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(54) Title: DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF CRISPR SYSTEMS AND COMPOSITIONS FOR GENOME EDITING AS TO HEMATOPOIETIC STEM CELLS (HSCS)

	Zinc Finger Nucleases	TALE Nucleases	CRISPR-Cas9
Recognition site	Typically 9 to 18 bp per ZFP. 18 to 36 bp per ZFN pair.	Typically 14 to 20 bp per TALE. 28 to 40 bp per TALEN.	20p (20bp guide sequence + 2bp PAM sequence); up to 44 bp for double nicking.
Specificity	Small number of positional mismatches tolerated.	Small number of positional mismatches tolerated.	Positional and multiple consecutive mismatches tolerated.
Targeting constraints	Difficult to target non-G-rich sequences.	5' targeted base must be a T.	Targeted sequence must precede a PAM.
Ease of engineering	Difficult, may require substantial protein engineering.	Moderate, requires complex molecular cloning methods.	Easily re-targeted using standard cloning procedures and oligo synthesis.
Immunogenicity	Likely low, as ZFNs are based on human transcription factor protein scaffold.	Unknown, protein derived from the bacteria <i>Xanthomonas</i> sp.	Unknown, protein derived from various bacterial species.
Ease of ex vivo delivery	Relatively easy through methods such as electroporation and lentiviral transduction.	Relatively easy through methods such as electroporation and lentiviral transduction.	Relatively easy through methods such as electroporation and lentiviral transduction.
Ease of in vivo delivery	Relatively easy due to small size of ZFN expression cassettes; allows use in a variety of viral vectors.	Difficult due to the large size of each TALEN and repetitive nature of DNA encoding TALENs, leading to unwanted recombination events.	Moderate. The commonly used Cas9 from <i>S. pyogenes</i> may be large enough to impose delivery problems, but other, smaller orthologous exist.
Ease of multiplexing	Low	Low	High

(57) Abstract: The invention provides for delivery, engineering and optimization of systems, methods, and compositions for manipulation of sequences and/or activities of target sequences. Provided are delivery systems and tissues or organ which are targeted as sites for delivery. Also provided are vectors and vector systems some of which encode one or more components of a CRISPR complex, as well as methods for the design and use of such vectors. Also provided are methods of directing CRISPR complex formation in eukaryotic cells to ensure enhanced specificity for target recognition and avoidance of toxicity and to edit or modify a target site in a genomic locus of interest to alter or improve the status of a disease or a condition.

Fig. 1



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DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF CRISPR SYSTEMS AND COMPOSITIONS FOR GENOME EDITING AS TO HEMATOPOIETIC STEM CELLS (HSCs)

RELATED APPLICATIONS AND INCORPORATION BY REFERENCE

[0001] This application claims priority from US application Serial No. 62/091,461, filed December 12, 2014. Reference is made to PCT/US2014/070127, filed December 12, 2014, which claims the benefit of US provisional patent applications 61/915,176; 61/915,192; 61/915,215; 61/915,107, 61/915,145; 61/915,148; and 61/915,153 each filed December 12, 2013. Reference is made to US patent application 14/705,719, filed May 6, 2015, which is a continuation of PCT/US2014/070057, filed December 12, 2014, and claims the benefit of US provisional patent applications 61/915,118, 61/915,148 and 61/915,215, each filed December 12, 2013, US provisional patent application 62/010,441, filed June 10, 2014, and US provisional patent application 62/054,490, filed September 24, 2014.

[0002] Reference is also made to US provisional patent application Serial Nos. 62/181,453, filed June 18, 2015, 62/207,312, filed August 19, 2015, 62/237,360, filed October 5, 2015, and 62/255,256, filed November 13, 2015, entitled CRISPR ENZYME MUTATIONS REDUCING OFF-TARGET EFFECTS.

[0003] The foregoing applications, and all documents cited therein or during their prosecution (“appln cited documents”) and all documents cited or referenced in the appln cited documents, and all documents cited or referenced herein (“herein cited documents”), and all documents cited or referenced in herein cited documents, together with any manufacturer’s instructions, descriptions, product specifications, and product sheets for any products mentioned herein or in any document incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention. More specifically, all referenced documents are incorporated by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

[0004] This invention was made with government support under Grant Nos. MH100706; MH110049 and DK097768 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

[0005] The present invention generally relates to the delivery, engineering, optimization and therapeutic applications of systems, methods, and compositions used for the control of gene expression involving sequence targeting, such as genome perturbation or gene-editing, that relate to Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and components thereof. The present invention relates to *in vitro*, *ex vivo* and/or *in vivo* systems, methods, and compositions for delivery of the CRISPR-Cas system(s) to achieve therapeutic benefits *via* genome editing in animals, including mammals.

BACKGROUND OF THE INVENTION

[0006] Recent advances in genome sequencing techniques and analysis methods have significantly accelerated the ability to catalog and map genetic factors associated with a diverse range of biological functions and diseases. Precise genome targeting technologies are needed to enable systematic reverse engineering of causal genetic variations by allowing selective perturbation of individual genetic elements, as well as to advance synthetic biology, biotechnological, and medical applications. Although genome-editing techniques such as designer zinc fingers (ZFN), transcription activator-like effectors (TALEs), or homing meganucleases are available for producing targeted genome perturbations, there remains a need for new genome engineering technologies that are affordable, easy to set up, scalable, and amenable to targeting multiple positions within the eukaryotic genome.

SUMMARY OF THE INVENTION

[0007] Despite valid therapeutic hypotheses and strong efforts in drug development, there have only been a limited number of successes using small molecules to treat diseases with strong genetic contributions. Thus, there exists a pressing need for alternative and robust systems for therapeutic strategies that are able to modify nucleic acids within disease-affected cells and tissues. Adding CRISPR-Cas system(s) to the repertoire of therapeutic genome engineering methods significantly simplifies the methodology and accelerates the ability to catalog and map genetic factors associated with a diverse range of biological functions and diseases, develop animal models for genetic diseases, and develop safe, effective therapeutic alternatives. To utilize the CRISPR-Cas system(s) effectively for genome editing without deleterious effects, it is critical to understand aspects of engineering, optimization and cell-type/tissue/organ specific delivery of

these genome engineering tools, which are aspects of the claimed invention. Aspects of this invention address this need and provide related advantages.

[0008] An exemplary CRISPR complex may comprise a CRISPR enzyme protein (*e.g.*, Cas9) complexed with a guide sequence hybridized to a target sequence within the target polynucleotide. The guide sequence is linked to a tracr mate sequence, which in turn hybridizes to a tracr sequence. Applicants have optimized components of the CRISPR-Cas genome engineering system, including using SaCas9 (Cas9 from *Staphylococcus aureus*). SaCas9 is preferred. Reference is made to PCT application PCT/US2014/070152, filed December 12, 2014, entitled, "ENGINEERING OF SYSTEMS, METHODS AND OPTIMIZED GUIDE COMPOSITIONS WITH NEW ARCHITECTURES FOR SEQUENCE MANIPULATION" (the "New Architecture PCT"), published as WO/2015/089473, incorporated herein by reference, which involves SaCas9. For instance, the New Architecture PCT involves provides a composition which is a non-naturally occurring or engineered CRISPR-Cas system dual guide RNA molecule (dgRNA) capable of effecting the manipulation of a target nucleic acid within a prokaryotic or eukaryotic cell when in complex within the cell with a CRISPR enzyme comprising a *Staphylococcus aureus* Cas9 enzyme (SaCas9); the dgRNA comprising: I. a chimeric RNA molecule comprising, in a tandem arrangement: a) a guide sequence, which is capable of hybridizing to a sequence of a target nucleic acid to be manipulated; and b) a tracr mate sequence, comprising a region of sense sequence; and II. a tracr RNA molecule, comprising a region of antisense sequence which is capable of hybridizing with the region of sense sequence of the tracr mate sequence; wherein the guide sequence comprises a length of 21 or more nucleotides. The New Architecture PCT also involves a non-naturally occurring or engineered CRISPR-Cas system chimeric single guide RNA molecule (sgRNA) capable of effecting the manipulation of a target nucleic acid within a prokaryotic or eukaryotic cell when in complex within the cell with a CRISPR enzyme comprising a CRISPR enzyme, or a non-naturally occurring or engineered composition comprising a CRISPR-Cas system comprising said sgRNA, the sgRNA comprising a guide sequence capable of hybridizing to a target sequence in a locus, *e.g.*, genomic locus of interest in a cell wherein architecture of the sgRNA is modified. The New Architecture PCT also involves a non-naturally occurring or engineered CRISPR-Cas system chimeric single guide RNA molecule (sgRNA) capable of effecting the manipulation of a target nucleic acid within a prokaryotic or eukaryotic cell when in complex within the cell with a CRISPR enzyme comprising a *Staphylococcus aureus* Cas9

enzyme (SaCas9); the sgRNA comprising, in a tandem arrangement: I. a guide sequence, which is capable of hybridizing to a sequence of the target nucleic acid to be manipulated; II. a tracr mate sequence, comprising a region of sense sequence; III. a linker sequence; and IV. a tracr sequence, comprising a region of antisense sequence which is positioned adjacent the linker sequence and which is capable of hybridizing with the region of sense sequence thereby forming a stem-loop; wherein the guide sequence comprises a length of 21 or more nucleotides. The dual guide RNA molecules, the dgRNAs, sgRNAs, DNA polynucleotide molecules, DNA expression vectors, delivery vectors, methods, systems, compositions or complexes of the New Architecture PCT, especially such comprising a *Staphylococcus aureus* Cas9 enzyme (SaCas9) or a fragment or derivative of *Staphylococcus aureus* Cas9 (e.g., such as an SaCas9 having an amino acid substitution or mutation such as N580A) are envisioned as being useful in the practice of the present invention; and, it is noted that in an aspect in the dgRNA or sgRNA the guide sequence can comprise a length of 22 or more nucleotides, 23 or more nucleotides, or 24 or more nucleotides, preferably wherein the guide sequence comprises a length of 21, 22, 23 or 24 nucleotides; and the linker sequence can commence after position +36 or less of the sense sequence, after position +25 or less of the sense sequence, or after position +21 or less of the sense sequence, or after position +18 or less of the sense sequence, or after position +15 or less of the sense sequence, or after position +14 or less of the sense sequence; or in the dgRNA the the tracr mate has a length of 36 nucleotides or less, or has a length of 25 nucleotides or less, or has a length of 21 nucleotides or less, or has a length of 18 nucleotides or less, or has a length of 15 nucleotides or less, or has a length of 14 nucleotides or less.

[0009] Various delivery means may be employed for delivering components of the CRISPR-Cas system to cells, tissues and organs, *ex vivo* and/or *in vivo*. Applicants have effectively packaged CRISPR-Cas system components (e.g., comprising SaCas9) into a viral delivery vector, e.g., AAV, and have demonstrated that it can be used to modify endogenous genome sequence in mammalian cells *in vivo*. Moreover, Applicants have modified HSCs with a particle system. A key feature of Applicants' present invention it that it effectively addresses the challenges of low efficiency of *in vivo* delivery (of therapeutic components) and low efficiency of homology directed repair (HDR) and in particular challenges associated with co-delivery are solved by the small Cas9, SaCas9 from *Staphylococcus aureus*, which can be readily packaged into a single Adeno-associated virus (AAV) vector to express both the Cas9 protein and its corresponding

sgRNA(s). Further, importantly, Applicants have shown that introduction of small SaCas9, has reduced the number of viral vectors required to perform HDR from 3 vectors to 2 vectors. And the number of particles to be contacted with HSCs can be one or two. In one aspect, the invention provides methods for using one or more elements of a CRISPR-Cas system. The CRISPR complex of the invention provides an effective means for modifying a target polynucleotide in a locus or loci, e.g., genomic locus or loci of an HSC. The CRISPR complex of the invention has a wide variety of utilities including modifying (e.g., deleting, inserting, translocating, inactivating, activating) a target polynucleotide in an HSC. As such the CRISPR complex of the invention has a broad spectrum of applications in, e.g., gene or genome editing, gene therapy, drug discovery, drug screening, disease diagnosis, and prognosis. Aspects of the invention relate to Cas9 enzymes having improved targeting specificity in a CRISPR-Cas9 system having guide RNAs having optimal activity, smaller in length than wild-type Cas9 enzymes and nucleic acid molecules coding therefor, and chimeric Cas9 enzymes, as well as methods of improving the target specificity of a Cas9 enzyme or of designing a CRISPR-Cas9 system comprising designing or preparing guide RNAs having optimal activity and/or selecting or preparing a Cas9 enzyme having a smaller size or length than wild-type Cas9 whereby packaging a nucleic acid coding therefor into a delivery vector is more advanced as there is less coding therefor in the delivery vector than for wild-type Cas9, and/or generating chimeric Cas9 enzymes. Also provided are uses of the present sequences, vectors, enzymes or systems, in medicine. Also provided are uses of the same in gene or genome editing.

[0010] In the invention, the Cas enzyme can be wildtype Cas9 including any naturally-occurring bacterial Cas9. Cas9 orthologs typically share the general organization of 3-4 RuvC domains and a HNH domain. The 5' most RuvC domain cleaves the non-complementary strand, and the HNH domain cleaves the complementary strand. All notations are in reference to the guide sequence. The catalytic residue in the 5' RuvC domain is identified through homology comparison of the Cas9 of interest with other Cas9 orthologs (from *S. pyogenes* type II CRISPR locus, *S. thermophilus* CRISPR locus 1, *S. thermophilus* CRISPR locus 3, and *Franciscilla novicida* type II CRISPR locus), and the conserved Asp residue (D10) is mutated to alanine to convert Cas9 into a complementary-strand nicking enzyme. Similarly, the conserved His and Asn residues in the HNH domains are mutated to Alanine to convert Cas9 into a non-complementary-strand nicking enzyme. In some embodiments, both sets of mutations may be

made, to convert Cas9 into a non-cutting enzyme. Accordingly, the Cas enzyme can be wildtype Cas9 including any naturally-occurring bacterial Cas9. The CRISPR, Cas or Cas9 enzyme can be codon optimized for HSC, or a modified version, including any chimaeras, mutants, homologs or orthologs. In an additional aspect of the invention, a Cas9 enzyme may comprise one or more mutations and may be used as a generic DNA binding protein with or without fusion to a functional domain. The mutations may be artificially introduced mutations or gain- or loss-of-function mutations. The mutations may include but are not limited to mutations in one of the catalytic domains (D10 and H840) in the RuvC and HNH catalytic domains, respectively. Further mutations have been characterized. In one aspect of the invention, the transcriptional activation domain may be VP64. In other aspects of the invention, the transcriptional repressor domain may be KRAB or SID4X. Other aspects of the invention relate to the mutated Cas 9 enzyme being fused to domains which include but are not limited to a nuclease, a transcriptional activator, repressor, a recombinase, a transposase, a histone remodeler, a demethylase, a DNA methyltransferase, a cryptochrome, a light inducible/controllable domain or a chemically inducible/controllable domain. The invention can involve sgRNAs or tracrRNAs or guide or chimeric guide sequences that allow for enhancing performance of these RNAs in cells. The CRISPR enzyme can be a type I or III CRISPR enzyme, preferably a type II CRISPR enzyme. This type II CRISPR enzyme may be any Cas enzyme. A preferred Cas enzyme may be identified as Cas9 as this can refer to the general class of enzymes that share homology to the biggest nuclease with multiple nuclease domains from the type II CRISPR system. Most preferably, the Cas9 enzyme is from, or is derived from, spCas9 or saCas9. By derived, Applicants mean that the derived enzyme is largely based, in the sense of having a high degree of sequence homology with, a wildtype enzyme, but that it has been mutated (modified) in some way as described herein

[0011] It will be appreciated that the terms Cas and CRISPR enzyme are generally used herein interchangeably, unless otherwise apparent. As mentioned above, many of the residue numberings used herein refer to the Cas9 enzyme from the type II CRISPR locus in *Streptococcus pyogenes*. However, it will be appreciated that this invention includes many more Cas9s from other species of microbes, such as SpCas9, SaCas9, St1Cas9 and so forth. Further examples are provided herein. The skilled person will be able to determine appropriate corresponding residues in Cas9 enzymes other than SpCas9 by comparison of the relevant amino acid sequences. Thus, where a specific amino acid replacement is referred to using the SpCas9 numbering, then, unless the

context makes it apparent this is not intended to refer to other Cas9 enzymes, the disclosure is intended to encompass corresponding modifications in other Cas9 enzymes. An example of a codon optimized sequence, in this instance optimized for humans (i.e. being optimized for expression in humans) is provided herein, see the SaCas9 human codon optimized sequence. Whilst this is preferred, it will be appreciated that other examples are possible and codon optimization for a host species is known. The invention comprehends methods wherein the Cas9 is a chimeric Cas9 proteins. These methods may comprise N-terminal fragment(s) of one Cas9 homolog with C-terminal fragment(s) of one or more other or another Cas9 homolog. It will be appreciated that in the present methods, where the organism is an animal, the modification may occur *ex vivo* or *in vitro*, for instance in a cell culture and in some instances not *in vivo*. In other embodiments, it may occur *in vivo*. The invention comprehends in some embodiments a composition of the invention or a CRISPR enzyme thereof (including or alternatively mRNA encoding the CRISPR enzyme), wherein the target sequence is flanked at its 3' end by a PAM (protospacer adjacent motif) sequence comprising 5'-motif, especially where the Cas9 is (or is derived from) *S. pyogenes* or *S. aureus* Cas9. For example, a suitable PAM is 5'-NRG or 5'-NNGRR (where N is any Nucleotide) for SpCas9 or SaCas9 enzymes (or derived enzymes). It will be appreciated that SpCas9 or SaCas9 are those from or derived from *S. pyogenes* or *S. aureus* Cas9.

[0012] The skilled person, from this disclosure and the knowledge in the art, can employ such proteins in the practice of the invention. For instance, the skilled person will be able to determine appropriate corresponding residues in CRISPR-Cas9 enzymes from the disclosure herein and the knowledge in the art for comparison of relevant amino acid sequences. For example, the skilled person will be able to determine appropriate corresponding residues in Cas9 enzymes other than SpCas9 by comparison of the relevant amino acid sequences. Thus, where a specific amino acid replacement is referred to using the SpCas9 numbering, then, unless the context makes it apparent this is not intended to refer to other Cas9 enzymes, the disclosure is intended to encompass corresponding modifications in other Cas9 enzymes. An example of a codon optimized sequence, in this instance optimized for humans (i.e. being optimized for expression in humans) is provided herein, see the SaCas9 human codon optimized sequence. Whilst this is preferred, it will be appreciated that other examples are possible and codon optimization for a host species is known. The invention comprehends methods wherein the CRISPR-Cas protein or Cas9 is a chimeric, e.g.,

a chimeric of two or more different CRISPR-Cas proteins or different Cas9 proteins. These methods may comprise N-terminal fragment(s) of one CRISPR-Cas protein or Cas9 homolog with C-terminal fragment(s) of one or more other or another CRISPR-Cas proteins or Cas9 homolog. It will be appreciated that in the present methods, where the organism is an animal, the modification may occur *ex vivo* or *in vitro*, for instance in a cell culture and in some instances not *in vivo*. In other embodiments, it may occur *in vivo*. The invention comprehends in some embodiments a composition of the invention or a CRISPR enzyme or protein thereof (including or alternatively mRNA encoding the CRISPR enzyme or protein), wherein the target sequence is flanked at its 3' end by a PAM (protospacer adjacent motif) sequence comprising 5'-motif, especially where the Cas9 is (or is derived from) *S. pyogenes* or *S. aureus* Cas9. For example, a suitable PAM is 5'-NRG or 5'-NNGRR (where N is any Nucleotide) for SpCas9 or SaCas9 enzymes (or derived enzymes). It will be appreciated that SpCas9 or SaCas9 are those from or derived from *S. pyogenes* or *S. aureus* Cas9.

[0013] In one aspect, the invention provides a method of modifying an organism or a non-human organism by manipulation of a target sequence in a locus, e.g., genomic locus of interest of a HSC, e.g., wherein the locus or genomic locus of interest is associated with a mutation associated with an aberrant protein expression or with a disease condition or state, comprising:

delivering to an HSC via a delivery system, e.g., via contacting an HSC with a particle containing, and/or one or more vectors expressing and/or having transcription and/or translation of, and/or one or more nucleic acid molecules (RNA, e.g., mRNA, and/or DNA giving rise to expression and/or transcription and/or translation *in vivo* in the HSC) a non-naturally occurring or engineered composition comprising:

- I. a CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, comprising:
 - (a) a guide sequence capable of hybridizing to a target sequence in a HSC,
 - (b) a tracr mate sequence, and
 - (c) a tracr sequence, and
- II. a CRISPR enzyme, optionally comprising at least one or more nuclear localization sequences,

wherein the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and

wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence;

and

the method may optionally include also delivering a HDR template, e.g., via the particle contacting the HSC containing or contacting the HSC with another particle containing, the HDR template wherein the HDR template provides expression of a normal or less aberrant form of the protein; wherein “normal” is as to wild type, and “aberrant” can be a protein expression that gives rise to a condition or disease state; and

optionally the method may include isolating or obtaining HSC from the organism or non-human organism, optionally expanding the HSC population, performing contacting of the particle(s) with the HSC to obtain a modified HSC population, optionally expanding the population of modified HSCs, and optionally administering modified HSCs to the organism or non-human organism.

[0014] The teachings provided herein provide effective methods for providing modified hematopoietic stem cells and progeny thereof, including but not limited to cells of the myeloid and lymphoid lineages of blood, including T cells, B cells, monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, dendritic cells, and megakaryocytes or platelets, and natural killer cells and their precursors and progenitors. Such cells can be modified by knocking out, knocking in, or otherwise modulating targets, for example to remove or modulate CD52 as described above, and other targets, such as, without limitation, CXCR4, and PD-1. Thus compositions, cells, and method of the invention can be used to can be to modulate immune responses and to treat, without limitation, malignancies, viral infections, and immune disorders.

[0015] In one aspect, the invention provides a method of modifying an organism or a non-human organism by manipulation of a target sequence in locus of interest, e.g., a genomic locus of interest of a HSC, e.g., wherein the locus of interest, e.g., genomic locus of interest is associated with a mutation associated with an aberrant protein expression or with a disease condition or state, comprising: delivering to an HSC, e.g., via a delivery system, e.g., via

contacting an HSC with a particle containing, and/or one or more vectors expressing and/or having transcription and/or translation of, and/or one or more nucleic acid molecules (RNA, e.g., mRNA, and/or DNA giving rise to expression and/or transcription and/or translation *in vivo* in the HSC), a non-naturally occurring or engineered composition comprising: I. (a) a guide sequence capable of hybridizing to a target sequence in a HSC, and (b) at least one or more tracr mate sequences, II. a CRISPR enzyme optionally having one or more NLSs, and III. a polynucleotide sequence comprising a tracr sequence, wherein the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence; and

the method may optionally include also delivering a HDR template, e.g., via the particle contacting the HSC containing or contacting the HSC with another particle containing, the HDR template wherein the HDR template provides expression of a normal or less aberrant form of the protein; wherein “normal” is as to wild type, and “aberrant” can be a protein expression that gives rise to a condition or disease state; and

optionally the method may include isolating or obtaining HSC from the organism or non-human organism, optionally expanding the HSC population, performing contacting of the particle(s) with the HSC to obtain a modified HSC population, optionally expanding the population of modified HSCs, and optionally administering modified HSCs to the organism or non-human organism.

[0016] The delivery system can be of one or more polynucleotides encoding any one or more or all of the CRISPR-complex, advantageously linked to one or more regulatory elements for *in vivo* expression, e.g. via particle(s), containing a vector containing the polynucleotide(s) operably linked to the regulatory element(s). Any or all of the polynucleotide sequence encoding a CRISPR enzyme or protein, and RNA therefor, e.g., guide sequence, and optionally where applicable tracr mate sequence or tracr sequence, may be RNA. It will be appreciated that where reference is made to a polynucleotide, which is RNA and is said to ‘comprise’ a feature such as for example, a tracr mate sequence, the RNA sequence includes the feature. Where the polynucleotide is DNA and is said to comprise a feature such as for example, a tracr mate sequence, the DNA sequence is or can be transcribed into the RNA including the feature at issue. Where the feature is a protein, such as

the CRISPR enzyme, the DNA or RNA sequence referred to is, or can be, translated (and in the case of DNA transcribed first).

[0017] In certain embodiments the invention provides a method of modifying an organism, e.g., mammal including human or a non-human mammal or organism by manipulation of a target sequence in an HSC e.g., a genomic locus of interest, such as wherein the target sequence or genomic locus of interest is associated with a mutation associated with an aberrant protein expression or with a disease condition or state, comprising delivering CRISPR-Cas system that targets the target sequence, e.g., via a delivery system.

[0018] For example, the delivering can comprise contacting of one or more non-naturally occurring or engineered compositions with the HSC or introducing one or more CRISPR-Cas systems into the HSC. A non-naturally occurring or engineered composition can comprise one or more particles containing the CRISPR-Cas system or components thereof.

[0019] A non-naturally occurring or engineered vector composition can comprise viral, plasmid or nucleic acid molecule vector(s) (e.g. RNA) operably encoding the CRISPR-Cas composition for expression thereof, wherein the vector composition comprises:

(A) I. a first regulatory element operably linked to a CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the polynucleotide sequence comprises (a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell, (b) a tracr mate sequence, and (c) a tracr sequence, and II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences (or optionally at least one or more nuclear localization sequences as some embodiments can involve no NLS), wherein (a), (b) and (c) are arranged in a 5' to 3' orientation, wherein components I and II are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, or (B) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising I. a first regulatory element operably linked to (a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell, and (b) at least one or more tracr mate sequences, II. a second regulatory element operably linked to an

enzyme-coding sequence encoding a CRISPR enzyme, and III. a third regulatory element operably linked to a tracr sequence, wherein components I, II and III are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence; the method may optionally include also delivering a HDR template, e.g., via the particle contacting the HSC containing or contacting the HSC with another particle containing, the HDR template wherein the HDR template provides expression of a normal or less aberrant form of the protein; wherein “normal” is as to wild type, and “aberrant” can be a protein expression that gives rise to a condition or disease state; and optionally the method may include isolating or obtaining HSC from the organism or non-human organism, optionally expanding the HSC population, performing contacting of the particle(s) with the HSC to obtain a modified HSC population, optionally expanding the population of modified HSCs, and optionally administering modified HSCs to the organism or non-human organism. In some embodiments, components I, II and III are located on the same vector. In other embodiments, components I and II are located on the same vector, while component III is located on another vector. In other embodiments, components I and III are located on the same vector, while component II is located on another vector. In other embodiments, components II and III are located on the same vector, while component I is located on another vector. In other embodiments, each of components I, II and III is located on different vectors. The invention also provides a viral or plasmid vector system as described herein.

[0020] By manipulation of a target sequence, Applicants also mean the epigenetic manipulation of a target sequence. This may be of the chromatin state of a target sequence, such as by modification of the methylation state of the target sequence (i.e. addition or removal of methylation or methylation patterns or CpG islands), histone modification, increasing or reducing accessibility to the target sequence, or by promoting 3D folding. It will be appreciated that where reference is made to a method of modifying an organism or mammal including human or a non-human mammal or organism by manipulation of a target sequence in a locus, e.g., genomic locus of interest, this may apply to the organism (or mammal) as a whole or just a single cell or population of cells from that organism (if the organism is multicellular). In the case of humans, for

instance, Applicants envisage, *inter alia*, a single cell or a population of cells and these may preferably be modified *ex vivo* and then introduced or re-introduced. In this case, a biopsy or other tissue or biological fluid sample may be necessary. Stem cells are also particularly preferred in this regard. But, of course, *in vivo* embodiments are also envisaged. And the invention is especially advantageous as to HSCs.

[0021] The invention in some embodiments comprehends a method of modifying an organism or a non-human organism by manipulation of a first and a second target sequence on opposite strands of a polynucleotide, e.g., DNA, duplex, for instance, in a locus, e.g., genomic locus of interest, in a HSC e.g., wherein the locus or genomic locus of interest is associated with a mutation associated with an aberrant protein expression or with a disease condition or state, comprising delivering, e.g., by contacting HSCs with particle(s) comprising a non-naturally occurring or engineered composition comprising :

- I. a first CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the first polynucleotide sequence comprises:
 - (a) a first guide sequence capable of hybridizing to the first target sequence,
 - (b) a first tracr mate sequence, and
 - (c) a first tracr sequence,

- II. a second CRISPR-Cas system chiRNA polynucleotide sequence, wherein the second polynucleotide sequence comprises:
 - (a) a second guide sequence capable of hybridizing to the second target sequence,
 - (b) a second tracr mate sequence, and
 - (c) a second tracr sequence, and

- III. a polynucleotide sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences and comprising one or more mutations, wherein (a), (b) and (c) are arranged in a 5' to 3' orientation; or

- IV. expression product(s) of one or more of I. to III., e.g., the the first and the second tracr mate sequence, the CRISPR enzyme;

[0022] wherein when transcribed, the first and the second tracr mate sequence hybridize to the first and second tracr sequence respectively and the first and the second guide sequence directs sequence-specific binding of a first and a second CRISPR complex to the first and second target sequences respectively, wherein the first CRISPR complex comprises the CRISPR enzyme complexed with (1) the first guide sequence that is hybridized to the first target sequence, and (2) the first tracr mate sequence that is hybridized to the first tracr sequence, wherein the second CRISPR complex comprises the CRISPR enzyme complexed with (1) the second guide sequence that is hybridized to the second target sequence, and (2) the second tracr mate sequence that is hybridized to the second tracr sequence, wherein the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA, and wherein the first guide sequence directs cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directs cleavage of the other strand near the second target sequence inducing a double strand break, thereby modifying the organism or the non-human organism; and the method may optionally include also delivering a HDR template, e.g., via the particle contacting the HSC containing or contacting the HSC with another particle containing, the HDR template wherein the HDR template provides expression of a normal or less aberrant form of the protein; wherein "normal" is as to wild type, and "aberrant" can be a protein expression that gives rise to a condition or disease state; and optionally the method may include isolating or obtaining HSC from the organism or non-human organism, optionally expanding the HSC population, performing contacting of the particle(s) with the HSC to obtain a modified HSC population, optionally expanding the population of modified HSCs, and optionally administering modified HSCs to the organism or non-human organism. In some methods of the invention any or all of the polynucleotide sequence encoding the CRISPR enzyme, the first and the second guide sequence, the first and the second tracr mate sequence or the first and the second tracr sequence, is/are RNA. In further embodiments of the invention the polynucleotides encoding the sequence encoding the CRISPR enzyme, the first and the second guide sequence, the first and the second tracr mate sequence or the first and the second tracr sequence, is/are RNA and are delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun; but, it is advantageous that the delivery is via a particle. In certain embodiments of the invention, the first and second tracr mate sequence share 100% identity and/or the first and second tracr sequence share 100% identity. In some embodiments, the polynucleotides may be comprised within a vector

system comprising one or more vectors. In preferred embodiments of the invention the CRISPR enzyme is a Cas9 enzyme, e.g. SpCas9. In an aspect of the invention the CRISPR enzyme comprises one or more mutations in a catalytic domain, wherein the one or more mutations, with reference to SpCas9 are selected from the group consisting of D10A, E762A, H840A, N854A, N863A and D986A, e.g., a D10A mutation. In preferred embodiments, the first CRISPR enzyme has one or more mutations such that the enzyme is a complementary strand nicking enzyme, and the second CRISPR enzyme has one or more mutations such that the enzyme is a non-complementary strand nicking enzyme. Alternatively the first enzyme may be a non-complementary strand nicking enzyme, and the second enzyme may be a complementary strand nicking enzyme. In preferred methods of the invention the first guide sequence directing cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directing cleavage of the other strand near the second target sequence results in a 5' overhang. In embodiments of the invention the 5' overhang is at most 200 base pairs, preferably at most 100 base pairs, or more preferably at most 50 base pairs. In embodiments of the invention the 5' overhang is at least 26 base pairs, preferably at least 30 base pairs or more preferably 34-50 base pairs.

[0023] The invention in some embodiments comprehends a method of modifying an organism or a non-human organism by manipulation of a first and a second target sequence on opposite strands of a DNA duplex in a genomic locus of interest in a HSC e.g., wherein the genomic locus of interest is associated with a mutation associated with an aberrant protein expression or with a disease condition or state, comprising delivering, e.g., by contacting HSCs with particle(s) comprising a non-naturally occurring or engineered composition comprising:

- I. a first regulatory element operably linked to
 - (a) a first guide sequence capable of hybridizing to the first target sequence, and
 - (b) at least one or more tracr mate sequences,
- II. a second regulatory element operably linked to
 - (a) a second guide sequence capable of hybridizing to the second target sequence, and
 - (b) at least one or more tracr mate sequences,

- III. a third regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, and
- IV. a fourth regulatory element operably linked to a tracr sequence,
- V. expression product(s) of one or more of I. to IV., e.g., the the first and the second tracr mate sequence, the CRISPR enzyme;

[0024] Components I, II, III and IV can be located on the same or different vectors and/or be contained in same or different particles of the system; advantageously they are in the same vector and/or same particle.

[0025] When transcribed, the first and the second guide sequence direct sequence-specific binding of a first and a second CRISPR complex to the first and second target sequences respectively. If present in and applicable to the CRISPR-Cas system, the tracr mate sequence hybridizes to the tracr sequence. A first CRISPR complex comprises the CRISPR enzyme complexed with the first guide sequence that is hybridized to the first target sequence; and optionally if present in and applicable to the CRISPR-Cas system, the tracr mate sequence that is hybridized to the tracr sequence. A second CRISPR complex comprises the CRISPR enzyme complexed with the second guide sequence that is hybridized to the second target sequence; and optionally if present in and applicable to the CRISPR-Cas system, the tracr mate sequence that is hybridized to the tracr sequence. The polynucleotide sequence encoding the CRISPR enzyme can be DNA or RNA. The first guide sequence directs cleavage of one strand of the polynucleotide, e.g., DNA, duplex near the first target sequence. The second guide sequence directs cleavage of the other strand near the second target sequence inducing a double strand break; advantageously the breaks have overhangs.

[0026] The method may optionally include also delivering a polynucleotide, e.g., HDR, template, e.g., via a delivery system used to deliver CRISPR-Cas system(s) or component(s) thereof, e.g., the particle contacting the HSC containing; or contacting the HSC with another particle containing, the template. In instances where a complexed or assembled CRISPR-Cas system is delivered via a particle, the particle can include the template, e.g., hybridization bonded or pre-annealed to RNA of the system. The template provides expression of a normal or less aberrant form of the protein; wherein "normal" is as to wild type, and "aberrant" can be a protein expression that gives rise to a condition or disease state. The template can be DNA or RNA, depending upon the CRISPR-Cas system enzyme or protein. Optionally the method may include

isolating or obtaining HSC from the organism or non-human organism . The method may also optionally include expanding the HSC population, e.g., performing a herein method to obtain a modified HSC population, and expanding the population of modified HSCs. The method can optionally include administering modified HSCs to the organism or non-human organism. HSCs can be obtained from a first organism or non-human organism, subjected to a herein method to obtain a modified HSC population, the modified HSC population may optionally be expanded, and modified HSCs can be administered to a second organism or non-human organism. In a personalized medicine therapy, the first and second organism or non-human organism can be the same. In other instances, the first and second organisms can be sufficiently related that cells from the first can be administered to the second (e.g., cells from a biological relative, a compatible donor)

[0027] The invention also provides a vector system as described herein. The system may comprise one, two, three or four different vectors. Components I, II, III and IV may thus be located on one, two, three or four different vectors, and all combinations for possible locations of the components are herein envisaged, for example: components I, II, III and IV can be located on the same vector; components I, II, III and IV can each be located on different vectors; components I, II, III and IV may be located on a total of two or three different vectors, with all combinations of locations envisaged, etc. In some methods of the invention any or all of the polynucleotide sequence encoding the CRISPR enzyme, the first and the second guide sequence, the first and the second tracr mate sequence if applicable to the CRISPR-Cas system or the first and the second tracr sequence if applicable to the CRISPR-Cas system, is/are RNA. In further embodiments of the invention the first and second tracr mate sequence if applicable to the CRISPR-Cas system share 100% identity and/or the first and second tracr sequence if applicable to the CRISPR-Cas system share 100% identity. In certain preferred embodiments of the invention the CRISPR enzyme is a Cas9 enzyme, e.g. SpCas 9 or SaCas9. In an aspect of the invention the CRISPR enzyme comprises one or more mutations in a catalytic domain, wherein the one or more mutations with reference to SpCas9 are selected from the group consisting of D10A, E762A, H840A, N854A, N863A and D986A; e.g., D10A mutation. In preferred embodiments, the first CRISPR enzyme has one or more mutations such that the enzyme is a complementary strand nicking enzyme, and the second CRISPR enzyme has one or more mutations such that the enzyme is a non-complementary strand nicking enzyme. Alternatively the first enzyme may be a

non-complementary strand nicking enzyme, and the second enzyme may be a complementary strand nicking enzyme. In a further embodiment of the invention, one or more of the viral vectors are delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun; but, particle delivery is advantageous.

[0028] In preferred methods of the invention the first guide sequence directing cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directing cleavage of other strand near the second target sequence results in a 5' overhang. In embodiments of the invention the 5' overhang is at most 200 base pairs, preferably at most 100 base pairs, or more preferably at most 50 base pairs. In embodiments of the invention the 5' overhang is at least 26 base pairs, preferably at least 30 base pairs or more preferably 34-50 base pairs.

[0029] The invention in some embodiments comprehends a method of modifying a genomic locus of interest in HSC e.g., wherein the genomic locus of interest is associated with a mutation associated with an aberrant protein expression or with a disease condition or state, by introducing into the HSC, e.g., by contacting HSCs with particle(s) comprising, a Cas protein having one or more mutations and two guide RNAs that target a first strand and a second strand of the DNA molecule respectively in the HSC, whereby the guide RNAs target the DNA molecule and the Cas protein nicks each of the first strand and the second strand of the DNA molecule, whereby a target in the HSC is altered; and, wherein the Cas protein and the two guide RNAs do not naturally occur together and the method may optionally include also delivering a HDR template, e.g., via the particle contacting the HSC containing or contacting the HSC with another particle containing, the HDR template wherein the HDR template provides expression of a normal or less aberrant form of the protein; wherein "normal" is as to wild type, and "aberrant" can be a protein expression that gives rise to a condition or disease state; and optionally the method may include isolating or obtaining HSC from the organism or non-human organism, optionally expanding the HSC population, performing contacting of the particle(s) with the HSC to obtain a modified HSC population, optionally expanding the population of modified HSCs, and optionally administering modified HSCs to the organism or non-human organism. In preferred methods of the invention the Cas protein nicking each of the first strand and the second strand of the DNA molecule results in a 5' overhang. In embodiments of the invention the 5' overhang is at most 200 base pairs, preferably at most 100 base pairs, or more preferably at most 50 base pairs. In embodiments of the invention the 5' overhang is at least 26 base pairs, preferably at least 30 base pairs or more preferably 34-50

base pairs. Embodiments of the invention also comprehend the guide RNAs comprising a guide sequence fused to a tracr mate sequence and a tracr sequence. In an aspect of the invention the Cas protein is codon optimized for expression in a eukaryotic cell, preferably a mammalian cell or a human cell. In further embodiments of the invention the Cas protein is a type II CRISPR-Cas protein, e.g. a Cas 9 protein. In a highly preferred embodiment the Cas protein is a Cas9 protein, e.g. SpCas9 or SaCas9. In aspects of the invention the Cas protein has one or more mutations in respect of SpCas9 selected from the group consisting of D10A, E762A, H840A, N854A, N863A and D986A; e.g., a D10A mutation. Aspects of the invention relate to the expression of a gene product being decreased or a template polynucleotide being further introduced into the DNA molecule encoding the gene product or an intervening sequence being excised precisely by allowing the two 5' overhangs to reanneal and ligate or the activity or function of the gene product being altered or the expression of the gene product being increased. In an embodiment of the invention, the gene product is a protein.

[0030] The invention in some embodiments comprehends a method of modifying a genomic locus of interest in HSC e.g., wherein the genomic locus of interest is associated with a mutation associated with an aberrant protein expression or with a disease condition or state, by introducing into the HSC, e.g., by contacting HSCs with particle(s) comprising,

- a) a first regulatory element operably linked to each of two CRISPR-Cas system guide RNAs that target a first strand and a second strand respectively of a double stranded DNA molecule of the HSC, and
- b) a second regulatory element operably linked to a Cas protein, or
- c) expression product(s) of a) or b),

[0031] wherein components (a) and (b) are located on same or different vectors of the system, whereby the guide RNAs target the DNA molecule of the HSC and the Cas protein nicks each of the first strand and the second strand of the DNA molecule of the HSC; and, wherein the Cas protein and the two guide RNAs do not naturally occur together; and the method may optionally include also delivering a HDR template, e.g., via the particle contacting the HSC containing or contacting the HSC with another particle containing, the HDR template wherein the HDR template provides expression of a normal or less aberrant form of the protein; wherein "normal" is as to wild type, and "aberrant" can be a protein expression that gives rise to a condition or disease state; and optionally the method may include isolating or obtaining HSC from the organism or non-human

organism, optionally expanding the HSC population, performing contacting of the particle(s) with the HSC to obtain a modified HSC population, optionally expanding the population of modified HSCs, and optionally administering modified HSCs to the organism or non-human organism. In aspects of the invention the guide RNAs may comprise a guide sequence fused to a tracr mate sequence and a tracr sequence. In an embodiment of the invention the Cas protein is a type II CRISPR-Cas protein. In an aspect of the invention the Cas protein is codon optimized for expression in a eukaryotic cell, preferably a mammalian cell or a human cell. In further embodiments of the invention the Cas protein is a type II CRISPR-Cas protein, e.g. a Cas 9 protein. In a highly preferred embodiment the Cas protein is a Cas9 protein, e.g. SpCas9 or SaCas9. In aspects of the invention the Cas protein has one or more mutations with reference to SpCas9 selected from the group consisting of D10A, E762A, H840A, N854A, N863A and D986A; e.g., the D10A mutation. Aspects of the invention relate to the expression of a gene product being decreased or a template polynucleotide being further introduced into the DNA molecule encoding the gene product or an intervening sequence being excised precisely by allowing the two 5' overhangs to reanneal and ligate or the activity or function of the gene product being altered or the expression of the gene product being increased. In an embodiment of the invention, the gene product is a protein. In preferred embodiments of the invention the vectors of the system are viral vectors. In a further embodiment, the vectors of the system are delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun; and particles are preferred. In one aspect, the invention provides a method of modifying a target polynucleotide in a HSC. In some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. In some embodiments, said cleavage comprises cleaving one or two strands at the location of the target sequence by said CRISPR enzyme. In some embodiments, said cleavage results in decreased transcription of a target gene. In some embodiments, the method further comprises repairing said cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein said repair results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of said target polynucleotide. In some

embodiments, said mutation results in one or more amino acid changes in a protein expressed from a gene comprising the target sequence. In some embodiments, the method further comprises delivering one or more vectors or expression product(s) thereof, e.g., via particle(s), to said HSC, wherein the one or more vectors drive expression of one or more of: the CRISPR enzyme, the guide sequence linked to the tracr mate sequence, and the tracr sequence. In some embodiments, said vectors are delivered to the HSC in a subject. In some embodiments, said modifying takes place in said HSC in a cell culture. In some embodiments, the method further comprises isolating said HSC from a subject prior to said modifying. In some embodiments, the method further comprises returning said HSC and/or cells derived therefrom to said subject.

[0032] In one aspect, the invention provides a method of generating a HSC comprising a mutated disease gene. In some embodiments, a disease gene is any gene associated with an increase in the risk of having or developing a disease. In some embodiments, the method comprises (a) introducing one or more vectors or expression product(s) thereof, e.g., via particle(s), into a HSC, wherein the one or more vectors drive expression of one or more of: a CRISPR enzyme, a guide sequence linked to a tracr mate sequence, and a tracr sequence; and (b) allowing a CRISPR complex to bind to a target polynucleotide to effect cleavage of the target polynucleotide within said disease gene, wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence within the target polynucleotide, and (2) the tracr mate sequence that is hybridized to the tracr sequence, thereby generating a HSC comprising a mutated disease gene. In some embodiments, said cleavage comprises cleaving one or two strands at the location of the target sequence by said CRISPR enzyme. In some embodiments, said cleavage results in decreased transcription of a target gene. In some embodiments, the method further comprises repairing said cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein said repair results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of said target polynucleotide. In some embodiments, said mutation results in one or more amino acid changes in a protein expression from a gene comprising the target sequence. In some embodiments the modified HSC is administered to an animal to thereby generate an animal model.

[0033] In one aspect, the invention provides for methods of modifying a target polynucleotide in a HSC. In some embodiments, the method comprises allowing a CRISPR complex to bind to

the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. In other embodiments, this invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell that arises from an HSC. The method comprises increasing or decreasing expression of a target polynucleotide by using a CRISPR complex that binds to the polynucleotide in the HSC; advantageously the CRISPR complex is delivered via particle(s).

