257). The enhancer region was found within intron 2 of the BCL11A gene, and three areas of DNAseI hypersensitivity (often indicative of a chromatin state that is associated with regulatory potential) in intron 2 were identified. These three areas were identified as "+62", "+58" and "+55" in accordance with the distance in kilobases from the transcription start site of BCL11A. These enhancer regions are roughly 350 (+55); 550 (+58); and 350 (+62) nucleotides in length (Bauer 2013, *ibid*).

[0015] When developing a nuclease for use in therapeutic treatments of humans, it is essential that the nuclease have the utmost safety characteristics. Specifically, the nucleases must have very low levels of off-target cleavage. Significant numbers of double strand cuts in locations other than the user-specified target can lead to repression of off-target genes, and in rare instances, the occurrence of chromosomal translocations (see Hoban and Bauer (2016) *Blood*, 127(21):2525-2535 and Tsang *et al.* (2017) *Nature Methods*, in press). Improvements in specificity can be achieved by eliminating non-specific interactions between the engineered nuclease and the genomic DNA (see U.S. Provisional Patent Application Nos. 62/378,978 and 62/443,981).

[0016] Thus, there remains a need for additional highly specific methods and compositions for the alteration of BCL11A gene expression, for example to treat hemoglobinopathies such as sickle cell disease and beta thalassemia.

SUMMARY

[0017] The present invention describes highly specific compositions and methods for use in gene therapy and genome engineering. Specifically, the methods and compositions described relate to inactivating (*e.g.*, by completely or partially abolishing its expression) a BCL11A gene, for example, a gene that acts as regulator of one or more additional genes. In particular, the invention describes methods and compositions for interfering with enhancer function in a BCL11A gene to diminish or knock out its activity in specific cell lineages (*e.g.*, erythroid). Additionally, the invention provides methods and compositions for interfering with BCL11A enhancer functions wherein the enhancer sequences are not located within the coding sequences of the BCL11A gene, and wherein the reagents provided exhibit highly specific activity. The resulting down-regulation of the BCL11A gene in these circumstances results in increased expression of gamma globin, and the number of off-target cleavage events is reduced.

[0018] In some examples, the invention comprises a non-naturally occurring zinc finger protein comprising a zinc finger protein (ZFP) comprising 4, 5 or 6 fingers, each finger comprising a recognition helix region that recognizes a DNA target subsite wherein the recognition helix regions comprise the sequences in the order shown in a single row of Table 1. Within each zinc finger, the 7 amino acid recognition helix region is numbered -1 to +6 within the zinc finger backbone (of approximately 30 residues, including zinc coordinating residues). In certain examples, 1, 2, 3 or more of the component zinc fingers of the zinc finger proteins described herein further comprise mutations to one or more residues outside the recognition helix region, including but not limited to mutations to amino acids at position -5, position -14 or at both positions -5 and -14 (numbering continuing from the -1 to +6 numbering used for the recognition helix region) are mutated. *See, e.g.,* Qm4 and Qm14 mutations described in U.S. Provisional Patent Applications 62/378,978 and 62/443,981. The component zinc fingers of the zinc finger protein can be linked by any linkers, for example as described in U.S. Patent No. 8,772,453. In certain examples, the ZFP comprises the recognition helixes as shown in Table 1 for the proteins designated as follows: 63014 (which binds to the target site shown in SEQ ID NO:1) and 65722 (which binds to the target site shown in SEQ ID NO:2).

[0019] In certain examples, the zinc finger proteins as described herein are fused to a functional domain (*e.g.,* transcriptional activation domain, transcriptional repression domain, cleavage domain (to form a zinc finger nuclease), etc.). Any linker may be used to operably link the cleavage domain and the zinc finger protein, including but not limited to linkers as described in U.S. Patent Nos. 9,394,531 and 9,567,609. Furthermore, when a FokI cleavage domain is used, further mutations in the catalytic domain, dimerization domain, to phosphate contact residues (not in the dimerization or catalytic domain), and combinations of mutations in any one of the catalytic domain, dimerization domain and to phosphate contact residues may be present, including but not limited to ELD or KKR mutations to the dimerization domain, mutations to residues 525 (K to S) of the FokI domain, and combinations of ELD or KKR mutations to the dimerization domain and mutations to residues 525 (K to S) of the FokI domain, numbered relative to wild-type. *See,* U.S. Patent Nos. 7,888,121; 7,914,796; 8,034,598; 8,623,618 and U.S. Patent Publication No. 2011/0201055 and U.S. Provisional Patent Application Nos. 62/378,978 and 62/443,981.

