attributes for cell therapy applications. As demonstrated in the examples below, primary cells edited in accordance with the processes provided herein achieved higher double stranded break (DSB) formation, higher combined homology direct repair (HR) and non-homologous end joining (NHEJ) frequency, a higher frequency of biallelic and monoallelic HR events, improved cell survival, proliferative capability, and cell fitness post gene editing, as well as shortened manufacturing time. Moreover, the processes provided herein required substantially lower levels of viral DNA donor vector needed to achieve levels of HR-mediated DNA integration observed with previous methods, which mitigates DDR otherwise resulting from viral capsid burden and may improve the safety and efficacy of gene-edited cellular drug products.

[0007] Thus in one aspect, provided herein is a process for genetically modifying a plurality of primary cells comprising: (a) isolating the plurality of primary cells from a subject; (b) culturing the plurality of primary cells in a cell culture device, wherein the cell culture device comprises culture media and a gas-permeable membrane that mediates passive diffusion of oxygen and carbon dioxide to cells residing on the bottom surface of the cell culture device; and (c) contacting the plurality of primary cells with one or more components of a gene-editing system, wherein the contacting comprises electroporation of the plurality of stem cells with the one or more components of the gene-editing system in a closed flow-through electroporation chamber having a capacity of at least 2 milliliters. In some examples, the process further comprises culturing the plurality of primary cells after electroporation in a cell culture device, wherein the cell culture device comprises culture media and a gas-permeable membrane that mediates passive diffusion of oxygen and carbon dioxide to cells residing on the bottom surface of the cell culture device.

[0008] In some examples, the cell culture device is a closed gas-permeable bioreactor, wherein media can be collected or exchanged through media lines integrated into the bioreactor. In some examples, the gas-permeable membrane is a silicone membrane. In some examples, the gas-permeable membrane ranges from about 1 cm^2 to about 1000 cm^2 in size. In some examples, the cell culture device comprises a media volume of about 5 ml to 10,000 ml. In some examples, the cell culture device is a G-REX bioreactor. In some examples, the G-REX bioreactor is selected from the group consisting of a G-REX 10M-CS, 100M-CS, and 500M-CS bioreactor. In some examples, the primary cells are cultured in the cell culture device for about 3 days prior to electroporation and for about 1 day after electroporation. In some examples, the primary cells are cultured in the cell culture device for about 2 days prior to

electroporation and for about 2 days after electroporation. In some examples, the primary cells are cultured in the gas-permeable cell culture device for about 2 days prior to electroporation and for about 1 day after electroporation. In some examples, the primary cells are cultured within the gas-permeable cell culture device at 37°C, 5%CO2, 5%O2 and 0% relative humidity. In some examples, the primary cells are cryopreserved and/or formulated as drug product following the cell culturing after electroporation.

[0009] In some examples, the closed flow-through electroporation chamber has a capacity of at least 3 milliliters. In some examples, the primary cells are electroporated in an electroporation chamber having a capacity range of 1 ml to 3.5 ml. In some examples, the primary cells are electroporated in an electroporation chamber having a capacity range of 5 ml to 20 ml. In some examples, the primary cells are electroporated in an electroporation chamber having a capacity range of 10 ml to 100 ml. In some examples, the electroporation is performed on a MaxCyte ExPERT GTx electroporator. In some examples, the closed flow-through electroporation chamber is a MaxCyte CL-2 electroporation cartridge. In some examples, the closed flow-through electroporation chamber is a MaxCyte CL-1.1 electroporation cartridge. In some examples, the closed flow-through electroporation chamber is a MaxCyte R-20K electroporation cartridge. In some examples, the primary cells are electroporated in a solution comprising a cell density of 25x10^6 to 100x10^6 cells/ml.

