

CONDITIONALLY IMMORTALIZED STEM CELLS AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 63/493,540 filed on March 31, 2023, the entire contents of which are incorporated herein by reference.

REFERENCE TO SEQUENCE LISTING

[0002] The Sequence Listing associated with this application is provided in text format in lieu of a paper copy and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is 31C7646_ST26.xml. The text file is 102,400 bytes, was created on March 23, 2024, and is being submitted electronically via Patent Center.

FIELD OF THE DISCLOSURE

[0003] The current disclosure provides conditionally immortalized stem cells and uses thereof.

BACKGROUND OF THE DISCLOSURE

[0004] Cell therapy is a promising field for the treatment of medical disorders. For example, the use of engineered cells for cellular immunotherapy allows for treatment of cancers or other diseases by leveraging various aspects of the immune system to target and destroy diseased or damaged cells. During cell therapy, cells from various sources can be transplanted into a subject for treatment of a disease. If primary cells are used for transplantation, continuous access to fresh tissue sources is required. Such therapies require cells in numbers sufficient for therapeutically relevant doses, however, it can be difficult to collect a desired number of cells especially if the cells are sourced from primary tissue. Normal human somatic cells, for example, have a finite duplication capacity and can reach cellular senescence especially when cultured in vitro.

[0005] Another drawback of cell therapy is derived from the use of an allogeneic cell product. An allogeneic cell product refers to cells that are obtained from individuals belonging to the same species but are genetically dissimilar. Use of these cells can result in an immune response upon transplantation into a subject in a process termed host versus graft rejection or the process termed graft versus host disease. In the former, the patient's existing immune system attacks the transplanted cells as foreign. In the latter, it is the transplanted cells that attack the patient's cells as foreign.

[0006] Furthermore, for cellular therapy, cell manufacture, and research purposes, it would be ideal to have a cell product that is pre-manufactured, well characterized, and available for immediate use. Because stems cells can be an important precursor to generate or regenerate

organs, repair tissues, prepare or deliver certain biological factors, or treat diseases or disorders, they may be a useful candidate in generating pre-manufactured cell lines for multiple purposes.

SUMMARY OF THE DISCLOSURE

[0007] The current disclosure provides conditionally immortalized stem cells and uses thereof.

[0008] Particular embodiments utilize stem cell-derived cell populations that are modified to include a conditional (e.g., drug-inducible) immortalization gene (e.g., TERT and SV40 large T antigen). In particular embodiments, the conditional immortalization gene prevents cell senescence when a growth controlling agent is administered to the stem cells. These embodiments are particularly useful to provide immortalized cell populations (e.g., differentiated cell populations) that can be maintained as immortal cell lines by the administration of a growth controlling agent (e.g., drug).

[0009] Particular embodiments utilize stem cells modified to include a conditional immortalization gene and factors that support use as feeder cells during cell culture. These embodiments are particularly useful to generate immortalized feeder cells. Feeder cells are cell that provide factors to help a cell population of interest to proliferate. Feeder cells can be adherent cells (e.g., mesenchymal stem cells) or suspension cells (e.g., CD34+ cells). Immortalized feeder cells can be genetically modified to support growth of particular cell types, such as expression of membrane-bound IL21 and/or knock-out of MHC Class I and/or Class II to support growth of natural killer (NK) cells. These embodiments may also include a suicide switch to reduce contamination of cell populations of interest with feeder cells.

[0010] Particular embodiments utilize stem cells modified to include a conditional immortalization gene and factors that support use as tester cells during research and development. These embodiments are particularly useful to generate immortalized tester cells. Examples include tester cells that express a cancer antigen or a viral antigen to test efficacy of antibodies, recombinant receptors, or similar therapeutic treatments under development. The expression of a viral antigen can be used as living vaccine that allows for extended antigenic presentation in a physiologically appropriate manner. When manufactured for *in vivo* use, these immortalized tester cells may also express a detectable label, such as fluorescent proteins and/or luciferase. These embodiments may also include a suicide switch.

[0011] Particular embodiments utilize stem cells modified to include a conditional immortalization gene and a suicide gene. These embodiments are particularly useful to generate immortalized cell populations (e.g., differentiated cell populations) for a therapeutic purpose. The immortalization gene can prevent cell senescence during cell manipulation and culture with the

administration of a growth controlling agent until administration of the cell population to a subject, at which point the growth controlling agent can be withdrawn. Furthermore, the suicide gene provides an additional safety feature by causing the apoptosis (programmed cell death) of genetically modified cells both during cell manufacture and/or after administration to a subject. For example, the suicide switch provides a safety feature allowing the removal of proliferating cells from cultured cells in vitro before use as a therapeutic cell population. Further, their effect can be canceled after administration to a subject. If an unwanted side effect of their administration were to occur. In particular embodiments, a therapeutic cell can be further genetically modified to include factors that support use as a therapeutic cell such as proteins, antibodies, or recombinant receptors (e.g., chimeric antigen receptors).

BRIEF DESCRIPTION OF THE FIGURES

[0012] Some of the drawings submitted herewith may be better understood in color. Applicant considers the color versions of the drawings as part of the original submission and reserves the right to present color images of the drawings in later proceedings.

[0013] FIG. 1. Schematic of insertion of cell division essential locus and a suicide gene.

[0014] FIGs. 2A-2D. Epitope Line Demonstration. (2A) In vitro assay was performed in PAN3 cell line or PLSX11 cell line with major histocompatibility complex (MHC) I and II knockout. (2B) Schematic of example genetic construct for transposon-based gene editing containing an example expression product and luciferase. (2C) Flow cytometry results of in vitro expression of genetic construct for CD19 and BCMA detection. The pBP plasmid successfully transduced cells. (2D) In vivo detection of luciferase expressing tumor cells.

[0015] FIG. 3. Using Feeder Lines to Expand Adult and cord blood-derived natural killer (NK) cells. The production of activated NK cells from naïve NK cells can traditionally include the addition of cytokines, autologous accessory cells, irradiated autologous feeder cells, and/or irradiated allogeneic feeder cells (e.g., modified K562 or EBV-LCL cells). The activated NK cells have increased cell number, enhanced natural cytotoxicity and antibody-dependent cell-mediated cytotoxicity, and enhanced secretory function.

[0016] FIG. 4. Characteristics of immortalized hiPSCs further edited. Morphology of parental iPSCs (SK005.3) and the immortalized line.

[0017] FIG. 5. Expression level analysis of FMC63 clonal lines by qPCR and FACS shows the transgene expression in clones. The successful insertion and expression of the transgenes, FMC63 and TK.007, were confirmed by FACS analysis of FMC63 protein as well as qPCR of FMC63 and TK.007 transcripts. The transgene copy number in each clone was determined via

digit-droplet PCR, ranging from 13 copies to 28 copies. The copy number correlates to both the mRNA and protein level of each of the transgene.

[0018] FIGs. 6A, 6B. (6A) Inducible immortalization expression vectors. The construct map and detailed plasmid map of inducible immortalization expression vectors. (6B) Work flow of editing a previously edited iPSC line genome to contain an inducible immortalization gene. The workflow of generating FMC63-IL15 chimeric antigen receptor (CAR)+ Thymidine Kinase expressing SK005.3 hiPSC and the further insertion of immortalization vectors in this edited iPSC. SK005.3 hiPSC were co-transfected with FMC63 CAR plasmid and PiggyBac (PBac) transposase plasmid using Lipo3000 transfection reagent. Clones that express high levels of transgenes were signal cell sorted and expanded. These clones were further transfected with the immortalization vector using the sleeping beauty transposon system. Clones with successful immortalization vector insertion were enriched via Neomycin drug selection and expanded.

[0019] FIG. 7. Expression analysis of Doxycycline (DOX) induction of inducible immortalization gene hTERT and SV40 LT in the previously edited iPSC lines. The successful induction of the expression of immortalization factors via Doxycycline treatment is shown. Under the Tet-inducible system, in the absence of doxycycline, very low levels of hTERT and SV40 large T antigen transcripts were detected by qPCR, likely due to the leakiness of the Tet-inducible system. In the presence of doxycycline, hTERT and SV40 transcripts levels increase significantly in comparison to no doxycycline and in a clear dose-dependent manner. In contrast, there is no difference in the transcript levels of rtTA and FMC63 CAR, which were not under Tet-inducible, in the presence or absence of doxycycline. Each data bar was an average of triplicate technical measurements. The error bar represents SEM. Nd: non-detectable.

[0020] FIGs 8A-8F. (8A) Representative phase contrast images (4x objective) of cell morphology for unmodified and TetON hTERT SV40 iPSC cultures upon expansion in iPSC culture medium for 3 passages, in the absence or presence of 0.1 μ M Doxycycline Hyclate (DOX) are shown. (8B) RT-PCR analysis is shown for the expression of hTERT, SV40, and rtTA transcripts in unmodified and hTERT SV40 iPSCs (normalized to the YWHAZ gene), after induction with 0.1 μ M Doxycycline Hyclate (DOX) for 12 days. DOX treatment increased the expression of inducible hTERT and SV40 transcripts in the hTERT SV40 iPSC line only, while the rtTA genes was constitutively expressed in the hTERT SV40 iPSC line but not in the unmodified control cell line. (8C) The diagram of the hematopoietic progenitor cell differentiation process is shown. iPSC are thawed and expanded before they are passaged into AggreWells to generate embryoid bodies (EBs). After 5 days of culture in AggreWells, the EBs are transferred to a 6-well plate. At day 12 of the differentiation, EBs are dissociated, positively-selected for CD34 expression and

phenotypically characterized for hematopoietic progenitor cells surface marker expression. (8D) Representative phase contrast images (4x objective) are shown of TetON hTERT SV40 iPSC-derived EBs in AggreWells on day 2 of the differentiation and EBs in 6-well plates on day 12 of the differentiation, before harvest. Doxycycline Hyclate (DOX) treatment increased the size of EB compared to the no treatment control, suggesting the induction of immortalization genes promotes cell proliferation during EB formation. (8E) The viability and the viable cell yield are shown of the cell fraction obtained after enrichment through CD34-positive selection following EB dissociation, for Unmodified and TetON hTERT SV40 iPSCs, with and without 0.1 μ M Doxycycline Hyclate (DOX) treatment. DOX treatment increased the viability and viable cell yield of the positively-selected cell fraction compared to the no treatment control. (8F) Flow cytometry histograms are shown for measurement of CD34 protein expression of cells stained with CD34-FITC antibody after CD34-positive selection and the percentage of CD34-positive cells for each of the Unmodified and TetON hTERT SV40 iPSC lines, with and without DOX treatment.

[0021] FIG. 9. The diagram of the natural killer (NK) cell differentiation process is shown. iPS cells are thawed and expanded before they are passaged into AggreWells to generate EBs. After 5 days of culture in AggreWells, the EBs are transferred to a 6-well plate. At day 12 of the differentiation, EBs are dissociated, positively-selected for CD34 expression, phenotypically characterized for hematopoietic progenitor cells surface marker expression, and seeded for Lymphoid Progenitor Cell differentiation. After 14 days of culture, Lymphoid Progenitor Cells are harvested, phenotypically characterized for cell surface marker expression and seeded for NK Cell differentiation.

[0022] FIG. 10. Generating iPSC lines with overexpression of CD19 and BCMA by PiggyBac Transposon. The workflow of generating hCD19 or BCMA transgene PAN3 and SK005.3 hIPSC line. hCD19 and BCMA were co-transfected PAN3 and SK005.3

[0023] FIG. 11. A list of CAR-T targeted antigens that could be used to make tester lines for evaluating CAR T cells.

[0024] FIG. 12. The piggyBac and Lentivirus vector design allows insertion of other CAR-T targeted antigens. A schematic of the piggyBac transposon vector and Lentivirus vector are shown.

[0025] FIGs. 13A, 13B. Fluorescence and Bioluminescence principle. (13A) Fluorescence achieved in target cells by expression of a Green Fluorescent Protein (GFP) driven by a ubiquitous promoter (CAG). Induction of light emission requires an excitation light. (13B) Bioluminescence achieved by expression of Luciferase driven by an ubiquitous promoter (CAG). Induction of luminescence requires the presence of the luciferin enzyme.

[0026] FIGs. 14A, 14B. Differentiation of immortalized iPSC line SK005.3-hTertSV40 to MSCs. (14A) Morphology change during differentiation of the immortalized iPSC line SK005.3-hTertSV40. (14B) Flow cytometry data showing the amount of expression of CD105 and CD73 for the differentiated cells.

[0027] FIGs. 15A, 15B. (15A) Schematic of cell lines used (Lines A, B, and C). (15B) Genotyping of lines used in NK assay.

[0028] FIG. 16. MSC phenotype was assessed for CD90 and CD105 expression on cell surface by Flow Cytometry. A limited panel of MSC markers, CD90 and CD105 were used to assess the phenotype of the cells from Lines A, B, and C. All lines were highly positive for the 2 essential MSC markers against unstained and isotype controls.

[0029] FIG. 17. HLA ABC surface expression. Class I/II knockout (KO) was assessed based on expression of HLA type ABC measured via flow cytometry. Line A was unedited and therefore was highly positive for HLA ABC, while Lines B and C were significantly lower in HLA ABC expression confirming the intended gene edit for Class I/II KO.

[0030] FIG. 18. State of co-culture at the beginning of assay (Day 0).

[0031] FIG. 19. State of co-culture on Day 3 of the co-culture assay.

[0032] FIG. 20. NK Activation and Expansion – Count and viabilities. Viability and count for NK control (black circles), iNK only (black square), NK on MSC (white circles), iNK on MSC (white square), and MSCs co-cultured with NK cells (black diamonds) are presented.

[0033] FIGs. 21A, 21B. Restimulation of Activated NK cells. (21A) NK cell number over 3 days of re-stimulation (Day 6 of co-culture). (21B) 4x magnification images of NK cells on feeder Line C at Day 3 before transfer on fresh feeders (left) and on Day 6 (right). The insert shows a 10x magnification of the same image.

DETAILED DESCRIPTION

[0034] The current disclosure provides conditionally immortalized stem cells and uses thereof. The current disclosure also provides immortalized cell lines generated from conditionally immortalized stem cells and uses thereof.

[0035] Particular embodiments utilize stem cells that are modified to include a conditional (e.g., drug-inducible) immortalization gene (e.g., TERT and SV40 large T antigen). In particular embodiments, the conditional immortalization gene prevents cell senescence when a growth controlling agent is administered to the stem cells. These embodiments are particularly useful to provide immortalized cell populations (e.g., differentiated cell populations) that can be maintained as immortal cell lines by the administration of a growth controlling agent (e.g., drug).

[0036] Particular embodiments utilize stem cells modified to include a conditional immortalization gene and sequences encoding an expression product that support use as feeder cells during cell culture. These embodiments are particularly useful to generate immortalized feeder cells. Feeder cells are cell that provide factors to help a cell population of interest to proliferate. Feeder cells can be adherent cells (e.g., mesenchymal stem cells) or suspension cells (e.g., CD34+ cells). Immortalized feeder cells can be genetically modified to support growth of particular cell types, such as expression of membrane-bound IL21 and/or knock-out of MHC Class I, MHC Class II, or MHC Class I and Class II to support growth of natural killer (NK) cells. These embodiments may also include a suicide switch to reduce contamination of cell populations of interest with feeder cells.

[0037] Particular embodiments utilize stem cells modified to include a conditional immortalization gene and sequences encoding an expression product that support use as tester cells during research and development. These embodiments are particularly useful to generate immortalized tester cells. Examples include tester cells that express a cancer antigen or a viral antigen to test efficacy of antibodies, recombinant receptors, or similar therapeutic treatments under development. The expression of a viral antigen can be used as living vaccine that allows for extended antigenic presentation in a physiologically appropriate manner. When manufactured for *in vivo* use, these immortalized tester cells may also express a detectable label, such as fluorescent proteins and/or luciferase. These embodiments may also include a suicide switch.

[0038] Particular embodiments utilize stem cell-derived cell populations modified to include a conditional immortalization gene and a suicide gene. These embodiments are particularly useful to generate immortalized cell populations (e.g., differentiated cell populations) for a therapeutic purpose. The immortalization gene can prevent cell senescence during cell manipulation and culture with the administration of a growth controlling agent until administration of the cell population to a subject, at which point the growth controlling agent can be withdrawn. Furthermore, the suicide gene provides an additional safety feature by causing the apoptosis (programmed cell death) of genetically modified cells both during cell manufacture and/or after administration to a subject. For example, the suicide switch provides a safety feature allowing the removal of proliferating cells from cultured cells *in vitro* before use as a therapeutic cell population. Further, their effect can be canceled after administration to a subject. If an unwanted side effect of their administration were to occur. In particular embodiments, a therapeutic cell can be further genetically modified to include factors that support use as a therapeutic cell such as proteins, antibodies, or recombinant receptors (e.g., chimeric antigen receptors).

[0039] Aspects of the current disclosure are now described with more supporting options and

detail as follows: (I) Stem Cell Types; (II) Differentiation of Stem Cells; (III) Genetic Modification of Cells; (III-A) Conditional Immobilization; (III-B) Expression Products; (III-B-1) Protein; (III-B-2) Recombinant Receptors; (III-B-3) Detectable Labels; (III-C) Major Histocompatibility Class (MHC) Molecules; (III-D) Suicide Gene; (III-E) Other Control Features; (IV) Culture and Storage of Cells; (V) Cell-based Formulations; (VI) Methods of Use; (VI-A) Feeder Cells for Cell Manufacturing; (VI-B) Tester Cells for Research and Development; (VI-C) Conditionally Immortal Therapeutic Cell Line; (VII) Exemplary Embodiments; (VIII) Experimental Examples; and (IX) Closing Paragraphs. These headings are provided for organizational purposes only and do not limit the scope or interpretation of the disclosure.

[0040] (I) Stem Cell Types. Stem cells are cells capable of differentiation into other cell types, including those having a particular, specialized function (e.g., tissue specific cells, parenchymal cells and progenitors thereof). There are various classes of stem cells, which can be characterized in their ability to differentiate into a desired cell/tissue type. For example, stem cells can be totipotent, pluripotent, multipotent, or unipotent.

[0041] As used herein, the term “totipotent” or “totipotency” refers to a cell's ability to divide and ultimately produce an entire organism including extra embryonic tissues *in vivo*. In one aspect, the term “totipotent” refers to the ability of the cell to progress through a series of divisions into a blastocyst *in vitro*. The blastocyst includes an inner cell mass (ICM) and a trophoblast. The cells found in the ICM give rise to pluripotent stem cells that possess the ability to proliferate indefinitely, or if properly induced, differentiate in all cell types contributing to an organism. Trophoblast cells generate extra-embryonic tissues, including placenta and amnion. Totipotent stem cells can include fertilized oocytes, cells of embryos at the two and four cell stages of development, that have the ability to differentiate into any type of cell of the particular species. For example, a single totipotent stem cell could give rise to a complete animal, as well as to any of the myriad of cell types found in the particular species (e.g., humans).

[0042] Totipotent stem cells are the source of pluripotent stem cells. As used herein, the term “pluripotent” refers to a cell's potential to differentiate into cells of the three germ layers: endoderm (e.g., interior stomach lining, gastrointestinal tract, the lungs), mesoderm (e.g., muscle, bone, blood, urogenital), or ectoderm (e.g., epidermal tissues and nervous system). Pluripotent stem cells can give rise to any fetal or adult cell type including germ cells. However, pluripotent stem cells alone cannot develop into a fetal or adult animal when transplanted *in utero* because they lack the potential to contribute to extra embryonic tissue (e.g., placenta *in vivo* or trophoblast *in vitro*). “Progenitor cells” can be either multipotent or pluripotent. Progenitor cells are cells that can give rise to different terminally differentiated cell types, and cells that are

capable of giving rise to various progenitor cells. A standard art-accepted test of pluripotency includes the ability to form a teratoma in 8-12 week old SCID mice; however identification of various pluripotent stem cell characteristics can also be used to detect pluripotent cells. "Pluripotent stem cell characteristics" refer to characteristics of a cell that distinguish pluripotent stem cells from other cells. The ability to give rise to progeny that can undergo differentiation, under the appropriate conditions, into cell types that collectively demonstrate characteristics associated with cell lineages from all of the three germinal layers (endoderm, mesoderm, and ectoderm) is a pluripotent stem cell characteristic. Expression or non-expression of certain combinations of molecular markers are also pluripotent stem cell characteristics. For example, human pluripotent stem cells express at least some, and in some embodiments, all of the markers from the following list: SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49/6E, ALP, Sox2, E-cadherin, UTF-1, Oct4, Rex1, and Nanog. Cell morphologies associated with pluripotent stem cells are also pluripotent stem cell characteristics. In particular embodiments, pluripotency can be verified by reviewing cell morphology, TRA1-60 live staining, performing flow cytometry for pluripotency markers, and/or alkaline phosphatase staining. In particular embodiments, reviewing the morphology of the cell includes looking for colonies with well-defined borders, looking for cells with an enlarged nucleus, and/or looking for cells with a high nucleus to cytosol ratio. In particular embodiments, performing flow cytometry for pluripotency markers includes performing flow cytometry for SSEA-4, Oct4, Nanog, and Sox2. Pluripotent stem cells include embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC).

[0043] The term "embryonic stem cell" used in the present invention is a cell cultured by separating and culturing the inner cell mass (inner cell mass) of the blastocyst, which is an early stage of development after fertilization. Although pluripotent human embryonic stem cells (hESC) derived from human blastocysts are promising sources for cell-based therapies to treat diseases and disorders such as Parkinson's disease, cardiac infarction, spinal cord injury, and diabetes mellitus, their clinical potential has been hampered by their immunogenicity and ethical concerns.

[0044] Cord blood stem cells refer to a population enriched in hematopoietic stem cells, or enriched in hematopoietic stem and progenitor cells, derived from human umbilical cord blood and/or human placental blood collected at birth. The hematopoietic stem cells, or hematopoietic stem and progenitor cells, can be positive for a specific marker expressed in increased levels on hematopoietic stem cells or hematopoietic stem and progenitor cells, relative to other types of hematopoietic cells. For example, such markers can be, but are not limited to CD34, CD43, CD45RO, CD45RA, CD59, CD90, CD109, CD117, CD133, CD166, HLA DR, or a combination

thereof. Also, the hematopoietic stem cells, or hematopoietic stem and progenitor cells, can be negative for an expressed marker, relative to other types of hematopoietic cells. For example, such markers can be, but are not limited to Lin, CD38, or a combination thereof.

[0045] The term "de-differentiated stem cell or induced pluripotent stem cell (iPSC)" as used herein refers to pluripotent cells induced by artificially dedifferentiating (reprogramming) the adult cells that have already been differentiated. The term "adult cell" as used herein refers to a cell derived from an adult that is born and alive, as opposed to an embryonic cell. As used herein, the term "differentiation" refers to a phenomenon in which structures or functions are specialized while cells divide and proliferate and grow, that is, a cell or tissue of an organism has a shape or function to perform a task given to each. iPSC can be reprogrammed from adult stem cells using any method known in the art. In particular embodiments, an iPSC includes PLSX11. In particular embodiments, a method for reprogramming adult cells into iPSC includes contacting the adult cell with an engineered expression construct (EEC) encoding a reprogramming factor (RF) operably linked to: i) a 5' untranslated region (UTR) including a minimal promoter, a mini-enhancer, and a Kozak sequence; and/or ii) a 3' UTR including a spacer and a stem loop structure. In particular embodiments, the stem loop structure includes hybridizing sequences and a loop segment. In particular embodiments, the loop segment includes 7-15 nucleotides. In particular embodiments, the 5' UTR additionally includes a start codon. In particular embodiments, the 3' UTR additionally includes a stop codon and/or a polyA tail. In particular embodiments, the RF includes Oct4, Sox2, Klf4, Nanog, Myc, SV40Tag, or Lin28. The components of the 5' UTR and 3' UTR can be any sequences known in the art including those provided in Table 1.

Table 1. 5' UTR and 3' UTR Components and Constructs.

SEQ ID NO:	Sequence	Description
N/A	CAUACUCA	Mini-enhancer sequence
N/A	GGGAGA	Minimal promoter (e.g., Mini-T7 promoter)
N/A	GCCRCCAUG	Kozak Consensus Sequence
N/A	[N ₁₋₃]AUA	Spacer
N/A	[N ₁₋₃]AAA	Spacer
N/A	UGCAUA	Spacer
N/A	UGCAAA	Spacer
N/A	CCUC and GAGG	Hybridizing Sequences

N/A	CUCC and GGAG	Hybridizing Sequences
1	UAACGGUCUU	Loop segment
2	GGGAGACAUACUCAGCC <u>A</u> CC	5' UTR Construct with "A" Kozak Sequence (no start codon)
3	GGGAGACAUACUCAGCC <u>G</u> CC	5' UTR Construct with "G" Kozak Sequence (no start codon)
4	GGGAGACAUACUCAGCC <u>A</u> CCAUG	5' UTR Construct with "A" Kozak Sequence (with start codon)
5	GGGAGACAUACUCAGCC <u>G</u> CCAUG	5' UTR Construct with "G" Kozak Sequence (with start codon)
6	[N ₁₋₃]AUACCUC[N ₇₋₁₅]GAGG	3' UTR Construct
7	[N ₁₋₃]AAACCUC[N ₇₋₁₅]GAGG	3' UTR Construct
8	[N ₁₋₃]AUAGGAG[N ₇₋₁₅]CUCC	3' UTR Construct
9	[N ₁₋₃]AAAGGAG[N ₇₋₁₅]CUCC	3' UTR Construct
10	[N ₁₋₃]AUAGAGG[N ₇₋₁₅]CCUC	3' UTR Construct
11	[N ₁₋₃]AAAGAGG[N ₇₋₁₅]CCUC	3' UTR Construct
12	Stop- UGCAUACCUCUAACGGUCUUGAGG- PolyA	3' UTR Construct
13	Stop- UGCAUAGGAGUAACGGUCUUCUCC- PolyA	3' UTR Construct
14	Stop- UGCAAAGGAGUAACGGUCUUCUCC- PolyA	3' UTR Construct
15	UAAUGCAUACCUCU <u>A</u> ACGGUCUUGAGG	3' UTR Construct
16	UGAUGCAUACCUCU <u>A</u> ACGGUCUUGAGG	3' UTR Construct
17	UAGUGCAUACCUCU <u>A</u> ACGGUCUUGAGG	3' UTR Construct

18	UAAUGCAUAGAGG <u>UAA</u> CGGUCUUCCUC	3' UTR Construct
19	UGAUGCAUAGAGG <u>UAA</u> CGGUCUUCCUC	3' UTR Construct
20	UAGUGCAUAGAGG <u>UAA</u> CGGUCUUCCUC	3' UTR Construct
21	UAAUGCAAAGAGG <u>UAA</u> CGGUCUUCCUC	3' UTR Construct
22	UGAUGCAAAGAGG <u>UAA</u> CGGUCUUCCUC	3' UTR Construct
23	UAGUGCAAAGAGG <u>UAA</u> CGGUCUUCCUC	3' UTR Construct

[0046] In comparison, a multipotent stem cell is capable of differentiating into a subset of cells compared to a pluripotent stem cell. Pluripotent stem cells give rise to multipotent stem cells through spontaneous differentiation or as a result of exposure to differentiation induction conditions in vitro. The term “multipotent” refers to a cell's potential to differentiate and give rise to a limited number of related, different cell types. These cells are characterized by their multi-lineage potential and the ability for self-renewal. In vivo, the pool of multipotent stem cells replenishes the population of mature functionally active cells in the body. Among the exemplary multipotent stem cell types are hematopoietic, mesenchymal, or neuronal stem cells.

[0047] Hematopoietic stem cells are immature cells found in the peripheral blood and bone marrow that can develop into all types of blood cells, including white blood cells, red blood cells, and platelets. Mesenchymal stem cells also known as mesenchymal stromal cells or medicinal signaling cells are multipotent stromal cells. Mesenchymal stem cells are more differentiated than pluripotent stem cells but retain the ability to differentiate into a variety of cell types, including osteoblasts, chondrocytes, myocytes and adipocytes. According to certain embodiments, mesenchymal stem cells are more readily reprogrammed than fully differentiated somatic cells. Neural stem cells (NSCs) are self-renewing, multipotent cells that generate the radial glial progenitor cells that generate the neurons and glia of the nervous system.