[0020] In certain examples, zinc finger nucleases (ZFNs) may be used in dimerizing pairs to cleave at or near one or both of the target sites for the ZFNs of the pair, for example, the "left partner" of Table 1 (*e.g.*, 63014) can form a dimer with the "right partner" of Table 1 (*e.g.*, 65722) to cleave BCL11A enhancer sequences. In certain examples, the pair of ZFNs comprises the following amino acid sequences: 63014:

MDYKDHDGDYKDHDIDYKDDDDKMAPKKKRKVGIHGVPAAMAERPFQCRI
CMQNFSDQSNLRAHIRTHTGEKPFACDICGRKFARNFSLTMHTKIHTGSQKPF
QCRICMQNFSSTGNLTNHIRTHTGEKPFACDICGRKFATSGSLTRHTKIHTHPR
APIPKPFQCRICMQNFSDQSNLRAHIRTHTGEKPFACDICGRKFAAQCCLFHHT
KIH– Linker -
ELEEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEFFMKVYGYRGK
HLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMERYVEENQ
TRDKHLNPNEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNHITNCNGA
VLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINFRS (SEQ ID NO:3); and
MDYKDHDGDYKDHDIDYKDDDDKMAPKKKRKVGIHGVPAAMAERPFQCRI
CMQKFARNDHRTTHTKIHTGEKPFQCRICMQNFSQKAHLIRHIRTHTGEKPFA
CDICGRKFAQKGTLGEHTKIHTGSQKPFQCRICMQNFSRGRDLSRHIRTHTGE
KPFACDICGRKFARRDNLHSHTKIH– Linker-
ELEEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEFFMKVYGYRGK
HLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVKENQ
TRNKHINPNEWWKVYPSSVTEFKFLFVSGHFSGNYKAQLTRLNRKTNCNGA
VLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINF (SEQ ID NO:4), wherein the Linker sequence can be any linker sequence known in the art, for example as described in U.S. Patent Nos. 9,394,531 and 9,567,609. In certain examples, the linker for 63014 comprises or consists of the L7c5 linker (LRGSISRARPLNPHP (SEQ ID NO:5)) and the linker used in 65722 comprises or consists of the L0 linker (LRGSQLVKS (SEQ ID NO:6), see U.S. Patent No. 9,567,609). The FokI cleavage domain sequence C-terminal to the Linker of the sequences shown above may also comprise alternative FokI domains operably linked to the zinc finger protein. In certain examples, the FokI cleavage domain may include alternate or additional mutations to the catalytic domain, the dimerization domain, phosphate contact residues, and combinations of mutations to any one of the catalytic domain, dimerization domain and phosphate contact residues.

[0021] In another example, the invention comprises delivery of at least one nuclease (*e.g.*, a nuclease that binds to a BCL11A enhancer sequence) to a human stem cell or precursor cell (HSC/PC) for the purpose of genome engineering. In certain examples, the nuclease comprises a zinc finger protein (ZFP) comprising 4, 5 or 6 fingers, each finger comprising a recognition helix region that recognizes a target subsite wherein the recognition helix regions comprise the sequences in the order shown in a single row of Table 1. In other examples, the ZFN nuclease comprises the pair of nucleases designated 63014/65722. The nuclease(s) as described herein may further comprise a linker (*e.g.*, between the DNA-binding domain and the cleavage domain), for example a linker as shown in 9,567,609, including but not limited to LRGSISRARPLNPHP (SEQ ID NO:5) or (LRGSQLVKS (SEQ ID NO:6)).

