

METHODS FOR CLINICAL-SCALE PRODUCTION OF GENETICALLY MODIFIED PRIMARY CELLS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of, and priority to, U.S. provisional patent application Ser. No. 63/505,647, filed June 1, 2023, which is hereby incorporated by reference herein in its entirety.

FIELD

[0002] Provided herein are processes useful for genetic modification of primary cells which utilize large scale cell culture and electroporation techniques to improve gene editing outcomes and cell viability.

BACKGROUND

[0003] The CRISPR-Cas9 RNA-guided nuclease system has proven to be a versatile and effective tool for gene editing primary cells *ex vivo* via the NHEJ (non-homologous end joining) repair pathway, with promising outcomes in the clinic where the edited primary cells are administered to a patient as part of a treatment regimen. However, genome editing strategies that aim to integrate a DNA donor sequence (*e.g.* for gene correction applications) via the homologous recombination (HR) repair pathway has yet to demonstrate clinical success. Unlike NHEJ repair modalities, cells undergoing genomic integration by homology directed repair (HDR) must be cultured prior to gene editing, as the HDR pathway is most prevalent during the S/G2 phase of the cell cycle, when the cell synthesizes DNA. The subsequent steps of nuclear delivery of the CRISPR-Cas9 complex by electroporation and addition of the DNA donor template can induce a DNA Damage Response (DDR) that can negatively impact the functionality and gene correction efficiency of the cells, which in turn, may impact the safety and efficacy of the therapeutic drug product. Consequently, there would be a need to substantially enhance the dose of gene modified cells to achieve the intended therapeutic effect. In the context of autologous hematopoietic stem and progenitor cell (HSPC) therapies, this would require isolation of higher quantities of HSPCs at the outset, which itself presents a risk to certain patients.

[0004] Recent efforts to improve gene-modified cell therapies have focused on optimization of the ex-vivo gene editing protocols which aim to maximize efficiency and specificity. However, there are still limited tools available to deliver therapeutic doses of these products to patients using GMP-compliant manufacturing processes for clinical trials and commercially licensed products. Therefore, there is a need for robust, clinically scalable manufacturing methods that can generate substantial doses of therapeutic primary cells, for example, hematopoietic stem and progenitor cells (HSPCs) comprising high levels of gene modification, while maintaining the functional attributes of HSPCs such as self-renewal and clonogenicity. Additionally, the manufacturing process must be feasible for a wide range of patient populations, as the dose of cells needed is based on patient weight, with some cell therapies (e.g. HSCT) requiring a minimal dose of $\geq 3 \times 10^6$ CD34+ cells/kg.

[0005] Hematopoietic stem and progenitor cells are stem cells present in the bone marrow that can give rise to all blood cell types, such as the white blood cells of the immune system and red blood cells. The therapeutic administration of HSPCs comprising one or more gene modifications have the potential to treat a variety of adverse conditions, including blood disorders like sickle cell disease (SCD) and transfusion dependent beta-thalassemia (TDT); severe immune disorders like X-linked Severe Combined Immunodeficiency (X-SCID) and X-linked Chronic granulomatous Disease (X-CGD); metabolic diseases such as mucopolysaccharidosis type 1/III (MPSI/ MPS III) and Gaucher's disease, as well as malignant cancers of the blood and bone marrow. The ability to produce sufficiently high doses of functional gene-modified hematopoietic stem and progenitor cells (HSPCs) from a patient's own mobilized peripheral blood is essential to ensure hematopoietic recovery and durable long term blood cell production for the lifetime of the patient. The ex vivo culture, gene editing and manipulation steps for HSPCs must be highly controlled to maintain their cell phenotype, viability, and functionality in order to generate safe and efficacious gene-modified HSPC drug products that, upon transplantation, are capable of reconstituting the entire immune system.

SUMMARY

[0006] Provided herein are processes that utilize high-capacity gas-permeable cell culture and flow-through electroporation devices to transfect primary cells with gene-editing reagents under conditions which improve gene editing performance, cell yield and drug product (DP) quality

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 embodiments, 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 embodiments, 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 embodiments, the gas-permeable membrane is a silicone membrane. In some embodiments, the gas-permeable membrane ranges from about 1 cm² to about 1000 cm² in size. In some embodiments, the cell culture device comprises a media volume of about 5 ml to 10,000 ml. In some embodiments, the cell culture device is a G-REX bioreactor. In some embodiments, the G-REX bioreactor is selected from the group consisting of a G-REX 10M-CS, 100M-CS, and 500M-CS bioreactor. In some embodiments, 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 embodiments, 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 embodiments, 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 embodiments, 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 embodiments, the primary cells are cryopreserved and/or formulated as drug product following the cell culturing after electroporation.

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

[0010] In some embodiments, the plurality of primary cells comprises primary blood cells, primary mesenchymal cells, or a combination thereof. In some embodiments, the plurality of primary cells comprises primary stem cells, primary progenitor cells, or primary somatic cells. In some embodiments, 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 embodiments, 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 embodiments, 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 embodiments, 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 embodiments, the plurality of primary cells comprises a heterogeneous population of primary cells.

[0011] In some embodiments, 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 embodiments, the CRISPR/Cas RNA-guided polypeptide is a base editor or a prime editor.

[0012] In some embodiments, 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 embodiments, the one or more components of the gene editing system further comprises a DNA donor polynucleotide. In some embodiments, 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 embodiments, 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 embodiments, the CRISPR nuclease is a Cas protein. In some embodiments, the Cas protein is Cas9 or a high-fidelity variant thereof. In some embodiments, the sgRNA and the CRISPR nuclease are formed in a ribonucleoprotein (RNP) complex. In some embodiments, the sgRNA comprises one or more chemically modified nucleotides. In some embodiments, 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 embodiments, a 5' end, a 3' end, or a combination thereof of the modified sgRNA comprises a modified nucleotide.

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

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

[0015] In some embodiments, the plurality of primary cells comprises CD34⁺ hematopoietic stem and progenitor cells (HSPCs). In some embodiments, the HSPCs are gene-edited at the HBB locus. In some embodiments, the subject is a mammal. In some embodiments, the mammal is a human. In some embodiments, 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 embodiments, 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 embodiments, 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 embodiments, the hemoglobinopathy is sickle cell disease, α -thalassemia, β -thalassemia, or δ -thalassemia. In some embodiments, integration of a donor polynucleotide sequence is capable of replacing a mutant allele in the primary cell with a wild-type allele. In some embodiments, the process further comprises administering gene-edited primary cells to a patient in need thereof. In some embodiments, 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 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 embodiments, the cell therapy is an autologous cell therapy. In some embodiments, the cell therapy is an allogenic cell therapy.

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 $\geq 100 \times 10^6$ 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.

[0022] **FIGURE 5** provides the experimental design and process flow diagram for assessing cell viability and proliferation when manufacturing gene edited HSCs under the V1.0 Process (in which HSCs were gene edited under low-volume electroporation conditions and cultured in culture bags) and the V1.1 Process (in which HSCs were gene edited under high-volume electroporation conditions and cultured in gas-permeable high-capacity bioreactors).

[0023] **FIGURE 6** provides a comparison of cell viabilities throughout manufacturing runs under the V1.0 Process (in which HSCs were gene edited under low-volume electroporation conditions and cultured in culture bags) and the V1.1 Process (in which HSCs were gene edited under high-volume electroporation conditions and cultured in gas-permeable high-capacity bioreactors). Cell viability was assessed at cell thaw, pre-electroporation (“pre-zap”), at media change following gene editing, and at harvest, as well as on the gene-edited product following the thaw of cryopreserved satellite vials. Measurements were obtained using AO/DAPI staining.

[0024] **FIGURE 7** provides a comparison of cell proliferation rates (expressed as % recovery from thaw) throughout manufacturing runs under the V1.0 Process, in which HSCs were gene edited under low-volume electroporation conditions and cultured in culture bags, and the V1.1 Process, in which HSCs were gene edited under high-volume electroporation conditions and cultured in gas-permeable high-capacity bioreactors (Batch 1, 2, 3, 4 and 4a). Cell counts were assessed following cell thaw, gene editing, media change following gene editing, and at harvest. Measurements were obtained using an NC-202 cell counter. 3+2: Culture in G-REX bioreactor three days prior to and two days following electroporation; 2+2: Culture in G-REX bioreactor 2 days prior to and two days following electroporation; 2+1: Culture in G-REX bioreactor 2 days prior to and one day following electroporation.

[0025] **FIGURE 8** provides drug product bulk allele distribution following CRISPR-mediated gene editing of the HBB locus in HSCs. 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) Editing performed using a recombinant AAV6 DNA donor under low-volume electroporation conditions and cultured in culture bags (V1.0; AAV MOI = 2500) or high-volume electroporation conditions and cultured in gas-permeable high-capacity bioreactors (V1.1; AAV MOI = 625, 1250). (B) Editing performed using a recombinant AAV6 DNA donor at an MOI of 2500 under high-volume electroporation conditions and cultured

in gas-permeable high-capacity bioreactors. PD-140, PD-144 and PD-148 represent three different engineering runs.

[0026] **FIGURE 9** provides distribution of colony forming units (CFU) from HSCs cultured in culture bags and subjected to low-volume electroporation (V1.0) versus HSCs cultured in gas-permeable bioreactors and subjected to high-volume electroporation (V1.1). HSCs were edited with RNP only (CRISPR/Cas precomplexed with sgRNA targeting the HBB gene; “RNP control”) or RNP plus an AAV6 DNA donor designed to integrate into HBB via homologous recombination, at multiplicities of infection of 625, 1250 or 2500. Untreated controls were subjected to electroporation but not gene editing.

DETAILED DESCRIPTION

Definitions

[0027] Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. The meaning and scope of the terms should be clear, however, in the event of any latent ambiguity, definitions provided herein take precedent over any dictionary or extrinsic definition. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. In this application, the use of “or” means “and/or” unless stated otherwise. Furthermore, the use of the term “including”, as well as other forms, such as “includes” and “included”, is not limiting.

[0028] Generally, nomenclatures used in connection with, and techniques of, cell and tissue culture, molecular biology, immunology, genomics and protein and nucleic acid chemistry described herein are those well-known and commonly used in the art. The methods and techniques of the present disclosure are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. Enzymatic reactions and purification techniques are performed according to manufacturer’s specifications, as commonly accomplished in the art or as described herein. The nomenclatures used in connection with, and the laboratory procedures and techniques of, genome engineering, gene and cell therapy, and medicinal and pharmaceutical chemistry described herein are those well-known and

commonly used in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

[0029] As used herein, the singular forms “a,” “an,” and “the” include the plural referents unless the context clearly indicates otherwise.

[0030] The terms “about” and “approximately” indicate and encompasses an indicated value and a range above and below that value. In certain embodiments, the term “about” indicates a range within 20%, within 15%, within 10%, within 9%, within 8%, within 7%, within 6%, within 5%, within 4%, within 3%, within 2%, within 1%, or less of a given value or range. In certain embodiments, the term “about” indicates the designated value $\pm$ one standard deviation of that value.

[0031] The term “combinations thereof” includes every possible combination of elements to which the term refers to.

[0032] As used herein, “primary cell” refers to any cell harvested directly from a living body. In some embodiments, the primary cell is a mammalian primary cell. In some embodiments, the primary cell is a rodent or mouse primary cell. In some embodiments, the primary cell is a human primary cell. Primary cells can be from any organ or tissue including, but not limited to, blood, brain, heart, liver, lung, pancreas, colon, stomach, epithelium, testis, ovary and muscle. In some embodiments, the primary cell is a hematopoietic stem cell.

[0033] As used herein, “stem cell” refers to any cell that has the ability to differentiate into more than one cell type. In some embodiments, the stem cell is a mammalian stem cell. In some embodiments, the stem cell is a rodent or mouse stem cell. In some embodiments, the stem cell is a human stem cell. Stem cells can be from any organ or tissue including, but not limited to, blood, brain, heart, liver, lung, pancreas, colon, stomach, epithelium, testis, ovary and muscle. In some embodiments, the stem cell is pluripotent or multipotent.