[0048] Unipotent stem cells are stem cells that produce one cell type but have the property of self-renewal that distinguishes stem cells from non-stem cells. Examples of unipotent stem cells includes germ line stem cells and epidermal stem cells. The term “precursor cell,” “progenitor cell,” and “stem cell” are used interchangeably in the art and refer either to a totipotent, pluripotent, multipotent or in some cases, a unipotent cell.

[0049] (II) Differentiation of Stem Cells. In particular embodiments, stem cells are differentiated, for example, for cell manufacturing (e.g., feeder cell), research and development (e.g., tester cell), or therapeutic purposes (e.g., therapeutic cell). Where differentiation of stem cells is desired, stem cells (e.g., iPSC) can be exposed to one or more activation factors (e.g., growth factors, differentiation factors, and/or survival factors) that promote differentiation into a more committed

cell type. A more differentiated stem cell is more committed in relation to a different stem cell type along a development pathway.

[0050] Stem cells of the present disclosure can differentiate into more specialized cell types such as committed progenitors as well as cells further along the differentiation and/or maturation pathway that are partly or fully matured or differentiated. “Committed progenitors” give rise to a fully differentiated cell of a specific cell lineage. Exemplary cells include mesenchymal stem cells (MSC) or hematopoietic stem cells (HSC). Exemplary differentiated cells include pancreatic cells (e.g., alpha, beta, and delta cells), epithelial cells, cardiac cells (e.g., cardiomyocytes), endothelial cells, liver cells (e.g., hepatocytes (HCs), hepatic stellate cells (HSCs), Kupffer cells (KCs), and liver sinusoidal endothelial cells (LSECs)), endocrine cells, connective tissue cells (e.g., fibroblasts), muscle cells (e.g., myoblasts), brain cells (e.g., neurons), bone cells (e.g., osteoblasts and osteoclasts), kidney cells, and immune cells (e.g., T-cells, NK cells, or macrophages).

[0051] Many activation factors and cell culture conditions that promote differentiation are known in the art (see, e.g., U.S. Patent No. 7,399,633 at Section 5.2 and Section 5.5). For example, stem cell factor (SCF) can be used in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin (IL)-7 to promote differentiation into myeloid stem/progenitor cells or lymphoid stem/progenitor cells, respectively. In particular embodiments, iPSC can be differentiated into a lymphoid stem/progenitor cell by exposing iPSC to 100 ng/ml of each of SCF and GM-CSF or IL-7. In particular embodiments, a retinoic acid receptor (RAR) agonist, or preferably all trans retinoic acid (ATRA) is used to promote the differentiation of iPSC. Differentiation into natural killer cells, e.g., can be achieved by exposing cultured iPSC to RPMI media supplemented with human serum, IL-2 at 50 U/mL and IL-15 at 500ng/mL. In additional embodiments, RPMI media can also be supplemented L-glutamine.

[0052] Mesenchymal stem cells (MSC) are multipotent adult stem cells that are able to differentiate into numerous cell types including cells in skeletal tissue, such as cartilage, bone and the fat in the bone marrow. Typically, MSC are derived from the bone marrow which can lead to a lack of accessibility to MSC despite the need for them in many mainstream clinical treatments. MSC can be identified by the presence of certain markers including CD73, CD90 and CD105, but the absence of CD14, CD20, CD34 or CD45. MSC can be generated from pluripotent stem cells such as ESC and iPSC by culturing on collagen type I-coated plates; forming embryoid bodies, culturing with PDGF AB, KSB-3, EGM-2MV, DMEM, or mTeSR1 medium supplemented with ROCK inhibitors (e.g., Y27632), and/or inhibiting pathways including the TGF- β pathway, or the bFGF pathway (see, e.g., Zhou et al., 2021, Stem Cell Research & Therapy. 12(175)).

Mesodermal induction can also be achieved by culture with IMDM/F12 with BMP4 and activin and culture with ES-Cult methylcellulose, StemLine II and ESFM with FGF2.

[0053] The CD34 molecule, belonging to the cadherin family, is a highly glycosylated single-pass transmembrane protein that is selectively expressed on the surface of human and other mammalian hematopoietic stem cells (HSC), hematopoietic progenitor cells (HPC), and vascular endothelial cells (ECs), and gradually diminishes to disappear as the cells mature. Many methods for inducing differentiation of stem cells into CD34+ cells exist, such as an Embryoid Bodies (EB) differentiation method, an adherent induced differentiation method, and differentiation by culturing cells in a combination of different cytokines and compounds. CD34+ cells have also been derived from human pluripotent stem cells by inhibition of mitogen-activated protein kinase (MAPK) extracellular signal-regulated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling and activation of bone morphogenic protein-4 (BMP4) signaling (Park et al., 2010, Blood, 116(25):5762-5772). Some medium supplements that can aid in the differentiation of stem cells to CD34+ cells includes BMP activator, bFGF, VEGF, SCF, IGF, EPO, IL6, and IL11; a ROCK inhibitor; a Wnt pathway activator; and/or a TGF β receptor/ALK inhibitor. In particular embodiments, iPSCs can be differentiated into CD34-positive hematopoietic progenitor cells using the StemDiff Hematopoietic Medium and Supplements (StemCell Technologies). For the differentiation, adherent iPSC cultures can be dissociated to single cells and plated at 3.5×10^6 cells/well to generate Embryoid Bodies (EBs). After culturing the EBs, EBs can be harvested and dissociated into single cells. CD34-positive cells can be isolated from the single cell suspension. Staining for CD34, CD45 and CD43 expression can be used to confirm that the cells are CD34 positive cells.

[0054] T cells are a type of white blood cell that plays an important role in the immune system. T cells can be differentiated from stem cells by differentiation into a CD34+ cell and then differentiation into a T cell. T cell differentiation protocols aim to copy the development of lymphocytes. Hence, stimulation by SCF, FLT3L, IL7, and Notch signaling (Moore and Zlotnik, 1997; Radtke et al., 1999, 2004; Politikos et al., 2015) can be useful in differentiating T cells from CD34+ cells. Differentiation effectiveness can be assessed by surface markers. T cells should stop expression of CD34 and should subsequently express CD7, CD5 and finally CD4 and CD8.

[0055] NK cells are important for body defense and tumor resistance, but the function of NK cells in tumor patients is usually damaged. Externally inputting NK cells with normal functions or enhanced functions through genetic modification, namely NK cell adoptive therapy, is a promising cancer treatment. However, NK cell immunotherapy requires a large number of NK cells and the main sources of NK cells are currently: NK cells (PB-NK) obtained by separating

autologous/allogeneic peripheral blood, NK cells (UCB-NK) obtained by separating autologous/allogeneic umbilical cord blood, NK cells (hESC-NK/iPSC-NK) obtained by differentiating embryonic stem cells/inducing pluripotent stem cells and NK cell lines such as NK-92. The production of NK cells from stem cells (e.g., iPSC) could greatly aid in NK cell adoptive therapy and can include culturing the stem cells in SPM-NK culture medium which includes Stempro-34 complete medium, DMEM/F12 medium, L-glutamine, ascorbic acid, ITS-X, SCF, Flt-3L, IL-3, IL-7, IL-15. In particular embodiments, iPSCs are differentiated into NK cells. In particular embodiments, iPSCs are differentiated into NK cells by first differentiating iPSCs into CD34-positive hematopoietic progenitor cells. Next, the CD34-positive cells are differentiated into CD5-positive and CD7-positive lymphoid progenitor cells, and the lymphoid progenitor cells are differentiated into CD56-positive cells. Staining for CD56, CD16 and CD3 expression can be used to confirm differentiation into NK cells.

[0056] Cardiomyocytes have been generated *in vitro* from a wide range of stem cells, including iPSC (see, e.g., Gai, *et al.*, 2009, *Cell. Biol. Int.* 33:1184-93; Kuzmenkin, *et al.*, 2009, *FASEB J.* 23:4168-80; Pfannkuche, *et al.*, 2009, *Cell Physiol. Biochem.* 24:73-86), ESCs (see, e.g., Beqqali, *et al.*, 2009, *Cell. Mol. Life Sci.* 66:800-13; Steel, *et al.*, 2009, *Curr. Opin. Drug Discov. Dev.* 12:133-40), HSPC (see, e.g., Choi, *et al.*, 2008, *Biotechnol. Lett.* 30:835-43; Antonitsis, *et al.*, 2008, *Thorac. Cardiovasc. Surg.* 56:77-82; Ge, *et al.*, 2009, *Biochem. Biophys. Res. Commun.* 381:317-21; Gwak, *et al.*, 2009, *Cell. Biochem. Funct.* 27:148-54), and cardiomyocyte progenitor cells (see, e.g., Smits, *et al.*, 2009, *Nat. Protoc.* 4:232-43). Mummery, *et al.*, 2012 July 20, *Circ. Res.* 111(3): 344-358 provides a summary of methods to differentiate iPSC and hESCs into cardiomyocytes. Methods to differentiate stem cells (e.g., iPSC) into cardiac cells are also described in, e.g., U.S. Publication No. 2015/0017718.

[0057] In particular embodiments, cardiomyocyte progenitors can be generated from embryoid bodies (EBs) treated with Activin A, BMP4 or with 2+Wnt3 and bFGF. These progenitors express Nkx2.5, Tbx5/20, Gata-4, Mef2c and Hand1/2. Their further differentiation to functional cardiomyocytes can be promoted with VEGF and Dkk1 (Vidarsson, *et al.*, 2010, *Stem Cell Rev.* 6:108-20).

[0058] A protocol for generating insulin producing beta-cells involves stepwise lineage restriction generating in sequence: definitive endodermal cells (Activin+Wnt3), primitive foregut endoderm (FGF10+KAAD-cyclopamine), posterior foregut endoderm (RA+FGF10+KAAD-cyclopamine), pancreatic endoderm and endocrine precursors (Extendin-4), and hormone producing cells (IGF1+HGF). Transcription factor profiles include: Sox17, CER, FoxA2, and the cytokine receptor CXCR4 (definitive endodermal cells), Hnf1B, Hnf4A (primitive foregut endoderm), Pdx1, Hnf6,

H1xB9 (posterior foregut endoderm), and Nkx6.1, Nkx2.2, Ngn3, Pax4 (pancreatic endoderm and endocrine precursors). See, e.g., D'Amour, *et al.*, 2006, *Nat. Biotechnol.* 24:1392-401; Kroon, *et al.*, 2008, *Nat. Biotechnol.* 26:443-52). Another method to induce stem cells (e.g., iPSC) to commit to definitive endoderm, then to pancreatic endoderm, to pancreatic endocrine/exocrine cells and finally to more mature islet cells is described in Jiang, *et al.*, 2007, *Stem Cells* 25(8): p. 1940-53.

[0059] Various types of retinal cells can be generated from stem cells (e.g., iPSC) (see, e.g., Lamba, *et al.*, 2006, *Proc. Natl. Acad. Sci. USA* 103:12769-74; Reh, *et al.*, 2010, *Methods Mol. Biol.* 636:139-53). EBs can be produced and thereafter treated with IGF1, Noggin (BMP inhibitor) and Dkk1 (Wnt inhibitor). This treatment with IGF1, Noggin (BMP inhibitor), and Dkk1 (Wnt inhibitor) can direct stem cells (e.g., iPSC) to adopt a retinal progenitor phenotype, expressing Pax6 and Chx10. Exposing these progenitors to N-(N-(3,5-difluorophenacetyl)-1-alanyl)-S-phenylglycine t-butyl ester (DAPT), a blocker of Notch signaling, promotes neuronal differentiation (Lamba, *et al.*, 2010, *PLoS One* 5:e8763). The decision to undergo photoreceptor differentiation is under the control of the transcription factor, Blimp1 (Brzezinski, *et al.*, 2010, *Development* 137:619-29).

[0060] In particular embodiments, neuronal differentiation can be achieved by replacing a stem cell culture media with a media including basic fibroblast growth factor (bFGF) heparin, and an N2 supplement (e.g., transferrin, insulin, progesterone, putrescine, and selenite). Two days later, differentiating cells can be attached by plating them onto dishes coated with laminin or polyornithine. After an additional 10–11 days in culture, primitive neuroepithelial cells will have formed. The identity of the cells can be confirmed by staining for PAX6 (paired box protein 6, a transcription factor), SOX2 (sex-determining region Y-box 2, another transcription factor), and N-cadherin (a calcium-dependent cell adhesion molecule specific to neural tissue). Neuroepithelial cells can be further differentiated into, e.g., motor neurons (see, e.g., Li, *et al.* 2005, *Nat. Biotechnol.* 23, 215–221), dopaminergic neurons (see, e.g., Yan, *et al.* 2005, *Stem Cells* 23, 781–790), and oligodendrocytes (Nistor, *et al.* 2005, *Glia* 49, 385–396).

[0061] Additional information regarding differentiation to motor neurons includes treatment with RA (Pax6 expressing primitive neuroepithelial cells), RA+Shh (Pax6/Sox1 expressing neuroepithelial cells), which gradually start to express the motor neuron progenitor marker Olig2. Reducing RA+Shh concentration promotes the emergence of motor neurons expressing HB9 and Islet1. The addition of brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), insulin-like growth factor-1 (IGF1), and cAMP promotes process outgrowth (see, e.g., Hu, *et al.*, 2009, *Nat. Protoc.* 4:1614-22; Hu, *et al.*, 2010, *Proc. Natl. Acad. Sci. USA*; 107:4335-40).

[0062] Additional information regarding differentiation to dopaminergic neurons includes overexpression of the transcription factor Nurr1 followed by exposure to Shh, FGF-8 and ascorbic acid (see, e.g., Lee, *et al.*, 2000 June, *Nat. Biotechnol.* 18(6):675-9; Kriks and Studer, 2009, *Adv. Exp. Med. Biol.* 651:101-11; Lindvall and Kokaia, 2009 May, *Trends Pharmacol. Sci.* 30(5):260-7.). The combination of stromal cell-derived factor 1 (SDF-1/CXCL12), pleiotrophin (PTN), insulin-like growth factor 2 (IGF2), and ephrin B1 (EFNB1) can induce stem cells (e.g., iPSC) to differentiate to TH-positive neurons *in vitro*, expressing midbrain specific markers, including Engrailed 1, Nurr1, Pitx3, and dopamine transporter (DAT). These neurons are capable of generating action potentials and forming functional synaptic connections (Vazin, *et al.*, 2009, *PLoS One* 4:e6606).

[0063] A protocol to produce mature myelinating oligodendrocytes includes directing stem cells (e.g., iPSC) toward neuroectoderm differentiation in the absence of growth factors for 2 weeks. These cells express neuroectoderm transcription factors, including Pax6 and Sox1. Next stem cells (e.g., iPSC) are exposed to the caudalizing factor retinoic acid (RA) and the ventralizing morphogen Shh for 10 days to begin expression of Olig2. To prevent the differentiation to motor neurons and promote the generation of oligodendrocyte precursor cells (OPC)s, cells are cultured with FGF2 for 10 days. By day 35, the Olig2 progenitors co-express Nkx2.2 and no longer give rise to motor neurons. The co-expression of Olig2 and Nkx2.2 reflects a stage prior to human OPCs (pre-OPCs). These pre-OPCs are finally cultured in a glia medium including triiodothyronine (T3), neurotrophin 3 (NT3), PDGF, cAMP, IGF-1 and biotin, which individually or synergistically can promote the survival and proliferation of the OPCs, for another 8 weeks to generate OPCs. These OPCs are bipolar or multipolar, express Olig2, Nkx2.2, Sox10 and PDGFR α , become motile and are able to differentiate to competent oligodendrocytes. WO2007/066338 also describes differentiation protocols for the generation of oligodendrocyte-like cells.

[0064] A protocol to produce glutamatergic neurons includes use of stem cells (e.g., iPSC) to produce cell aggregates which are then treated for 8 days with RA. This results in Pax6 expressing radial glial cells, which after additional culturing in N2 followed by "complete" medium results in 95% glutamate neurons (Bibel, *et al.*, 2007, *Nat. Protoc.* 2:1034-43).

[0065] A protocol to produce GABAergic neurons includes exposing EBs for 3 days to all-trans-RA. After subsequent culture in serum-free neuronal induction medium including Neurobasal medium supplemented with B27, bFGF and EGF, 95% GABA neurons develop (see, e.g., Chatzi, *et al.*, 2009, *Exp. Neurol.* 217:407-16).

[0066] U.S. Publication No. 2013/0330306 describes compositions and methods to induce

differentiation and proliferation of neural precursor cells or neural stem cells into neural cells using umbilical cord blood-derived mesenchymal stem cells; U.S. Publication No. 2007/0179092 describes use of pituitary adenylate cyclase activating polypeptide (PACAP) to enhance neural stem cell proliferation, differentiation and survival; U.S. Publication No. 2012/0329714 describes use of prolactin to increase neural stem cell numbers; while U.S. Publication No. 2012/0308530 describes a culture surface with amino groups that promotes neuronal differentiation into neurons, astrocytes and oligodendrocytes. U.S. Publication No. 2006/211109 describes improved methods for efficiently producing neuroprogenitor cells and differentiated neural cells such as dopaminergic neurons and serotonergic neurons from pluripotent stem cells, e.g., iPSCs.

[0067] Thus, the fate of neural stem cells can be controlled by a variety of extracellular factors. Commonly used factors include amphiregulin; BMP-2 (U.S. Pat. Nos. 5,948,428 and 6,001,654); brain derived growth factor (BDNF; Shetty and Turner, 1998, *J. Neurobiol.* 35:395-425); neurotrophins (e.g., Neurotrophin-3 (NT-3) and Neurotrophin-4 (NT-4); Caldwell, *et al.*, 2001, *Nat. Biotechnol.* 1;19:475-9); ciliary neurotrophic factor (CNTF); cyclic adenosine monophosphate; epidermal growth factor (EGF); dexamethasone (glucocorticoid hormone); fibroblast growth factor (bFGF; U.S. Pat. NO.5,766,948; FGF-1, FGF-2); forskolin; GDNF family receptor ligands; growth hormone; interleukins; insulin-like growth factors; isobutyl 3-methylxanthine; leukemia inhibitory growth factor (LIF; U.S. Patent No. 6,103,530); Notch antagonists (U.S. Patent No. 6,149,902); platelet derived growth factor (PDGF; U.S. Patent No. 5,753,506); potassium; retinoic acid (U.S. Patent No. 6,395,546); somatostatin; tetanus toxin; and transforming growth factor- α and TGF- β (U.S. Pat. Nos. 5,851,832 and 5,753,506).

[0068] In particular embodiments, preferred proliferation-inducing neural growth factors include BDNF, EGF and FGF-1 or FGF-2. Growth factors can be usually added to the culture medium at concentrations ranging between 1 fg/ml of a pharmaceutically acceptable composition (including, e.g., CNS compatible carriers, excipients and/or buffers) to 1 mg/ml. Growth factor expanded stem cells (e.g., iPSC) can also differentiate into neurons and glia after mitogen withdrawal from a culture medium.

[0069] Additionally, WO 2004/046348 describes differentiation protocols for the generation of neural-like cells from bone marrow-derived stem cells. WO 2006/134602 describes differentiation protocols for the generation of neurotrophic factor secreting cells. Commercial kits are also available from Life Technologies and include PSC Neural Induction Medium, Geltrex™ LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix, and a Human Neural Stem Cell Immunocytochemistry kit. Stem cells (e.g., iPSC) differentiated into neural cells using the Life Technology kits can be further terminally differentiated into neurons, astrocytes and

oligodendrocytes using Life Technologies' B-27® supplements, with N-2 supplement and NEUROBASAL® Medium.

[0070] Additional methods to assist with stem cell (e.g., iPSC) differentiation protocols include, e.g., culture vessels with a portion including an oxygen permeable substrate at least partially coated with a synthetic matrix having an average thickness of less than 100 nm. See, e.g., U.S. Publication No. 2014/0370598.

[0071] U.S. Publication No. 2013/0251690 describes methods to support stem cell (e.g., iPSC) differentiation in elderly populations.

[0072] A number of different differentiation methods have been described. Additional methods that can be used within the teaching of the current disclosure can be found in the art by those with ordinary skill. Furthermore, and as indicated, differentiation of stem cells (e.g., iPSC) can be confirmed by measuring cellular markers expressed by the desired differentiated cell.

[0073] The foregoing discussion describes *in vitro* or *ex vivo* differentiation methods. Modified stem cells (e.g., iPSC) disclosed herein can also differentiate *in vivo* following administration, as described elsewhere herein.

[0074] (III) Genetic Modification of Cells. Desired genes disclosed herein can be introduced into or deleted from cells by any method known in the art. Methods of modifying a desired gene can include transfection, electroporation, microinjection, lipofection, calcium phosphate mediated transfection, infection with a viral or bacteriophage vector including the gene sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, *in vivo* nanoparticle-mediated delivery, etc. Numerous techniques are known in the art for the modification genes in cells (see e.g., Loeffler and Behr, 1993, *Meth. Enzymol.* 217:599-618; Cohen, *et al.*, 1993, *Meth. Enzymol.* 217:618-644; Cline, 1985, *Pharmac. Ther.* 29:69-92) and may be used, provided that the necessary developmental and physiological functions of the recipient cells are not unduly disrupted. The technique can provide for the stable transfer of the gene to the cell, so that the gene is expressible by the cell and, in certain instances, preferably heritable and expressible by its cell progeny. The technique can also provide the stable deletion of the gene from the cell and its cell progeny.

[0075] The term "gene" refers to a nucleic acid sequence (used interchangeably with polynucleotide or nucleotide sequence) that encodes a desired gene as described herein. This definition includes various sequence polymorphisms, mutations, and/or sequence variants wherein such alterations do not substantially affect the function of the encoded gene. The term "gene" may include not only coding sequences but also regulatory regions such as promoters, enhancers, and termination regions. The term further can include all introns and other DNA

sequences spliced from an mRNA transcript, along with variants resulting from alternative splice sites. Gene sequences encoding the molecule can be DNA or RNA that directs the expression of a protein encoded by the gene. These nucleic acid sequences may be a DNA strand sequence that is transcribed into RNA or an RNA sequence that is translated into protein. The nucleic acid sequences include both the full-length nucleic acid sequences as well as non-full-length sequences derived from the full-length protein. The sequences can also include degenerate codons of the native sequence or sequences that may be introduced to provide codon preference in a specific cell type. Portions of complete gene sequences are referenced throughout the disclosure as is understood by one of ordinary skill in the art.

[0076] Genes described herein can be readily prepared by synthetic or recombinant methods. In embodiments, the gene sequence encoding any of these sequences can also have one or more restriction enzyme sites at the 5' and/or 3' ends of the coding sequence in order to provide for easy excision and replacement of the gene sequence encoding the sequence with another gene sequence encoding a different sequence. In embodiments, the gene sequence encoding the sequences can be codon optimized for expression in mammalian cells.

[0077] "Encoding" refers to the property of specific sequences of nucleotides in a gene, such as a cDNA, or an mRNA, to serve as templates for synthesis of other macromolecules such as a defined sequence of amino acids. Thus, a gene codes for a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. A "gene sequence encoding a protein" includes all nucleotide sequences that are degenerate versions of each other and that code for the same amino acid sequence or amino acid sequences of substantially similar form and function.

[0078] Polynucleotide gene sequences encoding more than one desired gene can include desired genes operably linked to each other and further include relevant regulatory sequences. For example, there can be a functional linkage between a regulatory sequence and an exogenous nucleic acid sequence resulting in expression of the latter. For another example, a first nucleic acid sequence can be operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary or helpful, join coding regions, into the same reading frame.

[0079] Targeted genetic engineering approaches can be used to insert, modify, and in some cases, delete targeted genes. Targeted genetic engineering approaches include transposon-based systems, the CRISPR/Cas nuclease system, zinc finger nucleases, and transcription

activator like effector nucleases can be used to incorporate desired genes into stem cells.

[0080] Particular embodiments can use transposon-based systems as gene editing agents to mediate the integration of a desired gene into a cell. Generally, such methods will involve introducing into cells (i) a first vector encoding a transposase (or a transposase polypeptide) and (ii) a second vector encoding a desired genetic element that is flanked by transposon repeats.

[0081] A transposase refers to an enzyme that is a component of a functional nucleic acid-protein complex capable of transposition and which is mediating transposition. Transposase also refers to integrases from retrotransposons or of retroviral origin. A transposition reaction includes a transposase and a transposon or an integrase enzyme. In particular embodiments, the efficiency of integration, the size of the DNA sequence that can be integrated, and the number of copies of a DNA sequence that can be integrated into a genome can be improved by using such transposable elements. Transposons include a short nucleic acid sequence with terminal repeat sequences upstream and downstream of a larger segment of DNA. Transposases bind the terminal repeat sequences and catalyze the movement of the transposon to another portion of the genome.

[0082] Several transposon/transposase systems have been adapted for genetic insertions of heterologous DNA sequences. Examples of such transposases include sleeping beauty ("SB", e.g., derived from the genome of salmonid fish); SB100X; piggyBac™ (e.g., derived from lepidopteran cells and/or the *Myotis lucifugus*); mariner (e.g., derived from *Drosophila*); frog prince (e.g., derived from *Rana pipiens*); Tol1; Tol2 (e.g., derived from medaka fish); TcBuster™ (e.g., derived from the red flour beetle *Tribolium castaneum*), Helraiser, Himar1, Passport, Minos, Ac/Ds, PIF, Harbinger, Harbinger3-DR, HSmr1, and spinON." Transposases and transposon systems are further described in U.S. Pat. Nos. 6,489,458; 7,148,203; 8,227,432; and 9,228,180. In particular embodiments, transposon-based systems are beneficial because they allow large payloads to be delivered allowing for dual or triple expression products.

[0083] The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated protein) nuclease system is an engineered nuclease system used for genetic engineering that is based on a bacterial system. Information regarding CRISPR-Cas systems and components thereof are described in, for example, US8697359, US8771945, US8795965, US8865406, US8871445, US8889356, US8889418, US8895308, US8906616, US8932814, US8945839, US8993233 and US8999641 and applications related thereto; and WO2014/018423, WO2014/093595, WO2014/093622, WO2014/093635, WO2014/093655, WO2014/093661, WO2014/093694, WO2014/093701, WO2014/093709, WO2014/093712, WO2014/093718, WO2014/145599, WO2014/204723, WO2014/204724, WO2014/204725,

WO2014/204726, WO2014/204727, WO2014/204728, WO2014/204729, WO2015/065964, WO2015/089351, WO2015/089354, WO2015/089364, WO2015/089419, WO2015/089427, WO2015/089462, WO2015/089465, WO2015/089473 and WO2015/089486, WO2016205711, WO2017/106657, WO2017/127807 and applications related thereto.

[0084] Particular embodiments utilize zinc finger nucleases (ZFNs) as gene editing agents. ZFNs are a class of site-specific nucleases engineered to bind and cleave DNA at specific positions. ZFNs are used to introduce double stranded breaks (DSBs) at a specific site in a DNA sequence which enables the ZFNs to target unique sequences within a genome in a variety of different cells. A zinc finger is a domain of 30 amino acids within the zinc finger binding domain whose structure is stabilized through coordination of a zinc ion. Examples of zinc fingers include C2H2 zinc fingers, C3H zinc fingers, and C4 zinc fingers. A designed zinc finger domain is a domain not occurring in nature whose design/composition results principally from rational criteria, e.g., application of substitution rules and computerized algorithms for processing information in a database storing information of existing ZFP designs and binding data. A well-known example of a ZFN is a fusion of the FokI nuclease with a zinc finger DNA binding domain. For additional information regarding ZFNs and ZFNs useful within the teachings of the current disclosure, see, e.g., US 6,534,261; US 6,607,882; US 6,746,838; US 6,794,136; US 6,824,978; 6,866,997; US 6,933,113; 6,979,539; US 7,013,219; US 7,030,215; US 7,220,719; US 7,241,573; US 7,241,574; US 7,585,849; US 7,595,376; US 6,903,185; US 6,479,626; US 2003/0232410 and US 2009/0203140 as well as Gaj *et al.*, Nat Methods, 2012, 9(8):805-7; Ramirez *et al.*, Nucl Acids Res, 2012, 40(12):5560-8; Kim *et al.*, Genome Res, 2012, 22(7): 1327-33; Urnov *et al.*, Nature Reviews Genetics, 2010, 11 :636-646; Miller, *et al.* Nature biotechnology 25, 778-785 (2007); Bibikova, *et al.* Science 300, 764 (2003); Bibikova, *et al.* Genetics 161, 1169-1175 (2002); Wolfe, *et al.* Annual review of biophysics and biomolecular structure 29, 183-212 (2000); Kim, *et al.* Proceedings of the National Academy of Sciences of the United States of America 93, 1156-1160 (1996); and Miller, *et al.* The EMBO journal 4, 1609-1614 (1985).

