, they provide clinical benefit in the heterozygotic state, and avoid the insertion of vector sequences.

[0025] Within a third embodiment, the present disclosure provides compositions and methods for recapitulating, via genome editing, one or more naturally-occurring mutation(s) within a patient's genome thereby providing clinical benefits including, for example, deletional or non-deletional forms of hereditary persistence of fetal hemoglobin (HPFH). More specifically, the present disclosure provides compositions and methods for achieving the direct correction of a thalassemia and/or a sickle cell disease (SCD) mutation through genome editing.

[0026] Within certain aspects of this embodiment, one or more homing endonuclease(s) is/are employed in combination with a normal or wild-type polynucleotide sequence (correction template) to permit the editing and/or repair of one or more genetic sequence, such as a β-like globin gene(s). These homing endonucleases permit the modification of key regulatory and/or coding sequences within a gene locus, exemplified herein by the human β-globin gene locus, through the transient expression of a polynucleotide that includes one or more naturally occurring mutation(s). Within related aspects, the compositions of these

embodiments comprise a polynucleotide that encodes one or more TALEN, one or more TALE-HE fusion protein, and/or one or more TREX2 protein.

[0027] More specifically, the present disclosure provides compositions and methods for genome editing, comprising one or more polynucleotides, each encoding a HE and a correction template, which may be employed to generate naturally-occurring mutations within stem cells, including, for example, hematopoietic stem cells (HSCs), embryonic stem (ES) cells, and induced pluripotent stem cells (iPSCs). Genome edited HSCs, ESs, and iPSCs, including autologous HSCs and iPSCs, may be transplanted into a patient to treat one or more hemoglobinopathies, such as a thalassemia and/or sickle cell disease.

[0028] The compositions and methods disclosed herein permit the efficient modification of HSCs, ESs, and iPSCs, through the transient expression of a polynucleotide encoding a HE with or without a targeting template, a Cas9 endonuclease, and/or an RNA guide strand, without the need for the persistent expression or insertion of an exogenous gene to achieve the amelioration of hemoglobinopathies in mature erythroid cells and in patient cells *in vivo*. Because these therapeutic methods do not require the integration and/or persistent expression of a transgene, the safety concerns associated with currently available gene therapy technologies are obviated.

[0029] Within a fourth embodiment, the present disclosure provides compositions and methods for the delivery of one or more homing endonuclease(s) and/or one or more Cas9 endonuclease(s) in combination with one or more RNA guide strands, each of which may be transiently expressed in targeted regions shown to have clinical benefit in humans. The endonuclease coding sequences described herein may be expressed in combination with, or fused to, a TAL effector nuclease (TALEN) coding sequence. Exemplified herein are TAL effector-HE (TALE-HE) fusion proteins and polynucleotides that encode those TALE-HE fusion proteins, which target critical genomic regions that influence fetal hemoglobin production.

[0030] Within certain aspects of these embodiments, a polynucleotide encoding one or more HE with or without a targeting template, one or more Cas9

endonuclease, one or more RNA guide strands, one or more TALEN, one or more TALE-HE fusion protein, and/or one or more TREX2 protein are operably linked to a promoter sequence within a viral vector to achieve the delivery and transient expression of a HE, a Cas9, an RNA guide strand, a TALEN, a TALE-HE fusion protein, and/or a TREX2 protein. Suitable viral vectors that may be satisfactorily employed for the delivery of HE, TALEN, TALE-HE fusion protein, and/or TREX2 protein may be selected from the group consisting of a cocal pseudotyped lentiviral vector, a foamy virus vector, an adenoviral vector, and an adeno-associated viral (AAV) vector.

[0031] Within a fifth embodiment, the present disclosure provides compositions and methods comprising *ex-vivo* expanded modified hematopoietic stem cells (HSCs), which allow for efficient engraftment of corrected cells and the use of induced pluripotent stem cells (iPSCs) for screening and clinical application. Within certain aspects of these embodiments are provided compositions and methods for the efficient expansion of autologous HSCs, autologous gene-modified HSCs, iPSC-derived HSCs, and ES cells. Cord blood expansion methodology may be employed, which methodology utilizes Delta1 in serum free media supplemented with hematopoietic growth factors using mobilized peripheral blood CD34+ cells obtained from normal donors. These compositions and methods may be used in combination with one or more additional reagent to enhance the survival and proliferation of hematopoietic stem/progenitor cells. Within other aspects, these compositions and methods may employ endothelial cell co-cultures for the enhanced expansion of long-term repopulating cells, including corrected iPSC-derived HSCs.

[0032] Within a sixth embodiment, the present disclosure provides compositions and methods for providing supportive care, which compositions and methods comprise off-the-shelf cellular therapies that abrogate post-transplant neutropenia and improve outcome following transplantation of gene-corrected autologous HSCs. *Ex vivo* expanded, cryopreserved cord blood (CB) stem/progenitor cells may, for example, be administered as a means of supportive care to patients with thalassemia and/or sickle cell disease who are undergoing myeloablative HCT with autologous CD34+ gene corrected cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0033] Certain aspects of the present disclosure will be better understood in view of the following figures:

[0034] Figure 1 shows targets to increase expression of β-globin-like genes in adult erythroid tissues. Factors that are implicated in regulating the switch from a fetal expression pattern (two γ genes) to an adult program (δ and β) are displayed. (Adapted from Wilber *et al.*, *Blood* 117(15):3945-3953 (2011)).

[0035] Figure 2 depicts three exemplary rare cleaving nuclease technologies.

[0036] Figure 3 is a graph showing that the risk of non-relapse mortality is highest among double CBT recipients. Non-relapse mortality after double CBT (DUCB), matched unrelated donor (MUD), mismatched unrelated donor (MMUD), and matched related donor (SIB) transplant.

[0037] Figure 4 shows that a culture of CB progenitors with Delta1$^{ext\text{-}IgG}$ results in more rapid neutrophil recovery in a myeloablative double CBT setting. The individual and median times (solid line) to ANC of ≥500/μl for patients receiving double unit CBT with two non-manipulated units ("conventional") versus with one *ex vivo* expanded unit and one non-manipulated unit ("expanded") is presented.

[0038] Figure 5 is a bar graph depicting the number of cord blood transplantations performed annually by disease indication.

[0039] Figure 6 (SEQ ID NO: 1) is the sequence of the 3.6 kb region for which the HbF silencing region falls, which spans chr11:5212342-5215944 in HG18.

[0040] Figure 7 (SEQ ID NO: 2) is the 350 base pair region spanning from a repeat element (chr11:5,213,912-5,214,261 in HG18), through the upstream French HPFH breakpoint known to disrupt the Bcl11a occupancy region within the HbF silencing region and that includes a GATA-1 binding motif, and from which exemplary homing endonucleases (HEs) of the present disclosure were designed.

[0041] Figure 8 (SEQ ID NO: 13) is the human beta-globin gene from 1 kb upstream of the cap through the polyA site, which spans from chr11:5203272-5205877 in HG18 (reverse strand).

[0042] Figure 9 (SEQ ID NO: 14) is a 606 bp region of the human beta-globin spanning from the promoter into Intron 2 (chr11:5204380-5204985 in HG18). This relatively small region contains the majority of mutations leading to severe thalassemia as well as the mutation causing sickle cell disease. This small region is readily amenable to homologous recombination resulting in gene correction.

[0043] Figure 10 (SEQ ID NO: 24) is the cDNA sequence for human Bcl11a cDNA (CCDS1862.1).

[0044] Figure 11 is a restriction map for the plasmid pET-21a(+).

[0045] Figure 12 is a restriction map for the plasmid pEndo (Doyon *et al.*, *J. Am. Chem. Soc.* 128(7):2477-2484 (2006).

[0046] Figure 13 is a schematic diagram of directed evolution for creating the BCL11A gene-targeting endonuclease. A constructed library was subjected to selection in IVC against a target site, a portion of which was replaced with the BCL11A gene target.

[0047] Figure 14 (SEQ ID NO: 28) is the nucleotide sequence of I-HjeMI (the parental enzyme for the BCL11A gene targeting nuclease), which is codon optimized for expression in *E. coli*.

[0048] Figure 15 (SEQ ID NO: 29) is the nucleotide sequence of I-HjeMI (the parental enzyme for the BCL11A gene targeting nuclease), which is codon optimized for mammalian expression.

[0049] Figure 16 (SEQ ID NO: 30) is the amino acid sequence of the homing endonuclease I-HjeMI.

[0050] Figure 17 (SEQ ID NO: 31) is the nucleotide sequence of a BCL11A gene targeting nuclease (Bcl11Ahje), which is based on the homing endonuclease I-HjeMI (obtained through directed evolution in IVC and in bacteria), which is codon optimized for expression in *E. coli*.

[0051] Figure 18 (SEQ ID NO: 32) is the nucleotide sequence of a BCL11A gene targeting nuclease based on the homing endonuclease I-HjeMI (obtained through directed evolution in IVC and in bacteria), which is codon optimized for mammalian expression.

[0052] Figure 19 (SEQ ID NO: 33) is the amino acid sequence of a BCL11A gene targeting nuclease based on the homing endonuclease I-HjeMI (obtained through directed evolution in IVC and in bacteria).

[0053] Figure 20 is a protein model showing the distribution of amino-acid residues different between the BCL11A gene-targeting endonuclease and its parental LHE I-HjeMI. Substituted residues of the BCL11A gene-targeting endonuclease are mapped on the crystal structure of I-HjeMI bound to its target site (PDB ID: 3UVF). D161 is deleted in the variant endonuclease.

[0054] Figure 21 is a bar graph showing the activity of a BCL11A gene-targeting endonuclease in a two-plasmid cleavage assay.

[0055] Figure 22A (SEQ ID NO: 34) nucleotide sequence of I-OnuI homing endonuclease (the parental enzyme for homing endonucleases targeting the HbF silencing region), codon optimized for expression in *E. coli*.

[0056] Figure 22B (SEQ ID NO: 15) is an amino acid sequence of I-OnuI homing endonuclease.

[0057] Figure 23 is an agarose gel showing the activity of an I-OnuI homing endonuclease targeting the HbF silencing region.

[0058] Figure 24 (SEQ ID NO: 35) is the nucleotide sequence of MegaTAL:5.5 RVD + Y2 I-AniI.

[0059] Figure 25 (SEQ ID NO: 36) is an amino acid sequence of MegaTAL:5.5 RVD + Y2 I-AniI.

[0060] Figure 26 (SEQ ID NO: 37) nucleotide sequence of Cas9 endonuclease (from Mali *et al.*, *Science* (2013)).

[0061] Figure 27 (SEQ ID NO: 38) is the nucleotide sequence of an RNA Guide Strand for use with Cas9 endonuclease (from Mali *et al.*, *Science* (2013)).

[0062] Figure 28 (SEQ ID NO: 62) is a nucleotide sequence of I-CpaMI homing endonuclease (ORF, codon optimized for mammalian expression).

[0063] Figure 29 (SEQ ID NO: 63) is an amino acid sequence of I-CpaMI homing endonuclease.

[0064] Figure 30 is an agarose gel showing the detection of targeted mutagenesis at the endogenous human BCL11A gene as described in Example 4.

[0065] Figure 31 is a restriction map for the plasmid pCcdB wt6 (Doyon *et al.*, *J. Am. Chem. Soc.* 128(7):2477-2484 (2006).

[0066] Figure 32 (SEQ ID NO: 64) is a nucleotide sequence of a BCL11A gene targeting nuclease-encoding plasmid (pExodusBCL11Ahje).

[0067] Figure 33 (SEQ ID NO: 65) is a nucleotide sequence of TREX2-encoding plasmid (pExodus CMV.Trex2).

[0068] Figure 34 is a restriction map for the plasmid pExodusBCL11Ahje.

[0069] Figure 35 is a restriction map for the plasmid pExodus CMV.Trex2.

DETAILED DESCRIPTION OF THE DISCLOSURE

[0070] The present disclosure is directed, generally, to compositions and methods for the treatment of a genetic disease, such as a hemoglobinopathy, by the transient or persistent expression of a polynucleotide that encodes one or more endonuclease(s) or endonuclease fusion protein(s), including one or more homing endonuclease(s) and/or homing endonuclease fusion protein(s) and/or one or more

Cas9 endonuclease(s) and/or Cas9 endonuclease fusion protein(s) in combination with one or more RNA guide strands: (a) to disrupt a Bcl11a coding region; (b) to disrupt a HbF silencing DNA regulatory element or pathway, such as a Bcl11a-regulated HbF silencing region; (c) to mutate one or more γ-globin gene promoter(s) to achieve increased expression of a γ-globin gene; (d) to mutate one or more δ-globin gene promoter(s) to achieve increased expression of a δ-globin gene; and/or (e) to correct one or more β-globin gene mutation(s). The compositions and methods disclosed herein find utility in the treatment of hemoglobinopathies, including β-thalassemia and sickle cell disease. The compositions and methods described herein may, optionally, comprise a polynucleotide that encodes one or more TALEN, one or more TALE-HE fusion protein, and/or one or more TREX2 protein.

[0071] The present disclosure will be better understood in view of the following definitions:

Definitions

[0072] As used herein, the term "hemoglobinopathy" refers to a class of genetic defects that result in an abnormal structure, abnormal function or altered expression of one or more of the globin chains of the hemoglobin molecule. Hemoglobinopathies are inherited single-gene disorders. Common hemoglobinopathies include thalassemias and sickle-cell disease.

[0073] As used herein, the term "thalassemia" refers to a hemoglobinopathy that results from an altered ratio of α-like to β-like globin polypeptide chains resulting in the underproduction of normal hemoglobin tetrameric proteins and the accrual of free or unpaired α- or β- chains.

[0074] As used herein, the term "sickle-cell disease" refers to a group of autosomal recessive genetic blood disorders, which results from mutations in a globin gene and which is characterized by red blood cells that assume an abnormal, rigid, sickle shape. They are defined by the presence of β^S –gene coding for a β-globin chain variant in which glutamic acid is substituted by valine at amino acid position 6 of the peptide, and second β-gene that has a mutation that allows for the

crystallization of HbS leading to a clinical phenotype. The term "sickle-cell anaemia" refers to a specific form of sickle-cell disease in patients who are homozygous for the mutation that causes HbS. Other common forms of sickle cell disease include HbS / β-thalassemia, HbS / HbC and HbS / HbD. Table 1 discloses the nucleotide sequences encoding the initial amino acids of a wild-type and sickle cell β-globin chains

Table 1

β-globin chain	Sequence	Sequence Identifier
Wild-type	GTGCACCTCACTCCAGAGGAG	SEQ ID NO: 3
Sickle	GTGCACCTCACTCCAGTGGAG	SEQ ID NO: 4

[0075] As used herein, the term "hereditary persistence of fetal hemoglobin" or "HPFH" refers to, a benign condition in which significant fetal hemoglobin (hemoglobin F) production continues well into adulthood, disregarding the normal shutoff point.