[0034] As used herein, the terms “subject”, “individual” or “patient” refer, interchangeably, to a warm-blooded animal such as a mammal. In particular embodiments, the term refers to a human. A subject may have, be suspected of having, or be predisposed to, a disease or disorder (e.g. a hemoglobinopathy) for which receiving cell therapy may be beneficial. The term also includes livestock, pet animals, or animals kept for study, including horses, cows, sheep, poultry, pigs, cats, dogs, zoo animals, goats, primates (e.g. cynomolgus macaques, or rhesus macaques), and rodents (e.g. mice and rats). A “subject in need thereof” refers to a subject that has one or more symptoms

of, that has received a diagnosis, or that is suspected of having or being predisposed to a disease or condition which may be treated with, and/or may potentially benefit from cell therapy as described herein.

[0035] The term “administering” as used herein refers to a method of giving a dosage of a composition (e.g., an antibody and/or cell therapy composition) to a subject. The method of administration can vary depending on various factors (e.g., the pharmaceutical composition being administered, and the severity of the condition, disease, or disorder being treated).

[0036] The term “treating” or “treatment” refers to any one of the following: ameliorating one or more symptoms of a disease or condition; preventing the manifestation of such symptoms before they occur; slowing down or completely preventing the progression of the disease or condition (as may be evident by longer periods between reoccurrence episodes, slowing down or prevention of the deterioration of symptoms, etc.); enhancing the onset of a remission period; slowing down the irreversible damage caused in the progressive-chronic stage of the disease or condition (both in the primary and secondary stages); delaying the onset of said progressive stage; or any combination thereof.

[0037] An “effective amount” refers to an amount of a compound or composition, as disclosed herein effective to achieve a particular biological, therapeutic, or prophylactic result.

[0038] Processes for Generating Genetically Modified Primary Cells

[0039] Primary Cells

[0040] Provided herein are processes useful for genetically modifying primary cells. In some embodiments, primary cells are isolated from sources such as from healthy volunteers, from patients, from patients having a particular disease or medical condition, regardless of clinical manifestation, i.e. patients having a certain genotype or phenotype. In some embodiments, primary cells are isolated from mammals. In some embodiments, primary cells are isolated from animals. In some embodiments, primary cells are isolated from humans. The primary cells can be isolated by any technique deemed useful to the person of skill.

[0041] Any type of primary cell may be of interest, such as a stem cell, e.g., embryonic stem cell, induced pluripotent stem cell, adult stem cell (e.g., hematopoietic stem cell, mesenchymal stem cell, neural stem cell, organ stem cell), a progenitor cell, a somatic cell (e.g., fibroblast, hepatocyte, heart cell, liver cell, pancreatic cell, muscle cell, skin cell, blood cell, neural cell, immune cell), and any other cell of the body, e.g., human body. The cells can be primary cells or

primary cell cultures derived from a subject, e.g., an animal subject or a human subject, and allowed to grow in vitro for a limited number of passages. In some embodiments, the cells are disease cells or derived from a subject with a disease. For instance, the cells can be cancer or tumor cells.

[0042] In some embodiments, the primary cell is selected from the group consisting of a primary blood cell, a primary mesenchymal cell, and a combination thereof. In some embodiments, the primary blood cell is selected from the group consisting of an immune cell, a red blood cell, a progenitor or stem cell thereof, and a combination thereof. In some instances, the immune cell is selected from the group consisting of a T cell, a B cell, a dendritic cell, a natural killer cell, a macrophage, a neutrophil, an eosinophil, a basophil, a mast cell, a precursor thereof, and a combination thereof. The progenitor or stem cell can be selected from the group consisting of a hematopoietic progenitor cell, a hematopoietic stem cell, and a combination thereof. In some cases, the red blood cell is a blood stem cell. In some instances, the primary mesenchymal cell is selected from the group consisting of a mesenchymal stem cell, a mesenchymal progenitor cell, a mesenchymal precursor cell, a differentiated mesenchymal cell, and a combination thereof. The differentiated mesenchymal cell can be selected from the group consisting of a bone cell, a cartilage cell, a muscle cell, an adipose cell, a stromal cell, a fibroblast, a dermal cell, and a combination thereof.

[0043] In some embodiments, the primary cell is isolated from a mammal prior to subjecting the primary cell to genetic modification, for example via electroporation with gene editing reagents as described below. For instance, the primary cell can be harvested from a human subject. In some instances, the primary cell or a progeny thereof is returned to the mammal after introducing the gene modification reagents into the primary cell. In other words, the genetically modified primary cell undergoes autologous transplantation. In other instances, the genetically modified primary cell undergoes allogeneic transplantation. For example, a primary cell that has not undergone stable gene modification is isolated from a donor subject, and then the genetically modified primary cell is transplanted into a recipient subject who is different than the donor subject.

[0044] The primary cell can comprise a population of primary cells. In some cases, the population of primary cells comprises a heterogeneous population of primary cells. In other cases, the population of primary cells comprises a homogeneous population of primary cells.

[0045] In particular embodiments of the methods and processes provided herein, the primary cell is a primary hematopoietic stem cell. The hematopoietic stem cells can be any hematopoietic stem cells deemed useful by the practitioner of skill. In certain embodiments, the exogenous hematopoietic stem cells, once engrafted, are capable of reconstituting hematopoiesis in the patient. Human hematopoiesis is defined by a cell surface marker expression-based hierarchy initiated by hematopoietic stem cells that both self-renew and differentiate into multipotent progenitors, which in turn give rise to lineage-restricted progenitors, and finally terminally differentiated blood cells (Baum et al., *PNAS* 89, 2804-2808 (1992); Majeti et al., *Cell Stem Cell* 1, 635-645 (2007); Doulatov et al., *Cell Stem Cell* 10, 120-136 (2012)). CD34⁺ expression defines the heterogeneous HSPC population, which can be further classified as a multipotent progenitor (CD34⁺/CD38⁻/CD45RA⁻), long-term repopulating cell in xenograft mice (CD34⁺/CD38⁻/CD90⁺), and a population highly enriched for hematopoietic stem cells (CD34⁺/CD38⁻/CD90⁺/CD45RA⁻).

[0046] In certain embodiments, the hematopoietic stem cells are of any subtype or colony forming unit. In certain embodiments, the hematopoietic stem cells are colony forming unit-granulocyte-erythrocyte-monocyte-megakaryocyte cells. In certain embodiments, the hematopoietic stem cells are colony forming unit-erythrocyte cells. In certain embodiments, the hematopoietic stem cells are colony forming unit-granulocyte-macrophage cells. In certain embodiments, the hematopoietic stem cells are colony forming unit-megakaryocyte cells. In certain embodiments, the hematopoietic stem cells are colony forming unit-basophil cells. In certain embodiments, the hematopoietic stem cells are colony forming unit-eosinophil cells.

[0047] The hematopoietic stem cells can be from any source deemed useful to the person of skill. In certain embodiments, the hematopoietic stem cells are from a donor. In certain embodiments, the donor is the patient. In certain embodiments, the donor is another subject of the same species, for instance another human. In certain embodiments, the hematopoietic stem cells are autologous. In certain embodiments, the hematopoietic stem cells are allogeneic. In certain embodiments, the hematopoietic stem cells are syngeneic.

[0048] Hematopoietic stem cells can be harvested by any technique deemed useful to the person of skill. In some embodiments, the donor subject is administered a hematopoietic stem cells mobilizing agent (e.g. plerixafor (Mozobil®), G-CSF, GM-CSF), prior to harvest. In certain embodiments, the hematopoietic stem cells are harvested from peripheral blood. In certain

embodiments, the hematopoietic stem cells are harvested from cord blood. In certain embodiments, the hematopoietic stem cells are harvested from bone marrow. In some embodiments, a population of donor cells can be obtained from a product that is collected from a subject, such as a patient or subject in need of an autologous HSCT. The product can be an apheresis product that contains a heterogeneous mixture of cells that have been collected from the subject. The heterogeneous mixture of cells can contain primary cells as well as primary CD34⁺ cells and/or human stem cells and/or progenitor cells (HSPCs). The CD34⁺ cells and/or HSPCs can be isolated or separated from the other cells in order to obtain a population of stem cells. Following the separation of CD34⁺ HSPCs, the resulting population of stem cells are substantially free of non-CD34⁺ cells and are ready for subsequent genetic manipulation.

[0049] In some embodiments, the harvested hematopoietic stem cells are separated from the population of primary cells using flow cytometry. In some instances, the flow cytometry comprises fluorescence-activated cell sorting (FACS). In certain other embodiments, the harvested hematopoietic stem cells are separated from the population of primary cells using magnetic bead separation. In some instances, the magnetic bead separation comprises magnetic-activated cell sorting (MACS). In certain other embodiments, the harvested hematopoietic stem cells are separated using a device configured for hematopoietic stem cell enrichment, such as the Miltenyi Biotec CliniMACS cell manufacturing platform.

[0050] Culture of Primary Cells in Gas-permeable High-Capacity Bioreactors

[0051] In some embodiments of the processes for genetically modifying primary cells provided herein, the primary cells are cultured in a cell culture device comprising a gas-permeable membrane. With typical two-dimensional (2-D) plastic cell culture devices, oxygen is generally supplied by diffusion from the top gas-liquid interface only, and stirring, rocking or perfusion is often required to enhance oxygen delivery throughout the vessel, which creates a risk of failure. In the absence of medium flow, dissolved oxygen at the bottom of the culture device may become rapidly exhausted, particularly with increasing cell densities.

[0052] Accordingly, in preferred embodiments of the processes provided herein, primary cells are cultured in cell culture devices utilizing a gas-permeable membrane at the bottom of the device. In some embodiments, the primary cells are cultured in a gas-permeable cell culture device prior to electroporation and gene-editing of the cells. In some embodiments, the primary cells are cultured in a gas-permeable cell culture device after electroporation and gene-editing of the cells.

In some embodiments, the primary cells are cultured in a gas-permeable cell culture device prior to and after electroporation and gene-editing of the cells.

[0053] The gas-permeable membrane enables oxygen supply thorough rapid equilibration of dissolved oxygen at the bottom of the culture device, in close proximity to the cells. The gas-permeable membrane reduces or eliminates the need to actively deliver oxygen and nutrients by agitation, thereby minimizing the need for manual manipulations. Additionally, gas-permeable culture devices can hold larger medium volumes, and hence more nutrients, which minimizes the need for media exchange and agitation to the cells. Larger media volumes also lead to dilution of metabolic waste that, when combined with enhanced oxygen delivery, creates a more physiologic, static environment for cells while cultured *ex vivo*.

[0054] In some embodiments, the gas-permeable cell culture device is a gas-permeable bioreactor. In some embodiments, the gas-permeable bioreactor is a closed system wherein media can be collected or exchanged through media lines integrated into the bioreactor, such that media collection can occur without disturbing the cells on the surface of the gas-permeable membrane at the bottom of the bioreactor. In some such embodiments, media or cell collection occurs in an automated or semi-automated fashion, for example, through the use of a collection device capable of pushing pressurized air into the bioreactor such that media or cells can be collected through a collection tube integrated into the bioreactor and connected to a collection bag or container.

[0055] In some embodiments, the gas-permeable culture device comprises a fluorinated ethylene propylene (FEP)-teplone and polycarbonate membrane (see, e.g. Amps et al. (2010), *Cryobiology*. 60: 344-350; and Salerno et al. (2018), *J. Memb. Sci.* 563: 694-707. In some embodiments, the gas-permeable culture device comprises a porous polystyrene membrane (see e.g., Luetchford et al. (2018), *J. Memb. Sci.* 565:425-438. In some embodiments, the gas-permeable culture device comprises a carbon fluoride membrane [20] (Menzel et al. (2017), *Biomed. Res. Int.* 2017: 5258196. In some embodiments, the gas-permeable culture device comprises a silicon membrane (Vera et al. (2010), *J. Immunother.* 33: 305-315.