[0034] In some methods, a target polynucleotide can be inactivated to effect the modification of the expression in a HSC. For example, upon the binding of a CRISPR complex to a target sequence in a cell, the target polynucleotide is inactivated such that the sequence is not transcribed, the coded protein is not produced, or the sequence does not function as the wild-type sequence does.

[0035] In some embodiments the RNA of the CRISPR-Cas system, e.g., the guide or sgRNA, can be modified; for instance to include an aptamer or a functional domain. An aptamer is a synthetic oligonucleotide that binds to a specific target molecule; for instance a nucleic acid molecule that has been engineered through repeated rounds of in vitro selection or SELEX (systematic evolution of ligands by exponential enrichment) to bind to various molecular targets such as small molecules, proteins, nucleic acids, and even cells, tissues and organisms. Aptamers are useful in that they offer molecular recognition properties that rival that of antibodies. In addition to their discriminate recognition, aptamers offer advantages over antibodies including that they elicit little or no immunogenicity in therapeutic applications. Accordingly, in the practice of the invention, either or both of the enzyme or the RNA can include a functional domain.

[0036] In some embodiments, the functional domain is a transcriptional activation domain, preferably VP64. In some embodiments, the functional domain is a transcription repression domain, preferably KRAB. In some embodiments, the transcription repression domain is SID, or concatemers of SID (eg SID4X). In some embodiments, the functional domain is an epigenetic modifying domain, such that an epigenetic modifying enzyme is provided. In some embodiments, the functional domain is an activation domain, which may be the P65 activation domain.

[0037] The invention further comprehends a composition of the invention or a CRISPR complex or enzyme thereof or RNA thereof (including or alternatively mRNA encoding the

CRISPR enzyme) for use in medicine or in therapy. In some embodiments the invention comprehends a composition according to the invention or components thereof for use in a method according to the invention. In some embodiments the invention provides for the use of a composition of the invention or a CRISPR complex or enzyme thereof or RNA thereof (including or alternatively mRNA encoding the CRISPR enzyme) in *ex vivo* gene or genome editing, especially in HSCs which optionally may then be introduced into an organism or non-human organism from which the HSCs were obtained or another organism or non-human organism of the same species. In certain embodiments the invention comprehends use of a composition of the invention or a CRISPR complex or enzyme thereof or RNA thereof (including or alternatively mRNA encoding the CRISPR enzyme) in the manufacture of a medicament for *ex vivo* gene or genome editing or for use in a method according to the invention. In certain embodiments the invention provides a method of treating or inhibiting a condition caused by a defect in a target sequence in a genomic locus of interest in a subject (e.g., mammal or human) or a non-human subject (e.g., mammal) in need thereof comprising modifying HSCs of the subject or a non-human subject by manipulation of the target sequence in the HSC and administering the modified HSCs to the subject or non-human subject, advantageously the modifying of the HSCs is through contacting the HSCs with a particle containing the CRISPR complex or the components thereof, advantageously in certain embodiments the particle also provides a HDR template or another particle or a vector provides the HDR template, and wherein the condition is susceptible to treatment or inhibition by manipulation of the target sequence.

[0038] Certain RNA of the CRISPR Cas complex is also known and referred to as sgRNA (single guide RNA). In advantageous embodiments RNA of the CRISPR Cas complex is sgRNA. The CRISPR-Cas9 system has been engineered to target genetic locus or loci in HSCs. Cas9 protein, advantageously codon-optimized for a eukaryotic cell and especially a mammalian cell, e.g., a human cell, for instance, HSC, and sgRNA targeting a locus or loci in HSC, e.g., the gene EMX1, were prepared. These were advantageously delivered via particles. The particles were formed by the Cas9 protein and the sgRNA being admixed. The sgRNA and Cas9 protein mixture was admixed with a mixture comprising or consisting essentially of or consisting of surfactant, phospholipid, biodegradable polymer, lipoprotein and alcohol, whereby particles containing the sgRNA and Cas9 protein were formed. The invention comprehends so making particles and particles from such a method as well as uses thereof. More generally, particles were formed using

an efficient process. First, Cas9 protein and sgRNA targeting the gene EMX1 or the control gene LacZ were mixed together at a suitable, e.g., 3:1 to 1:3 or 2:1 to 1:2 or 1:1 molar ratio, at a suitable temperature, e.g., 15-30C, e.g., 20-25C, e.g., room temperature, for a suitable time, e.g., 15-45, such as 30 minutes, advantageously in sterile, nuclease free buffer, e.g., 1X PBS. Separately, particle components such as or comprising: a surfactant, e.g., cationic lipid, e.g., 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP); phospholipid, e.g., dimyristoylphosphatidylcholine (DMPC); biodegradable polymer, such as an ethylene-glycol polymer or PEG, and a lipoprotein, such as a low-density lipoprotein, e.g., cholesterol were dissolved in an alcohol, advantageously a C₁₋₆ alkyl alcohol, such as methanol, ethanol, isopropanol, e.g., 100% ethanol. The two solutions were mixed together to form particles containing the Cas9-sgRNA complexes. In certain embodiments the particle can contain an HDR template. That can be a particle co-administered with sgRNA+Cas9 protein-containing particle, or i.e., in addition to contacting an HSC with an sgRNA+Cas9 protein-containing particle, the HSC is contacted with a particle containing an HDR template; or the HSC is contacted with a particle containing all of the sgRNA, Cas9 and the HDR template. The HDR template can be administered by a separate vector, whereby in a first instance the particle penetrates an HSC cell and the separate vector also penetrates the cell, wherein the HSC genome is modified by the sgRNA+Cas9 and the HDR template is also present, whereby a genomic loci is modified by the HDR; for instance, this may result in correcting a mutation. The particle in the herein discussion is advantageously obtained or obtainable from admixing an sgRNA(s) and Cas9 protein mixture (optionally containing HDR template(s) or such mixture only containing HDR template(s) when separate particles as to template(s) is desired) with a mixture comprising or consisting essentially of or consisting of surfactant, phospholipid, biodegradable polymer, lipoprotein and alcohol (wherein one or more sgRNA targets the genetic locus or loci in the HSC).

[0039] In herein discussions concerning the target being associated with a mutation or with a disease condition, such mutation or disease condition can be, for instance Hemophilia B, SCID, SCID-X1, ADA-SCID, Hereditary tyrosinemia, β -thalassemia, X-linked CGD, Wiskott-Aldrich syndrome, Fanconi anemia, adrenoleukodystrophy (ALD), metachromatic leukodystrophy (MLD), or HIV/AIDS; or more generally an Immunodeficiency disorder, Hematologic condition, or genetic lysosomal storage disease.

[0040] Hematopoietic stem cells treatments are also relied on to alleviate certain aspects of genetic disorders. Alpha-mannosidosis is inherited in an autosomal recessive fashion and is caused by a spectrum of mutations in the *MAN2B1* gene located on chromosome 19 (19 p13.2-q12). One study of 39 families identified eight splicing, six missense, and three nonsense mutations, as well as two small insertions and two small deletions (see Berg et al., Jan 1999, Am. J. Hum. Genet. 64(1): 77–88. Grewall et al., (J Pediatr. 2004 May;144(5):569-73) treated alpha-mannosidosis by transplanting allogeneic hematopoietic stem cell. Alpha-mannosidosis can be treated by transplantation of a subjects own hematopoietic stem cells prepared according to the invention to correct deficient expression of *MAN2B1*.

[0041] Leukodystrophies are a group on inherited diseases in which molecular abnormalities of glial cells are responsible defects in myelin formation and/or maintenance within the central and peripheral nervous system. In globoid cell leukodystrophy (Krabbe Disease) there is an inherited metabolic disorder of the central nervous system caused by deficiency of the lysosomal enzyme galactocerebrosidase (GALC). The mutational spectrum comprises missense mutations in evolutionarily conserved residues as well as frameshift mutations, nonsense mutations, and mutations that mediate exon skipping. Hematopoietic stem cell transplantation has provided an effective treatment. The engraftment from normal donors provides cells able to correct the metabolic defect see Tappino et al., Dec. 2010, Hum Mutat. 31(12):E1894-914. doi: 10.1002/humu.21367. Leukodystrophies can be treated by transplantation of a subjects own hematopoietic stem cells prepared according to the invention.

[0042] Polycythemia vera (PCV), being is caused by neoplastic proliferation of erythroid, megakaryocytic and granulocytic cells. PCV is associated with a mutation in the tyrosine kinase JAK2 (usually V617F), which acts in signaling pathways of the EPO-receptor, rendering those cells hypersensitive to EPO. (See, Lussana et al., May 2014, Haematologica, 99(5):916-21. doi: 10.3324/haematol.2013.094284. Epub 2014 Jan 3). Allogeneic stem cell transplantation is currently the only potentially curative treatment. PCV can be treated by transplantation of a subjects own hematopoietic stem cells prepared according to the invention. Calreticulin (CALR) mutations are the second most common cause of myeloproliferative neoplasms. Consequences in PCV include double the normal number of erythrocytes per cubic millimeter of blood, elevated hematocrit, and twice normal blood volume. Familial essential thrombocythaemia (ET) is another congenital disease inherited in an autosomal dominant manner. ET is characterized by

overproduction of platelets by megakaryocytes in the bone marrow, and alterations of the TPO receptor encoded for by the c-mpl gene are associated. According to the invention, HSCs obtained from subjects afflicted with such myeloproliferative disorders are subject to gene editing to correct the causal mutation. The patient's own gene edited stem cells may then be administered to the patient.

[0043] As to a CRISPR-Cas system of the invention being engineered or non-naturally occurring, the RNA of the system can be engineered, e.g., to target a nucleic acid molecule in an HSC and/or the protein and/or the nucleic acid molecule encoding it can be engineered, e.g., codon optimized for expression in a HSC and/or a having a functionalized effector, e.g., NLS, Fok I, etc and/or having one or more a mutation(s) or truncation(s), e.g., as to having the protein become a nicking enzyme or an enzyme that binds without significant cutting (e.g., less than 5% cutting activity) and/or wherein the protein is a chimeric of two or more CRISPR-Cas proteins.

[0044] Accordingly, it is an object of the invention to not encompass within the invention any previously known product, process of making the product, or method of using the product such that Applicants reserve the right and hereby disclose a disclaimer of any previously known product, process, or method. It is further noted that the invention does not intend to encompass within the scope of the invention any product, process, or making of the product or method of using the product, which does not meet the written description and enablement requirements of the USPTO (35 U.S.C. §112, first paragraph) or the EPO (Article 83 of the EPC), such that Applicants reserve the right and hereby disclose a disclaimer of any previously described product, process of making the product, or method of using the product.

[0045] It is noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as "comprises", "comprised", "comprising" and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean "includes", "included", "including", and the like; and that terms such as "consisting essentially of" and "consists essentially of" have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention. It may be advantageous in the practice of the invention to be in compliance with Art. 53(c) EPC and Rule 28(b) and (c) EPC. Nothing herein is to be construed as a promise.

[0046] These and other embodiments are disclosed or are obvious from and encompassed by, the following Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0047] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0048] Figure 1 shows comparison of different programmable nuclease platforms.

[0049] Figure 2 shows Types of Therapeutic Genome Modifications. The specific type of genome editing therapy depends on the nature of the mutation causing disease. **a**, In gene disruption, the pathogenic function of a protein is silenced by targeting the locus with NHEJ. Formation of indels on the gene of interest often result in frameshift mutations that create premature stop codons and a non-functional protein product, or non-sense mediated decay of transcripts, suppressing gene function. **b**, HDR gene correction can be used to correct a deleterious mutation. DSB is targeted near the mutation site in the presence of an exogenously provided, corrective HDR template. HDR repair of the break site with the exogenous template corrects the mutation, restoring gene function. **c**, An alternative to gene correction is gene addition. This mode of treatment introduces a therapeutic transgene into a safe-harbor locus in the genome. DSB is targeted to the safe-harbor locus and an HDR template containing homology to the break site, a promoter and a transgene is introduced to the nucleus. HDR repair copies the promoter-transgene cassette into the safe-harbor locus, recovering gene function, albeit without true physiological control over gene expression.

[0050] Figure 3 shows *Ex vivo* vs. *in vivo* editing therapy. In *ex vivo* editing therapy cells are removed from a patient, edited and then re-engrafted (top panel). For this mode of therapy to be successful, target cells must be capable of survival outside the body and homing back to target tissues post-transplantation. *In vivo* therapy involves genome editing of cells *in situ* (bottom panels). For *in vivo* systemic therapy, delivery agents that are relatively agnostic to cell identity or state would be used to effect editing in a wide range of tissue types. Although this mode of editing therapy may be possible in the future, no delivery systems currently exist that are efficient enough to make this feasible. *In vivo* targeted therapy, where delivery agents with tropism for specific organ systems are administered to patients are feasible with clinically relevant viral vectors.

[0051] Figure 4 shows a schematic representation of gene therapy *via* Cas9 Homologous Recombination (HR) vectors.

[0052] The figures herein are for illustrative purposes only and are not necessarily drawn to scale.

DETAILED DESCRIPTION OF THE INVENTION

[0053] With respect to general information on CRISPR-Cas Systems, components thereof, and delivery of such components, including methods, materials, delivery vehicles, vectors, particles, AAV, and making and using thereof, including as to amounts and formulations, all useful in the practice of the instant invention, reference is made to: US Patents Nos. 8,697,359, 8,771,945, 8,795,965, 8,865,406, 8,871,445, 8,889,356, 8,889,418 and 8,895,308; US Patent Publications US 2014-0310830 (US APP. Ser. No. 14/105,031), US 2014-0287938 A1 (U.S. App. Ser. No. 14/213,991), US 2014-0273234 A1 (U.S. App. Ser. No. 14/293,674), US2014-0273232 A1 (U.S. App. Ser. No. 14/290,575), US 2014-0273231 (U.S. App. Ser. No. 14/259,420), US 2014-0256046 A1 (U.S. App. Ser. No. 14/226,274), US 2014-0248702 A1 (U.S. App. Ser. No. 14/258,458), US 2014-0242700 A1 (U.S. App. Ser. No. 14/222,930), US 2014-0242699 A1 (U.S. App. Ser. No. 14/183,512), US 2014-0242664 A1 (U.S. App. Ser. No. 14/104,990), US 2014-0234972 A1 (U.S. App. Ser. No. 14/183,471), US 2014-0227787 A1 (U.S. App. Ser. No. 14/256,912), US 2014-0189896 A1 (U.S. App. Ser. No. 14/105,035), US 2014-0186958 (U.S. App. Ser. No. 14/105,017), US 2014-0186919 A1 (U.S. App. Ser. No. 14/104,977), US 2014-0186843 A1 (U.S. App. Ser. No. 14/104,900), US 2014-0179770 A1 (U.S. App. Ser. No. 14/104,837) and US 2014-0179006 A1 (U.S. App. Ser. No. 14/183,486), US 2014-0170753 (US App Ser No 14/183,429); European Patent Applications EP 2 771 468 (EP13818570.7), EP 2 764 103 (EP13824232.6), and EP 2 784 162 (EP14170383.5); and PCT Patent Publications WO 2014/093661 (PCT/US2013/074743), WO 2014/093694 (PCT/US2013/074790), WO 2014/093595 (PCT/US2013/074611), WO 2014/093718 (PCT/US2013/074825), WO 2014/093709 (PCT/US2013/074812), WO 2014/093622 (PCT/US2013/074667), WO 2014/093635 (PCT/US2013/074691), WO 2014/093655 (PCT/US2013/074736), WO 2014/093712 (PCT/US2013/074819), WO 2014/093701 (PCT/US2013/074800), WO 2014/018423 (PCT/US2013/051418) , WO 2014/204723 (PCT/US2014/041790), WO 2014/204724 (PCT/US2014/041800), WO 2014/204725 (PCT/US2014/041803), WO 2014/204726 (PCT/US2014/041804), WO 2014/204727 (PCT/US2014/041806), WO 2014/204728 (PCT/US2014/041808), and WO 2014/204729 (PCT/US2014/041809). Reference is also made to US provisional patent applications 61/758,468; 61/802,174; 61/806,375; 61/814,263;

61/819,803 and 61/828,130, filed on January 30, 2013; March 15, 2013; March 28, 2013; April 20, 2013; May 6, 2013 and May 28, 2013 respectively. Reference is also made to US provisional patent application 61/836,123, filed on June 17, 2013. Reference is additionally made to US provisional patent applications 61/835,931, 61/835,936, 61/836,127, 61/836, 101, 61/836,080 and 61/835,973, each filed June 17, 2013. Further reference is made to US provisional patent applications 61/862,468 and 61/862,355 filed on August 5, 2013; 61/871,301 filed on August 28, 2013; 61/960,777 filed on September 25, 2013 and 61/961,980 filed on October 28, 2013. Reference is yet further made to: PCT Patent applications Nos: PCT/US2014/041803, PCT/US2014/041800, PCT/US2014/041809, PCT/US2014/041804 and PCT/US2014/041806, each filed June 10, 2014 6/10/14; PCT/US2014/041808 filed June 11, 2014; and PCT/US2014/62558 filed October 28, 2014, and US Provisional Patent Applications Serial Nos.: 61/915,150, 61/915,301, 61/915,267 and 61/915,260, each filed December 12, 2013; 61/757,972 and 61/768,959, filed on January 29, 2013 and February 25, 2013; 61/835,936, 61/836,127, 61/836,101, 61/836,080, 61/835,973, and 61/835,931, filed June 17, 2013; 62/010,888 and 62/010,879, both filed June 11, 2014; 62/010,329 and 62/010,441, each filed June 10, 2014; 61/939,228 and 61/939,242, each filed February 12, 2014; 61/980,012, filed April 15, 2014; 62/038,358, filed August 17, 2014; 62/054,490, 62/055,484, 62/055,460 and 62/055,487, each filed September 25, 2014; and 62/069,243, filed October 27, 2014. Reference is also made to US provisional patent applications Nos. 62/055,484, 62/055,460, and 62/055,487, filed September 25, 2014; US provisional patent application 61/980,012, filed April 15, 2014; and US provisional patent application 61/939,242 filed February 12, 2014. Reference is made to PCT application designating, inter alia, the United States, application No. PCT/US14/41806, filed June 10, 2014. Reference is made to US provisional patent application 61/930,214 filed on January 22, 2014. Reference is made to US provisional patent applications 61/915,251; 61/915,260 and 61/915,267, each filed on December 12, 2013. Reference is made to US provisional patent application USSN 61/980,012 filed April 15, 2014. Reference is made to PCT application designating, inter alia, the United States, application No. PCT/US14/41806, filed June 10, 2014. Reference is made to US provisional patent application 61/930,214 filed on January 22, 2014. Reference is made to US provisional patent applications 61/915,251; 61/915,260 and 61/915,267, each filed on December 12, 2013.

[0054] Mention is also made of US application 62/091,455, filed, 12-Dec-14, PROTECTED GUIDE RNAS (PGRNAS); US application 62/096,708, 24-Dec-14, PROTECTED GUIDE RNAS (PGRNAS); US application 62/091,462, 12-Dec-14, DEAD GUIDES FOR CRISPR TRANSCRIPTION FACTORS; US application 62/096,324, 23-Dec-14, DEAD GUIDES FOR CRISPR TRANSCRIPTION FACTORS; US application 62/091,456, 12-Dec-14, ESCORTED AND FUNCTIONALIZED GUIDES FOR CRISPR-CAS SYSTEMS; US application 62/091,461, 12-Dec-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR GENOME EDITING AS TO HEMATOPOETIC STEM CELLS (HSCs); US application 62/094,903, 19-Dec-14, UNBIASED IDENTIFICATION OF DOUBLE-STRAND BREAKS AND GENOMIC REARRANGEMENT BY GENOME-WISE INSERT CAPTURE SEQUENCING; US application 62/096,761, 24-Dec-14, ENGINEERING OF SYSTEMS, METHODS AND OPTIMIZED ENZYME AND GUIDE SCAFFOLDS FOR SEQUENCE MANIPULATION; US application 62/098,059, 30-Dec-14, RNA-TARGETING SYSTEM; US application 62/096,656, 24-Dec-14, CRISPR HAVING OR ASSOCIATED WITH DESTABILIZATION DOMAINS; US application 62/096,697, 24-Dec-14, CRISPR HAVING OR ASSOCIATED WITH AAV; US application 62/098,158, 30-Dec-14, ENGINEERED CRISPR COMPLEX INSERTIONAL TARGETING SYSTEMS; US application 62/151,052, 22-Apr-15, CELLULAR TARGETING FOR EXTRACELLULAR EXOSOMAL REPORTING; US application 62/054,490, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR TARGETING DISORDERS AND DISEASES USING PARTICLE DELIVERY COMPONENTS; US application 62/055,484, 25-Sep-14, SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/087,537, 4-Dec-14, SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/054,651, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR MODELING COMPETITION OF MULTIPLE CANCER MUTATIONS IN VIVO; US application 62/067,886, 23-Oct-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR MODELING COMPETITION OF MULTIPLE CANCER MUTATIONS IN VIVO; US

application 62/054,675, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS IN NEURONAL CELLS/TISSUES; US application 62/054,528, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS IN IMMUNE DISEASES OR DISORDERS; US application 62/055,454, 25-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR TARGETING DISORDERS AND DISEASES USING CELL PENETRATION PEPTIDES (CPP); US application 62/055,460, 25-Sep-14, MULTIFUNCTIONAL-CRISPR COMPLEXES AND/OR OPTIMIZED ENZYME LINKED FUNCTIONAL-CRISPR COMPLEXES; US application 62/087,475, 4-Dec-14, FUNCTIONAL SCREENING WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/055,487, 25-Sep-14, FUNCTIONAL SCREENING WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/087,546, 4-Dec-14, MULTIFUNCTIONAL CRISPR COMPLEXES AND/OR OPTIMIZED ENZYME LINKED FUNCTIONAL-CRISPR COMPLEXES; and US application 62/098,285, 30-Dec-14, CRISPR MEDIATED IN VIVO MODELING AND GENETIC SCREENING OF TUMOR GROWTH AND METASTASIS.

[0055] Each of these patents, patent publications, and applications, and all documents cited therein or during their prosecution (“appln cited documents”) and all documents cited or referenced in the appln cited documents, together with any instructions, descriptions, product specifications, and product sheets for any products mentioned therein or in any document therein and incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention. All documents (e.g., these patents, patent publications and applications and the appln cited documents) are incorporated herein by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

[0056] Also with respect to general information on CRISPR-Cas Systems, mention is made of the following (also hereby incorporated herein by reference):

- *Multiplex genome engineering using CRISPR/Cas systems.* Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., & Zhang, F. *Science* Feb 15;339(6121):819-23 (2013);
- *RNA-guided editing of bacterial genomes using CRISPR-Cas systems.* Jiang W., Bikard D., Cox D., Zhang F, Marraffini LA. *Nat Biotechnol* Mar;31(3):233-9 (2013);

- *One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR/Cas-Mediated Genome Engineering.* Wang H., Yang H., Shivalila CS., Dawlaty MM., Cheng AW., Zhang F., Jaenisch R. *Cell* May 9;153(4):910-8 (2013);
- *Optical control of mammalian endogenous transcription and epigenetic states.* Konermann S, Brigham MD, Trevino AE, Hsu PD, Heidenreich M, Cong L, Platt RJ, Scott DA, Church GM, Zhang F. *Nature*. 2013 Aug 22;500(7463):472-6. doi: 10.1038/Nature12466. Epub 2013 Aug 23;
- *Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity.* Ran, FA., Hsu, PD., Lin, CY., Gootenberg, JS., Konermann, S., Trevino, AE., Scott, DA., Inoue, A., Matoba, S., Zhang, Y., & Zhang, F. *Cell* Aug 28. pii: S0092-8674(13)01015-5. (2013);
- *DNA targeting specificity of RNA-guided Cas9 nucleases.* Hsu, P., Scott, D., Weinstein, J., Ran, FA., Konermann, S., Agarwala, V., Li, Y., Fine, E., Wu, X., Shalem, O., Cradick, TJ., Marraffini, LA., Bao, G., & Zhang, F. *Nat Biotechnol* doi:10.1038/nbt.2647 (2013);
- *Genome engineering using the CRISPR-Cas9 system.* Ran, FA., Hsu, PD., Wright, J., Agarwala, V., Scott, DA., Zhang, F. *Nature Protocols* Nov;8(11):2281-308. (2013);
- *Genome-Scale CRISPR-Cas9 Knockout Screening in Human Cells.* Shalem, O., Sanjana, NE., Hartenian, E., Shi, X., Scott, DA., Mikkelsen, T., Heckl, D., Ebert, BL., Root, DE., Doench, JG., Zhang, F. *Science* Dec 12. (2013). [Epub ahead of print];
- *Crystal structure of cas9 in complex with guide RNA and target DNA.* Nishimasu, H., Ran, FA., Hsu, PD., Konermann, S., Shehata, SI., Dohmae, N., Ishitani, R., Zhang, F., Nureki, O. *Cell* Feb 27. (2014). 156(5):935-49;
- *Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells.* Wu X., Scott DA., Kriz AJ., Chiu AC., Hsu PD., Dadon DB., Cheng AW., Trevino AE., Konermann S., Chen S., Jaenisch R., Zhang F., Sharp PA. *Nat Biotechnol*. (2014) Apr 20. doi: 10.1038/nbt.2889,
- *CRISPR-Cas9 Knockin Mice for Genome Editing and Cancer Modeling,* Platt et al., *Cell* 159(2): 440-455 (2014) DOI: 10.1016/j.cell.2014.09.014,
- *Development and Applications of CRISPR-Cas9 for Genome Engineering,* Hsu et al., *Cell* 157, 1262-1278 (June 5, 2014) (Hsu 2014),
- *Genetic screens in human cells using the CRISPR/Cas9 system,* Wang et al., *Science*. 2014 January 3; 343(6166): 80–84. doi:10.1126/science.1246981,
- *Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation,* Doench et al., *Nature Biotechnology* published online 3 September 2014; doi:10.1038/nbt.3026, and

- *In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9*, Swiech et al, Nature Biotechnology ; published online 19 October 2014; doi:10.1038/nbt.3055.
- *Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex*, Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, Hsu PD, Habib N, Gootenberg JS, Nishimasu H, Nureki O, Zhang F., Nature. Jan 29;517(7536):583-8 (2015).
- *A split-Cas9 architecture for inducible genome editing and transcription modulation*, Zetsche B, Volz SE, Zhang F., (published online 02 February 2015) Nat Biotechnol. Feb;33(2):139-42 (2015);
- *Genome-wide CRISPR Screen in a Mouse Model of Tumor Growth and Metastasis*, Chen S, Sanjana NE, Zheng K, Shalem O, Lee K, Shi X, Scott DA, Song J, Pan JQ, Weissleder R, Lee H, Zhang F, Sharp PA. Cell 160, 1246–1260, March 12, 2015 (multiplex screen in mouse), and
- *In vivo genome editing using Staphylococcus aureus Cas9*, Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV, Sharp PA, Zhang F., (published online 01 April 2015), Nature. Apr 9;520(7546):186-91 (2015).
- *High-throughput functional genomics using CRISPR-Cas9*, Shalem et al., Nature Reviews Genetics 16, 299-311 (May 2015).
- *Sequence determinants of improved CRISPR sgRNA design*, Xu et al., Genome Research 25, 1147-1157 (August 2015).
- *A Genome-wide CRISPR Screen in Primary Immune Cells to Dissect Regulatory Networks*, Parnas et al., Cell 162, 675-686 (July 30, 2015).
- *CRISPR/Cas9 cleavage of viral DNA efficiently suppresses hepatitis B virus*, Ramanan et al., Scientific Reports 5:10833. doi: 10.1038/srep10833 (June 2, 2015).
- *Crystal Structure of Staphylococcus aureus Cas9*, Nishimasu et al., Cell 162, 1113-1126 (Aug. 27, 2015).
- *BCL11A enhancer dissection by Cas9-mediated in situ saturating mutagenesis*, Canver et al., Nature 527(7577):192-7 (Nov. 12, 2015) doi: 10.1038/nature15521. Epub 2015 Sep 16. each of which is incorporated herein by reference, and discussed briefly below:

- Cong *et al.* engineered type II CRISPR/Cas systems for use in eukaryotic cells based on both *Streptococcus thermophilus* Cas9 and also *Streptococcus pyogenes* Cas9 and demonstrated that Cas9 nucleases can be directed by short RNAs to induce precise cleavage of DNA in human and mouse cells. Their study further showed that Cas9 as converted into a nicking enzyme can be used to facilitate homology-directed repair in eukaryotic cells with minimal mutagenic activity. Additionally, their study demonstrated that multiple guide sequences can be encoded into a single CRISPR array to enable simultaneous editing of several at endogenous genomic loci sites within the mammalian genome, demonstrating easy programmability and wide applicability of the RNA-guided nuclease technology. This ability to use RNA to program sequence specific DNA cleavage in cells defined a new class of genome engineering tools. These studies further showed that other CRISPR loci are likely to be transplantable into mammalian cells and can also mediate mammalian genome cleavage. Importantly, it can be envisaged that several aspects of the CRISPR/Cas system can be further improved to increase its efficiency and versatility.
- Jiang *et al.* used the clustered, regularly interspaced, short palindromic repeats (CRISPR)–associated Cas9 endonuclease complexed with dual-RNAs to introduce precise mutations in the genomes of *Streptococcus pneumoniae* and *Escherichia coli*. The approach relied on dual-RNA:Cas9-directed cleavage at the targeted genomic site to kill unmutated cells and circumvents the need for selectable markers or counter-selection systems. The study reported reprogramming dual-RNA:Cas9 specificity by changing the sequence of short CRISPR RNA (crRNA) to make single- and multinucleotide changes carried on editing templates. The study showed that simultaneous use of two crRNAs enabled multiplex mutagenesis. Furthermore, when the approach was used in combination with recombineering, in *S. pneumoniae*, nearly 100% of cells that were recovered using the described approach contained the desired mutation, and in *E. coli*, 65% that were recovered contained the mutation.
- Wang *et al.* (2013) used the CRISPR/Cas system for the one-step generation of mice carrying mutations in multiple genes which were traditionally generated in multiple steps by sequential recombination in embryonic stem cells and/or time-consuming intercrossing

of mice with a single mutation. The CRISPR/Cas system will greatly accelerate the *in vivo* study of functionally redundant genes and of epistatic gene interactions.

- Konermann *et al.* addressed the need in the art for versatile and robust technologies that enable optical and chemical modulation of DNA-binding domains based CRISPR Cas9 enzyme and also Transcriptional Activator Like Effectors.
- Ran *et al.* (2013-A) described an approach that combined a Cas9 nickase mutant with paired guide RNAs to introduce targeted double-strand breaks. This addresses the issue of the Cas9 nuclease from the microbial CRISPR-Cas system being targeted to specific genomic loci by a guide sequence, which can tolerate certain mismatches to the DNA target and thereby promote undesired off-target mutagenesis. Because individual nicks in the genome are repaired with high fidelity, simultaneous nicking via appropriately offset guide RNAs is required for double-stranded breaks and extends the number of specifically recognized bases for target cleavage. The authors demonstrated that using paired nicking can reduce off-target activity by 50- to 1,500-fold in cell lines and to facilitate gene knockout in mouse zygotes without sacrificing on-target cleavage efficiency. This versatile strategy enables a wide variety of genome editing applications that require high specificity.
- Hsu *et al.* (2013) characterized SpCas9 targeting specificity in human cells to inform the selection of target sites and avoid off-target effects. The study evaluated >700 guide RNA variants and SpCas9-induced indel mutation levels at >100 predicted genomic off-target loci in 293T and 293FT cells. The authors that SpCas9 tolerates mismatches between guide RNA and target DNA at different positions in a sequence-dependent manner, sensitive to the number, position and distribution of mismatches. The authors further showed that SpCas9-mediated cleavage is unaffected by DNA methylation and that the dosage of SpCas9 and sgRNA can be titrated to minimize off-target modification. Additionally, to facilitate mammalian genome engineering applications, the authors reported providing a web-based software tool to guide the selection and validation of target sequences as well as off-target analyses.
- Ran *et al.* (2013-B) described a set of tools for Cas9-mediated genome editing *via* non-homologous end joining (NHEJ) or homology-directed repair (HDR) in mammalian cells, as well as generation of modified cell lines for downstream functional studies. To minimize off-target cleavage, the authors further described a double-nicking strategy using

the Cas9 nickase mutant with paired guide RNAs. The protocol provided by the authors experimentally derived guidelines for the selection of target sites, evaluation of cleavage efficiency and analysis of off-target activity. The studies showed that beginning with target design, gene modifications can be achieved within as little as 1–2 weeks, and modified clonal cell lines can be derived within 2–3 weeks.

- Shalem *et al.* described a new way to interrogate gene function on a genome-wide scale. Their studies showed that delivery of a genome-scale CRISPR-Cas9 knockout (GeCKO) library targeted 18,080 genes with 64,751 unique guide sequences enabled both negative and positive selection screening in human cells. First, the authors showed use of the GeCKO library to identify genes essential for cell viability in cancer and pluripotent stem cells. Next, in a melanoma model, the authors screened for genes whose loss is involved in resistance to vemurafenib, a therapeutic that inhibits mutant protein kinase BRAF. Their studies showed that the highest-ranking candidates included previously validated genes NF1 and MED12 as well as novel hits NF2, CUL3, TADA2B, and TADA1. The authors observed a high level of consistency between independent guide RNAs targeting the same gene and a high rate of hit confirmation, and thus demonstrated the promise of genome-scale screening with Cas9.
- Nishimasu *et al.* reported the crystal structure of *Streptococcus pyogenes* Cas9 in complex with sgRNA and its target DNA at 2.5 Å resolution. The structure revealed a bilobed architecture composed of target recognition and nuclease lobes, accommodating the sgRNA:DNA heteroduplex in a positively charged groove at their interface. Whereas the recognition lobe is essential for binding sgRNA and DNA, the nuclease lobe contains the HNH and RuvC nuclease domains, which are properly positioned for cleavage of the complementary and non-complementary strands of the target DNA, respectively. The nuclease lobe also contains a carboxyl-terminal domain responsible for the interaction with the protospacer adjacent motif (PAM). This high-resolution structure and accompanying functional analyses have revealed the molecular mechanism of RNA-guided DNA targeting by Cas9, thus paving the way for the rational design of new, versatile genome-editing technologies.
- Wu *et al.* mapped genome-wide binding sites of a catalytically inactive Cas9 (dCas9) from *Streptococcus pyogenes* loaded with single guide RNAs (sgRNAs) in mouse embryonic

stem cells (mESCs). The authors showed that each of the four sgRNAs tested targets dCas9 to between tens and thousands of genomic sites, frequently characterized by a 5-nucleotide seed region in the sgRNA and an NGG protospacer adjacent motif (PAM). Chromatin inaccessibility decreases dCas9 binding to other sites with matching seed sequences; thus 70% of off-target sites are associated with genes. The authors showed that targeted sequencing of 295 dCas9 binding sites in mESCs transfected with catalytically active Cas9 identified only one site mutated above background levels. The authors proposed a two-state model for Cas9 binding and cleavage, in which a seed match triggers binding but extensive pairing with target DNA is required for cleavage.

- Platt *et al.* established a Cre-dependent Cas9 knockin mouse. The authors demonstrated *in vivo* as well as *ex vivo* genome editing using adeno-associated virus (AAV)-, lentivirus-, or particle-mediated delivery of guide RNA in neurons, immune cells, and endothelial cells.
- Hsu *et al.* (2014) is a review article that discusses generally CRISPR-Cas9 history from yogurt to genome editing, including genetic screening of cells.
- Wang *et al.* (2014) relates to a pooled, loss-of-function genetic screening approach suitable for both positive and negative selection that uses a genome-scale lentiviral single guide RNA (sgRNA) library.
- Doench *et al.* created a pool of sgRNAs, tiling across all possible target sites of a panel of six endogenous mouse and three endogenous human genes and quantitatively assessed their ability to produce null alleles of their target gene by antibody staining and flow cytometry. The authors showed that optimization of the PAM improved activity and also provided an on-line tool for designing sgRNAs.
- Swiech *et al.* demonstrate that AAV-mediated SpCas9 genome editing can enable reverse genetic studies of gene function in the brain.
- Konermann *et al.* (2015) discusses the ability to attach multiple effector domains, e.g., transcriptional activator, functional and epigenomic regulators at appropriate positions on the guide such as stem or tetraloop with and without linkers.
- Zetsche *et al.* demonstrates that the Cas9 enzyme can be split into two and hence the assembly of Cas9 for activation can be controlled.
- Chen *et al.* relates to multiplex screening by demonstrating that a genome-wide *in vivo* CRISPR-Cas9 screen in mice reveals genes regulating lung metastasis.

- Ran *et al.* (2015) relates to SaCas9 and its ability to edit genomes and demonstrates that one cannot extrapolate from biochemical assays. Shalem *et al.* (2015) described ways in which catalytically inactive Cas9 (dCas9) fusions are used to synthetically repress (CRISPRi) or activate (CRISPRa) expression, showing advances using Cas9 for genome-scale screens, including arrayed and pooled screens, knockout approaches that inactivate genomic loci and strategies that modulate transcriptional activity.
- Shalem *et al.* (2015) described ways in which catalytically inactive Cas9 (dCas9) fusions are used to synthetically repress (CRISPRi) or activate (CRISPRa) expression, showing advances using Cas9 for genome-scale screens, including arrayed and pooled screens, knockout approaches that inactivate genomic loci and strategies that modulate transcriptional activity.
- Xu *et al.* (2015) assessed the DNA sequence features that contribute to single guide RNA (sgRNA) efficiency in CRISPR-based screens. The authors explored efficiency of CRISPR/Cas9 knockout and nucleotide preference at the cleavage site. The authors also found that the sequence preference for CRISPRi/a is substantially different from that for CRISPR/Cas9 knockout.
- Parnas *et al.* (2015) introduced genome-wide pooled CRISPR-Cas9 libraries into dendritic cells (DCs) to identify genes that control the induction of tumor necrosis factor (Tnf) by bacterial lipopolysaccharide (LPS). Known regulators of Tlr4 signaling and previously unknown candidates were identified and classified into three functional modules with distinct effects on the canonical responses to LPS.
- Ramanan *et al.* (2015) demonstrated cleavage of viral episomal DNA (cccDNA) in infected cells. The HBV genome exists in the nuclei of infected hepatocytes as a 3.2kb double-stranded episomal DNA species called covalently closed circular DNA (cccDNA), which is a key component in the HBV life cycle whose replication is not inhibited by current therapies. The authors showed that sgRNAs specifically targeting highly conserved regions of HBV robustly suppresses viral replication and depleted cccDNA.
- Nishimasu *et al.* (2015) reported the crystal structures of SaCas9 in complex with a single guide RNA (sgRNA) and its double-stranded DNA targets, containing the 5'-TTGAAT-3' PAM and the 5'-TTGGGT-3' PAM. A structural comparison of SaCas9 with SpCas9

highlighted both structural conservation and divergence, explaining their distinct PAM specificities and orthologous sgRNA recognition.

- Slaymaker et al (2015) reported the use of structure-guided protein engineering to improve the specificity of *Streptococcus pyogenes* Cas9 (SpCas9). The authors developed "enhanced specificity" SpCas9 (eSpCas9) variants which maintained robust on-target cleavage with reduced off-target effects.

[0057] Mention is also made of Tsai et al, "Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing," *Nature Biotechnology* 32(6): 569-77 (2014) which is not believed to be prior art to the instant invention or application, but which may be considered in the practice of the instant invention. Mention is also made of Konermann et al., "Genome-scale transcription activation by an engineered CRISPR-Cas9 complex," doi:10.1038/nature14136, incorporated herein by reference.

[0058] In general, the CRISPR-Cas or CRISPR system is as used in the foregoing documents, such as WO 2014/093622 (PCT/US2013/074667) and refers collectively to transcripts and other elements involved in the expression of or directing the activity of CRISPR-associated ("Cas") genes, including sequences encoding a Cas gene, a *tracr* (trans-activating CRISPR) sequence (e.g. *tracr*RNA or an active partial *tracr*RNA), a *tracr*-mate sequence (encompassing a "direct repeat" and a *tracr*RNA-processed partial direct repeat in the context of an endogenous CRISPR system), a guide sequence (also referred to as a "spacer" in the context of an endogenous CRISPR system), or "RNA(s)" as that term is herein used (e.g., RNA(s) to guide Cas9, e.g. CRISPR RNA and transactivating (*tracr*) RNA or a single guide RNA (sgRNA) (chimeric RNA)) or other sequences and transcripts from a CRISPR locus. In general, a CRISPR system is characterized by elements that promote the formation of a CRISPR complex at the site of a target sequence (also referred to as a protospacer in the context of an endogenous CRISPR system). In the context of formation of a CRISPR complex, "target sequence" refers to a sequence to which a guide sequence is designed to have complementarity, where hybridization between a target sequence and a guide sequence promotes the formation of a CRISPR complex. A target sequence may comprise any polynucleotide, such as DNA or RNA polynucleotides. In some embodiments, a target sequence is located in the nucleus or cytoplasm of a cell. In some embodiments, direct repeats may be identified *in silico* by searching for repetitive motifs that fulfill any or all of the following criteria: 1. found in a 2Kb window of genomic sequence flanking the type II CRISPR locus; 2. span from

20 to 50 bp; and 3. interspaced by 20 to 50 bp. In some embodiments, 2 of these criteria may be used, for instance 1 and 2, 2 and 3, or 1 and 3. In some embodiments, all 3 criteria may be used. In some embodiments it may be preferred in a CRISPR complex that the tracr sequence has one or more hairpins and is 30 or more nucleotides in length, 40 or more nucleotides in length, or 50 or more nucleotides in length; the guide sequence is between 10 to 30 nucleotides in length, the CRISPR/Cas enzyme is a Type II Cas9 enzyme. In embodiments of the invention the terms guide sequence and guide RNA are used interchangeably as in foregoing cited documents such as WO 2014/093622 (PCT/US2013/074667). In general, a guide sequence is any polynucleotide sequence having sufficient complementarity with a target polynucleotide sequence to hybridize with the target sequence and direct sequence-specific binding of a CRISPR complex to the target sequence. In some embodiments, the degree of complementarity between a guide sequence and its corresponding target sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more. Optimal alignment may be determined with the use of any suitable algorithm for aligning sequences, non-limiting example of which include the Smith-Waterman algorithm, the Needleman-Wunsch algorithm, algorithms based on the Burrows-Wheeler Transform (e.g. the Burrows Wheeler Aligner), ClustalW, Clustal X, BLAT, Novoalign (Novocraft Technologies; available at www.novocraft.com), ELAND (Illumina, San Diego, CA), SOAP (available at soap.genomics.org.cn), and Maq (available at maq.sourceforge.net). In some embodiments, a guide sequence is about or more than about 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 75, or more nucleotides in length. In some embodiments, a guide sequence is less than about 75, 50, 45, 40, 35, 30, 25, 20, 15, 12, or fewer nucleotides in length. Preferably the guide sequence is 10 - 30 nucleotides long. The ability of a guide sequence to direct sequence-specific binding of a CRISPR complex to a target sequence may be assessed by any suitable assay. For example, the components of a CRISPR system sufficient to form a CRISPR complex, including the guide sequence to be tested, may be provided to a host cell having the corresponding target sequence, such as by transfection with vectors encoding the components of the CRISPR sequence, followed by an assessment of preferential cleavage within the target sequence, such as by Surveyor assay as described herein. Similarly, cleavage of a target polynucleotide sequence may be evaluated in a test tube by providing the target sequence, components of a CRISPR complex, including the guide sequence to be tested and a control guide

sequence different from the test guide sequence, and comparing binding or rate of cleavage at the target sequence between the test and control guide sequence reactions. Other assays are possible, and will occur to those skilled in the art. A guide sequence may be selected to target any target sequence. In some embodiments, the target sequence is a sequence within a genome of a cell. Exemplary target sequences include those that are unique in the target genome. For example, for the *S. pyogenes* Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNNXGG where NNNNNNNNNNNNNXGG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. A unique target sequence in a genome may include an *S. pyogenes* Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNNXGG where NNNNNNNNNNNNNXGG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. For the *S. thermophilus* CRISPR1 Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNNXXAGAAW where NNNNNNNNNNNNXXAGAAW (N is A, G, T, or C; X can be anything; and W is A or T) has a single occurrence in the genome. A unique target sequence in a genome may include an *S. thermophilus* CRISPR1 Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNNXXAGAAW where NNNNNNNNNNNNXXAGAAW (N is A, G, T, or C; X can be anything; and W is A or T) has a single occurrence in the genome. For the *S. pyogenes* Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNNXGGXG where NNNNNNNNNNNNNXGGXG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. A unique target sequence in a genome may include an *S. pyogenes* Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNNXGGXG where NNNNNNNNNNNNXGGXG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. In each of these sequences "M" may be A, G, T, or C, and need not be considered in identifying a sequence as unique. In some embodiments, a guide sequence is selected to reduce the degree secondary structure within the guide sequence. In some embodiments, about or less than about 75%, 50%, 40%, 30%, 25%, 20%, 15%, 10%, 5%, 1%, or fewer of the nucleotides of the guide sequence participate in self-complementary base pairing when optimally folded. Optimal folding may be determined by any suitable polynucleotide folding algorithm. Some programs are based on calculating the minimal Gibbs free energy. An example of one such algorithm is mFold, as described by Zuker and Stiegler (Nucleic Acids Res. 9 (1981), 133-148). Another example folding

algorithm is the online webserver RNAfold, developed at Institute for Theoretical Chemistry at the University of Vienna, using the centroid structure prediction algorithm (see e.g. A.R. Gruber et al., 2008, Cell 106(1): 23-24; and PA Carr and GM Church, 2009, Nature Biotechnology 27(12): 1151-62).