[0076] As used herein, the term "globin" refers to a family of heme-containing proteins that are involved in the binding and transport of oxygen.

[0077] As used herein, the term "homing endonuclease" or "HE" refers to a class of restriction endonucleases that are characterized by recognition sequences that are long enough to occur only once in a genome and randomly with a very low probability (e.g., once every 7×10^{10} bp).

[0078] As used herein, the term "Transcription Activator-Like Effector Nuclease" or "TAL effector nuclease" or "TALEN" refers to a class of artificial restriction endonucleases that are generated by fusing a TAL effector DNA binding domain to a DNA cleavage domain.

[0079] As used herein, the term "three prime repair exonuclease 2" or "TREX2" refers to a nuclease having 3' exonuclease activity, which is typically involved in DNA replication, repair, and recombination.

[0080] As used herein, the term "Cas9 endonuclease" refers to an endonuclease that uses an RNA guide strand to target the site of endonuclease cleavage. The term "CRISPR endonuclease" refers to a Cas9 endonuclease in

combination with an RNA guide strand. *See*, Jinek *et al.*, *Science* 337:816-821 (2013); Cong *et al.*, *Science* (Jan. 3, 2013) (Epub ahead of print); and Mali *et al.*, *Science* (Jan. 3, 2013) (Epub ahead of print).

[0081] It will be understood that, unless indicated to the contrary, terms intended to be "open" (*e.g.*, the term "including" should be interpreted as "including but not limited to," the term "having" should be interpreted as "having at least," the term "includes" should be interpreted as "includes but is not limited to," etc.). Phrases such as "at least one," and "one or more," and terms such as "a" or "an" include both the singular and the plural.

[0082] It will be further understood that where features or aspects of the disclosure are described in terms of Markush groups, the disclosure is also intended to be described in terms of any individual member or subgroup of members of the Markush group. Similarly, all ranges disclosed herein also encompass all possible sub-ranges and combinations of sub-ranges and that language such as "between," "up to," "at least," "greater than," "less than," and the like include the number recited in the range and includes each individual member.

[0083] All references cited herein, whether *supra* or *infra*, including, but not limited to, patents, patent applications, and patent publications, whether U.S., PCT, or non-U.S. foreign, and all technical and/or scientific publications are hereby incorporated by reference in their entirety.

[0084] While various embodiments have been disclosed herein, other embodiments will be apparent to those skilled in the art. The various embodiments disclosed herein are for purposes of illustration and are not intended to be limiting, with the true scope and spirit being indicated by the claims.

Homing Endonucleases for Achieving High-efficiency, Multiplex Gene Disruption and Gene Editing Functions

[0085] As discussed above and exemplified below, the present disclosure provides compositions and methods comprising a polynucleotide that encodes one or more endonuclease(s), including one or more homing endonuclease(s) (HE(s)), such as one or more I-HjeMI homing endonuclease(s), I-CpaMI homing endonuclease(s),

and/or I-OnuI homing endonuclease(s), and/or one or more Cas9 endonuclease in combination with one or more RNA guide strands, which may be transiently or persistently expressed in targeted cells shown to have clinical benefit in humans. Exemplary endonucleases target critical genomic regions that influence fetal hemoglobin production by: (a) disrupting a Bcl11a coding region or a Bcl11a gene regulatory region; (b) disrupting a HbF silencing DNA regulatory element or pathway, such as a Bcl11a-regulated HbF silencing region; (c) mutating one or more γ-globin gene promoter(s) to achieve increased expression of a γ-globin gene; (d) mutating one or more δ-globin gene promoter(s) to achieve increased expression of a δ-globin gene; and/or (e) correcting one or more β-globin gene mutation(s). The compositions and methods disclosed herein find utility in the treatment of hemoglobinopathies, including β-thalassemia and sickle cell disease.

[0086] The endonuclease coding sequences described herein may be expressed in combination with, or fused to, a DNA binding domain coding sequence, such as a TAL effector (TALE) coding sequence or a nuclease coding sequence such as a three prime repair exonuclease 2 (TREX2) coding sequence. Exemplified herein are TALE-HE fusion proteins and polynucleotides that encode one or more TALE-HE fusion protein(s).

[0087] Four protein scaffolds are known in the art for achieving targeted gene modification and disruption in eukaryotes: zinc finger nucleases (ZFNs), TAL effector nucleases (TALENs), homing endonucleases (HEs), and Cas9 endonucleases in combination with a RNA guide strand. The present disclosure employs TAL effector nucleases, homing endonucleases, and/or Cas9 endonucleases either alone or in combination. TAL nucleases offer more straightforward modular design and higher DNA recognition specificity than zinc finger nucleases while homing endonucleases, such as LAGLIDADG homing endonucleases (LHEs), offer highly specific cleavage profiles, compact structures, and, because they are compact monomeric proteins that do not require dimerization as do ZFNs and TALENs, the ability to be used in multiplex combinations. Accordingly, HEs and CRISPR endonucleases (*i.e.* Cas9 endonucleases in combination with one or more RNA guide strands) are extremely efficient in mediating gene disruption. Stoddard, *supra* and Mali *et al.*, *Science* (2013), *supra.*

[0088] As part of the present disclosure, a critical region within the β-globin locus that suppresses HbF function has been identified. This region provides multiple targets for HE- and Cas9-mediated cleavage. Specifically-designed nucleases may be tested for activity against a cognate target site and for off-target activity against any closely related genomic targets. These HEs and Cas9 endonucleases in combination with one or more RNA guide strands may be engineered to avoid off-target genomic cleavage using the methods described in Stoddard, *Structure* 19:7-15 (2011) and Mali *et al.*, *Science* (2013). HEs and Cas9 endonucleases in combination with one or more RNA guide strands that are disclosed herein are capable of directly targeting the γ- and δ-promoters and replacing a 606 bp region (SEQ ID NO: 14) that spans the majority of thalassemia mutations as well as the HbS mutation.

[0089] To facilitate the generation of large deletions spanning the HbF silencing region or subsets thereof one or more HEs and/or one or more Cas9 endonucleases in combination with one or more RNA guide strands may be co-transduced with a bridging oligonucleotide, which spans from the endonuclease cleavage site to the end of the target region. Chen *et al.*, *Nat. Methods* 8(9):753-5 (2011). Higher frequency genome editing may be achieved by employing one or more HEs that bind to and cleave a sequence that flanks each side of the target region. Similarly, a HE and a mutagenizing oligonucleotide may be used to introduce promoter region mutations, which leads to elevated expression of a gamma or delta gene.

[0090] The presently disclosed HEs may be first evaluated in an erythroid cell line and in human CD34+ cells that are induced to differentiate to erythroid cells, thereby confirming the ability to alter globin gene expression. Depending upon the precise application contemplated, one, two, three, or more HEs may be delivered to facilitate the generation of larger deletions. The suitability of individual HEs can be assessed in additional culture and animal model assays to confirm their ability to target HSCs without compromising pluripotency and expansion potential, and to assess clinical benefit in hemoglobinopathy models. One or more HEs and one or more exonuclease, such as a TREX2 exonuclease or a TAL effector

exonuclease, may be delivered to CD34+ HSC for the induction of targeted genetic deletions in critical regions for HbF.

[0091] Individual nucleases may be tested against a series of targets in a 350 bp region (SEQ ID NO: 2) defined by a region initiating at the edge of a repeat element and spanning through the upstream French HPFH breakpoint known to disrupt the Bcl11a occupancy region within the HbF silencing region and that includes a GATA-1 binding motif. Initial analyses have identified seven targets, evenly distributed throughout the region, which comprise DNA sequence modules for which pools of highly active endonuclease variants have been isolated and sequenced. Notably, one target overlaps with a potential Bcl11a binding motif and is adjacent to the GATA-1 motif. Successively larger deletions of a target region may be achieved by transducing two, three, or more HEs. Alternatively, multiple targets for disrupting the Bcl11a gene have been identified on the 5'-end of the gene to ensure the elimination of gene function. Similarly, multiple optimal targets for Cas9 / RNA guide mediated disruption have been identified through the area that can be used singly, or in combination leading to larger deletions.

[0092] Individual HEs may be tested in transfected human cell lines using integrated genomic reporters, and may further employ additional selection steps to further optimize cleavage and gene conversion activities using protocols as described in Stoddard, *supra*. The validation and delivery of individual targeted HEs that are active against targets in the globin locus may be followed by vectorization of the nucleases in expression systems. For example, an expression system may be employed that links each HE to the coexpression of a nuclease, such as a TALEN and/or a TREX exonuclease, to achieve greatly enhanced gene disruption efficiency in transduced cells.

[0093] The present disclosure also provides TALE-HE fusion proteins, and polynucleotides that encode TALE-HE fusion proteins, which exhibit the desired feature of restricting the recruitment and activity of engineered HEs to the desired target site, such as within a globin locus, through the synergistic recognition of adjacent DNA targets by the TALE and HE scaffolds. Such TALE-HE fusions combine the most favorable properties of each scaffold (*i.e.*, modular assembly of

TALEs and nuclease specificity of HEs) while reducing nonspecific nuclease activity that is associated with traditional TALENs or zinc finger nucleases (ZFNs).

[0094] The high-resolution crystal structures have been determined for ten separate LAGLIDADG homing endonucleases (LHEs) in complex with their cognate DNA target sites. Stoddard, *Structure* 19:7-15 (2011) and Takeuchi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 108:13077-13082 (2011). Chimeric 'hybrids' of those LHEs have been constructed that, collectively, provide a broad range of LHE targeting proteins for gene-specific applications. Baxter *et al.*, *Nucl. Acids Res.* 40(16):7985-8000 (2012).

[0095] HEs having suitable target sequence specificity may be identified by a yeast surface display strategy, combined with high-throughput cell sorting for desirable DNA cleavage specificity. A series of protein-DNA 'modules', which correspond to sequential pockets of contacts that extend across the entire target site, may be systematically randomized in separate libraries. Each library may then be systematically sorted for populations of enzymes that can specifically cleave each possible DNA variant within each module, and each sorted population deep-sequenced and archived for subsequent enzyme assembly and design. HEs that may be suitably employed in the compositions and methods of the present disclosure are commercially available (Pregenen, Seattle, WA).

[0096] Within certain aspects, the compositions and methods described herein may employ the co-expression of one or more HE, including, for example, one or more LHE, with a TREX2 3' exonuclease. In contrast to the 5' overhangs left by current versions of ZFNs and TALENs, HEs generate 3' overhangs at the site of targeted double-strand breaks, which results in an enhanced rate of end processing following HE cleavage. Near complete modification of a double strand break site in primary cells can be achieved through HE/TREX2 co-expression. Because of the way HE/TREX2 co-expression influences break processing, this combination achieves multiple targeted deletions in one region and increases the safety of nuclease-induced targeted gene disruption by diminishing break persistence and reducing the potential for large scale translocations mediated through alternative end joining pathways.

[0097] The crystal structure of a TAL effector (PthXo1) bound to its DNA target site has recently been determined. Mak *et al.*, *Science* 335(6069):716-9 2012; e-pub 05Jan12 PubMed PMID: 22223736. These crystal structure data permit the precise definition of the boundaries of DNA recognition region and facilitates strategies for the creation of well-behaved TALEN-HE, or other TALEN-nuclease fusion construct, which may be applied to achieve a variety of complex genomic manipulations.

Genome Disruption to Bcl11a Gene Expression

[0098] Knockout of Bcl11a in a sickle cell mouse model ameliorates disease, supporting the clinical relevance of this pathway. Xu *et al.*, *Science* 334:993-6 (2011). In addition, mice containing a YAC transgene spanning the human β-globin locus are used to model perturbations in Bcl11a mediated silencing of HbF. Heterozygous and homozygous knockout of the endogenous Bcl11a gene in these mice results in γ-globin mRNA comprising 20 and 76% of total β-like mRNA respectively, compared to 0.24% in controls. Sankaran *et al.*, *Nature* 460:1093-7 (2009). This suggests Bcl11a acts as rheostat, modulating the degree of HbF suppression. Consistent with this, decrease of function mutations in Bcl11a result in elevated levels of HbF and a lessening of the clinical thalassemia and/or sickle cell disease phenotype. Galanello *et al.*, *Blood* 114:3935-7 (2009).

[0099] Within certain embodiments, the present disclosure provides compositions and methods that comprise one or more endonuclease(s), including one or more homing endonuclease(s) (HE(s)), such as one or more I-HjeMI homing endonuclease(s), I-CpaMI homing endonuclease(s), and/or I-OnuI homing endonuclease(s), and/or one or more Cas9 endonuclease(s) to achieve the disruption of a sequence that encodes Bcl11a or its key regulatory sequences. As described in greater detail and exemplified herein, compositions and methods comprising one or more Cas9 endonucleases further comprise one or more Bcl11a gene-specific RNA guide strands to mediate the targeting of the Cas9 endonuclease to a Bcl11a gene sequence.

[00100] The Bcl11a gene has multiple exons spanning over 100kb and results in several splice variants that lead to proteins associated with different activities. As

part of the present disclosure, several DNA targets have been identified that are transcribed into multiple Bcl11a splice variants. All disrupt the long (L) and extra-long (XL) forms, which are associated with the greatest HbF silencing activity, while one disrupts all forms of Bcl11a. These targets comprise DNA sequence modules for which pools of highly active endonuclease variants have been isolated and sequenced. The human Bcl11a cDNA sequence (CCDS1862.1) is presented herein as SEQ ID NO: 24 (Figure 10).

[00101] Thus, within certain aspects, the present disclosure provides compositions for achieving therapeutic levels of HbF, which compositions comprise a polynucleotide encoding one or more homing endonuclease (HE), which is capable of mediating the disruption of the nucleotide sequence within this 1.3 kb region, thereby preventing the binding of Bcl11a, and the formation of the corresponding repressive complex, and de-repressing γ-globin expression.

Genome Disruption to Block Bcl11a-mediated Silencing of HbF

[00102] As summarized above, within certain embodiments, the present disclosure provides compositions and methods for treating and/or ameliorating a genetic disease, such as a hemoglobinopathy, including a thalassemia and/or sickle cell disease. Certain aspects of these embodiments include the transient expression of a polynucleotide encoding one or more homing endonuclease(s) to disrupt a HbF silencing element or pathway within a β-globin gene locus or a δ-globin gene locus thereby increasing to therapeutic levels the expression of an endogenous gene such as a γ- or δ-globin gene.

[00103] The compositions disclosed herein comprise a polynucleotide encoding one or more homing endonuclease(s) (HE(s)) and or one or more Cas9 endonuclease in combination with one or more RNA guide strands and, optionally, one or more transcription activator-like (TAL) effector(s), to achieve the targeted disruption of key regulatory sequences within the β-globin gene locus. More specifically, the compositions and methods disclosed herein achieve an increase in γ-globin gene expression, and consequent HbF protein production, by removing essential elements for Bcl11a binding to the HbF silencing region(s) within the β-globin gene locus.