[0056] In some embodiments, the gas-permeable bioreactor comprises a membrane size from about 1 cm² to about 1000 cm². In some such embodiments, the gas-permeable bioreactor comprises a media volume from about 5 ml to 10,000 ml. In some embodiments, the gas-permeable bioreactor comprises a membrane size of about 10 cm². In some such embodiments, the gas-permeable bioreactor comprises a media volume of about 100 ml. In some embodiments, the gas-

permeable bioreactor is a G-REX[®] (Wilson Wolf) 10M-CS Bioreactor. In some embodiments, the gas-permeable bioreactor comprises a membrane size of about 100 cm². In some such embodiments, the gas-permeable bioreactor comprises a media volume of about 1000 ml. In some embodiments, the gas-permeable bioreactor is a G-REX[®] (Wilson Wolf) 100M-CS Bioreactor. In some embodiments, the gas-permeable bioreactor comprises a membrane size of about 500 cm². In some such embodiments, the gas-permeable bioreactor comprises a media volume of about 5000 ml. In some embodiments, the gas-permeable bioreactor is a G-REX[®] (Wilson Wolf) 500M-CS Bioreactor. In some embodiments, the gas-permeable 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. Other useful gas-permeable bioreactors are known in the art, including but not limited to those described in U.S. Patent Nos. 8,158,426; 8,158,427; 8,168,432; 8,956,860; 9,255,243; 9,441,192; and 9,410,114.

[0057] In some embodiments, primary cells are seeded within the gas-permeable cell culture device at a surface density of about 1×10^6 – 5×10^6 cells / cm² of the membrane. In some embodiments, the cells are seeded at a surface density of about 1×10^6 , 1.5×10^6 , 2×10^6 , 2.5×10^6 , 3×10^6 , 3.5×10^6 , 4×10^6 , 4.5×10^6 , or 5×10^6 cells / cm² of the membrane. In some embodiments, primary cells are cultured within the gas-permeable cell culture device at 37°C, 5%CO₂ + 5%O₂ + 0% relative humidity.

[0058] In some embodiments of the processes provided herein, primary cells (e.g. primary CD34⁺ HSCs) are cultured in the gas-permeable cell culture device for no more than 4 total days of culturing spanning pre-electroporation (e.g. pre-stimulation), electroporation and recovery. In some embodiments, the primary cells are cultured in the gas-permeable cell culture device for about 2 days prior to electroporation. In some embodiments, the primary cells are cultured in the gas-permeable cell culture device for about 3 days prior to electroporation. In some embodiments, the primary cells are cultured in the gas-permeable cell culture device for about 2 days after electroporation. In some embodiments, the primary cells are cultured in the gas-permeable cell culture device for about 1 day prior to electroporation. In some embodiments, the primary cells are cultured in the gas-permeable cell culture device for about 3 days prior to electroporation and for about 1 day after electroporation. In some embodiments, the primary cells are cultured in the gas-permeable cell culture device for about 2 days prior to electroporation and for about 2 days

after electroporation. In some embodiments, 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 embodiments, the primary cells are cryopreserved and/or formulated as drug product following the cell culturing after electroporation, as described further below.

[0059] Large Volume Electroporation of Primary Cells

[0060] According to the processes provided herein, following culturing of the primary cells in a gas-permeable culture device, one or more gene editing reagents are introduced into the primary cells via electroporation. Electroporation is a process that significantly increases cellular plasma membrane permeability and electrical conductivity by using an externally applied electric field, which permits introduction of large highly charged molecules such as DNA, RNA or proteins, which may not passively diffuse across the hydrophobic bilayer core of the cell. In some embodiments, the electroporation can be carried out with an electroporator capable of electroporating a large volume of primary cells. Manufacturing of autologous cell therapy products often requires manufacturing scales that are dependent on patient weight. For example, typical subject weights for autologous gene-edited HSC therapies range from 40kg to >90kg which demands the ability to process cells at ranges of 3.2×10^8 CD34+ cells to $>1.2 \times 10^9$ CD34+ cells (320 million to 1.2 billion cells). Electroporation systems that operate with a ≤ 1 mL electroporation cartridge would require multiple cartridges to perform electroporation at such large scales and presents manufacturing risks such as variability from cartridge to cartridge at the time of electroporation and can increase probability of operator errors. Accordingly, in some embodiments of the processes provided herein, the primary cells are electroporated in an electroporation chamber having at least a 2 ml capacity. In some embodiments, the primary cells are electroporated in an electroporation chamber having at least a 2.5 ml capacity. In some embodiments, the primary cells are electroporated in an electroporation chamber having at least a 3 ml capacity. In some embodiments, the primary cells are electroporated in an electroporation chamber having a capacity of greater than 3 ml. In some embodiments, the primary cells are electroporated in an electroporation chamber having a capacity range of 1 ml to 3.5 ml. In some embodiments, the primary cells are electroporated in an electroporation chamber having a capacity range of 5 ml to 20 ml. In some embodiments, the primary cells are electroporated in an electroporation chamber having a capacity range of 10 ml to 100 ml. In some embodiments, the electroporation chamber comprises a closed flow-through electroporation cartridge.

[0061] In some embodiments, the electroporator is capable of processing large volumes of cells by utilizing a closed flow-through process. In an exemplary flow-through process, primary cells (for example, harvested from a gas-permeable bioreactor), are mixed with a gene-editing reagent (for example a CRISPR/sgRNA RNP complex) under aseptic conditions and suspended in a suitable electroporation buffer. This electroporation solution is then transferred to an electroporation assembly for electroporation. In some embodiments, electroporation is performed on a computer controlled electroporator, which comprises computer-controlled valves and an air pressure system that regulates the flow of cells from the electroporation solution through the electroporation chamber in an automated fashion. Within the chamber, the cells are subjected to a series of electrical pulses while passing between electrodes, which effectively transfects the gene-editing reagent(s) into the cells. A discrete volume of the electroporation solution containing the cells and editing reagents is repeatedly passed through the electroporation cartridge until the entirety of the cell suspension has been electroporated under the same conditions. Following electroporation, the transfected cells can be aseptically collected in a collection container, then subject to recovery and/or further cell culturing in a gas-permeable bioreactor.

[0062] Commercial electroporators capable of closed flow-through electroporation include but are not limited to the MaxCyte® ExPERT GTX®. In some such embodiments, the primary cells are electroporated in a MaxCyte CL-2™ electroporation assembly. In some embodiments, the primary cells are electroporated in a MaxCyte CL-1.1 electroporation cartridge. In some embodiments, the primary cells are electroporated in a MaxCyte R-20K electroporation cartridge. In some embodiments, the primary cells are hematopoietic stem cells, wherein the HSCs are electroporated with an electroporation protocol selected from MaxCyte Protocol's HSC-1, HSC-2, HSC-3, HSC-4, HSC-5 and HSC-6. In some embodiments, the HSCs are electroporated with MaxCyte electroporation protocol HSC-4. In some embodiments, the HSCs are electroporated with MaxCyte electroporation protocol HSC-5. In some embodiments, the HSCs are electroporated with MaxCyte electroporation protocol HSC-6.

[0063] In some embodiments, the electroporation system is capable of processing 8 ml of electroporation solution comprising primary cells and gene editing reagents per one (1) minute of processing time. In some embodiments, the electroporation system is capable of processing of up to 20 billion cells in 30 minutes. In some embodiments, the electroporation system is capable of electroporating a cell range of about 5×10^8 to about 2×10^{10} cells from a single electroporation

solution comprising primary cells and gene editing reagents. In some embodiments, the electroporation system is capable of processing about 5-100 ml of a single electroporation solution comprising primary cells and gene editing reagents. In some embodiments, the electroporation system is capable of processing about 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or about 100 ml of a single electroporation solution comprising primary cells and gene editing reagents.

[0064] In some embodiments, the concentration of primary cells in the electroporation solution is 25×10^6 to 100×10^6 cells/ml. In some embodiments, the concentration of primary cells in the electroporation mix is about 25×10^6 cells/ml, about 30×10^6 cells/ml, about 35×10^6 cells/ml, about 40×10^6 cells/ml, about 45×10^6 cells/ml, about 50×10^6 cells/ml, about 55×10^6 cells/ml, about 60×10^6 cells/ml, about 65×10^6 cells/ml, about 70×10^6 cells/ml, about 75×10^6 cells/ml, about 80×10^6 cells/ml, about 85×10^6 cells/ml, about 90×10^6 cells/ml, about 95×10^6 cells/ml, or about 100×10^6 cells/ml. In other embodiments, the concentration of primary cells in the electroporation mix is 100×10^6 to 2×10^8 cells/ml. In some embodiments, the primary cells are hematopoietic stem cells.

[0065] It should be understood that the conditions set forth herein are illustrative and not restrictive. One of ordinary skill in the art can readily adjust parameters based on, for example, the size and quantity of the molecule(s) to be introduced, the type of primary cell, and the like.

[0066] Genetic Modification of Primary Cells

[0067] In some embodiments of the processes provided herein, the electroporation step is used to deliver a nucleic acid to the primary cell (e.g., a DNA and/or RNA). The nucleic acid can be any nucleic acid of interest, e.g., the nucleic acid can be linear or circular, and can be a plasmid, a viral genome, an RNA (e.g., a coding RNA such as an mRNA or a non-coding RNA such as a guide RNA, a short interfering RNA (siRNA), a short hairpin RNA (shRNA), a microRNA (miRNA), and the like), a DNA, etc. In some embodiments, the nucleic acid is an RNAi agent (e.g., an shRNA, an siRNA, a miRNA, etc.) or a DNA template encoding an RNAi agent. In some embodiments, the nucleic acid is an siRNA molecule (e.g., one that targets an mRNA, one that targets a miRNA). In some embodiments, the nucleic acid is an LNA molecule (e.g., one that targets a miRNA). In some embodiments, the nucleic acid is a miRNA. In some embodiments, the nucleic acid comprises an mRNA that encodes a therapeutic protein of interest, or a gene editing endonuclease as further described below. In some embodiments, the nucleic acid comprises a non-

coding RNA (e.g., an RNAi agent, a CRISPR/Cas guide RNA, etc.) and/or a DNA molecule encoding the non-coding RNA. Other useful nucleic acids include but are not limited to: species of RNA and DNA including mRNA, mRNA modified mRNA (monomethylation at position 1 of Adenosine), siRNA, miRNA, aptamers, shRNA, AAV-derived nucleic acids and scaffolds, morpholino RNA, peptide and peptide nucleic acids, cDNA, DNA origami, DNA and RNA with synthetic nucleotides, DNA and RNA with predefined secondary structures, multimers and oligomers of the aforementioned, and payloads whose sequence may encode other products such as any protein or polypeptide whose expression is desired.