[0059] In general, a tracr mate sequence includes any sequence that has sufficient complementarity with a tracr sequence to promote one or more of: (1) excision of a guide sequence flanked by tracr mate sequences in a cell containing the corresponding tracr sequence; and (2) formation of a CRISPR complex at a target sequence, wherein the CRISPR complex comprises the tracr mate sequence hybridized to the tracr sequence. In general, degree of complementarity is with reference to the optimal alignment of the tracr mate sequence and tracr sequence, along the length of the shorter of the two sequences. Optimal alignment may be determined by any suitable alignment algorithm, and may further account for secondary structures, such as self-complementarity within either the tracr sequence or tracr mate sequence. In some embodiments, the degree of complementarity between the tracr sequence and tracr mate sequence along the length of the shorter of the two when optimally aligned is about or more than about 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 97.5%, 99%, or higher. In some embodiments, the tracr sequence is about or more than about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, or more nucleotides in length. In some embodiments, the tracr sequence and tracr mate sequence are contained within a single transcript, such that hybridization between the two produces a transcript having a secondary structure, such as a hairpin. In an embodiment of the invention, the transcript or transcribed polynucleotide sequence has at least two or more hairpins. In preferred embodiments, the transcript has two, three, four or five hairpins. In a further embodiment of the invention, the transcript has at most five hairpins. In a hairpin structure the portion of the sequence 5' of the final "N" and upstream of the loop corresponds to the tracr mate sequence, and the portion of the sequence 3' of the loop corresponds to the tracr sequence. Further non-limiting examples of single polynucleotides comprising a guide sequence, a tracr mate sequence, and a tracr sequence are as follows (listed 5' to 3'), where "N" represents a base of a guide sequence, the first block of lower case letters represent the tracr mate sequence, and the second block of lower case letters represent the tracr sequence, and the final poly-T sequence represents the transcription terminator: (1)

NNNNNNNNNNNNNNNNNNNNggttttgtactctcaagatttaGAAAtaatcttgagaagctacaaagataa

ggcttcatgccgaaatcaacaccctgtcattttatggcagggtgttttcgtatttaaTTTTTT; (2)

NNNNNNNNNNNNNNNNNNNNNNNNNNgtttttgtactctcaGAAAtgcagaagctacaaagataaggcttcatgccg
aatcaacaccctgtcattttatggcagggtgttttcgtatttaaTTTTTT; (3)

NNNNNNNNNNNNNNNNNNNNNNNNNNgtttttgtactctcaGAAAtgcagaagctacaaagataaggcttcatgccg
aatcaacaccctgtcattttatggcagggtgtTTTTTT; (4)

NNNNNNNNNNNNNNNNNNNNNNNNNNgttttagagctaGAAAtagcaagttaaataaggctagccgttatcaact
gaaaaagtggcaccgagtcggtgcTTTTTT; (5)

NNNNNNNNNNNNNNNNNNNNNNNNNNgttttagagctaGAAATAGcaagttaaataaggctagccgttatcaac
ttgaaaaagtgtTTTTTT; and (6)

NNNNNNNNNNNNNNNNNNNNNNNNNNgttttagagctagAAATAGcaagttaaataaggctagccgttatcaTT
TTTTTT. In some embodiments, sequences (1) to (3) are used in combination with Cas9 from *S. thermophilus* CRISPR1. In some embodiments, sequences (4) to (6) are used in combination with Cas9 from *S. pyogenes*. In some embodiments, the tracr sequence is a separate transcript from a transcript comprising the tracr mate sequence.

[0060] In some embodiments, candidate tracrRNA may be subsequently predicted by sequences that fulfill any or all of the following criteria: 1. sequence homology to direct repeats (motif search in Geneious with up to 18-bp mismatches); 2. presence of a predicted Rho-independent transcriptional terminator in direction of transcription; and 3. stable hairpin secondary structure between tracrRNA and direct repeat. In some embodiments, 2 of these criteria may be used, for instance 1 and 2, 2 and 3, or 1 and 3. In some embodiments, all 3 criteria may be used.

[0061] In some embodiments, chimeric synthetic guide RNAs (sgRNAs) designs may incorporate at least 12 bp of duplex structure between the direct repeat and tracrRNA.

[0062] For minimization of toxicity and off-target effect, it will be important to control the concentration of CRISPR enzyme mRNA and guide RNA delivered. Optimal concentrations of CRISPR enzyme mRNA and guide RNA can be determined by testing different concentrations in a cellular or non-human eukaryote animal model and using deep sequencing to analyze the extent of modification at potential off-target genomic loci. For example, for the guide sequence targeting 5'-GAGTCCGAGCAGAAGAAGAA-3' in the EMX1 gene of the human genome, deep sequencing can be used to assess the level of modification at the following two off-target loci, 1: 5'-GAGTCCTAGCAGGAGAAGAA-3' and 2: 5'-GAGTCTAAGCAGAAGAAGAA-3'. The

concentration that gives the highest level of on-target modification while minimizing the level of off-target modification should be chosen for in vivo delivery. Alternatively, to minimize the level of toxicity and off-target effect, CRISPR enzyme nickase mRNA (for example *S. pyogenes* Cas9 with the D10A mutation) can be delivered with a pair of guide RNAs targeting a site of interest. The two guide RNAs need to be spaced as follows. Guide sequences and strategies to minimize toxicity and off-target effects can be as in WO 2014/093622 (PCT/US2013/074667).

[0063] The CRISPR system is derived advantageously from a type II CRISPR system. In some embodiments, one or more elements of a CRISPR system is derived from a particular organism comprising an endogenous CRISPR system, such as *Streptococcus pyogenes*. In preferred embodiments of the invention, the CRISPR system is a type II CRISPR system and the Cas enzyme is Cas9, which catalyzes DNA cleavage. Non-limiting examples of Cas proteins include Cas1, Cas1B, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8, Cas9 (also known as Csn1 and Csx12), Cas10, Csy1, Csy2, Csy3, Cse1, Cse2, Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16, CsaX, Csx3, Csx1, Csx15, Csf1, Csf2, Csf3, Csf4, homologues thereof, or modified versions thereof.

[0064] In some embodiments, the unmodified CRISPR enzyme has DNA cleavage activity, such as Cas9. In some embodiments, the CRISPR enzyme directs cleavage of one or both strands at the location of a target sequence, such as within the target sequence and/or within the complement of the target sequence. In some embodiments, the CRISPR enzyme directs cleavage of one or both strands within about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, 200, 500, or more base pairs from the first or last nucleotide of a target sequence. In some embodiments, a vector encodes a CRISPR enzyme that is mutated to with respect to a corresponding wild-type enzyme such that the mutated CRISPR enzyme lacks the ability to cleave one or both strands of a target polynucleotide containing a target sequence. For example, an aspartate-to-alanine substitution (D10A) in the RuvC I catalytic domain of Cas9 from *S. pyogenes* converts Cas9 from a nuclease that cleaves both strands to a nickase (cleaves a single strand). Other examples of mutations that render Cas9 a nickase include, without limitation, H840A, N854A, and N863A. As a further example, two or more catalytic domains of Cas9 (RuvC I, RuvC II, and RuvC III or the HNH domain) may be mutated to produce a mutated Cas9 substantially lacking all DNA cleavage activity. In some embodiments, a D10A mutation is combined with one or more of H840A,

N854A, or N863A mutations to produce a Cas9 enzyme substantially lacking all DNA cleavage activity. In some embodiments, a CRISPR enzyme is considered to substantially lack all DNA cleavage activity when the DNA cleavage activity of the mutated enzyme is about no more than 25%, 10%, 5%, 1%, 0.1%, 0.01%, or less of the DNA cleavage activity of the non-mutated form of the enzyme; an example can be when the DNA cleavage activity of the mutated form is nil or negligible as compared with the non-mutated form. Where the enzyme is not SpCas9, mutations may be made at any or all residues corresponding to positions 10, 762, 840, 854, 863 and/or 986 of SpCas9 (which may be ascertained for instance by standard sequence comparison tools). In particular, any or all of the following mutations are preferred in SpCas9: D10A, E762A, H840A, N854A, N863A and/or D986A; as well as conservative substitution for any of the replacement amino acids is also envisaged. The same (or conservative substitutions of these mutations) at corresponding positions in other Cas9s are also preferred. Particularly preferred are D10 and H840 in SpCas9. However, in other Cas9s, residues corresponding to SpCas9 D10 and H840 are also preferred. For instance, an N580 or N580A mutation in SaCas9. Orthologs of SpCas9 can be used in the practice of the invention. A Cas enzyme may be identified Cas9 as this can refer to the general class of enzymes that share homology to the biggest nuclease with multiple nuclease domains from the type II CRISPR system. Most preferably, the Cas9 enzyme is from, or is derived from, spCas9 (*S. pyogenes* Cas9) or saCas9 (*S. aureus* Cas9). StCas9[™] refers to wild type Cas9 from *S. thermophilus*, the protein sequence of which is given in the SwissProt database under accession number G3ECR1. Similarly, *S. pyogenes* Cas9 or spCas9 is included in SwissProt under accession number Q99ZW2. By derived, Applicants mean that the derived enzyme is largely based, in the sense of having a high degree of sequence homology with, a wildtype enzyme, but that it has been mutated (modified) in some way as described herein. It will be appreciated that the terms Cas and CRISPR enzyme are generally used herein interchangeably, unless otherwise apparent. As mentioned above, many of the residue numberings used herein refer to the Cas9 enzyme from the type II CRISPR locus in *Streptococcus pyogenes*. However, it will be appreciated that this invention includes many more Cas9s from other species of microbes, such as SpCas9, SaCa9, St1Cas9 and so forth. Enzymatic action by Cas9 derived from *Streptococcus pyogenes* or any closely related Cas9 generates double stranded breaks at target site sequences which hybridize to 20 nucleotides of the guide sequence and that have a protospacer-adjacent motif (PAM) sequence (examples include NGG/NRG or a PAM that can be determined as

described herein) following the 20 nucleotides of the target sequence. CRISPR activity through Cas9 for site-specific DNA recognition and cleavage is defined by the guide sequence, the tracr sequence that hybridizes in part to the guide sequence and the PAM sequence. More aspects of the CRISPR system are described in Karginov and Hannon, *The CRISPR system: small RNA-guided defence in bacteria and archaea*, *Mole Cell* 2010, January 15; 37(1): 7. The type II CRISPR locus from *Streptococcus pyogenes* SF370, which contains a cluster of four genes Cas9, Cas1, Cas2, and Csn1, as well as two non-coding RNA elements, tracrRNA and a characteristic array of repetitive sequences (direct repeats) interspaced by short stretches of non-repetitive sequences (spacers, about 30bp each). In this system, targeted DNA double-strand break (DSB) is generated in four sequential steps. First, two non-coding RNAs, the pre-crRNA array and tracrRNA, are transcribed from the CRISPR locus. Second, tracrRNA hybridizes to the direct repeats of pre-crRNA, which is then processed into mature crRNAs containing individual spacer sequences. Third, the mature crRNA:tracrRNA complex directs Cas9 to the DNA target consisting of the protospacer and the corresponding PAM via heteroduplex formation between the spacer region of the crRNA and the protospacer DNA. Finally, Cas9 mediates cleavage of target DNA upstream of PAM to create a DSB within the protospacer. A pre-crRNA array consisting of a single spacer flanked by two direct repeats (DRs) is also encompassed by the term "tracr-mate sequences"). In certain embodiments, Cas9 may be constitutively present or inducibly present or conditionally present or administered or delivered. Cas9 optimization may be used to enhance function or to develop new functions, one can generate chimeric Cas9 proteins. And Cas9 may be used as a generic DNA binding protein.

[0065] Typically, in the context of an endogenous CRISPR system, formation of a CRISPR complex (comprising a guide sequence hybridized to a target sequence and complexed with one or more Cas proteins) results in cleavage of one or both strands in or near (e.g. within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, or more base pairs from) the target sequence. Without wishing to be bound by theory, the tracr sequence, which may comprise or consist of all or a portion of a wild-type tracr sequence (e.g. about or more than about 20, 26, 32, 45, 48, 54, 63, 67, 85, or more nucleotides of a wild-type tracr sequence), may also form part of a CRISPR complex, such as by hybridization along at least a portion of the tracr sequence to all or a portion of a tracr mate sequence that is operably linked to the guide sequence.

[0066] An example of a codon optimized sequence, is in this instance a sequence optimized for expression in a eukaryote, e.g., humans (i.e. being optimized for expression in humans), or for

another eukaryote, animal or mammal as herein discussed; see, e.g., SaCas9 human codon optimized sequence in WO 2014/093622 (PCT/US2013/074667). Whilst this is preferred, it will be appreciated that other examples are possible and codon optimization for a host species other than human, or for codon optimization for specific organs is known. In some embodiments, an enzyme coding sequence encoding a CRISPR enzyme is codon optimized for expression in particular cells, such as eukaryotic cells. The eukaryotic cells may be those of or derived from a particular organism, such as a mammal, including but not limited to human, or non-human eukaryote or animal or mammal as herein discussed, e.g., mouse, rat, rabbit, dog, livestock, or non-human mammal or primate. In some embodiments, processes for modifying the germ line genetic identity of human beings and/or processes for modifying the genetic identity of animals which are likely to cause them suffering without any substantial medical benefit to man or animal, and also animals resulting from such processes, may be excluded. In general, codon optimization refers to a process of modifying a nucleic acid sequence for enhanced expression in the host cells of interest by replacing at least one codon (e.g. about or more than about 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more codons) of the native sequence with codons that are more frequently or most frequently used in the genes of that host cell while maintaining the native amino acid sequence. Various species exhibit particular bias for certain codons of a particular amino acid. Codon bias (differences in codon usage between organisms) often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, among other things, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization. Codon usage tables are readily available, for example, at the "Codon Usage Database" available at www.kazusa.or.jp/codon/ and these tables can be adapted in a number of ways. See Nakamura, Y., et al. "Codon usage tabulated from the international DNA sequence databases: status for the year 2000" *Nucl. Acids Res.* 28:292 (2000). Computer algorithms for codon optimizing a particular sequence for expression in a particular host cell are also available, such as Gene Forge (Aptagen; Jacobus, PA), are also available. In some embodiments, one or more codons (e.g. 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more, or all codons) in a sequence encoding a CRISPR enzyme correspond to the most frequently used codon for a particular amino acid.

[0067] In some embodiments, a vector encodes a CRISPR enzyme comprising one or more nuclear localization sequences (NLSs), such as about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs. In some embodiments, the CRISPR enzyme comprises about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs at or near the amino-terminus, about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs at or near the carboxy-terminus, or a combination of these (e.g. zero or at least one or more NLS at the amino-terminus and zero or at one or more NLS at the carboxy terminus). When more than one NLS is present, each may be selected independently of the others, such that a single NLS may be present in more than one copy and/or in combination with one or more other NLSs present in one or more copies. In a preferred embodiment of the invention, the CRISPR enzyme comprises at most 6 NLSs. In some embodiments, an NLS is considered near the N- or C-terminus when the nearest amino acid of the NLS is within about 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50, or more amino acids along the polypeptide chain from the N- or C-terminus. Non-limiting examples of NLSs include an NLS sequence derived from: the NLS of the SV40 virus large T-antigen, having the amino acid sequence PKKKRKV; the NLS from nucleoplasmin (e.g. the nucleoplasmin bipartite NLS with the sequence KRPAATKKAGQAKKKK); the c-myc NLS having the amino acid sequence PAAKRVKLD or RQRRNELKRSP; the hRNPA1 M9 NLS having the sequence NQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGQGY; the sequence RMRIZFKNKGKDTAELRRRRVEVSVELRKAKKDEQILKRRNV of the IBB domain from importin-alpha; the sequences VSRKRPRP and PPKKARED of the myoma T protein; the sequence POPKKKPL of human p53; the sequence SALIKKKKKMAP of mouse c-abl IV; the sequences DRLRR and PKQKKRK of the influenza virus NS1; the sequence RKLKKKIKKL of the Hepatitis virus delta antigen; the sequence REKKKFLKRR of the mouse Mx1 protein; the sequence KRKGDEVDGVDEVAKKKSKK of the human poly(ADP-ribose) polymerase; and the sequence RKCLQAGMNLEARKTKK of the steroid hormone receptors (human) glucocorticoid. In general, the one or more NLSs are of sufficient strength to drive accumulation of the CRISPR enzyme in a detectable amount in the nucleus of a eukaryotic cell. In general, strength of nuclear localization activity may derive from the number of NLSs in the CRISPR enzyme, the particular NLS(s) used, or a combination of these factors. Detection of accumulation in the nucleus may be performed by any suitable technique. For example, a detectable marker may be fused to the CRISPR enzyme, such that location within a cell may be visualized, such as in combination with a

means for detecting the location of the nucleus (e.g. a stain specific for the nucleus such as DAPI). Cell nuclei may also be isolated from cells, the contents of which may then be analyzed by any suitable process for detecting protein, such as immunohistochemistry, Western blot, or enzyme activity assay. Accumulation in the nucleus may also be determined indirectly, such as by an assay for the effect of CRISPR complex formation (e.g. assay for DNA cleavage or mutation at the target sequence, or assay for altered gene expression activity affected by CRISPR complex formation and/or CRISPR enzyme activity), as compared to a control not exposed to the CRISPR enzyme or complex, or exposed to a CRISPR enzyme lacking the one or more NLSs.

[0068] Aspects of the invention relate to the expression of the gene product being decreased or a template polynucleotide being further introduced into a DNA or RNA molecule encoding the gene product or an intervening sequence being excised precisely by allowing the two 5' overhangs to reanneal and ligate or the activity or function of the gene product being altered or the expression of the gene product being increased. In an embodiment of the invention, the gene product is a protein. In certain Cas9 systems, sgRNA pairs creating 5' overhangs with less than 8bp overlap between the guide sequences (offset greater than -8 bp) were able to mediate detectable indel formation ; and each guide used in these assays is able to efficiently induce indels when paired with wildtype Cas9, indicating that the relative positions of the guide pairs are the most important parameters in predicting double nicking activity. Since Cas9n and Cas9H840A nick opposite strands of DNA, substitution of Cas9n with Cas9H840A with a given sgRNA pair should have resulted in the inversion of the overhang type; but no indel formation is observed as with Cas9H840A indicating that Cas9H840A is a CRISPR enzyme substantially lacking all DNA cleavage activity (which is when the DNA cleavage activity of the mutated enzyme is about no more than 25%, 10%, 5%, 1%, 0.1%, 0.01%, or less of the DNA cleavage activity of the non-mutated form of the enzyme; whereby an example can be when the DNA cleavage activity of the mutated form is nil or negligible as compared with the non-mutated form, e.g., when no indel formation is observed as with Cas9H840A in the eukaryotic system in contrast to the biochemical or prokaryotic systems). Nonetheless, a pair of sgRNAs that will generate a 5' overhang with Cas9n should in principle generate the corresponding 3' overhang instead, and double nicking. Therefore, sgRNA pairs that lead to the generation of a 3' overhang with Cas9n can be used with another mutated Cas9 to generate a 5' overhang, and double nicking. Accordingly, in some embodiments, a recombination template is also provided. A recombination template may be a

component of another vector as described herein, contained in a separate vector, or provided as a separate polynucleotide. In some embodiments, a recombination template is designed to serve as a template in homologous recombination, such as within or near a target sequence nicked or cleaved by a CRISPR enzyme as a part of a CRISPR complex. A template polynucleotide may be of any suitable length, such as about or more than about 10, 15, 20, 25, 50, 75, 100, 150, 200, 500, 1000, or more nucleotides in length. In some embodiments, the template polynucleotide is complementary to a portion of a polynucleotide comprising the target sequence. When optimally aligned, a template polynucleotide might overlap with one or more nucleotides of a target sequences (e.g. about or more than about 1, 5, 10, 15, 20, or more nucleotides). In some embodiments, when a template sequence and a polynucleotide comprising a target sequence are optimally aligned, the nearest nucleotide of the template polynucleotide is within about 1, 5, 10, 15, 20, 25, 50, 75, 100, 200, 300, 400, 500, 1000, 5000, 10000, or more nucleotides from the target sequence.

[0069] In some embodiments, one or more vectors driving expression of one or more elements of a CRISPR system are introduced into a host cell such that expression of the elements of the CRISPR system direct formation of a CRISPR complex at one or more target sites. For example, a Cas enzyme, a guide sequence linked to a tracr-mate sequence, and a tracr sequence could each be operably linked to separate regulatory elements on separate vectors. Or, RNA(s) of the CRISPR System can be delivered to a transgenic Cas9 animal or mammal, e.g., an animal or mammal that constitutively or inducibly or conditionally expresses Cas9; or an animal or mammal that is otherwise expressing Cas9 or has cells containing Cas9, such as by way of prior administration thereto of a vector or vectors that code for and express *in vivo* Cas9. Alternatively, two or more of the elements expressed from the same or different regulatory elements, may be combined in a single vector, with one or more additional vectors providing any components of the CRISPR system not included in the first vector. CRISPR system elements that are combined in a single vector may be arranged in any suitable orientation, such as one element located 5' with respect to ("upstream" of) or 3' with respect to ("downstream" of) a second element. The coding sequence of one element may be located on the same or opposite strand of the coding sequence of a second element, and oriented in the same or opposite direction. In some embodiments, a single promoter drives expression of a transcript encoding a CRISPR enzyme and one or more of the guide sequence, tracr mate sequence (optionally operably linked to the guide sequence), and a tracr

sequence embedded within one or more intron sequences (e.g. each in a different intron, two or more in at least one intron, or all in a single intron). In some embodiments, the CRISPR enzyme, guide sequence, tracr mate sequence, and tracr sequence are operably linked to and expressed from the same promoter. Delivery vehicles, vectors, particles, nanoparticles, formulations and components thereof for expression of one or more elements of a CRISPR system are as used in herein-cited documents, such as WO 2014/093622 (PCT/US2013/074667). In some embodiments, a vector comprises one or more insertion sites, such as a restriction endonuclease recognition sequence (also referred to as a "cloning site"). In some embodiments, one or more insertion sites (e.g. about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more insertion sites) are located upstream and/or downstream of one or more sequence elements of one or more vectors. In some embodiments, a vector comprises an insertion site upstream of a tracr mate sequence, and optionally downstream of a regulatory element operably linked to the tracr mate sequence, such that following insertion of a guide sequence into the insertion site and upon expression the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell. In some embodiments, a vector comprises two or more insertion sites, each insertion site being located between two tracr mate sequences so as to allow insertion of a guide sequence at each site. In such an arrangement, the two or more guide sequences may comprise two or more copies of a single guide sequence, two or more different guide sequences, or combinations of these. When multiple different guide sequences are used, a single expression construct may be used to target CRISPR activity to multiple different, corresponding target sequences within a cell. For example, a single vector may comprise about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, or more guide sequences. In some embodiments, about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more such guide-sequence-containing vectors may be provided, and optionally delivered to a cell. In some embodiments, a vector comprises a regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, such as a Cas protein. CRISPR enzyme or CRISPR enzyme mRNA or CRISPR guide RNA or RNA(s) can be delivered separately; and advantageously at least one of these is delivered via a particle complex. CRISPR enzyme mRNA can be delivered prior to the guide RNA to give time for CRISPR enzyme to be expressed. CRISPR enzyme mRNA might be administered 1-12 hours (preferably around 2-6 hours) prior to the administration of guide RNA. Alternatively, CRISPR enzyme mRNA and guide RNA can be administered together. Advantageously, a second booster dose of guide RNA can be administered

1-12 hours (preferably around 2-6 hours) after the initial administration of CRISPR enzyme mRNA + guide RNA. Additional administrations of CRISPR enzyme mRNA and/or guide RNA might be useful to achieve the most efficient levels of genome modification.

[0070] In one aspect, the invention provides methods for using one or more elements of a CRISPR system. The CRISPR complex of the invention provides an effective means for modifying a target polynucleotide. The CRISPR complex of the invention has a wide variety of utility including modifying (e.g., deleting, inserting, translocating, inactivating, activating) a target polynucleotide in a multiplicity of cell types. As such the CRISPR complex of the invention has a broad spectrum of applications in, e.g., gene therapy, drug screening, disease diagnosis, and prognosis. An exemplary CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within the target polynucleotide. The guide sequence is linked to a tracr mate sequence, which in turn hybridizes to a tracr sequence. In one embodiment, this invention provides a method of cleaving a target polynucleotide. The method comprises modifying a target polynucleotide using a CRISPR complex that binds to the target polynucleotide and effect cleavage of said target polynucleotide. Typically, the CRISPR complex of the invention, when introduced into a cell, creates a break (e.g., a single or a double strand break) in the genome sequence. For example, the method can be used to cleave a disease gene in a cell. The break created by the CRISPR complex can be repaired by a repair processes such as the error prone non-homologous end joining (NHEJ) pathway or the high fidelity homology-directed repair (HDR). During these repair process, an exogenous polynucleotide template can be introduced into the genome sequence. In some methods, the HDR process is used modify genome sequence. For example, an exogenous polynucleotide template comprising a sequence to be integrated flanked by an upstream sequence and a downstream sequence is introduced into a cell. The upstream and downstream sequences share sequence similarity with either side of the site of integration in the chromosome. Where desired, a donor polynucleotide can be DNA, e.g., a DNA plasmid, a bacterial artificial chromosome (BAC), a yeast artificial chromosome (YAC), a viral vector, a linear piece of DNA, a PCR fragment, a naked nucleic acid, or a nucleic acid complexed with a delivery vehicle such as a liposome or poloxamer. The exogenous polynucleotide template comprises a sequence to be integrated (e.g., a mutated gene). The sequence for integration may be a sequence endogenous or exogenous to the cell. Examples of a sequence to be integrated include polynucleotides encoding a protein or a non-coding RNA (e.g., a microRNA). Thus, the sequence

for integration may be operably linked to an appropriate control sequence or sequences. Alternatively, the sequence to be integrated may provide a regulatory function. The upstream and downstream sequences in the exogenous polynucleotide template are selected to promote recombination between the chromosomal sequence of interest and the donor polynucleotide. The upstream sequence is a nucleic acid sequence that shares sequence similarity with the genome sequence upstream of the targeted site for integration. Similarly, the downstream sequence is a nucleic acid sequence that shares sequence similarity with the chromosomal sequence downstream of the targeted site of integration. The upstream and downstream sequences in the exogenous polynucleotide template can have 75%, 80%, 85%, 90%, 95%, or 100% sequence identity with the targeted genome sequence. Preferably, the upstream and downstream sequences in the exogenous polynucleotide template have about 95%, 96%, 97%, 98%, 99%, or 100% sequence identity with the targeted genome sequence. In some methods, the upstream and downstream sequences in the exogenous polynucleotide template have about 99% or 100% sequence identity with the targeted genome sequence. An upstream or downstream sequence may comprise from about 20 bp to about 2500 bp, for example, about 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300, 2400, or 2500 bp. In some methods, the exemplary upstream or downstream sequence have about 200 bp to about 2000 bp, about 600 bp to about 1000 bp, or more particularly about 700 bp to about 1000 bp. In some methods, the exogenous polynucleotide template may further comprise a marker. Such a marker may make it easy to screen for targeted integrations. Examples of suitable markers include restriction sites, fluorescent proteins, or selectable markers. The exogenous polynucleotide template of the invention can be constructed using recombinant techniques (see, for example, Sambrook et al., 2001 and Ausubel et al., 1996). In a method for modifying a target polynucleotide by integrating an exogenous polynucleotide template, a double stranded break is introduced into the genome sequence by the CRISPR complex, the break is repaired via homologous recombination an exogenous polynucleotide template such that the template is integrated into the genome. The presence of a double-stranded break facilitates integration of the template. In other embodiments, this invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell. The method comprises increasing or decreasing expression of a target polynucleotide by using a CRISPR complex that binds to the polynucleotide. In some methods, a target polynucleotide can be inactivated to effect the modification of the expression in a cell. For

example, upon the binding of a CRISPR complex to a target sequence in a cell, the target polynucleotide is inactivated such that the sequence is not transcribed, the coded protein is not produced, or the sequence does not function as the wild-type sequence does. For example, a protein or microRNA coding sequence may be inactivated such that the protein or microRNA or pre-microRNA transcript is not produced. In some methods, a control sequence can be inactivated such that it no longer functions as a control sequence. As used herein, "control sequence" refers to any nucleic acid sequence that effects the transcription, translation, or accessibility of a nucleic acid sequence. Examples of a control sequence include, a promoter, a transcription terminator, and an enhancer are control sequences. The target polynucleotide of a CRISPR complex can be any polynucleotide endogenous or exogenous to the eukaryotic cell. For example, the target polynucleotide can be a polynucleotide residing in the nucleus of the eukaryotic cell. The target polynucleotide can be a sequence coding a gene product (e.g., a protein) or a non-coding sequence (e.g., a regulatory polynucleotide or a junk DNA). Examples of target polynucleotides include a sequence associated with a signaling biochemical pathway, e.g., a signaling biochemical pathway-associated gene or polynucleotide. Examples of target polynucleotides include a disease associated gene or polynucleotide. A "disease-associated" gene or polynucleotide refers to any gene or polynucleotide which is yielding transcription or translation products at an abnormal level or in an abnormal form in cells derived from a disease-affected tissues compared with tissues or cells of a non disease control. It may be a gene that becomes expressed at an abnormally high level; it may be a gene that becomes expressed at an abnormally low level, where the altered expression correlates with the occurrence and/or progression of the disease. A disease-associated gene also refers to a gene possessing mutation(s) or genetic variation that is directly responsible or is in linkage disequilibrium with a gene(s) that is responsible for the etiology of a disease. The transcribed or translated products may be known or unknown, and may be at a normal or abnormal level. The target polynucleotide of a CRISPR complex can be any polynucleotide endogenous or exogenous to the eukaryotic cell. For example, the target polynucleotide can be a polynucleotide residing in the nucleus of the eukaryotic cell. The target polynucleotide can be a sequence coding a gene product (e.g., a protein) or a non-coding sequence (e.g., a regulatory polynucleotide or a junk DNA). Without wishing to be bound by theory, it is believed that the target sequence should be associated with a PAM (protospacer adjacent motif); that is, a short sequence recognized by the CRISPR complex. The precise sequence and length requirements for the PAM differ depending

on the CRISPR enzyme used, but PAMs are typically 2-5 base pair sequences adjacent the protospacer (that is, the target sequence) Examples of PAM sequences are given throughout, and the skilled person will be able to identify further PAM sequences for use with a given CRISPR enzyme. In some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. In one aspect, the invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the polynucleotide such that said binding results in increased or decreased expression of said polynucleotide; wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. Similar considerations and conditions apply as above for methods of modifying a target polynucleotide. In fact, these sampling, culturing and re-introduction options apply across the aspects of the present invention. In one aspect, the invention provides for methods of modifying a target polynucleotide in a eukaryotic cell, which may be *in vivo*, *ex vivo* or *in vitro*. In some embodiments, the method comprises sampling a cell or population of cells from a human or non-human animal, and modifying the cell or cells. Culturing may occur at any stage *ex vivo*. The cell or cells may even be re-introduced into the non-human animal or plant. For re-introduced cells it is particularly preferred that the cells are stem cells.

[0071] Indeed, in some aspects of the invention, the CRISPR complex may comprise a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence, wherein said guide sequence may be linked to a tracr mate sequence which in turn may hybridize to a tracr sequence.

[0072] The invention relates to the engineering and optimization of systems, methods and compositions used for the control of gene expression involving sequence targeting, such as genome perturbation or gene-editing, that relate to the CRISPR-Cas system and components thereof. In advantageous embodiments, the Cas enzyme is Cas9. An advantage of the present methods is that the CRISPR system minimizes or avoids off-target binding and its resulting side

effects. This is achieved using systems arranged to have a high degree of sequence specificity for the target DNA.

Self-inactivating systems

[0073] Once intended alterations have been introduced, such as by editing intended copies of a gene in the genome of a cell, continued CRISPR- Cas9 system expression in that cell is no longer necessary. Indeed, sustained expression would be undesirable in certain casein case of off-target effects at unintended genomic sites, etc. Thus time-limited expression would be useful. Inducible expression offers one approach, but in addition Applicants have engineered a Self-Inactivating CRISPR-Cas9 system that relies on the use of a non-coding guide target sequence within the CRISPR vector itself. Thus, after expression begins, the CRISPR system will lead to its own destruction, but before destruction is complete it will have time to edit the genomic copies of the target gene (which, with a normal point mutation in a diploid cell, requires at most two edits). Simply, the self inactivating CRISPR-Cas system includes additional RNA (i.e., guide RNA) that targets the coding sequence for the CRISPR enzyme itself or that targets one or more non-coding guide target sequences complementary to unique sequences present in one or more of the following: (a) within the promoter driving expression of the non-coding RNA elements, (b) within the promoter driving expression of the Cas9 gene, (c) within 100bp of the ATG translational start codon in the Cas9 coding sequence, (d) within the inverted terminal repeat (iTR) of a viral delivery vector, e.g., in an AAV genome.

[0074] In embodiments of the invention the terms guide sequence and guide RNA are used interchangeably as in herein cited documents such as WO 2014/093622 (PCT/US2013/074667). In general, a guide sequence is any polynucleotide sequence having sufficient complementarity with a target polynucleotide sequence to hybridize with the target sequence and direct sequence-specific binding of a CRISPR complex to the target sequence. In some embodiments, the degree of complementarity between a guide sequence and its corresponding target sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more. Optimal alignment may be determined with the use of any suitable algorithm for aligning sequences, non-limiting example of which include the Smith-Waterman algorithm, the Needleman-Wunsch algorithm, algorithms based on the Burrows-Wheeler Transform (e.g., the Burrows Wheeler Aligner), ClustalW, Clustal X, BLAT, Novoalign (Novocraft Technologies; available at www.novocraft.com), ELAND

(Illumina, San Diego, CA), SOAP (available at soap.genomics.org.cn), and Maq (available at maq.sourceforge.net). In some embodiments, a guide sequence is about or more than about 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 75, or more nucleotides in length. In some embodiments, a guide sequence is less than about 75, 50, 45, 40, 35, 30, 25, 20, 15, 12, or fewer nucleotides in length. Preferably the guide sequence is 10 - 30 nucleotides long. The ability of a guide sequence to direct sequence-specific binding of a CRISPR complex to a target sequence may be assessed by any suitable assay. For example, the components of a CRISPR system sufficient to form a CRISPR complex, including the guide sequence to be tested, may be provided to a host cell having the corresponding target sequence, such as by transfection with vectors encoding the components of the CRISPR sequence, followed by an assessment of preferential cleavage within the target sequence, such as by Surveyor assay as described herein (for DNA cleavage) or RT-qPCR analysis to determine RNA interference. Similarly, cleavage of a target polynucleotide sequence may be evaluated in a test tube by providing the target sequence, components of a CRISPR complex, including the guide sequence to be tested and a control guide sequence different from the test guide sequence, and comparing binding or rate of cleavage at the target sequence between the test and control guide sequence reactions. Other assays are possible, and will occur to those skilled in the art. A guide sequence may be selected to target any target sequence. In some embodiments, the target sequence is a sequence within a genome of a cell. Exemplary target sequences include those that are unique in the target genome.

[0075] In an embodiment, a guide RNA molecule can be targeted to a known transcription response elements (e.g., promoters, enhancers, etc.), a known upstream activating sequences, and/or sequences of unknown or known function that are suspected of being able to control expression of the target DNA.

[0076] In some methods, a target polynucleotide can be inactivated to effect the modification of the expression in a cell. For example, upon the binding of a CRISPR complex to a target sequence in a cell, the target polynucleotide is inactivated such that the sequence is not transcribed, the coded protein is not produced, or the sequence does not function as the wild-type sequence does. For example, a protein or microRNA coding sequence may be inactivated such that the protein is not produced.

[0077] In certain embodiments, the CRISPR enzyme comprises one or more mutations selected from the group consisting of D917A, E1006A and D1225A and/or the one or more mutations is in a RuvC domain of the CRISPR enzyme or is a mutation as otherwise as discussed herein. In some embodiments, the CRISPR enzyme has one or more mutations in a catalytic domain, wherein when transcribed, the direct repeat sequence forms a single stem loop and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the enzyme further comprises a functional domain. In some embodiments, the functional domain is a transcriptional activation domain, preferably VP64. In some embodiments, the functional domain is a transcription repression domain, preferably KRAB. In some embodiments, the transcription repression domain is SID, or concatemers of SID (eg SID4X). In some embodiments, the functional domain is an epigenetic modifying domain, such that an epigenetic modifying enzyme is provided. In some embodiments, the functional domain is an activation domain, which may be the P65 activation domain.

Delivery generally

[0078] Through this disclosure and the knowledge in the art, CRISPR-Cas system, or components thereof or nucleic acid molecules thereof (including, for instance HDR template) or nucleic acid molecules encoding or providing components thereof may be delivered by a delivery system herein described both generally and in detail.

[0079] Vector delivery, e.g., plasmid, viral delivery: The CRISPR enzyme, for instance a Cas9, and/or any of the present RNAs, for instance a guide RNA, can be delivered using any suitable vector, e.g., plasmid or viral vectors, such as adeno associated virus (AAV), lentivirus, adenovirus or other viral vector types, or combinations thereof. Cas9 and one or more guide RNAs can be packaged into one or more vectors, e.g., plasmid or viral vectors. In some embodiments, the vector, e.g., plasmid or viral vector is delivered to the tissue of interest by, for example, an intramuscular injection, while other times the delivery is via intravenous, transdermal, intranasal, oral, mucosal, or other delivery methods. Such delivery may be either via a single dose, or multiple doses. One skilled in the art understands that the actual dosage to be delivered herein may vary greatly depending upon a variety of factors, such as the vector choice, the target cell, organism, or tissue, the general condition of the subject to be treated, the degree of transformation/modification sought, the administration route, the administration mode, the type of transformation/modification sought, etc.

[0080] Such a dosage may further contain, for example, a carrier (water, saline, ethanol, glycerol, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, etc.), a diluent, a pharmaceutically-acceptable carrier (e.g., phosphate-buffered saline), a pharmaceutically-acceptable excipient, and/or other compounds known in the art. The dosage may further contain one or more pharmaceutically acceptable salts such as, for example, a mineral acid salt such as a hydrochloride, a hydrobromide, a phosphate, a sulfate, etc.; and the salts of organic acids such as acetates, propionates, malonates, benzoates, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, gels or gelling materials, flavorings, colorants, microspheres, polymers, suspension agents, etc. may also be present herein. In addition, one or more other conventional pharmaceutical ingredients, such as preservatives, humectants, suspending agents, surfactants, antioxidants, anticaking agents, fillers, chelating agents, coating agents, chemical stabilizers, etc. may also be present, especially if the dosage form is a reconstitutable form. Suitable exemplary ingredients include microcrystalline cellulose, carboxymethylcellulose sodium, polysorbate 80, phenylethyl alcohol, chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, parachlorophenol, gelatin, albumin and a combination thereof. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991) which is incorporated by reference herein.

[0081] In an embodiment herein the delivery is via an adenovirus, which may be at a single booster dose containing at least 1×10^5 particles (also referred to as particle units, pu) of adenoviral vector. In an embodiment herein, the dose preferably is at least about 1×10^6 particles (for example, about 1×10^6 - 1×10^{12} particles), more preferably at least about 1×10^7 particles, more preferably at least about 1×10^8 particles (e.g., about 1×10^8 - 1×10^{11} particles or about 1×10^8 - 1×10^{12} particles), and most preferably at least about 1×10^9 particles (e.g., about 1×10^9 - 1×10^{10} particles or about 1×10^9 - 1×10^{12} particles), or even at least about 1×10^{10} particles (e.g., about 1×10^{10} - 1×10^{12} particles) of the adenoviral vector. Alternatively, the dose comprises no more than about 1×10^{14} particles, preferably no more than about 1×10^{13} particles, even more preferably no more than about 1×10^{12} particles, even more preferably no more than about 1×10^{11} particles, and most preferably no more than about 1×10^{10} particles (e.g., no more than about 1×10^9 articles). Thus, the dose may contain a single dose of adenoviral vector with, for example, about 1×10^6 particle units (pu), about 2×10^6 pu, about 4×10^6 pu, about 1×10^7 pu, about 2×10^7 pu, about $4 \times$

10^7 pu, about 1×10^8 pu, about 2×10^8 pu, about 4×10^8 pu, about 1×10^9 pu, about 2×10^9 pu, about 4×10^9 pu, about 1×10^{10} pu, about 2×10^{10} pu, about 4×10^{10} pu, about 1×10^{11} pu, about 2×10^{11} pu, about 4×10^{11} pu, about 1×10^{12} pu, about 2×10^{12} pu, or about 4×10^{12} pu of adenoviral vector. See, for example, the adenoviral vectors in U.S. Patent No. 8,454,972 B2 to Nabel, et. al., granted on June 4, 2013; incorporated by reference herein, and the dosages at col 29, lines 36-58 thereof. In an embodiment herein, the adenovirus is delivered via multiple doses.

[0082] In an embodiment herein, the delivery is via an AAV. A therapeutically effective dosage for in vivo delivery of the AAV to a human is believed to be in the range of from about 20 to about 50 ml of saline solution containing from about 1×10^{10} to about 1×10^{10} functional AAV/ml solution. The dosage may be adjusted to balance the therapeutic benefit against any side effects. In an embodiment herein, the AAV dose is generally in the range of concentrations of from about 1×10^5 to 1×10^{50} genomes AAV, from about 1×10^8 to 1×10^{20} genomes AAV, from about 1×10^{10} to about 1×10^{16} genomes, or about 1×10^{11} to about 1×10^{16} genomes AAV. A human dosage may be about 1×10^{13} genomes AAV. Such concentrations may be delivered in from about 0.001 ml to about 100 ml, about 0.05 to about 50 ml, or about 10 to about 25 ml of a carrier solution. Other effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves. See, for example, U.S. Patent No. 8,404,658 B2 to Hajjar, et al., granted on March 26, 2013, at col. 27, lines 45-60.

[0083] In an embodiment herein the delivery is via a plasmid. In such plasmid compositions, the dosage should be a sufficient amount of plasmid to elicit a response. For instance, suitable quantities of plasmid DNA in plasmid compositions can be from about 0.1 to about 2 mg, or from about 1 μ g to about 10 μ g per 70 kg individual. Plasmids of the invention will generally comprise (i) a promoter; (ii) a sequence encoding a CRISPR enzyme, operably linked to said promoter; (iii) a selectable marker; (iv) an origin of replication; and (v) a transcription terminator downstream of and operably linked to (ii). The plasmid can also encode the RNA components of a CRISPR complex, but one or more of these may instead be encoded on a different vector.

[0084] The doses herein are based on an average 70 kg individual. The frequency of administration is within the ambit of the medical or veterinary practitioner (e.g., physician, veterinarian), or scientist skilled in the art. It is also noted that mice used in experiments are typically about 20g and from mice experiments one can scale up to a 70 kg individual.

[0085] In some embodiments the RNA molecules of the invention are delivered in liposome or lipofectin formulations and the like and can be prepared by methods well known to those skilled in the art. Such methods are described, for example, in U.S. Pat. Nos. 5,593,972, 5,589,466, and 5,580,859, which are herein incorporated by reference. Delivery systems aimed specifically at the enhanced and improved delivery of siRNA into mammalian cells have been developed, (see, for example, Shen et al FEBS Let. 2003, 539:111-114; Xia et al., Nat. Biotech. 2002, 20:1006-1010; Reich et al., Mol. Vision. 2003, 9: 210-216; Sorensen et al., J. Mol. Biol. 2003, 327: 761-766; Lewis et al., Nat. Gen. 2002, 32: 107-108 and Simeoni et al., NAR 2003, 31, 11: 2717-2724) and may be applied to the present invention. siRNA has recently been successfully used for inhibition of gene expression in primates (see for example. Tolentino et al., Retina 24(4):660 which may also be applied to the present invention.

[0086] Indeed, RNA delivery is a useful method of *in vivo* delivery. It is possible to deliver Cas9 and gRNA (and, for instance, HR repair template) into cells using liposomes or particles or nanoparticles. Thus delivery of the CRISPR enzyme, such as a Cas9 and/or delivery of the RNAs of the invention may be in RNA form and via microvesicles, liposomes or particles or nanoparticles. For example, Cas9 mRNA and gRNA can be packaged into liposomal particles for delivery *in vivo*. Liposomal transfection reagents such as lipofectamine from Life Technologies and other reagents on the market can effectively deliver RNA molecules into the liver.