[00104] During normal development, expression of an embryonic β-like gene (ε-globin) is sequentially replaced by a pair of γ-globin genes in the fetus and the δ- and β-globin genes in the adult. In adult erythroid tissues, the zinc finger protein Bcl11a binds to a region between the γ-globin and δ-globin genes within the β-globin gene locus thereby silencing the production of HbF. The importance of Bcl11a-mediated silencing of HbF is supported by knockdown of Bcl11a mRNA in human CD34 cells, which increases HbF levels to 24-36% of total β-like proteins. Sankaran *et al.*, *Science* 322:1839-42 (2008). Removal of this region in the deletion form of HPFH, as well as the knockdown of Bcl11a, blocks Bcl11a-mediated HbF silencing and results in an elevated level of γ-globin gene expression and HbF protein production in adult erythroid tissues (Sankaran *et al.*, *N. Engl. J. Med.* 365:807-14 (2011)).

[00105] While multiple mechanisms contribute to an elevation in HbF protein levels, it has been shown that a 3.6 kb region is key for HbF silencing (SEQ ID NO: 1). Sankaran *et al.*, *N. Engl. J. Med.* 365:807-14 (2011). While there are several peaks of Bcl11a enrichment in the β-globin locus, the single peak in the 3.6 kb HbF silencing region stands out as proteins known to form a repressive complex with Bcl11a are bound in this region (GATA-1 and HDAC-1) and the chromatin is enriched for the repressive histone mark trimethylation of histone H3 on lysine27.

[00106] Notably this 3.6 kb region contains a single peak of Bcl11a binding downstream of the γ-gene. Multiple point mutations have been identified in the γ-globin promoters that result in HbF levels of 20-30% as a heterozygote, ameliorating thalassemia and SCD. These point mutations cluster to three regions all within 200 bp of the γ-cap sites: (1) -200, a GC-rich region bound by SP1 and a stage specific protein complex; (2) -175, bound by GATA-1; and (3) Oct1 and a CCAAT motif at -117 bound by several addition factors. Forget, *Ann. NY Acad. Sci.* 850:38-44 (1998).

[00107] Mutations within these three regions block the binding of a repressive complex in adult erythroid cells. Consequently, these regions are suitable targets for HE-mediated disruption and targeted mutation by the compositions and methods disclosed herein. The disruption of these regions leads to a decrease in repressive

complexes, which results in an elevated level of γ-globin gene expression, and a corresponding increase in HbF protein production to levels that are sufficient to achieve therapeutic efficacy in methods for the treatment of hemoglobinopathies, including β-thalassemia and sickle cell disease.

[00108] A single peak of Bcl11a occupancy is present within the 3.6 kb HbF silencing region (Sankaran *et al.*, *N. Engl. J. Med.* 365:807-14 (2011)) ((SEQ ID NO: 1). This region of Bcl11a occupancy is disrupted by the upstream breakpoint of French HPFH Sankaran *et al.*, *N. Engl. J. Med.* 365:807-14 (2011). Described herein is a 350 bp region initiating at the edge of a repeat element and spanning through the upstream French HPFH breakpoint known to disrupt the Bcl11a occupancy region within the HbF silencing region and that includes a GATA-1 binding motif (SEQ ID NO: 2). The base before the upstream French HPFH deletion is HG18 chr11:5,214,023. The GATA-1 motif spans chr11:5,214,200-5,214,206 Without being limited by theory, it is believed that GATA-1 and HDAC-1 form a repressive complex with Bcl11a when Bcl11a is bound within this 350 bp region and this leads to the formation of a repressive complex that inhibits the expression of the γ-globin genes and, thereby, reduces cellular levels of HbF protein.

[00109] The HE-mediated disruption, which is achieved by the compositions and methods disclosed herein, occurs at high efficiency. Unlike shRNA knockdown approaches that are known in the art, the highly sequence specific disruption of the HbF silencing region, which is mediated by the homing endonucleases disclosed herein, avoids off target effects at other Bcl11a binding sites in the genome, and in other cell types, especially within B-cells where Bcl11a binding is required for normal development.

[00110] Thus, the homing endonucleases described herein exhibit unique advantages over conventional gene targeting nucleases. Because they are broadly efficacious regardless of genotype, the homing endonucleases described herein are not patient specific and provide clinical benefit in the heterozygotic state.

Recapitulation of Genetic Modifications for Correcting a Thalassemia or Sickle Cell Disease Mutation

[00111] Within other embodiments, the present disclosure provides compositions and methods for recapitulating, via genome editing, one or more naturally-occurring mutation(s) within a patient's genome to provide clinical benefits. More specifically, the present disclosure provides compositions and methods for achieving the direct correction of a thalassemia and/or sickle cell disease (SCD) mutation through genome editing.

[00112] The compositions and methods disclosed herein employ a correction template to achieve gene editing and correction to ameliorate hemoglobinopathies, including thalassemias and sickle cell disease, by enhancing the rate of homologous recombination (HR) between the correction template and the corresponding mutated sequence within a patient's genome. Exemplified herein are compositions and methods for correcting an underlying β-globin mutation, which provide clinical benefit in the heterozygotic state while avoiding the insertion of vector sequences. These compositions and methods may be used independently from or in combination with the compositions and methods described above for the disruption of Bcl11a-mediated gene silencing.

[00113] The present disclosure provides a robust set of technologies for genome editing that exploits the advantages of HEs, as compared to alternative platforms that are available in the art. These HEs can be combined with a TAL effector modular DNA binding platform to achieve additional therapeutic advantages.

[00114] While homologous recombination (HR) to edit genomes is powerful, it is inefficient. Introduction of a double stranded break at the region to be modified results in a tremendous increase in HR efficiency. Simultaneous introduction of a polynucleotide encoding a HE and a correction template, wherein the correction template comprises as little as 100 bp of flanking homology, allows an increased frequency of HR, thereby permitting genome editing as the corrective template is introduced.

[00115] The transduction of cells with a short synthesized correction template may also be employed for the efficient introduction of defined single base-pair mutations. Such approaches typically exploit a single HE. Alternatively HEs may be transduced that flank the region targeted for modification. Correction templates may be transduced by optimized methods as described herein. The design, transduction, and evaluation of HEs may be performed, as discussed in detail below, according to the methodology described Certo *et al.*, *Nat Methods* 8:671-6 (2011) and Jarjour *et al.*, *Nucleic Acids Res* 37:6871-80 (2009).

[00116] Within certain aspects of these embodiments, one or more homing endonuclease(s) is/are employed in combination with a normal or wild-type polynucleotide sequence to permit the editing and/or repair of one or more β-like globin gene(s). For example, the present disclosure provides compositions and methods for the treatment of hemoglobinopathies, which compositions and methods permit the modification of key regulatory and/or coding sequences within a gene locus, exemplified herein by the human β-globin gene locus, through the transient expression of a polynucleotide that includes one or more naturally occurring mutation(s).

[00117] More specifically, the present disclosure provides compositions and methods for genome editing, which may be employed to generate mutations that recapitulate naturally-occurring mutations within stem cells, including, for example, hematopoietic stem cells (HSCs), embryonic stem (ES) cells, and induced pluripotent stem cells (iPSCs). Genome edited HSCs, ESs, and iPSCs, including autologous HSCs and iPSCs, may be transplanted into a patient to treat one or more hemoglobinopathies, such as a thalassemia and/or sickle cell disease. The compositions and methods disclosed herein permit the efficient modification of HSCs, ESs, and iPSCs, without the need for the persistent expression or insertion of an exogenous gene to achieve the amelioration of hemoglobinopathies in mature erythroid cells and in patient cells *in vivo*.

[00118] Because these therapeutic methods do not require the integration and/or persistent expression of a transgene, the safety concerns associated with currently available gene therapy technologies are obviated. Within certain aspects

of these embodiments, the compositions and methods employ one or more polynucleotide for the targeted disruption of Bcl11a-mediated silencing of HbF.

[00119] Exemplified herein are compositions and methods that permit the recapitulation of genetic modifications within one or more HbF silencing region(s) that is/are responsible for hereditary persistence of HbF (HPFH). Because such genetic modifications lead to increased expression of a therapeutically effective gene, the recapitulated genetic modifications need only be present as a heterozygote to achieve therapeutic efficacy.

[00120] The compositions and methods for ameliorating thalassemia and sickle cell disease that are disclosed herein achieve therapeutic efficacy by introducing one or more mutation that result in increased HbF and/or HbA_2 and/or HbA protein production. Exemplified herein are compositions and methods for recapitulating one or more naturally-occurring deletion(s) of the β-globin gene and/or regions, which activate γ-globin gene expression thereby increasing levels of fetal hemoglobin. Because a modest increase in HbF and/or HbA_2 protein production is sufficient to ameliorate these disease phenotypes, heterozygotic mutations are sufficient to achieve substantial therapeutic benefit.

[00121] Within certain aspects, the delivery of a correction template may be done in conjunction with the delivery of a selectable marker gene thereby permitting the selection of corrected cells *ex vivo* and *in vivo*, although such an approach requires long-term expression via integration of the selectable marker gene. Beard *et al.*, *J. Clin. Invest.* 120:2345-54 (2010) and Munoz *et al.*, *Nucleic Acids Res.* 39(2):729-743 (2011).

[00122] Activation of β-globin expression in adult tissues depends upon binding of KLF-1 at a CACCC box in its promoter. The δ-globin promoter lacks an intact CACCC box, KLF-1 is not bound and expression is limited to 2% of β-globin. Mutations of the δ-promoter that recapitulate the β-globin promoter by, for example, introducing an intact CACCC box, allow KLF-1 binding, and result in a therapeutically efficacious increase in δ-globin expression.

[00123] Within certain aspects of these embodiments, a non-deletion HPFH γ-globin promoter mutation may be generated. Only a single base pair must be modified to achieve efficacy. For example, a -175 T→C mutation (SEQ ID NO: 21) may be recapitulated to maximize the levels of HbF. Mutation of any of the four γ-globin genes will provide benefit, thus increasing potential targets.

Delivery of Homing Endonucleases, Cas9 Endonuclease, TAL Effector Nucleases, and TREX2 Exonucleases

[00124] Within further embodiments, the present disclosure provides systems, in particular non-integrating vector systems, for the delivery of one or more HE, Cas9, TALEN, and/or TREX2 nuclease described herein. There are three major challenges to the therapeutic gene editing of hematopoietic stem cells (HSCs): (1) nuclease reagents must be transiently delivered to HSCs; (2) gene editing efficiency in cells receiving a nuclease must be high; and (3) gene-edited HSCs must engraft to a level sufficient for therapeutic effect. These challenges may be overcome by employing various vectorization approaches.

[00125] Exemplified herein are cocal pseudotyped lentiviral vectors and foamy virus vectors for the efficient gene transfer to HSCs. Trobridge *et al.*, *Mol Ther* 18:725-33 (2008). Alternatively, adenoviral vectors may be modified as previously described for use in gene transfer to HSCs. Wang *et al.*, *Exp. Hematol.* 36:823-31 (2008) and Wang *et al.*, *Nat. Med.* 17:96-104 (2011). Within other aspects of these embodiments, AAV-based vector systems may also be employed for the delivery of HEs, Cas9 (and/or RNA guide strands), TALE-HE, TALENs, and/or TREX2 nucleases.

[00126] AAV6-serotype recombinant AAV vectors provide a 4.5 kb payload, sufficient to deliver a promoter-HE-exonuclease or a promoter-TAL-HE fusion-exonuclease cassette in addition to a small recombination template. Alternatively, it can carry the small Cas9 polypeptide and guide RNAs. AAV6 provides efficient transduction of human CD34+ umbilical cord blood cells of all known AAV capsids and is able to mediate significant levels of transient gene expression in HSC. Self-complementary and single stranded AAV6 vectors may be employed for both gene

knockout and recombination-based gene editing in HSC in cell lines and in primary CD34+ cells.

[00127] Adenoviral vectors with hybrid capsids are capable of efficiently transducing many types of hematopoietic cells including CD34+ cells. Improved transduction may be achieved with a chimeric adenoviral vector using the serotype 35 fiber (Ad5-F35) and the serotype 11 fiber (Ad5-F11) for efficient transduction of hematopoietic cells. Helper-dependent adenoviral vectors offer up to a 30 kb payload, along with transient gene expression in HSC, and can be used to deliver multiple HE/exonuclease cassettes, HE-TAL fusions, as well as very large recombination templates. Alternatively it can carry the small Cas9 polypeptide and guide RNAs. These modified chimeric adenovirus vectors may, therefore, be employed for both gene knockout and recombination-based gene editing in HSC.

[00128] Integration-deficient lentiviral and foamyviral vectors (IDLV and IDFV) provide 6 kb (IDLV) to 9 kb (IDFV) payloads, and have well documented capabilities to transduce human HSCs. Within certain aspects, both IDLV and IDFV vectors may be employed for gene knockout and recombination-based gene editing in HSC. IDLV with alternative promoter GFP cassettes provide efficient and high level expression in CD34+ HSC. High titer stocks may be achieved using a TFF purification step. Vectors with a set of promoter/GFP cassettes may be used to provide efficient and high level HE expression in CD34+ HSC and may be generated to express individual HEs, HE/Trex2, multiplex-HE (*i.e.*, two, three, or four HEs that are co-expressed), and multiplex-HE/TREX2 combinations. Multiplex HE expression permits multiple cleavage events in a critical region, which depending upon the precise application, may be desired to create increased HbF de-repression. Such multiplex strategies are feasible with LHEs, because they function autonomously, and may be satisfactorily employed in combination with TREX2 co-expression to permit highly efficient and synchronous processing of closely-targeted double strand breaks. Alternatively it can carry the small Cas9 polypeptide and guide RNAs.

[00129] The efficiency of gene targeting, levels of globin gene expression in individual targeted cells as well as populations of cells and of their progeny, the

effect of targeting on erythropoiesis and on stem cell function, and on hematologic parameters and organ function may be confirmed in model organisms.

[00130] Transductions may be followed by single-cell and bulk population assessments of modification efficiency and expression of β-like genes at the RNA and protein levels. Alterations in factor binding and chromatin structure may be assessed, as well as morphology, the extent of ineffective erythropoiesis and apoptosis. Candidates that score well in initial screens may be further assessed for effects on HSC pluripotency as well as the ability to ameliorate disease specific phenotypes *in vitro* and *in vivo*.