[0022] In some examples, the nuclease is delivered as a peptide, while in others it is delivered as a nucleic acid encoding the at least one nuclease. In some examples, more than one nuclease is used. In some preferred examples, the nucleic acid encoding the nuclease is an mRNA, and in some instances, the mRNA is protected. In some examples, the mRNA may be chemically modified (*See e.g.* Kormann *et al.* (2011) *Nature Biotechnology* 29(2):154-157). In other examples, the mRNA may comprise an ARCA cap (see U.S. Patent Nos. 7,074,596 and 8,153,773). In further examples, the mRNA may comprise a mixture of unmodified and modified nucleotides (see U.S. Patent Publication No. 2012/0195936). In a preferred example, the nucleic acid encoding the nuclease(s) is delivered to the HSC/PC via electroporation. In some examples, the nuclease cleaves at or near the binding site of a transcription factor. In some examples, the transcription factor is GATA-1.

[0023] In other examples, the invention comprises a cell or cell line in which an endogenous BCL11A enhancer sequence is genetically modified by a nuclease as described herein (*e.g.*, shown in Table 1), for example as compared to the wild-type sequence of the cell. The genetic modification to the BCL11A enhancer results in modification of globin (beta and gamma) gene expression. Nuclease-modified cells or cell lines as described herein are distinguishable in structure, function, and combinations of both structure and function from wild-type. The genetically modified cell or cell lines may be heterozygous or homozygous for the modification. The modifications may comprise insertions (*e.g.*, transgene insertion) deletions, and combinations of insertions and deletions; such insertions, deletions, and combinations of insertions and deletions are commonly referred to as "indels". In some preferred

examples, indels result in the destruction of a transcription factor binding site. In certain examples, the modification is at or near the nuclease(s) binding site(s), cleavage site(s), and combinations of binding and cleavage sites, for example, within 1-300 (or any value therebetween) base pairs upstream or downstream of the site(s) of cleavage, more preferably within 1-100 base pairs (or any value therebetween) of either side of the binding site(s), cleavage site(s), and combinations of binding and cleavage sites shown in Table 1, even more preferably within 1 to 50 base pairs (or any value therebetween) on either side of the binding site(s), cleavage site(s), and combinations of binding and cleavage sites. In certain examples, the genetic modification of the BCL11A enhancer sequence is within and/or between sequences shown in Table 1 (target sites). The modification may also include modifications to one or more nucleotides in the cleavage sites. The modification may also include modifications to one or more nucleotides in the binding sites. The modification may further include modifications to one or more nucleotides in the cleavage sites, and in one or more of the binding sites. In certain examples, one or more of the nuclease target site(s) is(are) not modified. In other examples, at least one of the target sites for the nuclease(s) is(are) modified. In certain examples, the modification is at or near the "+58" region of the BCL11A enhancer, for example, at or near a nuclease binding site shown in any of SEQ ID NO:1 and SEQ ID NO:2. Any cell or cell line may be modified by the nucleases as described herein, for example a stem cell (hematopoietic stem cell such as a CD34+ hematopoietic stem cell) or red blood cell (RBC) precursor cell.

[0024] Also described are cells or cell lines obtained following modification by a nuclease as described herein, for example cells or cell lines descended from a nuclease-modified cell or cell line as described herein. Partially or fully differentiated cells descended from the modified stem cells as described herein are also provided (*e.g.*, RBCs or RBC precursor cells). The cells descended from the nuclease-modified cells may be propagated, differentiated, and combinations of both propagated and differentiated *in vitro* (culture) or may differentiate within a live subject, for example following *ex vivo* administration of a nuclease-modified stem cell. Any of the genetically modified cells or cell lines disclosed herein may show increased expression of gamma globin. Compositions such as pharmaceutical compositions comprising the genetically modified cells as described herein are also provided.

