PROTEIN DELIVERY IN PRIMARY HEMATOPOIETIC CELLS

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Nos. 62/110,187, filed January 30, 2015, and 62/209,711, filed August 25, 2015, the contents of each of which are hereby incorporated by reference in the entirety for all purposes.

BACKGROUND OF THE INVENTION

[0002] Methods, compositions, reaction mixtures, kits, and devices, for precise and efficient manipulation primary cells hold great promise for development of cell-based therapeutics, as well as basic research into the function of various cells, tissues, organs, and systems in the body. For example, recent advances in the generation and use of primary antigen-specific T cells holds great promise for immunotherapy against cancer and infectious diseases. As another example, the ability to precisely target regulatory genes in primary cells can be used to study the phenotypic results of such modulation.

BRIEF SUMMARY OF THE INVENTION

[0003] Disclosed is a method of editing the genome of a cell, wherein the cell is a primary hematopoietic cell or a primary hematopoietic stem cell, the method comprising: a) providing a reaction mixture comprising a Cas9 nuclease domain (*e.g.*, a Cas9 apo protein) and the cell; and b) introducing the Cas9 nuclease domain inside the cell, wherein the Cas9 nuclease domain forms a complex with a guide RNA inside the cell. In one aspect, the present invention provides a method of editing the genome of a cell in vitro or ex vivo, wherein the cell is a primary hematopoietic cell or a primary hematopoietic stem cell, the method comprising: a) providing a reaction mixture comprising a Cas9 ribonucleoprotein complex and the cell, wherein the Cas9 ribonucleoprotein complex comprises a Cas9 nuclease domain and a guide RNA, wherein the guide RNA specifically hybridizes to a target region of the genome of the cell; and b) introducing the Cas9 ribonucleoprotein complex inside the cell. In some

examples, the guide RNA inside the cell is encoded by a guide RNA gene inside the cell, wherein the guide RNA gene comprises DNA. In some embodiments, the cell does not contain a nucleic acid encoding the Cas9 nuclease domain. In some examples, the efficiency of Cas9 delivery is at least about 20% or 30%. In some examples, the primary hematopoietic cell or a primary hematopoietic stem cell is modified to express a heterologous protein either before, during, or after the genome of the cell is edited as described above or elsewhere herein. In some examples, the heterologous protein is

encoded by a viral (e.g., a lentiviral) vector. In some examples, the heterologous protein is a chimeric antigen receptor (CAR) protein or a heterologous T-cell Receptor (TCR), including but not limited to a rearranged TCR.

[0004] In some examples, the present disclosure provides a method of editing the genome of a cell, wherein the cell is a primary hematopoietic cell or a primary hematopoietic stem cell, the method comprising: a) providing a reaction mixture comprising a Cas9 ribonucleoprotein complex and the cell, wherein the Cas9 ribonucleoprotein complex comprises a Cas9 nuclease domain and a guide RNA, wherein the guide RNA specifically hybridizes to a target region of the genome of the cell; and b) introducing the Cas9 ribonucleoprotein complex inside the cell. In another aspect, the present invention provides a method of editing the genome of a cell *in vitro* or *ex vivo*, wherein the cell is a primary hematopoietic cell or a primary hematopoietic stem cell, the method comprising: a) providing a reaction mixture comprising a Cas9 nuclease domain and the cell; and b) introducing the Cas9 nuclease domain inside the cell, wherein the Cas9 nuclease domain forms a complex with a guide RNA inside the cell. In some examplesthe method provides an efficiency of genome editing of at least about 20%. In some examples, the cell does not contain a nucleic acid encoding the Cas9 and/or a DNA nucleic acid encoding a guide RNA.

[0005] In some examples, prior to the providing of a) the cell is not immortalized or transformed. In some cases, after the introducing of b) the cell is not immortalized or transformed. In some examples, the cell has not been passaged prior to the providing of a). In some cases, prior to the providing of a), the cell has been directly isolated from a host organism or tissue and cultured. In some cases, prior to the providing of a), the cell has been directly isolated from a host organism or tissue and has not been cultured.