[00131] Initial screening of HE candidates and delivery systems may be performed in a mouse erythroleukemia cell line containing a single intact human chromosome 11 (N-MEL) and clinical grade CD34+ normal human HSCs with endpoints of assessing targeted mutation efficiency and globin gene expression. Both cell types can be induced to differentiate along an erythroid path during which expression of β-like genes is highly induced with high β- to □- and δ- ratios allowing a quantitative assessment of effects globin gene regulation at a single-cell and population level. Second level assessments may include an analysis of the pluripotency of transduced CD34+ cells and erythropoiesis. Suitable assay systems may include culturing to assess long-term proliferative potential, analysis of myeloid and erythroid colonies for clonal analysis and transplant into NOD SCID gamma (NSG) mice followed by assessment of multilineage engraftment of primary and secondary recipients. Clinical effectiveness may be assessed simultaneously *in vitro* and *in vivo*.

[00132] Knockout of murine Bcl11a leads to a dramatic dose-dependent increase in γ-globin in mice containing a human β-globin locus and ameliorates the sickle phenotype in humanized mouse models. While both systems allow the analysis of globin gene expression, the sickle mice allow for the assessment of the improvement of phenotype in these mice with special attention to the hematologic parameters, liver and lung pathology, renal function and spleen size. Phenotypic improvement may be correlated to the number of HbF containing cells, the HbF/HbS ratio and expression patterns in single cell assays.

[00133] In addition, erythrocyte lifespan and morphology may be assessed by transducing human CD34+ HSCs from hemoglobinopathy patients. Cultured thalassemic cells show minimal expansion, a lack of hemoglobinization, evidence of ineffective erythropoiesis and increased apoptosis compared to normals. These features permit the quantitative assessment of expression levels and degree of erythropoiesis post-targeting. The degree of sickling of erythroid progeny of CD34+ cells under hypoxic conditions may also be assessed. CD34+ cells from patients may be transplanted into NSG mice, after which several features of abnormal erythropoiesis are be recapitulated, allowing assessment of the effect of targeted mutagenesis.

Expansion of Autologous HSCs, ESs, and iPSC-derived HSCs

[00134] Within further embodiments, the present disclosure provides compositions and methods for the *ex-vivo* expansion of modified hematopoietic stem cells (HSCs) to allow for efficient engraftment of corrected cells and the use of induced pluripotent stem cells (iPSCs) for screening and clinical application. Within certain aspects of these embodiments are provided compositions and methods for the efficient expansion of autologous HSCs, autologous gene-modified HSCs, ESs, and iPSC-derived HSCs. Cord blood expansion methodology may be employed, which methodology utilizes Delta1 in serum free media supplemented with hematopoietic growth factors using mobilized peripheral blood CD34+ obtained from normal donors. These compositions and methods may be used in combination with one or more additional reagent to enhance the survival and proliferation of hematopoietic stem/progenitor cells. Within other aspects, these compositions and methods may employ endothelial cell co-cultures for the enhanced expansion of long-term repopulating cells, including corrected iPSC-derived HSCs.

[00135] For effective clinical translation of the presently disclosed gene correction strategies, the present disclosure provides methods for the *ex vivo* expansion of the absolute number of corrected autologous HSCs. Gene correction procedures are generally more efficient if done in a smaller scale and often only limited numbers of HSCs are available for correction. Thus, it is contemplated by the present disclosure that expansion methods may be employed to permit clinically

feasible *ex vivo* expansion of corrected HSCs, ESs, and/or HSCs derived from induced pluripotent stem cells (iPSCs). Within certain aspects, the present disclosure provides methods for expanding hematopoietic stem/progenitor cells for therapeutic application by exploiting the role of Notch signaling in determining stem cell fate. Dahlberg *et al., Blood* 117:6083-90 (2010); Delaney *et al., Nat Med* 16:232-6 (2010); and Varnum-Finney *et al., Nat Med* 6:1278-81 (2000).

[00136] These methods permit the clinically relevant *ex vivo* expansion of cord blood stem/progenitor cells, and an expanded cellular therapy for treatment of myelosuppression in patients undergoing cord blood transplantation, by first using a partially HLA-matched fresh product (harvested post-culture and infused directly) and/or by using a previously expanded and cryopreserved product as an off-the-shelf non-HLA matched cellular therapy.

[00137] *Ex vivo* expansion of gene-corrected autologous HSCs enhances the safety and effectiveness of HSC-based gene therapy by permitting the transplantation of greater numbers of appropriately corrected repopulating cells to allow for rapid repopulation and ensures predominance of gene-corrected cells *in vivo*. Accordingly, the present disclosure provides compositions and methods for the supportive care via a third-party, non HLA-matched, donor *ex vivo* expanded stem/progenitor cell, which is capable of providing rapid but transient myeloid recovery, essential to reduce the risk of early transplant related mortality secondary to infections that is observed after myeloablative T cell depleted autologous transplants. Delaney *et al., Nat Med* 16:232-6 (2010).

[00138] Agents that inhibit differentiation (*e.g.*, the Notch ligand) may be combined with compositions and methods that enhance the proliferation and survival of early stem/progenitor cells thereby achieving improved Notch-mediated *ex vivo* expansion. Enhanced proliferation of cord blood stem/progenitor cells may be achieved by combining the Notch ligand, Delta1, with the aryl hydrocarbon receptor inhibitor (SR1) (Boitano *et al., Science* 329:1345-8 (2011)) or HoxB4 (Watts *et al., Blood* 116:5859-66 (2010) and Zhang *et al., PLoS Med* 3:e173 (2006)) to enhance proliferation and self-renewal of hematopoietic precursors, and with angiopoietin-like 5 to enhance their survival. Essential to the clinical application of

gene therapy is the ability to expand long-term repopulating cells, assuring longevity of the corrected cell graft.

[00139] Akt-activated endothelial cells may be employed in co-culture systems to confirm expansion of gene-corrected cells. Butler *et al.*, *Cell Stem Cell* 6:251-64 (2011). Expansion of gene-corrected cells depends upon endothelial cell-induced activation of Notch signaling in the hematopoietic precursors. A second critical aspect for clinical application is the genetic and epigenetic fidelity of the derived cells as compared to their normal counterparts to ensure appropriate behavior and lack of oncogenic potential *in vivo*. Importantly, genome-wide assessment of expanded cord blood stem/progenitor cells exhibit fidelity of the transcriptome, chromatin structure, and the DNA methylome in comparison with primary isolated CD34+ cells.

[00140] Expansion strategies in normal CD34+ cells may be employed in conjunction with defined methods that utilize CD34+cells from patients with hemoglobinopathies. Cord blood expansion methodology may utilize Delta1 in serum free media supplemented with hematopoietic growth factors using mobilized peripheral blood CD34+ obtained from normal donors. Optimized *ex vivo* expansion conditions using established *in vitro* assays (immunophenotyping, growth, etc) and *in vivo* repopulating ability may be assessed using the NSG mouse model. Optimized conditions may be used in combination with compositions that include SR1 (aryl hydrocarbon receptor inhibitor), Hox proteins, or angiopoietins to enhance the proliferation and survival of early stem/progenitor cells. Promising combinations may be evaluated in progenitor cell *in vitro* assays and in the immunodeficient mouse model (NSG mice) and then extended from expansion of CD34+ from normal individuals to evaluate these methods for expansion of CD34+ cells from patients with thalassemia (and other hemoglobinopathies).

[00141] The transcriptional, genetic, and epigenetic fidelity of expanded cells with their normal counterpart HSCs may be assessed using genome wide approaches to assess the oncogenic potential of the generated cells. Following growth *in vivo* (after infusion), cells may be used to determine whether there are functionally

significant aberrations that enhance *in vivo* growth of any affected clone(s), thereby allowing selective expansion and detection of rare cells.

Cellular Therapies to Abrogate Post-transplant Neutropenia and to Improve Outcome Following Transplantation of Gene-corrected Autologous HSCs

[00142] Within another embodiment, the present disclosure provides compositions and methods for providing supportive care, which compositions and methods comprise off-the-shelf cellular therapies that abrogate post-transplant neutropenia and improve outcome following transplantation of gene-corrected autologous HSCs. *Ex vivo* expanded, cryopreserved cord blood (CB) stem/progenitor cells may, for example, be administered as a means of supportive care to patients with thalassemia and/or sickle cell disease who are undergoing myeloablative HCT with autologous CD34+ gene corrected cells.

[00143] In studies aimed at developing an economically feasible "off-the-shelf" source of progenitor cells capable of providing rapid neutrophil recovery, a bank of pre-expanded, cryopreserved hematopoietic stem/progenitor cell products was generated -- each being derived from a single CB unit that can be held for future clinical use.

[00144] The safety of administering this "off-the-shelf" non-HLA matched product to adults was demonstrated immediately following first salvage chemotherapy for relapsed/refractory AML, as well as in the myeloablative CBT setting in pediatric and adult patients with hematologic malignancy.

[00145] It has been hypothesized that this expanded cell product, which is devoid of T cells, can be infused as an off-the-shelf cellular therapy to provide rapid but temporary myeloid engraftment and to potentially facilitate autologous hematopoietic recovery in patients undergoing myeloablative HCT with autologous gene-corrected stem cell grafts, thereby reducing the infectious complications and risk of mortality.

[00146] Critical is the question of whether HLA-matching is required for safe infusion of an "off-the-shelf" non-HLA matched product, which is devoid of T cells.

Without the need for HLA matching, fresh CB units can be collected for immediate *ex vivo* expansion and the final product cryopreserved for future on demand use. Patient access to an off-the-shelf expanded CB product is dramatically enhanced as all of the expanded products banked would be potentially available for any given patient, regardless of HLA typing, race/ethnicity or location of the patient.

[00147] Moreover, the ability to create an off-the-shelf universal donor expanded cell therapy is not only promising to shorten the duration of severe neutropenia post HCT, it is also likely to enhance more broad areas of investigation outside of stem cell transplantation, *e.g.*, as a way of providing temporary myeloid engraftment for treatment of chemotherapy induced severe neutropenia, any acquired severe neutropenia or accidental radiation exposure.

[00148] *Ex vivo* expansion abrogates the risks of CBT by overcoming delayed hematopoietic recovery and a significant improvement in overall survival will result. A reduced risk of relapse has been observed in patients undergoing double CBT, and chronic GVHD is lower despite highly mismatched grafts. If the risk of early transplant related mortality can be reduced by infusion of *ex vivo* expanded cord blood progenitors to enhance hematopoietic recovery, overall survival is likely to exceed that seen with conventional unrelated donors.

[00149] Within further embodiments, the present disclosure provides cellular therapies to abrogate post-transplant neutropenia and to improve outcome following transplantation of gene-corrected autologous HSCs. Patients with thalassemia who undergo myeloablative HCT with autologous gene corrected cells are at increased risk of infections and mortality secondary to limiting numbers of CD34+ cells in the infused graft (until *ex vivo* expansion of these gene corrected cells to clinically feasible numbers are achieved). Infusion of a previously expanded and cryopreserved cord blood progenitor cell product as an off-the-shelf supportive care measure can be employed to reduce the risk of mortality by contributing to early, but transient, myeloid recovery until the long term graft contributes to hematopoietic recovery.

[00150] Patients who undergo myeloablative HCT experience severe pancytopenia as a direct consequence of the conditioning regimen, and all patients

are at increased risk of infection and bleeding during this time. The time to hematopoietic recovery (of neutrophil and platelets) is directly influenced by the CD34+ cell dose, and thus, for those patients undergoing myeloablative HCT with umbilical cord blood where the stem cell dose is $1/10^{th}$ of a conventional donor graft or with autologous CD34 enriched low cell dose grafts, the risk of transplant related mortality due to delayed hematopoietic recovery is even greater.

[00151] To overcome these risks and to increase the safety of these HCT approaches, there is a great need for novel therapies that can abrogate prolonged pancytopenia and facilitate more rapid hematopoietic recovery. As discussed above, such a strategy has been developed wherein the absolute number of marrow repopulating cord blood (CB) hematopoietic stem/progenitor cells (HSPC) can be increased by culture with the Notch ligand Delta1. Infusion of these partially HLA-matched *ex vivo* expanded CB cells into children or adults undergoing cord blood transplantation (CBT) has been demonstrated to be safe and can significantly shorten the time to reach an initial absolute neutrophil count of 500 from 26 to 11 days, as a result of rapid myeloid engraftment contributed by the expanded cells.

[00152] In more recent studies aimed at developing an economically feasible "off-the-shelf" source of progenitor cells capable of providing rapid neutrophil recovery, we have generated a bank of pre-expanded cryopreserved hematopoietic stem/progenitor cell products, each derived from a single CB unit that can be held for future clinical use. We have now also demonstrated the safety of administering this "off-the-shelf" non-HLA matched product to adults immediately following first salvage chemotherapy for relapsed/refractory AML, as well as in the myeloablative CBT setting in pediatric and adult patients with hematologic malignancy. We hypothesize that this expanded cell product which is devoid of T cells can be infused as an off-the-shelf cellular therapy to provide rapid but temporary myeloid engraftment and to potentially facilitate autologous hematopoietic recovery in patients undergoing myeloablative HCT with autologous gene-corrected stem cell grafts, thereby reducing the infectious complications and risk of mortality.

[00153] Using the defined optimal methods for generation of *ex vivo* expanded cord blood stem/progenitor cells, a bank of off-the-shelf expanded cell

products may be employed to determine the safety of infusing these cells as supportive care in an autologous gene-corrected HCT.

[00154] The present disclosure will be best understood in view of the following non-limiting Examples.

EXAMPLES

Example 1
Selection of Bcl11a Gene Targeting Homing Endonucleases Based on I-HjeMI, I-CpaMI, and I-OnuI Using In vitro Compartmentalization (IVC)

[00155] The open reading frame (ORF) of a parental LAGLIDADG homing endonuclease (LHE), I-HjeMI (Fig. 14; SEQ ID NO: 28; Jacoby *et al.*, *Nucl. Acids Res.* 40(11):4954-4964 (2012); Taylor *et al.*, *Nucl. Acids Res.* 40(Web Server issue):W110-6 (2012)), codon optimized for expression in *E. coli*, was cloned between the NcoI and NotI restriction sites of pET21-a(+) (Fig. 11; EMD Millipore (Novagen) division of Merck KGaA. To introduce site-directed saturation mutagenesis into the ORF of I-HjeMI, DNA fragments containing its partial ORF with approximately 20 base pairs of a region overlapped with flanking fragments on both sides were PCR-amplified using primers that contained degenerate codon 5'-NNK-3'(coding all 20 amino acids). Amino-acid residues mutated using such PCR primers are shown in Table 2. PCR products were purified by extraction from an agarose gel, and assembled in a subsequent round of PCR with a sequence containing 2 copies of target sites for variant endonucleases to be selected. Successfully assembled DNA fragment was again purified by gel extraction, and used as a library in *in vitro* compartmentalization (IVC).

