fragile than normal RBCs, and tend towards hemolysis, eventually leading to anemia in the patient.

[0013] Treatment and management of sickle cell patients is a life-long proposition involving antibiotic treatment, pain management and transfusions during acute episodes. One approach is the use of hydroxyurea, which exerts its effects in part by increasing the production of γ globin. Long term side effects of chronic hydroxyurea therapy are still unknown, however, and treatment gives unwanted side effects and can have variable efficacy from patient to patient. Despite an increase in the efficacy of sickle cell treatments, the life expectancy of patients is still only in the mid to late 50's and the associated morbidities of the disease have a profound impact on a patient's quality of life.

[0014] Thus, there remains a need for additional methods and compositions that can be used for genome editing, to correct an aberrant gene or alter the expression of others for example to treat hemoglobinopathies such as sickle cell disease.

SUMMARY

[0015] In one aspect, the present invention refers to a genetically modified cell comprising a genomic modification within or between SEQ ID NO:23 or SEQ ID NO:24 of an endogenous human beta-hemoglobin (Hbb) gene following double-stranded break by a pair of zinc finger nucleases, the pair comprising the zinc finger proteins designated 47773 and 47817, wherein the genomic modification comprises an insertion and/or deletion. In another aspect, the present invention refers to a genetically modified differentiated cell descended from the stem cell of the present invention. In yet another aspect, the present invention refers to pharmaceutical composition comprising the genetically modified cell of the present invention. In another aspect, the present invention refers to a composition comprising a pair of zinc finger nucleases, each zinc finger nuclease comprising a zinc finger protein and a cleavage domain or cleavage half-domain, wherein the zinc finger protein comprises 5 or 6 zinc finger domains comprising a recognition helix region, wherein the pair comprises the zinc finger proteins designated 33488 and 33501 or 47773 and 47817. In another aspect, the present invention refers to a isolated cell comprising the composition of any of the present invention. In another aspect, the present invention refers to a kit comprising a composition according to the present invention or a cell

according to the present invention. In yet another aspect, the present invention refers to an *in vitro* method of altering Hbb expression in a cell, the method comprising: introducing, into the cell, one or more compositions according to the present invention, under conditions such that the pair of zinc finger nucleases is expressed and expression of the Hbb gene is altered. In still another aspect, the present invention refers to an *in vitro* method of producing a genetically modified cell comprising a genomic modification within an endogenous Hbb gene, the method comprising the steps of: a) contacting a cell with a composition according to the present invention, b) subjecting the cell to conditions conducive to expressing the pair of zinc finger nuclease; and c) modifying the endogenous Hbb gene with the expressed fusion protein sufficient to produce the genetically modified cell. In one other aspect, the present invention refers to the use of the isolated cell of the present invention in the manufacture of a medicament for treating a patient with sickle cell anemia. Also disclosed herein are methods and compositions for altering the expression or for correcting one or more genes encoding proteins involved in a genetic disease (*e.g.*, producing proteins lacking, deficient or aberrant in the disease and/or proteins that regulate these proteins) such as sickle cell disease. The present disclosure describes compositions and methods for use in gene therapy and genome engineering.

[0016] Alteration of such proteins can result in the treatment of these genetic diseases. In particular, genome editing is used to correct an aberrant gene, insert a wild type gene, or change the expression of an endogenous gene. One approach involves the use of gene correction where a faulty endogenous β globin gene is targeted and the mutant sequence replaced. One approach further involves the use of modification of a stem cell (*e.g.*, hematopoietic stem cell or RBC precursor), which stem cell can then be used to engraft into a patient, for treatment of a hemoglobinopathy.

[0017] In one example, described herein is a genetically modified cell or cell line, for example as compared to the wild-type genomic sequence of the same type of cell or cell line. In certain examples, the cells comprise genetically modified