[0068] In some embodiments, the electroporation step of the processes described herein is used to introduce a protein into the primary cell. Examples of useful proteins that can be introduced to the primary cell include, but are not limited to: programmable gene editing proteins (e.g., transcription activator-like (TAL) effectors (TALEs), TALE nucleases (TALENs), zinc-finger proteins (ZFPs), zinc-finger nucleases (ZFNs), DNA-guided polypeptides such as *Natronobacterium gregoryi* Argonaute (NgAgo), CRISPR/Cas RNA-guided polypeptides such as Cas9, CasX, CasY, Cpf1, and the like); transposons (e.g., a Class I or Class II transposon—e.g., piggybac, sleeping beauty, Tc1/mariner, Tol2, PIF/harbinger, hAT, mutator, merlin, transib, helitron, maverick, frog prince, minos, Himar1 and the like); meganucleases (e.g., I-SceI, I-CeuI, I-CreI, I-DmoI, I-ChuI, I-DirI, I-FlmI, I-FlmII, I-AniI, I-SceIV, I-CsmI, I-PanI, I-PanII, I-PanMI, I-SceII, I-PpoI, I-SceIII, I-LtrI, I-GpiI, I-GZeI, I-OnuI, I-HjeMI, I-MsoI, I-TevI, I-TevII, I-TevIII, PI-MleI, PI-MtuI, PI-PspI, PI-Tli I, PI-Tli II, PI-SceV, and the like); megaTALs (see, e.g., Boissel et al., *Nucleic Acids Res.* 2014 February; 42(4): 2591-2601); SCF; BCL-XL; Foxp3; HoxB4; and SiRT6. For any of the above proteins, its introduction into the primary cell can include a nucleic acid (DNA and/or mRNA) encoding the protein, and/or can include the actual protein.

[0069] In some embodiments, the electroporation step is used to introduce a gene editing tool (i.e., a component of a gene editing system, e.g., a site-specific gene editing system such as a programmable gene editing system). For example, a nucleic acid payload can include one or more of: (i) a CRISPR/Cas guide RNA, (ii) a DNA encoding a CRISPR/Cas guide RNA, (iii) a DNA and/or RNA encoding a programmable gene editing protein such as a zinc finger protein (ZFP) (e.g., a zinc finger nuclease—ZFN), a transcription activator-like effector (TALE) protein (e.g., fused to a nuclease—TALEN), a DNA-guided polypeptide such as *Natronobacterium gregoryi* Argonaute (NgAgo), and/or a CRISPR/Cas RNA-guided polypeptide (e.g., Cas9, CasX,

CasY, Cpf1, and the like); (iv) a DNA donor template; (v) a nucleic acid molecule (DNA, RNA) encoding a site-specific recombinase (e.g., Cre recombinase, Dre recombinase, Flp recombinase, KD recombinase, B2 recombinase, B3 recombinase, R recombinase, Hin recombinase, Tre recombinase, PhiC31 integrase, Bxb1 integrase, R4 integrase, lambda integrase, HK022 integrase, HP1 integrase, and the like); (vi) a DNA encoding a resolvase and/or invertase (e.g., Gin, Hin, $\gamma\delta 3$, Tn3, Sin, Beta, and the like); and (vii) a transposon and/or a DNA derived from a transposon (e.g., bacterial transposons such as Tn3, Tn5, Tn7, Tn9, Tn10, Tn903, Tn1681, and the like; eukaryotic transposons such as Tc1/mariner super family transposons, PiggyBac superfamily transposons, hAT superfamily transposons, PiggyBac, Sleeping Beauty, Frog Prince, Minos, Himar1, and the like). In some cases a subject delivery vehicle is used to deliver a protein payload, e.g., a gene editing protein such as a ZFP (e.g., ZFN), a TALE (e.g., TALEN), a DNA-guided polypeptide such as *Natronobacterium gregoryi* Argonaute (NgAgo), a CRISPR/Cas RNA-guided polypeptide (e.g., Cas9, CasX, CasY, Cpf1, and the like), a site-specific recombinase (e.g., Cre recombinase, Dre recombinase, Flp recombinase, KD recombinase, B2 recombinase, B3 recombinase, R recombinase, Hin recombinase, Tre recombinase, PhiC31 integrase, Bxb1 integrase, R4 integrase, lambda integrase, HK022 integrase, HP1 integrase, and the like), a resolvase/invertase (e.g., Gin, Hin, $\gamma\delta 3$, Tn3, Sin, Beta, and the like); and/or a transposase (e.g., a transposase related to transposons such as bacterial transposons such as Tn3, Tn5, Tn7, Tn9, Tn10, Tn903, Tn1681, and the like; or eukaryotic transposons such as Tc1/mariner super family transposons, PiggyBac superfamily transposons, hAT superfamily transposons, PiggyBac, Sleeping Beauty, Frog Prince, Minos, Himar1, and the like). In some embodiments, the electroporation step is used to deliver nucleic acid and protein, and in some such embodiments, includes a ribonucleoprotein complex (RNP).

[0070] In particular embodiments of the processes described herein, primary cells are genetically modified to comprise therapeutic heterologous donor polynucleotide sequences. Donor polynucleotide sequences described herein may be incorporated within a wide variety of gene therapy constructs, e.g., to deliver a nucleic acid encoding a protein to a subject in need thereof. A vector construct refers to a polynucleotide molecule including all or a portion of a viral genome and an exogenous polynucleotide sequence. In some instances, gene transfer can be mediated by a DNA viral vector, such as an adenovirus (Ad) or adeno-associated virus (AAV). Other vectors useful in methods of gene therapy are known in the art. For example, a construct of the present

disclosure can include an alphavirus, herpesvirus, retrovirus, lentivirus, or vaccinia virus. The exogenous sequences generally encode recombinant molecules to be expressed in the cells, e.g., for use in cell therapy. Processing steps of the methods can also or alternatively include all or a portion of cell washing, dilution, selection, isolation, separation, cultivation, stimulation, packaging, and/or formulation. The methods generally allow for the processing, e.g., selection or separation and/or transduction, of cells on a large scale (such as in compositions of volumes greater than or at about 50 mL).

[0071] The processes provided herein can be utilized to introduce into primary cells small insertions or deletions (INDELS, e.g., of one or more base pairs), insertion of a heterologous DNA fragment (e.g. to correct one or more mutations) or an entire coding sequence, or deletion of an endogenous DNA sequence or fragment. The genetic modification introduced into the primary cell may result in a gain-of-function or loss-of-function of a targeted allele, and different mechanisms may be utilized to modify the primary cell, including but not limited, to non-homologous end joining (NHEJ), homology-directed repair (HDR), or homologous recombination (HR).

[0072] In some embodiments, primary cells are genetically modified using gene editing applications which utilize site-specific nucleases for knock-out of targeted genomic sequences or knock-in of exogenous sequences, and for transferring exogenous sequences to the cells by viral transduction through the use of recombinant viral vectors. In some such embodiments, primary cells are collected by apheresis, enriched from the apheresis product, then cryopreserved prior to performing any gene editing method (e.g., gene knock-out, gene knock-in, gene correction). Cryopreservation may be introduced after mobilization, collection (e.g. by apheresis) and/or selection of the primary cells. Following cryopreservation, an assessment can be made on whether the threshold number of primary cells has been collected from the donor to proceed with the gene editing steps that follow. If a threshold number of cells has not been reached from a single round of mobilization, collection, selection and cryopreservation, subsequent rounds may be performed until the threshold number of cells has been reached. Threshold numbers of primary cells to be collected may vary depending on a number of factors, including but not limited to, the gene editing procedure performed (e.g., gene knock-out, gene knock-in, gene correction), the targeted gene to be edited, the mechanism by which the targeted gene is modified (e.g., homology dependent repair (HDR)), the efficiency of the editing procedure (e.g. HDR efficiency) and the therapeutic threshold for treatment of a specific disease. In some embodiments, the threshold number of primary cells

to be collected from a donor prior to gene editing is about 1×10^4 to 1×10^5 , 1×10^5 to 1×10^6 , 1×10^6 to 1×10^7 cells/kg or more. In some embodiments, at least about 1×10^5 to 1×10^7 cells/kg are collected prior to gene editing. In some embodiments, at least about 1×10^4 , 2×10^4 , 3×10^4 , 4×10^4 , 5×10^4 , 6×10^4 , 7×10^4 , 8×10^4 , 9×10^4 , 1×10^5 , 2×10^5 , 3×10^5 , 4×10^5 , 5×10^5 , 6×10^5 , 7×10^5 , 8×10^5 , 9×10^5 , 1×10^6 , 2×10^6 , 3×10^6 , 4×10^6 , 5×10^6 , 6×10^6 , 7×10^6 , 8×10^6 , 9×10^6 , 1×10^7 , 2×10^7 , 3×10^7 , 4×10^7 , 5×10^7 , 6×10^7 , 7×10^7 , 8×10^7 , 9×10^7 , or about 1×10^8 primary cells /kg are collected prior to proceeding with gene editing of the collected cells. Once the threshold number of primary cells are mobilized, collected, selected for, and cryopreserved, the cells can then proceed to thaw, culture and gene editing in accordance with the processes described herein.

[0073] In some embodiments, the gene editing utilizes a nuclease introduced to the cell that is capable of causing a double-strand break near or within a genomic target site, which may be useful for increasing the frequency of homologous recombination and HDR at or near the cleavage site. In preferred embodiments, the recognition sequence for the nuclease is present in the host cell genome only at the target site, thereby minimizing any off-target genomic binding and cleavage by the nuclease. Gene-editing nucleases useful for the methods provided herein include but are not limited to a TAL-effector DNA binding domain-nuclease fusion protein (TALEN), a site-specific recombinase (for example, serine recombinase or a tyrosine recombinase, integrase (FLP, Cre, lambda integrase) or resolvase; a transposase, a zinc-finger nuclease (ZFN), and a clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) protein. 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, homologs thereof, or modified versions thereof.

[0074] In other embodiments, the primary cells are genetically modified using gene editing applications which utilize base editors. Base editing is a CRISPR-Cas9-based genome editing technology that allows the introduction of point mutations in the DNA without generating DSBs. Two major classes of base editors have been developed: cytidine base editors or CBEs allowing C>T conversions and adenine base editors or ABEs allowing A>G conversions (see e.g. Rees et al. (2018) *Nat Rev Genet* 19:770-788).

[0075] In other embodiments, the primary cells are genetically modified using gene editing applications which utilize prime editors. Prime editors (PE) consist of nCas9 fused to a reverse transcriptase used in combination with a prime editing RNA (pegRNA, a guide RNA that includes a template region for reverse transcription). Prime editing allows introduction of insertions, deletions (indels) and 12 base-to-base conversions. Prime editing relies on the ability of a reverse transcriptase (RT), fused to a Cas nickase variant, to convert RNA sequence brought by a prime editing guide RNA (pegRNA) into DNA at the nick site generated by the Cas protein. The DNA flap generated from this process is then included or not in the targeted DNA sequence. See, e.g. Anzalone et al. (2019) *Nature* 576:149-157. Non-limiting examples of prime editing systems include PE1, PE1-M1, PE1-M2, PE1-M3, PE1-M6, PE1-M15, PE1-M3inv, PE2, PE3, PE3b.

[0076] In some embodiments, genetically modified primary cells are generated by introducing a CRISPR-associated Cas nuclease capable of generating double-strand breaks (e.g. Cas9), a guide RNA polynucleotide, and a donor polynucleotide sequence into the primary cells. Through introduction of these components into the cell, a double stranded break can be introduced at a specific site as directed by the guide polynucleotide sequence and the CRISPR-associated Cas9 nuclease. A donor polynucleotide containing a sequence of interest can be further introduced into the cell and through homology directed recombination, the sequence of interest can be inserted into the cell. The transfer of the donor polynucleotide sequence can be carried out by transduction. The methods for viral transfer, e.g., transduction, generally involve at least initiation of transduction by incubating in a centrifugal chamber an input composition comprising the cells to be transduced and viral vector particles containing the vector, under conditions whereby cells are transduced or transduction is initiated in at least some of the cells in the input composition, wherein the method produces an output composition comprising the transduced cells.

[0077] In some embodiments the Cas nuclease can be in the form of a protein. In some embodiments, the Cas nuclease can be in the form of a plasmid, thereby allowing a cell that carries this expression construct to then express the Cas nuclease. In other embodiments, the Cas nuclease is pre-complexed with a guide RNA and introduced into the cell as a ribonucleoprotein (RNP). In some embodiments, the Cas nuclease and the guide polynucleotide sequence is introduced into the primary cell through electroporation.