[00156] Three rounds of IVC were conducted after each round of site-directed saturation mutagenesis in order to enrich variant nuclease genes with altered specificity. The oil-surfactant mixture (2% ABIL EM 90 (Evonik Industries AG Personal Care, Essen, North Rhine-Westphalia, Germany), 0.05% Triton X-100 in light mineral oil) was thoroughly mixed with the saturation buffer (100 mM potassium glutamate (pH 7.5), 10 mM magnesium acetate (pH 7.5), 1 mM dithiothreitol and 5 mg/ml bovine serum albumin), incubated at 37°C for 20

minutes, and centrifuged at 16,000 x g for 15 minutes at 4°C. Five hundred microliters of the upper phase was used to emulsify 30 µl of the *in vitro* protein synthesis mixture (25 µl of PURExpress (New England Biolabs, Ipswich, MA), 20 units of RNase inhibitor, 1 mg/ml bovine serum albumin, and 8 ng of a DNA library) by constant stirring at 1,400 r.p.m. for three and a half minutes on ice. The emulsion was incubated at 30°C for 4 hours, and then heated at 75°C for 15 minutes. Emulsified droplets were collected by centrifugation at 16,000 x g for 15 min at 4°C, and broken by an addition of phenol/chloroform/isoamyl alcohol. Nucleic acids were recovered by ethanol precipitation, and treated with RNase cocktail (Life Technologies Corporation (Invitrogen), Grand Island, New York). After purification using QIAquick PCR purification kit (Qiagen, Hilden, Germany), a DNA library was ligated with a DNA adaptor with a 4-base 3' overhang sequence complementary to the cohesive end of a target site generated by endonuclease variants expressed in emulsified droplets, and added to PCR mixture containing a pair of primers, one of which was specific for the ligated DNA adaptor in order to enrich genes of variant endonucleases linked to a cleaved target site. A PCR amplicon was gel-purified and the ORF of variant genes was further PCR-amplified to prepare a DNA library to be used in the subsequent round of IVC.

[00157] In the second round of IVC, an emulsion was made with 1 ng of a reconstructed library, and incubated at 42°C for 75 minutes before quenching *in vitro* transcription/translation reaction by heating at 75°C. The DNA library was recovered and active endonuclease genes were specifically enriched by PCR following ligation with a DNA adaptor as described above.

[00158] In the third round of IVC, an *in vitro* protein synthesis mixture containing 0.5 ng of a library fragment was emulsified in 4.5% Span 80/0.5 %Triton X-100/light mineral oil. The reaction ran at 42°C for 45 minutes and was heat-inactivated at 75°C. After extraction from emulsion, cleaved target site-associated endonuclease genes were PCR-amplified and subjected to the subsequent round of site-directed mutagenesis.

[00159] To redesign I-HjeMI variants that recognized the (-) and (+) half sites of the BCL11A gene target, 4 and 2 rounds of site-directed saturation mutagenesis

were carried out, respectively (Fig. 13). A pool of variant nucleases targeting the former half-site was subjected to an additional (fifth) round of mutagenesis on the surface opposite to the protein-DNA interface, followed by 3 rounds of IVC (Table 2).

Table 2
Amino-acid Positions Subjected to Saturation Mutagenesis in IVC

Round	Target Site*	Sequence Identifier	Amino Acid Residues
1	TTGAGGAGATGTCTCTGTTAAT	SEQ ID NO: 55	R61, R63, N64, E65, I66, M68, S70
2	TTGAGGTGATGTCTCTGTTAAT	SEQ ID NO: 56	Y20, S22, E33, G35, E37, S59, R61, R70, R72
3	TTGAAGTGATGTCTCTGTTAAT	SEQ ID NO: 57	Y20, S22, T24, T31, E33, G35, R72, R74
4	TCCAAGTGATGTCTCTGTTAAT	SEQ ID NO: 58	Y20, S22, T24, K26, G27, K28, T31, E33
5	TCCAAGTGATGTCTCTGTTAAT	SEQ ID NO: 59	S109, N110, A121, S123, N124, N135, S137
1	TTGAGGAGGTTTCTGTGTTAAT	SEQ ID NO: 60	S154, S168, D170, I193, L195, R202, K204
2	TTGAGGAGGTTTCTCGGTGGTG	SEQ ID NO: 61	S154, L158, N159, D162, D163, I166, I168, K204, T206

* Underlined nucleotides differ from those in the target site for the parental LHE I-HjeMI.

DNA fragments that encoded the N-terminal and the C-terminal half-domains of I-HjeMI variant endonucleases responsible for the (-) and (+) half sites of the BCL11A gene target were assembled, and a pool of nucleases that cleaved the full BCL11A gene target site were selected through 3 rounds of IVC (Fig. 13).

[00160] Table 3 presents exemplary BCL11a target sequences, which comprise DNA sequence modules for which pools of highly active endonuclease variants (based upon homing endonucleases I-CpaMI and I-OnuI) have been isolated and their sequences determined.

Table 3

Base Homing Endonuclease I-CpaMI		
f 683/2508 chr2:60,542,847-60,542,868 Disrupts Bcl11a-X and XL forms	ATGGGATTCATATTGCAGACAA	SEQ ID NO: 25
Base Homing Endonuclease I-OnuI		
r 1588/2508 chr2:60,542,630-60,542,651 Disrupts Bcl11a-X and XL forms	AGCCATTGGATTCAACCGCAGC	SEQ ID NO: 26
f 525/2508 chr2:60,543,005-60,543,026 Disrupts all Bcl11a forms	caaaCAgccATTCAcCagTgcA	SEQ ID NO: 27

Example 2

Optimization of Activity of BCL11A Gene-Targeting I-HjeMI Variants Using Two-plasmid Gene Elimination Cleavage Assay in Bacteria

[00161] The activity of I-HjeMI variants obtained in selection using IVC display selections (disclosed in Example 1, above) was optimized using a two-plasmid selection system in bacterial cells according to the methodology of Doyon *et al., J. Am. Chem. Soc.* 128(7):2477-2484 (2006). The ORF of the endonuclease genes was inserted between NcoI and NotI sites of the pENDO (Fig. 12, Doyon *et al., J. Am. Chem. Soc.* 128(7):2477-2484 (2006))expression plasmid. NovaXGF (EMD Millipore (Novagen)) competent cells harboring the pCcdB reporter plasmid (Fig. 31, Doyon *et al., J. Am. Chem. Soc.* 128(7):2477-2484 (2006); Takeuchi *et al., Nucl. Acids Res.* 37(3):877-890 (2009); and Takeuchi *et al., Proc. Natl. Acad. Sci. U.S.A.* 10.1073/pnas.1107719108 (2011)) containing 4 copies of the BCL11A gene target were transformed with a pool of the pEndo plasmid encoding I-HjeMI variants. The transformants were grown in 2 x YT medium (16 g/L tryptone, 10 g/L yeast extract, and 5 g/L NaCl) at 37 °C for 30 min and then diluted 10-fold with 2 x YT medium supplemented with 100 μg/mL carbenicillin and 0.02 % *L*-arabinose (in order to preinduce expression of I-HjeMI variants). After the culture was grown at

30°C for 15 hours, the cells were harvested, resuspended in sterile water and spread on both nonselective (1 x M9 salt, 1 % glycerol, 0.8 % tryptone, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 2 μg/mL thiamine, and 100 μg/mL carbenicillin) and selective plates (*i.e.* nonselective plates supplemented with 0.02% *L*-arabinose and 0.4 mM IPTG to induce expression of the toxic CcdB protein). After incubation at 30°C for 30-40 hours, the pEndo plasmid was extracted from the surviving colonies on the selective plates.

[00162] The ORFs encoding active I-HjeMI variants were amplified via error-prone PCR using the Gene Morph II Random Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). After digestion with NcoI, NotI, and DpnI, the resulting fragments were recloned into the pEndo vector. The plasmid was subjected to 2 rounds of selection under the conditions where variant endonucleases were expressed at 30°C for 4 hours before plating. The N-terminal half and C-terminal half domains of the selected genes were shuffled using overlapping PCR, and again cloned into the pEndo vector. Transformed cells carrying both the pEndo plasmid and the pCcdB reporter were grown in 2 x YT medium containing 0.02 % L-arabonise at 37°C for an hour and then spread on selective plates at 37°C for 16-20 hours. After 2 rounds of selection at the same level of stringency, the pEndo plasmid was extracted from surviving colonies on the selective plates, and ORFs of the variant genes carried on the plasmid were sequenced.

Example 3
Activity of BCL11A Gene-targeting Endonucleases Tested in a Two-plasmid Cleavage Assay

[00163] Activity of an exemplary BCL11A gene-targeting endonuclease (BCL11Ahje; Fig. 17, SEQ ID NO: 31), its catalytically inactive variant (BCL11Ahje D18N), and its parental LHE I-HjeMI (Fig. 14, SEQ ID NO: 28) was measured in bacterial cells that harbor the pCcdB reporter plasmid (Doyon *et al.*, *J. Am. Chem. Soc.* 128(7):2477-2484 (2006)) containing 4 copies of either the target site for I-HjeMI (I-HjeMI target) or the BCL11A gene target (TCCAAGTGATGTCTCGGTGGTG (SEQ ID NO: 39; underlined nucleotides differ from those in the target site for the parental LHE I-HjeMI). The pCcdB reporter plasmid encodes "control of cell death B" ("ccdB", a toxic protein in

bacteria, which is inducible by an addition of IPTG). Cleavage of the target sites in the reporter plasmid leads to RecBCD-mediated degradation of the reporter plasmid and corresponding cell survival on the selective medium containing IPTG. The survival rate was determined by dividing the number of colonies on the selective plates by that on the nonselective plates. Error bars refer ± S.D. of 3 independent experiments.

Example 4

Detection of Targeted Mutagenesis at the Endogenous Human BCL11A Gene

[00164] HEK 293T cells (1.6 x 10^5) were seeded 24 hours prior to transfection in 12-well plates, and transfected with 0.5 ug each of expression plasmids for the BCL11A gene targeting nuclease and TREX2. At 48 hours post transfection, transfected cells were lysed and genomic DNA was extracted using Quick-gDNA MiniPrep kit (Zymo Research). Approximately 500-bp fragment spanning the BCL11A gene target was PCR-amplified from 50 ng of the extracted genomes using a pair of the following primers: Bcl11A_up1, 5'- GCT GGA ATG GTT GCA GTA AC -3' (SEQ ID NO: 66); Bcl11A_down1, 5'- CAA ACA GCC ATT CAC CAG TG -3' (SEQ ID NO: 67). The PCR amplicon was incubated in 1 x NEB buffer 4 plus 1 x BSA (New England Biolabs) with or without 0.5 uM of the BCL11A gene targeting nuclease that was purified from E. coli overexpressing the recombinant protein at 37 °C for 2 hours. The reaction was terminated by adding 5 x Stop solution (50 mM Tris-HCl (pH7.5), 5 mM EDTA, 0.5 % SDS, 25 % glycerol, 0.1 orange G and 0.5 mg/mL proteinase K). After incubation at room temperature for 15 minutes, a half of each sample was separated on a 1.6 % agarose gel containing ethidium bromide in TAE (upper panels). The rest of each sample was purified using DNA Clean & Concentrator-5 kit (Zymo Research), and used as a template in the second round of PCR with a pair of the following primers: Bcl11A_up2, 5'- CTG CCA GCT CTC TAA GTC TCC -3' (SEQ ID NO: 68); Bcl11A_down2, 5'- TGC AAC ACG CAC AGA ACA CTC -3' (SEQ ID NO: 69). The PCR product was again digested with the BCL11A gene targeting nuclease under the conditions described above, and analyzed on a 1.6 % agarose gel (lower panels) (See Figure 30).

Example 5

Selection of Fetal Hemoglobin Silencing Region Targeting Endonucleases based on I-OnuI using In vitro Compartmentalization

[00165] Exemplary homing endonuclease (HE) target sequences, which are evenly distributed throughout the 350 bp region (SEQ ID NO: 2) that includes the region of Bcl11a occupancy within the HbF silencing region in adult erythroid cells that is disrupted in the French HPFH deletion, are presented in Table 4. These target sequences comprise DNA sequence modules for which pools of highly active endonuclease variants have been isolated and sequenced.

Table 4

Position	Chromosomal Location	Sequence	Sequence Identifier
Wild Type	N/A	TTTCCACTTATTCAACCTTTTA	SEQ ID NO:5
f 13/303	chr11:5,214,235-5,214,256	TGTGGCCCTATTCTTGTGTTCA	SEQ ID NO:6
f 79/303	chr11:5,214,169-5,214,190	CATTGTCACTTTCTTCCCTACT	SEQ ID NO:7
f 143/303	chr11:5,214,105-5,214,126	TAAAATACATTTCTTCACTAAG	SEQ ID NO:8
f 124/303	chr11:5,214,089-5,214,110	ACTAAGTGAGAATAATCTTTTA	SEQ ID NO:9
f 200/303	chr11:5,214,048-5,214,069	GCCACCACCTTTCTTGAATTAT	SEQ ID NO:10
f 211/303	chr11:5,214,037-5,214,058	TCTTGAATTATTCAATATCTTT	SEQ ID NO:11
f 274/303	chr11:5,213,974-5,213,995	TTAAAGGTCATTCATGGCTCCT	SEQ ID NO:12

[00166] Table 5 presents a region from -100 bp to 210 bp upstream of globin genes, which is identical for both Aγ- and Gγ-globin genes and which contains many of the non-deletion HPFH mutations. Gene editing resulting in these mutations leads to decreased repression, thus activation, of a gamma gene and results in increased HbF.