[0025] In other examples, the invention comprises delivery of a donor nucleic acid to a target cell to provide a genetically modified cell in which the donor is integrated into the cell. The donor may be delivered prior to, after, or along with the nucleic acid encoding the nuclease(s) of Table 1. The donor nucleic acid may comprise an exogenous sequence (transgene) to be integrated into the genome of the cell, for example, an endogenous locus. In some examples, the donor may comprise a full-length gene or fragment thereof flanked by regions of homology with the targeted cleavage site. In some examples, the donor lacks homologous regions and is integrated into a target locus through homology independent mechanism (*i.e.* NHEJ). The donor may comprise any nucleic acid sequence, for example a nucleic acid that, when used as a substrate for homology-directed repair of the nuclease-induced double-strand break, leads to a donor-specified deletion to be generated at the endogenous chromosomal locus (*e.g.*, BCL11A enhancer region) or, alternatively (or in addition to), novel allelic forms of (*e.g.*, point mutations that ablate a transcription factor binding site) the endogenous locus to be created. In some examples, the donor nucleic acid is an oligonucleotide wherein integration leads to a gene correction event, or a targeted deletion.

[0026] In other examples, the nuclease, donor, and combinations of both the nuclease and donor is(are) delivered by viral, non-viral, and combinations of viral and non-viral gene transfer methods. In preferred 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.

[0027] In some examples, deletions comprising regions within the DNAseI hypersensitive regions of the enhancer (*e.g.*, the +58 region of the BCL11A enhancer) are made using one or more nucleases as shown in Table 1. These deletions can comprise from about 1 nucleotide to about 551 nucleotides. Thus, the deletions can comprise, 1, 5, 10, 15, 20, 25, 30, 40, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550 nucleotides, or any value therebetween. In some examples, the deletions comprise binding regions for one or more transcription factors. In some preferred examples, the deletions comprise a GATA-1 binding site, or the binding site for GATA-1 in combination with other factors.

[0028] In some examples, the DNA binding domains of Table 1 are fused to a functional domain. Some examples include fusion of the DNA binding domains with domains capable of regulating the expression of a gene. In some examples, the fusion

proteins comprise the DNA binding domain of Table 1 fused to a gene expression modulatory domain where the modulator represses gene expression.

[0029] In some examples, the HSC/PC cells are contacted with the nucleases, the DNA binding proteins, and combinations of the nucleases and DNA binding proteins of the invention (*i.e.,* ZFPs as shown in Table 1). In some examples, the nucleases, the DNA binding proteins, and combinations of the nucleases and DNA binding proteins are delivered as nucleic acids and in other examples, they are delivered as proteins. In some examples, the nucleic acids are mRNAs encoding the, the DNA binding proteins, and combinations of the nucleases and DNA binding proteins, and in further examples, the mRNAs may be protected. In some examples, the mRNA may be chemically modified, may comprise an ARCA cap, a mixture of unmodified and modified nucleotides, and combinations of an ARCA cap and a mixture of unmodified and modified nucleotides. Cells or cell lines descended from these cells are also provided, including partially or fully differentiated cells.

[0030] In some examples, the HSC/PC are contacted with the nucleases, the DNA binding proteins, and combinations of the nucleases and DNA binding proteins of the invention *ex vivo*, following apheresis of the HSC/PC from a subject, or purification from harvested bone marrow. In some examples, the nucleases described herein cause modifications within the BCL11A enhancer regions, for example resulting a genetically modified cell that is structurally, functionally, and combinations of structurally and functionally distinct from wild-type cells, other modified (*e.g.,* nuclease-modified) cells, and combinations of wild-type and other modified cells. In further examples, the HSC/PC containing the BCL11A enhancer region modifications are introduced back into the subject. In some instances, the HSC/PC containing the BCL11A enhancer region modifications are expanded prior to introduction. In other examples, the genetically modified HSC/PCs are given to the subject in a bone marrow transplant wherein the HSC/PC engraft, differentiate and mature *in vivo*. In some examples, the HSC/PC are isolated from the subject following G-CSF-induced mobilization, plerixafor-induced mobilization, and combinations of G-CSF- and plerixafor-induced mobilization, and in others, the cells are isolated from human bone marrow or human umbilical cords. In some examples, the subject is treated to a mild myeloablative procedure prior to introduction of the graft comprising the modified HSC/PC, while in other examples, the subject is treated with a vigorous myeloablative conditioning regimen. In some examples, the methods and

compositions of the invention are used to treat or prevent a hemoglobinopathy. In some examples, the hemoglobinopathy is a thalassemia. In some examples, the hemoglobinopathy is a beta thalassemia, while in other examples, the hemoglobinopathy is sickle cell disease.