[0085] Particular embodiments can use transcription activator like effector nucleases (TALENs) as gene editing agents. TALENs refer to fusion proteins including a transcription activator-like effector (TALE) DNA binding protein and a DNA cleavage domain. TALENs are used to edit genes and genomes by inducing double DSBs in the DNA, which induce repair mechanisms in cells. Generally, two TALENs must bind and flank each side of the target DNA site for the DNA cleavage domain to dimerize and induce a DSB. For additional information regarding TALENs, see US 8,440,431; US 8,440,432; US 8,450,471; US 8,586,363; and US 8,697,853; as well as Joung and Sander, Nat Rev Mol Cell Biol, 2013, 14(1):49-55; Beurdeley *et al.*, Nat Commun, 2013, 4: 1762;

Scharenberg et al., *Curr Gene Ther*, 2013, 13(4):291-303; Gaj et al., *Nat Methods*, 2012, 9(8):805-7; Miller, et al. *Nature biotechnology* 29, 143-148 (2011); Christian, et al. *Genetics* 186, 757-761 (2010); Boch, et al. *Science* 326, 1509-1512 (2009); and Moscou, & Bogdanove, *Science* 326, 1501 (2009).

[0086] Particular embodiments can utilize MegaTALs as gene editing agents. MegaTALs have a rare-cleaving nuclease structure in which a TALE is fused with the DNA cleavage domain of a meganuclease. Meganucleases, also known as homing endonucleases, are single peptide chains that have both DNA recognition and nuclease function in the same domain. In contrast to the TALEN, the megaTAL only requires the delivery of a single peptide chain for functional activity.

[0087] Nanoparticles that result in selective in vivo genetic modification of targeted cell types have been described and can be used to deliver desired genes to a stem cell. In particular embodiments, the nanoparticles can be those described in WO2014153114, WO2017181110, and WO201822672.

[0088] Vectors and viral vectors can also be used to deliver desired genes to a stem cell. A "vector" is a nucleic acid molecule that is capable of transporting another nucleic acid. Vectors may be, e.g., plasmids (DNA plasmids or RNA plasmids), transposon-based systems, cosmids, bacterial artificial chromosomes, viruses, or phage. An "expression vector" is a vector that is capable of directing the expression of a protein encoded by one or more genes carried by the vector when it is present in the appropriate environment.

[0089] "Lentivirus" refers to a genus of retroviruses that are capable of infecting dividing and non-dividing cells. Several examples of lentiviruses include HIV (human immunodeficiency virus: including HIV type 1, and HIV type 2); equine infectious anemia virus; feline immunodeficiency virus (FIV); bovine immune deficiency virus (BIV); and simian immunodeficiency virus (SIV).

[0090] A lentiviral vector is a vector derived from at least a portion of a lentivirus genome, including especially a self-inactivating lentiviral vector as provided in Milone et al., *Mol. Ther.* 17(8): 1453-1464 (2009). Other examples of lentivirus vectors that may be used in the clinic, include: the LENTIVECTOR® gene delivery technology from Oxford BioMedica, the LENTIMAX™ vector system from Lentigen and the like. Nonclinical types of lentiviral vectors are also available and would be known to one skilled in the art. In particular embodiments, cells are genetically engineered to express desired genes using a lentivirus or lentiviral vector.

[0091] "Retroviruses" are viruses having an RNA genome. "Gammaretrovirus" refers to a genus of the retroviridae family. Exemplary gammaretroviruses include mouse stem cell virus, murine leukemia virus, feline leukemia virus, feline sarcoma virus, and avian reticuloendotheliosis viruses.

[0092] Retroviral vectors (see Miller, *et al.*, 1993, *Meth. Enzymol.* 217:581-599) can be used. In such embodiments, the gene to be expressed is cloned into the retroviral vector for its delivery into cells. In particular embodiments, a retroviral vector includes all of the cis-acting sequences necessary for the packaging and integration of the viral genome, i.e., (a) a long terminal repeat (LTR), or portions thereof, at each end of the vector; (b) primer binding sites for negative and positive strand DNA synthesis; and (c) a packaging signal, necessary for the incorporation of genomic RNA into virions. More detail about retroviral vectors can be found in Boesen, *et al.*, 1994, *Biotherapy* 6:291-302; Clowes, *et al.*, 1994, *J. Clin. Invest.* 93:644-651; Kiem, *et al.*, 1994, *Blood* 83:1467-1473; Salmons and Gunzberg, 1993, *Human Gene Therapy* 4:129-141; and Grossman and Wilson, 1993, *Curr. Opin. in Genetics and Devel.* 3:110-114. Adenoviruses, adeno-associated viruses (AAV) and alphaviruses can also be used. See Kozarsky and Wilson, 1993, *Current Opinion in Genetics and Development* 3:499-503, Rosenfeld, *et al.*, 1991, *Science* 252:431-434; Rosenfeld, *et al.*, 1992, *Cell* 68:143-155; Mastrangeli, *et al.*, 1993, *J. Clin. Invest.* 91:225-234; Walsh, *et al.*, 1993, *Proc. Soc. Exp. Biol. Med.* 204:289-300; and Lundstrom, 1999, *J. Recept. Signal Transduct. Res.* 19: 673-686. Other methods of gene delivery include use of mammalian artificial chromosomes (Vos, 1998, *Curr. Op. Genet. Dev.* 8:351-359); liposomes (Tarahovsky and Ivanitsky, 1998, *Biochemistry (Mosc)* 63:607-618); ribozymes (Branch and Klotman, 1998, *Exp. Nephrol.* 6:78-83); and triplex DNA (Chan and Glazer, 1997, *J. Mol. Med.* 75:267-282).

[0093] There are a large number of available, suitable viral vectors including those identified for human gene therapy applications (see Pfeifer and Verma, 2001, *Ann. Rev. Genomics Hum. Genet.* 2:177). Methods of using retroviral and lentiviral viral vectors and packaging cells for transducing mammalian host cells with desired genes are described in, e.g., US 8,119,772; Walchli, *et al.*, 2011, *PLoS One* 6:327930; Zhao, *et al.*, 2005, *J. Immunol.* 174:4415; Engels, *et al.*, 2003, *Hum. Gene Ther.* 14:1155; Frecha, *et al.*, 2010, *Mol. Ther.* 18:1748; and Verhoeyen, *et al.*, 2009, *Methods Mol. Biol.* 506:97. Retroviral and lentiviral vector constructs and expression systems are also commercially available.

[0094] In particular embodiments, the transposon-based system is used to genetically modify cells to include a gene. In particular embodiments, the CRISPR/Cas system is used to genetically modify cells to knockout a gene.

[0095] (III-A) Conditional Immobilization. Genetic tools have been created that not only immortalize a cell but offer regulatory control to switch cell proliferation on and off using external factors as controlled by the operator through a growth controlling agent. Herein, cell immortalization refers to the modification of a cell such that the cell can be cultured indefinitely

and does not undergo cell senescence. Cell senescence refers to the process by which cells eventually stop multiplying or dividing. Cell senescence is thought to be an antitumor mechanism and typically occurs in response to various cell stressors, such as telomere erosion, DNA damage, oxidative stress, and oncogenic activation. Several methods exist for immortalizing cells in culture including expression of viral genes (e.g., SV40 large T antigen), expression of telomerase reverse transcriptase protein (TERT), and other methods for the conditional expression of telomerase.

[0096] Viral genes, including Epstein-Barr virus (EBV), Simian virus 40 (SV40) T antigen, adenovirus E1A and E1B, and human papilloma virus (HPV) E6 and E7 can induce immortalization by a process known as viral transformation. Generally, these viral genes achieve immortalization of the cell by inactivating the tumor suppressor genes that put cells into a replicative senescent state. Occasionally, these cells may become genetically unstable (aneuploid) and lose the properties of the primary cell. In particular embodiments, it is desirable that such viral-induced immortalization does not also result in transformation of the cells into a tumor cell phenotype.

[0097] SV40 large T antigen (Simian Vacuolating Virus 40 Tag) is a hexamer protein that is an oncogene derived from the polyomavirus SV40 which is capable of transforming a variety of cell types. The transforming activity of SV40 large T antigen is due in large part to its perturbation of the retinoblastoma (pRB) and p53 tumor suppressor proteins. In addition, SV40 large T antigen binds to several other cellular factors, including the transcriptional co-activators p300 and CBP, which may contribute to its transformation function.

[0098] Cells can also be immortalized by expression of the telomerase reverse transcriptase protein (TERT), particularly in those cells most affected by telomere length (e.g., human cells). The term "TERT" as used herein refers to a polypeptide sequence possessing telomerase catalytic activity. Telomerase is an enzyme that adds specific DNA sequence repeats ("TTAGGG" in all vertebrates) to the 3' end of DNA strands in the telomere regions, which are found at the ends of eukaryotic chromosomes. The telomeres contain condensed DNA material, giving stability to the chromosomes. The enzyme is a reverse transcriptase that carries its own RNA molecule, which is used as a template when it elongates telomeres, which are shortened after each replication cycle. It includes two molecules each of telomerase catalytic subunit also referred to as Telomerase Reverse Transcriptase (TERT); Telomerase RNA (hTR or TERC); and dyskerin. TERT is a reverse transcriptase, which creates single-stranded DNA using single-stranded RNA as a template. This protein is inactive in most somatic cells, but when TERT is exogenously expressed, the cells are able to maintain telomere lengths sufficient to avoid

replicative senescence. Analysis of several telomerase-immortalized cell lines has verified that the cells maintain a stable genotype and retain critical phenotypic markers.

[0099] Other methods to conditionally immortalize cells include conditional expression of telomerase using the pHUSH vector system, the transposon-based gene trap system, and/or conditional gene expression using tamoxifen-dependent Cre recombinase-loxP site-mediated recombination. Skilled artisans are familiar with such techniques. For example, in the expression of telomerase, lentiviral vectors containing the drug-controllable expression of polymerase (Pol) II promoter-driven expression of transgenes (i.e. telomerase) or Pol III promoter-controlled sequences encoding small inhibitory hairpin RNAs (shRNAs) are suitable methodologies for creating immortalized cells (Szulc, J., et al., *Nature Methods* 2006 3(2):109-116). The pHUSH vector system can be used to conditionally immortalize cells. This inducible expression vector system is used for regulated expression of shRNA, miRNA or cDNA cassettes on a single viral vector (Gray, D. C., et al., *BMC Biotechnology* 2007, 7:61). The transposon-based gene trap system incorporates the doxycycline-repressive Tet-Off (tTA) system that is capable of activating the expression of a gene (for example telomerase) which is under control of a Tet response element (TRE) promoter (Geurts, A. M., et al., *BMC Biotechnology* 2006, 6:30). Tamoxifen-dependent Cre recombinase-loxP site-mediated recombination and bicistronic gene-trap expression vectors allow for transgene (i.e. telomerase) expression from endogenous promoters (Vallier, L., et al., *PNAS* 2001 98(5):2467-2472).

[0100] In particular embodiments, cells are conditionally immortalized by expression of TERT and/or SV40 large T antigen. In particular embodiments, the conditional immortalization gene can be turned on by administration of a growth controlling agent (e.g., drug) and can be turned off by stopping administration of the growth controlling agent. A “growth controlling agent” as described herein, refers to Inducible immortalization of cells allows for the control of proliferation of modified cells responsive to an inducing agent or drug. Exemplary non-limiting examples of such inducible systems are the Tet-on/off systems which utilize tetracycline/doxycycline as the inducing agent. Other inducible systems are also contemplated for carrying out the methods described herein. Examples of non-Tet inducible systems include the coumermycin inducible expression system, the RheoSwitch® (RheoGene, Inc., Noristown, PA) Mammalian Inducible Expression system, estrogen receptor inducible systems, cumate-inducible systems, and Cre-Lox recombinase systems. In some cases, cell lines are generated that have stably incorporated the inducible systems or constructs described herein. Alternatively, cells can be modulated to transiently express the inducible systems or constructs described herein (e.g., via transient transfection of at least one construct).

[0101] A Tet-on or Tet-off system typically utilizes a tetracycline transactivator protein. TetO sequences are typically positioned upstream of any open reading frame (ORF) whose expression is sought to be controlled using the Tet system. A promoter and the TetO sequence(s) can make up a tetracycline response element (TRE). In some cases, the TRE includes TetO sequence(s) and is placed upstream of a promoter and the ORF(s) for one or more genes of interest. In the Tet-on system, the transactivator protein has a strong binding affinity for TetO operator sequence(s) when it is not bound by tetracycline (or a derivative such as doxycycline). In the absence of tetracycline, the transactivator protein does not bind to the tetracycline response element (TRE). When tetracycline is added, it binds to the transactivator protein and causes the transactivator protein to bind to the TRE to induce expression of downstream ORF(s). In a Tet-off system, the transactivator protein has a strong binding affinity for TetO operator sequence(s) only when it is not bound by tetracycline. In the absence of tetracycline, the transactivator protein binds the TetO sequences and promotes expression of the downstream ORF(s). Added tetracycline binds to the transactivation protein causing a conformational change that results in decreased or loss of binding to the TRE, resulting in reduced expression of the downstream ORF(s). In particular embodiments, the drug includes tetracycline or doxycycline. In particular embodiments, doxycycline includes doxycycline hyclate. In particular embodiments, media is supplemented with the drug at 0.01 μM to 5 μM . In particular embodiments, media is supplemented with the drug at 0.1 μM to 1 μM . In particular embodiments, media is supplemented with the drug at 0.1 μM , 0.3 μM , 0.6 μM , or 1 μM . In particular embodiments, the drug is added to the culture and differentiation medium throughout the culture and/or differentiation. In particular embodiments, the drug is added to the differentiation medium at the initiation of the differentiation stage.

[0102] (III-B) Expression Products. In particular embodiments, it is useful for a cell to express a specific molecule or factor that is useful to support its desired function as a feeder cell, tester cell, or therapeutic cell. As referred to herein, an expression product is a molecule expressed by a cell that supports the cells use for a desired function (e.g., as a feeder cell, tester cell, or therapeutic cell). An expression product can include a protein (e.g., an antibody, an antigen, a detectable label, and/or a recombinant receptor), DNA, or RNA (e.g., mRNA). The expression product can be secreted by the cell into the extracellular matrix or can be expressed on the surface of the cell. In particular embodiments, the expression product is directed to the cell surface or directed for secretion by a signal peptide which is encoded by a signal sequence.

[0103] The term “signal peptide” or “signal peptide sequence” is defined herein as a peptide sequence usually present at the N-terminal end of newly synthesized secretory or membrane

polypeptide which directs the polypeptide across or into a cell membrane of the cell (the plasma membrane in prokaryotes and the endoplasmic reticulum membrane in eukaryotes). It is usually subsequently removed. In particular, said signal peptide may be capable of directing the polypeptide into a cell's secretory pathway. The signal sequence can be foreign or native. A native signal sequence is naturally present in relation to the encoded protein. A foreign signal sequence is a signal peptide that is not native to the encoded protein, i.e. it originates from another gene than the encoded protein. Example membrane-spanning signal peptides include glycoprotein C signal peptide, foamy virus Env signal peptide, CD8 signal peptide, or granulocyte-macrophage colony-stimulating factor (GM-CSF) signal peptide. An example secretory signal peptide includes mouse mammary tumor virus (MMTV) envelope protein signal peptide.

[0104] Depending on the use of the cell, the cell may be genetically modified to express a specific expression product. For example, in a feeder cell, expression products could include proteins (e.g., antigens, or antibodies that stimulate the activation and expansion of a desired cell type). In a tester cell, expression products could include antigens (e.g., cancer antigens) such that the tester cell can be used to test a new therapeutic treatment. In a therapeutic cell, expression products could include antibodies or recombinant receptors such that the therapeutic cell can target and kill an undesired cell type. Alternatively, a therapeutic cell could express a protein or antibody such that the therapeutic cell can be used to replace a deficient protein or antibody within a subject.

[0105] Any useful protein (e.g., recombinant receptor or detectable label) or can be used.

[00106] (III-B-1) Protein. A protein is molecule made of one or more chains of amino acids. A protein can include a peptide, an antigen, an antibody, an enzyme, etc. The protein can be a secreted protein a non-secreted protein, or a membrane-bound protein. An antigen is a type of protein and refers to any substance that specifically binds to a selected antibody. The term "antibody" includes (in addition to antibodies having two full-length heavy chains and two full-length light chains as described above) variants, derivatives, and fragments thereof. Furthermore, unless explicitly excluded, antibodies can include monoclonal antibodies, human or humanized antibodies, bispecific antibodies, trispecific antibodies, tetraspecific antibodies, multi-specific antibodies, polyclonal antibodies, linear antibodies, minibodies, domain antibodies, synthetic antibodies, chimeric antibodies, antibody fusions, and fragments thereof, respectively. In particular embodiments, antibodies can include oligomers or multiplexed versions of antibodies.

[0107] Any protein useful for cell manufacture, research and development, or therapeutic treatment can be used. For example, proteins useful in cell differentiation or cell proliferation can

be expressed. For example, in the culture of NK cells, membrane-bound IL21 is important to promote cell maturation, proliferation, increase cytotoxic effect, and enhance anti-tumor activity. For the culture of NK cells, the IL21 can be made membrane-bound by expressing it on an exosome, membrane lysate, bead, or by engineering cells to express IL21 on its surface.

[0108] In tumor immunotherapy, activation of cytotoxicity of NK cells and CD8 + T cells is key, and many studies have shown that IL21 plays an important role in this procedure. IL21 promotes maturation of NK cells to produce IFN- γ and perforin, and induces NK cell-mediated anti-tumor cytotoxicity to target NKG2D ligands on the surface of tumor cells, and increasing the lethality of NK cells via the antibody-dependent cell-mediated cytotoxicity (ADCC) (see literature: "Spolski, R. & Leonard, W J Interleukin-21: a double-edged sword with therapeutic potential. Nat. Rev. Drug Discov. 13, 379-395 (2014)."). Secondly, IL21 can induce the proliferation of CD8+ T cells, induce the generation of memory T cells, and promote the secretion of IFN γ /granzyme to enhance the killing of tumors by CD8 + T cells and contribute to the memory immune response to recurrent tumor cells.

[0109] In particular embodiments, IL21 includes the sequence:

MRSSPGNMERIVICLMVIFLGLTVHKSSSQGQDRHMIRMRLIDIVDQLKNYVNDLVPEFLPAP
EDVETNCEWSAFSCFQKAQLKSANTGNNERIINVSIIKKLRKPPSTNAGRRQKHRLTCPSCDS
YEKKPPKEFLERFKSLLQKMIHQHLSSRTHGSEDS (SEQ ID NO: 24).

[0110] In particular embodiments, stem cells can be engineered to express a cancer cell antigen. Cancer cell antigens are molecules preferentially expressed by cancer cells. "Preferentially expressed" means that a cancer cell antigen is found at higher levels on cancer cells as compared to other cell types. In some instances, the cancer antigen is expressed on the cancer cell types at least 25%, 35%, 45%, 55%, 65%, 75%, 85%, 95%, 96%, 97%, 98%, 99%, or 100% more than on non-cancer cells.

[0111] The following provides examples of cancer antigens that are more likely to be co-expressed in particular cancers: BCMA, CD4, CD5, CD7, CD19, CD20, CD22, CD33, CD73, CD123, CD133, CD138, CD244, CD276, CS1, EGFR, EGFRVIII, EpCAM, FLT3, GD2, GPA7, GPC3, HER2, Mesothelin, MUC1, NKG2D, PSMA, PSCA, or TF.

[0112] The following table provides examples of cancer antigens that are more likely to be co-expressed in particular cancer types:

Cancer Antigens Likely to be Co-Expressed	Cancer Type
CD138, CS1, B-cell maturation antigen (BCMA), PD-L1, EGFR	Multiple Myeloma
CD22, CD19, CD4	Lymphoma
CD7, CD19, CD5, FLT3	Acute lymphocytic leukemia (ALL)

CD33, CD19, CD4, CD123	Acute myelocytic leukemia (AML)
CD19	Chronic lymphocytic leukemia (CLL)
PSMA, WT1, Prostate Stem Cell antigen (PSCA), SV40 T, PD-L1, EFGR	Prostate Cancer
HER2, EpCAM, TF, ERBB2, ROR1, PD-L1, EFGR, MUC16, folate receptor (FOLR), CEA	Breast Cancer
CD133, PD-L1, EFGR	Stem Cell Cancer
HER1, L1-CAM, MUC16, FOLR, Lewis Y, ROR1, mesothelin, WT-1, PD-L1, EFGR, CD56	Ovarian Cancer
HER2	Renal cell carcinoma (RCC)
EGFR, EGFRVIII, CD73, HER2	Glioblastoma
mesothelin, PD-L1, EFGR	Mesothelioma
carboxy-anhydrase-IX (CAIX); PD-L1, EFGR	Renal Cell Carcinoma
GPA7, GD2, PD-L1, EFGR	Melanoma
mesothelin, CEA, CD24, ROR1, PD-L1, EFGR, MUC16	Pancreatic Cancer
ROR1, PD-L1, EFGR, mesothelin, MUC16, FOLR, CEA, CD56	Lung Cancer
mesothelin, PD-L1, EFGR	Cholangiocarcinoma
MUC16, PD-L1, EFGR,	Bladder Cancer
GD2, CD244, CD276, ROR1, glypican-2, CD56, disialoganglioside, PD-L1, EFGR,	Neuroblastoma
HER2, EpCAM, NKG2D, CEA, PD-L1, EFGR	Colorectal Cancer
GD2	Ewing sarcoma
GPC3	Hepatocellular cancer (HCC)
CD56, PD-L1, EFGR,	Merkel Cell Carcinoma

[0113] In particular embodiments, the protein can be a viral, bacterial, fungal, or parasitic antigen. In particular embodiments, viral antigens include viral entry proteins. Examples of viral entry proteins include [virus (entry protein)]: Chikungunya (E1 Env and E2 Env); Ebola glycoprotein (EBOV GP); Hendra (F glycoprotein and G glycoprotein); hepatitis B (large (L), middle (M), and small (S)); hepatitis C (glycoprotein E1 and glycoprotein E2); HIV envelope (Env); influenza hemagglutinin (HA); Lassa virus envelope glycoprotein (GPC); measles (hemagglutinin glycoprotein (H) and fusion glycoprotein F0 (F)); MERS-CoV (Spike (S)); Nipah (fusion glycoprotein F0 (F) and glycoprotein G); Rabies virus glycoprotein (RABV G); RSV (fusion glycoprotein F0 (F) and glycoprotein G); and SARS-CoV (Spike (S)); among many others.

[0114] Additional HIV proteins include gene products of the gag, pol, and env genes such as HIV gp32, HIV gp41, HIV gp120, HIV gp160, HIV P17/24, HIV P24, HIV P55 GAG, HIV P66 POL, and HIV GP36. Other HIV proteins of interest include the Nef protein and other accessory proteins such as Vpr, Vpu, Tat, and Rev. Very particular examples of specific viral proteins and strains include BF520.W14.C2; BG505.W6M.C2.T332N; BG505 SOSIP Env trimer;

BL035.W6M.ENV.C1; SF162; ZM109F.PB4; C2-94UG114; HIV-BAL, HIV-LAI, SIV/mac239; MN gp41 monomer; ectodomain ZA.1197/MB; Q23; QA013.70I.Env.H1; QA013.385M.Env.R3 677; QB850.73P.C14; QB850.632P.B10; Q461.D1; and QC406.F3. Numerous additional proteins/strains are known to one of ordinary skill in the art.

[0115] As further particular examples of viral antigens, cytomegaloviral antigens include envelope glycoprotein B and CMV pp65; Epstein-Barr antigens include EBV EBNA1, EBV P18, and EBV P23; hepatitis antigens include the S, M, and L proteins of hepatitis B virus, the pre-S antigen of hepatitis B virus, HBCAG DELTA, HBV HBE, hepatitis C viral RNA, HCV NS3 and HCV NS4; herpes simplex viral antigens include immediate early proteins and glycoprotein D; influenza antigens include hemagglutinin and neuraminidase; Japanese encephalitis viral antigens include proteins E, M-E, M-E-NS1, NS1, NS1-NS2A and 80% E; measles antigens include the measles virus fusion protein; rabies antigens include rabies glycoprotein and rabies nucleoprotein; respiratory syncytial viral antigens include the RSV fusion protein and the M2 protein; rotaviral antigens include VP7sc; rubella antigens include proteins E1 and E2; and varicella zoster viral antigens include gpl and gpII.

[0116] Bacterial antigen can include: anthrax antigens include anthrax protective antigen; gram-negative bacilli antigens include lipopolysaccharides; diptheria antigens include diptheria toxin; Mycobacterium tuberculosis antigens include mycolic acid, heat shock protein 65 (HSP65), the 30 kDa major secreted protein and antigen 85A; pertussis toxin antigens include hemagglutinin, pertactin, FIM2, FIM3 and adenylate cyclase; pneumococcal antigens include pneumolysin and pneumococcal capsular polysaccharides; rickettsiae antigens include rompA; streptococcal antigens include M proteins; and tetanus antigens include tetanus toxin.

[0117] Fungal antigens can include: coccidioides antigens include spherule antigens; cryptococcal antigens include capsular polysaccharides; histoplasma antigens include heat shock protein 60 (HSP60); leishmania antigens include gp63 and lipophosphoglycan; plasmodium falciparum antigens include merozoite surface antigens, sporozoite surface antigens, circumsporozoite antigens, gametocyte/gamete surface antigens, protozoal and other parasitic antigens including the blood-stage antigen pf 155/RESA; schistosomae antigens include glutathione-S-transferase and paramyosin; tinea fungal antigens include trichophytin; toxoplasma antigens include SAG-1 and p30; and Trypanosoma cruzi antigens include the 75-77 kDa antigen and the 56 kDa antigen.

[0118] In particular embodiments, the protein can include an enzyme or protein useful for a therapeutic treatment. For example, there are several proteins that are deficient in certain diseases that could be replaced by administering cells that can produce said protein. For

example, insulin can be useful for the treatment of diabetes; factor VIII, factor IX, or factor XI for the treatment of clotting disorders; alpha-1 antitrypsin (A1AT) for the treatment of chronic obstructive pulmonary disease (COPD) and liver disorders; and glucocerebrosidase (GC), acid sphingomyelinase, mucopolysaccharides, acid alpha-glucosidase, aspartylglucosaminidase, alpha-galactosidase A, palmitoyl protein thioesterase, tripeptidyl peptidase, lysosomal transmembrane protein, cysteine transporter, acid ceramidase, acid alpha-L-fucosidase, cathepsin A, acid beta-glucosidase, acid beta-galactosidase, iduronate-2-sulfatase, alpha-L-iduronidase, galactocerebrosidase, acid alpha-mannosidase, acid beta-mannosidase, arylsulfatase B, arylsulfatase A, N-acetylgalactosamine-6-sulfate, N-acetylglucosamine-1-phosphotransferase, acid sphingomyelinase, NPC-1, alpha-glucosidase, beta-hexosaminidase B, heparan N-sulfatase, alpha-N-acetylglucosaminidase, acetyl-CoA: alpha-glucosaminide, N-acetylglucosamine-6-sulfate, alpha-N-acetylgalactosaminidase, alpha-neuramidase, beta-glucuronidase, beta-hexosaminidase A, and/or acid lipase can be useful for the treatment of lysosomal storage diseases.

[0119] (III-B-2) Recombinant Receptors. Cells described herein can be genetically modified to express a recombinant receptor. In particular embodiments, a recombinant receptor includes a chimeric antigen receptor (CAR) and/or an engineered T cell receptor (eTCR).