[0006] In some examples, the introducing comprises electroporation. In some examples, the introducing comprises: coating a nanowire or nanotube with the Cas9 ribonucleoprotein complex or Cas9 apo protein; contacting the cell with the nanowire or nanotube coated with the Cas9 ribonucleoprotein complex or Cas9 apo protein; and piercing a cell membrane of the cell with the nanowire or nanotube coated with the Cas9 ribonucleoprotein complex or Cas9 apo protein. In some examples, the introducing comprises: forcing the reaction mixture through a cell deforming constriction that

is smaller than the diameter of the cell, wherein the forcing introduces transient pores into a cell membrane of the cell; and allowing the Cas9 ribonucleoprotein complex or Cas9 apo protein to enter the cell through the transient pores.

[0007] In some examples, the Cas9 ribonucleoprotein complex or Cas9 apo protein comprises a ligand for an extracellular receptor on the cell, and the introducing comprises receptor mediated internalization of the Cas9 ribonucleoprotein complex or Cas9 apo protein. In some examples, the Cas9 ribonucleoprotein complex or Cas9 apo protein comprises a

cell penetrating peptide, and the introducing comprises contacting the cell penetrating peptide to the cell.

[0008] In some cases, the electroporation comprises positioning the reaction mixture into a chamber between a cathode and an anode, and applying a voltage potential between the cathode and the anode of from about 20 kV/m to about 100 kV/m. In some cases, the voltage potential is applied as a pulse having a length of from about 5 ms to about 100 ms. In some cases, the method further comprises repeating the application of the voltage potential pulse from 2 to 10 times. In some cases, the chamber is a hollow member having a longitudinal length and a horizontal cross sectional area; the chamber comprises a first and second distal end separated by the longitudinal length; and the chamber has: a first electrode at the first distal end; and a reservoir containing an electrolytic solution in fluid communication with the second distal end of the chamber, said reservoir having a second electrode. In some cases, the chamber has a ratio of longitudinal length to horizontal cross-sectional area in the range of 50 to 10,000.

[0009] In some examples, the Cas9 ribonucleoprotein complex or the Cas9 apo protein in the reaction mixture is at a concentration of from about 0.25 μM to about 5 μM. In some examples, the Cas9 ribonucleoprotein complex or the Cas9 apo protein in the reaction mixture is at a concentration of from about 0.9 μM to about 1.8 μM. In some examples, the reaction mixture contains from about 1×10^5 to about 4×10^5 primary hematopoietic cells or primary hematopoietic stem cells or from about 0.9×10^4 to about 3.6×10^4 primary hematopoietic cells or primary hematopoietic stem cells per μL. In some examples, the reaction mixture contains from about 2×10^5 to about 2.5×10^5 primary hematopoietic cells or primary hematopoietic stem cells or 1.8×10^4 to about 2.2×10^4 primary hematopoietic cells or primary hematopoietic stem cells per μL. In some examples, the cell is a primary hematopoietic cell.

[0010] In some cases, the primary hematopoietic cell is an immune cell. In some cases, the immune cell is a T cell. In some cases, the T cell is a regulatory T cell, an effector T cell, or a naïve T cell. In some cases, the regulatory T cell, effector T cell, or naïve T cell is a $CD4^+$ T cell. In some cases, the T cell is a $CD4^+CD25^{hi}CD127^{lo}$ regulatory T cell. In some cases, the T cell is a $FOXP3^+$ T cell. In some cases, the T cell is a $CD4^+CD25^{lo}CD127^{hi}$ effector T cell. In some cases, the T cell is a $CD4^+CD25^{lo}CD127^{hi}CD45RA^{hi}CD45RO^-$ naïve T cell. In some cases, the T cell is a $CD8^+$ T cell. In some cases, the T cell is a $CD4^+CD8^+$ T cell. In

some cases, prior to the providing of a), the T cell is pre-activated. In some cases, prior to the providing of a), the T cell is unstimulated. In some cases, the T cell comprises a recombinant antigen receptor.