Table 5

	Sequence	Sequence Identifier
Wild-type	TGGGGGCCCCTTCCCCACACTATCTCAATGCAAATATCTG TCTGAAACGGTCCCTGGCTAAACTCCACCCATGGGTTGGC CAGCCTTGCCTTGACCAATAGCCTTGACAA	SEQ ID NO:16
G-gamma - 202 C→G	TGGGGGCGCCTTCCCCACACTATCTCAATGCAAATATCTG TCTGAAACGGTCCCTGGCTAAACTCCACCCATGGGTTGGC CAGCCTTGCCTTGACCAATAGCCTTGACAA	SEQ ID NO:17
G-gamma - 175 T→C	TGGGGGCCCCTTCCCCACACTATCTCAATGCAAACATCTG TCTGAAACGGTCCCTGGCTAAACTCCACCCATGGGTTGGC CAGCCTTGCCTTGACCAATAGCCTTGACAA	SEQ ID NO:18
G-gamma - 114 C→T	TGGGGGCCCCTTCCCCACACTATCTCAATGCAAATATCTG TCTGAAACGGTCCCTGGCTAAACTCCACCCATGGGTTGGC CAGCCTTGCCTTGACTAATAGCCTTGACAA	SEQ ID NO:19
A-gamma - 196 C→T	TGGGGGCCCCTTCTCCACACTATCTCAATGCAAATATCTG TCTGAAACGGTCCCTGGCTAAACTCCACCCATGGGTTGGC CAGCCTTGCCTTGACCAATAGCCTTGACAA	SEQ ID NO:20
A-gamma - 175 T→C	TGGGGGCCCCTTCCCCACACTATCTCAATGCAAACATCTG TCTGAAACGGTCCCTGGCTAAACTCCACCCATGGGTTGGC CAGCCTTGCCTTGACCAATAGCCTTGACAA	SEQ ID NO:21
A-gamma - 117 G→A	TGGGGGCCCCTTCCCCACACTATCTCAATGCAAATATCTG TCTGAAACGGTCCCTGGCTAAACTCCACCCATGGGTTGGC CAGCCTTGCCTTAACCAATAGCCTTGACAA	SEQ ID NO:22
A-gamma - 114→-102 deleted (deleted bases in bold)	TGGGGGCCCCTTCCCCACACTATCTCAATGCAAATATCTG TCTGAAACGGTCCCTGGCTAAACTCCACCCATGGGTTGGC CAGCCTTGCCTTGACCAATAGCCTTGACAA	SEQ ID NO:23

[00167] Table 6 presents amino acid positions within a parental LHE I-OnuI homing endonuclease (Figure 22A, SEQ ID NO: 34) that were subjected to saturation mutagenesis in IVC (as described in Example 1, above) to create homing endonucleases that are targeted against a human fetal globin silencing region.

Table 6

Amino-acid Positions Subjected to Saturation Mutagenesis in IVC to Create Targeted Homing Endonucleases against a Human Fetal Globin Silencing Region

Round	Target site*	Sequence Identifier	Amino-acid residues
1	TTTCCAATTATTCAACCTTTTA	SEQ ID NO:	L26, G44, Q46, A70,

		40	S72, S78, K80
2	TCTTGAATTATTCAACCTTTTA	SEQ ID NO: 41	L26, R28, R30, N32, S40, E42, G44, K80, T82
1	TTTCCATTTATTCAATATTTTA	SEQ ID NO: 42	F182, N184, V199, S201, K225, K227, D236, V238
2	TTTCCATTTATTCAATATCTTT	SEQ ID NO: 43	F182, N184, I186, S190, K191, Q197, V199, V238, T240

*Underlined nucleotides differ from those in the target site for the parental LHE I-OnuI.

[00168] Figure 23 presents the results of a of a cleavage assay with 'half-targets' from a human fetal globin silencing region. The amplified bands contain both the cleaved half-sites (captured by ligation with complementary duplex oligonucleotides and corresponding overhanging ssDNA) and the sequences of the enzyme variants that are responsible for generation of cleaved DNA products. The final step upon completion of enrichment of the 'half-site' endonuclease libraries includes the assembly of DNA fragments that encode the N-terminal and C-terminal half domains of I-OnuI homing endonuclease, which are responsible for the left (L) and right (R) half sites of the gGlobin silencing region target. Active I-OnuI endonucleases are selected from a pool that cleaves the full-length human fetal globin silencing region target.

Example 6

MegaTALs Homing Endonucleases with N-terminal Fusions of TAL Anchors to Increase Specificity and Activity of a Gene-targeted Endonuclease

[00169] N-terminal fusions of TAL anchors can be employed to increase the specificity and activity of a gene-targeted endonuclease, including one or more homing endonucleases such as one or more of the I-HjeMI, I-CpaMI, and I-OnuI homing endonucleases. MegaTALs are constructed using the Golden Gate assembly strategy described by Cermak *et al., Nucl. Acids Res.* 39:e82–e82 (2011), using an RVD plasmid library and destination vector (see, Fig. 24, SEQ ID NO: 35 and Fig.

25, SEQ ID NO: 36 for the nucleotide and amino acid sequences of MegaTAL:5.5 RVD + Y21-AniI).

[00170] Plasmids are modified to allow the assembly of TAL effector containing 1.5 to 10.5 TAL repeats and their corresponding RVDs ('Repeat Variable Diresidues,' which contact consecutive DNA bases in the 5' region of the target site and thereby define the cognate DNA sequence in that region). The pthX01 destination vector was modified to include a hemagglutinin (HA) tag immediately downstream of the NLS and to yield a TALEN scaffold that begins at residue 154 (relative to wild-type PthXo1 TAL effector) and ends 63 residues beyond the final 'half TAL repeat' sequence.

[00171] TAL effectors are built using the following RVDs to target each specific nucleotide: A – NI, C – HD, G – NN and T – NG. Following cloning of the TAL effector repeats into the destination vector, an individual protein linker ('Zn4'; VGGS) and the engineered homing endonuclease variants are cloned in place of the FokI nuclease catalytic domain, between engineered Xba-I and Sal-I restriction sites.

[00172] This is a 'model test case' MegaTAL (a fusion of a TAL effector at the N-terminal end of a single protein chain, fused via a flexible linker to the wild-type Y2 I-AniI homing endonuclease, which was originally described in Takeuchi *et al.*, *Nucl. Acids Res.* 37(3): 877-890 (2009).

Example 7

A Cas9-based Endonuclease System for Disrupting a Bcl11a-regulated Fetal Hemoglobin (HbF) Silencing Region

[00173] The recent mechanistic understanding of the clustered regularly interspaced short palindromic repeat (CRISPR) system that bacteria use for adaptive immunity has led to the development of a powerful tool that allows for genome editing of mammalian cells, which can be employed in the compositions and methods for the treatment of hemoglobinopathies that are disclosed herein: (a) to disrupt a Bcl11a coding region; (b) to disrupt a HbF silencing DNA regulatory element or pathway, such as a Bcl11a-regulated HbF silencing region; (c) to mutate one or more γ-globin gene promoter(s) to achieve increased expression of a γ-globin

gene; (d) to mutate one or more δ-globin gene promoter(s) to achieve increased expression of a δ-globin gene; and/or (e) to correct one or more β-globin gene mutation(s). The bacterial CRISPR system is described in Jinek *et al.*, *Science* 337:816-821 (2013); Cong *et al.*, *Science* (Jan. 3, 2013) (Epub ahead of print); and Mali *et al.*, *Science* (Jan. 3, 2013) (Epub ahead of print).

[00174] The Cas9 protein generates a double stranded break at a specific site the location of which is determined by an RNA-guide sequence. All guide RNAs contain the same scaffold sequence that binds Cas9, as well as a variable targeting sequence having the structure G-N_{20}-GG, which provides Cas9-RNA complex cleavage specificity. Co-expression of the Cas9 protein and a guide RNA results in the efficient cleavage and disruption at a sequence-specific location within the human genome, which sequence-specific cleavage is defined by the guide RNA sequence. Co-expression of Cas9 and guide RNAs that are specific to multiple targets leads to efficient deletion of the intervening region between target sites.

[00175] Thus, within certain aspects of the present disclosure, Cas9-meditated genome editing is employed: (1) to disrupt the Bcl11a binding site within the HbF silencing region, (2) to disrupt Bcl11a gene function, and (3) to delete the entire HbF silencing region. Target regions are identified and guide RNAs are designed and generated based on consideration of the optimal guide RNA target sequences. Exemplified herein are guide RNAs that target the Bcl11a binding region within the HbF silencing region as well as the single GATA-1 binding motif. These guide RNAs are used singly or in combination to achieve the targeted disruption of the HbF silencing region. Cas9 with guide RNAs to additional regions within the HbF silencing regions that correspond to Bcl11a peaks of occupancy are also used singly and in combination. Several pairs of guide RNAs flanking the Bcl11a binding site and GATA-1 motif, as well as the entire footprint, can also be co-expressed with Cas9 in order to generate deletions within the HbF silencing region.

[00176] The sequence of a human codon optimized Cas9 from Mali *et al.*, *Science* (Jan. 3, 2013) is presented in Fig. 26, SEQ ID NO: 37. The generic sequence of a guide RNA (Mali *et al.*) is presented in Fig. 27, SEQ ID NO: 38, the key sequence elements of which are presented in Table 7. Exemplary Cas9 Guide

RNAs sequences of target-specific binding to and cleavage of the human fetal hemoglobin (HbF) silencing region (Fig. 6, SEQ ID NO: 1 and Fig. 7, SEQ ID NO: 2) are presented in Table 8.

Table 7

Sequence Elements of a Generic Cas9 Guide RNA

Description	Sequence Identifier	Nucleotide Sequence
U6 Promoter Sequence	SEQ ID NO: 44	GGACGAAACACC
Generic Target-specific Sequence	SEQ ID NO: 45	GNNNNNNNNNNNNNNNNNNN
Guide RNA Scaffold Sequence	SEQ ID NO: 46	GTTTTAGAGCTAGAAATAGC AAGTTAAAATAAGGCTAGTC CGTTATCAACTTGAAAAAGT GGCACCGAGTCGGTGCT
Poly T Tail	SEQ ID NO: 47	NNNNTTTTTT

Table 8

Target-specific Sequences for Exemplary Cas9 Guide RNAs

Designation	Description	Use	Sequence Identifier	Nucleotide Sequence
GGN20GG-B	Targets the GATA-1 recognition motif	Used singly, with #C to delete the putative Bcl11a and GATA-1motifs jointly, or with #E, #F or #G to delete the entire Bcl11a ChIP peak and surrounding sequences	SEQ ID NO: 48	GCCATTTCTATTA TCAGACTTGG
GGN20GG-C	Targets the putative Bcl11a recognition motif	Used singly, with #B to delete the putative Bcl11a and GATA-1motifs jointly	SEQ ID NO: 49	GCTGGGCTTCTGT TGCAGTAGGG
GGN20GG-D	Targets immediately adjacent to the putative Bcl11a recognition motif	Used singly, with #B to delete the putative Bcl11a and GATA-1motifs jointly	SEQ ID NO: 50	GAAAATGGGAGAC AAATAGCTGG

GGN20GG-E	Targets downstream of the Bcl11a binding peak	Used with #B and/or #H to delete the entire Bcl11a ChIP peak and surrounding sequences	SEQ ID NO: 51	GAATAATTCAAGA AAGGTGGTGG
GGN20GG-F	Targets downstream of the Bcl11a binding peak	Used with #B and/or #H to delete the entire Bcl11a ChIP peak and surrounding sequences	SEQ ID NO: 52	GATATTGAATAAT TCAAGAAAGG
GGN20GG-G	Targets downstream of the Bcl11a binding peak	Used with #B and/or #H to delete the entire Bcl11a ChIP peak and surrounding sequences	SEQ ID NO: 53	GCCTGAGATTCTG ATCACAAGGG
GGN20GG-H	Targets upstream of the Bcl11a binding peak	Used with #E, #F or #G to delete the entire Bcl11a ChIP peak and surrounding sequences	SEQ ID NO: 54	GGTAAATTCTTAA GGCCATGAGG

Example 8

Vector Systems for Expressing Endonucleases

[00177] For NSG, sickle cell and thalassemia mouse models, human CD34 cells or mouse bone marrow nucleated cells are transduced along with a fluorescent marker allowing flow cytometry-based enrichment of cells prior to transplantation. Suitable transduction methods include the following:

[00178] *AAV6 vectors*. AAV6-serotype recombinant AAV vectors provide a 4.5 kb payload, sufficient to deliver a promoter-HE-exonuclease or promoter-TAL-HE fusion-exonuclease cassettes in addition to a small recombination template. Alternatively they can carry Cas9 and a guide RNA. In addition, we have preliminary data that show AAV6 provides the most efficient transduction of human CD34+ umbilical cord blood cells of all known AAV capsids, and is able to mediate significant levels of transient gene expression in HSC.

[00179] *Modified Adenovirus vectors*. Adenoviral vectors with hybrid capsids are capable of efficiently transducing many types of hematopoietic cells

including CD34+ cells. Improved transduction with the chimeric vector using the serotype 35 fiber (Ad5-F35) was demonstrated by Dr. Rawlings (SCH) and more recent data suggest that the serotype 11 fiber (Ad5-F11) may be even more efficient in hematopoietic cells. Helper-dependent adenoviral vectors offer up to a 30 kb payload, along with transient gene expression in HSC, and can be used to deliver multiple HE/exonuclease cassettes, HE-TAL fusions, as well as very large recombination templates or a Cas9 expression cassette and multiple guide RNAs.

[00180] *Integration-deficient Lentiviral and Foamyviral Vectors (IDLV and IDFV)*. These vectors provide 6 kb (IDLV) to 9kb (IDFV) payloads, and have well documented capabilities to transduce human HSCs. Both IDLV and IDFV vectors can be used for gene knockout and recombination-based gene editing in HSC. Drs. Rawlings (SCH) and Kiem (FHCRC) have generated and evaluated a series of IDLV with alternative promoter GFP cassettes and have determined constructs that provide efficient and high level expression in CD34+ HSC.

[00181] *Direct nucleofection of plasmid and mRNA*. Conditions for efficient transduction of N-MEL and CD34 cells have been defined using the Amaxa nucleofection system. Benefits include the lack of integration, and the ability to transduce multiple expression plasmids or RNA species simultaneously.

[00182] In parallel, sorted and un-sorted cells will be transplanted into separate mice. While the later transplants may contain low numbers of modified cells, human studies of post-transplant chimeras suggest that these cells will have a selective survival advantage and be enriched in the periphery. Regardless, single reticulocyte RNA and F-Cell analysis will allow assessment of gene disruption in cells even if present in low abundance.

[00183] Second level assessments will focus on the pluripotency of transduced CD34+ cells and erythropoiesis. Assays will include culturing to assess long-term proliferative potential, analysis of myeloid and erythroid colonies for clonal analysis and transplant into NOD *scid* gamma (NSG) mice followed by assessment of multi-lineage engraftment of primary and secondary recipients.

[00184] Typically stem cells are infused via tail vein injection after total body

irradiation (275 rads for NSG mice or 1000 rads for C57 mice). Though efficient, this is effective, for most studies we will inject stem cells directly into the mouse femur as 50 fold fewer cells are required, ideal or assaying a potentially limited number of flow cytometry sorted and/or modified cells. After anesthesia and local analgesia are provided and anatomic landmarks defined, 0.5-1 milion cells are directly injected into the femurs marrow space.

Example 9

Characterization of Homing and Cas9 Endonucleases for Efficient Gene Targeting

[00185] For clinical impact efficient gene targeting is demonstrated by assessing levels of globin gene expression in individual targeted cells and in populations of cells, the effect of targeting on erythropoiesis and on stem cell function, and impact hematologic parameters and organ function in model organisms.

[00186] Transductions are followed by single cell and bulk population assessments of gene targeting efficiency and expression of all β-like genes at the RNA and protein levels. Alterations in factor binding and chromatin structure are assessed, as cell morphology and the extent of ineffective erythropoiesis and apoptosis. Candidate endonucleases that score well in initial screens are further assessed for effects on HSC pluripotency as well as the ability to ameliorate disease specific phenotypes *in vitro* and *in vivo*.