[0087] Means of delivery of RNA also preferred include delivery of RNA via particles (Cho, S., Goldberg, M., Son, S., Xu, Q., Yang, F., Mei, Y., Bogatyrev, S., Langer, R. and Anderson, D., Lipid-like nanoparticles for small interfering RNA delivery to endothelial cells, Advanced Functional Materials, 19: 3112-3118, 2010) or exosomes (Schroeder, A., Levins, C., Cortez, C., Langer, R., and Anderson, D., Lipid-based nanotherapeutics for siRNA delivery, Journal of Internal Medicine, 267: 9-21, 2010, PMID: 20059641). Indeed, exosomes have been shown to be particularly useful in delivery siRNA, a system with some parallels to the CRISPR system. For instance, El-Andaloussi S, et al. ("Exosome-mediated delivery of siRNA in vitro and in vivo." Nat Protoc. 2012 Dec;7(12):2112-26. doi: 10.1038/nprot.2012.131. Epub 2012 Nov 15.) describe how exosomes are promising tools for drug delivery across different biological barriers and can be harnessed for delivery of siRNA in vitro and in vivo. Their approach is to generate targeted exosomes through transfection of an expression vector, comprising an exosomal protein fused with a peptide ligand. The exosomes are then purified and characterized from transfected cell

supernatant, then RNA is loaded into the exosomes. Delivery or administration according to the invention can be performed with exosomes, in particular but not limited to the brain. Vitamin E (α -tocopherol) may be conjugated with CRISPR Cas and delivered to the brain along with high density lipoprotein (HDL), for example in a similar manner as was done by Uno et al. (HUMAN GENE THERAPY 22:711–719 (June 2011)) for delivering short-interfering RNA (siRNA) to the brain. Mice were infused via Osmotic minipumps (model 1007D; Alzet, Cupertino, CA) filled with phosphate-buffered saline (PBS) or free TocsiBACE or Toc-siBACE/HDL and connected with Brain Infusion Kit 3 (Alzet). A brain-infusion cannula was placed about 0.5mm posterior to the bregma at midline for infusion into the dorsal third ventricle. Uno et al. found that as little as 3 nmol of Toc-siRNA with HDL could induce a target reduction in comparable degree by the same ICV infusion method. A similar dosage of CRISPR Cas conjugated to α -tocopherol and co-administered with HDL targeted to the brain may be contemplated for humans in the present invention, for example, about 3 nmol to about 3 μ mol of CRISPR Cas targeted to the brain may be contemplated. Zou et al. ((HUMAN GENE THERAPY 22:465-475 (April 2011)) describes a method of lentiviral-mediated delivery of short-hairpin RNAs targeting PKC γ for in vivo gene silencing in the spinal cord of rats. Zou et al. administered about 10 μ l of a recombinant lentivirus having a titer of 1×10^9 transducing units (TU)/ml by an intrathecal catheter. A similar dosage of CRISPR Cas expressed in a lentiviral vector targeted to the brain may be contemplated for humans in the present invention, for example, about 10-50 ml of CRISPR Cas targeted to the brain in a lentivirus having a titer of 1×10^9 transducing units (TU)/ml may be contemplated.

[0088] In terms of local delivery to the brain, this can be achieved in various ways. For instance, material can be delivered intrastrially *e.g.* by injection. Injection can be performed stereotactically via a craniotomy.

[0089] Enhancing NHEJ or HR efficiency is also helpful for delivery. It is preferred that NHEJ efficiency is enhanced by co-expressing end-processing enzymes such as Trex2 (Dumitrache et al. Genetics. 2011 August; 188(4): 787–797). It is preferred that HR efficiency is increased by transiently inhibiting NHEJ machineries such as Ku70 and Ku86. HR efficiency can also be increased by co-expressing prokaryotic or eukaryotic homologous recombination enzymes such as RecBCD, RecA.

Packaging and Promoters generally

[0090] Ways to package Cas9 coding nucleic acid molecules, e.g., DNA, into vectors, e.g., viral vectors, to mediate genome modification *in vivo* include:

To achieve NHEJ-mediated gene knockout:

Single virus vector:

Vector containing two or more expression cassettes:

Promoter-Cas9 coding nucleic acid molecule -terminator

Promoter-gRNA1-terminator

Promoter-gRNA2-terminator

Promoter-gRNA(N)-terminator (up to size limit of vector)

Double virus vector:

Vector 1 containing one expression cassette for driving the expression of Cas9

Promoter-Cas9 coding nucleic acid molecule-terminator

Vector 2 containing one more expression cassettes for driving the expression of one or more guideRNAs

Promoter-gRNA1-terminator

Promoter-gRNA(N)-terminator (up to size limit of vector)

To mediate homology-directed repair.

In addition to the single and double virus vector approaches described above, an additional vector is used to deliver a homology-direct repair template.

[0091] The promoter used to drive Cas9 coding nucleic acid molecule expression can include:

AAV ITR can serve as a promoter: this is advantageous for eliminating the need for an additional promoter element (which can take up space in the vector). The additional space freed up can be used to drive the expression of additional elements (gRNA, etc.). Also, ITR activity is relatively weaker, so can be used to reduce potential toxicity due to over expression of Cas9.

For ubiquitous expression, can use promoters: CMV, CAG, CBh, PGK, SV40, Ferritin heavy or light chains, etc.

For brain or other CNS expression, can use promoters: SynapsinI for all neurons, CaMKIIalpha for excitatory neurons, GAD67 or GAD65 or VGAT for GABAergic neurons, etc.

For liver expression, can use Albumin promoter.

For lung expression, can use SP-B.

For endothelial cells, can use ICAM.

For hematopoietic cells can use IFNbeta or CD45.

For Osteoblasts can use OG-2.

[0092] The promoter used to drive guide RNA can include:

Pol III promoters such as U6 or H1

Use of Pol II promoter and intronic cassettes to express gRNA

Adeno associated virus (AAV)

[0093] Cas9 and one or more guide RNA can be delivered using adeno associated virus (AAV), lentivirus, adenovirus or other plasmid or viral vector types, in particular, using formulations and doses from, for example, US Patents Nos. 8,454,972 (formulations, doses for adenovirus), 8,404,658 (formulations, doses for AAV) and 5,846,946 (formulations, doses for DNA plasmids) and from clinical trials and publications regarding the clinical trials involving lentivirus, AAV and adenovirus. For examples, for AAV, the route of administration, formulation and dose can be as in US Patent No. 8,454,972 and as in clinical trials involving AAV. For Adenovirus, the route of administration, formulation and dose can be as in US Patent No. 8,404,658 and as in clinical trials involving adenovirus. For plasmid delivery, the route of administration, formulation and dose can be as in US Patent No 5,846,946 and as in clinical studies involving plasmids. Doses may be based on or extrapolated to an average 70 kg individual (*e.g.* a male adult human), and can be adjusted for patients, subjects, mammals of different weight and species. Frequency of administration is within the ambit of the medical or veterinary practitioner (*e.g.*, physician, veterinarian), depending on usual factors including the age, sex, general health, other conditions of the patient or subject and the particular condition or symptoms being addressed. The viral vectors can be injected into the tissue of interest. For cell-type specific genome modification, the expression of Cas9 can be driven by a cell-type specific promoter. For example, liver-specific expression might use the Albumin promoter and neuron-specific expression (*e.g.* for targeting CNS disorders) might use the Synapsin I promoter.

[0094] In terms of *in vivo* delivery, AAV is advantageous over other viral vectors for a couple of reasons:

Low toxicity (this may be due to the purification method not requiring ultra centrifugation of cell particles that can activate the immune response)

Low probability of causing insertional mutagenesis because it doesn't integrate into the host genome.

[0095] AAV has a packaging limit of 4.5 or 4.75 Kb. This means that Cas9 as well as a promoter and transcription terminator have to be all fit into the same viral vector. Constructs larger than 4.5 or 4.75 Kb will lead to significantly reduced virus production. SpCas9 is quite large, the gene itself is over 4.1 Kb, which makes it difficult for packing into AAV. Therefore embodiments of the invention include utilizing homologs of Cas9 that are shorter. For example:

Species	Cas9 Size
<i>Corynebacter diphtheriae</i>	3252
<i>Eubacterium ventriosum</i>	3321
<i>Streptococcus pasteurianus</i>	3390
<i>Lactobacillus farciminis</i>	3378
<i>Sphaerochaeta globus</i>	3537
<i>Azospirillum B510</i>	3504
<i>Gluconacetobacter diazotrophicus</i>	3150
<i>Neisseria cinerea</i>	3246
<i>Roseburia intestinalis</i>	3420
<i>Parvibaculum lavamentivorans</i>	3111
<i>Staphylococcus aureus</i>	3159
<i>Nitratifactor salsuginis</i> DSM 16511	3396
<i>Campylobacter lari</i> CF89-12	3009
<i>Streptococcus thermophilus</i> LMD-9	3396

[0096] These species are therefore, in general, preferred Cas9 species.

[0097] As to AAV, the AAV can be AAV1, AAV2, AAV5 or any combination thereof. One can select the AAV of the AAV with regard to the cells to be targeted; e.g., one can select AAV serotypes 1, 2, 5 or a hybrid capsid AAV1, AAV2, AAV5 or any combination thereof for targeting brain or neuronal cells; and one can select AAV4 for targeting cardiac tissue. AAV8 is useful for delivery to the liver. The herein promoters and vectors are preferred individually. A tabulation of certain AAV serotypes as to these cells (see Grimm, D. et al, J. Virol. 82: 5887-5911 (2008)) is as follows:

Cell Line	AAV-1	AAV-2	AAV-3	AAV-4	AAV-5	AAV-6	AAV-8	AAV-9
Huh-7	13	100	2.5	0.0	0.1	10	0.7	0.0
HEK293	25	100	2.5	0.1	0.1	5	0.7	0.1
HeLa	3	100	2.0	0.1	6.7	1	0.2	0.1
HepG2	3	100	16.7	0.3	1.7	5	0.3	ND
Hep1A	20	100	0.2	1.0	0.1	1	0.2	0.0
911	17	100	11	0.2	0.1	17	0.1	ND
CHO	100	100	14	1.4	333	50	10	1.0
COS	33	100	33	3.3	5.0	14	2.0	0.5

MeWo	10	100	20	0.3	6.7	10	1.0	0.2
NIH3T3	10	100	2.9	2.9	0.3	10	0.3	ND
A549	14	100	20	ND	0.5	10	0.5	0.1
HT1180	20	100	10	0.1	0.3	33	0.5	0.1
Monocytes	1111	100	ND	ND	125	1429	ND	ND
Immature DC	2500	100	ND	ND	222	2857	ND	ND
Mature DC	2222	100	ND	ND	333	3333	ND	ND

Lentivirus

[0098] Lentiviruses are complex retroviruses that have the ability to infect and express their genes in both mitotic and post-mitotic cells. The most commonly known lentivirus is the human immunodeficiency virus (HIV), which uses the envelope glycoproteins of other viruses to target a broad range of cell types.

[0099] Lentiviruses may be prepared as follows. After cloning pCasES10 (which contains a lentiviral transfer plasmid backbone), HEK293FT at low passage (p=5) were seeded in a T-75 flask to 50% confluence the day before transfection in DMEM with 10% fetal bovine serum and without antibiotics. After 20 hours, media was changed to OptiMEM (serum-free) media and transfection was done 4 hours later. Cells were transfected with 10 µg of lentiviral transfer plasmid (pCasES10) and the following packaging plasmids: 5 µg of pMD2.G (VSV-g pseudotype), and 7.5µg of psPAX2 (gag/pol/rev/tat). Transfection was done in 4mL OptiMEM with a cationic lipid delivery agent (50uL Lipofectamine 2000 and 100ul Plus reagent). After 6 hours, the media was changed to antibiotic-free DMEM with 10% fetal bovine serum. These methods use serum during cell culture, but serum-free methods are preferred.

[00100] Lentivirus may be purified as follows. Viral supernatants were harvested after 48 hours. Supernatants were first cleared of debris and filtered through a 0.45µm low protein binding (PVDF) filter. They were then spun in a ultracentrifuge for 2 hours at 24,000 rpm. Viral pellets were resuspended in 50ul of DMEM overnight at 4C. They were then aliquotted and immediately frozen at -80°C.

[00101] In another embodiment, minimal non-primate lentiviral vectors based on the equine infectious anemia virus (EIAV) are also contemplated, especially for ocular gene therapy (see, e.g., Balagaan, *J Gene Med* 2006; 8: 275 – 285). In another embodiment, RetinoStat®, an equine infectious anemia virus-based lentiviral gene therapy vector that expresses angiostatic proteins endostatin and angiostatin that is delivered via a subretinal injection for the treatment of the web

form of age-related macular degeneration is also contemplated (see, e.g., Binley et al., HUMAN GENE THERAPY 23:980–991 (September 2012)) and this vector may be modified for the CRISPR-Cas system of the present invention.

[00102] In another embodiment, self-inactivating lentiviral vectors with an siRNA targeting a common exon shared by HIV tat/rev, a nucleolar-localizing TAR decoy, and an anti-CCR5-specific hammerhead ribozyme (see, e.g., DiGiusto et al. (2010) Sci Transl Med 2:36ra43) may be used/and or adapted to the CRISPR-Cas system of the present invention. A minimum of 2.5×10^6 CD34+ cells per kilogram patient weight may be collected and prestimulated for 16 to 20 hours in X-VIVO 15 medium (Lonza) containing 2 $\mu\text{mol/L}$ -glutamine, stem cell factor (100 ng/ml), Flt-3 ligand (Flt-3L) (100 ng/ml), and thrombopoietin (10 ng/ml) (CellGenix) at a density of 2×10^6 cells/ml. Prestimulated cells may be transduced with lentiviral at a multiplicity of infection of 5 for 16 to 24 hours in 75-cm² tissue culture flasks coated with fibronectin (25 mg/cm²) (RetroNectin, Takara Bio Inc.).

[00103] Lentiviral vectors have been disclosed as in the treatment for Parkinson's Disease, see, e.g., US Patent Publication No. 20120295960 and US Patent Nos. 7303910 and 7351585. Lentiviral vectors have also been disclosed for the treatment of ocular diseases, see e.g., US Patent Publication Nos. 20060281180, 20090007284, US20110117189; US20090017543; US20070054961, US20100317109. Lentiviral vectors have also been disclosed for delivery to the brain, see, e.g., US Patent Publication Nos. US20110293571; US20110293571, US20040013648, US20070025970, US20090111106 and US Patent No. US7259015.

RNA delivery

[00104] RNA delivery: The CRISPR enzyme, for instance a Cas9, and/or any of the present RNAs, for instance a guide RNA, can also be delivered in the form of RNA. Cas9 mRNA can be generated using *in vitro* transcription. For example, Cas9 mRNA can be synthesized using a PCR cassette containing the following elements: T7_promoter-kozak sequence (GCCACC)-Cas9-3' UTR from beta globin-polyA tail (a string of 120 or more adenines). The cassette can be used for transcription by T7 polymerase. Guide RNAs can also be transcribed using *in vitro* transcription from a cassette containing T7_promoter-GG-guide RNA sequence.

[00105] To enhance expression and reduce possible toxicity, the CRISPR enzyme-coding sequence and/or the guide RNA can be modified to include one or more modified nucleoside *e.g.* using pseudo-U or 5-Methyl-C.

[00106] mRNA delivery methods are especially promising for liver delivery currently.

[00107] Much clinical work on RNA delivery has focused on RNAi or antisense, but these systems can be adapted for delivery of RNA for implementing the present invention. References below to RNAi *etc.* should be read accordingly.

Particle delivery systems and/or formulations:

[00108] Several types of particle delivery systems and/or formulations are known to be useful in a diverse spectrum of biomedical applications. In general, a particle is defined as a small object that behaves as a whole unit with respect to its transport and properties. Particles are further classified according to diameter. Coarse particles cover a range between 2,500 and 10,000 nanometers. Fine particles are sized between 100 and 2,500 nanometers. Ultrafine particles, or nanoparticles, are generally between 1 and 100 nanometers in size. The basis of the 100-nm limit is the fact that novel properties that differentiate particles from the bulk material typically develop at a critical length scale of under 100 nm.

[00109] As used herein, a particle delivery system/formulation is defined as any biological delivery system/formulation which includes a particle in accordance with the present invention. A particle in accordance with the present invention is any entity having a greatest dimension (e.g. diameter) of less than 100 microns (μm). In some embodiments, inventive particles have a greatest dimension of less than 10 μm . In some embodiments, inventive particles have a greatest dimension of less than 2000 nanometers (nm). In some embodiments, inventive particles have a greatest dimension of less than 1000 nanometers (nm). In some embodiments, inventive particles have a greatest dimension of less than 900 nm, 800 nm, 700 nm, 600 nm, 500 nm, 400 nm, 300 nm, 200 nm, or 100 nm. Typically, inventive particles have a greatest dimension (e.g., diameter) of 500 nm or less. In some embodiments, inventive particles have a greatest dimension (e.g., diameter) of 250 nm or less. In some embodiments, inventive particles have a greatest dimension (e.g., diameter) of 200 nm or less. In some embodiments, inventive particles have a greatest dimension (e.g., diameter) of 150 nm or less. In some embodiments, inventive particles have a greatest dimension (e.g., diameter) of 100 nm or less. Smaller particles, e.g., having a greatest dimension of 50 nm or less are used in some embodiments of the invention. In some embodiments, inventive particles have a greatest dimension ranging between 25 nm and 200 nm.

[00110] Particle characterization (including e.g., characterizing morphology, dimension, etc.) is done using a variety of different techniques. Common techniques are electron microscopy (TEM,

SEM), atomic force microscopy (AFM), dynamic light scattering (DLS), X-ray photoelectron spectroscopy (XPS), powder X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), ultraviolet-visible spectroscopy, dual polarisation interferometry and nuclear magnetic resonance (NMR). Characterization (dimension measurements) may be made as to native particles (i.e., preloading) or after loading of the cargo (herein cargo refers to e.g., one or more components of CRISPR-Cas system e.g., CRISPR enzyme or mRNA or guide RNA, or any combination thereof, and may include additional carriers and/or excipients) to provide particles of an optimal size for delivery for any *in vitro*, *ex vivo* and/or *in vivo* application of the present invention. In certain preferred embodiments, particle dimension (e.g., diameter) characterization is based on measurements using dynamic laser scattering (DLS). Mention is made of US Patent No. 8,709,843; US Patent No. 6,007,845; US Patent No. 5,855,913; US Patent No. 5,985,309; US Patent No. 5,543,158; and the publication by James E. Dahlman and Carmen Barnes et al. Nature Nanotechnology (2014) published online 11 May 2014, doi:10.1038/nnano.2014.84, concerning particles, methods of making and using them and measurements thereof.

[00111] Particles delivery systems within the scope of the present invention may be provided in any form, including but not limited to solid, semi-solid, emulsion, or colloidal particles. As such any of the delivery systems described herein, including but not limited to, e.g., lipid-based systems, liposomes, micelles, microvesicles, exosomes, or gene gun may be provided as particle delivery systems within the scope of the present invention.

Particles

[00112] CRISPR enzyme mRNA and guide RNA may be delivered simultaneously using particles or lipid envelopes; for instance, CRISPR enzyme and RNA of the invention, e.g., as a complex, can be delivered via a particle as in Dahlman et al., WO2015089419 A2 and documents cited therein, such as 7C1 (see, e.g., James E. Dahlman and Carmen Barnes et al. Nature Nanotechnology (2014) published online 11 May 2014, doi:10.1038/nnano.2014.84), e.g., delivery particle comprising lipid or lipidoid and hydrophilic polymer, e.g., cationic lipid and hydrophilic polymer, for instance wherein the the cationic lipid comprises 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) or 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (DMPC) and/or wherein the hydrophilic

polymer comprises ethylene glycol or polyethylene glycol (PEG); and/or wherein the particle further comprises cholesterol (e.g., particle from formulation 1 = DOTAP 100, DMPC 0, PEG 0, Cholesterol 0; formulation number 2 = DOTAP 90, DMPC 0, PEG 10, Cholesterol 0; formulation number 3 = DOTAP 90, DMPC 0, PEG 5, Cholesterol 5), wherein particles are formed using an efficient, multistep process wherein first, effector protein and RNA are mixed together, e.g., at a 1:1 molar ratio, e.g., at room temperature, e.g., for 30 minutes, e.g., in sterile, nuclease free 1X PBS; and separately, DOTAP, DMPC, PEG, and cholesterol as applicable for the formulation are dissolved in alcohol, e.g., 100% ethanol; and, the two solutions are mixed together to form particles containing the complexes).

[00113] For example, Su X, Fricke J, Kavanagh DG, Irvine DJ (“In vitro and in vivo mRNA delivery using lipid-enveloped pH-responsive polymer nanoparticles” *Mol Pharm.* 2011 Jun 6;8(3):774-87. doi: 10.1021/mp100390w. Epub 2011 Apr 1) describes biodegradable core-shell structured particles with a poly(β -amino ester) (PBAE) core enveloped by a phospholipid bilayer shell. These were developed for in vivo mRNA delivery. The pH-responsive PBAE component was chosen to promote endosome disruption, while the lipid surface layer was selected to minimize toxicity of the polycation core. Such are, therefore, preferred for delivering RNA of the present invention.

[00114] Use of nanoparticles and particles to modulate immune responses at the single cell level falls into three general categories (1) particle directly attached to or embedded by an immune cell *ex vivo*, enabling the particle to deliver “cargo” or a therapeutic upon injection in vivo, thereby releasing the therapeutic directly; (2) leverage the natural propensity of particles to target or be scavenged by phagocytic cells *in vivo*; (3) actively target a specific cell in vivo by modulating the ligands or antibodies on the surface of the particle for specificity. (Irvine, D. J., Hanson, M. C., Rakhra, K., & Tokatlian, T. (2015). Synthetic Nanoparticles for Vaccines and Immunotherapy. *Chemical Reviews*, 115(19), 11109–11146.)

[00115] Generally, gold nanoparticles (which range in size from 10 to 100 nm), are inert particles which are biocompatible and possess optical properties which make the use of gold nanoparticles ideal for diagnostic and photothermal applications, such as in the case of applications in deep tissue. However, gold nanoparticles possess challenges which include prolonged retention in the hepatobiliary system and they are nonbiodegradable, both of which may lead to issues as to toxicity. (Irvine, D. J. et al. (2015) *Id.*)

[00116] In another example, use of amphiphilic gold nanoparticles, which comprise an approximately 2.3 nm gold core and an amphiphilic ligand shell, readily embedded within erythrocyte membranes, indicating that amphiphilic gold particles may serve as a vector for delivering a therapeutic into red blood cells directly or other cells. (Atukorale, P. et al. "Influence of the Glycocalyx and Plasma Membrane Composition on Amphiphilic Gold Nanoparticle Association with Erythrocytes," *Nanoscale* DOI: 10.1039/C5NR01355K (2015).)

[00117] In one embodiment, particles based on self assembling bioadhesive polymers are contemplated, which may be applied to oral delivery of peptides, intravenous delivery of peptides and nasal delivery of peptides, all to the brain. Other embodiments, such as oral absorption and ocular delivery of hydrophobic drugs are also contemplated. The molecular envelope technology involves an engineered polymer envelope which is protected and delivered to the site of the disease (see, e.g., Mazza, M. et al. *ACS Nano*, 2013. 7(2): 1016-1026; Siew, A., et al. *Mol Pharm*, 2012. 9(1):14-28; Lalatsa, A., et al. *J Contr Rel*, 2012. 161(2):523-36; Lalatsa, A., et al., *Mol Pharm*, 2012. 9(6):1665-80; Lalatsa, A., et al. *Mol Pharm*, 2012. 9(6):1764-74; Garrett, N.L., et al. *J Biophotonics*, 2012. 5(5-6):458-68; Garrett, N.L., et al. *J Raman Spect*, 2012. 43(5):681-688; Ahmad, S., et al. *J Royal Soc Interface* 2010. 7:S423-33; Uchegbu, I.F. *Expert Opin Drug Deliv*, 2006. 3(5):629-40; Qu, X., et al. *Biomacromolecules*, 2006. 7(12):3452-9 and Uchegbu, I.F., et al. *Int J Pharm*, 2001. 224:185-199). Doses of about 5 mg/kg are contemplated, with single or multiple doses, depending on the target tissue.

[00118] In one embodiment, particles that can deliver RNA to a cancer cell to stop tumor growth developed by Dan Anderson's lab at MIT may be used/and or adapted to the CRISPR Cas system of the present invention. In particular, the Anderson lab developed fully automated, combinatorial systems for the synthesis, purification, characterization, and formulation of new biomaterials and nanoformulations. See, e.g., Alabi et al., *Proc Natl Acad Sci U S A*. 2013 Aug 6;110(32):12881-6; Zhang et al., *Adv Mater*. 2013 Sep 6;25(33):4641-5; Jiang et al., *Nano Lett*. 2013 Mar 13;13(3):1059-64; Karagiannis et al., *ACS Nano*. 2012 Oct 23;6(10):8484-7; Whitehead et al., *ACS Nano*. 2012 Aug 28;6(8):6922-9 and Lee et al., *Nat Nanotechnol*. 2012 Jun 3;7(6):389-93.

[00119] U.S. patent application 20110293703 relates to lipidoid compounds are also particularly useful in the administration of polynucleotides, which may be applied to deliver the CRISPR Cas system of the present invention. In one aspect, the aminoalcohol lipidoid compounds

are combined with an agent to be delivered to a cell or a subject to form microparticles, nanoparticles, liposomes, or micelles. The agent to be delivered by the particles, liposomes, or micelles may be in the form of a gas, liquid, or solid, and the agent may be a polynucleotide, protein, peptide, or small molecule. The aminoalcohol lipidoid compounds may be combined with other aminoalcohol lipidoid compounds, polymers (synthetic or natural), surfactants, cholesterol, carbohydrates, proteins, lipids, etc. to form the particles. These particles may then optionally be combined with a pharmaceutical excipient to form a pharmaceutical composition.

[00120] US Patent Publication No. 20110293703 also provides methods of preparing the aminoalcohol lipidoid compounds. One or more equivalents of an amine are allowed to react with one or more equivalents of an epoxide-terminated compound under suitable conditions to form an aminoalcohol lipidoid compound of the present invention. In certain embodiments, all the amino groups of the amine are fully reacted with the epoxide-terminated compound to form tertiary amines. In other embodiments, all the amino groups of the amine are not fully reacted with the epoxide-terminated compound to form tertiary amines thereby resulting in primary or secondary amines in the aminoalcohol lipidoid compound. These primary or secondary amines are left as is or may be reacted with another electrophile such as a different epoxide-terminated compound. As will be appreciated by one skilled in the art, reacting an amine with less than excess of epoxide-terminated compound will result in a plurality of different aminoalcohol lipidoid compounds with various numbers of tails. Certain amines may be fully functionalized with two epoxide-derived compound tails while other molecules will not be completely functionalized with epoxide-derived compound tails. For example, a diamine or polyamine may include one, two, three, or four epoxide-derived compound tails off the various amino moieties of the molecule resulting in primary, secondary, and tertiary amines. In certain embodiments, all the amino groups are not fully functionalized. In certain embodiments, two of the same types of epoxide-terminated compounds are used. In other embodiments, two or more different epoxide-terminated compounds are used. The synthesis of the aminoalcohol lipidoid compounds is performed with or without solvent, and the synthesis may be performed at higher temperatures ranging from 30-100 °C., preferably at approximately 50-90 °C. The prepared aminoalcohol lipidoid compounds may be optionally purified. For example, the mixture of aminoalcohol lipidoid compounds may be purified to yield an aminoalcohol lipidoid compound with a particular number of epoxide-derived compound tails. Or the mixture may be purified to yield a particular stereo- or regioisomer. The

aminoalcohol lipidoid compounds may also be alkylated using an alkyl halide (e.g., methyl iodide) or other alkylating agent, and/or they may be acylated.

[00121] US Patent Publication No. 20110293703 also provides libraries of aminoalcohol lipidoid compounds prepared by the inventive methods. These aminoalcohol lipidoid compounds may be prepared and/or screened using high-throughput techniques involving liquid handlers, robots, microtiter plates, computers, etc. In certain embodiments, the aminoalcohol lipidoid compounds are screened for their ability to transfect polynucleotides or other agents (e.g., proteins, peptides, small molecules) into the cell.

[00122] US Patent Publication No. 20130302401 relates to a class of poly(beta-amino alcohols) (PBAs) has been prepared using combinatorial polymerization. The inventive PBAs may be used in biotechnology and biomedical applications as coatings (such as coatings of films or multilayer films for medical devices or implants), additives, materials, excipients, non-biofouling agents, micropatterning agents, and cellular encapsulation agents. When used as surface coatings, these PBAs elicited different levels of inflammation, both in vitro and in vivo, depending on their chemical structures. The large chemical diversity of this class of materials allowed us to identify polymer coatings that inhibit macrophage activation in vitro. Furthermore, these coatings reduce the recruitment of inflammatory cells, and reduce fibrosis, following the subcutaneous implantation of carboxylated polystyrene microparticles. These polymers may be used to form polyelectrolyte complex capsules for cell encapsulation. The invention may also have many other biological applications such as antimicrobial coatings, DNA or siRNA delivery, and stem cell tissue engineering. The teachings of US Patent Publication No. 20130302401 may be applied to the CRISPR Cas system of the present invention.

[00123] In another embodiment, lipid particles (LNPs) are contemplated. An antitransthyretin small interfering RNA has been encapsulated in lipid particles and delivered to humans (see, e.g., Coelho et al., N Engl J Med 2013;369:819-29), and such a system may be adapted and applied to the CRISPR Cas system of the present invention. Doses of about 0.01 to about 1 mg per kg of body weight administered intravenously are contemplated. Medications to reduce the risk of infusion-related reactions are contemplated, such as dexamethasone, acetaminophen, diphenhydramine or cetirizine, and ranitidine are contemplated. Multiple doses of about 0.3 mg per kilogram every 4 weeks for five doses are also contemplated.

[00124] LNPs have been shown to be highly effective in delivering siRNAs to the liver (see, e.g., Taberero et al., *Cancer Discovery*, April 2013, Vol. 3, No. 4, pages 363-470) and are therefore contemplated for delivering RNA encoding CRISPR Cas to the liver. A dosage of about four doses of 6 mg/kg of the LNP every two weeks may be contemplated. Taberero et al. demonstrated that tumor regression was observed after the first 2 cycles of LNPs dosed at 0.7 mg/kg, and by the end of 6 cycles the patient had achieved a partial response with complete regression of the lymph node metastasis and substantial shrinkage of the liver tumors. A complete response was obtained after 40 doses in this patient, who has remained in remission and completed treatment after receiving doses over 26 months. Two patients with RCC and extrahepatic sites of disease including kidney, lung, and lymph nodes that were progressing following prior therapy with VEGF pathway inhibitors had stable disease at all sites for approximately 8 to 12 months, and a patient with PNET and liver metastases continued on the extension study for 18 months (36 doses) with stable disease.

[00125] However, the charge of the LNP must be taken into consideration. As cationic lipids combined with negatively charged lipids to induce nonbilayer structures that facilitate intracellular delivery. Because charged LNPs are rapidly cleared from circulation following intravenous injection, ionizable cationic lipids with pKa values below 7 were developed (see, e.g., Rosin et al., *Molecular Therapy*, vol. 19, no. 12, pages 1286-2200, Dec. 2011). Negatively charged polymers such as RNA may be loaded into LNPs at low pH values (e.g., pH 4) where the ionizable lipids display a positive charge. However, at physiological pH values, the LNPs exhibit a low surface charge compatible with longer circulation times. Four species of ionizable cationic lipids have been focused upon, namely 1,2-dilinoyleyl-3-dimethylammonium-propane (DLinDAP), 1,2-dilinoyleyl-3-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinoyleyl-keto-N,N-dimethyl-3-aminopropane (DLinKDMA), and 1,2-dilinoyleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLinKC2-DMA). It has been shown that LNP siRNA systems containing these lipids exhibit remarkably different gene silencing properties in hepatocytes in vivo, with potencies varying according to the series DLinKC2-DMA>DLinKDMA>DLinDMA>>DLinDAP employing a Factor VII gene silencing model (see, e.g., Rosin et al, *Molecular Therapy*, vol. 19, no. 12, pages 1286-2200, Dec. 2011). A dosage of 1 µg/ml of LNP or CRISPR-Cas RNA in or associated with the LNP may be contemplated, especially for a formulation containing DLinKC2-DMA.

[00126] Preparation of LNPs and CRISPR Cas encapsulation may be used/and or adapted from Rosin et al, *Molecular Therapy*, vol. 19, no. 12, pages 1286-2200, Dec. 2011). The cationic lipids 1,2-dilinoyleoyl-3-dimethylammonium-propane (DLinDAP), 1,2-dilinoyleoxy-3-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinoyleoxyketo-N,N-dimethyl-3-aminopropane (DLinK-DMA), 1,2-dilinoyleoyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLinKC2-DMA), (3-*o*-[2''-(methoxypolyethyleneglycol 2000) succinoyl]-1,2-dimyristoyl-sn-glycol (PEG-S-DMG), and R-3-[(ω -methoxy-poly(ethylene glycol)2000) carbamoyl]-1,2-dimyristyloxylpropyl-3-amine (PEG-C-DOMG) may be provided by Tekmira Pharmaceuticals (Vancouver, Canada) or synthesized. Cholesterol may be purchased from Sigma (St Louis, MO). The specific CRISPR Cas RNA may be encapsulated in LNPs containing DLinDAP, DLinDMA, DLinK-DMA, and DLinKC2-DMA (cationic lipid:DSPC:CHOL: PEGS-DMG or PEG-C-DOMG at 40:10:40:10 molar ratios). When required, 0.2% SP-DiOC18 (Invitrogen, Burlington, Canada) may be incorporated to assess cellular uptake, intracellular delivery, and biodistribution. Encapsulation may be performed by dissolving lipid mixtures comprised of cationic lipid:DSPC:cholesterol:PEG-c-DOMG (40:10:40:10 molar ratio) in ethanol to a final lipid concentration of 10 mmol/l. This ethanol solution of lipid may be added drop-wise to 50 mmol/l citrate, pH 4.0 to form multilamellar vesicles to produce a final concentration of 30% ethanol vol/vol. Large unilamellar vesicles may be formed following extrusion of multilamellar vesicles through two stacked 80 nm Nuclepore polycarbonate filters using the Extruder (Northern Lipids, Vancouver, Canada). Encapsulation may be achieved by adding RNA dissolved at 2 mg/ml in 50 mmol/l citrate, pH 4.0 containing 30% ethanol vol/vol drop-wise to extruded preformed large unilamellar vesicles and incubation at 31 °C for 30 minutes with constant mixing to a final RNA/lipid weight ratio of 0.06/1 wt/wt. Removal of ethanol and neutralization of formulation buffer were performed by dialysis against phosphate-buffered saline (PBS), pH 7.4 for 16 hours using Spectra/Por 2 regenerated cellulose dialysis membranes. Particle size distribution may be determined by dynamic light scattering using a NICOMP 370 particle sizer, the vesicle/intensity modes, and Gaussian fitting (Nicomp Particle Sizing, Santa Barbara, CA). The particle size for all three LNP systems may be ~70 nm in diameter. RNA encapsulation efficiency may be determined by removal of free RNA using VivaPureD MiniH columns (Sartorius Stedim Biotech) from samples collected before and after dialysis. The encapsulated RNA may be extracted from the

eluted particles and quantified at 260 nm. RNA to lipid ratio was determined by measurement of cholesterol content in vesicles using the Cholesterol E enzymatic assay from Wako Chemicals USA (Richmond, VA). In conjunction with the herein discussion of LNPs and PEG lipids, PEGylated liposomes or LNPs are likewise suitable for delivery of a CRISPR-Cas system or components thereof.

[00127] Preparation of large LNPs may be used/and or adapted from Rosin et al, *Molecular Therapy*, vol. 19, no. 12, pages 1286-2200, Dec. 2011. A lipid premix solution (20.4 mg/ml total lipid concentration) may be prepared in ethanol containing DLinKC2-DMA, DSPC, and cholesterol at 50:10:38.5 molar ratios. Sodium acetate may be added to the lipid premix at a molar ratio of 0.75:1 (sodium acetate:DLinKC2-DMA). The lipids may be subsequently hydrated by combining the mixture with 1.85 volumes of citrate buffer (10 mmol/l, pH 3.0) with vigorous stirring, resulting in spontaneous liposome formation in aqueous buffer containing 35% ethanol. The liposome solution may be incubated at 37 °C to allow for time-dependent increase in particle size. Aliquots may be removed at various times during incubation to investigate changes in liposome size by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK). Once the desired particle size is achieved, an aqueous PEG lipid solution (stock = 10 mg/ml PEG-DMG in 35% (vol/vol) ethanol) may be added to the liposome mixture to yield a final PEG molar concentration of 3.5% of total lipid. Upon addition of PEG-lipids, the liposomes should their size, effectively quenching further growth. RNA may then be added to the empty liposomes at an RNA to total lipid ratio of approximately 1:10 (wt:wt), followed by incubation for 30 minutes at 37 °C to form loaded LNPs. The mixture may be subsequently dialyzed overnight in PBS and filtered with a 0.45- μ m syringe filter.

[00128] Spherical Nucleic Acid (SNATM) constructs and other particles (particularly gold particles) are also contemplated as a means to delivery CRISPR-Cas system to intended targets. Significant data show that AuraSense Therapeutics' Spherical Nucleic Acid (SNATM) constructs, based upon nucleic acid-functionalized gold particles, are useful.

[00129] Literature that may be employed in conjunction with herein teachings include: Cutler et al., *J. Am. Chem. Soc.* 2011 133:9254-9257, Hao et al., *Small*. 2011 7:3158-3162, Zhang et al., *ACS Nano*. 2011 5:6962-6970, Cutler et al., *J. Am. Chem. Soc.* 2012 134:1376-1391, Young et al., *Nano Lett.* 2012 12:3867-71, Zheng et al., *Proc. Natl. Acad. Sci. USA*. 2012 109:11975-80, Mirkin, *Nanomedicine* 2012 7:635-638 Zhang et al., *J. Am. Chem. Soc.* 2012 134:16488-1691,

Weintraub, *Nature* 2013 495:S14-S16, Choi et al., *Proc. Natl. Acad. Sci. USA*. 2013 110(19):7625-7630, Jensen et al., *Sci. Transl. Med.* 5, 209ra152 (2013) and Mirkin, et al., *Small*, 10:186-192.

[00130] Self-assembling particles with RNA may be constructed with polyethyleneimine (PEI) that is PEGylated with an Arg-Gly-Asp (RGD) peptide ligand attached at the distal end of the polyethylene glycol (PEG). This system has been used, for example, as a means to target tumor neovasculature expressing integrins and deliver siRNA inhibiting vascular endothelial growth factor receptor-2 (VEGF R2) expression and thereby achieve tumor angiogenesis (see, e.g., Schiffelers et al., *Nucleic Acids Research*, 2004, Vol. 32, No. 19). Nanoplexes may be prepared by mixing equal volumes of aqueous solutions of cationic polymer and nucleic acid to give a net molar excess of ionizable nitrogen (polymer) to phosphate (nucleic acid) over the range of 2 to 6. The electrostatic interactions between cationic polymers and nucleic acid resulted in the formation of polyplexes with average particle size distribution of about 100 nm, hence referred to here as nanoplexes. A dosage of about 100 to 200 mg of CRISPR Cas is envisioned for delivery in the self-assembling particles of Schiffelers et al.

[00131] The nanoplexes of Bartlett et al. (*PNAS*, September 25, 2007, vol. 104, no. 39) may also be applied to the present invention. The nanoplexes of Bartlett et al. are prepared by mixing equal volumes of aqueous solutions of cationic polymer and nucleic acid to give a net molar excess of ionizable nitrogen (polymer) to phosphate (nucleic acid) over the range of 2 to 6. The electrostatic interactions between cationic polymers and nucleic acid resulted in the formation of polyplexes with average particle size distribution of about 100 nm, hence referred to here as nanoplexes. The DOTA-siRNA of Bartlett et al. was synthesized as follows: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid mono(N-hydroxysuccinimide ester) (DOTA-NHSester) was ordered from Macrocyclics (Dallas, TX). The amine modified RNA sense strand with a 100-fold molar excess of DOTA-NHS-ester in carbonate buffer (pH 9) was added to a microcentrifuge tube. The contents were reacted by stirring for 4 h at room temperature. The DOTA-RNAsense conjugate was ethanol-precipitated, resuspended in water, and annealed to the unmodified antisense strand to yield DOTA-siRNA. All liquids were pretreated with Chelex-100 (Bio-Rad, Hercules, CA) to remove trace metal contaminants. Tf-targeted and nontargeted siRNA particles may be formed by using cyclodextrin-containing polycations. Typically, particles were formed in water at a charge ratio of 3 (+/-) and an siRNA concentration of 0.5 g/liter. One percent

of the adamantane-PEG molecules on the surface of the targeted particles were modified with Tf (adamantane-PEG-Tf). The particles were suspended in a 5% (wt/vol) glucose carrier solution for injection.

[00132] Davis et al. (Nature, Vol 464, 15 April 2010) conducts a RNA clinical trial that uses a targeted particle-delivery system (clinical trial registration number NCT00689065). Patients with solid cancers refractory to standard-of-care therapies are administered doses of targeted particles on days 1, 3, 8 and 10 of a 21-day cycle by a 30-min intravenous infusion. The particles consist of a synthetic delivery system containing: (1) a linear, cyclodextrin-based polymer (CDP), (2) a human transferrin protein (TF) targeting ligand displayed on the exterior of the particle to engage TF receptors (TFR) on the surface of the cancer cells, (3) a hydrophilic polymer (polyethylene glycol (PEG) used to promote particle stability in biological fluids), and (4) siRNA designed to reduce the expression of the RRM2 (sequence used in the clinic was previously denoted siR2B+5). The TFR has long been known to be upregulated in malignant cells, and RRM2 is an established anti-cancer target. These particles (clinical version denoted as CALAA-01) have been shown to be well tolerated in multi-dosing studies in non-human primates. Although a single patient with chronic myeloid leukaemia has been administered siRNA by liposomal delivery, Davis et al.'s clinical trial is the initial human trial to systemically deliver siRNA with a targeted delivery system and to treat patients with solid cancer. To ascertain whether the targeted delivery system can provide effective delivery of functional siRNA to human tumours, Davis et al. investigated biopsies from three patients from three different dosing cohorts; patients A, B and C, all of whom had metastatic melanoma and received CALAA-01 doses of 18, 24 and 30 mg m⁻² siRNA, respectively. Similar doses may also be contemplated for the CRISPR Cas system of the present invention. The delivery of the invention may be achieved with particles containing a linear, cyclodextrin-based polymer (CDP), a human transferrin protein (TF) targeting ligand displayed on the exterior of the particle to engage TF receptors (TFR) on the surface of the cancer cells and/or a hydrophilic polymer (for example, polyethylene glycol (PEG) used to promote particle stability in biological fluids).

[00133] In terms of this invention, it is preferred to have one or more components of CRISPR complex, e.g., CRISPR enzyme or mRNA or guide RNA or sgRNA or if present HDR template may be delivered using one or more particles or particles or lipid envelopes. Other delivery systems or vectors are may be used in conjunction with the particle aspects of the invention.

[00134] In general, a "nanoparticle" refers to any particle having a diameter of less than 1000 nm. In certain preferred embodiments, nanoparticles of the invention have a greatest dimension (e.g., diameter) of 500 nm or less. In other preferred embodiments, nanoparticles of the invention have a greatest dimension ranging between 25 nm and 200 nm. In other preferred embodiments, nanoparticles of the invention have a greatest dimension of 100 nm or less. In other preferred embodiments, nanoparticles of the invention have a greatest dimension ranging between 35 nm and 60 nm.

[00135] Particles encompassed in the present invention may be provided in different forms, e.g., as solid particles (e.g., metal such as silver, gold, iron, titanium), non-metal, lipid-based solids, polymers), suspensions of particles, or combinations thereof. Metal, dielectric, and semiconductor particles may be prepared, as well as hybrid structures (e.g., core-shell particles). Particles made of semiconducting material may also be labeled quantum dots if they are small enough (typically sub 10 nm) that quantization of electronic energy levels occurs. Such nanoscale particles are used in biomedical applications as drug carriers or imaging agents and may be adapted for similar purposes in the present invention.

[00136] Semi-solid and soft particles have been manufactured, and are within the scope of the present invention. A prototype particle of semi-solid nature is the liposome. Various types of liposome particles are currently used clinically as delivery systems for anticancer drugs and vaccines. Particles with one half hydrophilic and the other half hydrophobic are termed Janus particles and are particularly effective for stabilizing emulsions. They can self-assemble at water/oil interfaces and act as solid surfactants.