[0011] In some examples, the reaction mixture further comprises a double or single-stranded oligonucleotide DNA template, and wherein the method comprises introducing the double or single-stranded oligonucleotide DNA template inside the cell. In some examples, the double or single-stranded oligonucleotide DNA template is at a concentration of from about 9 μM to about 180 μM. In some cases, the double or single-stranded oligonucleotide DNA template is at a concentration of about 45 μM. In some cases, the method provides an efficiency of primary hematopoietic cell (*e.g.*, stimulated or unstimulated T cell) or primary hematopoietic stem cell genome editing (*e.g.*, by nick repair, non-homologous end joining repair, or homology directed repair of Cas9 single or double-stranded cleavage sites) of at least about 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, or 80%. .

[0012] In some cases, the method provides an efficiency of primary hematopoietic cell (*e.g.*, stimulated or unstimulated T cell) or primary hematopoietic stem cell genome editing (*e.g.*, by nick repair, non-homologous end joining repair, or homology directed repair of Cas9 single or double-stranded cleavage sites) of from about 20% to about 80%, from about 25%, to about 70%, from about 30% to about 75%, from about 40% to about 75%, from about 50% to about 70%, from about 20% to about 70%, from about 25% to about 65%, from about 30% to about 60%, or from about 35% to about 55%.

[0013] In some cases, the method provides an efficiency of primary hematopoietic cell (*e.g.*, stimulated or unstimulated T cell) or primary hematopoietic stem cell template directed genome editing of at least about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, or 75%.

[0014] In some cases, the method provides an efficiency of primary hematopoietic cell (*e.g.*, stimulated or unstimulated T cell) or primary hematopoietic stem cell template directed genome editing of from about 5% to about 30%, from about 7% to about 25%, from about 10% to about 20%, from about 5%, to about 25%, from about 10% to about 25%, from about 5% to about 20%, from about 5% to about 15%, or from about 10% to about 15. In some cases, the single stranded oligonucleotide DNA template encodes a recombinant antigen receptor, a portion thereof, or a component thereof.

[0015] In some examples, the cell is a T cell, and the method further comprises: c) after the introducing of b), transferring the reaction mixture to a culture medium containing a CD3 agonist and a CD28 agonist and culturing the cells. In some cases, the CD3 agonist or the CD28 agonist are immobilized on a solid surface, or the CD3 agonist and the CD28 agonist are immobilized on a solid surface (*e.g.*, immobilized on a bead or separate beads or on a surface of a culture plate or well). In some cases, the CD3 agonist is an anti-CD3 antibody. In some cases, the CD28 agonist is an anti-CD28 antibody. In some cases, the method further comprises: c) after the culturing of c), transferring the reaction mixture to a culture medium that does not contain a CD3 agonist or a CD28 agonist and culturing the cells.

[0016] In some cases, the anti-CD3 antibody (*e.g.*, immobilized or soluble) is at a concentration of about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 μg/mL. In some cases, the anti-CD3 antibody (*e.g.*, immobilized or soluble) is at a concentration of from about 0.5 to about 25 μg/mL, from about 1 to about 20 μg/mL, from about 2 to about 15 μg/mL, from about 5 to about 15 μg/mL, or from about 5 to about 10 μg/mL. In some cases, the anti-CD28 antibody (*e.g.*, immobilized or soluble) is at a concentration of about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 μg/mL. In some cases, the anti-CD28 antibody (*e.g.*, immobilized or soluble) is at a concentration of from about 0.5 to about 15 μg/mL, from about 1 to about 15 μg/mL, from about 2 to about 10 μg/mL, from about 1 to about 7.5 μg/mL, or from about 2 to about 5 μg/mL.

[0017] In some examples, the Cas9 ribonucleoprotein complex or Cas9 apo protein comprises a Cas9 nuclease. In some examples, the Cas9 ribonucleoprotein complex or Cas9 apo protein comprises a Cas9 nickase. In some examples, the Cas9 ribonucleoprotein complex or Cas9 apo protein comprises a Cas9 nuclease domain fused to a restriction endonuclease or nickase. In some examples, the Cas9 ribonucleoprotein

complex or Cas9 apo protein comprises a Cas9 nuclease domain fused to a transcriptional modulator or a chromatin modifier.