[00187] Initial screening of endonuclease candidates and delivery systems is performed in a mouse erythroleukemia cell line containing a single intact human chromosome 11 (N-MEL) and clinical grade CD34+ normal human HSCs with endpoints of assessing targeted mutation efficiency and globin gene expression. Both cell types can be induced to differentiate along an erythroid path during which expression of β-like genes is highly induced with a low □-globin / □- + β-globin RNA ratio. Using a HbF specific antibody, the percent of "F-cells" can be quantified. These low ratios are ideal as the systems are sensitive for detecting and quantifying even small increases in □-globin mRNA as well as HbF expression at the single cell and population level.

[00188] N-MEL cells are a derivative of murine erythroleukemia cells that contain a single intact human chromosome 11 that contains the β-globin locus. This erythroid cell line normally expresses low levels of mouse and human β-like globin genes, but can be induced to differentiate at which time globin expression is greatly increased.

[00189] N-MEL cells are efficiently tranduced using the Amaxa nucleofection system. Using 2 μg of plasmid DNA, Kit "L" and program A20 25% of cells are transduced. Infection at multiplicity of infection (MOI) of 20 with a RSCS-MCS-PG-WZ based lentiviral vector containing a homing endonuclease designed to disrupt the Bcl11a gene yields approximately 40% of N-MEL cells being transduced.

[00190] The efficiency of targeted disruption is assayed using the Cel-1 assay. The target region is amplified by PCR, heat-denatured, re-annealed and exposed to the enzyme Cel-1 that efficiently cleaves bubbles from mismatched regions as small as one base pair. If the targeted region has been mutated in any way, heteroduplexes of wild type and mutant strands are cleaved and detected by gel electrophoresis. This assay can be used on bulk populations of cells to estimate the efficiency of mutation, or on flow cytometry sorted individual cells in which case analysis of multiple cells provides an accurate assessment of mutation frequency.

[00191] Using routine quantitative Taqman RT-PCR (qRT-PCR) assays for RNA from a bulk population of cells, β-globin expression is induced 11-fold with differentiation and with a □-globin / □- + β-globin ratio of 0.1%. After infection with a Bcl11a knockdown vector a 30-fold increase in this ratio can be obtained, thus demonstrating that this cell culture system provides an accurate readout of disruption of the BCl11a mediated HbF silencing pathway.

[00192] Due to concerns that altering Bcl11a pathways may lead to a relative increase in □-globin RNA but a decrease in globin gene expression, flow cytometry can be used to sort 1000 cell pools of cells that are lysed and the above qRT-PCR assays can be performed. Because RNA is directly compared from the same number of cells, any diminution in the amount of globin RNA is reflected by an increase in

the Ct, providing a direct measure of both the level of β- and □- expression, as well as the ratio of □-globin / □- + β-globin.

[00193] To determine the percent of cells that show an altered □-globin / □- + β-globin ratio after manipulation and to determine the range of change in expression observed, single cell assays are performed. Flow cytometry sorted individual cells are subjected to routine RT-PCR of □- and β-globin RNA simultaneously for several cycles and once adequate material is present to allow for accurate splitting of the sample, □- and β-globin are assessed by qRT-PCR as above.

[00194] Ultimately it is levels of HbF protein, not RNA that are therapeutic, thus single cell and bulk cell HbF assays are performed. Bulk populations of cells are assayed using HPLC after cell lysis and elution on a hemoglobin-dedicated column and the ratios of HbF to HbA and HbA2 are determined. The number of cells expressing HbF are compared pre- and post-transduction using gluteraldehyde fixing cells, permeabilizing with detergent and adding an HbF specific antibody followed by quantitation by flow cytometry.

[00195] Effects of mutations are assessed in modified cloned cells after isolation of single cells by flow cytometry. The above assays are performed. In addition, to show that targeted mutations disrupt binding of the Bcl11a repressive complex, cells are fixed in formaldehyde, chromatin isolated and sheared by sonication. Chromatin immune-precipitation (ChIP) is performed using commercially available antibodies to Bcl11a, GATA-1 and HDAC-1. Binding of these proteins to a target region in N-MEL cells, as well as erythroid-differentiated CD34 cells is described below. A lack of binding is assessed after targeted disruption.

Example 10
Clinical Grade CD34+ Hematopoietic Stem Cells

[00196] CD34+ cells from normal human donors are adhered to culture dishes with fibronectin peptide CH-296 and infected at an MOI of 20 twice, 8 hours apart, in media containing G-CSF, SCF, IL-3, IL-6, FLT-3, and TPO, with RSCS-MCS-

PG-WZ based lentiviral vectors described above. This results in ~80% of cells being infected. Transduced cells are differentiated to erythroid cells using the protocol of Douy. Giarratana *et al.*, *Nat. Biotechnol.* 23(1):69-74 (2005). The qRT-PCR assays above reveal a □-globin / □- + β-globin ratio of 4%. This low ratio allows for the sensitive detection of increases that are secondary to genome editing.

[00197] To assess alterations in differentiation state, flow cytometry using multiple cell surface markers including CD34, CD71, and glycophorin are performed, as well as assessment of cell growth and morphology of cytospins after Wright-Giemsa staining. Disruption efficiency is assessed by Cel-1 assays on bulk populations and single cells as above. Additional assessment of globin gene expression and HbF is performed as above using qRT-PCR on1000 cell pools and individual cells as well as HPLC and F-cell assays. ChIP is employed to assess binding of Bcl11a, GATA-1, and HDAC-1 to the target region.

[00198] To assess clinical effectiveness in human cells CD34+ human, HSCs from hemoglobinopathy patients are transduced and cultured using the same methods. In addition to the routine analysis for normal cells, additional disease-specific assessments are performed. Cultured thalassemic cells show minimal expansion, a lack of hemoglobinization, evidence of ineffective erythropoiesis, and increased apoptosis compared to normal cells. This allows for quantitative assessments of improvements in expression and erythropoiesis post-targeting. Similarly, the degree of sickling of erythroid progeny of CD34 cells under hypoxic conditions is assessed. These transduced CD34+ cells from hemogobinopathy patients are transplanted into NSG mice, after which several features of abnormal erythropoiesis are recapitulated, allowing assessment of the effect of targeted mutagenesis.

[00199] Clinical effectiveness is assessed *in vivo* in mouse models of hemoglobinopathies. Knockout of murine Bcl11a leads to a dramatic dose-dependent increase in □-globin in mice containing a human β-globin locus on a transgene and ameliorates the sickle phenotype in humanized mouse models. While both systems allow the analysis of globin gene expression, the sickle mice allow for the assessment of the improvement of phenotype in these mice with special attention

to the hematologic parameters especially the hematocrit, liver and lung pathology, renal function and spleen size. This can be correlated to the number of HbF containing cells, the HbF/S ratio and expression patterns in single cell assays similar to the above.

[00200] For RNA analysis total blood is used as it contains sufficient RNA-containing reticulocytes for analysis. For single cell analyses blood is stained with thiazole orange and RNA containing cells with the forward and side scatter profiles of red cells are collected. The erythrocyte lifespan is significantly reduced in the sickle cell mice and improvement in lifespan is assessed after intra-peritoneal injection of NHS-biotin with labels 100% of RBC. At time points, a microliter of blood is stained with strep-avidin FITC and the remaining percent of labeled RBC is assessed by flow cytometry. This is done with the mice from our BERK sickle cell mice. Pászty *et al.*, *Science* 278(5339):876-878 (1997). In addition, the □-globin / □- + β-globin ratio is assessed in mice containing the A25 and A85 human β-globin YACs. Porcu *et al.*, *Blood* 90(11):4602-4609 (1997).

[00201] Two thalassemic models are assessed, the Th-3 mouse heterozygotes (Yang *et al.*, Proc. Natl. Acad. Sci. U.S.A. 92(25):11608-11612 (1995)) and mice heterozygous for a deletion of the LCR (Bender *et al.*, *Mol Cell.* 5(2):387-393 (2000)). In each case, the focus of analysis for thalassemic mice will be hematocrit, assessment of ineffective erythropoiesis, spleen size, iron overload and organ morphology.

CLAIMS

What is claimed is:

1. A composition for the treatment of a hemoglobinopathy, comprising one or more endonuclease(s) selected from the group consisting of a homing endonuclease (HE) and a CRISPR endonuclease, wherein each of said endonuclease(s) binds to a nucleotide sequence selected from the group consisting of a Bcl11a coding region, a Bcl11a gene regulatory region, an adult human β-globin locus, a fetal hemoglobin (HbF) silencing region, a Bcl11a-regulated HbF silencing region, a γ-globin gene promoter, a δ-globin gene promoter, and a site of a β-globin gene mutation.

2. The composition of claim 1 wherein said endonuclease is a HE that binds to said Bcl11a coding region or said Bcl11a gene regulatory region.

3. The composition of claim 2 wherein said HE is selected from the group consisting of an I-HjeMI homing endonuclease, an I-CpaMI homing endonuclease, and an I-OnuI homing endonuclease.

4. The composition of claim 3 wherein said HE is an I-HjeMI homing endonuclease.

5. The composition of claim 4 wherein said I- HjeMI homing endonuclease comprises one or more amino acid substitutions within the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 28 or SEQ ID NO: 29, wherein said amino acid substitution is selected from the group consisting of Y20, S22, T24, K26, G27, K28, T31, E33, G35, E37, S59, R61, R63, N64, E65, I66, M68, S70, R70, R72, R74, S109, N110, A121, S123, N124, N135, S137, S154, L158, N159, D162, D163, I166, I168, S168, D170, I193, L195, R202, K204, and T206.

6. The composition of claim 5 wherein said I-HjeMI homing endonuclease comprises the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 31 or SEQ ID NO: 32.

7. The composition of claim 6 wherein said I-HjeMI homing endonuclease comprises the amino acid sequence of SEQ ID NO: 33 or a variant thereof wherein said I-HjeMI homing endonuclease can specifically bind to a Bcl11a coding region.

8. The composition of claim 1 wherein said endonuclease is a CRISPR endonuclease wherein said CRISPR comprises an RNA guide strand and a Cas9 endonuclease wherein said RNA guide strand mediates the binding of said Cas9 endonuclease to a fetal hemoglobin (HbF) silencing region.

9. The composition of claim 8 wherein said RNA guide strand comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, and SEQ ID NO: 54.

10. The composition of claim 9 wherein said Cas9 endonuclease is encoded by the nucleotide sequence of SEQ ID NO: 37 or a variant thereof which encodes a functional Cas9 endonuclease.

11. The composition of claim 1 wherein said endonuclease is a HE that can specifically bind to a fetal hemoglobin (HbF) silencing region.

12. The composition of claim 11 wherein said HE is selected from the group consisting of an I-HjeMI homing endonuclease, an I-CpaMI homing endonuclease, and an I-OnuI homing endonuclease.

13. The composition of claim 12 wherein said HE is an I-OnuI homing endonuclease.

14. The composition of claim 13 wherein said I-OnuI homing endonuclease comprises the amino acid sequence encoded by a variant of the nucleotide sequence of SEQ ID NO: 34 that encodes an I-OnuI homing endonuclease that can specifically bind to said fetal hemoglobin (HbF) silencing region.

15. The composition of claim 14 wherein said I-OnuI homing endonuclease comprises one or more amino acid substitutions within the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 34, wherein said amino acid substitution is selected from the group consisting of L26, R28, R30, N32, S40, E42, G44, Q46, A70, S72, S78, K80, and T82.

16. The composition of claim 15 wherein said I-OnuI homing endonuclease comprises one or more amino acid substitutions within the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 34, wherein said amino acid substitution is selected from the group consisting of F182, N184, I186, S190, K191, Q197, V199, S201, K225, K227, D236, V238, and T240.

17. The composition of claim 1, further comprising a TAL effector nuclease (TALEN), a TAL-HE, or a TREX2 protein.

18. A composition for the treatment of a hemoglobinopathy, comprising one or more polynucleotide encoding one or more endonuclease(s) selected from the group consisting of a homing endonuclease (HE) and a Cas9 endonuclease, wherein each of said endonuclease(s) binds to a nucleotide sequence selected from the group consisting of a Bcl11a coding region, a Bcl11a gene regulatory region, an adult human β-globin locus, a fetal hemoglobin (HbF) silencing region, a Bcl11a-regulated HbF silencing region, a γ-globin gene promoter, a δ-globin gene promoter, and a site of a β-globin gene mutation.

19. The composition of claim 18 wherein said endonuclease is a HE that binds to said Bcl11a coding region or said Bcl11a gene regulatory region.

20. The composition of claim 19 wherein said HE is selected from the group consisting of an I-HjeMI homing endonuclease, an I-CpaMI homing endonuclease, and an I-OnuI homing endonuclease.

21. The composition of claim 20 wherein said HE is an I-HjeMI homing endonuclease.

22. The composition of claim 21 wherein said I- HjeMI homing endonuclease comprises one or more amino acid substitutions within the amino acid

sequence encoded by the nucleotide sequence of SEQ ID NO: 28 or SEQ ID NO: 29, wherein said amino acid substitution is selected from the group consisting of Y20, S22, T24, K26, G27, K28, T31, E33, G35, E37, S59, R61, R63, N64, E65, I66, M68, S70, R70, R72, R74, S109, N110, A121, S123, N124, N135, S137, S154, L158, N159, D162, D163, I166, I168, S168, D170, I193, L195, R202, K204, and T206.

23. The composition of claim 22 wherein said I-HjeMI homing endonuclease comprises the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 31 or SEQ ID NO: 32.

24. The composition of claim 23 wherein said I-HjeMI homing endonuclease comprises the amino acid sequence of SEQ ID NO: 33 or a variant thereof wherein said I-HjeMI homing endonuclease can specifically bind to a Bcl11a coding region.

25. The composition of claim 24 wherein said endonuclease is a CRISPR endonuclease wherein said CRISPR comprises an RNA guide strand and a Cas9 endonuclease wherein said RNA guide strand mediates the binding of said Cas9 endonuclease to a fetal hemoglobin (HbF) silencing region.

26. The composition of claim 25 wherein said RNA guide strand comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, and SEQ ID NO: 54.

27. The composition of claim 26 wherein said Cas9 endonuclease is encoded by the nucleotide sequence of SEQ ID NO: 37 or a variant thereof which encodes a functional Cas9 endonuclease.

28. The composition of claim 18 wherein said endonuclease is a HE that can specifically bind to a fetal hemoglobin (HbF) silencing region.

29. The composition of claim 28 wherein said HE is selected from the group consisting of an I-HjeMI homing endonuclease, an I-CpaMI homing endonuclease, and an I-OnuI homing endonuclease.