[00137] US Patent No. 8,709,843, incorporated herein by reference, provides a drug delivery system for targeted delivery of therapeutic agent-containing particles to tissues, cells, and intracellular compartments. The invention provides targeted particles comprising comprising polymer conjugated to a surfactant, hydrophilic polymer or lipid. US Patent No. 6,007,845, incorporated herein by reference, provides particles which have a core of a multiblock copolymer formed by covalently linking a multifunctional compound with one or more hydrophobic polymers and one or more hydrophilic polymers, and contain a biologically active material. US Patent No. 5,855,913, incorporated herein by reference, provides a particulate composition having aerodynamically light particles having a tap density of less than 0.4 g/cm³ with a mean diameter of between 5 μ m and 30 μ m, incorporating a surfactant on the surface thereof for drug delivery to the

pulmonary system. U.S. Patent No. 5,985,309, incorporated herein by reference, provides particles incorporating a surfactant and/or a hydrophilic or hydrophobic complex of a positively or negatively charged therapeutic or diagnostic agent and a charged molecule of opposite charge for delivery to the pulmonary system. US. Patent No. 5,543,158, incorporated herein by reference, provides biodegradable injectable particles having a biodegradable solid core containing a biologically active material and poly(alkylene glycol) moieties on the surface. WO2012135025 (also published as US20120251560), incorporated herein by reference, describes conjugated polyethyleneimine (PEI) polymers and conjugated aza-macrocycles (collectively referred to as “conjugated lipomer” or “lipomers”). In certain embodiments, it can be envisioned that such methods and materials of herein-cited documents, e.g., conjugated lipomers, can be used in the context of the CRISPR-Cas system to achieve *in vitro*, *ex vivo* and *in vivo* genomic perturbations to modify gene expression, including modulation of protein expression.

[00138] In one embodiment, the particle may be epoxide-modified lipid-polymer, advantageously 7C1 (see, e.g., James E. Dahlman and Carmen Barnes et al. *Nature Nanotechnology* (2014) published online 11 May 2014, doi:10.1038/nnano.2014.84). C71 was synthesized by reacting C15 epoxide-terminated lipids with PEI600 at a 14:1 molar ratio, and was formulated with C14PEG2000 to produce particles (diameter between 35 and 60 nm) that were stable in PBS solution for at least 40 days. An epoxide-modified lipid-polymer may be utilized to deliver the CRISPR-Cas system of the present invention to pulmonary, cardiovascular or renal cells, however, one of skill in the art may adapt the system to deliver to other target organs. Dosage ranging from about 0.05 to about 0.6 mg/kg are envisioned. Dosages over several days or weeks are also envisioned, with a total dosage of about 2 mg/kg.

Exosomes

[00139] Exosomes are endogenous nano-vesicles that transport RNAs and proteins, and which can deliver RNA to the brain and other target organs. To reduce immunogenicity, Alvarez-Erviti et al. (2011, *Nat Biotechnol* 29: 341) used self-derived dendritic cells for exosome production. Targeting to the brain was achieved by engineering the dendritic cells to express Lamp2b, an exosomal membrane protein, fused to the neuron-specific RVG peptide. Purified exosomes were loaded with exogenous RNA by electroporation. Intravenously injected RVG-targeted exosomes delivered GAPDH siRNA specifically to neurons, microglia, oligodendrocytes in the brain, resulting in a specific gene knockdown. Pre-exposure to RVG exosomes did not attenuate

knockdown, and non-specific uptake in other tissues was not observed. The therapeutic potential of exosome-mediated siRNA delivery was demonstrated by the strong mRNA (60%) and protein (62%) knockdown of BACE1, a therapeutic target in Alzheimer's disease.

[00140] To obtain a pool of immunologically inert exosomes, Alvarez-Erviti et al. harvested bone marrow from inbred C57BL/6 mice with a homogenous major histocompatibility complex (MHC) haplotype. As immature dendritic cells produce large quantities of exosomes devoid of T-cell activators such as MHC-II and CD86, Alvarez-Erviti et al. selected for dendritic cells with granulocyte/macrophage-colony stimulating factor (GM-CSF) for 7 d. Exosomes were purified from the culture supernatant the following day using well-established ultracentrifugation protocols. The exosomes produced were physically homogenous, with a size distribution peaking at 80 nm in diameter as determined by nanoparticle tracking analysis (NTA) and electron microscopy. Alvarez-Erviti et al. obtained 6-12 μg of exosomes (measured based on protein concentration) per 10^6 cells.

[00141] Next, Alvarez-Erviti et al. investigated the possibility of loading modified exosomes with exogenous cargoes using electroporation protocols adapted for nanoscale applications. As electroporation for membrane particles at the nanometer scale is not well-characterized, nonspecific Cy5-labeled RNA was used for the empirical optimization of the electroporation protocol. The amount of encapsulated RNA was assayed after ultracentrifugation and lysis of exosomes. Electroporation at 400 V and 125 μF resulted in the greatest retention of RNA and was used for all subsequent experiments.

[00142] Alvarez-Erviti et al. administered 150 μg of each BACE1 siRNA encapsulated in 150 μg of RVG exosomes to normal C57BL/6 mice and compared the knockdown efficiency to four controls: untreated mice, mice injected with RVG exosomes only, mice injected with BACE1 siRNA complexed to an in vivo cationic liposome reagent and mice injected with BACE1 siRNA complexed to RVG-9R, the RVG peptide conjugated to 9 D-arginines that electrostatically binds to the siRNA. Cortical tissue samples were analyzed 3 d after administration and a significant protein knockdown (45%, $P < 0.05$, versus 62%, $P < 0.01$) in both siRNA-RVG-9R-treated and siRNARVG exosome-treated mice was observed, resulting from a significant decrease in BACE1 mRNA levels (66% [+ or -] 15%, $P < 0.001$ and 61% [+ or -] 13% respectively, $P < 0.01$). Moreover, Applicants demonstrated a significant decrease (55%, $P < 0.05$) in the total [beta]-amyloid 1-42 levels, a main component of the amyloid plaques in Alzheimer's pathology, in

the RVG-exosome-treated animals. The decrease observed was greater than the β -amyloid 1-40 decrease demonstrated in normal mice after intraventricular injection of BACE1 inhibitors. Alvarez-Erviti et al. carried out 5'-rapid amplification of cDNA ends (RACE) on BACE1 cleavage product, which provided evidence of RNAi-mediated knockdown by the siRNA.

[00143] Finally, Alvarez-Erviti et al. investigated whether RNA-RVG exosomes induced immune responses in vivo by assessing IL-6, IP-10, TNF α and IFN- α serum concentrations. Following exosome treatment, nonsignificant changes in all cytokines were registered similar to siRNA-transfection reagent treatment in contrast to siRNA-RVG-9R, which potently stimulated IL-6 secretion, confirming the immunologically inert profile of the exosome treatment. Given that exosomes encapsulate only 20% of siRNA, delivery with RVG-exosome appears to be more efficient than RVG-9R delivery as comparable mRNA knockdown and greater protein knockdown was achieved with fivefold less siRNA without the corresponding level of immune stimulation. This experiment demonstrated the therapeutic potential of RVG-exosome technology, which is potentially suited for long-term silencing of genes related to neurodegenerative diseases. The exosome delivery system of Alvarez-Erviti et al. may be applied to deliver the CRISPR-Cas system of the present invention to therapeutic targets, especially neurodegenerative diseases. A dosage of about 100 to 1000 mg of CRISPR Cas encapsulated in about 100 to 1000 mg of RVG exosomes may be contemplated for the present invention.

[00144] El-Andaloussi et al. (Nature Protocols 7,2112–2126(2012)) discloses how exosomes derived from cultured cells can be harnessed for delivery of RNA in vitro and in vivo. This protocol first describes the generation of targeted exosomes through transfection of an expression vector, comprising an exosomal protein fused with a peptide ligand. Next, El-Andaloussi et al. explain how to purify and characterize exosomes from transfected cell supernatant. Next, El-Andaloussi et al. detail crucial steps for loading RNA into exosomes. Finally, El-Andaloussi et al. outline how to use exosomes to efficiently deliver RNA in vitro and in vivo in mouse brain. Examples of anticipated results in which exosome-mediated RNA delivery is evaluated by functional assays and imaging are also provided. The entire protocol takes ~3 weeks. Delivery or administration according to the invention may be performed using exosomes produced from self-derived dendritic cells. From the herein teachings, this can be employed in the practice of the invention.

[00145] In another embodiment, the plasma exosomes of Wahlgren et al. (Nucleic Acids Research, 2012, Vol. 40, No. 17 e130) are contemplated. Exosomes are nano-sized vesicles (30–90nm in size) produced by many cell types, including dendritic cells (DC), B cells, T cells, mast cells, epithelial cells and tumor cells. These vesicles are formed by inward budding of late endosomes and are then released to the extracellular environment upon fusion with the plasma membrane. Because exosomes naturally carry RNA between cells, this property may be useful in gene therapy, and from this disclosure can be employed in the practice of the instant invention.

[00146] Exosomes from plasma can be prepared by centrifugation of buffy coat at 900g for 20 min to isolate the plasma followed by harvesting cell supernatants, centrifuging at 300g for 10 min to eliminate cells and at 16 500g for 30 min followed by filtration through a 0.22 mm filter. Exosomes are pelleted by ultracentrifugation at 120 000g for 70 min. Chemical transfection of siRNA into exosomes is carried out according to the manufacturer's instructions in RNAi Human/Mouse Starter Kit (Quiagen, Hilden, Germany). siRNA is added to 100 ml PBS at a final concentration of 2 mmol/ml. After adding HiPerFect transfection reagent, the mixture is incubated for 10 min at RT. In order to remove the excess of micelles, the exosomes are re-isolated using aldehyde/sulfate latex beads. The chemical transfection of CRISPR Cas into exosomes may be conducted similarly to siRNA. The exosomes may be co-cultured with monocytes and lymphocytes isolated from the peripheral blood of healthy donors. Therefore, it may be contemplated that exosomes containing CRISPR Cas may be introduced to monocytes and lymphocytes of and autologously reintroduced into a human. Accordingly, delivery or administration according to the invention may be performed using plasma exosomes.

Liposomes

[00147] Delivery or administration according to the invention can be performed with liposomes. Liposomes are spherical vesicle structures composed of a uni- or multilamellar lipid bilayer surrounding internal aqueous compartments and a relatively impermeable outer lipophilic phospholipid bilayer. Liposomes have gained considerable attention as drug delivery carriers because they are biocompatible, nontoxic, can deliver both hydrophilic and lipophilic drug molecules, protect their cargo from degradation by plasma enzymes, and transport their load across biological membranes and the blood brain barrier (BBB) (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review).

[00148] Liposomes can be made from several different types of lipids; however, phospholipids are most commonly used to generate liposomes as drug carriers. Although liposome formation is spontaneous when a lipid film is mixed with an aqueous solution, it can also be expedited by applying force in the form of shaking by using a homogenizer, sonicator, or an extrusion apparatus (see, e.g., Spuch and Navarro, *Journal of Drug Delivery*, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review).

[00149] Several other additives may be added to liposomes in order to modify their structure and properties. For instance, either cholesterol or sphingomyelin may be added to the liposomal mixture in order to help stabilize the liposomal structure and to prevent the leakage of the liposomal inner cargo. Further, liposomes are prepared from hydrogenated egg phosphatidylcholine or egg phosphatidylcholine, cholesterol, and dicetyl phosphate, and their mean vesicle sizes were adjusted to about 50 and 100 nm. (see, e.g., Spuch and Navarro, *Journal of Drug Delivery*, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review).

[00150] A liposome formulation may be mainly comprised of natural phospholipids and lipids such as 1,2-distearoyl-sn-glycero-3-phosphatidyl choline (DSPC), sphingomyelin, egg phosphatidylcholines and monosialoganglioside. Since this formulation is made up of phospholipids only, liposomal formulations have encountered many challenges, one of the ones being the instability in plasma. Several attempts to overcome these challenges have been made, specifically in the manipulation of the lipid membrane. One of these attempts focused on the manipulation of cholesterol. Addition of cholesterol to conventional formulations reduces rapid release of the encapsulated bioactive compound into the plasma or 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) increases the stability (see, e.g., Spuch and Navarro, *Journal of Drug Delivery*, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review).

[00151] In a particularly advantageous embodiment, Trojan Horse liposomes (also known as Molecular Trojan Horses) are desirable and protocols may be found at <http://cshprotocols.cshlp.org/content/2010/4/pdb.prot5407.long>. These particles allow delivery of a transgene to the entire brain after an intravascular injection. Without being bound by limitation, it is believed that neutral lipid particles with specific antibodies conjugated to surface allow crossing of the blood brain barrier via endocytosis. Applicant postulates utilizing Trojan Horse Liposomes

to deliver the CRISPR family of nucleases to the brain via an intravascular injection, which would allow whole brain transgenic animals without the need for embryonic manipulation. About 1-5 g of DNA or RNA may be contemplated for in vivo administration in liposomes.

[00152] In another embodiment, the CRISPR Cas system or components thereof may be administered in liposomes, such as a stable nucleic-acid-lipid particle (SNALP) (see, e.g., Morrissey et al., *Nature Biotechnology*, Vol. 23, No. 8, August 2005). Daily intravenous injections of about 1, 3 or 5 mg/kg/day of a specific CRISPR Cas targeted in a SNALP are contemplated. The daily treatment may be over about three days and then weekly for about five weeks. In another embodiment, a specific CRISPR Cas encapsulated SNALP) administered by intravenous injection to at doses of about 1 or 2.5 mg/kg are also contemplated (see, e.g., Zimmerman et al., *Nature Letters*, Vol. 441, 4 May 2006). The SNALP formulation may contain the lipids 3-N-[(w-methoxypoly(ethylene glycol) 2000) carbamoyl] -1,2-dimyristyloxy-propylamine (PEG-C-DMA), 1,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane (DLinDMA), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol, in a 2:40:10:48 molar per cent ratio (see, e.g., Zimmerman et al., *Nature Letters*, Vol. 441, 4 May 2006).

[00153] In another embodiment, stable nucleic-acid-lipid particles (SNALPs) have proven to be effective delivery molecules to highly vascularized HepG2-derived liver tumors but not in poorly vascularized HCT-116 derived liver tumors (see, e.g., Li, *Gene Therapy* (2012) 19, 775–780). The SNALP liposomes may be prepared by formulating D-Lin-DMA and PEG-C-DMA with distearoylphosphatidylcholine (DSPC), Cholesterol and siRNA using a 25:1 lipid/siRNA ratio and a 48/40/10/2 molar ratio of Cholesterol/D-Lin-DMA/DSPC/PEG-C-DMA. The resulted SNALP liposomes are about 80–100 nm in size. In yet another embodiment, a SNALP may comprise synthetic cholesterol (Sigma-Aldrich, St Louis, MO, USA), dipalmitoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA), 3-N-[(w-methoxy poly(ethylene glycol)2000)carbamoyl]-1,2-dimyrestyloxypropylamine, and cationic 1,2-dilinoleyloxy-3-N,Ndimethylaminopropane (see, e.g., Geisbert et al., *Lancet* 2010; 375: 1896-905). A dosage of about 2 mg/kg total CRISPR Cas per dose administered as, for example, a bolus intravenous infusion may be contemplated. In yet another embodiment, a SNALP may comprise synthetic cholesterol (Sigma-Aldrich), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC; Avanti Polar Lipids Inc.), PEG-cDMA, and 1,2-dilinoleyloxy-3-(N;N-dimethyl)aminopropane (DLinDMA) (see, e.g., Judge, *J. Clin. Invest.*

119:661-673 (2009)). Formulations used for in vivo studies may comprise a final lipid/RNA mass ratio of about 9:1.

[00154] The safety profile of RNAi nanomedicines has been reviewed by Barros and Gollob of Alnylam Pharmaceuticals (see, e.g., *Advanced Drug Delivery Reviews* 64 (2012) 1730–1737). The stable nucleic acid lipid particle (SNALP) is comprised of four different lipids — an ionizable lipid (DLinDMA) that is cationic at low pH, a neutral helper lipid, cholesterol, and a diffusible polyethylene glycol (PEG)-lipid. The particle is approximately 80 nm in diameter and is charge-neutral at physiologic pH. During formulation, the ionizable lipid serves to condense lipid with the anionic RNA during particle formation. When positively charged under increasingly acidic endosomal conditions, the ionizable lipid also mediates the fusion of SNALP with the endosomal membrane enabling release of RNA into the cytoplasm. The PEG-lipid stabilizes the particle and reduces aggregation during formulation, and subsequently provides a neutral hydrophilic exterior that improves pharmacokinetic properties.

[00155] To date, two clinical programs have been initiated using SNALP formulations with RNA. Tekmira Pharmaceuticals recently completed a phase I single-dose study of SNALP-ApoB in adult volunteers with elevated LDL cholesterol. ApoB is predominantly expressed in the liver and jejunum and is essential for the assembly and secretion of VLDL and LDL. Seventeen subjects received a single dose of SNALP-ApoB (dose escalation across 7 dose levels). There was no evidence of liver toxicity (anticipated as the potential dose-limiting toxicity based on preclinical studies). One (of two) subjects at the highest dose experienced flu-like symptoms consistent with immune system stimulation, and the decision was made to conclude the trial.

[00156] Alnylam Pharmaceuticals has similarly advanced ALN-TTR01, which employs the SNALP technology described above and targets hepatocyte production of both mutant and wild-type TTR to treat TTR amyloidosis (ATTR). Three ATTR syndromes have been described: familial amyloidotic polyneuropathy (FAP) and familial amyloidotic cardiomyopathy (FAC) — both caused by autosomal dominant mutations in TTR; and senile systemic amyloidosis (SSA) cause by wildtype TTR. A placebo-controlled, single dose-escalation phase I trial of ALN-TTR01 was recently completed in patients with ATTR. ALN-TTR01 was administered as a 15-minute IV infusion to 31 patients (23 with study drug and 8 with placebo) within a dose range of 0.01 to 1.0 mg/kg (based on siRNA). Treatment was well tolerated with no significant increases in liver function tests. Infusion-related reactions were noted in 3 of 23 patients at ≥ 0.4 mg/kg; all

responded to slowing of the infusion rate and all continued on study. Minimal and transient elevations of serum cytokines IL-6, IP-10 and IL-1ra were noted in two patients at the highest dose of 1 mg/kg (as anticipated from preclinical and NHP studies). Lowering of serum TTR, the expected pharmacodynamics effect of ALN-TTR01, was observed at 1 mg/kg.

[00157] In yet another embodiment, a SNALP may be made by solubilizing a cationic lipid, DSPC, cholesterol and PEG-lipid e.g., in ethanol, e.g., at a molar ratio of 40:10:40:10, respectively (see, Semple et al., Nature Nanotechnology, Volume 28 Number 2 February 2010, pp. 172-177). The lipid mixture was added to an aqueous buffer (50 mM citrate, pH 4) with mixing to a final ethanol and lipid concentration of 30% (vol/vol) and 6.1 mg/ml, respectively, and allowed to equilibrate at 22 °C for 2 min before extrusion. The hydrated lipids were extruded through two stacked 80 nm pore-sized filters (Nuclepore) at 22 °C using a Lipex Extruder (Northern Lipids) until a vesicle diameter of 70–90 nm, as determined by dynamic light scattering analysis, was obtained. This generally required 1–3 passes. The siRNA (solubilized in a 50 mM citrate, pH 4 aqueous solution containing 30% ethanol) was added to the pre-equilibrated (35 °C) vesicles at a rate of ~5 ml/min with mixing. After a final target siRNA/lipid ratio of 0.06 (wt/wt) was reached, the mixture was incubated for a further 30 min at 35 °C to allow vesicle reorganization and encapsulation of the siRNA. The ethanol was then removed and the external buffer replaced with PBS (155 mM NaCl, 3 mM Na₂HPO₄, 1 mM KH₂PO₄, pH 7.5) by either dialysis or tangential flow diafiltration. siRNA were encapsulated in SNALP using a controlled step-wise dilution method process. The lipid constituents of KC2-SNALP were DLin-KC2-DMA (cationic lipid), dipalmitoylphosphatidylcholine (DPPC; Avanti Polar Lipids), synthetic cholesterol (Sigma) and PEG-C-DMA used at a molar ratio of 57.1:7.1:34.3:1.4. Upon formation of the loaded particles, SNALP were dialyzed against PBS and filter sterilized through a 0.2 µm filter before use. Mean particle sizes were 75–85 nm and 90–95% of the siRNA was encapsulated within the lipid particles. The final siRNA/lipid ratio in formulations used for in vivo testing was ~0.15 (wt/wt). LNP-siRNA systems containing Factor VII siRNA were diluted to the appropriate concentrations in sterile PBS immediately before use and the formulations were administered intravenously through the lateral tail vein in a total volume of 10 ml/kg. This method and these delivery systems may be extrapolated to the CRISPR Cas system of the present invention.

Other Lipids

[00158] Other cationic lipids, such as amino lipid 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (DLin-KC2-DMA) may be utilized to encapsulate CRISPR Cas system or components thereof or nucleic acid molecule(s) coding therefor e.g., similar to siRNA (see, e.g., Jayaraman, *Angew. Chem. Int. Ed.* 2012, 51, 8529 – 8533), and hence may be employed in the practice of the invention. A preformed vesicle with the following lipid composition may be contemplated: amino lipid, distearoylphosphatidylcholine (DSPC), cholesterol and (R)-2,3-bis(octadecyloxy) propyl-1-(methoxy poly(ethylene glycol)2000)propylcarbamate (PEG-lipid) in the molar ratio 40/10/40/10, respectively, and a FVII siRNA/total lipid ratio of approximately 0.05 (w/w). To ensure a narrow particle size distribution in the range of 70–90 nm and a low polydispersity index of 0.11 ± 0.04 ($n=56$), the particles may be extruded up to three times through 80 nm membranes prior to adding the CRISPR Cas RNA. Particles containing the highly potent amino lipid 16 may be used, in which the molar ratio of the four lipid components 16, DSPC, cholesterol and PEG-lipid (50/10/38.5/1.5) which may be further optimized to enhance in vivo activity.

[00159] Michael S D Kormann et al. ("Expression of therapeutic proteins after delivery of chemically modified mRNA in mice: *Nature Biotechnology*, Volume:29, Pages: 154–157 (2011)) describes the use of lipid envelopes to deliver RNA. Use of lipid envelopes is also preferred in the present invention.

[00160] In another embodiment, lipids may be formulated with the CRISPR Cas system of the present invention to form lipid nanoparticles (LNPs). Lipids include, but are not limited to, DLin-KC2-DMA4, C12-200 and colipids disteroylphosphatidyl choline, cholesterol, and PEG-DMG may be formulated with CRISPR Cas instead of siRNA (see, e.g., Novobrantseva, *Molecular Therapy–Nucleic Acids* (2012) 1, e4; doi:10.1038/mtna.2011.3) using a spontaneous vesicle formation procedure. The component molar ratio may be about 50/10/38.5/1.5 (DLin-KC2-DMA or C12-200/disteroylphosphatidyl choline/cholesterol/PEG-DMG). The final lipid:siRNA weight ratio may be ~12:1 and 9:1 in the case of DLin-KC2-DMA and C12-200 lipid nanoparticles (LNPs), respectively. The formulations may have mean particle diameters of ~80 nm with >90% entrapment efficiency. A 3 mg/kg dose may be contemplated.

[00161] Tekmira has a portfolio of approximately 95 patent families, in the U.S. and abroad, that are directed to various aspects of LNPs and LNP formulations (see, e.g., U.S. Pat. Nos. 7,982,027; 7,799,565; 8,058,069; 8,283,333; 7,901,708; 7,745,651; 7,803,397; 8,101,741;

8,188,263; 7,915,399; 8,236,943 and 7,838,658 and European Pat. Nos 1766035; 1519714; 1781593 and 1664316), all of which may be used and/or adapted to the present invention.

[00162] The CRISPR Cas system or components thereof or nucleic acid molecule(s) coding therefor may be delivered encapsulated in PLGA Microspheres such as that further described in US published applications 20130252281 and 20130245107 and 20130244279 (assigned to Moderna Therapeutics) which relate to aspects of formulation of compositions comprising modified nucleic acid molecules which may encode a protein, a protein precursor, or a partially or fully processed form of the protein or a protein precursor. The formulation may have a molar ratio 50:10:38.5:1.5-3.0 (cationic lipid: fusogenic lipid: cholesterol: PEG lipid). The PEG lipid may be selected from, but is not limited to PEG-c-DOMG, PEG-DMG. The fusogenic lipid may be DSPC. See also, Schrum et al., Delivery and Formulation of Engineered Nucleic Acids, US published application 20120251618.

[00163] Nanomerics' technology addresses bioavailability challenges for a broad range of therapeutics, including low molecular weight hydrophobic drugs, peptides, and nucleic acid based therapeutics (plasmid, siRNA, miRNA). Specific administration routes for which the technology has demonstrated clear advantages include the oral route, transport across the blood-brain-barrier, delivery to solid tumours, as well as to the eye. See, e.g., Mazza et al., 2013, ACS Nano. 2013 Feb 26;7(2):1016-26; Uchegbu and Siew, 2013, J Pharm Sci. 102(2):305-10 and Lalatsa et al., 2012, J Control Release. 2012 Jul 20; 161(2):523-36.

[00164] U.S. Patent Publication No. 20050019923 describes cationic dendrimers for delivering bioactive molecules, such as polynucleotide molecules, peptides and polypeptides and/or pharmaceutical agents, to a mammalian body. The dendrimers are suitable for targeting the delivery of the bioactive molecules to, for example, the liver, spleen, lung, kidney or heart (or even the brain). Dendrimers are synthetic 3-dimensional macromolecules that are prepared in a step-wise fashion from simple branched monomer units, the nature and functionality of which can be easily controlled and varied. Dendrimers are synthesised from the repeated addition of building blocks to a multifunctional core (divergent approach to synthesis), or towards a multifunctional core (convergent approach to synthesis) and each addition of a 3-dimensional shell of building blocks leads to the formation of a higher generation of the dendrimers. Polypropylenimine dendrimers start from a diaminobutane core to which is added twice the number of amino groups by a double Michael addition of acrylonitrile to the primary amines followed by the hydrogenation

of the nitriles. This results in a doubling of the amino groups. Polypropylenimine dendrimers contain 100% protonable nitrogens and up to 64 terminal amino groups (generation 5, DAB 64). Protonable groups are usually amine groups which are able to accept protons at neutral pH. The use of dendrimers as gene delivery agents has largely focused on the use of the polyamidoamine, and phosphorous containing compounds with a mixture of amine/amide or N--P(O₂)S as the conjugating units respectively with no work being reported on the use of the lower generation polypropylenimine dendrimers for gene delivery. Polypropylenimine dendrimers have also been studied as pH sensitive controlled release systems for drug delivery and for their encapsulation of guest molecules when chemically modified by peripheral amino acid groups. The cytotoxicity and interaction of polypropylenimine dendrimers with DNA as well as the transfection efficacy of DAB 64 has also been studied.

[00165] U.S. Patent Publication No. 20050019923 is based upon the observation that, contrary to earlier reports, cationic dendrimers, such as polypropylenimine dendrimers, display suitable properties, such as specific targeting and low toxicity, for use in the targeted delivery of bioactive molecules, such as genetic material. In addition, derivatives of the cationic dendrimer also display suitable properties for the targeted delivery of bioactive molecules. See also, Bioactive Polymers, US published application 20080267903, which discloses "Various polymers, including cationic polyamine polymers and dendrimeric polymers, are shown to possess anti-proliferative activity, and may therefore be useful for treatment of disorders characterised by undesirable cellular proliferation such as neoplasms and tumours, inflammatory disorders (including autoimmune disorders), psoriasis and atherosclerosis. The polymers may be used alone as active agents, or as delivery vehicles for other therapeutic agents, such as drug molecules or nucleic acids for gene therapy. In such cases, the polymers' own intrinsic anti-tumour activity may complement the activity of the agent to be delivered." The disclosures of these patent publications may be employed in conjunction with herein teachings for delivery of CRISPR Cas system(s) or component(s) thereof or nucleic acid molecule(s) coding therefor.

Supercharged proteins

[00166] Supercharged proteins are a class of engineered or naturally occurring proteins with unusually high positive or negative net theoretical charge and may be employed in delivery of CRISPR Cas system(s) or component(s) thereof or nucleic acid molecule(s) coding therefor. Both supernegatively and superpositively charged proteins exhibit a remarkable ability to withstand

thermally or chemically induced aggregation. Superpositively charged proteins are also able to penetrate mammalian cells. Associating cargo with these proteins, such as plasmid DNA, RNA, or other proteins, can enable the functional delivery of these macromolecules into mammalian cells both in vitro and in vivo. David Liu's lab reported the creation and characterization of supercharged proteins in 2007 (Lawrence et al., 2007, *Journal of the American Chemical Society* 129, 10110–10112).

[00167] The nonviral delivery of RNA and plasmid DNA into mammalian cells are valuable both for research and therapeutic applications (Akinc et al., 2010, *Nat. Biotech.* 26, 561–569). Purified +36 GFP protein (or other superpositively charged protein) is mixed with RNAs in the appropriate serum-free media and allowed to complex prior addition to cells. Inclusion of serum at this stage inhibits formation of the supercharged protein-RNA complexes and reduces the effectiveness of the treatment. The following protocol has been found to be effective for a variety of cell lines (McNaughton et al., 2009, *Proc. Natl. Acad. Sci. USA* 106, 6111–6116). However, pilot experiments varying the dose of protein and RNA should be performed to optimize the procedure for specific cell lines.

(1) One day before treatment, plate 1×10^5 cells per well in a 48-well plate.

(2) On the day of treatment, dilute purified +36 GFP protein in serumfree media to a final concentration 200nM. Add RNA to a final concentration of 50nM. Vortex to mix and incubate at room temperature for 10min.

(3) During incubation, aspirate media from cells and wash once with PBS.

(4) Following incubation of +36 GFP and RNA, add the protein-RNA complexes to cells.

(5) Incubate cells with complexes at 37 °C for 4h.

(6) Following incubation, aspirate the media and wash three times with 20 U/mL heparin PBS. Incubate cells with serum-containing media for a further 48h or longer depending upon the assay for activity.

(7) Analyze cells by immunoblot, qPCR, phenotypic assay, or other appropriate method.

[00168] David Liu's lab has further found +36 GFP to be an effective plasmid delivery reagent in a range of cells. As plasmid DNA is a larger cargo than siRNA, proportionately more +36 GFP protein is required to effectively complex plasmids. For effective plasmid delivery Applicants

have developed a variant of +36 GFP bearing a C-terminal HA2 peptide tag, a known endosome-disrupting peptide derived from the influenza virus hemagglutinin protein. The following protocol has been effective in a variety of cells, but as above it is advised that plasmid DNA and supercharged protein doses be optimized for specific cell lines and delivery applications.

- (1) One day before treatment, plate 1×10^5 per well in a 48-well plate.
- (2) On the day of treatment, dilute purified β 36 GFP protein in serumfree media to a final concentration 2 mM. Add 1mg of plasmid DNA. Vortex to mix and incubate at room temperature for 10min.
- (3) During incubation, aspirate media from cells and wash once with PBS.
- (4) Following incubation of β 36 GFP and plasmid DNA, gently add the protein-DNA complexes to cells.
- (5) Incubate cells with complexes at 37 C for 4h.
- (6) Following incubation, aspirate the media and wash with PBS. Incubate cells in serum-containing media and incubate for a further 24–48h.
- (7) Analyze plasmid delivery (e.g., by plasmid-driven gene expression) as appropriate.

[00169] David Liu's lab has further found +36 GFP to be an effective plasmid delivery reagent in a range of cells. As plasmid DNA is a larger cargo than siRNA, proportionately more +36 GFP protein is required to effectively complex plasmids. For effective plasmid delivery Applicants have developed a variant of +36 GFP bearing a C-terminal HA2 peptide tag, a known endosome-disrupting peptide derived from the influenza virus hemagglutinin protein. The following protocol has been effective in a variety of cells, but as above it is advised that plasmid DNA and supercharged protein doses be optimized for specific cell lines and delivery applications:

- (1) One day before treatment, plate 1×10^5 per well in a 48-well plate.
- (2) On the day of treatment, dilute purified β 36 GFP protein in serumfree media to a final concentration 2 mM. Add 1mg of plasmid DNA. Vortex to mix and incubate at room temperature for 10min.
- (3) During incubation, aspirate media from cells and wash once with PBS.
- (4) Following incubation of β 36 GFP and plasmid DNA, gently add the protein-DNA complexes to cells.
- (5) Incubate cells with complexes at 37 C for 4h.
- (6) Following incubation, aspirate the media and wash with PBS. Incubate cells in serum-containing media and incubate for a further 24–48h.
- (7) Analyze plasmid delivery (e.g., by plasmid-driven gene expression) as appropriate. See also, e.g., McNaughton et al., Proc. Natl. Acad. Sci. USA 106, 6111-6116 (2009); Cronican et al., ACS Chemical Biology 5, 747-752

(2010); Cronican et al., *Chemistry & Biology* 18, 833-838 (2011); Thompson et al., *Methods in Enzymology* 503, 293-319 (2012); Thompson, D.B., et al., *Chemistry & Biology* 19 (7), 831-843 (2012). The methods of the super charged proteins may be used and/or adapted for delivery of the CRISPR Cas system of the present invention. These systems of Dr. Lui and documents herein in conjunction with herein teachings can be employed in the delivery of CRISPR Cas system(s) or component(s) thereof or nucleic acid molecule(s) coding therefor.

Cell Penetrating Peptides (CPPs)

[00170] In yet another embodiment, cell penetrating peptides (CPPs) are contemplated for the delivery of the CRISPR Cas system. CPPs are short peptides that facilitate cellular uptake of various molecular cargo (from nanosize particles to small chemical molecules and large fragments of DNA). The term "cargo" as used herein includes but is not limited to the group consisting of therapeutic agents, diagnostic probes, peptides, nucleic acids, antisense oligonucleotides, plasmids, proteins, nanoparticles, liposomes, chromophores, small molecules and radioactive materials. In aspects of the invention, the cargo may also comprise any component of the CRISPR Cas system or the entire functional CRISPR Cas system. Aspects of the present invention further provide methods for delivering a desired cargo into a subject comprising: (a) preparing a complex comprising the cell penetrating peptide of the present invention and a desired cargo, and (b) orally, intraarticularly, intraperitoneally, intrathecally, intrarterially, intranasally, intraparenchymally, subcutaneously, intramuscularly, intravenously, dermally, intrarectally, or topically administering the complex to a subject. The cargo is associated with the peptides either through chemical linkage via covalent bonds or through non-covalent interactions.

[00171] The function of the CPPs are to deliver the cargo into cells, a process that commonly occurs through endocytosis with the cargo delivered to the endosomes of living mammalian cells. Cell-penetrating peptides are of different sizes, amino acid sequences, and charges but all CPPs have one distinct characteristic, which is the ability to translocate the plasma membrane and facilitate the delivery of various molecular cargoes to the cytoplasm or an organelle. CPP translocation may be classified into three main entry mechanisms: direct penetration in the membrane, endocytosis-mediated entry, and translocation through the formation of a transitory structure. CPPs have found numerous applications in medicine as drug delivery agents in the treatment of different diseases including cancer and virus inhibitors, as well as contrast agents for cell labeling. Examples of the latter include acting as a carrier for GFP, MRI contrast agents, or

quantum dots. CPPs hold great potential as in vitro and in vivo delivery vectors for use in research and medicine. CPPs typically have an amino acid composition that either contains a high relative abundance of positively charged amino acids such as lysine or arginine or has sequences that contain an alternating pattern of polar/charged amino acids and non-polar, hydrophobic amino acids. These two types of structures are referred to as polycationic or amphipathic, respectively. A third class of CPPs are the hydrophobic peptides, containing only apolar residues, with low net charge or have hydrophobic amino acid groups that are crucial for cellular uptake. One of the initial CPPs discovered was the trans-activating transcriptional activator (Tat) from Human Immunodeficiency Virus 1 (HIV-1) which was found to be efficiently taken up from the surrounding media by numerous cell types in culture. Since then, the number of known CPPs has expanded considerably and small molecule synthetic analogues with more effective protein transduction properties have been generated. CPPs include but are not limited to Penetratin, Tat (48-60), Transportan, and (R-Ahx-R4) (Ahx=aminohexanoyl).

[00172] U.S. Patent 8,372,951, provides a CPP derived from eosinophil cationic protein (ECP) which exhibits highly cell-penetrating efficiency and low toxicity. Aspects of delivering the CPP with its cargo into a vertebrate subject are also provided. Further aspects of CPPs and their delivery are described in U. S. patents 8,575,305; 8,614,194 and 8,044,019. CPPs can be used to deliver the CRISPR-Cas system or components thereof. That CPPs can be employed to deliver the CRISPR-Cas system or components thereof is also provided in the manuscript "Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA", by Suresh Ramakrishna, Abu-Bonsrah Kwaku Dad, Jagadish Beloor, et al. *Genome Res.* 2014 Apr 2. [Epub ahead of print], incorporated by reference in its entirety, wherein it is demonstrated that treatment with CPP-conjugated recombinant Cas9 protein and CPP-complexed guide RNAs lead to endogenous gene disruptions in human cell lines. In the paper the Cas9 protein was conjugated to CPP via a thioether bond, whereas the guide RNA was complexed with CPP, forming condensed, positively charged nanoparticles. It was shown that simultaneous and sequential treatment of human cells, including embryonic stem cells, dermal fibroblasts, HEK293T cells, HeLa cells, and embryonic carcinoma cells, with the modified Cas9 and guide RNA led to efficient gene disruptions with reduced off-target mutations relative to plasmid transfections.

Implantable devices

[00173] In another embodiment, implantable devices are also contemplated for delivery of the CRISPR Cas system or component(s) thereof or nucleic acid molecule(s) coding therefor. For example, U.S. Patent Publication 20110195123 discloses an implantable medical device which elutes a drug locally and in prolonged period is provided, including several types of such a device, the treatment modes of implementation and methods of implantation. The device comprising of polymeric substrate, such as a matrix for example, that is used as the device body, and drugs, and in some cases additional scaffolding materials, such as metals or additional polymers, and materials to enhance visibility and imaging. An implantable delivery device can be advantageous in providing release locally and over a prolonged period, where drug is released directly to the extracellular matrix (ECM) of the diseased area such as tumor, inflammation, degeneration or for symptomatic objectives, or to injured smooth muscle cells, or for prevention. One kind of drug is RNA, as disclosed above, and this system may be used/and or adapted to the CRISPR Cas system of the present invention. The modes of implantation in some embodiments are existing implantation procedures that are developed and used today for other treatments, including brachytherapy and needle biopsy. In such cases the dimensions of the new implant described in this invention are similar to the original implant. Typically a few devices are implanted during the same treatment procedure.

[00174] As in U.S. Patent Publication 20110195123, there is provided a drug delivery implantable or insertable system, including systems applicable to a cavity such as the abdominal cavity and/or any other type of administration in which the drug delivery system is not anchored or attached, comprising a biostable and/or degradable and/or bioabsorbable polymeric substrate, which may for example optionally be a matrix. It should be noted that the term "insertion" also includes implantation. The drug delivery system is preferably implemented as a "Loder" as described in U.S. Patent Publication 20110195123.

[00175] The polymer or plurality of polymers are biocompatible, incorporating an agent and/or plurality of agents, enabling the release of agent at a controlled rate, wherein the total volume of the polymeric substrate, such as a matrix for example, in some embodiments is optionally and preferably no greater than a maximum volume that permits a therapeutic level of the agent to be reached. As a non-limiting example, such a volume is preferably within the range of 0.1 m^3 to 1000 mm^3 , as required by the volume for the agent load. The Loder may optionally be larger, for

example when incorporated with a device whose size is determined by functionality, for example and without limitation, a knee joint, an intra-uterine or cervical ring and the like.

[00176] The drug delivery system (for delivering the composition) is designed in some embodiments to preferably employ degradable polymers, wherein the main release mechanism is bulk erosion; or in some embodiments, non degradable, or slowly degraded polymers are used, wherein the main release mechanism is diffusion rather than bulk erosion, so that the outer part functions as membrane, and its internal part functions as a drug reservoir, which practically is not affected by the surroundings for an extended period (for example from about a week to about a few months). Combinations of different polymers with different release mechanisms may also optionally be used. The concentration gradient at the surface is preferably maintained effectively constant during a significant period of the total drug releasing period, and therefore the diffusion rate is effectively constant (termed "zero mode" diffusion). By the term "constant" it is meant a diffusion rate that is preferably maintained above the lower threshold of therapeutic effectiveness, but which may still optionally feature an initial burst and/or may fluctuate, for example increasing and decreasing to a certain degree. The diffusion rate is preferably so maintained for a prolonged period, and it can be considered constant to a certain level to optimize the therapeutically effective period, for example the effective silencing period.

[00177] The drug delivery system optionally and preferably is designed to shield the nucleotide based therapeutic agent from degradation, whether chemical in nature or due to attack from enzymes and other factors in the body of the subject.

[00178] The drug delivery system of U.S. Patent Publication 20110195123 is optionally associated with sensing and/or activation appliances that are operated at and/or after implantation of the device, by non and/or minimally invasive methods of activation and/or acceleration/deceleration, for example optionally including but not limited to thermal heating and cooling, laser beams, and ultrasonic, including focused ultrasound and/or RF (radiofrequency) methods or devices.

[00179] According to some embodiments of U.S. Patent Publication 20110195123, the site for local delivery may optionally include target sites characterized by high abnormal proliferation of cells, and suppressed apoptosis, including tumors, active and or chronic inflammation and infection including autoimmune diseases states, degenerating tissue including muscle and nervous

tissue, chronic pain, degenerative sites, and location of bone fractures and other wound locations for enhancement of regeneration of tissue, and injured cardiac, smooth and striated muscle.

[00180] The site for implantation of the composition, or target site, preferably features a radius, area and/or volume that is sufficiently small for targeted local delivery. For example, the target site optionally has a diameter in a range of from about 0.1 mm to about 5 cm.

[00181] The location of the target site is preferably selected for maximum therapeutic efficacy. For example, the composition of the drug delivery system (optionally with a device for implantation as described above) is optionally and preferably implanted within or in the proximity of a tumor environment, or the blood supply associated thereof.

[00182] For example the composition (optionally with the device) is optionally implanted within or in the proximity to pancreas, prostate, breast, liver, via the nipple, within the vascular system and so forth.

[00183] The target location is optionally selected from the group consisting of (as non-limiting examples only, as optionally any site within the body may be suitable for implanting a Loder): 1. brain at degenerative sites like in Parkinson or Alzheimer disease at the basal ganglia, white and gray matter; 2. spine as in the case of amyotrophic lateral sclerosis (ALS); 3. uterine cervix to prevent HPV infection; 4. active and chronic inflammatory joints; 5. dermis as in the case of psoriasis; 6. sympathetic and sensoric nervous sites for analgesic effect; 7. Intra osseous implantation; 8. acute and chronic infection sites; 9. Intra vaginal; 10. Inner ear--auditory system, labyrinth of the inner ear, vestibular system; 11. Intra tracheal; 12. Intra-cardiac; coronary, epicardiac; 13. urinary bladder; 14. biliary system; 15. parenchymal tissue including and not limited to the kidney, liver, spleen; 16. lymph nodes; 17. salivary glands; 18. dental gums; 19. Intra-articular (into joints); 20. Intra-ocular; 21. Brain tissue; 22. Brain ventricles; 23. Cavities, including abdominal cavity (for example but without limitation, for ovary cancer); 24. Intra esophageal and 25. Intra rectal.

[00184] Optionally insertion of the system (for example a device containing the composition) is associated with injection of material to the ECM at the target site and the vicinity of that site to affect local pH and/or temperature and/or other biological factors affecting the diffusion of the drug and/or drug kinetics in the ECM, of the target site and the vicinity of such a site.

[00185] Optionally, according to some embodiments, the release of said agent could be associated with sensing and/or activation appliances that are operated prior and/or at and/or after

insertion, by non and/or minimally invasive and/or else methods of activation and/or acceleration/deceleration, including laser beam, radiation, thermal heating and cooling, and ultrasonic, including focused ultrasound and/or RF (radiofrequency) methods or devices, and chemical activators.

[00186] According to other embodiments of U.S. Patent Publication 20110195123, the drug preferably comprises a RNA, for example for localized cancer cases in breast, pancreas, brain, kidney, bladder, lung, and prostate as described below. Although exemplified with RNAi, many drugs are applicable to be encapsulated in Loder, and can be used in association with this invention, as long as such drugs can be encapsulated with the Loder substrate, such as a matrix for example, and this system may be used and/or adapted to deliver the CRISPR Cas system of the present invention.

[00187] As another example of a specific application, neuro and muscular degenerative diseases develop due to abnormal gene expression. Local delivery of RNAs may have therapeutic properties for interfering with such abnormal gene expression. Local delivery of anti apoptotic, anti inflammatory and anti degenerative drugs including small drugs and macromolecules may also optionally be therapeutic. In such cases the Loder is applied for prolonged release at constant rate and/or through a dedicated device that is implanted separately. All of this may be used and/or adapted to the CRISPR Cas system of the present invention.

[00188] As yet another example of a specific application, psychiatric and cognitive disorders are treated with gene modifiers. Gene knockdown is a treatment option. Loders locally delivering agents to central nervous system sites are therapeutic options for psychiatric and cognitive disorders including but not limited to psychosis, bi-polar diseases, neurotic disorders and behavioral maladies. The Loders could also deliver locally drugs including small drugs and macromolecules upon implantation at specific brain sites. All of this may be used and/or adapted to the CRISPR Cas system of the present invention.