[0018] In some examples, the reaction mixture comprises at least two structurally different Cas9 ribonucleoprotein complexes or at least two structurally different Cas9 apo proteins. In some cases, the at least two structurally different Cas9 ribonucleoprotein complexes contain structurally different sgRNAs. In some cases, the at least two structurally different Cas9 ribonucleoprotein complexes or at least two different Cas9 apo proteins contain structurally different Cas9 domains.

[0019] In yet another aspect, the present invention provides a plurality of primary hematopoietic cells or primary hematopoietic stem cells, wherein the plurality of cells do not contain a nucleic acid encoding Cas9 and/or a DNA nucleic acid encoding a guide RNA, and wherein at least 20% of the plurality of cells contains a Cas9 ribonucleoprotein complex. In some examples, at least 30% of the plurality of cells contains a Cas9 ribonucleoprotein complex. In some examples, at least 20% of the plurality of cells contains a Cas9 ribonucleoprotein complex and a single stranded oligonucleotide DNA template. In some examples, the plurality of cells have not enriched for the presence of the Cas9 ribonucleoprotein complex. In some examples, at least 20% or 30% of the plurality of cells contains a double stranded break, or an NHEJ or HDR repaired double stranded break at a target genomic region. In some examples, the primary hematopoietic cells or a primary hematopoietic stem cells are modified to express a heterologous protein (e.g., a chimeric antigen receptor (CAR)) either before, during, or after the genome of the cell is edited as described above or elsewhere herein. The heterologous protein can be encodes by a viral vector (e.g., a lentiviral vector) introduced into the cells.

DEFINITIONS

[0020] As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

[0021] The term “nucleic acid” or “polynucleotide” refers to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless

WHAT IS CLAIMED IS:

1. A method of editing the genome of a cell *in vitro* or *ex vivo*, wherein the cell is a primary hematopoietic cell or a primary hematopoietic stem cell, the method comprising:

a) providing a reaction mixture comprising a Cas9 ribonucleoprotein complex and the cell, wherein the Cas9 ribonucleoprotein complex comprises a Cas9 nuclease domain and a guide RNA, wherein the guide RNA specifically hybridizes to a target region of the genome of the cell; and

b) introducing the Cas9 ribonucleoprotein complex inside the cell.

2. The method of claim 1, wherein the method provides an efficiency of genome editing of at least about 20%.

3. The method of claim 1, wherein prior to the providing of a) the cell is not immortalized or transformed, and wherein after the introducing of b) the cell is not immortalized or transformed.

4. The method of claim 1, wherein the introducing comprises electroporation.

5. The method of claim 1, wherein the introducing comprises:

coating a nanowire or nanotube with the Cas9 ribonucleoprotein complex;

contacting the cell with the nanowire or nanotube coated with the Cas9 ribonucleoprotein complex; and

piercing a cell membrane of the cell with the nanowire or nanotube coated with the Cas9 ribonucleoprotein complex.

6. The method of claim 1, wherein the introducing comprises:

forcing the reaction mixture through a cell deforming constriction that is smaller than the diameter of the cell, wherein the forcing introduces transient pores into a cell membrane of the cell; and

allowing the Cas9 ribonucleoprotein complex to enter the cell through the transient pores.

7. The method of claim 1, wherein the Cas9 ribonucleoprotein complex comprises a ligand for an extracellular receptor on the cell, and the introducing comprises receptor mediated internalization of the Cas9 ribonucleoprotein complex.

8. The method of claim 1, wherein the Cas9 ribonucleoprotein complex comprises a cell penetrating peptide, and the introducing comprises contacting the cell penetrating peptide to the cell.