RBC precursors (hematopoietic stem cells known as "HSCs"). The cell or cell line may be heterozygous or homozygous for the modification. The modifications may comprise insertions, deletions and/or combinations thereof. In certain examples, the HSCs are modified with an engineered nuclease and a donor nucleic acid such that a wild type gene (*e.g.*, globin gene) is inserted and expressed and/or an endogenous aberrant gene is corrected. In certain examples, the modification (*e.g.*, insertion) is at or near the nuclease(s) binding and/or cleavage site(s), including but not limited to, modifications to sequences within 1-300 (or any number of base pairs therebetween) base pairs upstream or downstream of the site(s) of cleavage and/or binding site; modifications within 1-100 base pairs (or any number of base pairs therebetween) of either side of the binding and/or cleavage site(s); modifications within 1 to 50 base pairs (or any number of base pairs therebetween) on either side of the binding and/or cleavage site(s); and/or modifications to one or more base pairs of the nuclease binding site and/or cleavage site. In certain examples, the modification is at or near (*e.g.*, 1-300 base pairs or any number of base pairs therebetween) SEQ ID NO:23 or 24. In other examples, the modification is 1-100 (or any number of base pairs therebetween) base pairs of SEQ ID NO:23 or 24. In certain examples, the modification is within SEQ ID NO:23 and/or SEQ ID NO:24, for example a modification of 1 or more base pairs in either SEQ ID NO:23 or 24. In some cases, the wild type gene sequence for insertion encodes a wild type β globin. In other cases, the endogenous aberrant gene is the β globin gene, for example one or more genomic modifications that correct at least one mutation in an endogenous aberrant human beta-hemoglobin (Hbb) gene. In some examples, the modification of the beta globin gene allows proper splicing of transcribed RNAs. In other examples, the modification of the beta globin gene corrects the mutation in the sickle allele such that codon GTG that codes for the incorrect valine amino acid at the sixth position in the polypeptide sequence is altered to GAG, encoding glutamic acid as is found in the wild type polypeptide. Partially or fully differentiated cells descended from the genetically modified stem cells as described herein are also provided (*e.g.*, RBCs or RBC precursor cells). Compositions such as pharmaceutical compositions comprising the genetically modified cells as described herein are also provided.

[0018] In another example, described herein is a zinc-finger protein (ZFP) that binds to target site in a region of interest (*e.g.*, a β globin gene) in a genome, wherein the ZFP comprises one or more engineered zinc-finger binding domains. In one example, the ZFP is a zinc-finger nuclease (ZFN) that cleaves a target genomic region of interest, wherein the ZFN comprises one or more engineered zinc-finger binding domains and a nuclease cleavage domain or cleavage half-domain. Cleavage domains and cleavage half domains can be obtained, for example, from various restriction endonucleases and/or homing endonucleases. In one example, the cleavage half-domains are derived from a Type IIS restriction endonuclease (*e.g.*, *Fok* I). In certain examples, the zinc finger domain recognizes a target site in a globin or safe harbor gene. In certain examples, the zinc finger domain comprises 5 or 6 zinc finger domains and recognizes a target site in a globin gene (*e.g.*, a zinc finger protein having 5 or 6 fingers with the recognition helix regions shown in Table 1).

[0019] In another example, described herein is a TALE protein (Transcription activator like) that binds to target site in a region of interest (*e.g.*, β globin gene) in a genome, wherein the TALE comprises one or more engineered TALE binding domains. In one example, the TALE is a nuclease (TALEN) that cleaves a target genomic region of interest, wherein the TALEN comprises one or more engineered TALE DNA binding domains and a nuclease cleavage domain or cleavage half-domain. Cleavage domains and cleavage half domains can be obtained, for example, from various restriction endonucleases and/or homing endonucleases. In one example, the cleavage half-domains are derived from a Type IIS restriction endonuclease (*e.g.*, *Fok* I).

[0020] In another example, described herein is a CRISPR/Cas or a TtAgo system that binds to target site in a region of interest (*e.g.*, a disease associated gene) in a genome, wherein the CRISPR/Cas system comprises a CRIPSR/Cas nuclease and an engineered crRNA/tracrRNA (or single guide RNA). In certain examples, the CRISPR/Cas or the TtAgo system recognizes a target in a globin gene.

[0021] In some examples, the nuclease which facilitates genomic engineering of the cell is delivered as a peptide, while in others it is delivered as a nucleic acid encoding the nuclease. In some examples, more than one nuclease is used and may be delivered in nucleic acid form, protein form, or combinations thereof. In some preferred examples, the nucleic acid(s) encoding the nuclease is

(are) an mRNA, and in some instances, the mRNA is protected. In further preferred examples, the mRNA may comprise an ARCA cap and/or may comprise a mixture of modified and unmodified nucleotides. The nuclease may comprise a zinc finger nuclease (ZFN), a TALE-nuclease (TALEN), TtAgo or a CRISPR/Cas nuclease system or a combination thereof. In a preferred example, the nucleic acid encoding the nuclease(s) is delivered via electroporation. In another example, described herein is a method for increasing targeted integration (*e.g.*, via HDR) following nuclease-mediated cleavage in a cell. In certain examples, the methods comprise the steps of: (i) introducing one or more nucleases (and/or mRNAs or expression constructs that express the nuclease(s) and one or more single guide RNA if needed) along with one or more donor molecules into a host cell and (ii) introducing one or more factors that affect and/or increase stem cell expansion without loss of stemness or differentiation into the cell and/or the culture media containing the cell before, during and/or after introduction of the nuclease(s). The donor may be delivered prior to, after, or along with the nucleic acid encoding the nuclease(s).