[0010] In some examples, the plurality of primary cells comprises primary blood cells, primary mesenchymal cells, or a combination thereof. In some examples, the plurality of primary cells comprises primary stem cells, primary progenitor cells, or primary somatic cells. In some examples, the stem cells are selected from the group consisting of embryonic stem cells, induced pluripotent stem cells, hematopoietic stem cells, mesenchymal stem cells, neural stem cells, and organ stem cells. In some examples, the progenitor cells are selected from the group consisting of hematopoietic progenitor cells, myeloid progenitor cells, lymphoid progenitor cells, multipotent progenitor cells, oligopotent progenitor cells, and lineage-restricted progenitor cells. In some examples, the somatic cells are selected from the group consisting of fibroblasts, hepatocytes, heart cells, liver cells, pancreatic cells, muscle cells, skin cells, blood cells, neural cells, and immune cells. In some examples, the immune cells are selected from the group consisting of T lymphocytes (T cells), B lymphocytes (B cells), small lymphocytes, natural killer cells (NK cells), natural killer T cells, macrophages, monocytes, monocyte-precursor cells,

eosinophils, neutrophils, basophils, megakaryocytes, myeloblasts, mast cells and dendritic cells. In some examples, the plurality of primary cells comprises a heterogeneous population of primary cells.

[0011] In some examples, the one or more components of the gene editing system is selected from the group consisting of: (i) a CRISPR/Cas guide RNA, (ii) a DNA molecule encoding a CRISPR/Cas guide RNA, (iii) a nucleic acid molecule encoding a CRISPR/Cas RNA-guided polypeptide, (iv) a CRISPR/Cas RNA-guided polypeptide, (v) a CRISPR/Cas guide RNA complexed with a CRISPR/Cas RNA-guided polypeptide, (vi) a nucleic acid molecule encoding a zinc finger protein (ZFP), (vii) a ZFP, (viii) a nucleic acid molecule encoding a transcription activator-like effector (TALE) protein, (ix) a TALE protein, and (x) a DNA donor polynucleotide. In some examples, the CRISPR/Cas RNA-guided polypeptide is a base editor or a prime editor.

[0012] In some examples, the one or more components of the gene editing system comprises a nuclease capable of generating a double-strand break within a gene locus of a cell. In some examples, the one or more components of the gene editing system further comprises a DNA donor polynucleotide. In some examples, the DNA donor polynucleotide comprises non-overlapping 5′ and 3′ homology arms, wherein each homology arm is homologous to a portion of the gene locus, whereupon generation of the double-strand break within the gene locus by the nuclease, the donor polynucleotide sequence is integrated into the gene locus by homology directed repair (HDR).

[0013] In some examples, the gene editing system comprises a CRISPR nuclease and a single guide RNA (sgRNA) capable of hybridizing to a target sequence within the gene locus, wherein the sgRNA guides the CRISPR nuclease to the target sequence. In some examples, the CRISPR nuclease is a Cas protein. In some examples, the Cas protein is Cas9 or a high-fidelity variant thereof. In some examples, the sgRNA and the CRISPR nuclease are formed in a ribonucleoprotein (RNP) complex. In some examples, the sgRNA comprises one or more chemically modified nucleotides. In some examples, the modified nucleotide is selected from the group consisting of: a 2'-O-methyl nucleotide, a 2′-O-methyl 3′-phosphorothioate nucleotide, and a 2'-O-methyl 3'-thioPACE nucleotide. In some examples, a 5′ end, a 3′ end, or a combination thereof of the modified sgRNA comprises a modified nucleotide.

[0014] In some examples, the donor polynucleotide is comprised in a viral vector, a plasmid, or a single-stranded oligodeoxynucleotide (ssODN). In some examples, the donor

polynucleotide is comprised in a recombinant adeno-associated viral (AAV) vector. In some examples, the AAV vector is selected from the group consisting of AAV1, AAV2, AAV3, AAV4, AAV3, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, and AAV12. In some examples, the AAV vector is an AAV6 vector. In some examples, the AAV vector is transduced at a multiplicity of infection (MOI) of 2500 or less. In some examples, the AAV vector is transduced at an MOI of 1250 or less. In some examples, the AAV vector is transduced at an MOI of 625 or less.