[00189] As another example of a specific application, silencing of innate and/or adaptive immune mediators at local sites enables the prevention of organ transplant rejection. Local delivery of RNAs and immunomodulating reagents with the Loder implanted into the transplanted organ and/or the implanted site renders local immune suppression by repelling immune cells such as CD8 activated against the transplanted organ. All of this may be used/and or adapted to the CRISPR Cas system of the present invention.

[00190] As another example of a specific application, vascular growth factors including VEGFs and angiogenin and others are essential for neovascularization. Local delivery of the factors, peptides, peptidomimetics, or suppressing their repressors is an important therapeutic modality; silencing the repressors and local delivery of the factors, peptides, macromolecules and small drugs stimulating angiogenesis with the Loder is therapeutic for peripheral, systemic and cardiac vascular disease.

[00191] The method of insertion, such as implantation, may optionally already be used for other types of tissue implantation and/or for insertions and/or for sampling tissues, optionally without modifications, or alternatively optionally only with non-major modifications in such methods. Such methods optionally include but are not limited to brachytherapy methods, biopsy, endoscopy with and/or without ultrasound, such as ERCP, stereotactic methods into the brain tissue, Laparoscopy, including implantation with a laparoscope into joints, abdominal organs, the bladder wall and body cavities.

[00192] Implantable device technology herein discussed can be employed with herein teachings and hence by this disclosure and the knowledge in the art, CRISPR-Cas system or components thereof or nucleic acid molecules thereof or encoding or providing components may be delivered via an implantable device.

Patient-specific screening methods

[00193] A CRISPR-Cas system that targets nucleotide, e.g., trinucleotide repeats can be used to screen patients or patient samples for the presence of such repeats. The repeats can be the target of the RNA of the CRISPR-Cas system, and if there is binding thereto by the CRISPR-Cas system, that binding can be detected, to thereby indicate that such a repeat is present. Thus, a CRISPR-Cas system can be used to screen patients or patient samples for the presence of the repeat. The patient can then be administered suitable compound(s) to address the condition; or, can be administered a CRISPR-Cas system to bind to and cause insertion, deletion or mutation and alleviate the condition.

HSC—Delivery to and Editing of Hematopoietic Stem Cells; and Particular Conditions

[00194] The term “Hematopoietic Stem Cell” or “HSC” is meant to include broadly those cells considered to be an HSC, e.g., blood cells that give rise to all the other blood cells and are derived from mesoderm; located in the red bone marrow, which is contained in the core of most bones. HSCs of the invention include cells having a phenotype of hematopoietic stem cells, identified by

small size, lack of lineage (lin) markers, and markers that belong to the cluster of differentiation series, like: CD34, CD38, CD90, CD133, CD105, CD45, and also c-kit, - the receptor for stem cell factor. Hematopoietic stem cells are negative for the markers that are used for detection of lineage commitment, and are, thus, called Lin⁻; and, during their purification by FACS, a number of up to 14 different mature blood-lineage markers, e.g., CD13 & CD33 for myeloid, CD71 for erythroid, CD19 for B cells, CD61 for megakaryocytic, etc. for humans; and, B220 (murine CD45) for B cells, Mac-1 (CD11b/CD18) for monocytes, Gr-1 for Granulocytes, Ter119 for erythroid cells, Il7Ra, CD3, CD4, CD5, CD8 for T cells, etc. Mouse HSC markers: CD34^{lo/-}, SCA-1⁺, Thy1.1^{+/lo}, CD38⁺, C-kit⁺, lin⁻, and Human HSC markers: CD34⁺, CD59⁺, Thy1/CD90⁺, CD38^{lo/-}, C-kit/CD117⁺, and lin⁻. HSCs are identified by markers. Hence in embodiments discussed herein, the HSCs can be CD34⁺ cells. HSCs can also be hematopoietic stem cells that are CD34⁻/CD38⁻. Stem cells that may lack c-kit on the cell surface that are considered in the art as HSCs are within the ambit of the invention, as well as CD133⁺ cells likewise considered HSCs in the art.

[00195] The CRISPR-Cas9 system has been engineered to target genetic locus or loci in HSCs. Cas9 protein, advantageously codon-optimized for a eukaryotic cell and especially a mammalian cell, e.g., a human cell, for instance, HSC, and sgRNA targeting a locus or loci in HSC, e.g., the gene EMX1, were prepared. These were advantageously delivered via particles. The particles were formed by the Cas9 protein and the sgRNA being admixed. The sgRNA and Cas9 protein mixture was admixed with a mixture comprising or consisting essentially of or consisting of surfactant, phospholipid, biodegradable polymer, lipoprotein and alcohol, whereby particles containing the sgRNA and Cas9 protein were formed. The invention comprehends so making particles and particles from such a method as well as uses thereof.

[00196] More generally, particles were formed using an efficient process. First, Cas9 protein and sgRNA targeting the gene EMX1 or the control gene LacZ were mixed together at a suitable, e.g., 3:1 to 1:3 or 2:1 to 1:2 or 1:1 molar ratio, at a suitable temperature, e.g., 15-30C, e.g., 20-25C, e.g., room temperature, for a suitable time, e.g., 15-45, such as 30 minutes, advantageously in sterile, nuclease free buffer, e.g., 1X PBS. Separately, particle components such as or comprising: a surfactant, e.g., cationic lipid, e.g., 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP); phospholipid, e.g., dimyristoylphosphatidylcholine (DMPC); biodegradable polymer, such as an ethylene-glycol polymer or PEG, and a lipoprotein, such as a low-density lipoprotein, e.g., cholesterol were dissolved in an alcohol, advantageously a C₁₋₆ alkyl alcohol, such as methanol,

ethanol, isopropanol, e.g., 100% ethanol. The two solutions were mixed together to form particles containing the Cas9-sgRNA complexes. In certain embodiments the particle can contain an HDR template. That can be a particle co-administered with sgRNA+Cas9 protein-containing particle, or i.e., in addition to contacting an HSC with an sgRNA+Cas9 protein-containing particle, the HSC is contacted with a particle containing an HDR template; or the HSC is contacted with a particle containing all of the sgRNA, Cas9 and the HDR template. The HDR template can be administered by a separate vector, whereby in a first instance the particle penetrates an HSC cell and the separate vector also penetrates the cell, wherein the HSC genome is modified by the sgRNA+Cas9 and the HDR template is also present, whereby a genomic loci is modified by the HDR; for instance, this may result in correcting a mutation.

[00197] After the particles were formed, HSCs in 96 well plates were transfected with 15ug Cas9 protein per well. Three days after transfection, HSCs were harvested, and the number of insertions and deletions (indels) at the EMX1 locus were quantified.

[00198] This demonstrates that HSCs can be modified using CRISPR-Cas9 targeting a genomic locus or loci of interest in the HSC. The HSCs that are to be modified can be in vivo, i.e., in an organism, for example a human or a non-human eukaryote, e.g., animal, such as fish, e.g., zebra fish, mammal, e.g., primate, e.g., ape, chimpanzee, macaque, rodent, e.g., mouse, rabbit, rat, canine or dog, livestock (cow / bovine, sheep / ovine, goat or pig), fowl or poultry, e.g., chicken. The HSCs that are to be modified can be in vitro, i.e., outside of such an organism. And, modified HSCs can be used ex vivo, i.e., one or more HSCs of such an organism can be obtained or isolated from the organism, optionally the HSC(s) can be expanded, the HSC(s) are modified by a composition comprising a CRISPR-Cas9 that targets a genetic locus or loci in the HSC, e.g., by contacting the HSC(s) with the composition, for instance, wherein the composition comprises a particle containing the CRISPR enzyme and one or more sgRNA that targets the genetic locus or loci in the HSC, such as a particle obtained or obtainable from admixing an sgRNA and Cas9 protein mixture with a mixture comprising or consisting essentially of or consisting of surfactant, phospholipid, biodegradable polymer, lipoprotein and alcohol (wherein one or more sgRNA targets the genetic locus or loci in the HSC), optionally expanding the resultant modified HSCs and administering to the organism the resultant modified HSCs. In some instances the isolated or obtained HSCs can be from a first organism, such as an organism from a same species as a second organism, and the second organism can be the organism to which the the resultant modified HSCs

are administered, e.g., the first organism can be a donor (such as a relative as in a parent or sibling) to the second organism. Modified HSCs can have genetic modifications to address or alleviate or reduce symptoms of a disease or condition state of an individual or subject or patient. Modified HSCs, e.g., in the instance of a first organism donor to a second organism, can have genetic modifications to have the HSCs have one or more proteins e.g. surface markers or proteins more like that of the second organism. Modified HSCs can have genetic modifications to simulate a disease or condition state of an individual or subject or patient and would be re-administered to a non-human organism so as to prepare an animal model. Expansion of HSCs is within the ambit of the skilled person from this disclosure and knowledge in the art, see e.g., Lee, "Improved ex vivo expansion of adult hematopoietic stem cells by overcoming CUL4-mediated degradation of HOXB4." *Blood*. 2013 May 16;121(20):4082-9. doi: 10.1182/blood-2012-09-455204. Epub 2013 Mar 21.

[00199] As indicated to improve activity, sgRNA may be pre-complexed with the Cas9 protein, before formulating the entire complex in a particle. Formulations may be made with a different molar ratio of different components known to promote delivery of nucleic acids into cells (e.g. 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (DMPC), polyethylene glycol (PEG), and cholesterol) For example DOTAP : DMPC : PEG : Cholesterol Molar Ratios may be DOTAP 100, DMPC 0, PEG 0, Cholesterol 0; or DOTAP 90, DMPC 0, PEG 10, Cholesterol 0; or DOTAP 90, DMPC 0, PEG 5, Cholesterol 5. DOTAP 100, DMPC 0, PEG 0, Cholesterol 0. The invention accordingly comprehends admixing sgRNA, Cas9 protein and components that form a particle; as well as particles from such admixing.

[00200] In a preferred embodiment, particles containing the Cas9-sgRNA complexes may be formed by mixing Cas9 protein and one or more sgRNAs together, preferably at a 1:1 molar ratio, enzyme: guide RNA. Separately, the different components known to promote delivery of nucleic acids (e.g. DOTAP, DMPC, PEG, and cholesterol) are dissolved, preferably in ethanol. The two solutions are mixed together to form particles containing the Cas9-sgRNA complexes. After the particles are formed, Cas9-sgRNA complexes may be transfected into cells (e.g. HSCs). Bar coding may be applied. The particles, the Cas-9 and/or the sgRNA may be barcoded.

[00201] The invention in an embodiment comprehends a method of preparing an sgRNA-and-Cas9 protein containing particle comprising admixing an sgRNA and Cas9 protein

mixture with a mixture comprising or consisting essentially of or consisting of surfactant, phospholipid, biodegradable polymer, lipoprotein and alcohol. An embodiment comprehends an sgRNA-and-Cas9 protein containing particle from the method. The invention in an embodiment comprehends use of the particle in a method of modifying a genomic locus of interest, or an organism or a non-human organism by manipulation of a target sequence in a genomic locus of interest, comprising contacting a cell containing the genomic locus of interest with the particle wherein the sgRNA targets the genomic locus of interest; or a method of modifying a genomic locus of interest, or an organism or a non-human organism by manipulation of a target sequence in a genomic locus of interest, comprising contacting a cell containing the genomic locus of interest with the particle wherein the sgRNA targets the genomic locus of interest. In these embodiments, the genomic locus of interest is advantageously a genomic locus in an HSC.

[00202] Considerations for Therapeutic Applications: A consideration in genome editing therapy is the choice of sequence-specific nuclease. Each nuclease platform possesses its own unique set of strengths and weaknesses, many of which must be balanced in the context of treatment to maximize therapeutic benefit (Figure 1). Thus far, two therapeutic editing approaches with nucleases have shown significant promise: gene disruption and gene correction. Gene disruption involves stimulation of NHEJ to create targeted indels in genetic elements, often resulting in loss of function mutations that are beneficial to patients (Figure 2A). In contrast, gene correction uses HDR to directly reverse a disease causing mutation, restoring function while preserving physiological regulation of the corrected element (Figure 2B). HDR may also be used to insert a therapeutic transgene into a defined 'safe harbor' locus in the genome to recover missing gene function (Figure 2C). For a specific editing therapy to be efficacious, a sufficiently high level of modification must be achieved in target cell populations to reverse disease symptoms. This therapeutic modification 'threshold' is determined by the fitness of edited cells following treatment and the amount of gene product necessary to reverse symptoms. With regard to fitness, editing creates three potential outcomes for treated cells relative to their unedited counterparts: increased, neutral, or decreased fitness. In the case of increased fitness, for example in the treatment of SCID-X1, modified hematopoietic progenitor cells selectively expand relative to their unedited counterparts. SCID-X1 is a disease caused by mutations in the IL2RG gene, the function of which is required for proper development of the hematopoietic lymphocyte lineage [Leonard, W.J., et al. Immunological reviews 138, 61-86 (1994); Kaushansky, K. & Williams, W.J. Williams

hematology, (McGraw-Hill Medical, New York, 2010)]. In clinical trials with patients who received viral gene therapy for SCID-X1, and a rare example of a spontaneous correction of SCID-X1 mutation, corrected hematopoietic progenitor cells were able to overcome this developmental block and expand relative to their diseased counterparts to mediate therapy [Bouso, P., et al. Proceedings of the National Academy of Sciences of the United States of America 97, 274-278 (2000); Hacein-Bey-Abina, S., et al. The New England journal of medicine 346, 1185-1193 (2002); Gaspar, H.B., et al. Lancet 364, 2181-2187 (2004)]. In this case, where edited cells possess a selective advantage, even low numbers of edited cells can be amplified through expansion, providing a therapeutic benefit to the patient. In contrast, editing for other hematopoietic diseases, like chronic granulomatous disorder (CGD), would induce no change in fitness for edited hematopoietic progenitor cells, increasing the therapeutic modification threshold. CGD is caused by mutations in genes encoding phagocytic oxidase proteins, which are normally used by neutrophils to generate reactive oxygen species that kill pathogens [Mukherjee, S. & Thrasher, A.J. Gene 525, 174-181 (2013)]. As dysfunction of these genes does not influence hematopoietic progenitor cell fitness or development, but only the ability of a mature hematopoietic cell type to fight infections, there would be likely no preferential expansion of edited cells in this disease. Indeed, no selective advantage for gene corrected cells in CGD has been observed in gene therapy trials, leading to difficulties with long-term cell engraftment [Malech, H.L., et al. Proceedings of the National Academy of Sciences of the United States of America 94, 12133-12138 (1997); Kang, H.J., et al. Molecular therapy : the journal of the American Society of Gene Therapy 19, 2092-2101 (2011)]. As such, significantly higher levels of editing would be required to treat diseases like CGD, where editing creates a neutral fitness advantage, relative to diseases where editing creates increased fitness for target cells. If editing imposes a fitness disadvantage, as would be the case for restoring function to a tumor suppressor gene in cancer cells, modified cells would be outcompeted by their diseased counterparts, causing the benefit of treatment to be low relative to editing rates. This latter class of diseases would be particularly difficult to treat with genome editing therapy.

[00203] In addition to cell fitness, the amount of gene product necessary to treat disease also influences the minimal level of therapeutic genome editing that must be achieved to reverse symptoms. Haemophilia B is one disease where a small change in gene product levels can result in significant changes in clinical outcomes. This disease is caused by mutations in the gene encoding

factor IX, a protein normally secreted by the liver into the blood, where it functions as a component of the clotting cascade. Clinical severity of haemophilia B is related to the amount of factor IX activity. Whereas severe disease is associated with less than 1% of normal activity, milder forms of the diseases are associated with greater than 1% of factor IX activity [Kaushansky, K. & Williams, W.J. Williams hematology, (McGraw-Hill Medical, New York, 2010); Lofqvist, T., et al. Journal of internal medicine 241, 395-400 (1997)]. This suggests that editing therapies that can restore factor IX expression to even a small percentage of liver cells could have a large impact on clinical outcomes. A study using ZFNs to correct a mouse model of haemophilia B shortly after birth demonstrated that 3-7% correction was sufficient to reverse disease symptoms, providing preclinical evidence for this hypothesis [Li, H., et al. Nature 475, 217-221 (2011)].

[00204] Disorders where a small change in gene product levels can influence clinical outcomes and diseases where there is a fitness advantage for edited cells, are ideal targets for genome editing therapy, as the therapeutic modification threshold is low enough to permit a high chance of success given the current technology. Targeting these diseases has now resulted in successes with editing therapy at the preclinical level and a phase I clinical trial. Improvements in DSB repair pathway manipulation and nuclease delivery are needed to extend these promising results to diseases with a neutral fitness advantage for edited cells, or where larger amounts of gene product are needed for treatment. The Table below shows some examples of applications of genome editing to therapeutic models, and the references of the below Table and the documents cited in those references are hereby incorporated herein by reference as if set out in full.

Disease Type	Nuclease Platform Employed	Therapeutic Strategy	References
Hemophilia B	ZFN	HDR-mediated insertion of correct gene sequence	Li, H., et al. Nature 475, 217-221 (2011)
SCID	ZFN	HDR-mediated insertion of correct gene sequence	Genovese, P., et al. Nature 510, 235-240 (2014)
Hereditary tyrosinemia	CRISPR	HDR-mediated correction of mutation in liver	Yin, H., et al. Nature biotechnology 32, 551-553 (2014)

[00205] Addressing each of the conditions of the foregoing table, using the CRISPR-Cas9 system to target by either HDR-mediated correction of mutation, or HDR-mediated insertion of correct gene sequence, advantageously via a delivery system as herein, e.g., a particle delivery system, is within the ambit of the skilled person from this disclosure and the knowledge in the art.

Thus, an embodiment comprehends contacting a Hemophilia B, SCID (e.g., SCID-X1, ADA-SCID) or Hereditary tyrosinemia mutation-carrying HSC with an sgRNA-and-Cas9 protein containing particle targeting a genomic locus of interest as to Hemophilia B, SCID (e.g., SCID-X1, ADA-SCID) or Hereditary tyrosinemia (e.g., as in Li, Genovese or Yin). The particle also can contain a suitable HDR template to correct the mutation; or the HSC can be contacted with a second particle or a vector that contains or delivers the HDR template. In this regard, it is mentioned that Haemophilia B is an X-linked recessive disorder caused by loss-of-function mutations in the gene encoding Factor IX, a crucial component of the clotting cascade. Recovering Factor IX activity to above 1% of its levels in severely affected individuals can transform the disease into a significantly milder form, as infusion of recombinant Factor IX into such patients prophylactically from a young age to achieve such levels largely ameliorates clinical complications. With the knowledge in the art and the teachings in this disclosure, the skilled person can correct HSCs as to Haemophilia B using a CRISPR-Cas9 system that targets and corrects the mutation (X-linked recessive disorder caused by loss-of-function mutations in the gene encoding Factor IX) (e.g., with a suitable HDR template that delivers a coding sequence for Factor IX); specifically, the sgRNA can target mutation that give rise to Haemophilia B, and the HDR can provide coding for proper expression of Factor IX. An sgRNA that targets the mutation-and-Cas9 protein containing particle is contacted with HSCs carrying the mutation. The particle also can contain a suitable HDR template to correct the mutation for proper expression of Factor IX; or the HSC can be contacted with a second particle or a vector that contains or delivers the HDR template. The so contacted cells can be administered; and optionally treated / expanded; cf. Cartier, discussed herein.

[00206] Severe Combined Immune Deficiency (SCID) results from a defect in lymphocytes T maturation, always associated with a functional defect in lymphocytes B (Cavazzana-Calvo et al., *Annu. Rev. Med.*, 2005, 56, 585-602; Fischer et al., *Immunol. Rev.*, 2005, 203, 98-109). Overall incidence is estimated to 1 in 75 000 births. Patients with untreated SCID are subject to multiple opportunist micro-organism infections, and do generally not live beyond one year. SCID can be treated by allogenic hematopoietic stem cell transfer, from a familial donor. Histocompatibility with the donor can vary widely. In the case of Adenosine Deaminase (ADA) deficiency, one of the SCID forms, patients can be treated by injection of recombinant Adenosine Deaminase enzyme. Since the ADA gene has been shown to be mutated in SCID patients (Giblett et al., *Lancet*, 1972,

2, 1067-1069), several other genes involved in SCID have been identified (Cavazzana-Calvo et al., *Annu. Rev. Med.*, 2005, 56, 585-602; Fischer et al., *Immunol. Rev.*, 2005, 203, 98-109). There are four major causes for SCID: (i) the most frequent form of SCID, SCID-X1 (X-linked SCID or X-SCID), is caused by mutation in the IL2RG gene, resulting in the absence of mature T lymphocytes and NK cells. IL2RG encodes the gamma C protein (Noguchi, et al., *Cell*, 1993, 73, 147-157), a common component of at least five interleukin receptor complexes. These receptors activate several targets through the JAK3 kinase (Macchi et al., *Nature*, 1995, 377, 65-68), which inactivation results in the same syndrome as gamma C inactivation; (ii) mutation in the ADA gene results in a defect in purine metabolism that is lethal for lymphocyte precursors, which in turn results in the quasi absence of B, T and NK cells; (iii) V(D)J recombination is an essential step in the maturation of immunoglobulins and T lymphocytes receptors (TCRs). Mutations in Recombination Activating Gene 1 and 2 (RAG1 and RAG2) and Artemis, three genes involved in this process, result in the absence of mature T and B lymphocytes; and (iv) Mutations in other genes such as CD45, involved in T cell specific signaling have also been reported, although they represent a minority of cases (Cavazzana-Calvo et al., *Annu. Rev. Med.*, 2005, 56, 585-602; Fischer et al., *Immunol. Rev.*, 2005, 203, 98-109). Since when their genetic bases have been identified, the different SCID forms have become a paradigm for gene therapy approaches (Fischer et al., *Immunol. Rev.*, 2005, 203, 98-109) for two major reasons. First, as in all blood diseases, an ex vivo treatment can be envisioned. Hematopoietic Stem Cells (HSCs) can be recovered from bone marrow, and keep their pluripotent properties for a few cell divisions. Therefore, they can be treated in vitro, and then re injected into the patient, where they repopulate the bone marrow. Second, since the maturation of lymphocytes is impaired in SCID patients, corrected cells have a selective advantage. Therefore, a small number of corrected cells can restore a functional immune system. This hypothesis was validated several times by (i) the partial restoration of immune functions associated with the reversion of mutations in SCID patients (Hirschhorn et al., *Nat. Genet.*, 1996, 13, 290-295; Stephan et al., *N. Engl. J. Med.*, 1996, 335, 1563-1567; Bousso et al., *Proc. Natl. Acad. Sci. USA*, 2000, 97, 274-278; Wada et al., *Proc. Natl. Acad. Sci. USA*, 2001, 98, 8697-8702; Nishikomori et al., *Blood*, 2004, 103, 4565-4572), (ii) the correction of SCID-X1 deficiencies in vitro in hematopoietic cells (Candotti et al., *Blood*, 1996, 87, 3097-3102; Cavazzana-Calvo et al., *Blood*, 1996, *Blood*, 88, 3901-3909; Taylor et al., *Blood*, 1996, 87, 3103-3107; Hacein-Bey et al., *Blood*, 1998, 92, 4090-4097), (iii) the correction of

SCID-X1 (Soudais et al., *Blood*, 2000, 95, 3071-3077; Tsai et al., *Blood*, 2002, 100, 72-79), JAK-3 (Bunting et al., *Nat. Med.*, 1998, 4, 58-64; Bunting et al., *Hum. Gene Ther.*, 2000, 11, 2353-2364) and RAG2 (Yates et al., *Blood*, 2002, 100, 3942-3949) deficiencies in vivo in animal models and (iv) by the result of gene therapy clinical trials (Cavazzana-Calvo et al., *Science*, 2000, 288, 669-672; Aiuti et al., *Nat. Med.*, 2002; 8, 423-425; Gaspar et al., *Lancet*, 2004, 364, 2181-2187). U.S. Patent Publication No. 20110182867 assigned to the Children's Medical Center Corporation and the President and Fellows of Harvard College relates to methods and uses of modulating fetal hemoglobin expression (HbF) in a hematopoietic progenitor cells via inhibitors of BCL11A expression or activity, such as RNAi and antibodies. The targets disclosed in U.S. Patent Publication No. 20110182867, such as BCL11A, may be targeted by the CRISPR Cas system of the present invention for modulating fetal hemoglobin expression. See also Bauer et al. (*Science* 11 October 2013: Vol. 342 no. 6155 pp. 253-257) and Xu et al. (*Science* 18 November 2011: Vol. 334 no. 6058 pp. 993-996) for additional BCL11A targets. Using a CRISPR-Cas9 system that targets and one or more of the mutations associated with SCID, for instance a CRISPR-Cas9 system having sgRNA(s) and HDR template(s) that respectively targets mutation of *IL2RG* that give rise to SCID and provide corrective expression of the gamma C protein. An sgRNA that targets the mutation(s) (e.g., one or more involved in SCID, for instance mutation as to *IL2RG* that encodes the gamma C protein)-and-Cas9 protein containing particle is contacted with HSCs carrying the mutation(s). The particle also can contain a suitable HDR template(s) to correct the mutation for proper expression of one or more of the proteins involved in SCID, e.g., gamma C protein; or the HSC can be contacted with a second particle or a vector that contains or delivers the HDR template. The so contacted cells can be administered; and optionally treated / expanded; cf. Cartier, discussed herein. Mention is also made of target sequences identified in US Patent Publication Nos. 20110225664, 20110091441, 20100229252, 20090271881 and 20090222937 that may be of interest as to the present invention.

[00207] In Cartier, "MINI-SYMPOSIUM: X-Linked Adrenoleukodystrophypa, Hematopoietic Stem Cell Transplantation and Hematopoietic Stem Cell Gene Therapy in X-Linked Adrenoleukodystrophy," *Brain Pathology* 20 (2010) 857-862, incorporated herein by reference along with the documents it cites, as if set out in full, there is recognition that allogeneic hematopoietic stem cell transplantation (HSCT) was utilized to deliver normal lysosomal enzyme to the brain of a patient with Hurler's disease, and a discussion of HSC gene therapy to treat ALD.

In two patients, peripheral CD34⁺ cells were collected after granulocyte-colony stimulating factor (G-CSF) mobilization and transduced with an myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer binding site substituted (MND)-ALD lentiviral vector. CD34⁺ cells from the patients were transduced with the MND-ALD vector during 16 h in the presence of cytokines at low concentrations. Transduced CD34⁺ cells were frozen after transduction to perform on 5% of cells various safety tests that included in particular three replication-competent lentivirus (RCL) assays. Transduction efficacy of CD34⁺ cells ranged from 35% to 50% with a mean number of lentiviral integrated copy between 0.65 and 0.70. After the thawing of transduced CD34⁺ cells, the patients were reinfused with more than $4 \cdot 10^6$ transduced CD34⁺ cells/kg following full myeloablation with busulfan and cyclophosphamide. The patient's HSCs were ablated to favor engraftment of the gene-corrected HSCs. Hematological recovery occurred between days 13 and 15 for the two patients. Nearly complete immunological recovery occurred at 12 months for the first patient, and at 9 months for the second patient. In contrast to using lentivirus, with the knowledge in the art and the teachings in this disclosure, the skilled person can correct HSCs as to ALD using a CRISPR-Cas9 system that targets and corrects the mutation (e.g., with a suitable HDR template); specifically, the sgRNA can target mutations in *ABCD1*, a gene located on the X chromosome that codes for ALD, a peroxisomal membrane transporter protein, and the HDR can provide coding for proper expression of the protein. An sgRNA that targets the mutation-and-Cas9 protein containing particle is contacted with HSCs, e.g., CD34⁺ cells carrying the mutation as in Cartier. The particle also can contain a suitable HDR template to correct the mutation for expression of the peroxisomal membrane transporter protein; or the HSC can be contacted with a second particle or a vector that contains or delivers the HDR template. The so contacted cells optionally can be treated as in Cartier. The so contacted cells can be administered as in Cartier.

[00208] Drakopoulou, "Review Article, The Ongoing Challenge of Hematopoietic Stem Cell-Based Gene Therapy for β -Thalassemia," *Stem Cells International*, Volume 2011, Article ID 987980, 10 pages, doi:10.4061/2011/987980, incorporated herein by reference along with the documents it cites, as if set out in full, discuss modifying HSCs using a lentivirus that delivers a gene for β -globin or γ -globin. In contrast to using lentivirus, with the knowledge in the art and the teachings in this disclosure, the skilled person can correct HSCs as to β -Thalassemia using a CRISPR-Cas9 system that targets and corrects the mutation (e.g., with a suitable HDR template

that delivers a coding sequence for β -globin or γ -globin, advantageously non-sickling β -globin or γ -globin); specifically, the sgRNA can target mutation that give rise to β -Thalassemia, and the HDR can provide coding for proper expression of β -globin or γ -globin. An sgRNA that targets the mutation-and-Cas9 protein containing particle is contacted with HSCs carrying the mutation. The particle also can contain a suitable HDR template to correct the mutation for proper expression of β -globin or γ -globin; or the HSC can be contacted with a second particle or a vector that contains or delivers the HDR template. The so contacted cells can be administered; and optionally treated / expanded; cf. Cartier. In this regard mention is made of: Cavazzana, "Outcomes of Gene Therapy for β -Thalassemia Major via Transplantation of Autologous Hematopoietic Stem Cells Transduced Ex Vivo with a Lentiviral β^{A-T87Q} -Globin Vector." tif2014.org/abstractFiles/Jean%20Antoine%20Ribeil_Abstract.pdf; Cavazzana-Calvo, "Transfusion independence and HMGA2 activation after gene therapy of human β -thalassaemia", *Nature* 467, 318–322 (16 September 2010) doi:10.1038/nature09328; Nienhuis, "Development of Gene Therapy for Thalassemia, Cold Spring Harbor Perspectives in Medicine, doi: 10.1101/cshperspect.a011833 (2012), LentiGlobin BB305, a lentiviral vector containing an engineered β -globin gene (β A-T87Q); and Xie et al., "Seamless gene correction of β -thalassaemia mutations in patient-specific iPSCs using CRISPR/Cas9 and piggyback" *Genome Research* gr.173427.114 (2014) <http://www.genome.org/cgi/doi/10.1101/gr.173427.114> (Cold Spring Harbor Laboratory Press); that is the subject of Cavazzana work involving human β -thalassaemia and the subject of the Xie work, are all incorporated herein by reference, together with all documents cited therein or associated therewith. In the instant invention, the HDR template can provide for the HSC to express an engineered β -globin gene (e.g., β A-T87Q), or β -globin as in Xie.

[00209] Song et al. (*Stem Cells Dev.* 2015 May 1;24(9):1053-65. doi: 10.1089/scd.2014.0347. Epub 2015 Feb 5) used CRISPR/ Cas9 to correct β -Thal iPSCs; gene-corrected cells exhibit normal karyotypes and full pluripotency as human embryonic stem cells (hESCs) showed no off-targeting effects. Then, Song et al. evaluated the differentiation efficiency of the gene-corrected β -Thal iPSCs. Song et al. found that during hematopoietic differentiation, gene-corrected β -Thal iPSCs showed an increased embryoid body ratio and various hematopoietic progenitor cell percentages. More importantly, the gene-corrected β -Thal iPSC lines restored HBB expression and reduced reactive oxygen species production compared with the uncorrected group.

Song et al.'s study suggested that hematopoietic differentiation efficiency of β -Thal iPSCs was greatly improved once corrected by the CRISPR-Cas9 system. Similar methods may be performed utilizing the CRISPR-Cas systems described herein, e.g. systems comprising Cas9 effector proteins.

[00210] Mention is made of WO 2015/148860, through the teachings herein the invention comprehends methods and materials of these documents applied in conjunction with the teachings herein. In an aspect of blood-related disease gene therapy, methods and compositions for treating beta thalassemia may be adapted to the CRISPR-Cas system of the present invention (see, e.g., WO 2015/148860). In an embodiment, WO 2015/148860 involves the treatment or prevention of beta thalassemia, or its symptoms, e.g., by altering the gene for B-cell CLL/lymphoma 11A (BCL11A). The BCL11A gene is also known as B-cell CLL/lymphoma 11A, BCL11A -L, BCL11A -S, BCL11AXL, CTIP 1, HBFQTL5 and ZNF. BCL11A encodes a zinc-finger protein that is involved in the regulation of globin gene expression. By altering the BCL11A gene (e.g., one or both alleles of the BCL11A gene), the levels of gamma globin can be increased. Gamma globin can replace beta globin in the hemoglobin complex and effectively carry oxygen to tissues, thereby ameliorating beta thalassemia disease phenotypes.

[00211] Sickle cell anemia is an autosomal recessive genetic disease in which red blood cells become sickle-shaped. It is caused by a single base substitution in the β -globin gene, which is located on the short arm of chromosome 11. As a result, valine is produced instead of glutamic acid causing the production of sickle hemoglobin (HbS). This results in the formation of a distorted shape of the erythrocytes. Due to this abnormal shape, small blood vessels can be blocked, causing serious damage to the bone, spleen and skin tissues. This may lead to episodes of pain, frequent infections, hand-foot syndrome or even multiple organ failure. The distorted erythrocytes are also more susceptible to hemolysis, which leads to serious anemia. As in the case of β -thalassaemia, sickle cell anemia can be corrected by modifying HSCs with the CRISPR/Cas9 system. The system allows the specific editing of the cell's genome by cutting its DNA and then letting it repair itself. The Cas9 protein is inserted and directed by a RNA guide to the mutated point and then it cuts the DNA at that point. Simultaneously, a healthy version of the sequence is inserted. This sequence is used by the cell's own repair system to fix the induced cut. In this way, the CRISPR/Cas9 allows the correction of the mutation in the previously obtained stem cells. With the knowledge in the art and the teachings in this disclosure, the skilled person can correct HSCs as

to sickle cell anemia using a CRISPR-Cas9 system that targets and corrects the mutation (e.g., with a suitable HDR template that delivers a coding sequence for β -globin, advantageously non-sickling β -globin); specifically, the sgRNA can target mutation that give rise to sickle cell anemia, and the HDR can provide coding for proper expression of β -globin. An sgRNA that targets the mutation-and-Cas9 protein containing particle is contacted with HSCs carrying the mutation. The particle also can contain a suitable HDR template to correct the mutation for proper expression of β -globin; or the HSC can be contacted with a second particle or a vector that contains or delivers the HDR template. The so contacted cells can be administered; and optionally treated / expanded; cf. Cartier. The HDR template can provide for the HSC to express an engineered β -globin gene (e.g., β A-T87Q), or β -globin as in Xie.

[00212] Mention is also made of WO 2015/148863 and through the teachings herein the invention comprehends methods and materials of these documents which may be adapted to the CRISPR-Cas system of the present invention. In an aspect of treating and preventing sickle cell disease, which is an inherited hematologic disease, WO 2015/148863 comprehends altering the BCL11A gene. By altering the BCL11A gene (e.g., one or both alleles of the BCL11A gene), the levels of gamma globin can be increased. Gamma globin can replace beta globin in the hemoglobin complex and effectively carry oxygen to tissues, thereby ameliorating sickle cell disease phenotypes.

[00213] Williams, "Broadening the Indications for Hematopoietic Stem Cell Genetic Therapies," Cell Stem Cell 13:263-264 (2013), incorporated herein by reference along with the documents it cites, as if set out in full, report lentivirus-mediated gene transfer into HSC/P cells from patients with the lysosomal storage disease metachromatic leukodystrophy disease (MLD), a genetic disease caused by deficiency of arylsulfatase A (ARSA), resulting in nerve demyelination; and lentivirus-mediated gene transfer into HSCs of patients with Wiskott-Aldrich syndrome (WAS) (patients with defective WAS protein, an effector of the small GTPase CDC42 that regulates cytoskeletal function in blood cell lineages and thus suffer from immune deficiency with recurrent infections, autoimmune symptoms, and thrombocytopenia with abnormally small and dysfunctional platelets leading to excessive bleeding and an increased risk of leukemia and lymphoma). In contrast to using lentivirus, with the knowledge in the art and the teachings in this disclosure, the skilled person can correct HSCs as to MLD (deficiency of arylsulfatase A (ARSA)) using a CRISPR-Cas9 system that targets and corrects the mutation (deficiency of arylsulfatase A (ARSA)) (e.g., with a suitable HDR template that delivers a coding sequence for ARSA);

specifically, the sgRNA can target mutation that gives rise to MLD (deficient ARSA), and the HDR can provide coding for proper expression of ARSA. An sgRNA that targets the mutation-and-Cas9 protein containing particle is contacted with HSCs carrying the mutation. The particle also can contain a suitable HDR template to correct the mutation for proper expression of ARSA; or the HSC can be contacted with a second particle or a vector that contains or delivers the HDR template. The so contacted cells can be administered; and optionally treated / expanded; cf. Cartier. In contrast to using lentivirus, with the knowledge in the art and the teachings in this disclosure, the skilled person can correct HSCs as to WAS using a CRISPR-Cas9 system that targets and corrects the mutation (deficiency of WAS protein) (e.g., with a suitable HDR template that delivers a coding sequence for WAS protein); specifically, the sgRNA can target mutation that gives rise to WAS (deficient WAS protein), and the HDR can provide coding for proper expression of WAS protein. An sgRNA that targets the mutation-and-Cas9 protein containing particle is contacted with HSCs carrying the mutation. The particle also can contain a suitable HDR template to correct the mutation for proper expression of WAS protein; or the HSC can be contacted with a second particle or a vector that contains or delivers the HDR template. The so contacted cells can be administered; and optionally treated / expanded; cf. Cartier.

[00214] In an aspect of the invention, methods and compositions which involve editing a target nucleic acid sequence, or modulating expression of a target nucleic acid sequence, and applications thereof in connection with cancer immunotherapy are comprehended by adapting the CRISPR-Cas system of the present invention. Reference is made to the application of gene therapy in WO 2015/161276 which involves methods and compositions which can be used to affect T-cell proliferation, survival and/or function by altering one or more T-cell expressed genes, e.g., one or more of FAS, BID, CTLA4, PDCD1, CBLB, PTPN6, TRAC and/or TRBC genes. In a related aspect, T-cell proliferation can be affected by altering one or more T-cell expressed genes, e.g., the CBLB and/or PTPN6 gene, FAS and/ or BID gene, CTLA4 and/or PDCD1 and/or TRAC and/or TRBC gene.

[00215] Chimeric antigen receptor (CAR)19 T-cells exhibit anti-leukemic effects in patient malignancies. However, leukemia patients often do not have enough T-cells to collect, meaning that treatment must involve modified T cells from donors. Accordingly, there is interest in establishing a bank of donor T-cells. Qasim et al. ("First Clinical Application of Talen Engineered Universal CAR19 T Cells in B-ALL" ASH 57th Annual Meeting and Exposition, Dec. 5-8, 2015,

Abstract 2046 (<https://ash.confex.com/ash/2015/webprogram/Paper81653.html> published online November 2015) discusses modifying CAR19 T cells to eliminate the risk of graft-versus-host disease through the disruption of T-cell receptor expression and CD52 targeting. Furthermore, CD52 cells were targeted such that they became insensitive to Alemtuzumab, and thus allowed Alemtuzumab to prevent host-mediated rejection of human leukocyte antigen (HLA) mismatched CAR19 T-cells. Investigators used third generation self-inactivating lentiviral vector encoding a 4g7 CAR19 (CD19 scFv-4-1BB-CD3 ζ) linked to RQR8, then electroporated cells with two pairs of TALEN mRNA for multiplex targeting for both the T-cell receptor (TCR) alpha constant chain locus and the CD52 gene locus. Cells which were still expressing TCR following ex vivo expansion were depleted using CliniMacs α/β TCR depletion, yielding a T-cell product (UCART19) with <1% TCR expression, 85% of which expressed CAR19, and 64% becoming CD52 negative. The modified CAR19 T cells were administered to treat a patient's relapsed acute lymphoblastic leukemia. The teachings provided herein provide effective methods for providing modified hematopoietic stem cells and progeny thereof, including but not limited to cells of the myeloid and lymphoid lineages of blood, including T cells, B cells, monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, dendritic cells, and megakaryocytes or platelets, and natural killer cells and their precursors and progenitors. Such cells can be modified by knocking out, knocking in, or otherwise modulating targets, for example to remove or modulate CD52 as described above, and other targets, such as, without limitation, CXCR4, and PD-1. Thus compositions, cells, and method of the invention can be used to can be to modulate immune responses and to treat, without limitation, malignancies, viral infections, and immune disorders, in conjunction with modification of administration of T cells or other cells to patients.

[00216] Watts, "Hematopoietic Stem Cell Expansion and Gene Therapy" *Cytotherapy* 13(10):1164–1171. doi:10.3109/14653249.2011.620748 (2011), incorporated herein by reference along with the documents it cites, as if set out in full, discusses hematopoietic stem cell (HSC) gene therapy, e.g., virus-mediated HSC gene thereapy, as an highly attractive treatment option for many disorders including hematologic conditions, immunodeficiencies including HIV/AIDS, and other genetic disorders like lysosomal storage diseases, including SCID-X1, ADA-SCID, β -thalassemia, X-linked CGD, Wiskott-Aldrich syndrome, Fanconi anemia, adrenoleukodystrophy (ALD), and metachromatic leukodystrophy (MLD).

[00217] With the knowledge in the art and the teachings in this disclosure, the skilled person can correct HSCs as to a genetic hematologic disorder, e.g., β -Thalassemia, Hemophilia, or a genetic lysosomal storage disease.

[00218] With the knowledge in the art and the teachings in this disclosure the skilled person can correct HSCs as to immunodeficiency condition such as HIV / AIDS comprising contacting an HSC with a CRISPR-Cas9 system that targets and knocks out CCR5. An sgRNA (and advantageously a dual guide approach, e.g., a pair of different sgRNAs; for instance, sgRNAs targeting of two clinically relevant genes, B2M and CCR5, in primary human CD4+ T cells and CD34+ hematopoietic stem and progenitor cells (HSPCs)) that targets and knocks out CCR5-and-Cas9 protein containing particle is contacted with HSCs. The so contacted cells can be administered; and optionally treated / expanded; cf. Cartier. *See also* Kiem, "Hematopoietic stem cell-based gene therapy for HIV disease," *Cell Stem Cell*. Feb 3, 2012; 10(2): 137–147; incorporated herein by reference along with the documents it cites; Mandal et al, "Efficient Ablation of Genes in Human Hematopoietic Stem and Effector Cells using CRISPR/Cas9," *Cell Stem Cell*, Volume 15, Issue 5, p643–652, 6 November 2014; incorporated herein by reference along with the documents it cites. Mention is also made of Ebina, "CRISPR/Cas9 system to suppress HIV-1 expression by editing HIV-1 integrated proviral DNA" *SCIENTIFIC REPORTS* | 3: 2510 | DOI: 10.1038/srep02510, incorporated herein by reference along with the documents it cites, as another means for combatting HIV/AIDS using a CRISPR-Cas9 system.

[00219] The rationale for genome editing for HIV treatment originates from the observation that individuals homozygous for loss of function mutations in CCR5, a cellular co-receptor for the virus, are highly resistant to infection and otherwise healthy, suggesting that mimicking this mutation with genome editing could be a safe and effective therapeutic strategy [Liu, R., et al. *Cell* 86, 367-377 (1996)]. This idea was clinically validated when an HIV infected patient was given an allogeneic bone marrow transplant from a donor homozygous for a loss of function CCR5 mutation, resulting in undetectable levels of HIV and restoration of normal CD4 T-cell counts [Hutter, G., et al. *The New England journal of medicine* 360, 692-698 (2009)]. Although bone marrow transplantation is not a realistic treatment strategy for most HIV patients, due to cost and potential graft vs. host disease, HIV therapies that convert a patient's own T-cells into CCR5 are desirable.

[00220] Early studies using ZFNs and NHEJ to knockout CCR5 in humanized mouse models of HIV showed that transplantation of CCR5 edited CD4 T cells improved viral load and CD4 T-cell counts [Perez, E.E., et al. *Nature biotechnology* 26, 808-816 (2008)]. Importantly, these models also showed that HIV infection resulted in selection for CCR5 null cells, suggesting that editing confers a fitness advantage and potentially allowing a small number of edited cells to create a therapeutic effect.

[00221] As a result of this and other promising preclinical studies, genome editing therapy that knocks out CCR5 in patient T cells has now been tested in humans [Holt, N., et al. *Nature biotechnology* 28, 839-847 (2010); Li, L., et al. *Molecular therapy : the journal of the American Society of Gene Therapy* 21, 1259-1269 (2013)]. In a recent phase I clinical trial, CD4+ T cells from patients with HIV were removed, edited with ZFNs designed to knockout the CCR5 gene, and autologously transplanted back into patients [Tebas, P., et al. *The New England journal of medicine* 370, 901-910 (2014)].