[0120] CAR include several distinct subcomponents that allow genetically modified cells (e.g., regulatory T cells) to recognize and kill cells expressing an antigen (e.g., a cancer antigen). The subcomponents include at least an extracellular component and an intracellular component. The extracellular component includes a binding domain that specifically binds an antigen epitope that is preferentially present on the surface of cells or in the area thereof. When the binding domain binds such epitopes, the intracellular component activates the cell to destroy the bound cell. CAR additionally include a transmembrane domain that directly or indirectly links the extracellular component to the intracellular component, and other subcomponents that can increase the CAR's function. For example, the inclusion of a spacer region and/or one or more linker sequences can allow the CAR to have additional conformational flexibility, often increasing the binding domain's ability to bind the targeted epitope.

[0121] A TCR is a heterodimeric fusion protein that typically includes an α and β chain. Each chain includes a variable region ($V\alpha$ and $V\beta$) and a constant region ($C\alpha$ and $C\beta$). In particular embodiments, an eTCR does not include the native TCR variable region but does include the native TCR constant region. In particular embodiments, the eTCR includes a binding domain (e.g., antibody) as the variable region of the α and/or β chain. In particular embodiments, eTCR include a $C\alpha$ and/or $C\beta$ chain sequence that is at least 90%, at least 91%, at least 92%, at least

93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or 100% identical to an amino acid sequence of a known or identified TCR C α or C β .

[0122] The binding domains of CAR and eTCR include a molecule that binds an antigen of interest. In many cases, the antigen of interest is a cancer antigen (see description of cancer antigens elsewhere herein). Other antigens of interest can include viral antigens, bacterial antigens, fungal antigens, etc. Antibodies are one example of binding domains and include whole antibodies or binding fragments of an antibody, e.g., Fv, Fab, Fab', F(ab')₂, and single chain (sc) forms and fragments thereof that specifically bind a cellular marker. Antibodies or antigen binding fragments can include all or a portion of polyclonal antibodies, monoclonal antibodies, human antibodies, humanized antibodies, synthetic antibodies, non-human antibodies, recombinant antibodies, chimeric antibodies, bispecific antibodies, mini bodies, and linear antibodies. Other binding fragments, such as Fv, Fab, Fab', F(ab')₂, can also be used within a CAR. Additional examples of antibody-based binding domain formats for use in a CAR include scFv-based grababodies and soluble VH domain antibodies. These antibodies form binding regions using only heavy chain variable regions. See, for example, Jespers et al., Nat. Biotechnol. 22:1161, 2004; Cortez-Retamozo et al., Cancer Res. 64:2853, 2004; Baral et al., Nature Med. 12:580, 2006; and Barthelemy et al., J. Biol. Chem. 283:3639, 2008.

[0123] In addition to binding domains, CAR and eTCR can additionally include transmembrane domains, intracellular effector domains, spacer regions, transduction markers, and tags.

[0124] Transmembrane domains typically have a three-dimensional structure that is thermodynamically stable in a cell membrane, and generally ranges in length from 15 to 30 amino acids. The structure of a transmembrane domain can include an α helix, a β barrel, a β sheet, a β helix, or any combination thereof. Transmembrane domains can include at least the transmembrane region(s) of the α , β or ζ chain of a T-cell receptor, CD28, CD27, CD3, CD45, CD4, CD5, CD8, CD9, CD16, CD22; CD45, CD37, CD64, CD80, CD86, CD134, CD137 and CD154.

[0125] A transmembrane domain can include one or more additional amino acids adjacent to the transmembrane region, e.g., one or more amino acid within the extracellular region of the expressed protein (e.g., up to 15 amino acids of the extracellular region) and/or one or more additional amino acids within the intracellular region of the expressed protein (e.g., up to 15 amino acids of the intracellular components).

[0126] Intracellular effector domains activate the expressing cell when the binding domain binds antigen. The term "effector domain" is thus meant to include any portion of the intracellular domain sufficient to transduce an activation signal.

[0127] An effector domain can include one, two, three or more intracellular signaling components (e.g., receptor signaling domains, cytoplasmic signaling sequences), co-stimulatory domains, or combinations thereof. Exemplary effector domains include signaling and stimulatory domains selected from: 4-1BB (CD137), CD3 γ , CD3 δ , CD3 ϵ , CD3 ζ , CD27, CD28, DAP10, ICOS, LAG3, NKG2D, NOTCH1, OX40, ROR2, SLAMF1, TCR α , TCR β , TRIM, Wnt, Zap70, or any combination thereof. In particular embodiments, exemplary effector domains include signaling and co-stimulatory domains selected from: CD86, Fc γ RIIa, DAP12, CD30, CD40, PD-1, lymphocyte function-associated antigen-1 (LFA-1), LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83, CD80, ICAM-1, GITR, BAFFR, SLAMF7, NKp80 (KLRF1), CD127, CD19, CD4, CD8 α , CD8 β , IL2R β , IL2R γ , IL7R α , ITGA4, VLA1, CD49a, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, ITGB7, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, GADS, PAG/Cbp, NKp44, NKp30, or NKp46.

[0128] Intracellular signaling component sequences that act in a stimulatory manner may include iTAMs. Examples of iTAMs including primary cytoplasmic signaling sequences include those derived from CD3 γ , CD3 δ , CD3 ϵ , CD3 ζ , CD5, CD22, CD66d, CD79a, CD79b, and common Fc γ R γ (FCER1G), Fc γ RIIa, FcR β (Fc ϵ Rib), DAP10, and DAP12. In particular embodiments, variants of CD3 ζ retain at least one, two, three, or all ITAM regions.

[0129] A co-stimulatory domain is a domain whose activation can be required for an efficient lymphocyte response to cellular marker binding. Some molecules are interchangeable as intracellular signaling components or co-stimulatory domains. Examples of costimulatory domains include CD27, CD28, 4-1BB (CD137), OX40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), NKG2C, and a ligand that specifically binds with CD83.

[0130] Spacer regions are used to create appropriate distances and/or flexibility between sub-components of a protein. Spacer regions typically include 10 to 250 amino acids, 10 to 200 amino acids, 10 to 150 amino acids, 10 to 100 amino acids, 10 to 50 amino acids, or 10 to 25 amino acids. Exemplary spacer regions include all or a portion of an immunoglobulin hinge region.

[0131] Transduction markers and tags can be helpful in identifying and isolating cells that have been successfully modified. Additional details about transduction makers and tags can be found elsewhere herein.

[0132] (III-B-3) Detectable Labels. In particular embodiments, stem cells are genetically modified to express a detectable label. Detectable labels can include any suitable label or detectable

group detectable by, for example, optical, spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Such detectable labels include fluorescent proteins, radiolabels, radioacoustic labels, enzyme labels, chemiluminescence labels, fluorescence labels, and biotin (with labeled avidin or streptavidin).

[0133] Fluorescent proteins can be particularly useful in cell staining, identification, and isolation uses. Exemplary fluorescent proteins include luciferase; blue fluorescent proteins (e.g. eBFP, eBFP2, Azurite, mKalama1, GFPuv, Sapphire, T-sapphire); cyan fluorescent proteins (e.g. eCFP, Cerulean, CyPet, AmCyan1, Midoriishi-Cyan, mTurquoise); green fluorescent proteins (e.g. GFP, GFP-2, tagGFP, turboGFP, EGFP, Emerald, Azami Green, Monomeric Azami Green (mAzamigreen)), CopGFP, AceGFP, avGFP, ZsGreen1, Oregon Green™ (Thermo Fisher Scientific)); orange fluorescent proteins (mOrange, mKO, Kusabira-Orange, Monomeric Kusabira-Orange, mTangerine, tdTomato); red fluorescent proteins (mKate, mKate2, mPlum, DsRed monomer, mCherry, mRuby, mRFP1, DsRed-Express, DsRed2, DsRed-Monomer, HcRed-Tandem, HcRed1, AsRed2, eqFP611, mRaspberry, mStrawberry, Jred, Texas Red™ (Thermo Fisher Scientific)); far red fluorescent proteins (e.g., mPlum and mNeptune); yellow fluorescent proteins (e.g., YFP, eYFP, Citrine, SYFP2, Venus, YPet, PhiYFP, ZsYellow1); and tandem conjugates. In particular embodiments, stem cells are genetically modified to express luciferase.

[0134] Radioisotopes can be used as a type of detectable label called a radiolabel. In particular embodiments, a radioisotope includes ^{131}I , ^{90}Y , and/or ^{211}At . In particular embodiments, a radioisotope is selected that includes a half-life ($t_{1/2}$) that enables high-yield radiolabeling and drug delivery. In particular embodiments, a radioisotope is selected that includes a half-life ($t_{1/2}$) of 7.2 hours. In particular embodiments, a radioisotope is selected that does not emit daughter radionuclides that cause organ toxicity. Exemplary radiolabels include ^{228}Ac , ^{111}Ag , ^{124}Am , ^{74}As , ^{211}At , ^{209}At , ^{194}Au , ^{128}Ba , ^7Be , ^{206}Bi , ^{245}Bk , ^{246}Bk , ^{76}Br , ^{11}C , ^{14}C , ^{47}Ca , ^{254}Cf , ^{242}Cm , ^{51}Cr , ^{67}Cu , ^{153}Dy , ^{157}Dy , ^{159}Dy , ^{165}Dy , ^{166}Dy , ^{171}Er , ^{250}Es , ^{254}Es , ^{147}Eu , ^{157}Eu , ^{52}Fe , ^{59}Fe , ^{251}Fm , ^{252}Fm , ^{253}Fm , ^{66}Ga , ^{72}Ga , ^{146}Gd , ^{153}Gd , ^{68}Ge , ^3H , ^{170}Hf , ^{171}Hf , ^{193}Hg , ^{193}mHg , ^{160}mHo , ^{130}I , ^{135}I , ^{114}mIn , ^{185}Ir , ^{42}K , ^{43}K , ^{76}Kr , ^{79}Kr , ^{81}mKr , ^{132}La , ^{262}Lr , ^{169}Lu , ^{174}mLu , ^{176}mLu , ^{257}Md , ^{260}Md , ^{28}Mg , ^{52}Mn , ^{90}Mo , ^{24}Na , ^{95}Nb , ^{138}Nd , ^{57}Ni , ^{66}Ni , ^{234}Np , ^{15}O , ^{182}Os , ^{189}mOs , ^{191}Os , ^{32}P , ^{201}Pb , ^{101}Pd , ^{143}Pr , ^{191}Pt , ^{243}Pu , ^{225}Ra , ^{81}Rb , ^{188}Re , ^{105}Rh , ^{211}Rn , ^{103}Ru , ^{35}S , ^{44}Sc , ^{72}Se , ^{153}Sm , ^{125}Sn , ^{91}Sr , ^{173}Ta , ^{154}Tb , ^{127}Te , ^{234}Th , ^{45}Ti , ^{166}Tm , ^{230}U , ^{237}U , ^{240}U , ^{48}V , ^{178}W , ^{181}W , ^{188}W , ^{125}Xe , ^{127}Xe , ^{133}Xe , ^{133}mXe , ^{135}Xe , ^{85}mY , ^{86}Y , ^{90}Y , ^{93}Y , ^{169}Yb , ^{175}Yb , ^{65}Zn , ^{71}mZn , ^{86}Zr , ^{95}Zr , and/or ^{97}Zr .

[0135] Exemplary enzyme labels include horseradish peroxidase, hydrolases, and alkaline phosphatase. Exemplary fluorescence labels include rhodamine, phycoerythrin, and fluorescein.

[0136] (III-C) Major Histocompatibility Class (MHC) Molecules. In particular embodiments, cells are genetically modified to knockout MHC. TCR ligands can be divided into two classes major histocompatibility complex class I (MHC I) and MHC class II (MHC II). Human MHC Is are complexes of human leukocyte antigens (HLAs: HLA-A, HLA-B, and HLA-C) and β 2-microglobulin while MHC IIs are heterodimers of several HLAs (HLA-DP, HLA-DQ, and HLA-DR). Antigen peptide-bound MHC I (pMHC-I) molecules can be presented on any nucleated cells recognized by CD8+ T cells. On the other hand, CD4+ T cells recognize antigen peptide-bound MHC II (pMHC-II) molecules that are presented on the antigen-presenting cells (APCs), such as B cells, macrophages, and dendritic cells (Wieczorek et al. *Front. Immunol.* 8, 292, 2017). Studies have shown that CD8 and CD4 molecules may play a role during the development of T cells by helping the TCR complex select a different class of MHC molecules (Tikhonova, et al. *Immunity* 36, 79–91, 2012).

[0137] T cells, through their T cell receptor (TCR) may recognize the T cell epitope in the context of an MHC class I molecule. MHC class I proteins can be expressed in all nucleated cells of higher vertebrates. The MHC class I molecule is a heterodimer composed of a 46-kDa heavy chain which is non-covalently associated with the 12-kDa light chain β 2-microglobulin (or P-2 - microglobulin or B2M). In the human genome, B2M is encoded by the b2m gene located on chromosome 15, while other MHC genes are present as gene clusters on chromosome 6. The human β 2-microglobulin protein has 119 amino acids (see UniProt database number P61769). In a model of mice lacking B2M, it can be demonstrated that B2M is essential for the presentation on the cell surface and the stability of the polypeptide binding groove of MHC class I molecules. Mismatches in MHC can cause immune rejection, resulting in graft destruction. Removal of MHC class I molecules on the cell surface by knocking out B2M genes can prevent mismatches.

[0138] The human MHC is also called the human leukocyte antigen (HLA) complex. In humans, there are several MHC class I alleles, such as, for example, HLA-A2, HLA-A1, HLA-A3, HLA-A24, HLA-A28, HLA-A31, HLA-A33, HLA-A34, HLA-B7, HLA-B45 and HLA-Cw8. In some cases, there can be differences in the frequency of subtypes between different populations.

[0139] In some embodiments, the TCR may recognize the T cell epitope in the context of an MHC class I or class II molecule. MHC class II proteins can be expressed in a subset of APCs. In humans, there are several MHC class II alleles, such as, for example, DR1, DR3, DR4, DR7, DR52, DQ1, DQ2, DQ4, DQ8 and DP1. In some embodiments, the MHC class II allele is an HLA-DRB 1*0101, an HLA-DRB*0301, an HLA-DRB*0701, an HLA-DRB*0401 or an HLA- DQB 1*0201 gene product.

[0140] MHC class II expression depends on CIITA and RFX, two transcription factors that are

highly selective for MHC class II genes. RFX is expressed ubiquitously, while CIITA expression is cell-specific and finely regulated. Hence, the pattern of MHC class II expression replicates faithfully the expression pattern of the gene encoding CIITA (MHC2TA). MHC2TA is expressed through a set of three cell-specific promoters, referred to as promoters I, III and IV. Promoters I and III are constitutively active in professional antigen-presenting cells, while in most other cell types CIITA expression is inducible with interferon gamma (IFN γ) through promoter IV. Expression of the MHC class II transactivator, CIITA, closely parallels that of class II MHC gene expression. It has also been shown that CIITA is induced by gamma interferon, and that transfection of CIITA alone into cells is sufficient to activate class II MHC.

[0141] The N-terminal of CIITA contains an acidic domain (amino acids 30-160), followed by domains rich in proline (amino acids 163-195), serine (amino acids 209-257), and threonine (amino acids 260-322). An acidic domain has been found in many transcription factors and has been shown to interact with basal transcriptional machinery in vitro and in vivo. However, it is likely that the acidic domain alone is not sufficient to activate the class II MHC promoter in CIITA, and that the acidic domains of other transcription factors behave differently from the CIITA acidic domain. H. Analysis of the primary amino acid sequence of CIITA does not show any homology to known conserved DNA-binding motif of transcription factors, and in vitro translated CIITA apparently does not interact with DNA.

[0142] Multiple HLA class I and class II proteins must be matched for histocompatibility in allogeneic recipients to avoid allogeneic rejection problems and immune responses. Provided herein are conditionally immortalized stem cells and cells differentiated therefrom with eliminated or substantially reduced expression of both HLA class I and HLA class II proteins. HLA class I deficiency can be achieved by functional deletion of any region of the HLA class I locus (chromosome 6p21), or deletion or reducing the expression level of HLA class-I associated genes including beta-2 microglobulin (B2M) gene, TAP1 gene, TAP2 gene and Tapasin. For example, the B2M gene encodes a common subunit essential for cell surface expression of all HLA class I heterodimers. B2M null cells are HLA-I deficient.

[0143] HLA class II deficiency can be achieved by functional deletion or reduction of HLA-II associated genes including RFXANK, CIITA, RFXS and RFXAP. CIITA is a transcriptional coactivator, functioning through activation of the transcription factor RFXS required for class II protein expression. CIITA null cells are HLA-II deficient.

[0144] In particular embodiments, undesirable immune responses to a cell is overcome by genetically modify or knocking out MHC genes. Various gene editing methods can be used to inactivate a gene encoding an MHC molecule or a subunit thereof, or inactivate a gene regulating

the expression of the gene encoding an MHC molecule or a subunit thereof. In particular embodiments, to genetically modify or knockout an MHC Class I molecule, the gene encoding the alpha chain and/or gene encoding B2M of the MHC Class I molecule can be targeted for genetic modification or knockout. In particular embodiments, the gene encoding B2M is knocked out. In particular embodiments, to genetically modify or knockout an MHC Class II molecule, the gene encoding CIITA is knocked out. In particular embodiments, an MHC null (HLA-I and HLA-II deficient) cell is produced by knocking out the gene encoding B2M and the gene encoding CIITA. **[0145]** In particular embodiments, B2M is knocked out using B2M guide RNA (gRNA). In particular embodiments, CIITA is knocked out using CIITA gRNA. Example gRNA sequences are presented in Table 2.

Table 2. gRNA targeting B2M and CIITA.

Target	Sequence	SEQ ID NO:
CIITA in mouse	CATTGCAGCTGGATACCAG	25
CIITA in mouse	ACTGAGACAGTAACGGCCA	26
CIITA in mouse	ATAGAGATCCCTGTAGAAGC	27
CIITA in human	TCAACTGCGACCAGTTCAGC	28
CIITA in human	CATCGCTGTTAAGAAGCTCC	29
CIITA in human	TTCCTACACAATGCGTTGCC	30
CIITA in human	GCCCCTAGAAGGTGGCTACC	31
CIITA in human	GATATTGGCATAAGCCTCCC	32
CIITA in human	TCCTACCTGTCTAGAGCCCCA	33
B2M in mouse	AGTATACTCACGCCACCCAC	34
B2M in mouse	AGTCGTCAGCATGGCTCGCT	35
B2M in mouse	CGTATGTATCAGTCTCAGTG	36
B2M in human	GAGTAGCGCGAGCACAGCTA	37
B2M in human	GGCCGAGATGTCTCGCTCCG	38
B2M in human	CAGTAAGTCAACTTCAATGT	39
B2M in human	CGTGAGTAAACCTGAATCTT	40
B2M in human	ACCCAGACACATAGCAATTC	41
B2M in human	ACTCACGCTGGATAGCCTCC	42

[0146] (III-D) Suicide Gene. Suicide genes can be used for the elimination of genetically engineered cells. Suicide genes are nucleic acid constructs encoding a protein (also referred to as a kill switch) that inducibly causes cell death or stops cell proliferation. The suicide gene is

inserted at a defined, specific target locus in the genome of an engineered cell, usually at both alleles of the target locus. The suicide gene is activated by contacting cells with an effective dose of a clinically acceptable orthologous activating agent. In particular embodiments, the orthologous activating agent can be a molecule (e.g., drugs, antibodies, small molecules), light, radiation, ultrasound, temperature, pH, or other means that causes activation of the kill switch. When activated, the kill switch causes the cell to stop proliferation, and in some embodiments activates apoptosis of the cell.

[0147] The suicide gene is inserted at a targeted site of the genome, where it is operably linked to the promoter of a gene of interest, without disrupting expression of the gene of interest. In some embodiments the suicide gene is integrated to replace the stop codon of the gene of interest. The kill switch protein in this embodiment may be flanked by self-cleaving peptide sequences to provide for cleavage of the gene of interest protein and the kill switch protein

[0148] In some embodiments, the protein encoded by the suicide gene is a protein that induces apoptosis upon dimerization. In some embodiments the protein is a human caspase protein, e.g., caspase 1, caspase 2, caspase 3, caspase 4, caspase 5, caspase 6, caspase 7, caspase 8, caspase 9, caspase 10, caspase 14, etc. In certain embodiments the protein is human caspase 9. The caspase protein is fused to a sequence that provides for chemically induced dimerization (CID), in which dimerization occurs only in the presence of the orthologous activating agent.

[0149] A popular kill switch for drug-induced cell apoptosis uses a modified human caspase 9 to the FK506 binding protein (FKBP) that dimerizes in the presence of a small-molecule drug. This kill switch is referred to as inducible caspase 9 or iCasp9 (Straathof et al., *Blood*. 2005, 105(11):4247-4254). This kill switch has shown efficacy in both preclinical and clinical contexts (Diaconu et al., *Mol Ther*. 2017, 25(3):580-592; and Stasi et al., *N Engl J Med*. 2011, 365(18):1673-1683). In particular embodiments, FDA-approved small molecules such as rapamycin can be used control iCasp9 kill switches (Stavrou et al., *mBio*. 2018, 9(3):e00923-18).

[0150] A suicide gene can be prepared by transcriptionally linking a cell division locus (CDL) and a sequence encoding a negative selectable marker. This allows a user to inducibly kill proliferating host cells including the suicide gene or inhibit the host cell's proliferation by killing at least a portion of proliferating cells by exposing the modified cells to an inducer of the negative selectable marker. A cell modified to include the suicide gene can be treated with an inducer (e.g., a drug) of the negative selectable marker in order to ablate proliferating cells or to inhibit cell proliferation by killing at least a portion of proliferating cells.

[0151] Example CDLs include CDK1, TOP2A, CENPA, BIRC5, and EEF2. Example negative selectable markers include Herpes Simplex Virus type 1 (HSV) thymidine kinase/ganciclovir (TK/GCV), deaminase/5-fluorocytosine (CD/5-FC), and carboxyl esterase/irinotecan (CE/CPT-11).

[0152] Host cells modified with the HSV-TK/GCV negative selectable marker will produce thymidine kinase (TK) and the TK protein will convert GCV into GCV monophosphate, which is then converted into GCV triphosphate by cellular kinases. GCV triphosphate incorporates into the replicating DNA during S phase, which leads to the termination of DNA elongation and cell apoptosis (Halloran and Fenton, 1998, *Cancer Res.* 58(17): 3855-65).

[0153] The CD/5-FC negative selectable marker system is a widely used suicide gene system. Cytosine deaminase (CD) is a non-mammalian enzyme that may be obtained from bacteria or yeast (e.g., from *Escherichia coli* or *Saccharomyces cerevisiae*, respectively) (Ramnaraine et al., 2003). CD catalyzes conversion of cytosine into uracil and is an important member of the pyrimidine salvage pathway in prokaryotes and fungi, but it does not exist in mammalian cells. 5-fluorocytosine (5-FC) is an antifungal prodrug that causes a low level of cytotoxicity in humans (Denny, 2003, *J Biomed Biotechnol*). CD catalyzes conversion of 5-FC into the genotoxic agent 5-FU, which has a high level of toxicity in humans (Ireton et al., 2002, *J Molec. Biol.* 315(4):687-697).

[0154] The CE/CPT-11 system is based on the carboxyl esterase enzyme, which is a serine esterase found in a different tissues of mammalian species (Humerickhouse et al., 2000, *Cancer Res.* 60(5):1189-92). The anti-cancer agent CPT-11 is a prodrug that is activated by CE to generate an active referred to as 7-ethyl-10-hydroxycamptothecin (SN-38), which is a strong mammalian topoisomerase I inhibitor (Wierdl et al., 2001). SN-38 induces accumulation of double-strand DNA breaks in dividing cells (Kojima et al., 1998, *Anal Chem.* 70(13):2446-53).

[0155] In particular embodiments, the suicide gene includes a CDK1 cell division locus transcriptionally linked to an HSV-TK/GCV negative selectable marker (CDK1/HSV-KT/GCV). In particular embodiments, the suicide gene includes a TOP2A cell division locus transcriptionally linked to an HSV-TK/GCV negative selectable marker (TOP2A/HSV-KT/GCV). In particular embodiments, the suicide gene includes CDK1/HSV-TK/GCV. In particular embodiments, the suicide gene includes TOP2A/HSV-KT/GCV.

[0156] (III-E) Other Control Features. In particular embodiments, cells can be genetically modified to include other control features. In particular embodiments, other control features include tag cassettes and transduction markers. In particular embodiments, genetic constructs for insertion into stem cells and described herein can include one or more tag cassettes and/or

transduction markers. Tag cassettes and transduction markers can be used to activate, promote proliferation of, detect, enrich for, isolate, track, deplete and/or eliminate genetically modified cells in vitro, in vivo and/or ex vivo. "Tag cassette" refers to a unique synthetic peptide sequence affixed to, fused to, or that is part of a genetic construct, to which a cognate binding molecule (e.g., ligand, antibody, or other binding partner) is capable of specifically binding where the binding property can be used to activate, promote proliferation of, detect, enrich for, isolate, track, deplete and/or eliminate the tagged protein and/or cells expressing the tagged protein. Transduction markers can serve the same purposes but are derived from naturally occurring molecules and are often expressed using a skipping element that separates the transduction marker from the rest of the genetic construct molecule.

[0157] Tag cassettes that bind cognate binding molecules include, for example, His tag (HHHHHH; SEQ ID NO: 43), Flag tag (DYKDDDDK; SEQ ID NO: 44), Xpress tag (DLYDDDDK; SEQ ID NO: 45), Avi tag (GLNDIFEAQKIEWHE; SEQ ID NO: 46), Calmodulin tag (KRRWKKNFIAVSAANRFKKISSSGAL; SEQ ID NO: 47), Polyglutamate tag, HA tag (YPYDVPDYA; SEQ ID NO: 48), Myc tag (EQKLISEEDL; SEQ ID NO: 49), Strep tag (which refers the original STREP® tag (WRHPQFGG; SEQ ID NO: 50), STREP® tag II (WSHPQFEK SEQ ID NO: 51 (IBA Institut für Bioanalytik, Germany); see, e.g., US 7,981,632), Softag 1 (SLAELLNAGLGGS; SEQ ID NO: 52), Softag 3 (TQDPSRVG; SEQ ID NO: 53), and V5 tag (GKPIPNPLLGLDST; SEQ ID NO: 54).

[0158] Conjugate binding molecules that specifically bind tag cassette sequences disclosed herein are commercially available. For example, His tag antibodies are commercially available from suppliers including Life Technologies, Pierce Antibodies, and GenScript. Flag tag antibodies are commercially available from suppliers including Pierce Antibodies, GenScript, and Sigma-Aldrich. Xpress tag antibodies are commercially available from suppliers including Pierce Antibodies, Life Technologies and GenScript. Avi tag antibodies are commercially available from suppliers including Pierce Antibodies, IsBio, and Genecopoeia. Calmodulin tag antibodies are commercially available from suppliers including Santa Cruz Biotechnology, Abcam, and Pierce Antibodies. HA tag antibodies are commercially available from suppliers including Pierce Antibodies, Cell Signal and Abcam. Myc tag antibodies are commercially available from suppliers including Santa Cruz Biotechnology, Abcam, and Cell Signal. Strep tag antibodies are commercially available from suppliers including Abcam, Iba, and Qiagen.

[0159] Transduction markers may be selected from at least one of a truncated CD19 (tCD19; see Budde et al., Blood 122: 1660, 2013); a truncated human EGFR (tEGFR; see Wang et al., Blood 118: 1255, 2011); an extracellular domain of human CD34; and/or RQR8 which combines

target epitopes from CD34 (see Fehse et al, Mol. Therapy 1(5 Pt 1); 448–456, 2000) and CD20 antigens (see Philip et al, Blood 124: 1277–1278).

[0160] Selection cassettes can be used for the selection of transformed cells. A selection cassette includes a selective marker gene. Selective marker genes are used to select transformed cells. Such selective markers may, for example, confer resistance to antibiotics, such as G418, hygromycin, blasticidin, neomycin, or puromycin. In other embodiments of the invention, the selective marker is operably linked to the inducible promoter, and the expression of the selective marker is toxic to the cell. Examples of such selective markers include xanthine / guanine phosphoribosyltransferase (gpt), hypoxanthine-guanine phosphoribosyltransferase (HGPRT) or thymidine kinase of the herpes simplex virus (HSV-TK). Polynucleotides encoding selective markers are functionally linked to the promoter active in the cell. In particular embodiments, the selection cassette includes a gene encoding neomycin resistance. In particular embodiments, the selection cassette includes a gene encoding puromycin resistance.