[0078] Introduction of the donor polynucleotide can occur through viral transduction using a delivery vector, such as adeno associated virus (AAV). AAV of any serotype or pseudotype can

be used. Certain AAV vectors are derived from single stranded (ss) DNA parvoviruses that are nonpathogenic for mammals. Briefly, rep and cap viral genes that can account for 96% of the archetypical wild-type AAV genome can be removed in the generation of certain AAV vectors, leaving flanking inverted terminal repeats (ITRs) that can be used to initiate viral DNA replication, packaging and integration. Wild type AAV integrates into the human host cell genome with preferential site specificity at chromosome 19q13.3. Alternatively, AAV can be maintained episomally. At least twelve human serotypes of AAV (AAV serotype 1 (AAV-1) to AAV-12) and more than 100 serotypes from nonhuman primates have been discovered to date. Any of these serotypes, as well as any combinations thereof, may be used within the scope of the present disclosure. A serotype of the viral vector can be selected from the group consisting of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, and AAV9. In some embodiments, the serotype is AAV6. In some embodiments, the AAV vector is transduced at a multiplicity of infection (MOI) of 2500 or less. In some embodiments, the AAV vector is transduced at an MOI of 1250 or less. In some embodiments, the AAV vector is transduced at an MOI of 625 or less. In some embodiments, the AAV vector is transduced at a multiplicity of infection (MOI) of 2500, 2450, 2400, 2350, 2300, 2250, 2200, 2150, 2100, 2050, 2000, 1950, 1900, 1850, 1800, 1750, 1700, 1650, 1600, 1550, 1500, 1450, 1400, 1350, 1300, 1250, 1200, 1150, 1100, 1050, 1000, 950, 900, 850, 800, 750, 700, 650, 600, 550, 500 or less than 500.

[0079] In some embodiments, the viral transduction occurs within 30 minutes of the electroporation. In some embodiments, the viral transduction occurs simultaneously with the electroporation. In some embodiments, the viral transduction occurs within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, or 30 minutes of the electroporation.

[0080] Pharmaceutical Compositions Comprising Genetically Modified Primary Cells

[0081] Also provided herein are methods, compositions and kits for use of the genetically modified primary cells prepared in accordance with the processes described herein, including pharmaceutical compositions, therapeutic methods, and methods of administration. Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to any animals.

[0082] In some embodiments, the pharmaceutical composition comprises a modified host cell that is genetically engineered to comprise an INDEL at a targeted gene locus of the host cell. In some embodiments, the pharmaceutical composition comprises a plurality of the genetically modified primary cells that have undergone nuclease cleavage resulting in INDELS at the target gene locus, and further comprises unmodified primary cells. In some embodiments, the pharmaceutical composition is comprised of at least 5% of the modified primary cells comprising an INDEL at the target gene locus. In some embodiments, the pharmaceutical composition is comprised of about 5% to 80% of the modified primary cells comprising an INDEL at the target gene locus. In some embodiments, the pharmaceutical composition is comprised of at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 11%, at least 12%, at least 13%, at least 14%, at least 15%, at least 16%, at least 17%, at least 18%, at least 19%, at least 20%, at least 21%, at least 22%, at least 23%, at least 24%, at least 25%, at least 26%, at least 27%, at least 28%, at least 29%, at least 30%, at least 31%, at least 32%, at least 33%, at least 34%, at least 35%, at least 36%, at least 37%, at least 38%, at least 39%, at least 40%, at least 41%, at least 42%, at least 43%, at least 44%, at least 45%, at least 46%, at least 47%, at least 48%, at least 49%, at least 50%, at least 51%, at least 52%, at least 53%, at least 54%, at least 55%, at least 56%, at least 57%, at least 58%, at least 59%, at least 60%, at least 61%, at least 62%, at least 63%, at least 64%, at least 65%, at least 66%, at least 67%, at least 68%, at least 69%, at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80% or more of the modified primary cells comprising an INDEL at the target gene locus.

[0083] In some embodiments, the pharmaceutical composition comprises a modified host cell that is genetically engineered to comprise an integrated donor sequence at a targeted gene locus of the host cell. In some embodiments, the modified host cell is genetically engineered to comprise an integrated functional donor sequence, for example, a SNP donor that corrects one or mutations in a target gene or inserts into or replaces some or all of the mutated allele with a wild-type allele. In particular embodiments, a functional donor sequence is integrated into the translational start site of the endogenous locus of the target gene. In particular embodiments, the functional donor sequence that is integrated into the host cell genome is expressed under control of the native promoter sequence of the target gene.

[0084] In some embodiments, the pharmaceutical composition comprises a plurality of the genetically modified primary cells, and further comprises unmodified primary cells and/or primary cells that have undergone nuclease cleavage resulting in INDELS at the target gene locus but not integration of the donor sequence. In some embodiments, the pharmaceutical composition is comprised of at least 5% of the modified primary cells comprising an integrated donor sequence. In some embodiments, the pharmaceutical composition is comprised of about 9% to 50% of the modified primary cells comprising an integrated donor sequence. In some embodiments, the pharmaceutical composition is comprised of at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 11%, at least 12%, at least 13%, at least 14%, at least 15%, at least 16%, at least 17%, at least 18%, at least 19%, at least 20%, at least 21%, at least 22%, at least 23%, at least 24%, at least 25%, at least 26%, at least 27%, at least 28%, at least 29%, at least 30%, at least 31%, at least 32%, at least 33%, at least 34%, at least 35%, at least 36%, at least 37%, at least 38%, at least 39%, at least 40%, at least 41%, at least 42%, at least 43%, at least 44%, at least 45%, at least 46%, at least 47%, at least 48%, at least 49%, at least 50%, at least 51%, at least 52%, at least 53%, at least 54%, at least 55%, at least 56%, at least 57%, at least 58%, at least 59%, at least 60%, at least 61%, at least 62%, at least 63%, at least 64%, at least 65%, at least 66%, at least 67%, at least 68%, at least 69%, at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80% or more of the modified primary cells comprising an integrated donor sequence.

[0085] The pharmaceutical compositions described herein may be formulated using one or more excipients to, *e.g.*: (1) increase stability; (2) alter the biodistribution (*e.g.*, target the cells to specific tissues or cell types, *e.g.* hematopoietic stem cells); and/or (3) enhance engraftment in the recipient. Formulations of the present disclosure can include, without limitation, saline, liposomes, lipid nanoparticles, polymers, peptides, proteins, and combinations thereof. Formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. As used herein the term “pharmaceutical composition” refers to compositions including at least one active ingredient (*e.g.*, exogenous hematopoietic stem cells) and optionally one or more pharmaceutically acceptable excipients. Pharmaceutical compositions of the present disclosure may be sterile.

[0086] Relative amounts of the active ingredient (*e.g.* the modified host cell), a pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition in

accordance with the present disclosure may vary, depending upon the identity, size, and/or condition of the subject being treated and further depending upon the route by which the composition is to be administered. For example, the composition may include between 0.1% and 99% (w/w) of the active ingredient. By way of example, the composition may include between 0.1% and 100%, *e.g.*, between 0.5 and 50%, between 1-30%, between 5-80%, or at least 80% (w/w) active ingredient.

[0087] Excipients, as used herein, include, but are not limited to, any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, and the like, as suited to the particular dosage form desired. Various excipients for formulating pharmaceutical compositions and techniques for preparing the composition are known in the art (see Remington: The Science and Practice of Pharmacy, 21st Edition, A. R. Gennaro, Lippincott, Williams & Wilkins, Baltimore, MD, 2006; incorporated herein by reference in its entirety). The use of a conventional excipient medium may be contemplated within the scope of the present disclosure, except insofar as any conventional excipient medium may be incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition.

[0088] Exemplary diluents include, but are not limited to, calcium carbonate, sodium carbonate, calcium phosphate, dicalcium phosphate, calcium sulfate, calcium hydrogen phosphate, sodium phosphate lactose, sucrose, cellulose, microcrystalline cellulose, kaolin, mannitol, sorbitol, inositol, sodium chloride, dry starch, cornstarch, powdered sugar, etc., and/or combinations thereof. Injectable formulations may be sterilized, for example, by filtration through a bacterial-retaining filter, and/or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[0089] *Dosing and administration*

[0090] In certain embodiments, the methods comprise administering to an individual in need of treatment a composition comprising an effective amount of genetically modified primary cells (*e.g.* genetically modified hematopoietic stem cells). Therapeutically effective doses of the genetically modified primary cells can be in the range of about one million to about 200 billion cells, such as, *e.g.*, 1 million to about 50 billion cells (*e.g.*, about 5 million cells, about 25 million cells, about 500 million cells, about 1 billion cells, about 5 billion cells, about 20 billion cells, about 30 billion

cells, about 40 billion cells, or a range defined by any two of the foregoing values), such as about 10 million to about 100 billion cells (*e.g.*, about 20 million cells, about 30 million cells, about 40 million cells, about 60 million cells, about 70 million cells, about 80 million cells, about 90 million cells, about 10 billion cells, about 25 billion cells, about 50 billion cells, about 75 billion cells, about 90 billion cells, or a range defined by any two of the foregoing values), and in some cases about 100 million cells to about 50 billion cells (*e.g.*, about 120 million cells, about 250 million cells, about 350 million cells, about 450 million cells, about 650 million cells, about 800 million cells, about 900 million cells, about 3 billion cells, about 30 billion cells, about 45 billion cells) or any value in between these ranges. In some embodiments, the method comprises administering between 2×10^6 and 2×10^8 viable hematopoietic stem cells per kg of body weight.

[0091] In certain embodiments, pharmaceutical compositions comprising genetically modified primary cells (*e.g.* genetically modified hematopoietic stem cells) in accordance with the present disclosure may be administered at dosage levels sufficient to deliver from, *e.g.*, about 1×10^4 to 1×10^5 , 1×10^5 to 1×10^6 , 1×10^6 to 1×10^7 , or more cells to the subject, or any amount sufficient to obtain the desired therapeutic or prophylactic, effect. The desired dosage of the modified host cell pharmaceutical compositions of the present disclosure may be administered one time or multiple times. In some embodiments, delivery of the modified host cell to a subject provides a therapeutic effect for at least 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 1 year, 13 months, 14 months, 15 months, 16 months, 17 months, 18 months, 19 months, 20 months, 20 months, 21 months, 22 months, 23 months, 2 years, 3 years, 4 years, 5 years, 6 years, 7 years, 8 years, 9 years, 10 years or more than 10 years. In some embodiments, only a single dose is needed to effect treatment or prevention of a disease or disorder described herein. In other embodiments, a subject in need thereof may receive more than one dose, for example, 2, 3, or more than 3 doses of a pharmaceutical genetically modified primary cells (*e.g.* genetically modified hematopoietic stem cells) compositions described herein to effect treatment or prevention of the disease or disorder. The genetically modified primary cells may be used in combination with one or more other therapeutic, prophylactic, research or diagnostic agents, or medical procedures, either sequentially or concurrently. In general, each agent will be administered at a dose and/or on a time schedule determined for that agent.

[0092] The infusion population and compositions thereof can be administered to an individual in need thereof using standard administration techniques, formulations, and/or devices. Provided are formulations and administration with devices, such as syringes and vials, for storage and administration of the compositions. Formulations or pharmaceutical composition comprising genetically modified primary cells include those for intravenous, intraperitoneal, subcutaneous, intramuscular, or pulmonary administration. Compositions of the genetically modified primary cells can be provided as sterile liquid preparations, *e.g.*, isotonic aqueous solutions, suspensions, emulsions, dispersions, or viscous compositions, which may in some aspects be buffered to a selected pH. Viscous compositions can be formulated within the appropriate viscosity range to provide longer contact periods with specific tissues. Liquid or viscous compositions can comprise carriers, which can be a solvent or dispersing medium containing, for example, water, saline, phosphate buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol) and suitable mixtures thereof. Sterile injectable solutions can be prepared by incorporating the genetically modified primary cells in a solvent, such as in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, dextrose, or the like.