[00222] In another study (Mandal et al., *Cell Stem Cell*, Volume 15, Issue 5, p643–652, 6 November 2014), CRISPR-Cas9 has targeted two clinical relevant genes, B2M and CCR5, in human CD4+ T cells and CD34+ hematopoietic stem and progenitor cells (HSPCs). Use of single RNA guides led to highly efficient mutagenesis in HSPCs but not in T cells. A dual guide approach improved gene deletion efficacy in both cell types. HSPCs that had undergone genome editing with CRISPR-Cas9 retained multilineage potential. Predicted on- and off-target mutations were examined via target capture sequencing in HSPCs and low levels of off-target mutagenesis were observed at only one site. These results demonstrate that CRISPR-Cas9 can efficiently ablate genes in HSPCs with minimal off-target mutagenesis, which have broad applicability for hematopoietic cell-based therapy.

[00223] Wang et al. (*PLoS One*, 2014 Dec 26;9(12):e115987. doi: 10.1371/journal.pone.0115987) silenced CCR5 via CRISPR associated protein 9 (Cas9) and single guided RNAs (guide RNAs) with lentiviral vectors expressing Cas9 and CCR5 guide RNAs. Wang et al. showed that a single round transduction of lentiviral vectors expressing Cas9 and CCR5 guide RNAs into HIV-1 susceptible human CD4+ cells yields high frequencies of CCR5 gene disruption. CCR5 gene-disrupted cells are not only resistant to R5-tropic HIV-1, including transmitted/founder (T/F) HIV-1 isolates, but also have selective advantage over CCR5 gene-undisrupted cells during R5-tropic HIV-1 infection. Genome mutations at potential off-target

sites that are highly homologous to these CCR5 guide RNAs in stably transduced cells even at 84 days post transduction were not detected by a T7 endonuclease I assay.

[00224] Fine et al. (Sci Rep. 2015 Jul 1;5:10777. doi: 10.1038/srep10777) identified a two-cassette system expressing pieces of the *S. pyogenes* Cas9 (SpCas9) protein which splice together in cellula to form a functional protein capable of site-specific DNA cleavage. With specific CRISPR guide strands, Fine et al. demonstrated the efficacy of this system in cleaving the HBB and CCR5 genes in human HEK-293T cells as a single Cas9 and as a pair of Cas9 nickases. The trans-spliced SpCas9 (tsSpCas9) displayed ~35% of the nuclease activity compared with the wild-type SpCas9 (wtSpCas9) at standard transfection doses, but had substantially decreased activity at lower dosing levels. The greatly reduced open reading frame length of the tsSpCas9 relative to wtSpCas9 potentially allows for more complex and longer genetic elements to be packaged into an AAV vector including tissue-specific promoters, multiplexed guide RNA expression, and effector domain fusions to SpCas9.

[00225] Li et al. (J Gen Virol. 2015 Aug;96(8):2381-93. doi: 10.1099/vir.0.000139. Epub 2015 Apr 8) demonstrated that CRISPR-Cas9 can efficiently mediate the editing of the CCR5 locus in cell lines, resulting in the knockout of CCR5 expression on the cell surface. Next-generation sequencing revealed that various mutations were introduced around the predicted cleavage site of CCR5. For each of the three most effective guide RNAs that were analyzed, no significant off-target effects were detected at the 15 top-scoring potential sites. By constructing chimeric Ad5F35 adenoviruses carrying CRISPR-Cas9 components, Li et al. efficiently transduced primary CD4+ T-lymphocytes and disrupted CCR5 expression, and the positively transduced cells were conferred with HIV-1 resistance.

[00226] Mention is made of WO 2015/148670 and through the teachings herein the invention comprehends methods and materials of this document applied in conjunction with the teachings herein. In an aspect of gene therapy, methods and compositions for editing of a target sequence related to or in connection with Human Immunodeficiency Virus (HIV) and Acquired Immunodeficiency Syndrome (AIDS) are comprehended. In a related aspect, the invention described herein comprehends prevention and treatment of HIV infection and AIDS, by introducing one or more mutations in the gene for C-C chemokine receptor type 5 (CCR5). The CCR5 gene is also known as CKR5, CCR-5, CD195, CKR-5, CCCKR5, CMKBR5, IDDM22, and CC-CKR-5. In a further aspect, the invention described herein comprehends provide for

prevention or reduction of HIV infection and/or prevention or reduction of the ability for HIV to enter host cells, e.g., in subjects who are already infected. Exemplary host cells for HIV include, but are not limited to, CD4 cells, T cells, gut associated lymphatic tissue (GALT), macrophages, dendritic cells, myeloid precursor cell, and microglia. Viral entry into the host cells requires interaction of the viral glycoproteins gp41 and gp120 with both the CD4 receptor and a co-receptor, e.g., CCR5. If a co-receptor, e.g., CCR5, is not present on the surface of the host cells, the virus cannot bind and enter the host cells. The progress of the disease is thus impeded. By knocking out or knocking down CCR5 in the host cells, e.g., by introducing a protective mutation (such as a CCR5 delta 32 mutation), entry of the HIV virus into the host cells is prevented.

[00227] One of skill in the art may utilize the above studies of, for example, Holt, N., et al. *Nature biotechnology* 28, 839-847 (2010), Li, L., et al. *Molecular therapy : the journal of the American Society of Gene Therapy* 21, 1259-1269 (2013), Mandal et al., *Cell Stem Cell*, Volume 15, Issue 5, p643–652, 6 November 2014, Wang et al. (*PLoS One*. 2014 Dec 26;9(12):e115987. doi: 10.1371/journal.pone.0115987), Fine et al. (*Sci Rep*. 2015 Jul 1;5:10777. doi: 10.1038/srep10777) and Li et al. (*J Gen Virol*. 2015 Aug;96(8):2381-93. doi: 10.1099/vir.0.000139. Epub 2015 Apr 8) for targeting CCR5 with the CRISPR Cas system of the present invention.

[00228] X-linked Chronic granulomatous disease (CGD) is a hereditary disorder of host defense due to absent or decreased activity of phagocyte NADPH oxidase. Using a CRISPR-Cas9 system that targets and corrects the mutation (absent or decreased activity of phagocyte NADPH oxidase) (e.g., with a suitable HDR template that delivers a coding sequence for phagocyte NADPH oxidase); specifically, the sgRNA can target mutation that gives rise to CGD (deficient phagocyte NADPH oxidase), and the HDR can provide coding for proper expression of phagocyte NADPH oxidase. An sgRNA that targets the mutation-and-Cas9 protein containing particle is contacted with HSCs carrying the mutation. The particle also can contain a suitable HDR template to correct the mutation for proper expression of phagocyte NADPH oxidase; or the HSC can be contacted with a second particle or a vector that contains or delivers the HDR template. The so contacted cells can be administered; and optionally treated / expanded; cf. Cartier.

[00229] Fanconi anemia: Mutations in at least 15 genes (*FANCA*, *FANCB*, *FANCC*, *FANCD1/BRCA2*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ/BACH1/BRIP1*, *FANCL/PHF9/POG*, *FANCM*, *FANCN/PALB2*, *FANCO/Rad51C*, and *FANCP/SLX4/BTBD12*)

can cause Fanconi anemia. Proteins produced from these genes are involved in a cell process known as the FA pathway. The FA pathway is turned on (activated) when the process of making new copies of DNA, called DNA replication, is blocked due to DNA damage. The FA pathway sends certain proteins to the area of damage, which trigger DNA repair so DNA replication can continue. The FA pathway is particularly responsive to a certain type of DNA damage known as interstrand cross-links (ICLs). ICLs occur when two DNA building blocks (nucleotides) on opposite strands of DNA are abnormally attached or linked together, which stops the process of DNA replication. ICLs can be caused by a buildup of toxic substances produced in the body or by treatment with certain cancer therapy drugs. Eight proteins associated with Fanconi anemia group together to form a complex known as the FA core complex. The FA core complex activates two proteins, called FANCD2 and FANCI. The activation of these two proteins brings DNA repair proteins to the area of the ICL so the cross-link can be removed and DNA replication can continue.

the FA core complex. More in particular, the FA core complex is a nuclear multiprotein complex consisting of FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM, functions as an E3 ubiquitin ligase and mediates the activation of the ID complex, which is a heterodimer composed of FANCD2 and FANCI. Once monoubiquitinated, it interacts with classical tumor suppressors downstream of the FA pathway including FANCD1/BRCA2, FANCN/PALB2, FANCI/BRIP1, and FANCO/Rad51C and thereby contributes to DNA repair via homologous recombination (HR). Eighty to 90 percent of FA cases are due to mutations in one of three genes, *FANCA*, *FANCC*, and *FANCG*. These genes provide instructions for producing components of the FA core complex. Mutations in such genes associated with the FA core complex will cause the complex to be nonfunctional and disrupt the entire FA pathway. As a result, DNA damage is not repaired efficiently and ICLs build up over time. Geiselhart, "Review Article, Disrupted Signaling through the Fanconi Anemia Pathway Leads to Dysfunctional Hematopoietic Stem Cell Biology: Underlying Mechanisms and Potential Therapeutic Strategies," *Anemia* Volume 2012 (2012), Article ID 265790, <http://dx.doi.org/10.1155/2012/265790> discussed FA and an animal experiment involving intrafemoral injection of a lentivirus encoding the FANCC gene resulting in correction of HSCs in vivo. Using a CRISPR-Cas9 system that targets and one or more of the mutations associated with FA, for instance a CRISPR-Cas9 system having sgRNA(s) and HDR template(s) that respectively targets one or more of the mutations of *FANCA*, *FANCC*, or *FANCG* that give rise to FA and provide corrective expression of one or more

of FANCA, FANCC or FANCG; e.g., the sgRNA can target a mutation as to FANCC, and the HDR can provide coding for proper expression of FANCC. An sgRNA that targets the mutation(s) (e.g., one or more involved in FA, such as mutation(s) as to any one or more of *FANCA*, *FANCC* or *FANCG*)-and-Cas9 protein containing particle is contacted with HSCs carrying the mutation(s). The particle also can contain a suitable HDR template(s) to correct the mutation for proper expression of one or more of the proteins involved in FA, such as any one or more of FANCA, FANCC or FANCG; or the HSC can be contacted with a second particle or a vector that contains or delivers the HDR template. The so contacted cells can be administered; and optionally treated / expanded; cf. Cartier.

[00230] The particle in the herein discussion (e.g., as to containing sgRNA(s) and Cas9, optionally HDR template(s), or HDR template(s); for instance as to Hemophilia B, SCID, SCID-X1, ADA-SCID, Hereditary tyrosinemia, β -thalassemia, X-linked CGD, Wiskott-Aldrich syndrome, Fanconi anemia, adrenoleukodystrophy (ALD), metachromatic leukodystrophy (MLD), HIV/AIDS, Immunodeficiency disorder, Hematologic condition, or genetic lysosomal storage disease) is advantageously obtained or obtainable from admixing an sgRNA(s) and Cas9 protein mixture (optionally containing HDR template(s) or such mixture only containing HDR template(s) when separate particles as to template(s) is desired) with a mixture comprising or consisting essentially of or consisting of surfactant, phospholipid, biodegradable polymer, lipoprotein and alcohol (wherein one or more sgRNA targets the genetic locus or loci in the HSC).

[00231] Indeed, the invention is especially suited for treating hematopoietic genetic disorders with genome editing, and immunodeficiency disorders, such as genetic immunodeficiency disorders, especially through using the particle technology herein-discussed. Genetic immunodeficiencies are diseases where genome editing interventions of the instant invention can be successful. The reasons include: Hematopoietic cells, of which immune cells are a subset, are therapeutically accessible. They can be removed from the body and transplanted autologously or allogeneically. Further, certain genetic immunodeficiencies, e.g., severe combined immunodeficiency (SCID), create a proliferative disadvantage for immune cells. Correction of genetic lesions causing SCID by rare, spontaneous 'reverse' mutations indicates that correcting even one lymphocyte progenitor may be sufficient to recover immune function in patients.../Users/t_kowalski/AppData/Local/Microsoft/Windows/Temporary Internet Files/Content.Outlook/GA8VY8LK/Treating SCID for Ellen.docx - _ENREF_1 See Bousso, P.,

et al. Diversity, functionality, and stability of the T cell repertoire derived *in vivo* from a single human T cell precursor. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 274-278 (2000). The selective advantage for edited cells allows for even low levels of editing to result in a therapeutic effect. This effect of the instant invention can be seen in SCID, Wiskott-Aldrich Syndrome, and the other conditions mentioned herein, including other genetic hematopoietic disorders such as alpha- and beta- thalassemia, where hemoglobin deficiencies negatively affect the fitness of erythroid progenitors.

[00232] The activity of NHEJ and HDR DSB repair varies significantly by cell type and cell state. NHEJ is not highly regulated by the cell cycle and is efficient across cell types, allowing for high levels of gene disruption in accessible target cell populations. In contrast, HDR acts primarily during S/G2 phase, and is therefore restricted to cells that are actively dividing, limiting treatments that require precise genome modifications to mitotic cells [Ciccia, A. & Elledge, S.J. *Molecular cell* **40**, 179-204 (2010); Chapman, J.R., et al. *Molecular cell* **47**, 497-510 (2012)].

[00233] The efficiency of correction via HDR may be controlled by the epigenetic state or sequence of the targeted locus, or the specific repair template configuration (single vs. double stranded, long vs. short homology arms) used [Hacein-Bey-Abina, S., et al. *The New England journal of medicine* **346**, 1185-1193 (2002); Gaspar, H.B., et al. *Lancet* **364**, 2181-2187 (2004); Beumer, K.J., et al. *G3* (2013)]. The relative activity of NHEJ and HDR machineries in target cells may also affect gene correction efficiency, as these pathways may compete to resolve DSBs [Beumer, K.J., et al. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 19821-19826 (2008)]. HDR also imposes a delivery challenge not seen with NHEJ strategies, as it requires the concurrent delivery of nucleases and repair templates. In practice, these constraints have so far led to low levels of HDR in therapeutically relevant cell types. Clinical translation has therefore largely focused on NHEJ strategies to treat disease, although proof-of-concept preclinical HDR treatments have now been described for mouse models of haemophilia B and hereditary tyrosinemia [Li, H., et al. *Nature* **475**, 217-221 (2011); Yin, H., et al. *Nature biotechnology* **32**, 551-553 (2014)].

[00234] Any given genome editing application may comprise combinations of proteins, small RNA molecules, and/or repair templates, making delivery of these multiple parts substantially more challenging than small molecule therapeutics. Two main strategies for delivery of genome editing tools have been developed: *ex vivo* and *in vivo*. In *ex vivo* treatments, diseased cells are

removed from the body, edited and then transplanted back into the patient (Figure 3, top panel). *Ex vivo* editing has the advantage of allowing the target cell population to be well defined and the specific dosage of therapeutic molecules delivered to cells to be specified. The latter consideration may be particularly important when off-target modifications are a concern, as titrating the amount of nuclease may decrease such mutations (Hsu et al., 2013). Another advantage of *ex vivo* approaches is the typically high editing rates that can be achieved, due to the development of efficient delivery systems for proteins and nucleic acids into cells in culture for research and gene therapy applications.

[00235] There may be drawbacks with *ex vivo* approaches that limit application to a small number of diseases. For instance, target cells must be capable of surviving manipulation outside the body. For many tissues, like the brain, culturing cells outside the body is a major challenge, because cells either fail to survive, or lose properties necessary for their function *in vivo*. Thus, in view of this disclosure and the knowledge in the art, *ex vivo* therapy as to tissues with adult stem cell populations amenable to *ex vivo* culture and manipulation, such as the hematopoietic system, by the CRISPR-Cas9 system are enabled. [Bunn, H.F. & Aster, J. Pathophysiology of blood disorders, (McGraw-Hill, New York, 2011)]

[00236] *In vivo* genome editing involves direct delivery of editing systems to cell types in their native tissues (Figure 3, bottom panels). *In vivo* editing allows diseases in which the affected cell population is not amenable to *ex vivo* manipulation to be treated. Furthermore, delivering nucleases to cells *in situ* allows for the treatment of multiple tissue and cell types. These properties probably allow *in vivo* treatment to be applied to a wider range of diseases than *ex vivo* therapies.

[00237] To date, *in vivo* editing has largely been achieved through the use of viral vectors with defined, tissue-specific tropism. Such vectors are currently limited in terms of cargo carrying capacity and tropism, restricting this mode of therapy to organ systems where transduction with clinically useful vectors is efficient, such as the liver, muscle and eye [Kotterman, M.A. & Schaffer, D.V. Nature reviews. Genetics 15, 445-451 (2014); Nguyen, T.H. & Ferry, N. Gene therapy 11 Suppl 1, S76-84 (2004); Boye, S.E., et al. Molecular therapy : the journal of the American Society of Gene Therapy 21, 509-519 (2013)].

[00238] A potential barrier for *in vivo* delivery is the immune response that may be created in response to the large amounts of virus necessary for treatment, but this phenomenon is not unique to genome editing and is observed with other virus based gene therapies [Bessis, N., et al. Gene

therapy 11 Suppl 1, S10-17 (2004)]. It is also possible that peptides from editing nucleases themselves are presented on MHC Class I molecules to stimulate an immune response, although there is little evidence to support this happening at the preclinical level. Another major difficulty with this mode of therapy is controlling the distribution and consequently the dosage of genome editing nucleases *in vivo*, leading to off-target mutation profiles that may be difficult to predict. However, in view of this disclosure and the knowledge in the art, including the use of virus- and particle-based therapies being used in the treatment of cancers, *in vivo* modification of HSCs, for instance by delivery by either particle or virus, is within the ambit of the the skilled person.

[00239] Ex Vivo Editing Therapy: The long standing clinical expertise with the purification, culture and transplantation of hematopoietic cells has made diseases affecting the blood system such as SCID, Fanconi anemia, Wiskott-Aldrich syndrome and sickle cell anemia the focus of *ex vivo* editing therapy. Another reason to focus on hematopoietic cells is that, thanks to previous efforts to design gene therapy for blood disorders, delivery systems of relatively high efficiency already exist. With these advantages, this mode of therapy can be applied to diseases where edited cells possess a fitness advantage, so that a small number of engrafted, edited cells can expand and treat disease. One such disease is HIV, where infection results in a fitness disadvantage to CD4+ T cells. The rationale for genome editing for HIV treatment originates from the observation that individuals homozygous for loss of function mutations in CCR5, a cellular co-receptor for the virus, are highly resistant to infection and otherwise healthy, suggesting that mimicking this mutation with genome editing could be a safe and effective therapeutic strategy [Liu, R., et al. Cell 86, 367-377 (1996)]. This idea was clinically validated when an HIV infected patient was given an allogeneic bone marrow transplant from a donor homozygous for a loss of function CCR5 mutation, resulting in undetectable levels of HIV and restoration of normal CD4 T-cell counts [Hutter, G., et al. The New England journal of medicine 360, 692-698 (2009)]. Although bone marrow transplantation is not a realistic treatment strategy for most HIV patients, due to cost and potential graft vs. host disease, HIV therapies that convert a patient's own T-cells into CCR5 are.

[00240] Early studies using ZFNs and NHEJ to knockout CCR5 in humanized mouse models of HIV showed that transplantation of CCR5 edited CD4 T cells improved viral load and CD4 T-cell counts [Perez, E.E., et al. Nature biotechnology 26, 808-816 (2008)]. Importantly, these models also showed that HIV infection resulted in selection for CCR5 null cells, suggesting that editing

confers a fitness advantage and potentially allowing a small number of edited cells to create a therapeutic effect.

[00241] As a result of this and other promising preclinical studies, genome editing therapy that knocks out CCR5 in patient T cells has now been tested in humans [Holt, N., et al. *Nature biotechnology* 28, 839-847 (2010); Li, L., et al. *Molecular therapy : the journal of the American Society of Gene Therapy* 21, 1259-1269 (2013)]. In a recent phase I clinical trial, CD4+ T cells from patients with HIV were removed, edited with ZFNs designed to knockout the CCR5 gene, and autologously transplanted back into patients [Tebas, P., et al. *The New England journal of medicine* 370, 901-910 (2014)]. Early results from this trial suggest that genome editing through ZFNs of the CCR5 locus is safe, although the follow up time is too short to fully understand the risks and efficacy of treatment.

[00242] *Ex vivo* editing therapy has been recently extended to include gene correction strategies. The barriers to HDR *ex vivo* were overcome in a recent paper from Genovese and colleagues, who achieved gene correction of a mutated IL2RG gene in hematopoietic stem cells (HSCs) obtained from a patient suffering from SCID-X1 [Genovese, P., et al. *Nature* 510, 235-240 (2014)]. Genovese *et. al.* accomplished gene correction in HSCs using a multimodal strategy. First, HSCs were transduced using integration-deficient lentivirus containing an HDR template encoding a therapeutic cDNA for IL2RG. Following transduction, cells were electroporated with mRNA encoding ZFNs targeting a mutational hotspot in IL2RG to stimulate HDR based gene correction. To increase HDR rates, culture conditions were optimized with small molecules to encourage HSC division. With optimized culture conditions, nucleases and HDR templates, gene corrected HSCs from the SCID-X1 patient were obtained in culture at therapeutically relevant rates. HSCs from unaffected individuals that underwent the same gene correction procedure could sustain long-term hematopoiesis in mice, the gold standard for HSC function. HSCs are capable of giving rise to all hematopoietic cell types and can be autologously transplanted, making them an extremely valuable cell population for all hematopoietic genetic disorders [Weissman, I.L. & Shizuru, J.A. *Blood* 112, 3543-3553 (2008)]. Gene corrected HSCs could, in principle, be used to treat a wide range of genetic blood disorders making this study an exciting breakthrough for therapeutic genome editing.

[00243] In Vivo Editing Therapy: *In vivo* editing can be used advantageously from this disclosure and the knowledge in the art. For organ systems where delivery is efficient, there have

already been a number of exciting preclinical therapeutic successes. The first example of successful *in vivo* editing therapy was demonstrated in a mouse model of haemophilia B [Li, H., et al. *Nature* 475, 217-221 (2011)]. As noted earlier, Haemophilia B is an X-linked recessive disorder caused by loss-of-function mutations in the gene encoding Factor IX, a crucial component of the clotting cascade. Recovering Factor IX activity to above 1% of its levels in severely affected individuals can transform the disease into a significantly milder form, as infusion of recombinant Factor IX into such patients prophylactically from a young age to achieve such levels largely ameliorates clinical complications [Lofqvist, T., et al. *Journal of internal medicine* 241, 395-400 (1997)]. Thus, only low levels of HDR gene correction are necessary to change clinical outcomes for patients. In addition, Factor IX is synthesized and secreted by the liver, an organ that can be transduced efficiently by viral vectors encoding editing systems.

[00244] Using hepatotropic adeno-associated viral (AAV) serotypes encoding ZFNs and a corrective HDR template, up to 7% gene correction of a mutated, humanized Factor IX gene in the murine liver was achieved [Li, H., et al. *Nature* 475, 217-221 (2011)]. This resulted in improvement of clot formation kinetics, a measure of the function of the clotting cascade, demonstrating for the first time that *in vivo* editing therapy is not only feasible, but also efficacious. As discussed herein, the skilled person is positioned from the teachings herein and the knowledge in the art, e.g., Li to address Haemophilia B with a particle-containing HDR template and a CRISPR-Cas9 system that targets the mutation of the X-linked recessive disorder to reverse the loss-of-function mutation.

[00245] Building on this study, other groups have recently used *in vivo* genome editing of the liver with CRISPR-Cas9 to successfully treat a mouse model of hereditary tyrosinemia and to create mutations that provide protection against cardiovascular disease. These two distinct applications demonstrate the versatility of this approach for disorders that involve hepatic dysfunction [Yin, H., et al. *Nature biotechnology* 32, 551-553 (2014); Ding, Q., et al. *Circulation research* 115, 488-492 (2014)]. Application of *in vivo* editing to other organ systems are necessary to prove that this strategy is widely applicable. Currently, efforts to optimize both viral and non-viral vectors are underway to expand the range of disorders that can be treated with this mode of therapy [Kotterman, M.A. & Schaffer, D.V. *Nature reviews. Genetics* 15, 445-451 (2014); Yin, H., et al. *Nature reviews. Genetics* 15, 541-555 (2014)]. As discussed herein, the skilled person is positioned from the teachings herein and the knowledge in the art, e.g., Yin to address hereditary

tyrosinemia with a particle-containing HDR template and a CRISPR-Cas9 system that targets the mutation.

[00246] Targeted deletion, therapeutic applications: Targeted deletion of genes may be preferred. Preferred are, therefore, genes involved in immunodeficiency disorder, hematologic condition, or genetic lysosomal storage disease, e.g., Hemophilia B, SCID, SCID-X1, ADA-SCID, Hereditary tyrosinemia, β -thalassemia, X-linked CGD, Wiskott-Aldrich syndrome, Fanconi anemia, adrenoleukodystrophy (ALD), metachromatic leukodystrophy (MLD), HIV/AIDS, other metabolic disorders, genes encoding mis-folded proteins involved in diseases, genes leading to loss-of-function involved in diseases; generally, mutations that can be targeted in an HSC, using any herein-discussed delivery system, with the particle system considered advantageous.

[00247] In the present invention, the immunogenicity of the CRISPR enzyme in particular may be reduced following the approach first set out in Tangri et al with respect to erythropoietin and subsequently developed. Accordingly, directed evolution or rational design may be used to reduce the immunogenicity of the CRISPR enzyme (for instance a Cas9) in the host species (human or other species).

[00248] Genome editing: The CRISPR/Cas9 systems of the present invention can be used to correct genetic mutations that were previously attempted with limited success using TALEN and ZFN and lentiviruses, including as herein discussed; see also WO2013163628 A2, Genetic Correction of Mutated Genes, published application of Duke University; US Patent Publication No. 20130145487 assigned to Cellectis.

[00249] Blood: The present invention also contemplates delivering the CRISPR-Cas system to the blood. The plasma exosomes of Wahlgren et al. (Nucleic Acids Research, 2012, Vol. 40, No. 17 e130) were previously described and may be utilized to deliver the CRISPR Cas system to the blood. The CRISPR Cas system of the present invention is also contemplated to treat hemoglobinopathies, such as thalassemias and sickle cell disease. See, e.g., International Patent Publication No. WO 2013/126794 for potential targets that may be targeted by the CRISPR Cas system of the present invention. Target sequences identified in US Patent Publication Nos. 20110225664, 20110091441, 20100229252, 20090271881 and 20090222937 may be of interest as to the present invention.

[00250] The target polynucleotide of a CRISPR complex may include a number of disease-associated genes and polynucleotides as well as signaling biochemical pathway-associated genes. Examples of target polynucleotides include a sequence associated with a signaling biochemical pathway, e.g., a signaling biochemical pathway-associated gene or polynucleotide. Examples of target polynucleotides include a disease associated gene or polynucleotide. A “disease-associated” gene or polynucleotide refers to any gene or polynucleotide which is yielding transcription or translation products at an abnormal level or in an abnormal form in cells derived from a disease-affected tissues compared with tissues or cells of a non disease control. It may be a gene that becomes expressed at an abnormally high level; it may be a gene that becomes expressed at an abnormally low level, where the altered expression correlates with the occurrence and/or progression of the disease. A disease-associated gene also refers to a gene possessing mutation(s) or genetic variation that is directly responsible or is in linkage disequilibrium with a gene(s) that is responsible for the etiology of a disease. The transcribed or translated products may be known or unknown, and may be at a normal or abnormal level. The target polynucleotide of a CRISPR complex can be any polynucleotide endogenous or exogenous to the eukaryotic cell. For example, the target polynucleotide can be a polynucleotide residing in the nucleus of the eukaryotic cell. The target polynucleotide can be a sequence coding a gene product (e.g., a protein) or a non-coding sequence (e.g., a regulatory polynucleotide or a junk DNA). The target can be a control element or a regulatory element or a promoter or an enhancer or a silencer. The promoter may, in some embodiments, be in the region of +200bp or even +1000 bp from the TTS. In some embodiments, the regulatory region may be an enhancer. The enhancer is typically more than +1000 bp from the TTS. More in particular, expression of eukaryotic protein-coding genes generally is regulated through multiple cis-acting transcription-control regions. Some control elements are located close to the start site (promoter-proximal elements), whereas others lie more distant (enhancers and silencers) Promoters determine the site of transcription initiation and direct binding of RNA polymerase II. Three types of promoter sequences have been identified in eukaryotic DNA. The TATA box, the most common, is prevalent in rapidly transcribed genes. Initiator promoters infrequently are found in some genes, and CpG islands are characteristic of transcribed genes. Promoter-proximal elements occur within ≈ 200 base pairs of the start site. Several such elements, containing up to ≈ 20 base pairs, may help regulate a particular gene. Enhancers, which are usually ≈ 100 – 200 base pairs in length, contain multiple 8- to 20-bp control elements. They may be located

from 200 base pairs to tens of kilobases upstream or downstream from a promoter, within an intron, or downstream from the final exon of a gene. Promoter-proximal elements and enhancers may be cell-type specific, functioning only in specific differentiated cell types. However, any of these regions can be the target sequence and are encompassed by the concept that the target can be a control element or a regulatory element or a promoter or an enhancer or a silencer. Without wishing to be bound by theory, it is believed that the target sequence should be associated with a PAM (protospacer adjacent motif); that is, a short sequence recognized by the CRISPR complex. The precise sequence and length requirements for the PAM differ depending on the CRISPR enzyme used, but PAMs are typically 2-5 base pair sequences adjacent the protospacer (that is, the target sequence) Examples of PAM sequences are given in the examples section below, and the skilled person will be able to identify further PAM sequences for use with a given CRISPR enzyme.

[00251] The Efficiency of HDR: Although the amount of genome modification in a target cell population required to create a therapeutic effect differs depending on the disease, the efficacy of most editing treatments are improved with increased editing rates. As previously noted, editing rates are controlled by the activity of DSB repair pathways and the efficiency of delivery to cells of interest. Therefore improvements to either one of these factors are likely to improve the efficacy of editing treatments. Attempts to increase the activity rates of DSB repair pathways have largely focused on HDR, as cell cycle regulation and the challenge of delivering an HDR template with nucleases makes strategies employing this pathway less efficient than NHEJ. Cell cycle regulation has now been somewhat by-passed for slowly cycling cell types through stimulation of mitosis with pharmacologic agents *ex vivo* [Kormann, M.S., et al. Nature biotechnology 29, 154-157 (2011)]. However, truly post-mitotic cells are unlikely to be amenable to such manipulation, limiting the applicability of this strategy. Attempts have been made to completely circumvent the need for HDR through direct ligation of DNA templates containing therapeutic transgenes into targeted DSBs. Such ligation events have been observed, but the rates are too low to be useful for therapy [Ran, F.A., et al. Cell 154, 1380-1389 (2013); Orlando, S.J., et al. Nucleic acids research 38, e152 (2010)]. Likely dramatically new approaches are necessary to improve HDR efficiency and increase the therapeutic efficacy of strategies requiring precise genomic correction.

[00252] Barcoding: In an aspect of the invention, barcoding techniques, e.g., of WO/2013/138585 A1, can be adapted or integrated into the practice of the invention. In an aspect

of the invention, barcoding techniques of WO/2013/138585 A1 can be adapted or integrated into the practice of the invention. WO/2013/138585 A1 provides methods for simultaneously determining the effect of a test condition on viability or proliferation of each of a plurality of genetically heterogeneous cell types. The methods include: providing a unitary sample comprising a plurality of, e.g., five, ten, twenty, twenty-five, or more, genetically heterogeneous cell types (each individual cell type is genetically homogeneous within itself, but differs from the others in the plurality), wherein each cell type further comprises: (i) an exogenous nucleic acid tag stably integrated into the genome of the cells, e.g., a tag comprising a core sequence that is unique to each cell type, and flanking amplification primer binding sequences that are the same in all of the cells of the plurality, and (ii) optionally, a marker, e.g., a selectable or detectable marker; and a known number of cells of each cell type is present in the sample; exposing the sample to a test condition for a selected time; and detecting a level of the exogenous nucleic acid tag in each cell type, wherein the level of the exogenous nucleic acid tag is proportional to the number of living cells in the sample after exposure to the test condition; and comparing the number of living cells in the sample after exposure to the test condition to a reference number of cells. The number of living cells in the sample after exposure to the test condition as compared to the reference number of cells indicates the effect of the test condition on viability or proliferation of each cell type. WO/2013/138585 A1 also provides methods for simultaneously determining the effect of a test condition on viability or proliferation of each of a plurality of genetically heterogeneous cell types, wherein the methods include providing a unitary sample comprising a plurality of, e.g., five, ten, twenty, twenty-five, or more, genetically heterogeneous cell types, wherein each cell type further comprises: (i) an exogenous nucleic acid tag stably integrated into the genome of the cells, e.g., comprising a core sequence that is unique to each cell type, and flanking amplification primer binding sequences that are the same in all of the cells of the plurality, and (ii) optionally, a selectable or detectable marker; and a known number of cells of each cell type is present in the sample; implanting the sample into a living animal; exposing the sample to a test condition for a selected time; harvesting the sample from the animal; and detecting a level of the exogenous nucleic acid tag in each cell type of the sample, wherein the level of the exogenous nucleic acid tag correlates to the number of living cells in the sample after exposure to the test condition; and comparing the number of living cells in the sample after exposure to the test condition to a reference number of cells. The number of living cells in the sample after exposure to the test

condition as compared to the reference number of cells indicates the effect of the test condition on viability or proliferation of each cell type. The tag can be Cas9 or another TAG or marker that is integrated into the genome of cells to be transplanted into or onto a non-human eukaryote, e.g., animal model, or that is integrated into the genome of the non-human transgenic eukaryote, e.g., animal, mammal, primate, rodent, mouse, rat, rabbit, etc (along with coding for Cas9). The test condition can be the administration or delivery of the RNA(s) to guide the Cas9 to induce one or more or a plurality, e.g., 3-50 or more, mutations. The test condition can be the administration, delivery or contacting with a putative chemical agent treatment and/or gene therapy treatment. The tag can also be the one or more or a plurality, e.g., 3-50 or more mutations, and the test condition can be the administration, delivery or contacting with a putative chemical agent treatment and/or gene therapy treatment.

Nucleic acids, amino acids and proteins, Regulatory sequences, Vectors, etc

[00253] Nucleic acids, amino acids and proteins: The invention uses nucleic acids to bind target DNA sequences. This is advantageous as nucleic acids are much easier and cheaper to produce than proteins, and the specificity can be varied according to the length of the stretch where homology is sought. Complex 3-D positioning of multiple fingers, for example is not required. The terms “polynucleotide”, “nucleotide”, “nucleotide sequence”, “nucleic acid” and “oligonucleotide” are used interchangeably. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three dimensional structure, and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, short interfering RNA (siRNA), short-hairpin RNA (shRNA), micro-RNA (miRNA), ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. The term also encompasses nucleic-acid-like structures with synthetic backbones, see, e.g., Eckstein, 1991; Baserga et al., 1992; Milligan, 1993; WO 97/03211; WO 96/39154; Mata, 1997; Strauss-Soukup, 1997; and Samstag, 1996. A polynucleotide may comprise one or more modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted

before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. As used herein the term “wild type” is a term of the art understood by skilled persons and means the typical form of an organism, strain, gene or characteristic as it occurs in nature as distinguished from mutant or variant forms. A “wild type” can be a base line. As used herein the term “variant” should be taken to mean the exhibition of qualities that have a pattern that deviates from what occurs in nature. The terms “non-naturally occurring” or “engineered” are used interchangeably and indicate the involvement of the hand of man. The terms, when referring to nucleic acid molecules or polypeptides mean that the nucleic acid molecule or the polypeptide is at least substantially free from at least one other component with which they are naturally associated in nature and as found in nature. “Complementarity” refers to the ability of a nucleic acid to form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick base pairing or other non-traditional types. A percent complementarity indicates the percentage of residues in a nucleic acid molecule which can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). “Perfectly complementary” means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. “Substantially complementary” as used herein refers to a degree of complementarity that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% over a region of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, or more nucleotides, or refers to two nucleic acids that hybridize under stringent conditions. As used herein, “stringent conditions” for hybridization refer to conditions under which a nucleic acid having complementarity to a target sequence predominantly hybridizes with the target sequence, and substantially does not hybridize to non-target sequences. Stringent conditions are generally sequence-dependent, and vary depending on a number of factors. In general, the longer the sequence, the higher the temperature at which the sequence specifically hybridizes to its target sequence. Non-limiting examples of stringent conditions are described in detail in Tijssen (1993), *Laboratory Techniques In Biochemistry And Molecular Biology-Hybridization With Nucleic Acid Probes Part I*, Second Chapter “Overview of principles of hybridization and the strategy of nucleic acid probe assay”, Elsevier, N.Y. Where reference is made to a polynucleotide sequence,

then complementary or partially complementary sequences are also envisaged. These are preferably capable of hybridising to the reference sequence under highly stringent conditions. Generally, in order to maximize the hybridization rate, relatively low-stringency hybridization conditions are selected: about 20 to 25° C lower than the thermal melting point (T_m). The T_m is the temperature at which 50% of specific target sequence hybridizes to a perfectly complementary probe in solution at a defined ionic strength and pH. Generally, in order to require at least about 85% nucleotide complementarity of hybridized sequences, highly stringent washing conditions are selected to be about 5 to 15° C lower than the T_m . In order to require at least about 70% nucleotide complementarity of hybridized sequences, moderately-stringent washing conditions are selected to be about 15 to 30° C lower than the T_m . Highly permissive (very low stringency) washing conditions may be as low as 50° C below the T_m , allowing a high level of mis-matching between hybridized sequences. Those skilled in the art will recognize that other physical and chemical parameters in the hybridization and wash stages can also be altered to affect the outcome of a detectable hybridization signal from a specific level of homology between target and probe sequences. Preferred highly stringent conditions comprise incubation in 50% formamide, 5×SSC, and 1% SDS at 42° C, or incubation in 5×SSC and 1% SDS at 65° C, with wash in 0.2×SSC and 0.1% SDS at 65° C. “Hybridization” refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson Crick base pairing, Hoogsteen binding, or in any other sequence specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi stranded complex, a single self-hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of PCR, or the cleavage of a polynucleotide by an enzyme. A sequence capable of hybridizing with a given sequence is referred to as the “complement” of the given sequence. As used herein, the term “genomic locus” or “locus” (plural loci) is the specific location of a gene or DNA sequence on a chromosome. A “gene” refers to stretches of DNA or RNA that encode a polypeptide or an RNA chain that has functional role to play in an organism and hence is the molecular unit of heredity in living organisms. For the purpose of this invention it may be considered that genes include regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. Accordingly, a gene includes, but is not necessarily limited to, promoter sequences, terminators,

translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites and locus control regions. As used herein, “expression of a genomic locus” or “gene expression” is the process by which information from a gene is used in the synthesis of a functional gene product. The products of gene expression are often proteins, but in non-protein coding genes such as rRNA genes or tRNA genes, the product is functional RNA. The process of gene expression is used by all known life - eukaryotes (including multicellular organisms), prokaryotes (bacteria and archaea) and viruses to generate functional products to survive. As used herein “expression” of a gene or nucleic acid encompasses not only cellular gene expression, but also the transcription and translation of nucleic acid(s) in cloning systems and in any other context. As used herein, “expression” also refers to the process by which a polynucleotide is transcribed from a DNA template (such as into and mRNA or other RNA transcript) and/or the process by which a transcribed mRNA is subsequently translated into peptides, polypeptides, or proteins. Transcripts and encoded polypeptides may be collectively referred to as “gene product.” If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell. The terms “polypeptide”, “peptide” and “protein” are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non amino acids. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a labeling component. As used herein the term “amino acid” includes natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics. As used herein, the term “domain” or “protein domain” refers to a part of a protein sequence that may exist and function independently of the rest of the protein chain. As described in aspects of the invention, sequence identity is related to sequence homology. Homology comparisons may be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs may calculate percent (%) homology between two or more sequences and may also calculate the sequence identity shared by two or more amino acid or nucleic acid sequences. In some preferred embodiments, the capping region of the dTALEs described herein have sequences that are at least 95% identical or share identity to the capping region amino acid sequences provided herein.

Sequence homologies may be generated by any of a number of computer programs known in the art, for example BLAST or FASTA, etc. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A; Devereux et al., 1984, *Nucleic Acids Research* 12:387). Examples of other software that may perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel et al., 1999 *ibid* – Chapter 18), FASTA (Atschul et al., 1990, *J. Mol. Biol.*, 403-410) and the GENWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel et al., 1999 *ibid*, pages 7-58 to 7-60). However it is preferred to use the GCG Bestfit program. Percentage (%) sequence homology may be calculated over contiguous sequences, i.e., one sequence is aligned with the other sequence and each amino acid or nucleotide in one sequence is directly compared with the corresponding amino acid or nucleotide in the other sequence, one residue at a time. This is called an “ungapped” alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues. Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion may cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without unduly penalizing the overall homology or identity score. This is achieved by inserting “gaps” in the sequence alignment to try to maximize local homology or identity. However, these more complex methods assign “gap penalties” to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - may achieve a higher score than one with many gaps. “Affinity gap costs” are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties may, of course, produce optimized alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example, when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension. Calculation of maximum % homology therefore first requires the production of an optimal alignment, taking into

consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (Devereux et al., 1984 *Nuc. Acids Research* 12 p387). Examples of other software that may perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel et al., 1999 *Short Protocols in Molecular Biology*, 4th Ed. – Chapter 18), FASTA (Altschul et al., 1990 *J. Mol. Biol.* 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel et al., 1999, *Short Protocols in Molecular Biology*, pages 7-58 to 7-60). However, for some applications, it is preferred to use the GCG Bestfit program. A new tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide sequences (see *FEMS Microbiol Lett.* 1999 174(2): 247-50; *FEMS Microbiol Lett.* 1999 177(1): 187-8 and the website of the National Center for Biotechnology information at the website of the National Institutes for Health). Although the final % homology may be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pair-wise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table, if supplied (see user manual for further details). For some applications, it is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62. Alternatively, percentage homologies may be calculated using the multiple alignment feature in DNASISTM (Hitachi Software), based on an algorithm, analogous to CLUSTAL (Higgins DG & Sharp PM (1988), *Gene* 73(1), 237-244). Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result. The sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of similarity in amino acid properties (such as polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues) and it is therefore useful to group amino acids together in functional groups. Amino acids may be grouped together based on the properties of their side chains alone. However, it is more useful to include mutation data as well. The sets of amino acids

thus derived are likely to be conserved for structural reasons. These sets may be described in the form of a Venn diagram (Livingstone C.D. and Barton G.J. (1993) "Protein sequence alignments: a strategy for the hierarchical analysis of residue conservation" *Comput. Appl. Biosci.* 9: 745-756) (Taylor W.R. (1986) "The classification of amino acid conservation" *J. Theor. Biol.* 119; 205-218). Conservative substitutions may be made, for example according to the table below which describes a generally accepted Venn diagram grouping of amino acids.

Set		Sub-set	
Hydrophobic	F W Y H K M I L V A G C	Aromatic	F W Y H
		Aliphatic	I L V
Polar	W Y H K R E D C S T N Q	Charged	H K R E D
		Positively charged	H K R
		Negatively charged	E D
Small	V C A G S P T N D	Tiny	A G S

[00254] Embodiments of the invention include sequences (both polynucleotide or polypeptide) which may comprise homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue or nucleotide, with an alternative residue or nucleotide) that may occur i.e., like-for-like substitution in the case of amino acids such as basic for basic, acidic for acidic, polar for polar, etc. Non-homologous substitution may also occur i.e., from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine. Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or β -alanine residues. A further form of variation, which involves the presence of one or more amino acid residues in peptoid form, may be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the α -carbon substituent group is on the residue's nitrogen atom rather than the α -carbon. Processes for

preparing peptides in the peptoid form are known in the art, for example Simon RJ et al., *PNAS* (1992) 89(20), 9367-9371 and Horwell DC, *Trends Biotechnol.* (1995) 13(4), 132-134.

[00255] For purpose of this invention, amplification means any method employing a primer and a polymerase capable of replicating a target sequence with reasonable fidelity. Amplification may be carried out by natural or recombinant DNA polymerases such as TaqGold™, T7 DNA polymerase, Klenow fragment of E.coli DNA polymerase, and reverse transcriptase. A preferred amplification method is PCR.

[00256] In certain aspects the invention involves vectors. As used herein, a “vector” is a tool that allows or facilitates the transfer of an entity from one environment to another. It is a replicon, such as a plasmid, phage, or cosmid, into which another DNA segment may be inserted so as to bring about the replication of the inserted segment. Generally, a vector is capable of replication when associated with the proper control elements. In general, the term “vector” refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Vectors include, but are not limited to, nucleic acid molecules that are single-stranded, double-stranded, or partially double-stranded; nucleic acid molecules that comprise one or more free ends, no free ends (e.g. circular); nucleic acid molecules that comprise DNA, RNA, or both; and other varieties of polynucleotides known in the art. One type of vector is a “plasmid,” which refers to a circular double stranded DNA loop into which additional DNA segments can be inserted, such as by standard molecular cloning techniques. Another type of vector is a viral vector, wherein virally-derived DNA or RNA sequences are present in the vector for packaging into a virus (e.g. retroviruses, replication defective retroviruses, adenoviruses, replication defective adenoviruses, and adeno-associated viruses (AAVs)). Viral vectors also include polynucleotides carried by a virus for transfection into a host cell. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g. bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively-linked. Such vectors are referred to herein as “expression vectors.” Common expression vectors of utility in recombinant DNA techniques are often in the form of plasmids.