[0161] Control features may be present in multiple copies in a genetic construct or can be expressed as distinct molecules with the use of a skipping element. Exemplary skipping elements include a self-cleaving polypeptide or IRES. In particular embodiments, a self-cleaving polypeptide includes a 2A peptide from porcine teschovirus-1 (P2A), Thosea asigna virus (T2A), equine rhinitis A virus (E2A), foot-and-mouth disease virus (F2A), or variants thereof. Further exemplary nucleic acid and amino acid sequences of 2A peptides are set forth in, for example, Kim et al. (PLOS One 6:e18556 (2011)).

[0162] For example, a genetic construct can have one, two, three, four, or five tag cassettes and/or one, two, three, four, or five transduction markers could also be expressed. Exemplary transduction markers and cognate pairs are described in US 13/463,247.

[0163] One advantage of including at least one control feature in a genetic construct is that cells expressing the genetic construct administered to a subject can be depleted using the cognate binding molecule to a tag cassette. In certain embodiments, the present disclosure provides a method for depleting a modified cell expressing a genetic construct by using an antibody specific for the tag cassette, using a cognate binding molecule specific for the control feature, or by using a second modified cell expressing an antibody or chimeric antigen receptor having specificity for the control feature. Elimination of modified cells may be accomplished using depletion agents specific for a control feature. For example, if tEGFR is used, then an anti-tEGFR binding domain (e.g., antibody, scFv) fused to or conjugated to a cell-toxic reagent (such as a toxin, radiometal) may be used, or an anti-tEGFR /anti-CD3 bispecific scFv, or an anti-tEGFR CAR T cell may be used.

[0164] In certain embodiments, modified cells may be detected or tracked in vivo by using antibodies that bind with specificity to a control feature (e.g., anti-Tag antibodies), or by other cognate binding molecules that specifically bind the control feature, which binding partners for the control feature are conjugated to a fluorescent dye, radio-tracer, iron-oxide nanoparticle or other imaging agent known in the art for detection by X-ray, CT scan, MRI-scan, PET-scan, ultrasound, flow-cytometry, near infrared imaging systems, or other imaging modalities (see, e.g., Yu, et al., *Theranostics* 2:3, 2012).

[0165] Thus, modified cells expressing at least one control feature can be, e.g., more readily identified, isolated, sorted, induced to proliferate, tracked, and/or eliminated as compared to a modified cell without a tag cassette.

[0166] (IV) Culture and Storage of Cells. In another embodiment, the disclosure provides methods of establishing and/or maintaining populations of cells (e.g., stem cells, engineered cells, and/or differentiated cells), or the progeny thereof, as well as mixed populations including both stem cells (e.g., iPSC) and progeny cells, and the populations of cells so produced. Once a culture of cells or a mixed culture of cells is established, the population of cells is mitotically expanded in vitro by passage to fresh medium as cell density dictates under conditions conducive to cell proliferation, with or without tissue formation. Such culturing methods for stem cells can include, for example, passaging the cells in culture medium lacking particular growth factors that induce differentiation (e.g., IGF, EGF, FGF, VEGF, and/or other growth factor), in the presence of an agent that stimulates (e.g., an agonist) of Klf, Oct4, Sox, Myc, SV40Tag, Nanog, Lin28 or any combination thereof, in the presence of Klf, Oct4, Sox, Myc, SV40Tag, Nanog, Lin28 or any combination thereof, or any combination of the foregoing. Mixed cultures or cultures containing differentiated and/or engineered cells can be cultured in fresh medium containing particular growth factors and supplements to aid in their maintenance and growth. Accordingly, appropriate passaging techniques can be used to control contact inhibition and quiescence. Thus, in one embodiment, for example, transferring a portion of the cells to a new culture vessel with fresh medium. Such removal or transfer can be done in any culture vessel.

[0167] In particular embodiments, the disclosure provides cell lines. As used herein a “cell line” means a culture of cells of the disclosure, or progeny cells thereof, that can be reproduced for an extended period of time, preferably indefinitely, and which term includes, for example, cells that are cultured, cryopreserved and re-cultured following cryopreservation. As used herein a “culture” means a population of cells grown in a medium and optionally passaged accordingly. A cell culture may be a primary culture (e.g., a culture that has not been passaged) or may be a secondary or subsequent culture (e.g., a population of cells which have been subcultured or

passaged one or more times).

[0168] Once the cells have been established in culture, as described above, they may be maintained or stored in cell “banks” including either continuous *in vitro* cultures of cells requiring regular transfer or cells which have been cryopreserved. In some embodiments, the banked cells are used for autologous treatment of a subject.

[0169] Cryopreservation of cells may be carried out according to known methods, such as those described in Doyle et al., (eds.), 1995, *Cell & Tissue Culture: Laboratory Procedures*, John Wiley & Sons, Chichester. For example, cells may be suspended in a “freeze medium” such as, for example, culture medium further including 15-20% fetal bovine serum (FBS) and 10% dimethylsulfoxide (DMSO), with or without 5-10% glycerol, at a density, for example, of $4-10 \times 10^6$ cells/ml. The cells are dispensed into glass or plastic vials which are then sealed and transferred to a freezing chamber of a programmable or passive freezer. The optimal rate of freezing may be determined empirically. For example, a freezing program that gives a change in temperature of -1°C/min through the heat of fusion may be used. Once vials containing the cells have reached -80°C , they are transferred to a liquid nitrogen storage area. Cryopreserved cells can be stored for a period of years, though they should be checked at least every 5 years for maintenance of viability.

[0170] The cryopreserved cells of the disclosure constitute a bank of cells, portions of which can be withdrawn by thawing and then used to produce a cell culture as needed. Thawing should generally be carried out rapidly, for example, by transferring a vial from liquid nitrogen to a 37°C . water bath. The thawed contents of the vial should be immediately transferred under sterile conditions to a culture vessel containing an appropriate medium. It is advisable that the cells in the culture medium be adjusted to an initial density of $1-3 \times 10^5$ cells/ml. Once in culture, the cells may be examined daily, for example, with an inverted microscope to detect cell proliferation, and subcultured as soon as they reach an appropriate density.

[0171] The cells of the disclosure may be withdrawn from a cell bank as needed, and used for the production of new cells, either *in vitro*, or *in vivo*, for example, by direct administration of cells to the subject.

[0172] Once established, a culture of cells may be used to produce progeny cells. Differentiation of stem cells (e.g., iPSC) to other cell types, followed by the production of tissue therefrom, can be triggered by specific exogenous growth factors or by changing the culture conditions (e.g., the density) of a stem cell (e.g., iPSC) culture. The cells can be used to reconstitute an irradiated subject and/or a subject treated with chemotherapy; or as a source of cells for specific lineages, by providing for their maturation, proliferation and differentiation into one or more selected

lineages. Examples of factors that can be used to induce differentiation include erythropoietin, colony stimulating factors, e.g., GM-CSF, G-CSF, or M-CSF, interleukins, e.g., IL-1, -2, -3, -4, -5, -6, -7, -8, and the like, Leukemia Inhibitory Factor (LIF), Steel Factor (Stf), or the like, coculture with tissue committed cells, or other lineage committed cells types to induce the stem cells (e.g., iPSC) into becoming committed to a particular lineage. Additional methods of differentiation are described in more detail elsewhere herein.

[0173] (V) Cell-based Formulations. In particular embodiments, stem cells, engineered cells, and/or differentiated cells of the present disclosure can be harvested from a culture medium and washed and concentrated into a carrier in a therapeutically-effective amount. Exemplary carriers include saline, buffered saline, physiological saline, water, Hanks' solution, Ringer's solution, Normosol-R (Abbott Labs), PLASMA-LYTE A® (Baxter Laboratories, Inc., Morton Grove, IL), and combinations thereof.

[0174] In particular embodiments, carriers can be supplemented with human serum albumin (HSA) or other human serum components or fetal bovine serum. In particular embodiments, a carrier for infusion includes buffered saline with 5% HSA or dextrose. Additional isotonic agents include polyhydric sugar alcohols including trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol, or mannitol.

[0175] Carriers can include buffering agents, such as citrate buffers, succinate buffers, tartrate buffers, fumarate buffers, gluconate buffers, oxalate buffers, lactate buffers, acetate buffers, phosphate buffers, histidine buffers, and/or trimethylamine salts.

[0176] Stabilizers refer to a broad category of excipients which can range in function from a bulking agent to an additive which helps to prevent cell adherence to container walls. Typical stabilizers can include polyhydric sugar alcohols; amino acids, such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, and threonine; organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xylitol, ribitol, myoinisitol, galactitol, glycerol, and cyclitols, such as inositol; PEG; amino acid polymers; sulfur-containing reducing agents, such as urea, glutathione, thiocetic acid, sodium thioglycolate, thioglycerol, alpha-monothioglycerol, and sodium thiosulfate; low molecular weight polypeptides (i.e., <10 residues); proteins such as HSA, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; monosaccharides such as xylose, mannose, fructose and glucose; disaccharides such as lactose, maltose and sucrose; trisaccharides such as raffinose, and polysaccharides such as dextran.

[0177] Where necessary or beneficial, formulations can include a local anesthetic such as lidocaine to ease pain at a site of injection.

[0178] Exemplary preservatives include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalkonium halides, hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, and 3-pentanol.

[0179] Therapeutically effective amounts of cells within formulations can be greater than 10^2 cells, greater than 10^3 cells, greater than 10^4 cells, greater than 10^5 cells, greater than 10^6 cells, greater than 10^7 cells, greater than 10^8 cells, greater than 10^9 cells, greater than 10^{10} cells, or greater than 10^{11} .

[0180] In formulations disclosed herein, cells are generally in a volume of a liter or less, 500 ml or less, 250 ml or less or 100 ml or less. Hence the density of administered cells is typically greater than 10^4 cells/ml, 10^7 cells/ml or 10^8 cells/ml.

[0181] The cell-based formulations disclosed herein can be prepared for administration by, *e.g.*, injection, infusion, perfusion, or lavage. The formulations can further be formulated for bone marrow, intravenous, intradermal, intraarterial, intranodal, intralymphatic, intraperitoneal, intralesional, intraprostatic, intravaginal, intrarectal, intrathecal, intratumoral, intramuscular, intravesicular, and/or subcutaneous injection. In particular embodiments, cell-based formulations disclosed herein can be cryopreserved for later use. In particular embodiments, cell-based formulation can be used for research and/or cell manufacturing protocols.

[0182] (VI) Methods of Use. The methods and formulations disclosed herein can be used for cell manufacturing, research, and therapeutic purposes. The methods and formulations disclosed herein can be used in vitro or in vivo.

[0183] (VI-A) Feeder Cells for Cell Manufacturing. Coculture with feeder cells can greatly enhance the proliferation and activation of some cell types such as T cells and NK cells. For example, in some cases, T cells do not receive a strong enough activation signal when the binding domain of an extracellular component binds a targeted cell marker, resulting in a failure to kill the bound cell. Further, administered T cell populations often do not proliferate sufficiently or persist in vivo for sufficient periods of time following administration to maintain on-going anti-target effects.

[0184] An engineered feeder cell is ideally designed to include characteristics necessary or beneficial for the activation and expansion of the cell it is designed to be cocultured with. A cocultured cell refers to the cell from a population of cells that is activated and expanded with the addition of an appropriate feeder cell. Considerations for a feeder cell include expression products to be expressed by the feeder cell, whether the feeder cell should be adherent or cultured in suspension, the MHC background of the feeder, and what differentiated cell type the

feeder cell should be.

[0185] T-cells can further be classified into helper cells (CD4+ T-cells) and cytotoxic T-cells (CTLs, CD8+ T-cells), which include cytolytic T-cells. T helper cells assist other white blood cells in immunologic processes, including maturation of B cells into plasma cells and activation of cytotoxic T-cells and macrophages, among other functions. These cells are also known as CD4+ T-cells because they express the CD4 protein on their surface. Helper T-cells become activated when they are presented with peptide antigens by MHC class II molecules that are expressed on the surface of antigen presenting cells (APCs). Once activated, they divide rapidly and secrete small proteins called cytokines that regulate or assist in the active immune response.

[0186] T cell activation can occur in the presence of trimeric CD70, α CD3, CD28, 4-1BB and/or other activation molecules. In one embodiment, expansion refers to a period following activation wherein CD3, CD27 or CD28 binding molecules are no longer present. In particular embodiments, expansion refers to a period following activation wherein activating α CD3 molecules and CD27 binding molecules are no longer present. This expansion period can last until the end of culture when, for example, the cells are formulated for administration. In certain embodiments, expansion may take place in the presence of α CD3 molecules, CD27 binding molecules (CD70 molecules), other co-stimulatory molecules, and/or cytokines. In some embodiments, T cell activation occurs using a CD28/CD3 stimulating molecule (e.g., DynaBeads).

[0187] NK cells also benefit from coculture with feeder cells. NK cells sense and kill target cells that lack major histocompatibility complex (MHC)-class I molecules. NK cell activating receptors include, among others, the natural cytotoxicity receptors (NKp30, NKp44 and NKp46), and lectin-like receptors NKG2D and DNAM-1. Their ligands are expressed on stressed, transformed, or infected cells but not on normal cells, making normal cells resistant to NK cell killing (Bottino, Castriconi et al. 2005; Gasser, Orsulic et al. 2005; Lanier 2005). NK cell activation is negatively regulated via inhibitory receptors, such as killer immunoglobulin (Ig)-like receptors (KIRs), NKG2A/CD94, and leukocyte Ig-like receptor-1 (LIR-1). Engagement of one inhibitory receptor may be sufficient to prevent target lysis (Bryceson, Ljunggren et al. 2009). Hence NK cells efficiently target cells that express many stress-induced ligands, and few MHC class I ligands.

[0188] NK cell effector agents can be a cytokine, an adhesion molecule, or an NK cell activating agent. In particular embodiments, the NK cell effector agent can be IL-15, IL-21, IL-2, 4-1BBL, IL-12, IL-18, MICA, 2B4, LFA-1, and BCM1/SLAMF2. Some cancer cell lines have been used as feeder cells such as genetically modified K562 cells (artificial antigen-presenting cells with membrane-bound MICA, 4-1BBL, membrane-bound IL-15 and IL-21) and Epstein-Barr virus-

transformed lymphoblastoid cell lines. Even though these methods allow for large-scale NK cell expansion, they have safety issues because of concerns of using cancer cell-based feeder cells in therapeutic methods.

[0189] The engineered feeder cell can be an adherent cell or a cell grown in suspension. This will also affect the specialized type of cell chosen or differentiated into to prepare the feeder cell. In particular embodiments, a conditionally immortalized stem cell is differentiated into an adherent, differentiated feeder cell. In particular embodiments, a conditionally immortalized stem cell is differentiated into suspended, differentiated feeder cell. In particular embodiments, when designing feeder cells for T cells, the engineered feeder cells can be adherent cells such as mesenchymal stem cells. In particular embodiments, when designing feeder cells for NK cells, the engineered feeder cells can be cells in suspension such as CD34+ cells.

[0190] The feeder cell can be engineered to express a similar MHC genetic background as the cocultured cell or can be engineered to be MHC null. In particular embodiments, the feeder cell can be engineered to be conditionally immortalized. In particular embodiments, the feeder cell can be engineered to include a suicide gene. In particular embodiments, the feeder cell can be engineered to express an expression product for the activation and/or expansion of the cocultured cell. For example, an engineered feeder cell for T cells can express CD70, α CD3, CD28, and/or 4-1BB. In particular embodiments, an engineered feeder cell for NK cells might express MICA, 4-1BBL, membrane-bound IL-15 and/or membrane-bound IL21.

[0191] In particular embodiments, a feeder cell is used in coculture with a cocultured cell for activation and/or expansion of the cocultured cell. In particular embodiments, a feeder cell includes a conditional immortalization gene. In particular embodiments, a feeder cell includes a conditional immortalization gene and a sequence encoding an expression product. In particular embodiments, the conditional immortalization gene includes a sequence encoding SV40 large T antigen and/or TERT. In particular embodiments, the expression product is important for cell activation and/or expansion. In particular embodiments, the expression product includes CD70, α CD3, CD28, and/or 4-1BB. In particular embodiments, the expression product includes MICA, 4-1BBL, membrane-bound IL-15 and/or membrane-bound IL21.

[0192] In particular embodiments, a feeder cell includes a conditional immortalization gene, a sequence encoding an expression product, and a suicide gene. In particular embodiments, the suicide gene includes CDK1/HSV-TK/GCV. In particular embodiments, the suicide gene includes TOP2A/HSV-TK/GCV.

[0193] In particular embodiments, a feeder cell includes a conditional immortalization gene and a sequence encoding an expression product, wherein the feeder cell is genetically modified to

knockout MHC expression. In particular embodiments, MHC expression includes B2M, CITA, and/or CIITA expression.

[0194] In particular embodiments, a feeder cell includes a conditional immortalization gene, a sequence encoding an expression product, and a suicide gene wherein the feeder cell is genetically modified to knockout MHC expression.

[0195] In particular embodiments, a feeder cell used in coculture with a T cell includes a conditional immortalization gene and a sequence encoding CD70, α CD3, CD28, and/or 4-1BB. In particular embodiments, a feeder cell used in coculture with a T cell includes a conditional immortalization gene; a sequence encoding CD70, α CD3, CD28, and/or 4-1BB; and a suicide gene. In particular embodiments, a feeder cell used in coculture with a T cell includes a conditional immortalization gene and a sequence encoding CD70, α CD3, CD28, and/or 4-1BB; wherein the feeder cell is genetically modified to knockout B2M and/or CIITA. In particular embodiments, a feeder cell used in coculture with a T cell includes a conditional immortalization gene; a sequence encoding CD70, α CD3, CD28, and/or 4-1BB; and a suicide gene; wherein the feeder cell is genetically modified to knockout B2M and/or CIITA (e.g., B2M and CIITA). In particular embodiments, the conditional immortalization gene includes a sequence encoding SV40 large T antigen and/or TERT. In particular embodiments, the suicide gene includes CDK1/HSV-TK/GCV. In particular embodiments, the suicide gene includes TOP2A/HSV-TK/GCV.

[0196] In particular embodiments, a feeder cell used in coculture with an NK cell includes a conditional immortalization gene and a sequence encoding MICA, 4-1BBL, membrane-bound IL-15 and/or membrane-bound IL21. In particular embodiments, a feeder cell used in coculture with an NK cell includes a conditional immortalization gene and a sequence encoding membrane-bound IL21. In particular embodiments, a feeder cell used in coculture with an NK cell includes a conditional immortalization gene; a sequence encoding membrane-bound IL21; and a suicide gene. In particular embodiments, a feeder cell used in coculture with an NK cell includes a conditional immortalization gene and a sequence encoding membrane-bound IL21; wherein the feeder cell is genetically modified to knockout B2M and/or CIITA (e.g., B2M and CIITA). In particular embodiments, a feeder cell used in coculture with an NK cell includes a conditional immortalization gene; a sequence encoding membrane-bound IL21; and a suicide gene; wherein the feeder cell is genetically modified to knockout B2M and/or CIITA. In particular embodiments, the conditional immortalization gene includes a sequence encoding SV40 large T antigen and/or TERT. In particular embodiments, the suicide gene includes CDK1/HSV-TK/GCV. In particular embodiments, the suicide gene includes TOP2A/HSV-TK/GCV.

[0197] In particular embodiments, a method of preparing a feeder cell includes differentiating a conditionally immortalized stem cell into a specialized cell type. In particular embodiments, the method further includes genetically modifying the stem cell or feeder cell to express an expression product. In particular embodiments, the expression product is important for cell activation and/or expansion. In particular embodiments, the expression product includes CD70, α CD3, CD28, 4-1BB, MICA, 4-1BBL, membrane-bound IL-15, and/or membrane-bound IL21. In particular embodiments, the method further includes genetically modifying the feeder cell or stem cell to knock-out MHC expression. In particular embodiments, the method further includes genetically modifying the feeder cell or stem cell to include a suicide gene.

[0198] (VI-B) Tester Cells for Research and Development. Methods and formulations described herein can also be used to produce immortalized cell lines for research and development purposes. These immortalized cell lines are referred to herein as tester cells. In addition to immortalized cell lines for feeder cells, it can be beneficial to have a standardized, pre-manufactured, well-characterized cell line for use in research. Cellular disease models for in vitro or in vivo use can be very helpful in assessing the success or outcomes of potential therapeutics, however variations in the cells used for the disease models can lead to significant variation from experiment to experiment. The present disclosure describes engineered cells that can be used as tester cells. These tester cells can be differentiated into the desired cell type and can be engineered to be conditionally immortal, express desired expression products (e.g., proteins), be MHC null, and/or include a suicide gene.

[0199] In particular embodiments, a conditionally immortalized stem cell is differentiated into a tester cell. In particular embodiments, stem cell or tester cell is genetically modified to include a a sequence encoding an expression product. In particular embodiments, a stem cell or tester cell is genetically modified to include a sequence encoding an expression product; and genetically modified to knockout MHC expression. In particular embodiments, a stem cell or tester cell is genetically modified to include a sequence encoding an expression product, and a suicide gene. In particular embodiments, a stem cell or tester cell is genetically modified to include a sequence encoding an expression product, and a suicide gene; and genetically modified to knockout MHC expression.

[0200] In particular embodiments, the conditionally immortalized stem cell is an iPSC. In particular embodiments, the conditionally immortalized stem cell can be differentiated into any useful cell type. In particular embodiments, the conditionally immortalized stem cell is differentiated into a pancreatic cell (e.g., alpha, beta, and delta cell), epithelial cell, cardiac cell (e.g., cardiomyocyte), endothelial cell, liver cell (e.g., hepatocyte, hepatic stellate cell, Kupffer

cell (KC), and liver sinusoidal endothelial cell (LSEC)), endocrine cell, connective tissue cell (e.g., fibroblast), muscle cell (e.g., myoblast), brain cell (e.g., neuron), bone cell (e.g., osteoblast and osteoclast), kidney cell, cartilage cell (e.g., chondrocyte), immune cell (e.g., T-cell, NK cell, or macrophage), other stem cell (e.g., mesenchymal stem cell, hematopoietic stem cell, CD34+ cell, neural stem cell), or any other useful cell. In particular embodiments, the conditional immortalization gene includes a sequence encoding SV40 large T antigen and/or TERT. In particular embodiments, the expression product includes a detectable label and/or a cancer antigen. In particular embodiments, the detectable label includes fluorescent protein, a radioisotope, an enzyme label, or a fluorescent label. In particular embodiments, the fluorescent protein includes luciferase. In particular embodiments, the cancer antigen includes BCMA, CD4, CD5, CD7, CD19, CD20, CD22, CD33, CD73, CD123, CD133, CD138, CD244, CD276, CS1, EGFR, EGFRVIII, EpCAM, FLT3, GD2, GPA7, GPC3, HER2, Mesothelin, MUC1, NKG2D, PSMA, PSCA, or TF. In particular embodiments, the tester cell is genetically modified to knockout MHC expression by knocking out B2M, CITA, and/or CIITA (e.g., B2M and CIITA). In particular embodiments, the suicide gene includes CDK1/HSV-TK/GCV, TOP2A/HSV-TK/GCV, or iCasp9.

[0201] In particular embodiments, a method of preparing a tester cell includes differentiating a conditionally immortalized stem cell into a tester cell. A method of preparing a tester cell includes differentiating a conditionally immortalized stem cell into a tester cell. In particular embodiments, a method of preparing a tester cell includes differentiating a conditionally immortalized stem cell into a tester cell; and genetically modifying the stem cell or tester cell to encode an expression product; and genetically modifying the stem cell or tester cell to knockout MHC expression. In particular embodiments, a method of preparing a tester cell includes differentiating a conditionally immortalized stem cell into a tester cell; and genetically modifying the stem cell or tester cell to include a sequence encoding an expression product and a suicide gene. In particular embodiments, a method of preparing a tester cell includes differentiating a conditionally immortalized stem cell into a tester cell; genetically modifying the stem cell or tester cell to encode an expression product, and a suicide gene; and genetically modifying the tester cell to knockout MHC expression.

[0202] (VI-C) Conditionally Immortal Therapeutic Cell Line. Another use of the methods and compositions disclosed herein includes the production of immortalized therapeutic cells for use in cell therapy. Cell therapy refers to the use of cells to replace or kill damaged or diseased cells. Herein, stem cells can be differentiated into therapeutic cells and can be further engineered to express desirable characteristics. For example, differentiated therapeutic cells can be

conditionally immortalized, express an expression product, and or include a suicide gene.

[0203] Therapeutic uses of the methods and formulations disclosed herein include treating subjects (humans, non-human primates, veterinary animals (dogs, cats, reptiles, birds, etc.) livestock (horses, cattle, goats, pigs, chickens, etc.) and research animals (monkeys, rats, mice, fish, etc.)) with formulations disclosed herein. Treating subjects includes delivering therapeutically effective amounts. Therapeutically effective amounts include those that provide effective amounts, prophylactic treatments and/or therapeutic treatments.

[0204] An "effective amount" is the amount of a formulation necessary to result in a desired physiological change in the subject. For example, an effective amount can provide an anti-cancer, anti-infection, anti-diabetic, or healing effect. Effective amounts are often administered for research purposes. Effective amounts disclosed herein can cause a statistically significant effect in an animal model or in vitro assay relevant to the assessment of a disease, disorder, or injury's development or progression.

[0205] A "prophylactic treatment" includes a treatment administered to a subject who does not display signs or symptoms of a disease, disorder, or injury or displays only early signs or symptoms of a disease, disorder, or injury such that treatment is administered for the purpose of diminishing or decreasing the risk of developing the disease, disorder, or injury further. Thus, a prophylactic treatment functions as a preventative treatment against a disease, disorder, or injury. In particular embodiments, prophylactic treatments reduce, delay, or prevent disease, disorder, or injury.

[0206] A "therapeutic treatment" includes a treatment administered to a subject who displays symptoms or signs of a disease, disorder, or injury and is administered to the subject for the purpose of diminishing or eliminating those signs or symptoms of the disease, disorder, or injury. The therapeutic treatment can reduce, control, or eliminate the presence or activity of the disease, disorder, or injury and/or reduce control or eliminate side effects of the disease, disorder, or injury.

[0207] Function as an effective amount, prophylactic treatment or therapeutic treatment are not mutually exclusive, and in particular embodiments, administered dosages may accomplish more than one treatment type.

[0208] Uses of the conditionally immortalized stem cell populations, progeny, or engineered progeny thereof include administration into subjects to treat a variety of pathological states including diseases and disorders resulting from cancers, neoplasms, injury, viral infections, diabetes and the like. Cells are introduced into a subject in need of such cells or in need of a molecule encoded or produced by the genetically altered cell.

[0209] The cells of the disclosure can be used in a variety of applications. These include:

transplantation or implantation of the cells either in a differentiated form, an undifferentiated form, a de-differentiated form. Such cells and tissues serve to repair, replace or augment tissue that has been damaged due to disease or trauma, or that failed to develop normally.

[0210] In one embodiment, a formulation including the cells of the disclosure is prepared for injection directly to the site where the production of new tissue is desired. For example, the cells of the disclosure may be suspended in a hydrogel solution for injection. Alternatively, the hydrogel solution containing the cells may be allowed to harden, for instance in a mold to form a matrix having cells dispersed therein prior to implantation. Once the matrix has hardened, the cell formations may be cultured so that the cells are mitotically expanded prior to implantation. A hydrogel is an organic polymer (natural or synthetic) which is cross-linked via covalent, ionic, or hydrogen bonds to create a three-dimensional open-lattice structure, which entraps water molecules to form a gel. Examples of materials which can be used to form a hydrogel include polysaccharides such as alginate and salts thereof, polyphosphazines, and polyacrylates, which are cross-linked ionically, polyethylene oxide-polypropylene glycol block copolymers which are cross-linked by temperature or pH, respectively. Methods of synthesis of the hydrogel materials, as well as methods for preparing such hydrogels, are known in the art.