[0015] In some examples, the plurality of primary cells comprises CD34+ hematopoietic stem and progenitor cells (HSPCs). In some examples, the HSPCs are gene-edited at the HBB locus. In some examples, the subject is a mammal. In some examples, the mammal is a human. In some examples, the gene-editing targets a gene locus of the primary cells that comprises one or more mutations associated with a disease or encodes an aberrant protein. In some examples, integration of a donor polynucleotide sequence into the target gene locus is capable of correcting a mutation in the primary cell that is associated with a disease. In some examples, the disease is selected from the group consisting of a hemoglobinopathy, a viral infection, X-linked severe combined immune deficiency, Fanconi anemia, hemophilia, neoplasia, cancer, alpha-1 antitrypsin deficiency, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, cystic fibrosis, blood diseases and disorders, inflammation, immune system diseases or disorders, metabolic diseases, liver diseases and disorders, kidney diseases and disorders, muscular diseases and disorders, bone or cartilage diseases and disorders, neurological and neuronal diseases and disorders, cardiovascular diseases and disorders, pulmonary diseases and disorders, and lysosomal storage disorders. In some examples, the hemoglobinopathy is sickle cell disease, α- thalassemia, β-thalassemia, or δ-thalassemia. In some examples, integration of a donor polynucleotide sequence is capable of replacing a mutant allele in the primary cell with a wild-type allele. In some examples, the process further comprises administering gene-edited primary cells to a patient in need thereof. In some examples, the gene-edited primary cells are administered back into the subject.

[0016] In another aspect, provided herein are genetically modified primary cells generated by the processes comprising cell culturing and high-volume electroporation methods described herein.

[0017] In another aspect, provided herein is the plurality of genetically modified primary cells as disclosed herein for use in a cell therapy. In one example, the present disclosure refers to a method of cell therapy comprising administering a plurality of genetically modified primary cells generated by the processes provided herein to a subject in need thereof. In some examples, the cell therapy is an autologous cell therapy. In some examples, the cell therapy is an allogenic cell therapy.

[0017a] In another aspect, provided herein is use of the plurality of genetically modified primary cells as disclosed herein in the manufacture of a medicament for treating a disease, wherein the disease is selected from the group consisting of a hemoglobinopathy, a viral infection, X-linked severe combined immune deficiency, Fanconi anemia, hemophilia, neoplasia, cancer, alpha-1 antitrypsin deficiency, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, cystic fibrosis, blood diseases and disorders, inflammation, immune system diseases or disorders, metabolic diseases, liver diseases and disorders, kidney diseases and disorders, muscular diseases and disorders, bone or cartilage diseases and disorders, neurological and neuronal diseases and disorders, cardiovascular diseases and disorders, pulmonary diseases and disorders, and lysosomal storage disorders.

BRIEF DESCRIPTION OF THE FIGURES

[0018] **FIGURE 1** provides a comparison of a previous process workflow for manufacturing gene-edited cells ("V1.0 Process") with a process workflow of the present disclosure ("V1.1 Process"). With V1.1, transition to a closed single use system mitigates risks associated with scaled up aseptic manipulations during clinical manufacturing. Closed transfers and larger electroporation volume minimizes cell losses and variability of gene editing efficiency.

[0019] **FIGURE 2** provides allele distribution following CRISPR-mediated gene editing (with AAV DNA donor at 2500 MOI) using the V1.0 process with either low-volume electroporation conditions (1 ml electroporation cartridge capacity; Lonza electroporator (LZ)) or high-volume electroporation conditions (3 ml electroporation cartridge capacity; MaxCyte GTx electroporator (MX)) programs. The frequency of corrective events is reported as the %HR, the frequency of mutations in the HBB locus is reported as the %INDELS, and the frequency of unmodified events is reported as the %WT. (A) Distribution of alleles in gene-edited HSCs manufactured using either low-volume (LZ) or high-volume (MX) electroporation conditions on 3 individual donor cells that were split for a side-by-side comparability at a scale of ≥100E+06 cells edited per condition. (B) Mean allele distribution suggests a high probability of achieving ~80% DSB (Sum of HR+INDEL) for improved nuclear delivery of CRISPR/Cas9 reagents and improved targeting efficiency.