[0093] Genetically modified primary cells included in the pharmaceutical compositions described above may be administered by any delivery route, systemic delivery or local delivery, which results in a therapeutically effective outcome. These include, but are not limited to, enteral, gastroenteral, epidural, oral, transdermal, intracerebral, intracerebroventricular, epicutaneous, intradermal, subcutaneous, nasal, intravenous, intra-arterial, intramuscular, intracardiac, intraosseous, intrathecal, intraparenchymal, intraperitoneal, intravesical, intravitreal, intracavernous), interstitial, intra-abdominal, intralymphatic, intramedullary, intrapulmonary, intraspinal, intrasynovial, intrathecal, intratubular, parenteral, percutaneous, periarticular, peridural, perineural, periodontal, rectal, soft tissue, and topical. In particular embodiments, the cells are administered intravenously. The pharmaceutical compositions may be administered to a subject using any amount and any route of administration effective for preventing, treating, or managing a disease described herein. The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the disease, the particular composition, its mode of administration, its mode of activity, and the like.

[0094] In some embodiments, cell therapy can be performed using cryopreserved populations of the genetically modified primary cells generated by the processes provided herein. Cells may be

cryopreserved following gene editing methods comprising the culturing and electroporation steps. The genetically modified primary cells can be stored by any technique deemed useful to the person of skill. In certain embodiments, the gene-edited cells are formulated in cryopreservation media and placed in cryogenic storage units such as liquid nitrogen freezers (-195°C) or ultra-low temperature freezers (-65°C , -80°C , or -120°C) for long term storage of at least one month, 2 months, 3 months, 4 months, 6 months, 1 year, 2 years, 3 years, or at least 5 years.

Methods of Treatment

[0095] Pharmaceutical compositions comprising genetically modified primary cells generated by the processes provided herein may be administered as part of a treatment regimen for a disease. The disease can be selected from the group consisting of a hemoglobinopathy, a viral infection, X-linked severe combined immune deficiency, Fanconi anemia, hemophilia, neoplasia, cancer, 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 instances, the hemoglobinopathy is sickle cell disease, α -thalassemia, β -thalassemia, or δ -thalassemia. In other instances, the viral infection is selected from the group consisting of a hepatitis B virus infection, hepatitis C virus infection, human papilloma virus infection, human immunodeficiency virus (HIV) infection, human T-lymphotrophic virus (HTLV) infection, Epstein-Barr virus infection, herpes virus infection, cytomegalovirus infection, and any other chronic viral infection. In yet other instances, the muscular diseases and disorders are selected from the group consisting of Becker muscular dystrophy, Duchenne muscular dystrophy, Emery-Dreifuss muscular dystrophy, facioscapulohumeral muscular dystrophy, any other muscular dystrophy, and muscular atrophy.

[0096] Pharmaceutical compositions comprising genetically modified primary hematopoietic stem cells generated by the processes provided herein may find particular use as part of a treatment regimen for any disease or condition for which hematopoietic stem cell transplantation (HSCT) is useful. HSCT may be used to treat a number of conditions, including congenital and acquired conditions. In some embodiments, acquired conditions treatable with HSCT include but are not limited to: (1) malignancies, including hematological malignancies such as leukemias (e.g. acute

lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML)), lymphomas (e.g. Hodgkin's disease, Non-Hodgkin's lymphoma), myelomas (e.g. multiple myeloma (Kahler's disease)); solid tumor cancers (e.g. neuroblastoma, desmoplastic small round cell tumor, Ewing's sarcoma, choriocarcinoma); (2) hematologic disease, including phagocyte disorders (e.g. chronic granulomatous disease), bone marrow failure disorders (e.g. myelodysplastic syndrome, Fanconi's anemia, dyskeratosis congenita), anemias (e.g. paroxysmal nocturnal hemoglobinuria, aplastic anemia, acquired pure red cell aplasia), myeloproliferative disorders (e.g. polycythemia vera, essential thrombocytosis, myelofibrosis); (3) metabolic disorders including amyloidosis (e.g. amyloid light chain (AL) amyloidosis); (4) environmentally-induced diseases such as radiation poisoning; (5) viral diseases (e.g. HTLV, HIV); and (5) autoimmune diseases such as multiple sclerosis.

[0097] In some embodiments, congenital conditions treatable with HSCT include but are not limited to: (1) lysosomal storage disorders, including lipidoses (disorders of lipid storage, such as neuronal ceroid lipofuscinoses (e.g. infantile neuronal ceroid lipofuscinosis (INCL, Santavuori disease) and Jansky–Bielschowsky disease (late infantile neuronal ceroid lipofuscinosis)); sphingolipidoses (e.g. Niemann–Pick disease and Gaucher disease), leukodystrophies (e.g. adrenoleukodystrophy, metachromatic leukodystrophy, Krabbe disease (globoid cell leukodystrophy); mucopolysaccharidoses (e.g. Hurler syndrome (MPS I H, α -L-iduronidase deficiency), Scheie syndrome (MPS I S), Hurler–Scheie syndrome (MPS I H-S), Hunter syndrome (MPS II, iduronidase sulfate deficiency), Sanfilippo syndrome (MPS III), Morquio syndrome (MPS IV), Maroteaux–Lamy syndrome (MPS VI), Sly syndrome (MPS VII)); glycoproteinoses (e.g. Mucopolipidosis II (I-cell disease), fucosidosis, aspartylglucosaminuria, alpha-mannosidosis); and Wolman disease (acid lipase deficiency); (2) immunodeficiencies, including T-cell deficiencies (e.g. ataxia-telangiectasia and DiGeorge syndrome), combined T- and B-cell deficiencies (e.g. severe combined immunodeficiency (SCID), all types), well-defined syndromes (e.g. Wiskott–Aldrich syndrome), phagocyte disorders (e.g. Kostmann syndrome, Shwachman–Diamond syndrome), immune dysregulation diseases (e.g. Griscelli syndrome, type II), innate immune deficiencies (e.g. NF-Kappa-B Essential Modulator (NEMO) deficiency (Inhibitor of Kappa Light Polypeptide Gene Enhancer in B Cells Gamma Kinase deficiency)); (3) hematologic diseases, including hemoglobinopathies (e.g. sickle cell disease, thalassemia (e.g. β thalassemia)), anemias (e.g. aplastic anemia such as Diamond–Blackfan anemia and Fanconi anemia), cytopenias

(e.g. Amegakaryocytic thrombocytopenia) and hemophagocytic syndromes (e.g. hemophagocytic lymphohistiocytosis (HLH)).

[0098] Following administration of genetically modified hematopoietic stem cells to a subject in need thereof, the recipient can be monitored for hematopoietic recovery, reconstitution and/or donor chimerism as indicators for successful engraftment. In some embodiments, engraftment is determined by assessing donor myeloid chimerism. In some embodiments, engraftment is determined by assessing lineage specific chimerism. In some embodiments, engraftment is determined by assessing naïve T cell production. Any method known in the art for assessing donor cell chimerism may be used with the disclosed methods (see e.g. Pinkel et al., *Proc Natl Acad Sci USA* (1996), 83: 2934–2938). In certain embodiments, following transplantation with donor stem cells, the recipient is a chimera or mixed chimera for the donor cells. Mixed chimerism (MC) is defined as the presence of more than 5% host-derived cells on more than one occasion in the whole blood. This is further categorized into high-level MC (95%-50% donor chimerism), low-level MC (49%-10% donor chimerism), or very low-level MC (< 10% donor chimerism).

[0099] 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. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

EXAMPLES

Example 1: Evaluation of Modified Cell Culture and Electroporation Conditions for Gene Editing of CD34+ Stem Cells

[0100] *Summary*

[0101] This study assessed the impact of incorporating gas-permeable bioreactors and large volume electroporation conditions on the quality of gene-edited CD34+ hematopoietic stem cells. Previously described CD34+ HSC manufacturing methods entailed cells cultured in culture bags and electroporated in an electroporation chamber having a 1 ml volume (hereinafter referred to as “Process Version 1.0” or “v1.0”). See e.g. International Patent Application Number

PCT/US2022/072014 (Publication No. 2022/232839) and Lattanzi et al. (*Sci Transl Med.* 2021 Jun 16; 13(598): eabf2444). In this study, a larger electroporation volume (≥ 2 ml), as well as the use of gas-permeable, high-capacity bioreactors, were incorporated into the cell manufacturing workflow, and the resulting drug product (DP) was compared to the original v1.0 process to determine the impact on gene editing performance, cell yield and drug product (DP) quality attributes.

[0102] For prospective hematopoietic stem cell therapy patients, subject weights can range from 40kg to >90kg, which demands the ability to process cells at ranges of $3.2E+08$ CD34⁺ cells to $>1.2E+09$ CD34⁺ cells in order to achieve target dosing. A 1 mL electroporation cuvette/cartridge would require multiple cartridges to perform electroporation at such large scales and presents manufacturing risks such as variability from cartridge to cartridge at the time of electroporation and can increase probability of operator errors. Accordingly, electroporation of gene-editing reagents using low and high electroporation volumes, respectively, were compared side by side across multiple donors of CD34⁺ enriched cells. As detailed below, gene-edited cells manufactured with the higher electroporation volume generated higher double stranded breaks (DSB) compared to cells electroporated with the lower electroporation volume. Higher trends in homologous recombination (HR) and Colony Forming units (CFU) were also observed with the higher electroporation volume.

[0103] Building on these results, the improved electroporation conditions were assessed in the context of cell culture in high-capacity gas-permeable bioreactors. While the use of culture bags during HSC manufacturing processes (e.g. before and after electroporation) is feasible in early stage clinical trials, scalability becomes a concern through progressive stages of DP development. Culture bags do not support ease and feasibility of critical process steps during larger scale manufacturing and lack the features of being closed and semi-automated that could otherwise enable manufacturing throughput, robustness, and scalability.

[0104] As shown in the results below, CD34⁺ enriched cells cultured in gas-permeable high-capacity bioreactors (G-Rex®, Wilson Wolf) prior to and after electroporation in ≥ 2 ml volume showed a significant improvement in cell yield, homologous recombination frequency, Colony Forming Units, and shortened culture compared to cells cultured in bags and electroporated in lower volume. These results demonstrate that the use of gas-permeable, high-capacity bioreactors that enable static cell culture, such as the G-Rex culture system, in combination with higher

electroporation volumes (e.g. ≥ 2 ml, Maxcyte electroporator) significantly improves cell health and enhances DP quality attributes with a higher probability of achieving manufacturing technical success at large scale.

[0105] A. Assessment of Higher Electroporation Volume

[0106] Experimental Design

[0107] A total of 3 donors were used to generate a large-scale comparison of manufacturing processes which utilized cell culture bags (V1.0) but differed by the electroporation volume and electroporation device (see **Table 1**). Donors 1 and 2 were thawed and cultured at the same time in cell culture bags for 72hrs pre-stimulation, then split evenly to be treated with either electroporation condition.

[0108] Table 1. Electroporation Volumes

Donor	Process Version	Electroporator	Electroporation Volume (ml)
Donor 1	V1.0 MX	MaxCyte	6.0
	V1.0 LZ	Lonza	2.0
Donor 2	V1.0 MX	MaxCyte	6.0
	V1.0 LZ	Lonza	2.0
Donor 3	V1.0 MX	MaxCyte	6.0
	V1.0 LZ	Lonza	2.0

[0109] The process flow is detailed below.