[0211] Such cell formulations may further include one or more other components, including selected extracellular matrix components, such as one or more types of collagen known in the art, and/or growth factors and drugs. Growth factors which may be usefully incorporated into the cell formulation include one or more tissue growth factors known in the art such as: any member of the transforming growth factor (TGF)- β family, insulin-like growth factor (IGF)-1 and -2, growth hormone, bone morphogenetic proteins (BMPs) such as BMP-13, and the like. Alternatively, the cells of the disclosure may be genetically engineered to express and produce growth factors such as BMP-13 or TGF- β . Other components may also be included in the formulation include, for example, buffers to provide appropriate pH and isotonicity, lubricants, viscous materials to retain the cells at or near the site of administration, (e.g., alginates, agars and plant gums) and other cell types that may produce a desired effect at the site of administration (e.g., enhancement or modification of the formation of tissue or its physicochemical characteristics, support for the viability of the cells, or inhibition of inflammation or rejection). The cells can be covered by an appropriate wound covering to prevent cells from leaving the site. Such wound coverings are known to those of skill in the art.

[0212] Alternatively, the formulations of the disclosure may be seeded onto a three-dimensional framework or scaffold and cultured to allow the cells to differentiate, grow and fill the matrix or immediately implanted in vivo, where the seeded cells will proliferate on the surface of the

framework and form a replacement tissue *in vivo* in cooperation with the cells of the subject. Such a framework can be implanted in combination with any one or more growth factors, drugs, additional cell types, or other components that stimulate formation or otherwise enhance or improve the practice of the disclosure.

[0213] The cells may be introduced directly into the peripheral blood or deposited within other locations throughout the body, e.g., a desired tissue, or on microcarrier beads in the peritoneum.

[0214] The cells of the disclosure may be used to treat subjects requiring the repair or replacement of tissue resulting from disease or trauma. Treatment may entail the use of the cells of the disclosure to produce new tissue, and the use of the tissue thus produced, according to any method presently known in the art or to be developed in the future. In one embodiment, administration includes the administration of genetically modified stem cells (e.g., iPSC). In particular embodiments, the administration includes the administration of differentiated stem cells. In particular embodiments, the administration includes the administration of differentiated, genetically modified stem cells.

[0215] In yet another embodiment, the formulations of the disclosure can be used in conjunction with a three-dimensional culture system in a “bioreactor” to produce tissue constructs which possess critical biochemical, physical and structural properties of native human tissue by culturing the cells and resulting tissue under environmental conditions which are typically experienced by native tissue. The bioreactor may include a number of designs. Typically, the culture conditions will include placing a physiological stress on the construct containing cells similar to what will be encountered *in vivo*.

[0216] For example, in one embodiment, the formulation can be administered to cancer patients who have undergone chemotherapy that have killed, reduced, or damaged stem cells or other cells of a subject, wherein the formulations replace the damaged or dead cells. Methods and compositions can provide stem cell bioreactors for the production of a desired polypeptide or may be used for gene delivery or gene therapy. In this embodiment, the cell-based formulation may be implanted or administered to a subject, or may be further differentiated to a desired cell type and implanted and delivered to the subject.

[0217] Formulations (e.g., therapeutic cells) which express a gene product of interest, or tissue produced *in vitro* therefrom, can be implanted into a subject who is otherwise deficient in that gene product. For example, genes that express products capable of preventing or ameliorating symptoms of various types of vascular diseases or disorders, or that prevent inflammatory disorders are of particular interest. In one embodiment, the cells of the disclosure are genetically engineered to express an anti-inflammatory gene product that would serve to reduce the risk of

failure of implantation or further degenerative change in tissue due to inflammatory reaction. For example, formulations of the disclosure can be genetically engineered to express one or more anti-inflammatory gene products including, for example, peptides or polypeptides corresponding to the idiotype of antibodies that neutralize granulocyte-macrophage colony stimulating factor (GM-CSF), tumor necrosis factor (TNF), IL-1, IL-2, or other inflammatory cytokines. IL-1 has been shown to decrease the synthesis of proteoglycans and collagens type II, IX, and XI (Tyler et al., 1985, *Biochem. J.* 227:69-878; Tyler et al., 1988, *Coll. Relat. Res.* 82:393-405; Goldring et al., 1988, *J. Clin. Invest.* 82:2026-2037; and Lefebvre et al., 1990, *Biophys. Acta.* 1052:366-72). TNF also inhibits synthesis of proteoglycans and type II collagen, although it is much less potent than IL-1 (Yaron, I., et al., 1989, *Arthritis Rheum.* 32:173-80; Ikebe, T., et al., 1988, *J. Immunol.* 140:827-31; and Saklatvala, J., 1986, *Nature* 322:547-49). Also, for example, the cells of the disclosure may be engineered to express the gene encoding the human complement regulatory protein that prevents rejection of a graft by the host. See, for example, McCurry et al., 1995, *Nature Medicine* 1:423-27.

[0218] It has been previously demonstrated that transplantation of beta islet cells provides therapy for patients with diabetes (Shapiro *et al.*, *N. Engl. J. Med.* 343:230-238, 2000). The formulations provide an alternative source of islet cells to prevent or treat diabetes. For example, stem cells of the disclosure can be generated, isolated and differentiated to a pancreatic cell type, genetically modified, and delivered to a subject. Alternatively, the conditionally immortalized stem cells can be genetically modified and delivered to the pancreas of the subject and differentiated to islet cells *in vivo*. Accordingly, the cells are useful for transplantation in order to prevent or treat the occurrence of diabetes.

[0219] In another embodiment, the formulations are genetically engineered to express genes for specific types of growth factors for successful and/or improved differentiation to fibroblasts, other stromal cells, or parenchymal cells and/or turnover either pre- or post-implantation.

[0220] The disclosure contemplates that the *in vitro* methods described herein can be used for non-autologous transplantations. The disclosure contemplates that the *in vitro* methods described herein can be used for autologous transplantation of stem cells or differentiated cells. In any of the foregoing embodiments, the disclosure contemplates that stem cells can be expanded in culture and stored for later retrieval and use. Similarly, the disclosure contemplates that differentiated cells can be expanded in culture and stored for later retrieval and use.

[0221] For administration, therapeutically effective amounts (also referred to herein as doses) can be initially estimated based on results from *in vitro* assays and/or animal model studies. Such information can be used to more accurately determine useful doses in subjects of interest. The

actual dose amount administered to a particular subject can be determined by a physician, veterinarian or researcher taking into account parameters such as physical and physiological factors including target, body weight, severity of condition, type of disease or injury, previous or concurrent therapeutic interventions, idiopathy of the subject and route of administration.

[0222] Therapeutically effective amounts of cell-based formulations can include 10^4 to 10^9 cells/kg body weight, or 10^3 to 10^{11} cells/kg body weight. Therapeutically effective amounts to administer can include greater than 10^2 cells, greater than 10^3 cells, greater than 10^4 cells, greater than 10^5 cells, greater than 10^6 cells, greater than 10^7 cells, greater than 10^8 cells, greater than 10^9 cells, greater than 10^{10} cells, or greater than 10^{11} .

[0223] Therapeutically effective amounts can be achieved by administering single or multiple doses during the course of a treatment regimen (e.g., daily, every other day, every 3 days, every 4 days, every 5 days, every 6 days, weekly, every 2 weeks, every 3 weeks, monthly, every 2 months, every 3 months, every 4 months, every 5 months, every 6 months, every 7 months, every 8 months, every 9 months, every 10 months, every 11 months or yearly). In particular embodiments, the treatment protocol may be dictated by a clinical trial protocol or an FDA-approved treatment protocol.

[0224] Therapeutically effective amounts can be administered by, e.g., injection, infusion, perfusion, or lavage. Routes of administration can include bolus intravenous, intradermal, intraarterial, intraparenteral, intranodal, intralymphatic, intraperitoneal, intralesional, intraprostatic, intravaginal, intrarectal, topical, intrathecal, intratumoral, intramuscular, intravesicular, and/or subcutaneous administration.

[0225] In certain embodiments, formulations are administered to a patient in conjunction with (e.g., before, simultaneously or following) any number of relevant treatment modalities. In particular embodiments, cells may be used in combination with chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAM PATH, anti-CD3 antibodies or other antibody therapies, cytoxin, fludarabine, cyclosporin, FK506, rapamycin, mycoplienolic acid, steroids, FR901228, cytokines, and irradiation.

[0226] Therapeutic cells can include any cell type that is useful in cell therapy including pancreatic cell (e.g., alpha, beta, and delta cell), epithelial cell, cardiac cell (e.g., cardiomyocyte), endothelial cell, liver cell (e.g., hepatocyte, hepatic stellate cell, Kupffer cell (KC), and liver sinusoidal endothelial cell (LSEC)), endocrine cell, connective tissue cell (e.g., fibroblast), muscle cell (e.g., myoblast), brain cell (e.g., neuron), bone cell (e.g., osteoblast and osteoclast), kidney cell, cartilage cell (e.g., chondrocyte), immune cell (e.g., T-cell, NK cell, or macrophage),

or other stem cell (e.g., mesenchymal stem cell, hematopoietic stem cell, CD34+ cell, neural stem cells, or any other useful cell. In particular embodiments, immune cells includes a T cell, a B cell, a natural killer (NK) cell, an NK-T cell, a monocyte/macrophage, a hematopoietic stem cells (HSC), or a hematopoietic progenitor cell (HPC).

[0227] In particular embodiments, a conditionally immortalized stem cell is differentiated into a therapeutic cell. In particular embodiments, the stem cell or therapeutic cell is genetically modified to express an expression product. In particular embodiments, the expression product can include a protein (e.g., an antigen, an antibody, a recombinant receptor, and/or a detectable label). In particular embodiments, the recombinant receptor includes a CAR or an eTCR. In particular embodiments, the detectable label is a fluorescent protein and/or luciferase.

[0228] In particular embodiments, a therapeutic cell is genetically modified to include a suicide gene. In particular embodiments, the suicide gene includes CDK1/HSV-TK/GCV, TOP2A/HSV-TK/GCV, or iCasp9. Although growth of the therapeutic cell can be controlled because of the conditional immortalization gene, a suicide gene provides an extra layer of safety by removing or killing the genetically modified cells.

[0229] In particular embodiments, a conditionally immortalized stem cell is differentiated into a T cell. In particular embodiments, the stem cell or T cell is genetically modified to express a CAR. In particular embodiments, the stem cell or T cell is genetically modified to include a suicide gene. In particular embodiments, the stem cell or T cell is genetically modified to encode a suicide gene and a CAR.

[0230] In particular embodiments, a conditionally immortalized stem cell is differentiated into an NK cell. In particular embodiments, the stem cell or NK cell is genetically modified to include a sequence encoding an expression product. In particular embodiments, the stem cell or NK cell is genetically modified to include a suicide gene. In particular embodiments, the stem cell or NK cell is genetically modified to include a a suicide gene and a sequence encoding an expression product.

[0231] In particular embodiments, a conditionally immortalized stem cell is differentiated into a liver cell. In particular embodiments, the stem cell or liver cell is genetically modified to include a sequence encoding an expression product. In particular embodiments, the stem cell or liver cell is genetically modified to include a suicide gene. In particular embodiments, the stem cell or liver cell is genetically modified to include a suicide gene, and a sequence encoding an expression product.

[0232] In particular embodiments, a conditionally immortalized stem cell is differentiated into a pancreatic beta cell. In particular embodiments, the stem cell or pancreatic beta cell is genetically

modified to include a sequence encoding insulin. In particular embodiments, the stem cell or pancreatic beta cell is genetically modified to include a suicide gene. In particular embodiments, the stem cell or pancreatic beta cell is genetically modified to include a suicide gene, and a sequence encoding insulin.

[0233] The Exemplary Embodiments and Experimental Examples below are included to demonstrate particular embodiments of the disclosure. Those of ordinary skill in the art should recognize in light of the present disclosure that many changes can be made to the specific embodiments disclosed herein and still obtain a like or similar result without departing from the spirit and scope of the disclosure.

[0234] (VII) Exemplary Embodiments.

1. A stem cell including a conditional immortalization gene.
2. The stem cell of embodiment 1, wherein the conditional immortalization gene encodes TERT.
3. The stem cell of embodiments 1 or 2, wherein the conditional immortalization gene encodes SV40 large T antigen.
4. The stem cell of any of embodiments 1-3, wherein the conditional immortalization gene includes TERT and SV40 large T antigen.
5. The stem cell of any of embodiments 1-4, wherein the conditional immortalization gene is induced by a drug.
6. The stem cell of embodiment 5, wherein the drug includes tetracycline and/or doxycycline.
7. The stem cell of any of embodiments 1-6, wherein the stem cell is a totipotent stem cell, a pluripotent stem cell, a multipotent stem cell, or a unipotent stem cell.
8. The stem cell of any of embodiments 1-7, wherein the pluripotent stem cell is an embryonic stem cell, a cord blood stem cell, or an induced pluripotent stem cells (iPSC).
9. The stem cell of any of embodiments 1-8, wherein the multipotent stem cell is a hematopoietic stem cell, a mesenchymal stem cell, or a neuronal stem cell.
10. The stem cell of any of embodiments 1-9, further including an exogenous sequence that encodes an expression product.
11. The stem cell of embodiment 10, wherein the expression product is a protein.
12. The stem cell of embodiment 11, wherein the protein includes a recombinant receptor, a detectable label, an antigen, an antibody, and/or an enzyme.
13. The stem cell of embodiments 11 or 12, wherein the protein includes CD70, α CD3, CD28, 4-1BB, MICA, 4-1BBL, membrane-bound interleukin (IL)-15, and/or membrane-bound IL21.
14. The stem cell of any of embodiments 11-13, wherein the protein includes membrane-bound IL21.

15. The stem cell of any of embodiments 11-14, wherein the protein includes a cancer antigen.
16. The stem cell of embodiment 15, wherein the cancer includes multiple myeloma, lymphoma, acute lymphocytic leukemia (ALL), acute myelocytic leukemia (AML), chronic lymphocytic leukemia (CLL), breast cancer, colorectal cancer, ovarian cancer, renal cell carcinoma (RCC), glioblastoma, prostate cancer, neuroblastoma, melanoma, Ewing sarcoma, and/or hepatocellular cancer (HCC).
17. The stem cell of embodiments 15 or 16, wherein the cancer antigen includes BCMA, CD4, CD5, CD7, CD19, CD20, CD22, CD33, CD73, CD123, CD133, CD138, CD244, CD276, CS1, EGFR, EGFRVIII, EpCAM, FLT3, GD2, GPA7, GPC3, HER2, Mesothelin, MUC1, NKG2D, PSMA, PSCA, and/or TF.
18. The stem cell of any of embodiments 15-17, wherein the cancer antigen includes BCMA, CD19, CD20, CD33, CD133, CD138, CS1, EGFR, EGFRVII, EpCAM, GD2, GPA7, HER2, NKG2D, MUC1, and/or PSCA.
19. The stem cell of any of embodiments 11-18, wherein the protein includes a viral, bacterial, fungal, and/or parasitic antigen.
20. The stem cell of any of embodiments 11-19, wherein the protein includes insulin, factor VIII, factor IX, factor XI, alpha-1 antitrypsin (A1AT), glucocerebrosidase (GC), acid sphingomyelinase, mucopolysaccharides, acid alpha-glucosidase, aspartylglucosaminidase, alpha-galactosidase A, palmitoyl protein thioesterase, tripeptidyl peptidase, lysosomal transmembrane protein, cysteine transporter, acid ceramidase, acid alpha-L-fucosidase, cathepsin A, acid beta-glucosidase, acid beta-galactosidase, iduronate-2-sulfatase, alpha-L-iduronidase, galactocerebrosidase, acid alpha-mannosidase, acid beta-mannosidase, arylsulfatase B, arylsulfatase A, N-acetylgalactosamine-6-sulfate, N-acetylglucosamine-1-phosphotransferase, acid sphingomyelinase, NPC-1, alpha-glucosidase, beta-hexosaminidase B, heparan N-sulfatase, alpha-N-acetylglucosaminidase, acetyl-CoA: alpha-glucosaminide, N-acetylglucosamine-6-sulfate, alpha-N-acetylgalactosaminidase, alpha-neuramidase, beta-glucuronidase, beta-hexosaminidase A, and/or acid lipase.
21. The stem cell of any of embodiments 12-20, wherein the recombinant receptor includes an extracellular component including a binding domain; an intracellular component including an effector domain; and a transmembrane domain linking the extracellular component to the intracellular component.
22. The stem cell of any of embodiments 12-21, wherein the recombinant receptor includes a chimeric antigen receptor and/or an engineered T cell receptor.
23. The stem cell of embodiments 21 or 22, wherein the binding domain of the recombinant

- receptor binds a cancer antigen, a viral antigen, a bacterial antigen, and/or a fungal antigen.
24. The stem cell of embodiment 23, wherein the cancer antigen includes BCMA, CD4, CD5, CD7, CD19, CD20, CD22, CD33, CD73, CD123, CD133, CD138, CD244, CD276, CS1, EGFR, EGFRVIII, EpCAM, FLT3, GD2, GPA7, GPC3, HER2, Mesothelin, MUC1, NKG2D, PSMA, PSCA, and/or TF.
 25. The stem cell of embodiments 23 or 24, wherein the cancer antigen includes BCMA, CD19, CD20, CD33, CD133, CD138, CS1, EGFR, EGFRVII, EpCAM, GD2, GPA7, HER2, NKG2D, MUC1, and/or PSCA.
 26. The stem cell of any of embodiments 21-25, wherein the effector domain includes all or a portion of the signaling domain of CD3 ζ and/or 4-1BB.
 27. The stem cell of any of embodiments 21-26, wherein the transmembrane domain includes a CD28 transmembrane domain.
 28. The stem cell of any of embodiments 12-27, wherein the recombinant receptor includes a CD19 binding domain.
 29. The stem cell of any of embodiments 12-28, wherein the recombinant receptor includes a BCMA binding domain.
 30. The stem cell of any of embodiments 12-29, wherein the detectable label includes a fluorescent protein, a radioisotope, an enzyme label, and/or a fluorescent label.
 31. The stem cell of embodiment 30, wherein the fluorescent protein includes luciferase.
 32. The stem cell of any of embodiments 1-31, wherein the stem cell is genetically modified to knockout a major histocompatibility complex (MHC).
 33. The stem cell of any of embodiments 1-32, wherein the stem cell is genetically modified to knockout β 2-microglobulin (B2M).
 34. The stem cell of any of embodiments 1-33, wherein the stem cell is genetically modified to knockout Class I Major Histocompatibility Complex Transactivator and/or Class II Major Histocompatibility Complex Transactivator.
 35. The stem cell of any of embodiments 1-34, wherein the stem cell is genetically modified to knockout B2M, CIITA, or B2M and CIITA.
 36. The stem cell of embodiments 33 or 35, wherein B2M is knocked out with the gRNA sequence as set forth in SEQ ID NOs: 34-42.
 37. The stem cell of embodiments 34 or 35, wherein CIITA is knocked out with the gRNA sequence as set forth in SEQ ID NOs: 25-33.
 38. The stem cell of any of embodiments 1-37, wherein the stem cell further includes a suicide gene.

39. The stem cell of embodiment 38, wherein the suicide gene includes CDK1 linked Herpes simplex virus-thymidine kinase/ganciclovir (CDK1/HSV-TK/GCV), TOP2A/HSV-TK/GCV, and/or inducible Casp9.
40. The stem cell of embodiments 38 or 39, wherein the suicide gene includes CDK1/HSV-TK/GCV.
41. The stem cell of any of embodiments 1-40, wherein the stem cell further includes a sequence encoding a tag cassette, a transduction marker, selection cassette, and/or a skipping element.
42. A cell line differentiated from the stem cell of any of embodiments 1-41.
43. The cell line of embodiment 42, wherein the cell line includes more differentiated stem cells than the stem cell of any of embodiments 1-41.
44. The cell line of embodiment 43, wherein the more differentiated stem cells are CD34+ hematopoietic stem cells or mesenchymal stem cells.
45. The cell line of any of embodiments 42-44, wherein the cell line includes pancreatic cells, epithelial cells, cardiac cells, endothelial cells, liver cells, endocrine cells, connective tissue cells, muscle cells, brain cells, bone cells, kidney cells, cartilage cells, or immune cells.
46. The cell line of embodiment 45, wherein the pancreatic cells include alpha cells, beta cells, or delta cells.
47. The cell line of embodiments 45 or 46, wherein the cardiac cells include cardiomyocytes.
48. The cell line of any of embodiments 45-47, wherein the liver cells include hepatocytes, hepatic stellate cells (HSCs), Kupffer cells (KCs), or liver sinusoidal endothelial cells (LSECs).
49. The cell line of any of embodiments 45-48, wherein the connective tissue cells include fibroblasts.
50. The cell line of any of embodiments 45-49, wherein the muscle cells include myoblasts.
51. The cell line of any of embodiments 45-50, wherein the brain cells include neurons.
52. The cell line of any of embodiments 45-51, wherein the bone cells include osteoblasts or osteoclasts.
53. The cell line of any of embodiments 45-52, wherein the cartilage cells include chondrocytes.
54. The cell line of any of embodiments 45-53, wherein the immune cells include T-cells, NK cells, or macrophages.
55. The cell line of any of embodiments 42-54, wherein cells within the cell line are genetically modified to express an expression product.
56. The cell line of embodiment 55, wherein the expression product is a protein.
57. The cell line of embodiment 56, wherein the protein includes a recombinant receptor, a detectable label, an antigen, an antibody, and/or an enzyme.

58. The cell line of embodiments 56 or 57, wherein the protein includes CD70, α CD3, CD28, 4-1BB, MICA, 4-1BBL, membrane-bound interleukin (IL)-15, and/or membrane-bound IL21
59. The cell line of any of embodiments 56-58, wherein the protein includes membrane-bound IL21.
60. The cell line of any of embodiments 56-59, wherein the protein includes a cancer antigen.
61. The cell line of embodiment 60, wherein the cancer includes multiple myeloma, lymphoma, acute lymphocytic leukemia (ALL), acute myelocytic leukemia (AML), chronic lymphocytic leukemia (CLL), breast cancer, colorectal cancer, ovarian cancer, renal cell carcinoma (RCC), glioblastoma, prostate cancer, neuroblastoma, melanoma, Ewing sarcoma, and/or hepatocellular cancer (HCC).
62. The cell line of embodiments 60 or 61, wherein the cancer antigen includes BCMA, CD4, CD5, CD7, CD19, CD20, CD22, CD33, CD73, CD123, CD133, CD138, CD244, CD276, CS1, EGFR, EGFRVIII, EpCAM, FLT3, GD2, GPA7, GPC3, HER2, Mesothelin, MUC1, NKG2D, PSMA, PSCA, and/or TF.
63. The cell line of any of embodiments 60-62, wherein the cancer antigen includes BCMA, CD19, CD20, CD33, CD133, CD138, CS1, EGFR, EGFRVII, EpCAM, GD2, GPA7, HER2, NKG2D, MUC1, and/or PSCA.
64. The cell line of any of embodiments 56-63, wherein the protein includes a viral, bacterial, fungal, and/or parasitic antigen.
65. The cell line of any of embodiments 56-64, wherein the protein includes insulin, factor VIII, factor IX, factor XI, alpha-1 antitrypsin (A1AT), glucocerebrosidase (GC), acid sphingomyelinase, mucopolysaccharides, acid alpha-glucosidase, aspartylglucosaminidase, alpha-galactosidase A, palmitoyl protein thioesterase, tripeptidyl peptidase, lysosomal transmembrane protein, cysteine transporter, acid ceramidase, acid alpha-L-fucosidase, cathepsin A, acid beta-glucosidase, acid beta-galactosidase, iduronate-2-sulfatase, alpha-L-iduronidase, galactocerebrosidase, acid alpha-mannosidase, acid beta-mannosidase, arylsulfatase B, arylsulfatase A, N-acetylgalactosamine-6-sulfate, N-acetylglucosamine-1-phosphotransferase, acid sphingomyelinase, NPC-1, alpha-glucosidase, beta-hexosaminidase B, heparan N-sulfatase, alpha-N-acetylglucosaminidase, acetyl-CoA: alpha-glucosaminide, N-acetylglucosamine-6-sulfate, alpha-N-acetylgalactosaminidase, alpha-neuramidase, beta-glucuronidase, beta-hexosaminidase A, and/or acid lipase.
66. The cell line of any of embodiments 57-65, wherein the recombinant receptor includes an extracellular component including a binding domain; an intracellular component including an effector domain; and a transmembrane domain linking the extracellular component to the

intracellular component.

67. The cell line of any of embodiments 57-66, wherein the recombinant receptor includes a chimeric antigen receptor and/or an engineered T cell receptor.
68. The cell line of embodiments 66 or 67, wherein the binding domain of the recombinant receptor binds a cancer antigen, a viral antigen, a bacterial antigen, and/or a fungal antigen.
69. The cell line of embodiment 68, wherein the cancer antigen includes BCMA, CD4, CD5, CD7, CD19, CD20, CD22, CD33, CD73, CD123, CD133, CD138, CD244, CD276, CS1, EGFR, EGFRV8, EpCAM, FLT3, GD2, GPA7, GPC3, HER2, Mesothelin, MUC1, NKG2D, PSMA, PSCA, and/or TF.
70. The cell line of embodiments 68 or 69, wherein the cancer antigen includes BCMA, CD19, CD20, CD33, CD133, CD138, CS1, EGFR, EGFRV7, EpCAM, GD2, GPA7, HER2, NKG2D, MUC1, and/or PSCA.
71. The cell line of any of embodiments 66-70, wherein the effector domain includes all or a portion of the signaling domain of CD3 ζ and/or 4-1BB.
72. The cell line of any of embodiments 66-71, wherein the transmembrane domain includes a CD28 transmembrane domain.
73. The cell line of any of embodiments 57-72, wherein the recombinant receptor includes a CD19 binding domain.
74. The cell line of any of embodiments 57-73, wherein the recombinant receptor includes a BCMA binding domain.
75. The cell line of any of embodiments 57-74, wherein the detectable label includes a fluorescent protein, a radioisotope, an enzyme label, and/or a fluorescent label.
76. The cell line of embodiment 75, wherein the fluorescent protein includes luciferase.
77. The cell line of any of embodiments 42-76, wherein cells within the cell line are genetically modified to knockout a major histocompatibility complex (MHC).
78. The cell line of any of embodiments 42-77, wherein cells within the cell line are genetically modified to knockout β 2-microglobulin (B2M).
79. The cell line of any of embodiments 42-78, wherein cells within the cell line are genetically modified to knockout Class I Major Histocompatibility Complex Transactivator and/or Class II Major Histocompatibility Complex Transactivator.
80. The cell line of any of embodiments 42-79, wherein cells within the cell line are genetically modified to B2M, CIITA, or B2M and CIITA.
81. The cell line of any of embodiments 78 - 80, wherein B2M is knocked out with the gRNA sequence as set forth in SEQ ID NOs: 34-42.

82. The cell line of embodiments 79 or 80, wherein CIITA is knocked out with the gRNA sequence as set forth in SEQ ID NOs: 25-33.
83. The cell line of any of embodiments 42-82, wherein cells within the cell line further include a suicide gene.
84. The cell line of embodiment 83, wherein the suicide gene includes CDK1 linked Herpes simplex virus-thymidine kinase/ganciclovir (CDK1/HSV-TK/GCV), TOP2A/HSV-TK/GCV, and/or inducible Casp9.
85. The cell line of embodiments 83 or 84, wherein the suicide gene includes CDK1/HSV-TK/GCV.
86. A method including genetically modifying a stem cell to include a conditional immortalization gene.
87. The method of embodiment 86, wherein the genetically modifying includes transfecting a stem cell with the conditional immortalization gene using the Tet inducible system.
88. The method of embodiments 86 or 87, wherein the conditional immortalization gene encodes TERT.
89. The method of any of embodiments 86-88, wherein the conditional immortalization gene encodes SV40 large T antigen.
90. The method of any of embodiments 86-89, wherein the conditional immortalization gene includes TERT and SV40 large T antigen.
91. The method of any of embodiments 86-90, wherein the conditional immortalization gene is induced by a drug.
92. The method of embodiment 91, wherein the drug includes tetracycline and/or doxycycline.
93. The method of any of embodiments 86-92, wherein the stem cell is a totipotent stem cell, a pluripotent stem cell, a multipotent stem cell, or a unipotent stem cell.
94. The method of embodiment 93, wherein the pluripotent stem cell is an embryonic stem cell, a cord blood stem cell, or an induced pluripotent stem cells (iPSC).
95. The method of embodiments 93 or 94, wherein the multipotent stem cell is a hematopoietic stem cell, a mesenchymal stem cell, or a neuronal stem cell.
96. The method of any of embodiments 86-95, further genetically modifying the stem cell to include an exogenous sequence that encodes an expression product.
97. The method of embodiment 96, wherein the genetically modifying the stem cell to include an exogenous sequence includes transfecting the stem cell with an expression construct using a transposon-based system and/or a lentivirus system.
98. The method of embodiments 96 or 97, wherein the genetically modifying the stem cell to

include an exogenous sequence includes transfecting the stem cell with an expression construct using a transposon-based system.