[0031] In some examples, the HSC/PC are further contacted with a donor molecule. In some examples, the donor molecule is delivered by a viral vector. The donor molecule may comprise one or more sequences encoding a functional polypeptide (*e.g.,* a cDNA or fragment thereof), with or without a promoter. Additional sequences (coding or non-coding sequences) may be included when a donor molecule is used for inactivation, including but not limited to, sequences encoding a 2A peptide, SA site, IRES, *etc.*

[0032] In one example, the methods and compositions of the invention comprise methods for contacting the HSC/PC *in vivo*. The nucleases, DNA binding proteins, or combination of nucleases and DNA binding proteins are delivered to HSC/PC *in situ* by methods known in the art. In some examples, the nucleases and/or DNA binding proteins of the invention comprise a viral particle that is administered to the subject in need, while in others, the nucleases, DNA binding proteins, or combination of nucleases and DNA binding proteins comprise a nanoparticle (*e.g.* liposome). In some examples, the viral particles, nanoparticles, or combination of viral particles and nanoparticles are delivered to the organ (*e.g.* bone marrow) wherein the HSC/PC reside.

[0033] In another example, described herein are methods of integrating a donor nucleic acid into the genome of a cell via homology-independent mechanisms. The methods comprise creating a double-stranded break (DSB) in the genome of a cell and cleaving the donor molecule using a nuclease as described herein, such that the donor nucleic acid is integrated at the site of the DSB. 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 that is engineered to bind a sequence within the region of interest, and a cleavage domain or a cleavage half-domain.

[0034] In one example, the donor may encode a regulatory protein of interest (*e.g.* ZFP TFs, TALE TFs or a CRISPR/Cas TF) that binds to, modulates expression of, or both binds to and modulates expression of a gene of interest. In one example, the regulatory proteins bind to a DNA sequence and prevents binding of other regulatory factors. In another example, the binding of the regulatory protein may modulate (*i.e.* induce or repress) expression of a target DNA.

[0035] In some examples, the transgenic HSC/PC cell, transgenic animal, or combination of transgenic HSC/PC cell and animal includes a transgene that encodes a human gene. In some instances, the transgenic animal comprises a knock out at the endogenous locus, and replacement of the endogenous gene with its human counterpart, 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.

[0036] In another example, provided herein is a method of altering gene expression (*e.g.*, BCL11A, a globin gene, and combinations of BCL11A and a globin gene) in a cell, the method comprising: introducing, into the cell, one or more nucleases as described herein (shown in Table 1), under conditions such that the one or more proteins are expressed and expression of the gene is altered. In certain examples, expression of a globin gene (*e.g.*, gamma globin or beta globin) is altered (*e.g.*, increased). Any of the methods described herein may further comprise integrating a donor sequence (*e.g.*, transgene or fragment thereof under the control of an exogenous or endogenous promoter) into the genome of the cell, for example integrating a donor at or near the site of nuclease cleavage in the BCL11A gene. The

donor sequence is introduced to the cell using a viral vector, as an oligonucleotide, on a plasmid, and combinations of one or more methods selected from using a viral vector, as an oligonucleotide, or on a plasmid. The cell in which gene expression is altered may be, for example, a red blood cell (RBC) precursor cell, a hematopoietic stem cell (*e.g.*, CD34+ cell), and combinations of RBC precursor cell and a hematopoietic stem cell.

[0037] In other examples, provided herein is a method of producing a genetically modified cell comprising a genomic modification within an endogenous BCL11A enhancer sequence (a modification to the nucleotide sequence of the BCL11A enhancer sequence), the method comprising the steps of: a) contacting a cell with a polynucleotide (*e.g.* DNA or mRNA) encoding a zinc finger nuclease comprising 4, 5, or 6 zinc finger domains in which each of the zinc finger domains comprises a recognition helix region in the order shown in a single row of Table 1; b) subjecting the cell to conditions conducive to expressing the zinc finger protein from the polynucleotide; and c) modifying the endogenous BCL11A enhancer sequence with the expressed zinc finger protein sufficient to produce the genetically modified cell. In certain examples, the cells are stimulated with at least one cytokine (*e.g.*, prior to step (a)). The polynucleotide may be contacted with the cell using any suitable method, including but not limited, via transfection, using a non-viral vector, using a viral vector, by chemical means or by exposure to an electric field (*e.g.*, electroporation).