[0022] The donor can be delivered by viral and/or non-viral gene transfer methods. In other examples, the donor is delivered to the cell via an adeno associated virus (AAV). In some instances, the AAV comprises LTRs that are of a heterologous serotype in comparison with the capsid serotype. In other examples, the donor is delivered to the cell via a lentivirus. In some instances, the lentivirus is an integrase defective lentivirus (IDLV).

[0023] In certain examples, the donor nucleic acid is integrated via non-homology dependent methods (*e.g.*, NHEJ). As noted above, upon *in vivo* cleavage the donor sequences can be integrated in a targeted manner into the genome of a cell at the location of a DSB. The donor sequence can include one or more of the same target sites for one or more of the nucleases used to create the DSB. Thus, the donor sequence may be cleaved by one or more of the same nucleases used to cleave the endogenous gene into which integration is desired. In certain examples, the donor sequence includes different nuclease target sites from the nucleases used to induce the DSB. DSBs in the genome of the target cell may be created by any mechanism. In certain examples, the DSB is created by one or more zinc-finger nucleases (ZFNs), fusion proteins comprising a zinc finger binding domain, which is engineered

to bind a sequence within the region of interest, and a cleavage domain or a cleavage half-domain. In other examples, the DSB is created by one or more TALE DNA-binding domains (naturally occurring or non-naturally occurring) fused to a nuclease domain (TALEN). In yet further examples, the DSB is created using a CRISPR/Cas nuclease system where an engineered single guide RNA or its functional equivalent is used to guide the nuclease to a targeted site in a genome. In yet further examples, the DSB is created using a TtAgo system.

[0024] In another example, the disclosure provides kits that are useful for increasing gene disruption and/or targeted integration (*e.g.* gene correction) following nuclease-mediated cleavage of a cell's genome (*e.g.* ZFNs, TAL-effector domain nuclease fusion proteins, a TtAgo system, or engineered homing endonucleases or engineered guide RNAs with the CRISPR/Cas system). The kits typically include one or more nucleases that bind to a target site, one or more factors that affect stem cell expansion and/or differentiation and instructions for introducing the nucleases and stem cell-affecting factors into the cells such that nuclease-mediated gene disruption and/or targeted integration is enhanced. Optionally, cells containing the target site(s) of the nuclease may also be included in the kits described herein. In certain examples, the kits comprise at least one construct with the target gene and a known nuclease capable of cleaving within the target gene. Such kits are useful for optimization of cleavage conditions in a variety of varying host cell types. Other kits contemplated by the disclosure may include a known nuclease capable of cleaving within a known target locus within a genome, and may additionally comprise a donor nucleic acid. In some examples, the donor DNA may encode a polypeptide, a regulatory region or a structural nucleic acid. In some examples, the polypeptide is a reporter gene (*e.g.* GFP). Such kits are useful for optimization of conditions for donor integration or for the construction of specifically modified cells, cell lines, and animals containing gene disruptions or targeted insertions.

[0025] In other examples, methods of administering a genetically modified stem cell as described herein to a subject are described. The genetically modified blood cell precursors ("HSC/PC") as described herein are typically given in a bone marrow transplant and the HSC/PC differentiate and mature *in vivo*. In some examples, the HSC/PC are isolated following G-CSF-induced mobilization, and in others, the cells are isolated from human bone marrow or umbilical cords. In some examples, the

HSC/PC are edited by treatment with a nuclease designed to knock out a specific gene or regulatory sequence. In other examples, the HSC/PC are modified with an engineered nuclease and a donor nucleic acid such that a wild type gene or other gene of interest is inserted and expressed and/or an endogenous aberrant gene is corrected. In some examples, the modified HSCs/PC are administered to the patient following mild myeloablative pre-conditioning. In other examples, the HSC/PC are administered after full myeloablation such that following engraftment, 100% of the hematopoietic cells are derived from the modified HSC/PC. Furthermore, the cell may be arrested in the G2 phase of the cell cycle.