99. The method of any of embodiments 96-98, wherein the expression product is a protein.
100. The method of embodiment 99, wherein the protein includes a recombinant receptor, a detectable label, an antigen, an antibody, and/or an enzyme.
101. The method of embodiment 99 or 100, wherein the protein includes CD70, α CD3, CD28, 4-1BB, MICA, 4-1BBL, membrane-bound interleukin (IL)-15, and/or membrane-bound IL21
102. The method of any of embodiments 99-101, wherein the protein includes membrane-bound IL21.
103. The method of any of embodiments 99-102, wherein the protein includes a cancer antigen.
104. The method of embodiment 103, wherein the cancer includes multiple myeloma, lymphoma, acute lymphocytic leukemia (ALL), acute myelocytic leukemia (AML), chronic lymphocytic leukemia (CLL), breast cancer, colorectal cancer, ovarian cancer, renal cell carcinoma (RCC), glioblastoma, prostate cancer, neuroblastoma, melanoma, Ewing sarcoma, and/or hepatocellular cancer (HCC).
105. The method of embodiments 103 or 104, wherein the cancer antigen includes BCMA, CD4, CD5, CD7, CD19, CD20, CD22, CD33, CD73, CD123, CD133, CD138, CD244, CD276, CS1, EGFR, EGFRVIII, EpCAM, FLT3, GD2, GPA7, GPC3, HER2, Mesothelin, MUC1, NKG2D, PSMA, PSCA, and/or TF.
106. The method of any of embodiments 103-105, wherein the cancer antigen includes BCMA, CD19, CD20, CD33, CD133, CD138, CS1, EGFR, EGFRVII, EpCAM, GD2, GPA7, HER2, NKG2D, MUC1, and/or PSCA.
107. The method of any of embodiments 99-106, wherein the protein includes a viral, bacterial, fungal, and/or parasitic antigen.
108. The method of any of embodiments 99-107, wherein the protein includes insulin, factor VIII, factor IX, factor XI, alpha-1 antitrypsin (A1AT), glucocerebrosidase (GC), acid sphingomyelinase, mucopolysaccharides, acid alpha-glucosidase, aspartylglucosaminidase, alpha-galactosidase A, palmitoyl protein thioesterase, tripeptidyl peptidase, lysosomal transmembrane protein, cysteine transporter, acid ceramidase, acid alpha-L-fucosidase, cathepsin A, acid beta-glucosidase, acid beta-galactosidase, iduronate-2-sulfatase, alpha-L-iduronidase, galactocerebrosidase, acid alpha-mannosidase, acid beta-mannosidase, arylsulfatase B, arylsulfatase A, N-acetylgalactosamine-6-sulfate, N-acetylglucosamine-1-phosphotransferase, acid sphingomyelinase, NPC-1, alpha-glucosidase, beta-hexosaminidase B, heparan N-sulfatase, alpha-N-acetylglucosaminidase, acetyl-CoA: alpha-

glucosaminide, N-acetylglucosamine-6-sulfate, alpha-N-acetylgalactosaminidase, alpha-neuramidase, beta-glucuronidase, beta-hexosaminidase A, and/or acid lipase.

109. The method of any of embodiments 100-108, wherein the recombinant receptor includes an extracellular component including a binding domain; an intracellular component including an effector domain; and a transmembrane domain linking the extracellular component to the intracellular component.
110. The method of any of embodiments 100-109, wherein the recombinant receptor includes a chimeric antigen receptor and/or an engineered T cell receptor.
111. The method of embodiments 109 or 110, wherein the binding domain of the recombinant receptor binds a cancer antigen, a viral antigen, a bacterial antigen, and/or a fungal antigen.
112. The method of embodiment 111, wherein the cancer antigen includes BCMA, CD4, CD5, CD7, CD19, CD20, CD22, CD33, CD73, CD123, CD133, CD138, CD244, CD276, CS1, EGFR, EGFRVIII, EpCAM, FLT3, GD2, GPA7, GPC3, HER2, Mesothelin, MUC1, NKG2D, PSMA, PSCA, and/or TF.
113. The method of embodiments 111 or 112, wherein the cancer antigen includes BCMA, CD19, CD20, CD33, CD133, CD138, CS1, EGFR, EGFRVII, EpCAM, GD2, GPA7, HER2, NKG2D, MUC1, and/or PSCA.
114. The method of any of embodiments 109-113, wherein the effector domain includes all or a portion of the signaling domain of CD3 ζ and/or 4-1BB.
115. The method of any of embodiments 109-114, wherein the transmembrane domain includes a CD28 transmembrane domain.
116. The method of any of embodiments 100-115, wherein the recombinant receptor includes a CD19 binding domain.
117. The method of any of embodiments 100-116, wherein the recombinant receptor includes a BCMA binding domain.
118. The method of any of embodiments 100-117, wherein the detectable label includes a fluorescent protein, a radioisotope, an enzyme label, and/or a fluorescent label.
119. The method of embodiment 118, wherein the fluorescent protein includes luciferase.
120. The method of any of embodiments 86-119, wherein the stem cells are genetically modified to knockout a major histocompatibility complex (MHC).
121. The method of any of embodiments 86-120, wherein the stem cells are genetically modified to knockout β 2-microglobulin (B2M).
122. The method of any of embodiments 86-121, wherein the stem cells are genetically modified to knockout Class I Major Histocompatibility Complex Transactivator and/or Class II

Major Histocompatibility Complex Transactivator.

123. The method of any of embodiments 86-122, wherein the stem cells are genetically modified to knockout B2M, CIITA, or B2M and CIITA.
124. The method of any of embodiments 121 - 123, wherein B2M is knocked out with the gRNA sequence as set forth in SEQ ID NOs: 34-42.
125. The method of embodiments 122 or 123, wherein CIITA is knocked out with the gRNA sequence as set forth in SEQ ID NOs: 25-33.
126. The method of any of embodiments 86-125, wherein the stem cells are further genetically modified to include a suicide gene.
127. The method of embodiment 126, wherein the suicide gene includes CDK1 linked Herpes simplex virus-thymidine kinase/ganciclovir (CDK1/HSV-TK/GCV), TOP2A/HSV-TK/GCV, and/or inducible Casp9.
128. The method of embodiments 126 or 127, wherein the suicide gene includes CDK1/HSV-TK/GCV.
129. A method including differentiating a stem cell of any of embodiments 1-41 into a more differentiated cell type.
130. The method of embodiment 129, wherein the more differentiated stem cells include CD34+ hematopoietic stem cells, mesenchymal stem cells, or neural stem cells.
131. The method of embodiments 129 or 130, wherein the more differentiated cell type includes more differentiated stem cells, pancreatic cells, epithelial cells, cardiac cells, endothelial cells, liver cells, endocrine cells, connective tissue cells, muscle cells, brain cells, bone cells, kidney cells, cartilage cells, cancer cells, or immune cells.
132. The method of embodiment 131, wherein the pancreatic cells include alpha cells, beta cells, or delta cells.
133. The method of embodiments 131 or 132, wherein the cardiac cells include cardiomyocytes.
134. The method of any of embodiments 131-133, wherein the liver cells include hepatocytes, hepatic stellate cells (HSCs), Kupffer cells (KCs), and liver sinusoidal endothelial cells (LSECs).
135. The method of any of embodiments 131-134, wherein the connective tissue cells include fibroblasts.
136. The method of any of embodiments 131-135, wherein the muscle cells include myoblasts.
137. The method of any of embodiments 131-136, wherein the brain cells include neurons.
138. The method of any of embodiments 131-137, wherein the bone cells include osteoblasts and osteoclasts.

139. The method of any of embodiments 131-138, wherein the cartilage cells include chondrocytes.
140. The method of any of embodiments 131-139, wherein the immune cells include T-cells, NK cells, or macrophages.
141. The method of any of embodiments 129-140, further including genetically modifying the more differentiated cell type to include an exogenous sequence that encodes an expression product.
142. The method of embodiment 141, wherein the expression product is a protein.
143. The method of embodiment 142, wherein the protein includes a recombinant receptor, a detectable label, an antigen, an antibody, and/or an enzyme.
144. The method of embodiments 142 or 143, wherein the protein includes CD70, α CD3, CD28, 4-1BB, MICA, 4-1BBL, membrane-bound interleukin (IL)-15, and/or membrane-bound IL21.
145. The method of any of embodiments 142-144, wherein the protein includes membrane-bound IL21.
146. The method of any of embodiments 142-145, wherein the protein includes a cancer antigen.
147. The method of embodiment 146, wherein the cancer includes multiple myeloma, lymphoma, acute lymphocytic leukemia (ALL), acute myelocytic leukemia (AML), chronic lymphocytic leukemia (CLL), breast cancer, colorectal cancer, ovarian cancer, renal cell carcinoma (RCC), glioblastoma, prostate cancer, neuroblastoma, melanoma, Ewing sarcoma, and/or hepatocellular cancer (HCC).
148. The method of embodiments 146 or 147, wherein the cancer antigen includes BCMA, CD4, CD5, CD7, CD19, CD20, CD22, CD33, CD73, CD123, CD133, CD138, CD244, CD276, CS1, EGFR, EGFRVIII, EpCAM, FLT3, GD2, GPA7, GPC3, HER2, Mesothelin, MUC1, NKG2D, PSMA, PSCA, and/or TF.
149. The method of any of embodiments 146-148, wherein the cancer antigen includes BCMA, CD19, CD20, CD33, CD133, CD138, CS1, EGFR, EGFRVII, EpCAM, GD2, GPA7, HER2, NKG2D, MUC1, and/or PSCA.
150. The method of any of embodiments 142-150, wherein the protein includes a viral, bacterial, fungal, and/or parasitic antigen.
151. The method of any of embodiments 142-151, wherein the protein includes insulin, factor VIII, factor IX, factor XI, alpha-1 antitrypsin (A1AT), glucocerebrosidase (GC), acid sphingomyelinase, mucopolysaccharides, acid alpha-glucosidase, aspartylglucosaminidase, alpha-galactosidase A, palmitoyl protein thioesterase, tripeptidyl peptidase, lysosomal

transmembrane protein, cysteine transporter, acid ceramidase, acid alpha-L-fucosidase, cathepsin A, acid beta-glucosidase, acid beta-galactosidase, iduronate-2-sulfatase, alpha-L-iduronidase, galactocerebrosidase, acid alpha-mannosidase, acid beta-mannosidase, arylsulfatase B, arylsulfatase A, N-acetylgalactosamine-6-sulfate, N-acetylglucosamine-1-phosphotransferase, acid sphingomyelinase, NPC-1, alpha-glucosidase, beta-hexosaminidase B, heparan N-sulfatase, alpha-N-acetylglucosaminidase, acetyl-CoA: alpha-glucosaminide, N-acetylglucosamine-6-sulfate, alpha-N-acetylglactosaminidase, alpha-neuramidase, beta-glucuronidase, beta-hexosaminidase A, and/or acid lipase.

152. The method of any of embodiments 143-151, wherein the recombinant receptor includes an extracellular component including a binding domain; an intracellular component including an effector domain; and a transmembrane domain linking the extracellular component to the intracellular component.
153. The method of embodiment 143-152, wherein the recombinant receptor includes a chimeric antigen receptor and/or an engineered T cell receptor.
154. The method of embodiments 152 or 153, wherein the binding domain of the recombinant receptor binds a cancer antigen, a viral antigen, a bacterial antigen, and/or a fungal antigen.
155. The method of embodiment 154, wherein the cancer antigen includes BCMA, CD4, CD5, CD7, CD19, CD20, CD22, CD33, CD73, CD123, CD133, CD138, CD244, CD276, CS1, EGFR, EGFRVIII, EpCAM, FLT3, GD2, GPA7, GPC3, HER2, Mesothelin, MUC1, NKG2D, PSMA, PSCA, and/or TF.
156. The method of embodiments 154 or 155, wherein the cancer antigen includes BCMA, CD19, CD20, CD33, CD133, CD138, CS1, EGFR, EGFRVII, EpCAM, GD2, GPA7, HER2, NKG2D, MUC1, and/or PSCA.
157. The method of any of embodiments 152-156, wherein the effector domain includes all or a portion of the signaling domain of CD3 ζ and/or 4-1BB.
158. The method of any of embodiments 152-157, wherein the transmembrane domain includes a CD28 transmembrane domain.
159. The method of any of embodiments 143-158, wherein the recombinant receptor includes a CD19 binding domain.
160. The method of any of embodiments 143-159, wherein the recombinant receptor includes a BCMA binding domain.
161. The method of any of embodiments 143-160, wherein the detectable label includes a fluorescent protein, a radioisotope, an enzyme label, and/or a fluorescent label.
162. The method of embodiment 161, wherein the fluorescent protein includes luciferase.

163. The method of any of embodiments 129-162, further including genetically modifying the more differentiated cell type to knockout major histocompatibility complex (MHC).
164. The method of embodiment 163, wherein the genetically modifying the more differentiated cell type to knockout MHC includes knocking out B2M and/or CIITA.
165. The method of embodiment 164, wherein the knocking out B2M and/or CIITA includes delivering the Cas9 nuclease, B2M gRNA, and CIITA gRNA to feeder cells.
166. The method of embodiment 165, wherein the B2M gRNA includes SEQ ID NOs: 34-42.
167. The method of embodiments 165 or 166, wherein the CIITA gRNA includes SEQ ID NOs: 25-33.
168. The method of any of embodiments 129-167, further including genetically modifying the more differentiated cell type to include a suicide gene.
169. The method of embodiment 168, wherein the suicide gene includes CDK1 linked Herpes simplex virus-thymidine kinase/ganciclovir (CDK1/HSV-TK/GCV), TOP2A/HSV-TK/GCV, and/or inducible Casp9.

[0235] (VIII) Experimental Examples.

[0236] Example 1.

[0237] Materials and Methods. Culture of induced pluripotent stem cells (iPSCs) – thawing, passage, cryopreservation. Non-genetically modified and genetically modified, including immortalized, iPSC lines were thawed, cultured and cryopreserved according to protocols developed by Stemcell Technologies (Stemcell Technologies 100-0276). Frozen vials were thawed quickly at 37°C, gently resuspended in mTeSR-plus (Stemcell Technologies 100-0276), centrifuged, resuspended in pre-warmed mTeSR-Plus supplemented with Y-27632 (Stemcell Technologies 72302), and plated on cell-culture plastic dishes coated with Geltrex (Thermo Fisher A1569601). Cultures were passages 1:5 – 1:10 every 4-6 days depending on cell growth, at an average of 75% confluency. Cultures were washed once with PBS and covered with ReLeSR (Stemcell Technologies 05872) for 1 minute. The ReLeSR was removed, the plates incubated for 2-4 minutes at 37°C, gently resuspended in mTeSR-Plus and added to GelTrex treated culture dishes. For cryo-preservation, cells were dissociated with ReLeaSR as described above, centrifuged and resuspended in CryoStore CS10 (Stemcell Technologies 07959). Vials were frozen at a rate on 1°C/min until reaching -80°C after which they were transferred to a -150°C freezer for long term storage.

[0238] Differentiation of iPSC to CD34-positive hematopoietic progenitor cells and characterization of CD34-positive hematopoietic progenitor cells. Control unmodified iPSCs and TetON hTERT SV40 IPSCs were differentiated to CD34-positive hematopoietic progenitor cells

using the StemDiff Hematopoietic Medium and Supplements (StemCell Technologies). For the TetON hTERT SV40 iPSC lines the iPSC culture and differentiation medium were supplemented with 0.1 μ M Doxycycline Hyclate (DOX, Sigma Aldrich). For the differentiation, adherent iPSC cultures were dissociated to single cells using Accutase (StemCell Technologies) and plated into AggreWell 6-well plates (StemCell Technologies) at 3.5×10^6 cells/well to generate Embryoid Bodies (EBs). After 5 days of culture the EBs were transferred to non-tissue culture treated 6-well plates and cultured for additional 7 days. On day 12 of differentiation, EBs were harvested and dissociated into single cells using Collagenase II (StemCell Technologies). CD34-positive cells were isolated from the single cell suspension using the EasySep Human CD34 Positive Selection Kit (StemCell Technologies) per the manufacturer's instructions. Freshly isolated CD34-positive cells were stained with anti-human CD34, CD45 and CD43 antibodies (Stem Cell Technologies) and analyzed via Flow Cytometry for surface marker expression using a CytoFlex flow cytometer (Beckman Coulter).

[0239] Differentiation of iPSC to natural killer (NK) cells and characterization of NK cells. Control unmodified iPSCs and TetON hTERT SV40 IPSCs were differentiated to NK cells using the StemDiff NK Cell Kit (StemCell Technologies) following a 3-step differentiation protocol per the manufacturer's instructions: iPSC differentiation to CD34-positive hematopoietic progenitor cells and selection of CD34-positive cells (12 days), differentiation of CD34-positive cells to CD5-positive and CD7-positive lymphoid progenitor cells (14 days), and differentiation of the lymphoid progenitor cells to CD56-positive cells (14 days). For the TetON hTERT SV40 IPSC lines the iPSC culture and differentiation medium were supplemented with 0.1 μ M Doxycycline Hyclate (DOX, Sigma Aldrich). In some instances, the Doxycycline Hyclate differentiation medium supplementation was performed at either the lymphoid progenitor differentiation stage and/or NK differentiation stage. At each stage of the differentiation, cells were harvested and characterized for surface marker expression via antibody staining (anti-human CD34, CD45 and CD43 antibodies (Stem Cell Technologies) for CD34-positive cells, anti-human CD7 antibody (BioLegend) and anti-human CD5 antibody (StemCell Technologies) for lymphoid progenitor cells, anti-human CD56, CD16 and CD3 antibodies (StemCell Technologies) for NK cells), followed by flow cytometry analysis (CytoFlex flow cytometer, Beckman Coulter).

[0240] Transposon-based transgenes delivery in iPSC lines using Lipofectamine 3000. Mammalian Expression plasmid of PiggyBac or Sleeping beauty transposases and transposon vectors of transgenes were designed in-house and synthesized by VectorBuilder. For gene delivery, iPSCs were cultured in Geltrex (Thermo fisher) coated culture wares with iPSC growth media (mTeSR plus, StemCell Technologies) for at least 2 passages before transfection. On the

day of the transfection, adherent iPSC cultures were dissociated to very small clumps using ReLeSR (StemCell Technologies) and plated into Geltrex-coated 6-well at 500,000 cells/ wells. The plasmids (transgene + transposase) and transfection reagents mix were prepared according to manufacture instructions (Lipofectamine 3000, Thermo Fisher). 2 hours after plating, plasmids/reagent mix was added to the corresponding well in the 6-well plate. For clones that were generated by the antibiotic selection, the antibiotics were introduced 24 hours (Neomycin) or 48 hours (Puromycin) after transfection. Antibiotic-resistant clones were collected 6-7 days (Puromycin), and 10 days (Neomycin) after the initial selection for downstream analysis. Antibiotic resistant clones that were pooled from each experiment and pooled cells were cultured with iPSC growth media for 72 hours. Then transfected cells were collected, stained with antibodies (anti-FMC63 CAR, BioLegend), and single cells sorted into 96-well plates using a cell sorter (CytoFlex flow cytometer, Beckman Coulter) to generate clonal lines.

[0241] Transgene copy number and expression level analysis. For genomic copy number analysis, genomic DNA from transgenic iPSC lines was extracted from cell pellets using DNeasy Blood & Tissue Kits (Qiagen). The transgene copy number in the transgenic iPSC line was measured using iCS-digital™ PSC kit. (Stemgenomics) Primer and probes used for ddPCR: Forward primer: 5'- GCTGCCGATTTCCAGAAGAAGA-3' (SEQ ID NO: 55; Reverse primer: 5'-GTCTGCGCTCCTGCTGAA-3' (SEQ ID NO: 56); probe: AAGGAGGATGTGAACTGA (SEQ ID NO: 57).

For analysis of transgene transcript level by qPCR, the RNA from transgenic iPSC lines was extracted from cell pellets using RNeasy Mini Kit (Qiagen). cDNA was synthesized from extracted RNA using QuantiTect Reverse Transcription Kit (Qiagen). qPCR was set up using SensiFast SYBR No-Rox kit (FroggaBio BIO-98020) performed on CFX384 Touch Real-Time PCR Detection System. (BioRad). qPCR primer used:

FMC63: Forward primer: 5'- TGGAGTGGCTGGGAGTAATA-3' (SEQ ID NO: 58); Reverse primer: 5'- ACTTGGCTCTTGGAGTTGTC-3' (SEQ ID NO: 59).

TK007: Forward primer: 5'- CAACATCTACACCACACAGCAC-3' (SEQ ID NO: 60); Reverse primer: 5'- CGGCATTCCCATTGTGATCTGG-3' (SEQ ID NO: 61).

YWHAZ: Forward primer: 5'- ACTTTTGGTACATTGTGGCTTCAA-3' (SEQ ID NO: 62); Reverse primer: 5'- CCGCCAGGACAAACCAGTAT-3' (SEQ ID NO: 63).

hTERT: Forward primer: 5'-CTCCATCCTGAAAGCCAAGAA-3' (SEQ ID NO: 64); Reverse primer: 5'- AGTCAGCTTGAGCAGGAATG-3' (SEQ ID NO: 65).

SV40LT: Forward primer: 5'-CCAGAAGAAGCAGAGGAAACTA -3' (SEQ ID NO: 66); Reverse primer: 5'- CCAAGTACATCCCAAGCAATAAC -3' (SEQ ID NO: 67).

rtTA: Forward primer: 5'- GGCCTGGAGAAACAGCTAAA-3' (SEQ ID NO: 68); Reverse primer: 5'- TCAAGGTCAAAGTCGTCAAGG-3' (SEQ ID NO: 69).

CD19: Forward primer: 5'- AGCTGTGACTTTGGCTTATCT-3' (SEQ ID NO: 70); Reverse primer: 5'- GGGTCAGTCATTCGCTTTCT-3' (SEQ ID NO: 71).

BCMA: Forward primer: 5'- GCGATTCTCTGGACCTGTTT-3' (SEQ ID NO: 72); Reverse primer: 5'- AGGAGACCTGATCCTGTGTT-3' (SEQ ID NO: 73).

Luciferase: Forward primer: 5'- GTGGTGTGCAGCGAGAATAG-3' (SEQ ID NO: 74); Reverse primer: 5'- CGCTCGTTGTAGATGTCGTTAG-3' (SEQ ID NO: 75).

For analysis of transgene protein level by FACS, 500,000 cells from transgenic iPSC lines were collected and stained with anti-CD19, anti-BCMA antibodies (363039, 357517, BioLegend). The expression level of the transgenes was measured using a CytoFlex flow cytometer (Beckman Coulter).

[0242] Dox induction of Tet inducible expression of immortalization genes. Transgenic iPSC lines that contain immortalization genes were cultured in iPSC growth media (mTeSR plus, Stemcell Technology) for at least two passages before Doxycycline induction. Doxycycline Hyclate (DOX, Sigma D9891) was diluted in iPSC growth media to a concentration of 0.3 μ M, 0.6 μ M, or 1 μ M. The transgenic iPSC lines were cultured in Doxycycline containing media for 72 hours. Cell pellet from each condition was collected for downstream analysis.

[0243] Example 2. Growth and characteristics of immortalized induced pluripotent stem cell (iPSC) lines.

[0244] In this example, it is demonstrated that human iPSCs genetically modified to harbor the inducible immortalization construct (see Example 3) can be grown with the same techniques as their unmodified parental line. It is further demonstrated that the immortalized iPSCs retain their typical morphology and pluripotency. Further, it is shown that immortalized iPSCs are capable to differentiate into all three embryonic lineages.

[0245] To this aim, the SK005.3 iPSC line was cultured in parallel with its immortalized progeny SK005.3-hTertSV40 under the same conditions outlined in the materials and methods section (See Example 1, Culture of iPSCs – thawing, passage, cryopreservation). Post thaw survival, growth rate and morphology was documented over several passages using cell counting and phase contrast photography (FIG. 4). It is also shown that the pluripotency of immortalized iPSC lines are maintained as evidence by alkaline phosphatase (AP) staining and expression of the pluripotency markers OCT4, NANOG, SOX2, SSEA3 and SSEA4. To this extent, both fluorescence-activated cell sorting (FACS) analysis using antibodies for each marker as well as global gene expression analysis (Thermo Fisher Pluritest) was used. Finally, the immortalized

iPSCs were demonstrated to be equally capable to differentiate into all three embryonic germ layers as their unmodified parental cell line. For this, in vitro differentiation was used followed by imaging and global gene expression analysis (Thermo Fisher Scorecard).

[0246] Example 3. Editing a wildtype (WT) or previously edited induced pluripotent stem cell (iPSC) line genome to contain an inducible immortalization gene. In this example, the ability of gene editing an human iPSC (hiPSC) line and further editing this previously edited iPSC line to contain inducible immortalization genes is demonstrated. To this aim, an hiPSC line, SK005.3, was cultured and passaged twice in iPSC growth media (mTeSR plus, StemCell Technologies) before co-transfection with a plasmid containing PiggyBac transposase and PiggyBac-FMC63-IL15 CAR-TK plasmid using Lipofectamine 3000 (Thermo Fisher). 72 hours after the transfection, transfected cells were stained with anti-FMC63 antibody, and cells with correct insertion and good FMC63 expression level were single-cell sorted via fluorescence-activated cell sorting (FACS). 4 clones were expanded, insertion copy number in each clone was analyzed by ddPCR, showed a range of 18 to 28 copies of inserted transgene. To further verify the expression of inserted transgenes, cDNA samples from each clone were collected and the expression analysis of FMC63 and TK.007 was performed by qPCR. In comparison to unedited wild-type SK005.3 cells, FIG. 5 demonstrates an increase in FMC63 and TK.007 transcript levels in edited clones. In addition to the analysis of the FMC63 transcript level, 500,000 cells from each clone were collected and stained with the anti-FMC63 antibody. The protein level in each clone was then measured by FACS. As shown in FIG. 5, compared with the unedited wild-type SK005.3 line, a significant increase in FMC63 protein level was observed, further evidencing the successful editing of the iPSC line.

[0247] There are various ways to immortalize cells. FIG. 6B shows the design for the inducible immortalization vector. It contains an inducible immortalization cassette that includes human Telomerase Transcriptase (hTERT) and SV40gp6 Large T Antigen (SV40 LT) under a Tetracycline (Tet)-inducible expression system. A Neomycin (Neo) resistant gene was placed downstream of the inducible immortalization genes and flanked by two loxP sites. This design allows the enrichment and selection of cells that have the correct insertion by culturing with Neo, and removal of the Neo gene from the final edited iPSC line. The inducible immortalization cassette was placed in a Sleeping Beauty (SB) transposon vector backbone. When co-transfected with SB transposase, the inducible immortalization can be effectively inserted by the SB transposon system.

[0248] Next, to demonstrate the ability to insert and activate the inducible immortalization genes, a pooled SK005.3 iPSC that express high-level FMC63 chimeric antigen receptor (CAR) and

TK.007 were cultured and passaged twice in iPSC growth media (mTeSR plus, StemCell Technologies) before co-transfected with the SB100x plasmid with the inducible immortalization vector using Lipofectamine 3000 (Thermo Fisher). 24 hours after transfection, transfected cells were cultured with iPSC growth media containing 150µg/mL Neo with daily media change. Neo-resistant colonies were collected and pooled 10 days after the selection. To test the activation of the Tet-inducible immortalization cassette, FMC63-Tet-hTERT-SV40 LT SK005.3 iPSCs were cultured without or with an increasing concentration of doxycycline (DOX) for 72 hours. cDNA from each condition and SK005.3 and TC1133 wildtype iPSC were collected and the transcript levels of hTERT, SV40 LT, rtTA, and FMC63 were measured by qPCR. As shown in FIG. 7, an increase in transcripts levels of two genes under Tet-On promotor (hTERT and SV40 LT) can be observed after 72 hours of DOX treatment, in a dose-dependent manner. In comparison, there are no significant changes in the transcript levels of rtTA and FMC63 in the presence or absence of DOX. Overall, these data demonstrate the ability to insert inducible immortalization genes to hiPSC and successful activation of the expression of the immortalization gene with DOX treatment.