[0020] **FIGURE 3** provides zygosity of editing outcomes in progeny from cells plated for a colony-forming unit assay (CFU). Corrective events are denoted by the different shades of green. HR/HR represents biallelic correction while HR/INDEL, HR/WT and HR/SNP are monoallelic and may contribute to curative outcomes in a sickle cell disease patient.

[0021] **FIGURE 4** provides the frequency of off-target INDELS at OT-1 when editing with a CRISPR RNP complex targeting the HBB gene under low-volume (LZ) or high-volume (MX) electroporation conditions. The percentage of mutations at OT-1 are reported as % INDELs at OT-1.

What is claimed:

1. A process for genetically modifying a plurality of primary cells comprising:
    (a) isolating the plurality of primary cells from a subject;
    (b) culturing the plurality of primary cells in a cell culture device, wherein the cell culture device comprises culture media and a gas-permeable membrane that mediates passive diffusion of oxygen and carbon dioxide to cells residing on the bottom surface of the cell culture device; and
    (c) contacting the plurality of primary cells with one or more components of a gene-editing system, wherein the contacting comprises electroporation of the plurality of stem cells with the one or more components of the gene-editing system in a closed flow-through electroporation chamber having a capacity of at least 2 milliliters.

2. The process of claim 1, further comprising culturing the plurality of primary cells after electroporation in a cell culture device, wherein the cell culture device comprises culture media and a gas-permeable membrane that mediates passive diffusion of oxygen and carbon dioxide to cells residing on the bottom surface of the cell culture device.

3. The process of claim 1 or 2, wherein the cell culture device is a closed gas-permeable bioreactor, wherein media can be collected or exchanged through media lines integrated into the bioreactor.

4. The process of any one of claims 1 to 3, wherein the gas-permeable membrane is a silicone membrane.

5. The process of any one of claims 1 to 4, wherein the gas-permeable membrane ranges from about 1 cm^2 to about 1000 cm^2 in size.

6. The process of any one of claims 1 to 4, wherein the cell culture device comprises a media volume of about 5 ml to 10,000 ml.

7. The process of any one of claims 1 to 6, wherein the cell culture device is a G-REX bioreactor selected from the group consisting of a 10M-CS, 100M-CS, and 500M-CS bioreactor.

8. The process of any one of claims 1 to 7, wherein the cell culture device is selected from the group consisting of G-REX 6M Well Plate, G-REX 6 well plate, G-Rex 24-well Plate, G-REX 10M Open system, G-REX 100 Open System, G-REX 100M Open System, G-REX100M Open System, AND G-Rex 500M Open System.

9. The process of claim 2, wherein the primary cells are cultured in the cell culture device for about 3 days prior to electroporation and for about 1 day after electroporation.

10. The process of claim 2, wherein the primary cells are cultured in the cell culture device for about 2 days prior to electroporation and for about 2 days after electroporation.

11. The process of claim 2, wherein the primary cells are cultured in the cell culture device for about 2 days prior to electroporation and for about 1 day after electroporation.

12. The process of any one of claims 1 to 11, wherein the primary cells are cryopreserved and/or formulated as drug product following the cell culturing after electroporation.

13. The process of any one of claims 1 to 12, wherein the primary cells are cultured within the gas-permeable cell culture device at 37°C, 5%CO2, 5%O2 and 0% relative humidity.

14. The process of any one of claims 1 to 13, wherein the closed flow-through electroporation chamber has a capacity of at least 3 milliliters.

15. The process of any one of claims 1 to 14, wherein the primary cells are electroporated in an electroporation chamber having a capacity range of 1 ml to 3.5 ml.

16. The process of any one of claims 1 to 14, wherein the primary cells are electroporated in an electroporation chamber having a capacity range of 5 ml to 20 ml.

17. The process of any one of claims 1 to 14, wherein the primary cells are electroporated in an electroporation chamber having a capacity range of 10 ml to 100 ml.

18. The process of any one of claims 1 to 17, wherein the electroporation is performed on a MaxCyte ExPERT GTx electroporator.

19. The process of claim 18, wherein the closed flow-through electroporation chamber is a MaxCyte CL-2 electroporation cartridge.

20. The process of claim 18, wherein the closed flow-through electroporation chamber is a MaxCyte R-20K electroporation cartridge.