9. The method of claim4, wherein the electroporation comprises positioning the reaction mixture into a chamber between a cathode and an anode, applying a voltage potential between the cathode and the anode of from about 20 kV/m to about 100 kV/m, and repeating the application of the voltage potential pulse from 2 to10 times, wherein the voltage potential is applied as a pulse having a length of from about 5 ms to about 100 ms.

10. The method of claim 1, wherein the Cas9 ribonucleoprotein complex in the reaction mixture is at a concentration of from about 0.25 μM to about 5 μM.

11. The method of claim 1, wherein the Cas9 ribonucleoprotein complex in the reaction mixture is at a concentration of from about 0.9 μM to about 1.8 μM.

12. The method of claim 1, wherein the reaction mixture contains from about 1×10^5 to about 4×10^5 primary hematopoietic cells or primary hematopoietic stem cells.

13. The method of claim 1, wherein the reaction mixture contains from about 2×10^5 to about 2.5×10^5 primary hematopoietic cells or primary hematopoietic stem cells.

14. The method of claim 1, wherein the cell is a primary hematopoietic cell, and the primary hematopoietic cell is an immune cell.

15. The method of claim 14, wherein the immune cell is a T cell, and wherein the T cell comprises a recombinant antigen receptor.

16. The method of claim 15, wherein the T cell is a regulatory T cell, an effector T cell, or a naïve T cell.

17. The method of claim 16, wherein the regulatory T cell, effector T cell, or naïve T cell is a $CD4^+$ T cell, or a $CD8^+$ T cell.

18. The method of claim 15, wherein the T cell is selected from the group consisting of a $CD4^+CD25^{hi}CD127^{lo}$ regulatory T cell, $FOXP3^+$ T cell, $CD4^+CD25^{lo}CD127^{hi}$ effector T cell, and $CD4^+CD25^{lo}CD127^{hi}CD45RA^{hi}CD45RO^-$ naïve T cell.

19. The method of claim 1, wherein the reaction mixture further comprises a single-stranded oligonucleotide DNA template, and wherein the method comprises introducing the single-stranded oligonucleotide DNA template inside the cell, wherein the single-stranded oligonucleotide DNA template is at a concentration of from about 9 μM to about 180 μM.

20. The method of claim 19, wherein the single-stranded oligonucleotide DNA template is at a concentration of about 45 μM.

21. The method of claim 19, wherein the single stranded oligonucleotide DNA template encodes a recombinant antigen receptor, a portion thereof, or a component thereof.

22. The method of claim 1, wherein the cell is a T cell, and the method further comprises:

c) after the introducing of b), transferring the reaction mixture to a culture medium containing a CD3 agonist and a CD28 agonist and culturing the cells.

23. The method of claim22, wherein the method further comprises:

c) after the culturing of c), transferring the reaction mixture to a culture medium that does not contain a CD3 agonist or a CD28 agonist and culturing the cells.

24. The method of claim 1, wherein the Cas9 ribonucleoprotein complex comprises a Cas9 nuclease or a Cas9 nickase.

25. The method of claim 1, wherein the Cas9 ribonucleoprotein complex comprises a Cas9 nuclease domain fused to a restriction endonuclease or nickase.

26. The method of claim 1, wherein the Cas9 ribonucleoprotein complex comprises a Cas9 nuclease domain fused to a transcriptional modulator or a chromatin modifier.

27. The method of claim 1, wherein the reaction mixture comprises at least two structurally different Cas9 ribnucleoprotein complexes.

28. A method of editing the genome of a cell *in vitro* or *ex vivo*, wherein the cell is a primary hematopoietic cell or a primary hematopoietic stem cell, the method comprising:

a) providing a reaction mixture comprising a Cas9 nuclease domain and the cell; and

b) introducing the Cas9 nuclease domain inside the cell,

wherein the Cas9 nuclease domain forms a complex with a guide RNA inside the cell.

29. A plurality of primary hematopoietic cells or primary hematopoietic stem cells, wherein the plurality of cells do not contain a nucleic acid encoding Cas9 and/or a DNA nucleic acid encoding a guide RNA, and wherein at least 20% of the plurality of cells contains a Cas9 ribonucleoprotein complex.