[0110] Day 0:

[0111] Cryopreserved CD34+ HSPCs were thawed at 37°C in a controlled water bath and diluted with Thaw Medium; Pulmozyme (DorNase Alfa) and Heparin Sodium. The cells from multiple CD34+ cell selections were pooled together and centrifuged to pellet, and the remaining supernatant was aspirated, and the cells were re-suspended to a concentration of $2.0 - 1.0 \times 10^6$ cells/mL in HSPC cytokine-rich media for pre-stimulation. The cell suspension was transferred to the calculated number of cell culture bags of appropriate size to culture within the range of $2.5 - 5.0 \times 10^5$ cells/mL. The cell culture was incubated in a humidified atmosphere of 5% CO₂ and 5% O₂ at 37°C for a pre-stimulation time ranging from 68-72 hours. USP Nitrogen 97 was used to maintain the 5% O₂ environment.

[0112] Day 3:

[0113] At the end of the cell culture time, the CD34+ enriched HSPCs were transferred to a centrifuge tube, and the cells were pelleted by centrifugation and resuspended in culture media. Post centrifugation, a sample of the cell suspension was taken for measurement of the cell count and cell viability. The CRISPR/Cas9 ribonucleoprotein complex (RNP) was formed by combining 46.2 μ L SpyFi Cas 9 nuclease and 123.3 μ L sgRNA targeting the HBB gene (per 1mL of Electroporation) at room temperature for a minimum of 10 minutes. The material was centrifuged, and the cell pellet was resuspended in electroporation buffer at a final concentration of 50×10^6 to 100×10^6 cells/mL. Another cell culture bag was prepared with HSPC cytokine-rich media, at 1:20 volume ratio of electroporated cell sample suspension to volume of media. An amount of AAV6 comprising a donor DNA sequence was added at a multiplicity of infection (MOI) of 2,500vg (or 1250vg or 625vg) to cells into virus bag. For high-volume electroporation conditions, the MaxCyte Closed Flow Cartridge Collection Bag was welded off and changed to a gas-permeable cell culture bag. The cell suspension, inclusive of RNP, was collected using a syringe and Blunt Cannula Needle with 2mL of air, then loaded into a MaxCyte Closed Flow Cartridge Sample Bag and was electroporated using HSC 6 program on the MaxCyte GTx Unit at 3mL per zap. The product was flowed into the attached cell culture bag. The cell culture bag was then sealed off and placed into an incubator with atmosphere of 5% CO₂ and 5% O₂ at 37°C for 10 minutes. After the 10-minute incubation, the cell culture bag was then welded to the virus containing cell culture bag and transferred using gravity drain technique, and the collection bag was washed twice. The transduced cell suspension was transferred to cell culture bags and cultured at a density of 2.5×10^5 – 5.0×10^5 cells/mL in a 5% CO₂ and 5% O₂ environment at 37°C.

[0114] *Day 4:*

[0115] At 16-20 hours post-gene editing, the cells were transferred to a centrifuge tube and pelleted by centrifugation. The supernatant was aspirated, and the cells were re-suspended in HSPC cytokine-rich media. The media exchange was performed to remove excess virus after transduction. The cells were cultured in 5% CO₂ and 5% O₂ at 37°C in sterile cell culture bags for an additional 16-24 hours.

[0116] *Day 5:*

[0117] After media exchange following gene correction, the cells were transferred to centrifuge tube(s) and were pelleted by centrifugation. The cell pellet(s) were washed with and re-suspended in PlasmaLyte-A buffer (or equivalent) with 2% (v/v) HSA. The cell suspensions, which

constituted drug substance, were combined in a single polypropylene centrifuge tube. No testing was performed prior to pooling the cells. An aliquot of the drug substance was sampled for cell count and viability on an automated cell counter.

[0118] Table 2. Process Changes between Low-Volume (v1.0 LZ) and High-Volume (v1.0 MX) Electroporation

Step/Parameter	V1.0 LZ	V1.0 MX	Rationale for Change	Impact
Day 3 Gene Correction Centrifugation speed	300xg for 10 minutes Brake 7	180xg for 7minutes Brake 3	Improved cell viability prior to EP	Maintains high cell viability essential for EP
Post-centrifugation resuspension buffer	HSPC Cytokine-Rich Media (Supplemented SCGM Media)	MaxCyte Electroporation Buffer	Washout of media components prior to EP	Improved transfection capability
Electroporation Density	50E+06- 200E+06	25E+06-125E+06	Flow through cartridge accommodates larger volumes; process optimized to these ranges	Improves robustness and manufacturing feasibility
RNP Formulation	2.5:1 sgRNA: SpyFiCas9	2.5:1 sgRNA: SpyFiCas9	No Change	Neutral/No change
AAV addition method	Open, AAV added to polypropylene tubes	Closed, AAV added to culture bag containing media, no interim transfer step and hold in polypropylene tubes	Improved manufacturing feasibility	Closed AAV transduction
Time to AAV Addition	Within 20 minutes but target 1-5 mins or as immediately as possible	10" post incubation of electroporated cells in a controlled incubator at 37°C	Allows recovery of cell membrane prior to culture steps	Neutral Reduces time to AAV contact in tubes
Maximum exposure time of cells to EP buffer	25minutes	60 minutes recommended	Manufacturer Recommendation, tested in PD	No Impact, extended exposure time allows washout of media components
Transfer of cell suspension to culture bags	Open transfer from tubes to bags in ISO 5 BSC	Closed transfer from Collection bag into culture bags in ISO 5 BSC	Transition to closed processing	Process Improvement
Day 4 Media Exchange Centrifugation speed	300xg for 10 minutes Brake 7	180xg for 7minutes Brake 3	Improved cell viability	Neutral
Day 5 Drug substance Formulation Centrifugation speed	300xg for 10 minutes Brake 7	180xg for 7minutes Brake 3	Improved cell viability	Neutral

[0119] Selection of Electroporators

[0120] For the high-volume electroporation condition, the Maxcyte GTX Electroporation System was used. This system is capable of closed flow-through electroporation to reduce variability in drug product and utilizes GMP closed processing assemblies which support electroporation ranges from 50uL-100mL, and which mitigate contamination risks compared to open processing assemblies. For the low-volume electroporation condition, the Lonza 4D Nucleofector Electroporation system was used. Similarities and differences between the Lonza and Maxcyte GTx System and processing components are shown in **Table 3**.

Table 3: Comparison of the Lonza and MaxCyte Systems

	Lonza 4D Nucleofector	MaxCyte GTx
EP Program Used for Manufacturing	DZ-100	HSC-4
Cartridge Type	1mL Nucleocuvette closed but not Flow-through	CL2 Closed Flow Through
EP Density Range	50E+06-200E+06	25E+06-125E+06
Volume	1mL	2mL-100mL
EP Buffer	THERAPEAK P3 Primary Nucleofector Solution Set (Solution + Supplement)	Hyclone MaxCyte Electroporation Buffer
EP Buffer Formulation	Operator formulates at point of use by addition of 5mL Supplement to 22.5mL P3 Primary Cell Nucleofector solution	Pre-formulated, no point of use formulation
Buffer Temperature at use	Room Temperature	Cooled at 2-8°C
cGMP Compliance	P3 Solution (Buffer) and supplement are GMP Nucleocuvette cartridge is not manufactured GMP 21CFR Part 11 Compliant software	All Components are GMP manufactured and are CE Marked for EU standards
Operating environment	ISO 5 BSC installation due to use of septum instead of tubing for cartridge loading and unloading Room Temperature	ISO 7 and 8 installations suitable due to use of fully closed processing assemblies and weldable tubing Room Temperature operation

[0121] Results

[0122] Efficiency of Gene Correction and Distribution of Alleles by NGS

[0123] A series of process development studies were performed on G-CSF + Plerixafor mobilized HSPCs to evaluate the distribution of alleles achieved in the drug products manufactured

using the Lonza (low-volume) electroporator as compared to the same process on the same healthy donors using the MaxCyte GTx (high-volume) electroporator. To eliminate donor-to-donor variability, G-CSF + Plerixafor mobilized CD34⁺ cells isolated from 3 donors were tested in a side-by-side study. Donors 1 and 2 were thawed and split on the same day of culturing into separate bags. Donor 3 cells were processed a few weeks apart to evaluate the two electroporators on the same process. On the day of cell thaw, the cell number in the bag was adjusted to match the starting cell number for both arms. For this study, cells from each donor were processed using the following processes:

[0124] End-to-End V1.0 Process with MaxCyte Electroporation (referred to as MX in the figures)

[0125] End-to-End V1.0 Process with Lonza Electroporation (Referred to as LZ in the figures)

[0126] The drug product batches generated were evaluated for cell viability, % CD34 purity, % homologous recombination and allele distribution, % CFU, % INDELS at an off-target site (OT-1) and frequency of translocations.

[0127] *Cell Viability*

[0128] The cell viability for these studies was measured daily in-process as well as on the drug products by thawing drug products generated from cryopreserved satellite vials followed by cell count using the NC-202 cell counter and AO/DAPI staining. The measurements of daily viability were included to identify whether any of the processing steps introduced a risk of low viability. The daily viability measurements did not exhibit any differences between the in-process viabilities that could be attributed to the electroporation steps. The batches generated for both arms demonstrated similar viability post thaw and were above release specification of 70%. The mean viability of the three V1.0 MX EP batches was 83.9% and the three V1.0 LZ EP batches was 84.5%. Post thaw drug product viability was predicted by measuring %Apoptosis using Annexin V staining kit and flow cytometry analysis. Cells were thawed and cultured using CRM for 48hrs and stained for Annexin V protein. Annexin V positive cells are determined to be in apoptotic stage. V1.0 MX drug product cultured for 48hrs post thaw had an average of 20.6% apoptotic cells and V1.0 LZ drug product had an average 17.03% apoptotic population.

[0129] *On-Target Allele Distribution*

[0130] Measurements for assessing allele distribution were performed by next-generation sequencing (NGS) using an amplicon sequencing assay, the results of which are shown in

FIGURE 2. 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 notable observation during these studies was that the targeting efficiency, measured by the sum of the HR and INDEL alleles (HR+INDELS), was increased using the high-volume electroporation condition (V1.0 MX) versus the low-volume electroporation condition (V1.0 LZ), which may be due to increased DSB formation. The improved targeting efficiency is indicative of a reduction of the unmodified WT alleles. Additionally, the higher targeting efficiency is likely to increase the frequency of biallelic HR/HR and monoallelic HR/INDEL and HR/WT events that would generate high frequencies of gene correction.

[0131] To estimate the frequency of electroporated cells with at least one corrected HR allele, bulk CFU clones were picked and analyzed by NGS for genotyping. Positive clones with HR alleles were identified by either biallelic corrective events (HR/HR) or monoallelic HR events (HR/INDEL, HR/WT, HR/SNP). For cells derived from a sickle cell patient, clones with at least 1 corrective HR event would produce functional HbA compared to those with non-curative HBB production (INDEL/INDEL, INDEL/SNP, INDEL/WT, WT/WT). As shown in **FIGURE 3**, cells that were edited under the high-volume electroporation condition (V1.0 MX) exhibited a higher frequency of biallelic and monoallelic HR events compared to cells that were edited under the low-volume electroporation condition (V1.0 LZ). An average 62% (n=3 donors) of colonies from the high-volume electroporation condition exhibited at least one corrective HR allele with an average of 30% presenting biallelic corrective HR events. >30% cell frequency of at least one corrected allele exceeds the 20% threshold for donor cell chimerism needed for hematologic cure after HSCT transplantation in SCD.

[0132] *Frequency of Off-Target INDELS at OT-1*

[0133] The safety of gene edited cell therapy drug products is in part assessed by monitoring editing-associated mutations at off-target loci. A target sequencing method to directly assess sequence changes at an off-target locus in connection with HBB gene editing has been developed and qualified. Briefly, previous studies as reported in Lattanzi et al. (*Sci Transl Med.* 2021 Jun 16; 13(598): eabf2444) used GUIDE-seq, CIRCLE-Seq and COSMID to identify OT (Off Target) sites using the msR02-HBB sgRNA used in the current study. The presence of the off-target sites was interrogated on toxicology lots where the single OT1 site was located in Chr9:101,833,584 to

101,833,606 in an intergenic region closest to the GRIN3A gene. The ranges of INDELS at OT-1 were noted at a range of 0.5% to 2.9% that demonstrated no genotoxicity.