[0038] Cells comprising one or a combination of the genomic modifications described herein are also provided, including cells descended from the cells produced by the methods described herein.

[0039] Also provided is a method of treating a patient in need of an increase in globin gene expression, the method comprising administering to the patient the pharmaceutical preparation, wherein the pharmaceutical preparation comprises genetically modified cells, proteins, polynucleotides, and combinations of one or more selected from genetically modified cells, proteins, and polynucleotides, as described herein in an amount sufficient to increase the globin gene expression in the patient. In certain examples, the patient is known to have, is suspected of having, or is at risk of developing a thalassemia or sickle cell disease.

[0040] A kit, comprising the nucleic acids, proteins, genetically modified cells, and combinations of one or more selected from the nucleic acids, proteins, and

genetically modified cells of the invention, is also provided. The kit may comprise nucleic acids encoding the nucleases, (*e.g.* RNA molecules or ZFN, TALEN or CRISPR/Cas system encoding genes contained in a suitable expression vector), aliquots of the nuclease proteins, donor molecules, suitable modifiers of stem cell self-renewal ("stemness"), cells, buffers, instructions (*e.g.*, for performing the methods of the invention) and the like, including various combinations of these kit components. The invention includes, but is not limited to, a genetically modified cell (*e.g.*, stem cell such as a hematopoietic (CD34+) stem cell or RBC precursor cell) comprising at least one genomic modification made by a nuclease (*e.g.*, as shown in a single row of Table 1), wherein the genomic modification is within an endogenous BCL11A enhancer sequence, and further wherein the genomic modification is selected from the group consisting of insertions, deletions and combinations thereof and comprises a modification at, near or between any of SEQ ID NO:1 and SEQ ID NO:2. In certain examples, the cell is a genetically modified differentiated cell descended from a stem cell as described herein (*e.g.*, a RBC descended from a hematopoietic stem cell or RBC precursor cell).

[0041] The nuclease may comprise at least one zinc finger nuclease (ZFN) (*e.g.*, as shown in Table 1), at least one TALEN, and combinations of at least one ZFN and at least one TALEN. The nuclease(s) may be introduced into the cell in protein form, as a polynucleotide encoding the nuclease(s), or as a combination of protein form and polynucleotide encoding the nuclease(s). In certain examples, the genomic modification comprises an insertion that comprises integration of a donor polynucleotide encoding a transgene. Also provided are pharmaceutical compositions comprising one or more of the genetically modified cells as described herein.

[0042] Also provided is a DNA-binding protein comprising a zinc finger protein comprising 4, 5 or 6 zinc finger domains comprising a recognition helix region, wherein the zinc finger proteins comprise the recognition helix regions in the order shown in a single row of Table 1. Also provided is a TALE protein comprising a plurality of repeats that bind to a sequence comprising a portion (*e.g.*, at least 4, 5, 6 or more) base pairs of the target sites shown in Table 1. A fusion protein comprising a zinc finger protein or TALE protein as described herein and a wild-type or engineered cleavage domain or cleavage half-domain is also provided as are polynucleotides encoding the proteins (ZFPs, TALEs, ZFNs, TALENs) as described herein. Cells (*e.g.*, isolated stem cells such as hematopoietic (CD34+) stem cells)

comprising one or more polynucleotides, proteins, and combinations of polynucleotides and proteins as described herein are also provided. Also provided are kits comprising one or more proteins, polynucleotides, cells, or combinations thereof as described herein.

[0043] A method of altering globin gene expression in a cell (*e.g.*, RBC precursor cell, hematopoietic stem cell and combinations of RBC precursor cell and hematopoietic stem cell) is also described, the method comprising: introducing, into the cell, one or more polynucleotides encoding one or more nucleases as described herein, under conditions such that the one or more proteins are expressed and expression of the globin gene (*e.g.*, gamma globin, beta globin, and combinations of gamma and beta globin) is altered (*e.g.*, increased). In certain examples, the methods further comprise integrating a donor sequence into the genome of the cell, for example using a viral vector, as an oligonucleotide or on a plasmid. The donor sequence may comprise a transgene under the control of an endogenous or exogenous promoter.