21. The process of claim 18, wherein the closed flow-through electroporation chamber is a MaxCyte CL1.1 electroporation cartridge.

22. The process of any one of claims 1 to 21, wherein the primary cells are electroporated in a solution comprising a cell density of 25x10^6 to 100x10^6 cells/ml.

23. The process of any one of claims 1 to 22, wherein the plurality of primary cells comprises primary blood cells, primary mesenchymal cells, or a combination thereof.

24. The process of any one of claims 1 to 22, wherein the plurality of primary cells comprises primary stem cells, primary progenitor cells, or primary somatic cells.

25. The process of claim 24, wherein the stem cells are selected from the group consisting of embryonic stem cells, induced pluripotent stem cells, hematopoietic stem cells, mesenchymal stem cells, neural stem cells, and organ stem cells.

26. The process of claim 24, wherein the progenitor cells are selected from the group consisting of hematopoietic progenitor cells, myeloid progenitor cells, lymphoid progenitor cells, multipotent progenitor cells, oligopotent progenitor cells, and lineage-restricted progenitor cells.

27. The process of claim 24, wherein the somatic cells are selected from the group consisting of fibroblasts, hepatocytes, heart cells, liver cells, pancreatic cells, muscle cells, skin cells, blood cells, neural cells, and immune cells.

28. The process of claim 27, wherein the immune cells are selected from the group consisting of T lymphocytes (T cells), B lymphocytes (B cells), small lymphocytes, natural killer cells (NK cells), natural killer T cells, macrophages, monocytes, monocyte-precursor cells, eosinophils, neutrophils, basophils, megakaryocytes, myeloblasts, mast cells and dendritic cells.

29. The process of any one of claims 1 to 28, wherein the plurality of primary cells comprises a heterogeneous population of primary cells.

30. The process of any one of claims 1 to 29, wherein the one or more components of the gene editing system is selected from the group consisting of: (i) a CRISPR/Cas guide RNA, (ii) a DNA molecule encoding a CRISPR/Cas guide RNA, (iii) a nucleic acid molecule encoding a CRISPR/Cas RNA-guided polypeptide, (iv) a CRISPR/Cas RNA-guided polypeptide, (v) a CRISPR/Cas guide RNA complexed with a CRISPR/Cas RNA-guided polypeptide, (vi) a nucleic acid molecule encoding a zinc finger protein (ZFP), (vii) a ZFP, (viii) a nucleic acid molecule encoding a transcription activator-like effector (TALE) protein, (ix) a TALE protein, and (x) a DNA donor polynucleotide.

31. The process of claim 30, wherein the CRISPR/Cas RNA-guided polypeptide is a base editor or a prime editor.

32. The process of any one of claims 1 to 30, wherein the one or more components of the gene editing system comprises a nuclease capable of generating a double-strand break within a gene locus of a cell.

33. The process of claim 32, wherein the one or more components of the gene editing system further comprises a DNA donor polynucleotide.

34. The process of claim 33, wherein the DNA donor polynucleotide comprises non-overlapping 5′ and 3′ homology arms, wherein each homology arm is homologous to a portion of the gene locus, whereupon generation of the double-strand break within the gene locus by the nuclease, the donor polynucleotide sequence is integrated into the gene locus by homology directed repair (HDR).

35. The process of any one of claims 1 to 34, wherein the gene editing system comprises a CRISPR nuclease and a single guide RNA (sgRNA) capable of hybridizing to a target sequence within the gene locus, wherein the sgRNA guides the CRISPR nuclease to the target sequence.

36. The process of any one of claims 30 to 34 wherein the CRISPR nuclease is a Cas protein.

37. The process of claim 36, wherein the Cas protein is Cas9 or a high-fidelity variant thereof.

38. The process of any of claims 35to 37, wherein the sgRNA and the CRISPR nuclease are formed in a ribonucleoprotein (RNP) complex.

39. The process of any one of claims 35 to 38, wherein the sgRNA comprises one or more chemically modified nucleotides.

40. The process of claim 39, wherein the modified nucleotide is selected from the group consisting of: a 2'-O-methyl nucleotide, a 2′-O-methyl 3′-phosphorothioate nucleotide, and a 2'-O-methyl 3'-thioPACE nucleotide.