[0026] In some examples, the transgenic HSC/PC cell and/or animal includes a transgene that encodes a human gene. In some instances, the transgenic animal comprises a knock out at the endogenous locus corresponding to exogenous transgene, thereby allowing the development of an *in vivo* system where the human protein may be studied in isolation. Such transgenic models may be used for screening purposes to identify small molecules or large biomolecules or other entities which may interact with or modify the human protein of interest. In some examples, the transgene is integrated into the selected locus (*e.g.*, safe-harbor) into a stem cell (*e.g.*, an embryonic stem cell, an induced pluripotent stem cell, a hematopoietic stem cell, etc.) or animal embryo obtained by any of the methods described herein, and then the embryo is implanted such that a live animal is born. The animal is then raised to sexual maturity and allowed to produce offspring wherein at least some of the offspring comprise edited endogenous gene sequence or the integrated transgene.

[0027] These and other aspects will be readily apparent to the skilled artisan in light of disclosure as a whole.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028] **Figures 1A through 1E** show cleavage and correction at the beta-globin locus in CD34+ cells using an IDLV donor. **Figure 1A** (SEQ ID NO:3) shows a portion of exon I DNA sequence of beta-globin showing the ZFN target sites (underlined) atop the start codon (bold) and the sickle mutation (bold, italic). **Figure 1B** is a gel showing targeted cleavage at the beta-globin locus in human umbilical cord blood CD34+ cells. Cells were analyzed three days after electroporation with *in vitro* transcribed mRNA encoding the ZFNs. 'Mock' represents untreated CD34+

CLAIMS

What is claimed is:

1. A genetically modified cell comprising a genomic modification within or between SEQ ID NO:23 or SEQ ID NO:24 of an endogenous human beta-hemoglobin (Hbb) gene following double-stranded break by a pair of zinc finger nucleases, the pair comprising the zinc finger proteins designated 47773 and 47817, wherein the genomic modification comprises an insertion and/or deletion.

2. The genetically modified cell of claim 1, wherein the cell is a stem cell.

3. The genetically modified cell of claim 2, wherein the stem cell is a hematopoietic stem cell.

4. The genetically modified cell of claim 3, wherein the hematopoietic stem cell is a CD34+ cell.

5. A genetically modified differentiated cell descended from the stem cell of any of claims 1 to 4.

6. The genetically modified cell of claim 5, wherein the cell is a red blood cell (RBC).

7. The genetically modified cell of any of claims 1 to 6, wherein the insertion comprises integration of a donor polynucleotide encoding a transgene.

8. The genetically modified cell of claim 7, wherein the donor polynucleotide corrects a sickle cell mutation.

9. A pharmaceutical composition comprising the genetically modified cell of any of claims 1 to 8.

10. A composition comprising a pair of zinc finger nucleases, each zinc finger nuclease comprising a zinc finger protein and a cleavage domain or cleavage half-domain, wherein the zinc finger protein comprises 5 or 6 zinc finger domains comprising a recognition helix region, wherein the pair comprises the zinc finger proteins designated 33488 and 33501 or 47773 and 47817.

11. The composition of claim 10, wherein the cleavage half-domain is a wild-type or engineered cleavage half-domain.

12. The composition of claim 10 or 11, wherein the composition comprises one or more polynucleotides encoding the pair of zinc finger nucleases.

13. An isolated cell comprising the composition of any of claims 10 to 12.

14. The cell of claim 13, wherein the cell is selected from the group consisting of a red blood cell (RBC) or a precursor cell (e.g., CD4+ hematopoietic stem cell).

15. A kit comprising a composition according to any of claims 10 to 12 or a cell according to claim 13 or 14.

16. An *in vitro* method of altering Hbb expression in a cell, the method comprising:

introducing, into the cell, one or more compositions according to claim 12, under conditions such that the pair of zinc finger nucleases is expressed and expression of the Hbb gene is altered.

17. The method of claim 16, wherein expression of the Hbb gene is increased.

18. The method of claim 16 or claim 17, further comprising integrating a donor sequence into the genome of the cell.

19. The method of any of claims 16 to 18, wherein the cell is selected from the group consisting of a red blood cell (RBC) precursor cell and a hematopoietic stem cell.

20. The method of any of claims 16 to 19, wherein the donor sequence comprises an oligonucleotide that corrects a mutation that alters the splice donor site for mRNA processing of the Hbb gene.

21. An *in vitro* method of producing a genetically modified cell comprising a genomic modification within an endogenous Hbb gene, the method comprising the steps of:

a) contacting a cell with a composition according to claim 12,

b) subjecting the cell to conditions conducive to expressing the pair of zinc finger nuclease; and

c) modifying the endogenous Hbb gene with the expressed fusion protein sufficient to produce the genetically modified cell.

22. The method of claim 21, wherein the method further comprises stimulating the cells with at least one cytokine.

23. Use of the isolated cell of claim 13 or 14 in the manufacture of a medicament for treating a patient with sickle cell anemia.