[0134] As shown in **FIGURE 4**, while the observed frequency of INDELS at OT-1 in gene-edited cells electroporated under the high-volume condition (V1.0 MX) was slightly higher than that observed in gene-edited cells electroporated under the low-volume condition (V1.0 LZ), the overall frequency of INDELS being within the range of 0.5-2.9% is not expected to cause genotoxicity based on previous toxicology studies in mice. Besides the frequency of INDELS, an assessment of the frequency of translocations by Digital Droplet PCR (ddPCR) showed that the frequency was below the detectable and previously reported threshold of 0.04% (see **Table 4** below).

[0135] *Impact on Cell Yield*

[0136] The cell yield of CD34+ stem cells, having undergone a gene correction process from thaw to drug product, can be influenced by several factors including cell growth kinetics, donor-to-donor variability, loss of cells during the gene correction process, recovery of the cells from the gene correction process and losses incurred during cell washing and from cryopreservation. To compare the yields using the two electroporation conditions, the recovery of cells from the gene correction step to drug product formulation was compared. The results, summarized in **Table 4** below, showed slightly higher %CD34 yields for HSCs processed under V1.0 MX compared to V1.0 LZ.

Table 4: Impact of high vs low-volume electroporation on gene editing performance

Donor	Electroporator	% Viability	% CD34	EP-DP Recovery	D0-D5 Yield	%HR	% INDELS	% WT	% SNP	% CFU	% HSC (CD34+CD38-CD45RA-CD90+)	INDELS at OT-1	Translocations by ddPCR
DN1	MaxCyte Lonza	84.3	83.93	88.67	88.7	45.37	36.53	13.88	4.22	26.4	20.3	1.39	Not detected
		84.5	81	147.33	147.3	37.45	35.48	22.64	4.43	28.8	17.58	1.15	Not detected
DN2	MaxCyte Lonza	81.2	89	151.33	151.3	49.77	32.39	12.92	4.92	29.2	21.01	1.31	Not detected
		81.6	89.2	142	142	44.06	30.82	21.17	3.95	23.8	21.04	1.37	Not detected
DN3	MaxCyte Lonza	86.3	92.9	103.73	103.7	45.92	32.8	16.62	4.66	30	27	1.5	Not detected
		87.5	87.7	139.86	139.9	35.75	33.41	25.98	4.86	23.9	20.05	0.92	Not detected

[0137] **B. Assessment of Gas-permeable High-Capacity Bioreactors in Combination with High-Volume Electroporation**

[0138] The optimized electroporation conditions described above (V1.0 MX) were further optimized to incorporate modified culture conditions to assess if the quality of gene-edited CD34+ HSCs could further be improved. Proof-of-concept process development studies were executed to

estimate the feasibility of culturing isolated CD34⁺ stem cells in gas-permeable bioreactors. G-Rex bioreactors (Wilson Wolf) were selected for featuring a completely closed culture system with semi-automation, which introduces less operation variabilities in the process. The gas-permeable membrane and vessel design eliminates the need to actively deliver oxygen and nutrients by agitation, minimizing the need for manual manipulations. Additionally, these bioreactors can hold larger medium volumes (more nutrients) which allows the cells to reach a maximum density without complexity or need for media exchange.

[0139] A series of small-scale optimization studies were performed to identify the most optimal parameters for culturing HSPCs for manufacturing of gene-edited cells. Table 5 represents a summary and rationale for these studies.

Table 5: Changes in process parameters with change in culture vessel for the production of gene-edited HSPCs

Conditions Optimized	Driver	Preliminary Operating Range/ Solutions Identified
G-REX Seeding Density	Seeding is not based on volumetric density (cells/ml), but rather the surface density (cells/cm ²)	2e6 – 4e6 cells/cm ²
Culture environment	Commercial cell therapy manufacturing facilities commonly restricts humidification	37C, 5%CO ₂ + 5%O ₂ + 0%RH
Culture duration with current media formulation	Maintain HSC Phenotype, literature	2+2 or 3+1
AAV Wash Out Step	Minimize cell manipulation and shear forces in system	90% Media replacement to remove cellular debris and process residuals; cells remain undisturbed on the membrane surface.
Closed Volume Transfers	Fully closed System	Use of gas-permeable bioreactors (e.g. G-Rex)

[0140] Proof of Concept (PoC) process development studies were executed to assess the ability to generate gene-edited HSPC drug product at large-scale (150 million+ cells) meeting key quality attributes for safety, identity, potency, and purity. Based on initial optimization in small scale, HSPCs from three donors were cultured at large scale in G-Rex 100M bioreactors. In addition to the change from culture bags to gas-permeable bioreactors, two additional process changes to the v1.0 process were implemented: (1) replacement of the open centrifugation steps prior to electroporation; and (2) large volume (>2ml) electroporation for the gene editing steps (as described above). These were performed on the Rotea CTS and MaxCyte, respectively, to enable

closed, semi-automated electroporation. This revised process is designated as “V1.1” in the figures referenced below. The resulting gene-edited HSPCs were evaluated for viability, cell yield, % homologous recombination (HR) and allele distribution, % Indels at OT-1, and % CFU.

[0141] Per the v1.0 process in culture bags, the cells are usually cultured for at least 72 hours to achieve 1.2x-1.5x proliferation prior to electroporation with gene editing reagents including CRISPR/Cas9 and AAV6 donor, then cultured for two additional days. Although this process worked well in culture bags, there were concerns about hyperproliferation and loss of HSC phenotype with this “3+2”-day culture regimen when using the G-REX system, due to its enhanced gas and nutrient distribution capabilities. Accordingly, two process variants were identified for the assessment of G-REX + high-volume electroporation at scale:

[0142] (1) 3 + 1: electroporate cells at 72 hours post thaw, and harvest the following day; and

[0143] (2) 2 + 2: electroporate cells at 48 hours post thaw, and harvest 48 hours post electroporation.

[0144] *Experimental Design*

[0145] Four healthy donor apheresis samples underwent CD34+ isolation via a LOVO and CliniMACS Plus selection procedure. Brief analytics were performed throughout the process as depicted in **FIGURE 5**.

[0146] **Results**

[0147] *Cell Growth Kinetics – Viability and Fold Increase*

[0148] The cell counts and viability were assessed at cell thaw, pre-electroporation (“pre-zap”), at media change following gene editing, and at harvest, as well as on the gene-edited product following the thaw of cryopreserved satellite vials. Measurements were obtained using NC-202 cell counter and AO/DAPI staining. The viability and cell counts were measured on each day to monitor cell growth and cycling to identify optimal electroporation day and harvest day. As shown in **FIGURE 6**, viability of cells subjected to V1.1, 2+2 conditions (Batches 1-4) was largely maintained throughout the process at above 90%, while cells subjected to V1.0 LZ, 3+2 showed viability that dropped to 80% at DP post-thaw.

[0149] As shown in **FIGURE 7**, cells showed higher proliferation when subjected to the V1.1, 2+2 process (Batches 1-4), exhibiting an average product yield of 176% with 1 less day in the manufacturing process, compared to 112% yield for the V1.0, 3+2 process engineering runs. These results are indicative of improved DP survival, proliferative capability, and cell fitness post gene

correction when utilizing gas-permeable high-capacity bioreactors and large volume electroporation. The improved yield with the shortened culture duration is promising and presents an opportunity to further alter culture conditions to improve HSC engraftment kinetics if needed. Alternatively, starting collection doses of CD34+ cells from SCD patients could be reduced from $8E+06$ CD34+ cells to $\geq 5E+06$ CD34 cells/kg with high probability of achieving drug product dose.

[0150] *On-Target Allele Distribution*

[0151] As shown in **FIGURE 8**, the allele distributions per NGS of the representative gene-edited product from HSCs cultured in gas-permeable bioreactors and electroporated at high volume (V1.1 process) exhibited higher homology direct repair (HR) and total editing events (INDEL + HR) compared to HSCs that were cultured in culture bags and electroporated under low-volume conditions (V1.0 LZ process). In particular, V1.0 LZ yielded HR rates of about 35%, and total editing rates (INDEL + HR) of about 75%, when using an AAV6 DNA donor at an MOI of 2500 (**FIGURE 8A**, left), while V1.1 yielded HR rates of between 55%-65%, and total editing rates (INDEL + HR) of about 90% (n=3) when using an AAV6 DNA donor MOI of 2500 (**FIGURE 8B**). The V1.1 process also enabled the use of lower amounts of the AAV6 donor (MOI=625, 1250; **FIGURE 8A**, center and right) while achieving the same or higher levels of editing compared to the V1.0 LZ process using the AAV6 donor at an MOI=2500. The frequency of off-target INDELs at OT-1 was very similar with both the V1.0 LZ and V1.1 processes (>2%), which supports safety and non-genotoxicity of the drug product.

[0152] *Colony Forming Units*

[0153] Colony forming units are a metric assessment for trilineage differentiation and clonogenicity potential of HSPCs. The drug product was thawed and plated to see the CFU potential. As shown in **FIGURE 9**, cells edited with either RNP only or RNP plus AAV donor at MOIs of 625, 1250 and 2500 under the V1.1 process showed comparatively higher CFU growth to cells edited under the V1.0 LZ process. The RNP only control represents clones generated in drug products without AAV6 addition, as increasing amounts of AAV was previously shown to exhibit decreased colony forming capability (Naldini et al., *Cell Stem Cell*, (2022); 29(10): 1428–1444.e9). As shown in **FIGURE 9A**, cells edited under V1.1 exhibited improved CFU across all editing conditions tested compared to cells edited under V1.0 LZ. Fold improvement in CFU exhibited in V1.1 controls compared to V1.0 LZ controls demonstrates overall improvement in the

cell culture and electroporation process when utilizing gas-permeable bioreactors and high-volume electroporation.

[0154] In summary, manufacturing processes which included high-volume electroporation and cell culture in gas-permeable high-capacity bioreactors demonstrated better cell viability and product yield compared to the V1.0 LZ process (low-volume electroporation, culture bags). Since the improved process had higher cell expansion, the manufacturing process could be reduced by one day. Higher %HR distribution and CFUs were observed in drug product generated using the improved process across all the donors tested. A summary of the study results is provided in Table 6.

[0155] Table 6: Improved gene editing, cell yield and drug product (DP) quality attributes following implementation of cell culture in gas-permeable high-capacity bioreactors and high-volume electroporation

Targets	V1.0 LZ (N=3; ENG 3, 4, 5)	V1.0 MX N=7 LS Runs	V1.1 (N=3; POC)
≥ 40% HR (>80% INDELS+HR) by NGS* (ddPCR currently used)	41	44	63
≥ Cell Viability in Drug Product	82	84	93
≥ 80% Cell Yield	112	134	176
Target HR:NHEJ ≥ 1	1.4	1.2	1.9
%CFU	26	30	34

[0156] All publications and patent, applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. While the claimed subject matter has been described in terms of various embodiments, the skilled artisan will appreciate that various modifications, substitutions, omissions, and changes may be made without departing from the spirit thereof. Accordingly, it is intended that the scope of the subject matter limited solely by the scope of the following claims, including equivalents thereof.

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 cm² to about 1000 cm² 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. In some embodiments, the gas-permeable 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 25×10^6 to 100×10^6 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 35 to 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, 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 administering gene-edited primary cells to a patient in need thereof.

59. The process of claim 58, wherein the gene-edited primary cells are 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. A method of cell therapy comprising administering the plurality of genetically modified primary cells of claim 59 to a subject in need thereof.

62. The method of claim 61, wherein the cell therapy is an autologous cell therapy.

63. The method of claim 61, wherein the cell therapy is an allogenic cell therapy.