41. The process of claim 39 or 40, wherein a 5′ end, a 3′ end, or a combination thereof of the modified sgRNA comprises a modified nucleotide.

42. The process of any one of claims 33 to 41, wherein the donor polynucleotide is comprised in a viral vector, a plasmid, or a single-stranded oligodeoxynucleotide (ssODN).

43. The process of claim 42, wherein the donor polynucleotide is comprised in an adeno-associated viral (AAV) vector.

44. The process of claim 43, wherein the AAV vector is selected from the group consisting of AAV1, AAV2, AAV3, AAV4, AAV3, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, and AAV12.

45. The process of claim 43, wherein the AAV vector is an AAV6 vector.

46. The process of any one of claims 43 to 45, wherein the AAV vector is transduced at an MOI of 2500 or less.

47. The process of any one of claims 43 to 45, wherein the AAV vector is transduced at an MOI of 1250 or less.

48. The process of any one of claims 43 to 45, wherein the AAV vector is transduced at an MOI of 625 or less.

49. The process of any one of claims 1 to 48, wherein the plurality of primary cells comprises CD34+ hematopoietic stem and progenitor cells (HSPCs)

50. The process of claim 49, wherein the HSPCs are gene-edited at the HBB locus.

51. The process of any one of claims 1 to 50, wherein the subject is a mammal.

52. The process of claim 41, wherein the mammal is a human.

53. The process of any one of claims 1 to 52, wherein the gene-editing targets a gene locus of the primary cells that comprises one or more mutations associated with a disease or encodes an aberrant protein.

54. The process of claim 53, wherein integration of a donor polynucleotide sequence into the target gene locus is capable of correcting a mutation in the primary cell that is associated with a disease.

55. The process of claim 54, wherein the disease is selected from the group consisting of a hemoglobinopathy, a viral infection, X-linked severe combined immune deficiency, Fanconi anemia, hemophilia, neoplasia, cancer, alpha-1 antitrypsin deficiency, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, cystic fibrosis, blood diseases and disorders, inflammation, immune system diseases or disorders, metabolic diseases, liver diseases and disorders, kidney diseases and disorders, muscular diseases and disorders, bone or cartilage

diseases and disorders, neurological and neuronal diseases and disorders, cardiovascular diseases and disorders, pulmonary diseases and disorders, and lysosomal storage disorders.

56. The process of claim 55, wherein the hemoglobinopathy is sickle cell disease, α-thalassemia, β-thalassemia, or δ-thalassemia.

57. The process of claim 53, wherein integration of a donor polynucleotide sequence is capable of replacing a mutant allele in the primary cell with a wild-type allele.

58. The process of any one of claims 1 to 57, further comprising the gene-edited primary cells to be administered to a patient in need thereof.

59. The process of claim 58, wherein the gene-edited primary cells are to be administered back into the subject.

60. A plurality of genetically modified primary cells generated by the process of any one of claims 1 to 59.

61. The plurality of genetically modified primary cells of claim 60 for use in a cell therapy.

62. The plurality of genetically modified primary cells of claim 61, wherein the cell therapy is an autologous cell therapy.

63. The plurality of genetically modified primary cells of claim 61, wherein the cell therapy is an allogenic cell therapy.

64. Use of the plurality of genetically modified primary cells of claim 61 in the manufacture of a medicament for treating a disease, wherein the disease is selected from the group consisting of a hemoglobinopathy, a viral infection, X-linked severe combined immune deficiency, Fanconi anemia, hemophilia, neoplasia, cancer, alpha-1 antitrypsin deficiency, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, cystic fibrosis, blood diseases and disorders, inflammation, immune system diseases or disorders, metabolic diseases, liver diseases and disorders, kidney diseases and disorders, muscular diseases and disorders, bone or cartilage

diseases and disorders, neurological and neuronal diseases and disorders, cardiovascular diseases and disorders, pulmonary diseases and disorders, and lysosomal storage disorders.