[0044] Also provided is a method of producing a genetically modified cell comprising a genomic modification within an endogenous BCL11A enhancer sequence (*e.g.*, target site as shown in Table 1), the method comprising the steps of: (a) contacting a cell with a polynucleotide encoding a fusion protein comprising a zinc finger nuclease comprising 4, 5, or 6 zinc finger domains in which each of the zinc finger domains comprises a recognition helix region in the order shown in a single row of Table 1; (b) subjecting the cell to conditions conducive to expressing the fusion protein from the polynucleotide; and (c) modifying the endogenous BCL11A enhancer sequence with the expressed fusion protein sufficient to produce the genetically modified cell. In certain examples, the method further comprises stimulating the cells with at least one cytokine. The polynucleotide(s) may be delivered inside the cell, for example using a non-viral delivery system, a viral delivery system, a delivery vehicle, and combinations selected from a non-viral delivery system, a viral delivery system, and a delivery vehicle and may comprise subjecting the cells to an electric field or employing cell membrane disruption as a delivery mechanism (so called 'Squeeze Technology', see *e.g.* Sharei *et al.* (2015) *PLOS ONE* doi: 10.1371/journal/pone.0118803).

[0045] Methods of treating a patient in need of an increase in globin gene expression (*e.g.*, a patient is known to have, is suspected of having, or is at risk of

developing a hemoglobinopathy such as a thalassemia (*e.g.*, β-thalassemia) or sickle cell disease are also provided, the method comprising administering to the patient the pharmaceutical composition as described herein (*e.g.*, proteins, polynucleotides, cells or a combination selected from proteins, polynucleotides and cells) in an amount sufficient to increase the globin gene expression in the patient.

[0045a] In another aspect, the present invention refers to a zinc finger nuclease (ZFN) comprising left and right ZFNs, the left ZFN comprising the amino acid sequence as shown in SEQ ID NO:29 and the right ZFN comprising the amino acid sequence as shown in SEQ ID NO:31.

[0045b] In yet another aspect, the present invention refers to the zinc finger nuclease or the polynucleotide as described herein for use in therapy for modifying an endogenous BCL11A enhancer sequence in a cell.

[0045c] In yet another aspect, the present invention refers to use of the zinc finger nuclease or the polynucleotide as described herein in the manufacture of a medicament for the treatment of hemoglobinopathy.

[0045d] In still another aspect, the present invention refers to the cell as described herein for use in therapy for increasing globin production in a subject.

[0045e] In still another aspect, the present invention refers to use of the cell as described herein in the manufacture of a medicament for the treatment of hemoglobinopathy, said use comprises that the cell is to be administered to a subject to increase globin production.

[0046] These and other examples will be readily apparent to the skilled artisan in light of disclosure as a whole.

BRIEF DESCRIPTION OF THE DRAWINGS

[0047] **Figures 1A and 1B** are schematics depicting an overview of the oligonucleotide duplex integration site assay. Figure 1 shows the first step of the assay, in which cells are treated with ZFNs in the presence of supplemental oligonucleotide duplex DNA, which is captured into a fraction of the resulting cleavage events. Figure 1B shows the subsequence step in which cells are cultured for seven days, genomic DNA is isolated, and segments of the genome flanking donor integration sites are amplified via adaptor-mediated PCR using a primer to the integrated oligonucleotide duplex. Amplicons are then sequenced to reveal candidate cleavage sites.

[0048] **Figure 2** depicts the location of 455 potential off-target loci identified by the oligonucleotide duplex integration site assay using the 51857/51949 original or parent ZFN pair. The top 63 loci are highlighted in gray and were analyzed in follow up indel analyses. Each locus is identified by the chromosome and base number indicating the most likely location of cleavage, as well as an indication of the number of integrants detected at that locus.