30. The composition of claim 29 wherein said HE is an I-OnuI homing endonuclease.

31. The composition of claim 30 wherein said I-OnuI homing endonuclease comprises the amino acid sequence encoded by a variant of the nucleotide sequence of SEQ ID NO: 34 that encodes an I-OnuI homing endonuclease that can specifically bind to said fetal hemoglobin (HbF) silencing region.

32. The composition of claim 31 wherein said I-OnuI homing endonuclease comprises one or more amino acid substitutions within the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 34, wherein said amino acid substitution is selected from the group consisting of L26, R28, R30, N32, S40, E42, G44, Q46, A70, S72, S78, K80, and T82.

33. The composition of claim 31 wherein said I-OnuI homing endonuclease comprises one or more amino acid substitutions within the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 34, wherein said amino acid substitution is selected from the group consisting of F182, N184, I186, S190, K191, Q197, V199, S201, K225, K227, D236, V238, and T240.

34. The composition of claim 18, further comprising a polynucleotide encoding a TAL effector nuclease (TALEN), a TALE-HE fusion protein, and/or a TREX2 nuclease.

35. A polynucleotide encoding an endonuclease selected from the group consisting of a homing endonuclease (HE) and a CRISPR endonuclease, wherein said endonuclease binds to a nucleotide sequence selected from the group consisting of a Bcl11a coding region, a Bcl11a gene regulatory region, an adult human β-globin locus, a fetal hemoglobin (HbF) silencing region, a Bcl11a-regulated HbF silencing region, a γ-globin gene promoter, a δ-globin gene promoter, and a site of a β-globin gene mutation.

36. The polynucleotide of claim 35 wherein said endonuclease is a HE that binds to said Bcl11a coding region or said Bcl11a gene regulatory region.

37. The polynucleotide of claim 36 wherein said HE is selected from the group consisting of an I-HjeMI homing endonuclease, an I-CpaMI homing endonuclease, and an I-OnuI homing endonuclease.

38. The polynucleotide of claim 37 wherein said HE is an I-HjeMI homing endonuclease.

39. The polynucleotide of claim 38 wherein said I- HjeMI homing endonuclease comprises one or more amino acid substitutions within the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 28 or SEQ ID NO: 29, wherein said amino acid substitution is selected from the group consisting of Y20, S22, T24, K26, G27, K28, T31, E33, G35, E37, S59, R61, R63, N64, E65, I66, M68, S70, R70, R72, R74, S109, N110, A121, S123, N124, N135, S137, S154, L158, N159, D162, D163, I166, I168, S168, D170, I193, L195, R202, K204, and T206. 25. The polynucleotide of claim 24 wherein said I-HjeMI homing endonuclease comprises the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 31 or SEQ ID NO: 32.

40. The polynucleotide of claim 39 wherein said I-HjeMI homing endonuclease comprises the amino acid sequence of SEQ ID NO: 33 or a variant thereof wherein said I-HjeMI homing endonuclease can specifically bind to a Bcl11a coding region.

41. The polynucleotide of claim 40 wherein said endonuclease is a HE that can specifically bind to a fetal hemoglobin (HbF) silencing region.

42. The polynucleotide of claim 41 wherein said HE is selected from the group consisting of an I-HjeMI homing endonuclease, an I-CpaMI homing endonuclease, and an I-OnuI homing endonuclease.

43. The polynucleotide of claim 35 wherein said HE is an I-OnuI homing endonuclease.

44. The polynucleotide of claim 43 wherein said I-OnuI homing endonuclease comprises the amino acid sequence encoded by a variant of the nucleotide sequence of SEQ ID NO: 34 that encodes an I-OnuI homing

endonuclease that can specifically bind to said fetal hemoglobin (HbF) silencing region.

45. The polynucleotide of claim 44 wherein said I-OnuI homing endonuclease comprises one or more amino acid substitutions within the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 34, wherein each of said amino acid substitutions is selected from the group consisting of L26, R28, R30, N32, S40, E42, G44, Q46, A70, S72, S78, K80, and T82.

46. The polynucleotide of claim 45 wherein said I-OnuI homing endonuclease comprises one or more amino acid substitutions within the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 34, wherein said amino acid substitution is selected from the group consisting of F182, N184, I186, S190, K191, Q197, V199, S201, K225, K227, D236, V238, and T240.

47. The polynucleotide of claim 35 further comprising a polynucleotide encoding a TAL effector nuclease (TALEN), a TALE-HE fusion protein, and/or a TREX2 nuclease.

48. A polynucleotide encoding an RNA guide strand that mediates the binding of a Cas9 endonuclease to a Bcl11a coding region, a Bcl11a gene regulatory region, an adult human β-globin locus, a fetal hemoglobin (HbF) silencing region, a Bcl11a-regulated HbF silencing region, a γ-globin gene promoter, a δ-globin gene promoter, and a site of a β-globin gene mutation.

49. The polynucleotide of claim 48 wherein said RNA guide strand mediates the specific binding of a Cas9 endonuclease to a fetal hemoglobin (HbF) silencing region.

50. The polynucleotide of claim 49 wherein said fetal hemoglobin (HbF) silencing region comprises the nucleotide sequence of SEQ ID NO: 1.

51. The polynucleotide of claim 50 wherein said fetal hemoglobin (HbF) silencing region comprises the nucleotide sequence of SEQ ID NO: 2.

52. The polynucleotide of claim 50 or claim 51 wherein said RNA guide strand comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, and SEQ ID NO: 54.

53. A vector system comprising a vector and a polynucleotide encoding an endonuclease selected from the group consisting of a homing endonuclease (HE) and a CRISPR endonuclease, wherein said endonuclease binds to a nucleotide sequence selected from the group consisting of a Bcl11a coding region, a Bcl11a gene regulatory region, an adult human β-globin locus, a fetal hemoglobin (HbF) silencing region, a Bcl11a-regulated HbF silencing region, a γ-globin gene promoter, a δ-globin gene promoter, and a site of a β-globin gene mutation.

54. The vector system of claim 53 wherein said vector is selected from the group consisting of an AAV6, a modified adenovirus vector, an integration-deficient lentiviral vector (IDLV), and an integration-deficient foamyviral vector (IDFV).

55. The vector system of claim 53 wherein said endonuclease is a HE that bind to said Bcl11a coding region or said Bcl11a gene regulatory region.

56. The vector system of claim 55 wherein said HE is selected from the group consisting of an I-HjeMI homing endonuclease, an I-CpaMI homing endonuclease, and an I-OnuI homing endonuclease.

57. The vector system of claim 56 wherein said HE is an I-HjeMI homing endonuclease.

58. The vector system of claim 57 wherein said I- HjeMI homing endonuclease comprises one or more amino acid substitutions within the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 28 or SEQ ID NO: 29, wherein said amino acid substitution is selected from the group consisting of Y20, S22, T24, K26, G27, K28, T31, E33, G35, E37, S59, R61, R63, N64, E65, I66, M68, S70, R70, R72, R74, S109, N110, A121, S123, N124, N135, S137, S154, L158, N159, D162, D163, I166, I168, S168, D170, I193, L195, R202, K204, and

T206. 25. The polynucleotide of claim 24 wherein said I-HjeMI homing endonuclease comprises the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 31 or SEQ ID NO: 32.

59. The vector system of claim 58 wherein said I-HjeMI homing endonuclease comprises the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 31 or SEQ ID NO: 32.

60. The vector system of claim 59 wherein said I-HjeMI homing endonuclease comprises the amino acid sequence of SEQ ID NO: 33 or a variant thereof wherein said I-HjeMI homing endonuclease can specifically bind to a Bcl11a coding region.

61. The vector system of claim 53 wherein said endonuclease is a HE that can specifically bind to a fetal hemoglobin (HbF) silencing region.

62. The vector system of claim 61 wherein said HE is selected from the group consisting of an I-HjeMI homing endonuclease, an I-CpaMI homing endonuclease, and an I-OnuI homing endonuclease.

63. The vector system of claim 62 wherein said HE is an I-OnuI homing endonuclease.

64. The vector system of claim 63 wherein said I-OnuI homing endonuclease comprises the amino acid sequence encoded by a variant of the nucleotide sequence of SEQ ID NO: 34 that encodes an I-OnuI homing endonuclease that can specifically bind to said fetal hemoglobin (HbF) silencing region.

65. The vector system of claim 64 wherein said I-OnuI homing endonuclease comprises one or more amino acid substitutions within the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 34, wherein said amino acid substitution is selected from the group consisting of L26, R28, R30, N32, S40, E42, G44, Q46, A70, S72, S78, K80, and T82.

66. The vector system of claim 64 wherein said I-OnuI homing endonuclease comprises one or more amino acid substitutions within the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 34, wherein said amino acid substitution is selected from the group consisting of F182, N184, I186, S190, K191, Q197, V199, S201, K225, K227, D236, V238, and T240.

67. The vector system of claim 53 further comprising a polynucleotide encoding a TAL effector nuclease (TALEN), a TALE-HE fusion protein, and/or a TREX2 nuclease.

68. A vector system comprising a vector and a polynucleotide encoding an RNA guide strand that mediates the binding of a Cas9 endonuclease to a nucleotide sequence selected from the group consisting of a Bcl11a coding region, a Bcl11a gene regulatory region, an adult human β-globin locus, a fetal hemoglobin (HbF) silencing region, a Bcl11a-regulated HbF silencing region, a γ-globin gene promoter, a δ-globin gene promoter, and a site of a β-globin gene mutation.

69. The vector system of claim 68 wherein said nucleotide sequence within an adult human β-globin locus is a fetal hemoglobin (HbF) silencing region.

70. The vector system of claim 69 wherein said fetal hemoglobin (HbF) silencing region comprises the nucleotide sequence of SEQ ID NO: 1.

71. The vector system of claim 69 wherein said fetal hemoglobin (HbF) silencing region comprises the nucleotide sequence of SEQ ID NO: 2.

72. The vector system of claim 70 or claim 71 wherein said RNA guide strand comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, and SEQ ID NO: 54.

73. A cell comprising a polynucleotide encoding one or more endonuclease(s) selected from the group consisting of a homing endonuclease (HE) and a CRISPR endonuclease, wherein each of said endonuclease(s) binds to a nucleotide sequence selected from the group consisting of a Bcl11a coding region, a

Bcl11a gene regulatory region, an adult human β-globin locus, a fetal hemoglobin (HbF) silencing region, a Bcl11a-regulated HbF silencing region, a γ-globin gene promoter, a δ-globin gene promoter, and a site of a β-globin gene mutation.

74. The cell of claim 73 wherein said cell is a stem cell.

75. The cell of claim 73 wherein said cell is a stem cell selected from the group consisting of a hematopoietic stem cell (HSC), an induced pluripotent stem cell (iPSC), an embryonic stem (ES) cell, and an erythroid progenitor cell.

76. The cell of claim 73 wherein said cell is a stem cell selected from the group consisting of a hematopoietic stem cell (HSC), an induced pluripotent stem cell (iPSC), and an erythroid progenitor cell.

77. The cell of claim 73 wherein said endonuclease is a HE that can specifically bind to said Bcl11a coding region.

78. The cell of claim 77 wherein said HE is selected from the group consisting of an I-HjeMI homing endonuclease, an I-CpaMI homing endonuclease, and an I-OnuI homing endonuclease.

79. The cell of claim 78 wherein said HE is an I-HjeMI homing endonuclease.

80. The cell of claim 79 wherein said I- HjeMI homing endonuclease comprises one or more amino acid substitutions within the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 28 or SEQ ID NO: 29, wherein said amino acid substitution is selected from the group consisting of Y20, S22, T24, K26, G27, K28, T31, E33, G35, E37, S59, R61, R63, N64, E65, I66, M68, S70, R70, R72, R74, S109, N110, A121, S123, N124, N135, S137, S154, L158, N159, D162, D163, I166, I168, S168, D170, I193, L195, R202, K204, and T206. 25.

81. The cell of claim 80 wherein said I-HjeMI homing endonuclease comprises the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 31 or SEQ ID NO: 32.

82. The cell of claim 81 wherein said I-HjeMI homing endonuclease comprises the amino acid sequence of SEQ ID NO: 33 or a variant thereof wherein said I-HjeMI homing endonuclease can specifically bind to a Bcl11a coding region.

83. The cell of claim 73 wherein said CRISPR endonuclease comprises an Cas9 endonuclease and an RNA guide strand wherein said RNA guide strand mediates the binding of said Cas9 endonuclease to a fetal hemoglobin (HbF) silencing region.

84. The cell of claim 83 wherein said RNA guide strand comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, and SEQ ID NO: 54.

85. The cell of claim 84 wherein said Cas9 endonuclease is encoded by the nucleotide sequence of SEQ ID NO: 37 or a variant thereof which encodes a functional Cas9 endonuclease.

86. The cell of claim 73 wherein said endonuclease is a HE that binds to a fetal hemoglobin (HbF) silencing region.

87. The cell of claim 86 wherein said HE is selected from the group consisting of an I-HjeMI homing endonuclease, an I-CpaMI homing endonuclease, and an I-OnuI homing endonuclease.

88. The cell of claim 87 wherein said HE is an I-OnuI homing endonuclease.

89. The cell of claim 88 wherein said I-OnuI homing endonuclease comprises the amino acid sequence encoded by a variant of the nucleotide sequence of SEQ ID NO: 34 that encodes an I-OnuI homing

endonuclease that can specifically bind to said fetal hemoglobin (HbF) silencing region.

90. The cell of claim 89 wherein said I-OnuI homing endonuclease comprises one or more amino acid substitutions within the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 34, wherein said amino acid substitution is selected from the group consisting of L26, R28, R30, N32, S40, E42, G44, Q46, A70, S72, S78, K80, and T82.

91. The cell of claim 89 wherein said I-OnuI homing endonuclease comprises one or more amino acid substitutions within the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 34, wherein said amino acid substitution is selected from the group consisting of F182, N184, I186, S190, K191, Q197, V199, S201, K225, K227, D236, V238, and T240.

92. The cell of claim 73, further comprising a polynucleotide encoding a TAL effector nuclease (TALEN), a TALE-HE fusion protein, and/or a TREX2 nuclease.

93. A genome edited stem cell, wherein said genome edited stem cell is generated by the introduction of a homing endonuclease and a correction template.

94. The genome edited stem cell of claim 93 wherein said correction template comprises a nucleotide sequence that permits the modification of key regulatory or coding sequences within a globin gene locus.

95. The genome edited stem cell of claim 93 wherein said stem cell is selected from the group consisting of a hematopoietic stem cell (HSC), an induced pluripotent stem cell (iPSC), an embryonic stem (ES) cell, and an erythroid progenitor cell.

96. The genome edited stem cell of claim 93 wherein said stem cell is selected from the group consisting of a hematopoietic stem cell (HSC), an induced pluripotent stem cell (iPSC), and an erythroid progenitor cell.