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[00257] Recombinant expression vectors can comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory elements, which may be selected on the basis of the host cells to be used for expression, that is operatively-linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, “operably linked” is intended to mean that the nucleotide sequence of interest is linked to the regulatory element(s) in a manner that allows for expression of the nucleotide sequence (e.g. in an in vitro transcription/translation system or in a host cell when the vector is introduced into the host cell). With regards to recombination and cloning methods, mention is made of U.S. patent application 10/815,730, published September 2, 2004 as US 2004-0171156 A1, the contents of which are herein incorporated by reference in their entirety.

[00258] Aspects of the invention relate to bicistronic vectors for chimeric RNA and Cas9. Bicistronic expression vectors for chimeric RNA and Cas9 are preferred. In general and particularly in this embodiment Cas9 is preferably driven by the CBh promoter. The chimeric RNA may preferably be driven by a Pol III promoter, such as a U6 promoter. Ideally the two are combined. The chimeric guide RNA typically consists of a 20bp guide sequence (Ns) and this may be joined to the tracr sequence (running from the first “U” of the lower strand to the end of the transcript). The tracr sequence may be truncated at various positions as indicated. The guide and tracr sequences are separated by the tracr-mate sequence, which may be GUUUUAGAGCUA. This may be followed by the loop sequence GAAA as shown. Both of these are preferred examples. Applicants have demonstrated Cas9-mediated indels at the human *EMXI* and *PVALB* loci by SURVEYOR assays. ChiRNAs are indicated by their “+n” designation, and crRNA refers to a hybrid RNA where guide and tracr sequences are expressed as separate transcripts. Throughout this application, chimeric RNA may also be called single guide, or synthetic guide RNA (sgRNA). The loop is preferably GAAA, but it is not limited to this sequence or indeed to being only 4bp in length. Indeed, preferred loop forming sequences for use in hairpin structures are four nucleotides in length, and most preferably have the sequence GAAA. However, longer or shorter loop sequences may be used, as may alternative sequences. The sequences preferably include a nucleotide triplet (for example, AAA), and an additional nucleotide (for example C or G). Examples of loop forming sequences include CAAA and AAAG. In practicing any of the methods disclosed herein, a suitable vector can be introduced to a cell or an embryo via one or

more methods known in the art, including without limitation, microinjection, electroporation, sonoporation, biolistics, calcium phosphate-mediated transfection, cationic transfection, liposome transfection, dendrimer transfection, heat shock transfection, nucleofection transfection, magnetofection, lipofection, impalefection, optical transfection, proprietary agent-enhanced uptake of nucleic acids, and delivery via liposomes, immunoliposomes, virosomes, or artificial virions. In some methods, the vector is introduced into an embryo by microinjection. The vector or vectors may be microinjected into the nucleus or the cytoplasm of the embryo. In some methods, the vector or vectors may be introduced into a cell by nucleofection.

[00259] The term “regulatory element” is intended to include promoters, enhancers, internal ribosomal entry sites (IRES), and other expression control elements (e.g. transcription termination signals, such as polyadenylation signals and poly-U sequences). Such regulatory elements are described, for example, in Goeddel, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Regulatory elements include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). A tissue-specific promoter may direct expression primarily in a desired tissue of interest, such as muscle, neuron, bone, skin, blood, specific organs (e.g. liver, pancreas), or particular cell types (e.g. lymphocytes). Regulatory elements may also direct expression in a temporal-dependent manner, such as in a cell-cycle dependent or developmental stage-dependent manner, which may or may not also be tissue or cell-type specific. In some embodiments, a vector comprises one or more pol III promoter (e.g. 1, 2, 3, 4, 5, or more pol III promoters), one or more pol II promoters (e.g. 1, 2, 3, 4, 5, or more pol II promoters), one or more pol I promoters (e.g. 1, 2, 3, 4, 5, or more pol I promoters), or combinations thereof. Examples of pol III promoters include, but are not limited to, U6 and H1 promoters. Examples of pol II promoters include, but are not limited to, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, e.g., Boshart et al, Cell, 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the β -actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1 α promoter. Also encompassed by the term “regulatory element” are enhancer elements, such as WPRE; CMV enhancers; the R-U5' segment in LTR of HTLV-I (Mol. Cell. Biol., Vol. 8(1), p. 466-472, 1988); SV40 enhancer; and the intron sequence between exons 2

and 3 of rabbit β -globin (Proc. Natl. Acad. Sci. USA., Vol. 78(3), p. 1527-31, 1981). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression desired, etc. A vector can be introduced into host cells to thereby produce transcripts, proteins, or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (e.g., clustered regularly interspersed short palindromic repeats (CRISPR) transcripts, proteins, enzymes, mutant forms thereof, fusion proteins thereof, etc.). With regards to regulatory sequences, mention is made of U.S. patent application 10/491,026, the contents of which are incorporated by reference herein in their entirety. With regards to promoters, mention is made of PCT publication WO 2011/028929 and U.S. application 12/511,940, the contents of which are incorporated by reference herein in their entirety.

[00260] Vectors can be designed for expression of CRISPR transcripts (e.g. nucleic acid transcripts, proteins, or enzymes) in prokaryotic or eukaryotic cells. For example, CRISPR transcripts can be expressed in bacterial cells such as *Escherichia coli*, insect cells (using baculovirus expression vectors), yeast cells, or mammalian cells. Suitable host cells are discussed further in Goeddel, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Alternatively, the recombinant expression vector can be transcribed and translated in vitro, for example using T7 promoter regulatory sequences and T7 polymerase.

[00261] Vectors may be introduced and propagated in a prokaryote or prokaryotic cell. In some embodiments, a prokaryote is used to amplify copies of a vector to be introduced into a eukaryotic cell or as an intermediate vector in the production of a vector to be introduced into a eukaryotic cell (e.g. amplifying a plasmid as part of a viral vector packaging system). In some embodiments, a prokaryote is used to amplify copies of a vector and express one or more nucleic acids, such as to provide a source of one or more proteins for delivery to a host cell or host organism. Expression of proteins in prokaryotes is most often carried out in *Escherichia coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, such as to the amino terminus of the recombinant protein. Such fusion vectors may serve one or more purposes, such as: (i) to increase expression of recombinant protein; (ii) to increase the solubility of the recombinant protein; and (iii) to aid in the purification of the recombinant protein by acting as a

ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Example fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith and Johnson, 1988. *Gene* 67: 31-40), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, N.J.) that fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein. Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amrann et al., (1988) *Gene* 69:301-315) and pET 11d (Studier et al., *GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY* 185, Academic Press, San Diego, Calif. (1990) 60-89). In some embodiments, a vector is a yeast expression vector. Examples of vectors for expression in yeast *Saccharomyces cerevisiae* include pYepSec1 (Baldari, et al., 1987. *EMBO J.* 6: 229-234), pMFa (Kuijan and Herskowitz, 1982. *Cell* 30: 933-943), pJRY88 (Schultz et al., 1987. *Gene* 54: 113-123), pYES2 (Invitrogen Corporation, San Diego, Calif.), and picZ (Invitrogen Corp, San Diego, Calif.). In some embodiments, a vector drives protein expression in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., SF9 cells) include the pAc series (Smith, et al., 1983. *Mol. Cell. Biol.* 3: 2156-2165) and the pVL series (Lucklow and Summers, 1989. *Virology* 170: 31-39).

[00262] In some embodiments, a vector is capable of driving expression of one or more sequences in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, 1987. *Nature* 329: 840) and pMT2PC (Kaufman, et al., 1987. *EMBO J.* 6: 187-195). When used in mammalian cells, the expression vector's control functions are typically provided by one or more regulatory elements. For example, commonly used promoters are derived from polyoma, adenovirus 2, cytomegalovirus, simian virus 40, and others disclosed herein and known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells see, e.g., Chapters 16 and 17 of Sambrook, et al., *MOLECULAR CLONING: A LABORATORY MANUAL*. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.

[00263] In some embodiments, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue-specific

regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert, et al., 1987. *Genes Dev.* 1: 268-277), lymphoid-specific promoters (Calame and Eaton, 1988. *Adv. Immunol.* 43: 235-275), in particular promoters of T cell receptors (Winoto and Baltimore, 1989. *EMBO J.* 8: 729-733) and immunoglobulins (Baneiji, et al., 1983. *Cell* 33: 729-740; Queen and Baltimore, 1983. *Cell* 33: 741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle, 1989. *Proc. Natl. Acad. Sci. USA* 86: 5473-5477), pancreas-specific promoters (Edlund, et al., 1985. *Science* 230: 912-916), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, e.g., the murine hox promoters (Kessel and Gruss, 1990. *Science* 249: 374-379) and the α -fetoprotein promoter (Campes and Tilghman, 1989. *Genes Dev.* 3: 537-546). With regards to these prokaryotic and eukaryotic vectors, mention is made of U.S. Patent 6,750,059, the contents of which are incorporated by reference herein in their entirety. Other embodiments of the invention may relate to the use of viral vectors, with regards to which mention is made of U.S. Patent application 13/092,085, the contents of which are incorporated by reference herein in their entirety. Tissue-specific regulatory elements are known in the art and in this regard, mention is made of U.S. Patent 7,776,321, the contents of which are incorporated by reference herein in their entirety. In some embodiments, a regulatory element is operably linked to one or more elements of a CRISPR system so as to drive expression of the one or more elements of the CRISPR system. In general, CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats), also known as SPIDRs (SPacer Interspersed Direct Repeats), constitute a family of DNA loci that are usually specific to a particular bacterial species. The CRISPR locus comprises a distinct class of interspersed short sequence repeats (SSRs) that were recognized in *E. coli* (Ishino et al., *J. Bacteriol.*, 169:5429-5433 [1987]; and Nakata et al., *J. Bacteriol.*, 171:3553-3556 [1989]), and associated genes. Similar interspersed SSRs have been identified in *Haloferax mediterranei*, *Streptococcus pyogenes*, *Anabaena*, and *Mycobacterium tuberculosis* (See, Groenen et al., *Mol. Microbiol.*, 10:1057-1065 [1993]; Hoe et al., *Emerg. Infect. Dis.*, 5:254-263 [1999]; Masepohl et al., *Biochim. Biophys. Acta* 1307:26-30 [1996]; and Mojica et al., *Mol. Microbiol.*, 17:85-93 [1995]). The CRISPR loci typically differ from other SSRs by the structure of the repeats, which have been termed short regularly spaced repeats (SRSRs) (Janssen et al., *OMICS J. Integ. Biol.*,

6:23-33 [2002]; and Mojica et al., *Mol. Microbiol.*, 36:244-246 [2000]). In general, the repeats are short elements that occur in clusters that are regularly spaced by unique intervening sequences with a substantially constant length (Mojica et al., [2000], supra). Although the repeat sequences are highly conserved between strains, the number of interspersed repeats and the sequences of the spacer regions typically differ from strain to strain (van Embden et al., *J. Bacteriol.*, 182:2393-2401 [2000]). CRISPR loci have been identified in more than 40 prokaryotes (See e.g., Jansen et al., *Mol. Microbiol.*, 43:1565-1575 [2002]; and Mojica et al., [2005]) including, but not limited to *Aeropyrum*, *Pyrobaculum*, *Sulfolobus*, *Archaeoglobus*, *Halocarcula*, *Methanobacterium*, *Methanococcus*, *Methanosarcina*, *Methanopyrus*, *Pyrococcus*, *Picrophilus*, *Thermoplasma*, *Corynebacterium*, *Mycobacterium*, *Streptomyces*, *Aquifex*, *Porphyromonas*, *Chlorobium*, *Thermus*, *Bacillus*, *Listeria*, *Staphylococcus*, *Clostridium*, *Thermoanaerobacter*, *Mycoplasma*, *Fusobacterium*, *Azarcus*, *Chromobacterium*, *Neisseria*, *Nitrosomonas*, *Desulfovibrio*, *Geobacter*, *Myxococcus*, *Campylobacter*, *Wolinella*, *Acinetobacter*, *Erwinia*, *Escherichia*, *Legionella*, *Methylococcus*, *Pasteurella*, *Photobacterium*, *Salmonella*, *Xanthomonas*, *Yersinia*, *Treponema*, and *Thermotoga*.

[00264] In some embodiments, the CRISPR enzyme is part of a fusion protein comprising one or more heterologous protein domains (e.g. about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more domains in addition to the CRISPR enzyme). A CRISPR enzyme fusion protein may comprise any additional protein sequence, and optionally a linker sequence between any two domains. Examples of protein domains that may be fused to a CRISPR enzyme include, without limitation, epitope tags, reporter gene sequences, and protein domains having one or more of the following activities: methylase activity, demethylase activity, transcription activation activity, transcription repression activity, transcription release factor activity, histone modification activity, RNA cleavage activity and nucleic acid binding activity. Non-limiting examples of epitope tags include histidine (His) tags, V5 tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Examples of reporter genes include, but are not limited to, glutathione-S-transferase (GST), horseradish peroxidase (HRP), chloramphenicol acetyltransferase (CAT) beta-galactosidase, beta-glucuronidase, luciferase, green fluorescent protein (GFP), HcRed, DsRed, cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), and autofluorescent proteins including blue fluorescent protein (BFP). A CRISPR enzyme may be fused to a gene sequence encoding a protein or a fragment of a protein that bind DNA molecules or

bind other cellular molecules, including but not limited to maltose binding protein (MBP), S-tag, Lex A DNA binding domain (DBD) fusions, GAL4 DNA binding domain fusions, and herpes simplex virus (HSV) BP16 protein fusions. Additional domains that may form part of a fusion protein comprising a CRISPR enzyme are described in US20110059502, incorporated herein by reference. In some embodiments, a tagged CRISPR enzyme is used to identify the location of a target sequence.

[00265] In some embodiments, a CRISPR enzyme may form a component of an inducible system. The inducible nature of the system would allow for spatiotemporal control of gene editing or gene expression using a form of energy. The form of energy may include but is not limited to electromagnetic radiation, sound energy, chemical energy and thermal energy. Examples of inducible system include tetracycline inducible promoters (Tet-On or Tet-Off), small molecule two-hybrid transcription activations systems (FKBP, ABA, etc), or light inducible systems (Phytochrome, LOV domains, or cryptochrome). In one embodiment, the CRISPR enzyme may be a part of a Light Inducible Transcriptional Effector (LITE) to direct changes in transcriptional activity in a sequence-specific manner. The components of a light may include a CRISPR enzyme, a light-responsive cytochrome heterodimer (e.g. from *Arabidopsis thaliana*), and a transcriptional activation/repression domain. Further examples of inducible DNA binding proteins and methods for their use are provided in US 61/736465 and US 61/721,283, which is hereby incorporated by reference in its entirety.

[00266] The practice of the present invention employs, unless otherwise indicated, conventional techniques of immunology, biochemistry, chemistry, molecular biology, microbiology, cell biology, genomics and recombinant DNA, which are within the skill of the art. See Sambrook, Fritsch and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R.I. Freshney, ed. (1987)).

Modifying a target

[00267] In one aspect, the invention provides for methods of modifying a target polynucleotide in a eukaryotic cell, which may be *in vivo*, *ex vivo* or *in vitro*. In some embodiments, the method

comprises sampling a cell or population of cells from a human or non-human animal, and modifying the cell or cells. Culturing may occur at any stage *ex vivo*. The cell or cells may even be re-introduced into the non-human animal or plant. For re-introduced cells it is particularly preferred that the cells are stem cells.

[00268] In some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized or hybridizable to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence.

[00269] In one aspect, the invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the polynucleotide such that said binding results in increased or decreased expression of said polynucleotide; wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized or hybridizable to a target sequence within said polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. Similar considerations and conditions apply as above for methods of modifying a target polynucleotide. In fact, these sampling, culturing and re-introduction options apply across the aspects of the present invention.

[00270] Indeed, in any aspect of the invention, the CRISPR complex may comprise a CRISPR enzyme complexed with a guide sequence hybridized or hybridizable to a target sequence, wherein said guide sequence may be linked to a tracr mate sequence which in turn may hybridize to a tracr sequence. Similar considerations and conditions apply as above for methods of modifying a target polynucleotide.

Kits

[00271] In one aspect, the invention provides kits containing any one or more of the elements disclosed in the above methods and compositions. Elements may be provided individually or in combinations, and may be provided in any suitable container, such as a vial, a bottle, or a tube. In some embodiments, the kit includes instructions in one or more languages, for example in more than one language.

[00272] In some embodiments, a kit comprises one or more reagents for use in a process utilizing one or more of the elements described herein. Reagents may be provided in any suitable container. For example, a kit may provide one or more reaction or storage buffers. Reagents may be provided in a form that is usable in a particular assay, or in a form that requires addition of one or more other components before use (e.g. in concentrate or lyophilized form). A buffer can be any buffer, including but not limited to a sodium carbonate buffer, a sodium bicarbonate buffer, a borate buffer, a Tris buffer, a MOPS buffer, a HEPES buffer, and combinations thereof. In some embodiments, the buffer is alkaline. In some embodiments, the buffer has a pH from about 7 to about 10. In some embodiments, the kit comprises one or more oligonucleotides corresponding to a guide sequence for insertion into a vector so as to operably link the guide sequence and a regulatory element. In some embodiments, the kit comprises a homologous recombination template polynucleotide. In some embodiments, the kit comprises one or more of the vectors and/or one or more of the polynucleotides described herein. The kit may advantageously allow to provide all elements of the systems of the invention.

EXAMPLES

[00273] The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion. The present examples, along with the methods described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses which are encompassed within the spirit of the invention as defined by the scope of the claims will occur to those skilled in the art.

Example 1: Particle-mediated delivery of CRISPR-Cas9 components into hematopoietic stem cells (HSCs)

[00274] Applicants have demonstrated that Cas9 can be delivered to cells via particles. In this instance, in that many nucleic therapeutics may require the delivery of both the one or more sgRNA and the Cas9 nuclease concurrently, we demonstrated the ability to deliver in this fashion.

[00275] The sgRNA was pre-complexed with the Cas9 protein, before formulating the entire complex in a particle. Twenty different particle formulations were generated and tested for CRISPR-Cas9 delivery efficiency into cells. Each formulation was made with a different molar ratio of four components known to promote delivery of nucleic acids into cells: 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP),

1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (DMPC), polyethylene glycol (PEG), and cholesterol (e.g. DOTAP : DMPC : PEG : Cholesterol Molar Ratios; Formulation number 1 = DOTAP 100, DMPC 0, PEG 0, Cholesterol 0; Formulation number 2 = DOTAP 90, DMPC 0, PEG 10, Cholesterol 0; Formulation number 3 = DOTAP 90, DMPC 0, PEG 5, Cholesterol 5).

[00276] Particles were formed using an efficient, multistep process. First, Cas9 protein and sgRNA targeting the gene EMX1 or the control gene LacZ were mixed together at a 1:1 molar ratio at room temperature for 30 minutes in sterile, nuclease free 1X PBS. Separately, DOTAP, DMPC, PEG, and cholesterol were dissolved in 100% ethanol. The two solutions were mixed together to form particles containing the Cas9-sgRNA complexes. After the particles were formed, HSCs in 96 well plates were transfected with 15ug Cas9 protein per well. Three days after transfection, HSCs were harvested, and the number of insertions and deletions (indels) at the EMX1 locus were quantified.

* * *

[00277] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention.

WHAT IS CLAIMED IS:

1. A method of modifying an organism or a non-human organism by manipulation of a target sequence in a hematopoietic stem cell (HSC), wherein the target sequence has an association with a mutation having an association with an aberrant protein expression or with a disease condition or state, wherein "normal" comprises wild type, and "aberrant" comprises expression that gives rise to a condition or disease state, said method comprising:

delivering to an HSC a non-naturally occurring or engineered composition comprising:

- I. a CRISPR-Cas9 system guide sequence RNA that hybridizes to a target nucleotide sequence in the HSC, or a polynucleotide encoding the guide sequence, operably for transcription in the HSC, and
- II. a Cas9 enzyme, optionally comprising at least one or more nuclear localization sequences, or a polynucleotide encoding the Cas9 enzyme, optionally comprising at least one or more nuclear localization sequences, operably for expression in the HSC,

to obtain an HSC population,

wherein the guide sequence directs sequence-specific binding of a CRISPR complex to the target nucleotide sequence in the HSC, and the CRISPR complex comprises the Cas9 enzyme complexed with the guide sequence;

optionally the method including also delivering a polynucleotide template, wherein the template provides expression of a normal or less aberrant form of a protein; and/or

optionally the method including, prior to the delivering, isolating or obtaining HSC from the organism or non-human organism, and/or

optionally the method including expanding the HSC population to obtain modified HSCs, and/or

optionally the method including administering HSCs from the HSC population or modified HSCs to the organism or non-human organism.

2. The method of claim 1 wherein the target sequence comprises a DNA sequence.

3. The method of claims 1, or 2 wherein the guide sequence RNA comprises a chimeric RNA (chiRNA) polynucleotide including a tracr mate sequence and a tracr sequence, or the polynucleotide encoding the guide sequence include coding for including the tracr mate

sequence and the tracr sequence, operably for transcription in the HSC, and in the CRISPR complex the tracr mate sequence hybridizes to the tracr sequence.

4. The method of any one of claims 1, 2 or 3 wherein the delivering comprises delivery of

- I. the CRISPR-Cas9 system guide, or the polynucleotide encoding the guide sequence, and at least one or more tracr mate sequences or at least one or more polynucleotides encoding the at least one or more tracr mate sequences operably for transcription in the HSC, or the polynucleotide encoding the guide sequence including at least one or more polynucleotides encoding the at least one or more tracr mate sequences operably for transcription in the HSC,
- II. the Cas9 enzyme, optionally comprising at least one or more nuclear localization sequences, or the polynucleotide encoding the Cas9 enzyme, optionally comprising at least one or more nuclear localization sequences, and
- III. tracr sequence or a polynucleotide sequence encoding the tracr sequence operably for transcription in the HSC, and

in the CRISPR complex the tracr mate sequence hybridizes to the tracr sequence.

5. The method of any one of claims 1-4 wherein delivering comprises delivery of

- I. the CRISPR-Cas system guide sequence RNA.

6. The method of any one of claims 1-5 wherein delivering comprises delivery of

- II. the Cas9 enzyme, optionally comprising at least one or more nuclear localization sequences.

7. The method of any one of claims 1-6 wherein delivering comprises delivery of

- I. the polynucleotide encoding the guide sequence.

8. The method of any one of claims 1-7 wherein delivering comprises delivery of

- II. the polynucleotide encoding the Cas9 enzyme, optionally comprising at least one or more nuclear localization sequences.

9. The method of claim 8 wherein delivering comprises delivery of the guide sequence RNA comprises the chimeric RNA (chiRNA) polynucleotide including the tracr mate sequence and the tracr sequence.

10. The method of claim 9 wherein delivering comprises delivery of the polynucleotide encoding the guide sequence include coding for including the tracr mate sequence and the tracr sequence, operably for transcription in the HSC, and in the CRISPR complex the tracr mate sequence hybridizes to the tracr sequence.

11. The method of any preceding claim wherein delivering comprises delivery of
II. the Cas9 enzyme, optionally comprising at least one or more nuclear localization sequences.

12. The method of any preceding claim wherein delivering comprises delivery of
II. the polynucleotide encoding the Cas9 enzyme, optionally comprising at least one or more nuclear localization sequences.

13. The method of any preceding claim wherein the delivering comprises delivery of
I. the CRISPR-Cas system guide sequence and at least one or more tracr mate sequences or at least one or more polynucleotides encoding the at least one or more tracr mate sequences.

14. The method of any preceding claim wherein the delivering comprises delivery of:
I. the CRISPR-Cas system guide sequence and at least one or more tracr mate sequences.

15. The method of any preceding claim wherein the delivering comprises delivery of
I. the polynucleotide encoding the CRISPR-Cas system guide sequence and at least one or more tracr mate sequences or at least one or more polynucleotides encoding the at least one or more tracr mate sequences.

16. The method of any preceding claim wherein the delivering comprises delivery of
III. the a polynucleotide sequence encoding the tracr sequence operably for transcription in the HSC.

17. The method of any preceding claim wherein the target sequence is on a strand of a polynucleotide duplex, and method obtains cleavage of the polynucleotide duplex whereby on one strand there is a 5' overhang and on the other strand there is a 3' overhang.

18. The method of any one of claims 1-16 wherein the target sequence is a first target sequence on a first strand of the polynucleotide duplex and there is a second target sequence on a second strand of the polynucleotide duplex, and the method comprises delivery of a first CRISPR-Cas system guide sequence RNA that hybridizes to the first target nucleotide sequence in

the HSC, or a polynucleotide encoding the first guide sequence, operably for transcription in the HSC, and delivery of a second CRISPR-Cas system guide sequence RNA that hybridizes to the second target nucleotide sequence in the HSC, or a polynucleotide encoding the second guide sequence, operably for transcription in the HSC.

19. The method of claim 18 wherein the delivery comprises delivering the first guide sequence.

20. The method of claim 18 wherein the delivery comprises delivering the polynucleotide encoding the first guide sequence.

21. The method of any one of claims 18-20 wherein the delivery comprises delivering the second guide sequence.

22. The method of any one of claims 18-20 wherein the delivery comprises delivering the polynucleotide encoding the second guide sequence.

23. The method of any one of claims 18-22 wherein either or both of the first or second guide sequence comprises a tracr mate sequence and a tracr sequence and the guide comprises a chimeric RNA (chiRNA) polynucleotide sequence.

24. The method of any one of claims 1-16 wherein the target sequence is a first target sequence on a first strand of the polynucleotide duplex and there is a second target sequence on a second strand of the polynucleotide duplex, and the method comprises delivery of: a first CRISPR-Cas system guide sequence RNA that hybridizes to the first target nucleotide sequence in the HSC including at least one or more tracr mate sequences, or a polynucleotide encoding the first guide sequence and at least one or more tracr mate sequences, operably for transcription in the HSC; and a second CRISPR-Cas system guide sequence RNA that hybridizes to the second target nucleotide sequence in the HSC including at least one or more tracr mate sequences, or a polynucleotide encoding the second guide sequence including at least one or more tracr mate sequences, operably for transcription in the HSC; and a tracr sequence or a polynucleotide encoding the tracr sequence, operably for transcription in the HSC.

25. The method of claim 24 wherein the delivery comprises delivering the first CRISPR-Cas system guide sequence RNA including at least one or more tracr mate sequences.

26. The method of claim 25 wherein the delivery comprises delivering the polynucleotide encoding the first guide sequence and at least one or more tracr mate sequences, operably for transcription in the HSC.

27. The method of any one of claims 24-26 wherein the delivery comprises delivering the second CRISPR-Cas system guide sequence RNA including at least one or more tracr mate sequences.

28. The method of any one of claims 24-26 wherein the delivery comprises delivering the polynucleotide encoding the second guide sequence including at least one or more tracr mate sequences, operably for transcription in the HSC.

29. The method of any one of claims 24-26 wherein the delivery comprises delivering the tracr sequence.

30. The method of any one of claims 24-26 wherein the delivery comprises delivering the polynucleotide encoding the tracr sequence, operably for transcription in the HSC.

31. The method of any of the preceding claims wherein the Cas9 is a nickase

32. The method of any of the preceding claims wherein the CRISPR-Cas system guide sequence RNA comprises an sgRNA.

33. The method of any of the preceding claims wherein the target sequence is associated with a genomic locus of interest associated with the mutation.

34. The method of any of the preceding claims wherein operably for transcription in the HSC includes the polynucleotide sequence operably linked to a regulatory element for expression or transcription of the polynucleotide sequence.

35. The method of any of the preceding claims wherein when there is a polynucleotide(s) encoding (a) the guide sequence, (b) the tracr mate sequence and (c) the tracr sequence, (a), (b) and (c) are arranged in a 5' to 3' orientation.

36. The method of any preceding claim wherein the organism or non-human organism is a mammal.

37. The method of claim 36 wherein the mammal is a human.

38. The method of any preceding claim wherein when the CRISPR complex arises from delivery of polynucleotide(s) for all components of the CRISPR complex.

39. The method of claim 38 wherein the polynucleotide(s) are delivered via a single vector or particle.

40. The method of claim 38 wherein the polynucleotide(s) are delivered via more than one vector.

41. The method of claim 38 wherein the polynucleotide(s) are delivered via a single vector.

42. The method of any preceding claim wherein delivery is via one or more particles contacting the HSC, wherein the one or more particles contain CRISPR complex component(s) and/or polynucleotide(s) therefor.

43. The method of claim 42 wherein the method comprises contacting the HSC with a particle containing the CRISPR complex.

44. The method of any one of claims 1-42 wherein delivery is via one or more vectors contacting the HSC or one or more particles containing one or more vectors contacting the HSC, wherein the one or more vectors contain the polynucleotide(s) encoding CRISPR complex component(s).

45. The method of claim 44 wherein the method comprises contacting the HSC with a vector containing the polynucleotide(s) encoding all CRISPR complex components, or a particle containing such a vector.

46. The method of any one of claims 44-45 wherein the vector comprises a viral, plasmid or nucleic acid molecule vector.

47. The method of any one of claims 42-46 wherein particle(s) is / are formed by a method comprising or consisting essentially of or consisting of admixing (i) a mixture of the CRISPR complex components with (ii) a mixture comprising or consisting essentially of or consisting of surfactant, phospholipid, biodegradable polymer, lipoprotein and alcohol, whereby particle(s) containing the CRISPR-complex are formed.

48. The method of claim 47 wherein the mixture (i) includes the polynucleotide template.

49. The method of any one of claims 1-47 including also delivering the polynucleotide template.

50. The method of claim 48 or 49 wherein the delivery of the polynucleotide template is via the template being included in a particle containing the CRISPR complex.

51. The method of claim 50 wherein the delivery of the polynucleotide template is via a particle.

52. The method of claim 51 wherein the particle containing the polynucleotide template is separate from particle(s) containing CRISPR complex component(s) and/or polynucleotide(s) coding therefor.

53. The method of claim 49 wherein the delivery of the polynucleotide template is via a vector.

54. The method of claim 53 wherein the vector is separate from vector(s) containing polynucleotide(s) coding for CRISPR complex component(s).

55. The method of claim 53 wherein the vector is the same as the vector containing polynucleotide(s) coding for CRISPR complex components.

56. The method of any one of the preceding claims wherein the polynucleotide encoding the CRISPR protein is codon optimized for expression in the HSC.

57. The method of any one of the preceding claims wherein the Cas9 comprises one or more mutations in a catalytic domain.

58. The method of claim 57, wherein the one or more mutations comprise, with reference to SpCas9, D10A, E762A, H840A, N854A, N863A or D986A, or an analogous mutation in a CRISPR protein other than SpCas9.

59. The method of claim 58 wherein the mutation comprises, with reference to SpCas9, a D10A mutation, or an analogous mutation in a Cas9 other than SpCas9.

60. The method of any one of claims 1-58 wherein the Cas9 comprises an SaCas9.

61. The method of any preceding claim including, prior to the delivering, isolating or obtaining HSC from the organism or non-human organism.

62. The method of any preceding claim including expanding the HSC population to obtain modified HSCs.

63. The method of claim 62 including administering HSCs from the HSC population or modified HSCs to the organism or non-human organism.

64. The method of any one of the preceding claims wherein the target or the genomic locus of interest is associated with Hemophilia B, sickle cell anemia, SCID, SCID-X1, ADA-SCID, Hereditary tyrosinemia, β -thalassemia, X-linked CGD, Wiskott-Aldrich syndrome, Fanconi anemia, adrenoleukodystrophy (ALD), metachromatic leukodystrophy (MLD), HIV/AIDS, Krabbe Disease, Polycythemia vera (PCV), myeloproliferative neoplasm, Familial essential thrombocythaemia (ET) or Alpha-mannosidosis.

65. The method of any one of the preceding claims wherein the target or the genomic locus of interest is associated with an Immunodeficiency disorder, Hematologic condition, a Leukodystrophy or genetic lysosomal storage disease.

66. Use of a CRISPR-Cas complex as defined in any of the foregoing claims in the preparation of a medicament for a patient in need of treatment for a disease condition or state, comprising performing a method as in any of the foregoing claims.

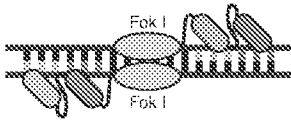
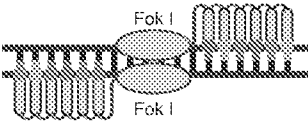
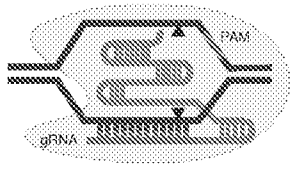
	Zinc Finger Nucleases	TALE Nucleases	CRISPR-Cas9
			
Recognition site	Typically 9 to 18 bp per ZFP, 18 to 36 bp per ZFN pair	Typically 14 to 20 bp per TALE, 28 to 40bp per TALEN	22bp (20bp guide sequence + 2bp PAM sequence); up to 44 bp for double nicking
Specificity	Small number of positional mismatches tolerated	Small number of positional mismatches tolerated	Positional and multiple consecutive mismatches tolerated
Targeting constraints	Difficult to target non-G-rich sequences	5' targeted base must be a T	Targeted sequence must precede a PAM
Ease of engineering	Difficult, may require substantial protein engineering	Moderate, requires complex molecular cloning methods	Easily re-targeted using standard cloning procedures and oligo synthesis
Immunogenicity	Likely low, as ZFs are based on human transcription factor protein scaffold	Unknown, protein derived from the bacteria <i>Xanthomonas sp.</i>	Unknown, protein derived from various bacterial species
Ease of <i>ex vivo</i> delivery	Relatively easy through methods such as electroporation and lentiviral transduction	Relatively easy through methods such as electroporation and lentiviral transduction	Relatively easy through methods such as electroporation and lentiviral transduction
Ease of <i>in vivo</i> delivery	Relatively easy due to small size of ZFN expression cassettes, allows use in a variety of viral vectors	Difficult due to the large size of each TALEN and repetitive nature of DNA encoding TALENs, leading to unwanted recombination events	Moderate: The commonly used Cas9 from <i>S. pyogenes</i> may be large enough to impose delivery problems, but other, smaller orthologues exist
Ease of multiplexing	Low	Low	High

Fig. 1

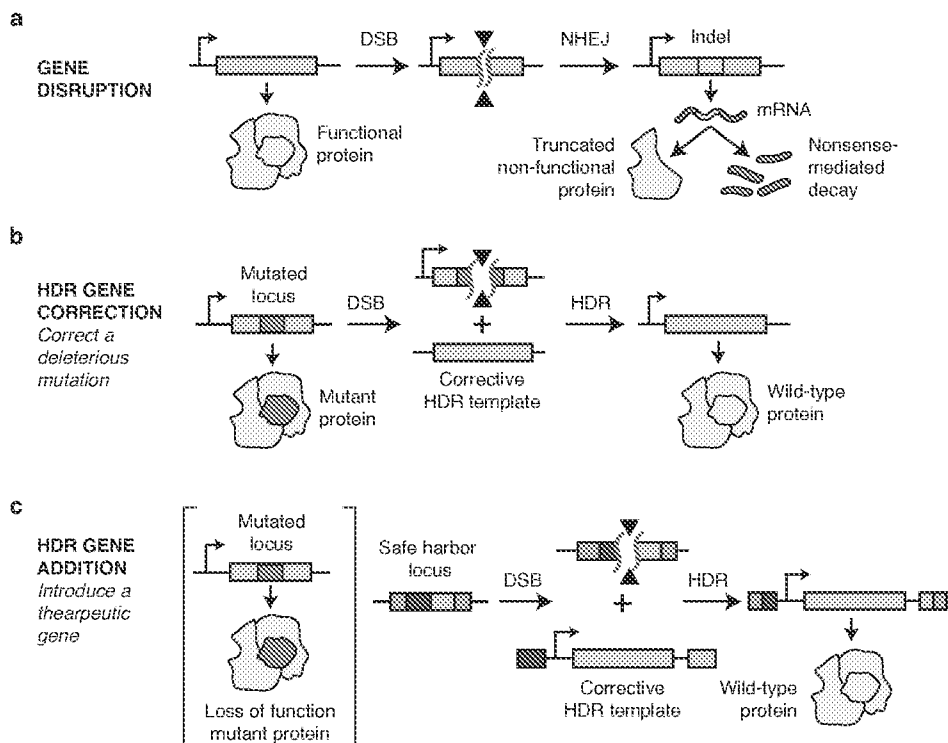


Fig. 2

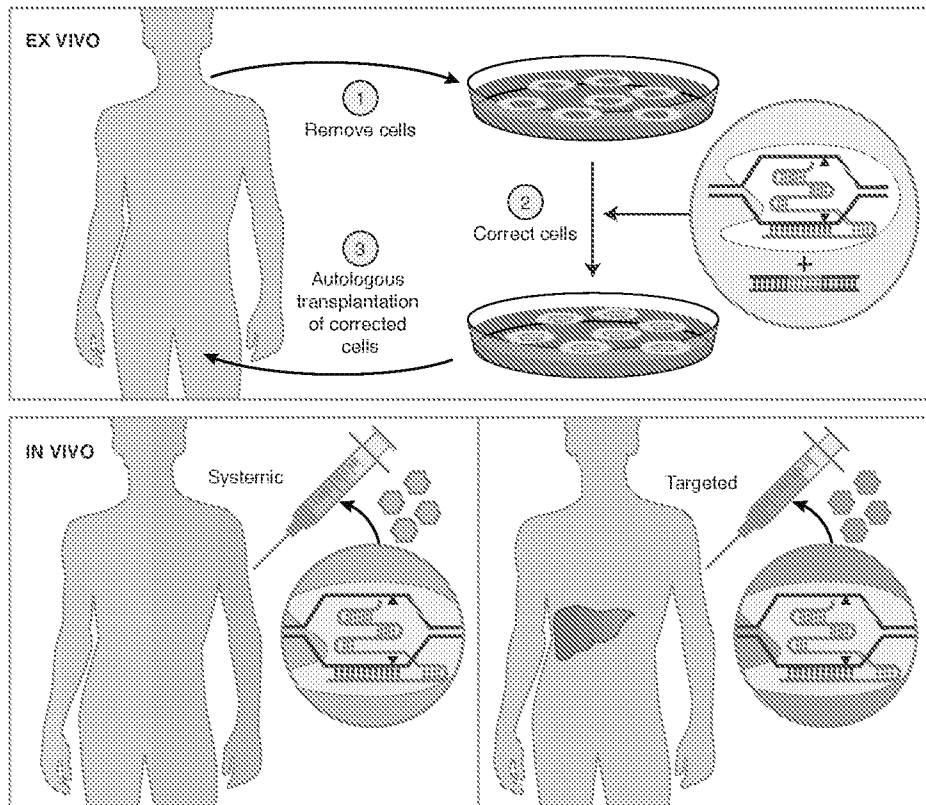


Fig. 3

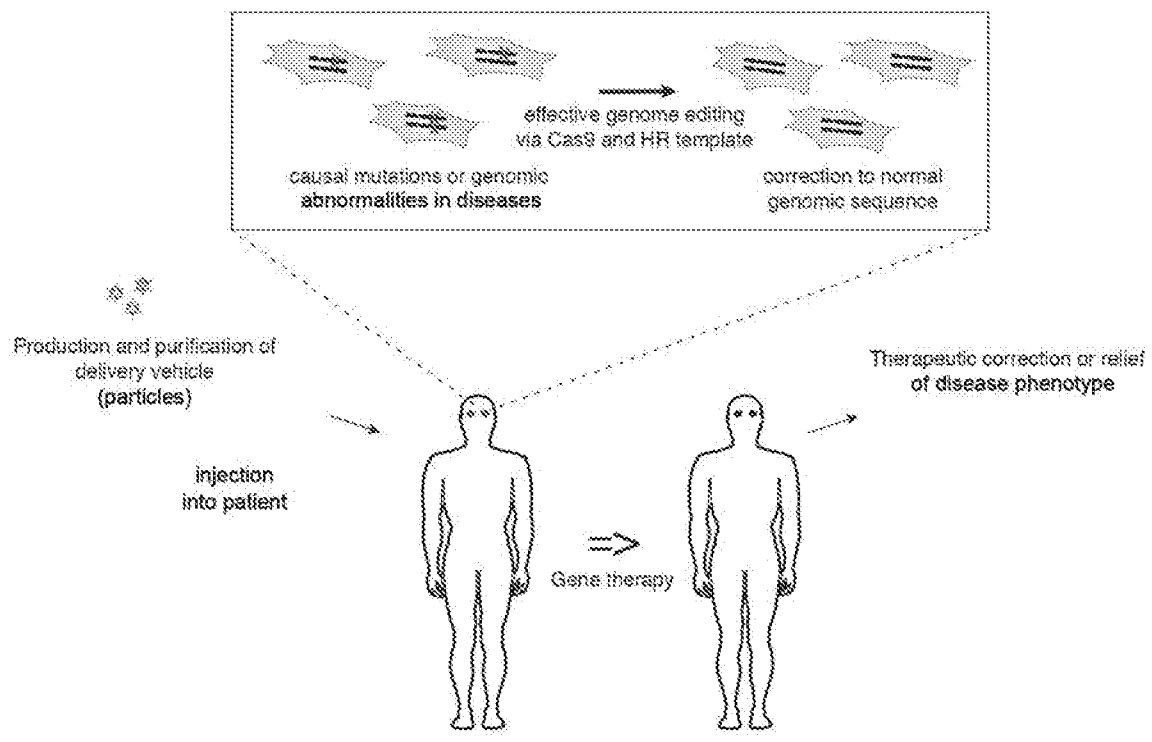
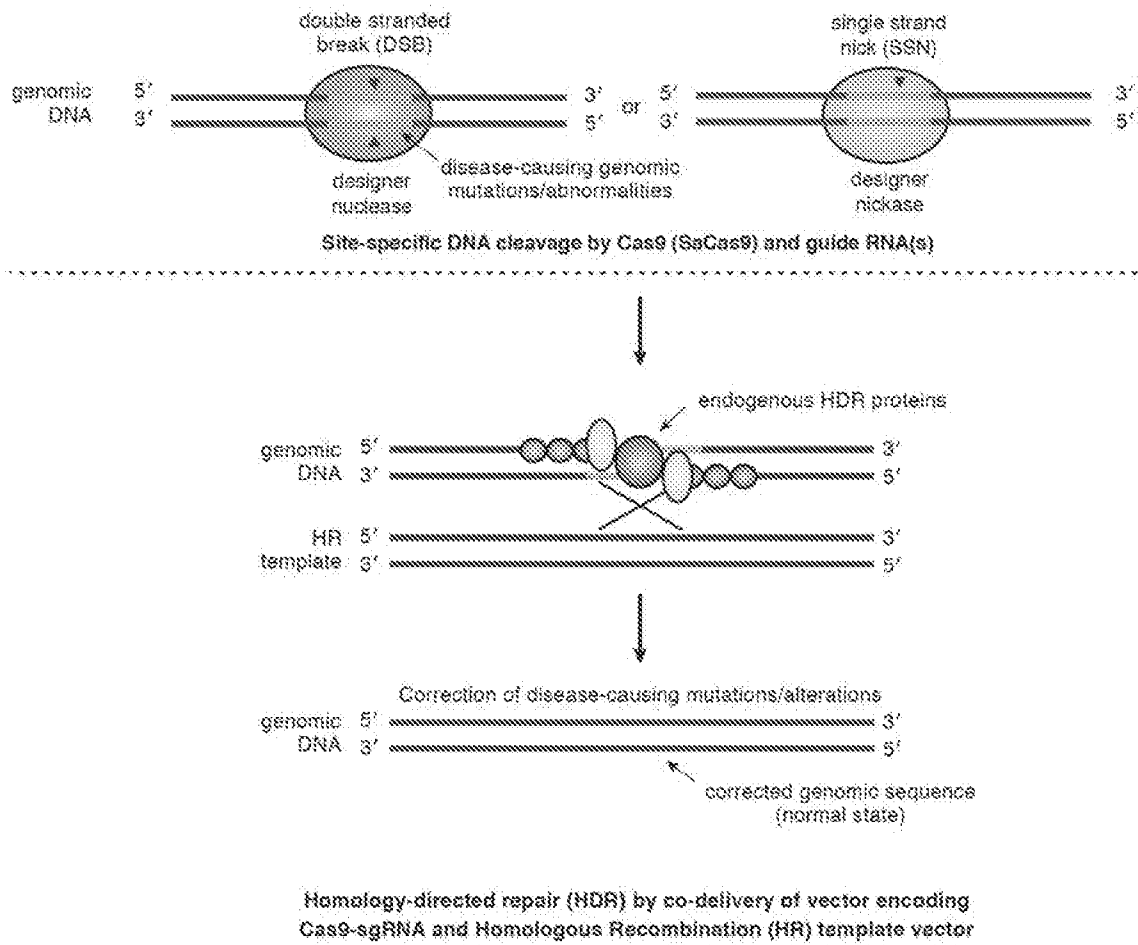


Fig. 4