[0049] **Figures 3A through 3C** depict exemplary phosphate contacting residues within the zinc finger scaffold. Figure 3A depicts a zinc finger interaction with a DNA molecule, and shows the location of the wild type arginine side chain and how it interacts with the phosphate backbone of the DNA molecule. Figure 3B (SEQ ID Nos:7 to 17) depicts exemplary ZFN sequences showing where this arginine is located in the primary sequence of each zinc finger (vertical row of 'R' residues

CLAIMS

1. A zinc finger nuclease (ZFN) comprising left and right ZFNs, the left ZFN comprising the amino acid sequence as shown in SEQ ID NO:29 and the right ZFN comprising the amino acid sequence as shown in SEQ ID NO:31.

2. A polynucleotide encoding one or more zinc finger nucleases according to claim 1.

3. The polynucleotide of claim 2, wherein the polynucleotide is mRNA.

4. The polynucleotide of claim 3 comprising SEQ ID NO:28 or SEQ ID NO:30.

5. A cell comprising the zinc finger nuclease of claim 1 or the polynucleotide of any of claims 2 to 4.

6. The cell of claim 5, wherein the cell is a stem cell or precursor cell.

7. The cell of claim 6, wherein the cell is a human cell.

8. The cell of any of claims 5 to 7, wherein the genome of the cell is modified by the zinc finger nuclease.

9. The cell of claim 8, wherein the genomic modification is selected from the group consisting of insertions, deletions and combinations thereof.

10. The cell of claim 8 or 9, wherein the genomic modification is within the +58 region of a BCL11A enhancer sequence.

11. A cell or cell line produced from the cell of any of claims 5 to 10.

12. A partially or fully differentiated cell descended from the cell or cell line of any of claims 5 to 11.

13. The cell of any of claims 5 to 12, wherein the cell exhibits increased expression of gamma and/or beta globin as compared to a cell without the genomic modification.

14. A pharmaceutical composition comprising the zinc finger nuclease according to claim 1, the polynucleotide of any of claims 2 to 4 or the cell of any of claims 5 to 13.

15. The zinc finger nuclease according to claim 1 or the polynucleotide of any of claims 2 to 4 for use in therapy for modifying an endogenous BCL11A enhancer sequence in a cell.

16. Use of the zinc finger nuclease according to claim 1 or the polynucleotide of any of claims 2 to 4 in the manufacture of a medicament for the treatment of hemoglobinopathy.

17. The use according to claim 16, wherein the zinc finger nuclease or the polynucleotide is to be administered to a cell, such that the endogenous BCL11A enhancer sequence is modified.

18. The zinc finger nuclease or the polynucleotide according to claim 15, or the use according to claim 16, further comprising introducing an exogenous sequence into the cell such that the exogenous sequence is inserted into the endogenous BCL11A enhancer sequence.

19. The use according to claim 16, wherein the modification comprises a deletion.

20. The cell of any of claims 5 to 13 for use in therapy for increasing globin production in a subject.

21. Use of the cell of any of claims 5 to 13 in the manufacture of a medicament for the treatment of hemoglobinopathy, said use comprises that the cell is to be administered to a subject to increase globin production.

22. The use according to claim 21, wherein the subject is a human and the cell is a human stem cell or human precursor cell.

23. The use according to claim 22, wherein the cell is infused into the patient and the cell engrafts, differentiates and matures in the subject.

24. The use according to any of claims 21 to 23, wherein the hemoglobinopathy is a beta-thalassemia or sickle cell disease.

25. A method of producing a genetically modified cell comprising a genomic modification within an endogenous BCL11A enhancer sequence, the method comprising the steps of:

a) contacting a cell with the polynucleotide of any of claims 2 to 4;

b) subjecting the cell to conditions conducive to expressing the fusion protein from the polynucleotide; and

c) modifying the endogenous BCL11A enhancer sequence with the expressed fusion protein sufficient to produce the genetically modified cell.

26. The method of claim 25, further comprising stimulating the cell with at least one cytokine.

27. A kit comprising the zinc finger nuclease of claim 1, the polynucleotide of any of claims 2 to 4 and/or the cell of any of claims 5 to 13.