[0249] Example 4. Differentiating an induced pluripotent stem cell (iPSC) line edited to contain an inducible immortalization gene into CD34+ cells. To demonstrate the ability of the gene-edited iPSC line containing the inducible immortalization gene to differentiate into specific phenotypes, unmodified iPSCs and TetON hTERT SV40 IPSCs were differentiated to CD34-positive hematopoietic progenitor cells. To this aim, unmodified and hTERT SV40 iPSC were cultured for three passages in iPSC growth medium (TeSR E8™ Medium, StemCell technologies), with and without 0.1 µM Doxycycline Hyclate (DOX) before initiation of hematopoietic progenitor cell differentiation. Both unmodified and TetON hTERT SV40 IPSCs displayed the typical iPSC morphology, with tightly packed colonies and well-defined colony borders, and the cell morphology was not affected by DOX treatment (FIG. 8A). To demonstrate the activation of the conditional immortalization construct, cell samples were collected from the iPSC cultures with and without DOX treatment and gene expression analysis was performed via RT-PCR. FIGs. 8A and 8B shows increased expression of the hTERT and SV40 transcripts upon DOX treatment (DOX-inducible) and constitutive expression of the rtTA transcript (always ON) in the hTERT SV40 IPSC line only, while no significant expression for these transcripts was measured in the unmodified iPSC line.

[0250] Next, unmodified iPSCs and TetON hTERT SV40 IPSCs were subjected to differentiation into hematopoietic progenitor cells following the timeline depicted in FIG. 8C. Briefly, iPSCs were seeded into AggreWell plates in StemDiff Hematopoietic Differentiation Medium (StemCell

Technologies) for embryoid bodies (EB) formation and cultured for 5 days. EBs were then transferred to 6-well plates and cultured for 7 days before harvest and selection of CD34-positive hematopoietic progenitor cells. FIG. 8D shows representative images of EBs generated from the TetON hTERT SV40 IPSC line cultured with and without DOX on day 2 of the differentiation in AggreWells and on day 12 of EB harvest. DOX treatment increased the size of EB compared to the no treatment control, suggesting the induction of immortalization genes promotes cell proliferation during EB formation. Upon EB harvest, dissociation and CD34-positive selection, the enriched CD34-positive cell fraction of the DOX-treated hTERT SV40 line showed higher viability and viable cell yield compared to the no treatment control (FIG. 8E). Further staining of the enriched fraction with CD34 antibody followed by flow cytometry analysis revealed a significantly higher percentage of CD34-positive cells upon DOX treatment compared to the unmodified line, with and without DOX, as shown in FIG. 8F (60.1% CD34-positive cells for the DOX-treated hTERT SV40 line compared to 28.8% CD34-positive cells for the DOX-treated unmodified line).

[0251] Overall, these data demonstrate that activation of the inducible immortalization construct increases the viability and yield of CD34-positive cells during hematopoietic differentiation.

[0252] Example 5. Differentiating an induced pluripotent stem cell (iPSC) line edited to contain an inducible immortalization gene into mesenchymal stem cells (MSC) and natural killer (NK) cells.

[0253] To demonstrate the ability of the gene-edited iPSC line containing the inducible immortalization gene to differentiate into specific phenotypes, unmodified iPSCs and TetON hTERT SV40 iPSCs were differentiated to NK cells using the StemDiff NK Cell Kit (StemCell Technologies) following a 3-step differentiation. FIG. 9 shows the diagram of the NK cell differentiation process. iPSCs are thawed and expanded before they are passaged into AggreWells to generate embryoid bodies (EBs). After 5 days of culture in AggreWells, the EBs are transferred to a 6-well plate. At day 12 of the differentiation, EBs are dissociated, positively-selected for CD34 expression, phenotypically characterized for hematopoietic progenitor cells surface marker expression, and seeded for Lymphoid Progenitor Cell differentiation. After 14 days of culture, Lymphoid Progenitor Cells are harvested, phenotypically characterized for cell surface marker expression and seeded for NK Cell differentiation.

[0254] In some instances, 0.1 μ M Doxycycline Hyclate (DOX) was added to the iPSC culture and differentiation medium throughout the differentiation process. In other instances, 0.1 μ M Doxycycline Hyclate was added to the differentiation medium at the initiation of either Lymphoid Progenitor Cell differentiation stage, or NK Cell differentiation stage. The presence of DOX in the differentiation medium stimulates the generation of a higher percentage of CD5-positive, CD7-positive lymphoid progenitor cells as demonstrated by flow cytometry analysis of the differentiated

population. Similarly, the presence of DOX increased the yield of CD56-positive NK cells at the end of the NK cell differentiation stage.

[0255] Together, these data demonstrate that activation of the inducible immortalization construct increases the yield of lymphoid progenitors and NK cells during the NK cell differentiation process.

[0256] Example 6. CD19 expressing mesenchymal stem cells (MSC)-derived induced pluripotent stem cell (iPSC) line edited to contain an inducible immortalization gene are useful in demonstrating that chimeric antigen receptor (CAR) T-cells are properly prepared.

[0257] CAR T cell therapy is a promising approach to cancer treatment that targets specific antigens expressed on the surface of cancer cells. One key step in CAR T cell preparation is to test whether the expanded CAR T cells are able to recognize and attack the cancer cells expressing the corresponding target antigen.

[0258] In this section, the ability to generate transgenic iPSCs that express CD19 and B-cell maturation antigen (BCMA) antigens and the potential of generating transgenic iPSCs that express an array of commonly targeted antigens for CAR-T cell quality control is demonstrated.

[0259] CD19 and BCMA are two commonly targeted antigens in treatments. CD19 is a cell surface antigen that is expressed on the surface of B cells. In cell therapy treatment of B-cell malignancies such as acute lymphoblastic leukemia and non-Hodgkin lymphoma, CD19 is a commonly targeted antigen. CAR T cells targeting CD19 have shown promising results in clinical trials, with high rates of complete remission in patients with relapsed or refractory B cell malignancies. BCMA, or B cell maturation antigen, is another cell surface antigen that is expressed on the surface of plasma cells, which produces antibodies. BCMA is a promising target for CAR T cell therapy in the treatment of multiple myeloma, a type of cancer that arises from abnormal plasma cells. CAR T cells targeting BCMA have shown high response rates in clinical trials, with some patients achieving complete remission.

[0260] First, a CD19 or BCMA expressing plasmid was designed in a PiggyBac plasmid backbone. As shown in FIG. 10, in addition to the antigen, a Puromycin (Puro) resistant gene was placed downstream of the antigen gene for the selection and enrichment of transfected cells. Furthermore, a Luciferase gene (Luc) was included in the design to facilitate cell tracking in downstream applications.

[0261] To generate the transgenic iPSC lines that express CD19 and BCMA, PAN3 and SK005.3 MHC class I/II KO hiPSC were cultured and passaged twice in iPSC growth media (mTeSR plus, StemCell Technologies) before co-transfected with a plasmid containing PiggyBac transposase and PiggyBac-CD19-Luc or PiggyBac- BCMA -Luc plasmid using Lipofectamine 3000 (Thermo Fisher). 48 hours after transfection, transfected cells were cultured with iPSC growth media

containing 0.8µg/mL Puro with daily media change. Puro-resistant colonies were pooled and passaged twice before frozen down. To verify the expression of inserted transgenes, cDNA samples from each pooled transfected cell from each condition were collected and the expression analysis of CD19 or BCMA was performed by qPCR. In comparison to unedited wild-type hiPSC lines, an increase in CD19 and BCMA transcript levels in edited pooled cells was demonstrated. In addition to the analysis of the transcript level, 500,000 cells from each clone were collected and stained with the anti-CD19 or anti-BCMA antibody. The protein level in each condition was then measured by FACS. Compared with the unedited wild-type hiPSC line, a significant increase in CD19 or BCMA protein level was observed, further evidencing the successful editing of the iPSC line. In this case, the insertion and expression of CD19 and BCMA were done in both unedited (PAN3) as well as previously edited hiPSC (SK005.3 MHC class I/II KO), highlighting the flexibility and adaptivity of the design and workflow.

[0262] In addition to CD19 and BCMA, an array of commonly targeted antigens has been identified as the target antigens for CAR-T cells for various diseases. FIG. 11 listed a panel of 12 common target antigens, including CD19 and BCMA. hiPSCs have the potential to differentiate into any cell type. Having hiPSCs that express these targeted antigens for CAR-T cells would greatly reduce the cost and time to verify the efficacy and specificity of CAR-T cell products in the QC phase. FIG. 12 shows two systems (PiggyBac- and lentivirus-based) which were designed for insertion and expression of CAR-T targeted antigen or other cargos into hiPSC, highlighting the capability of generating edited iPSC lines for QCing various clinical CAR T cells beyond just CD19 and BCMA.

[0263] Example 7. The induced pluripotent stem cell (iPSC) line containing an inducible immortalization gene can be further manipulated to express luciferase or green fluorescent protein (GFP).

[0264] Introduction. Fluorescence and bioluminescence have been widely used in biomedical research for decades. Both provide means of detecting cells expressing a protein as an emitted light that can be captured and analyzed to provide detailed data on specific gene expression. Fluorescence is found in a large variety in nature, ranging from minerals and marine organisms to butterflies and arachnids and is based on the absorption of light of a specific wavelength (excitation light) and the subsequent emission of a lower frequency (emission light) (FIG. 13A). The resulting emission of the fluorochrome-specific wavelength can be captured with cameras equipped with the corresponding filters. Fluorochromes exist in a wide array of colors, the most widely used being GFP (Prasher 1992, Gene, 111(2):229-33, PMID: 1347277). Bioluminescence refers to the direct emission of visible light without the need for excitation.

Luciferase is an enzyme that catalyzes a light-producing biochemical reaction when it is in the presence the substrate luciferin. Bioluminescence is found in nature (such as the firefly and the angler fish) (Shimomura 1995, Bio Bull. 189(1):1-5, PMID:7654844). Capturing this phenomenon for biomedical research has allowed the detection of transgenes in living organisms using Bioluminescent Light Imaging (BLI) (FIG. 13B). Here the benefits of each system was combined, optimizing their utilities. While GFP was used as a tool to visualize transgene expression *in vitro* and *ex-vivo*, Luciferase allows us to rack immortalized iPSCs in *in vivo* pre-clinical studies using appropriate mouse models.

[0265] DNA transposons are designed to move from one genomic location to another by a cut-and-paste mechanism. They are powerful forces of genetic change and have played a significant role in the evolution of many genomes. As genetic tools, they can be used to introduce foreign DNA into a genome. To obtain high levels of transgene expression, the piggyBAC and Sleeping Beauty transposon systems were used as briefly described in Example 3. Both piggyBAC and Sleeping Beauty (Chen et al., Nature Biotechnology, 2020, 38:165-168) has been used for many years in biotechnology.

[0266] Human iPSCs that carry the inducible immortalization transgene hTertSV40 display the same phenotype, pluripotency, ability to differentiate into all three embryonic lineages as are their unmodified. These cells can be further modified both with transgene constructs using the transposon technology as well as targeted genomic modifications (Knock-In and Knock-out) utilizing CRISPR/Cas9. In case of the former, the transposon system is used that was not utilized for the immortalization step (piggyBAC/Sleeping Beauty).

[0267] The order of engineering can be done in reverse: immortalizing previously gene edited iPSC lines.

[0268] The current disclosure provides immortalized cell lines generated from immortal cells and uses thereof.

[0269] Particular embodiments utilize stem cells modified to include a drug-inducible growth system (e.g., Tert and SV40). These embodiments are particularly useful to differentiate into immortalized differentiated cell populations that can be maintained as immortal through administration of the growth controlling drug.

[0270] Particular embodiments utilize stem cells modified to include a drug-inducible growth system and a suicide switch. These embodiments are particularly useful to differentiate into immortalized differentiated cell populations for a therapeutic purpose, the suicide switch providing an *in vivo* safety feature. Further, the suicide switch embodiment may be especially useful to provide a safety feature allowing the removal of proliferating cells from cultured cells (*in vitro*)

before use as a therapeutic, and after application as a therapeutic (in vivo).

[0271] Particular embodiments utilize stem cells modified to include a drug-inducible growth system and factors that support use as feeder cells during cell culture. These embodiments are particularly useful to differentiate into immortalized differentiated feeder cells. Examples include adherent cells (e.g., mesenchymal stem cells) or suspension cells (e.g. CD34+ cells). Differentiated immortalized feeder cells can be genetically modified to support growth of particular cell types, such as expression of membrane-bound IL21 and MHC Class I and Class II knock-out to support growth of natural killer (NK) cells. These embodiments may also include a suicide switch to reduce contamination of cell populations of interest with feeder cells. Further, these embodiments may also utilize cells that express a viral antigen that can be used as a living vaccine allowing for extended antigenic presentation in a physiologically appropriate manner. These embodiments may also express a reporter, such as fluorescent proteins and/or luciferase.

[0272] Particular embodiments utilize stem cells modified to include a drug-inducible growth system and factors that support use as tester cells during research and development. These embodiments are particularly useful to differentiate into immortalized differentiated tester cells. Examples include tester cells that express a cancer antigen or a viral antigen to test efficacy of antibodies, chimeric antigen receptors, or similar recombinant molecules under development. When manufactured for in vivo use, these immortalized differentiated tester cells may also express a reporter, such as luciferase. These embodiments may also include a suicide switch.

[0273] Example 8. Mesenchymal stem cell (MSC) line used in natural killer (NK) assays.

[0274] In this example, the method and results from functional tests on IL-21 expressing MSCs (also called feeder cells) are described. The functional test involves co-culture of NK cells with feeder lines that express IL-21 on the surface membrane. If IL-21 is properly expressed on the cell surface, and it can be recognized properly by NK cells, that leads to activation and expansion of NK cells when co-cultured.

[0275] In the following, the methods to confirm the phenotype and genotype of these feeder cell lines are described and results are presented. Then, the method of NK cell co-culture is described, and the results are presented.

[0276] To demonstrate the function of IL-21 expression in activating and expanding NK cells, 3 lines were used in NK activation assays as listed in Table 3 and shown in FIG. 15A.

Table 3. List of MSC lines used in NK activation and expansion functional test.

Cell Line	Description
Line A	SK005 immortalized MSC
Line B	MSC, SK5 Class I/II null MB-IL21 iPSC-derived
Line C	"Immortalized" MSC, SK5 Class I/II null MB-IL21 iPSC-derived

[0277] Line A served as the negative control for both IL-21 expression (i.e., had no expression of IL-21) and for Class I/II null (i.e., expressed Class I/II major histocompatibility class (MHC)). Line B served as control for immortalization (i.e., lacks hTert-SV40 expression). Line C is the target product and is positive for expression of IL-21 and immortalization cassettes as well as negative for expression of Class I/II MHC. In all tests and assays, Lines A and B were used as controls for Line C.

[0278] The genotype of the cells were confirmed via qPCR (FIG. 15B). In brief, genomic DNA was extracted via commercial kits (RNeasy® Plus Mini Kit, Qiagen, Germantown, MD) and qPCR protocols were performed using the primer sets in Table 4.

Table 4. Primers used for confirming genotype of the MSC lines for NK assay.

Primers	Forward primer	Reverse Primer
MB-IL21 qPCR F1	CGGCACCAGAAGATGTAGAAA (SEQ ID NO: 76)	TCCTCTCGTTATTTCCCGTATTG (SEQ ID NO: 77)
MB-IL21 qPCR F2	TGTCCCTCTTGCGATTCTTAC (SEQ ID NO: 78)	TCACTACCGTGTGTCCTACT (SEQ ID NO: 79)
hTERT qPCR F1	CTCCATCCTGAAAGCCAAGAA (SEQ ID NO: 80)	AGTCAGCTTGAGCAGGAATG (SEQ ID NO: 81)
SV40LT qPCR F1	CCAGAAGAAGCAGAGGAACTA (SEQ ID NO: 82)	CCAAGTACATCCCAAGCAATAAC (SEQ ID NO: 83)

[0279] The genotype of the cells were confirmed via qPCR (FIG. 15B) and Flow Cytometry (FIGs. 16 and 17). A summary of results can be found in Table 5.

Table 5: Summary of phenotyping and genotyping results for cells used in the NK assay.

	Phenotype	Expected Genotype			Expected Phenotype		
		hTert	SV40	MB-IL21	CD90	CD105	HLA ABC
Line A	Immortalized	+	+	-	+	+	+
Line B	MB-IL21, Class I/II null	-	-	+	+	+	-
Line C	Immortalized, MB-IL21, Class I/II null	+	+	+	+	+	-

[0280] NK Activation and Expansion Assay. The intended function of membrane-bound IL-21 (mbIL-21) expressing MSCs (feeder lines) was assessed via co-culture with primary human NK cells following the method described herein.

[0281] Negatively-selected, cryopreserved, primary human NK cells were purchased from a commercial vendor (BloodWork NW, Seattle, US). On Day -1 of co-culture (i.e. one day prior to start of co-culture), the MSC cells Lines A, B, and C were seeded at 1.75e5/well/line in 6 well plates in StemXvivo media (R&D Systems, US). Also on Day -1, the NK cells were thawed and seeded at 0.7e5/mL in NK Xpander media (Thermo Fisher, US) supplemented with 500 IU IL-2

and 5% FBS.

[0282] In a side arm, iNK cells generated from iPSC lines described in Example 5, were thawed and seeded with MSCs similarly to the primary NK cells as described above.

[0283] On Day 0 of culture, MSC line cell counts were assessed via counting of cells from a representative plate, and the NK cells were seeded on MSCs at 5:1 MSC-to-NK ratio in NK cell media. A control plate of MSCs was cultured in NK media, without NK co-culture, to assess the impact of NK media on MSC cells over the course of the experiment. Another control plate was seeded with NK cells only in the NK media (no co-culture). All co-culture and control plates were incubated at 37°C and 5% CO₂.

[0284] On Days 1 to 3 of co-culture, a respective plate containing the co-cultured cells was removed from the incubator, and the cells were imaged on a microscope to document the killing and activation/expansion on NK cells. Then, a sample of cell suspension was collected, and cell count was performed to assess viability and count of cells in suspension. The plates were then washed with PBS and the MSC cells were lifted using a lifting reagent (Accutase, Thermo, US), and were enumerated on the cell counter.

[0285] For re-stimulation test, all NK cells from Day 3 co-culture were removed, resuspended in fresh NK culture media, and were reseeded on a fresh plate of Line B and Line C MSC. Cells were imaged and counted on Day 6 (Day 3 of re-stimulation).

[0286] Results. FIGs. 18 and 19 are representative images of the state of the cells at the beginning (Day 0) and the end (Day 3) of 3-day co-culture assay. Cell viability and counts over the course of co-culture experiment are presented in FIGs. 20, 21A, and 21B. Due to impact of handling on viability of NK, total nucleated cell count (TNC) is reported and used to calculate normalized numbers instead of total viable count (TVC). It appears that the NK cells could be too fragile for sampling manipulation after activation, even when gently sampled. As such, the reported viabilities appear to be very low. This also seems consistent with iNK cells. Due to impact of handling on viability of NK, TNC is reported and used to calculate normalized numbers instead of TVC.

[0287] The results in FIG. 20 suggest that, in the absence of IL-21 expression, NK cells do not change in number by Day 3 (120%) compared to those on Lines B (145%) and C (185%). In comparison, NK cells in NK media without MSC co-culture maintain the viability and count through the 3-day assay period. This also suggests that when not activated, NK cells are more resilient against the sampling procedure, hence maintaining the viability, while those that were activated show fragility to pipetting as exhibited in low viability numbers for the co-cultured NK cells.

[0288] iNK cells will be assessed on Day 3 of co-culture. The iNK cells are not expected to

demonstrate any appreciable killing effect within the time window of this assay and the specific ratio in which they will be seeded on MSCs. It is expected that iNK cells will also demonstrate the killing effect if seeded at a higher ratio such as 1:1 or 5:1 NK to MSC.

[0289] The MSCs on the other hand, significantly decrease in number for Lines B and C compared to Line A which shows an increase instead of decrease. This suggests the killing action of NK cells on MSCs that expressed IL-21, while those which do not express IL-21 (Line A) continue expanding in culture by Day 3.

[0290] After restimulation, a significant increase in cell number was observed as documented by imaging and after performing cell count on the supernatant (FIGs. 21A, 21B). Cells activated on feeder Line B and Line C expanded significantly by multiple folds (13 to 16 folds) after 3 days of re-stimulation on fresh Line B and Line C feeders.

[0291] Example 9. Mesenchymal stem cell (MSC) lines lacking Class I or Class II HLA expression (individually, Class I null or Class II null) are tested in three different types of potency assays: natural killer (NK), natural killer T-cells (NKT), and T-cells assays.

[0292] In this example, the method and results from functional potency tests on four different IL-21 expressing MSCs (also called feeder cells) are described. The functional test is described in Example 8 and involves co-culture of Lymphocytes (NK, NKT, or T) cells with feeder lines that express IL-21 on the surface membrane.

[0293] The methods to confirm the phenotype and genotype of these feeder cell lines are also described in Example 8 and expected results are presented below. The method of Lymphocytic cell co-culture is described in Example 8, and the expected results are presented below.

[0294] To demonstrate the function of IL-21 expression in activating and expanding NK, NKT, and T-cells in the absence of MHC Class I or Class II expression, 5 lines are used in lymphocytic activation assays as listed in Table 3.

Table 6. List of MSC lines to be used in NK NKT, and T-cell activation and expansion functional tests.

Cell Line	Description
Line A	SK005 immortalized MSC
Line D	MSC, SK5 Class I null MB-IL21 iPSC-derived
Line E	"Immortalized" MSC, SK5 Class I null MB-IL21 iPSC-derived
Line F	MSC, SK5 Class II null MB-IL21 iPSC-derived
Line G	"Immortalized" MSC, SK5 Class II null MB-IL21 iPSC-derived

[0295] Line A serves as the negative control for both IL-21 expression (i.e., should have no expression of IL-21) and HLA expression of both Class I and II major histocompatibility complexes (MHC)). Line D serves as control for immortalization (i.e., lacks hTert-SV40 expression) and is

lacking proper Class I MHC expression (Class I null). Line E is the immortalized Class I null target product and is positive for expression of IL-21 and immortalization cassettes as well as negative for expression of Class I MHC. Line F serves as control for immortalization (i.e., lacks hTert-SV40 expression) and is lacking proper Class II MHC expression (Class II null). Line G is the immortalized Class II null target product and is positive for expression of IL-21 and immortalization cassettes as well as negative for expression of Class II MHC. In all tests and assays, Lines A and D are used as controls for Line E and Lines A and F are used as controls for line G.

[0296] The genotype of the cells are confirmed via qPCR as described in Example 8. In brief, genomic DNA is extracted via commercial kits (RNeasy® Plus Mini Kit, Qiagen, Germantown, MD) and qPCR protocols are performed using the primer sets in Table 4.

Table 7. Primers to be used for confirming genotype of the MSC lines for NK, NKT, and T-cell assays.

Primers	Forward primer	Reverse Primer
MB-IL21 qPCR F1	CGGCACCAGAAGATGTAGAAA (SEQ ID NO: 76)	TCCTCTCGTTATTTCCCGTATTG (SEQ ID NO: 77)
MB-IL21 qPCR F2	TGTCCCTCTTGCGATTCTTAC (SEQ ID NO: 78)	TCACTACCGTGTGTCCTACT (SEQ ID NO: 79)
hTERT qPCR F1	CTCCATCCTGAAAGCCAAGAA (SEQ ID NO: 80)	AGTCAGCTTGAGCAGGAATG (SEQ ID NO: 81)
SV40LT qPCR F1	CCAGAAGAAGCAGAGGAACTA (SEQ ID NO: 82)	CCAAGTACATCCCAAGCAATAAC (SEQ ID NO: 83)

[0297] The genotype of the cells are confirmed via qPCR and Flow Cytometry. A summary of expected results can be found in Table 8.

Table 8: Summary of phenotyping and genotyping expected results for cells to be used in the NK, NKT, and T-cell potency assays.

	Phenotype	Expected Genotype			Expected Phenotype			
		hTert	SV40	MB-IL21	CD90	CD105	HLA-ABC (MHC class I)	HLA-D (MHC class II)
Line A	Immortalized	+	+	-	+	+	+	+
Line D	MB-IL21, Class I null	-	-	+	+	+	-	+
Line E	Immortalized, MB-IL21, Class I null	+	+	+	+	+	-	+
Line F	MB-IL21, Class II null	-	-	+	+	+	+	-
Line G	Immortalized, MB-IL21, Class II null	+	+	+	+	+	+	-

[0298] NK, NKT, T-cell Activation and Expansion Assays. The intended function of membrane-bound IL-21 (mbIL-21) expressing MSCs (feeder lines) in the Class I only and Class II only background is assessed via co-culture with primary human NK cells, primary human NKT, and primary human T-cell following the method described herein.

[0299] Negatively-selected, cryopreserved, primary human cytotoxic lymphocytes (NK, NKT, or T-cells) are purchased from a commercial vendor (BloodWork NW, Seattle, US). On Day -1 of co-culture (i.e. one day prior to start of co-culture), the MSC cell Lines A, B, and C are seeded at 1.75×10^5 /well/line in 6 well plates in StemXvivo media (R&D Systems, US). Also on Day -1, the NK cells are thawed and seeded at 0.7×10^5 /mL in NK Xpander media (Thermo Fisher, US) supplemented with 500 IU IL-2 and 5% FBS.

[0300] In a side arm, cytotoxic lymphocyte cells (iNK, iNKT, or iT-cells) generated from iPSC lines described in Example 5, are thawed and seeded with MSCs similarly to the primary cytotoxic lymphocytes as described above.

[0301] On Day 0 of culture, MSC line cell counts are assessed via counting of cells from a representative plate, and the cytotoxic lymphocytes cells are seeded on MSCs at 5:1 MSC-to-cytotoxic lymphocytes ratio in cytotoxic lymphocyte cell media. A control plate of MSCs is cultured in cytotoxic lymphocyte media, without co-culture, to assess the impact of cytotoxic lymphocyte media on MSC cells over the course of the experiment. Another control plate is seeded with cytotoxic lymphocyte cells only in cytotoxic lymphocyte media (no co-culture). All co-culture and control plates are incubated at 37°C and 5% CO₂.

[0302] On Days 1 to 3 of co-culture, a respective plate containing the co-cultured cells is removed from the incubator, and the cells are imaged on a microscope to document the killing and activation/expansion on cytotoxic lymphocyte cells. Then, a sample of cell suspension is collected, and cell count is performed to assess viability and count of cells in suspension. The plates are then washed with PBS and the MSC cells are lifted using a lifting reagent (Accutase, Thermo, US), and are enumerated on the cell counter.

[0303] For re-stimulation test, all cytotoxic lymphocyte cells from Day 3 co-culture are removed, resuspended in fresh cytotoxic lymphocyte culture media, and are reseeded on a fresh plate of Line D, E, F, or G MSCs. Cells are imaged and counted on Day 6 (Day 3 of re-stimulation).

[0304] Results. FIGs. 18 and 19 represent anticipated images of the state of the cells at the beginning (Day 0) and the end (Day 3) of 3-day co-culture assay. Cell viability and counts over the course of co-culture experiment are conducted over the course of the 3-day potency assay. Due to impact of handling on viability of cytotoxic lymphocytes, total nucleated cell count (TNC) will be reported and used to calculate normalized numbers instead of total viable count (TVC).

Due to impact of handling on viability of cytotoxic lymphocytes, TNC is reported and used to calculate normalized numbers instead of TVC.

[0305] The results will show that, in the absence of IL stimulation, cytotoxic lymphocytes cells do not change in number by Day 3 compared to those cultured on Lines D E, F, and G, which is expected to be between 150% to 200%. In comparison, cytotoxic lymphocyte cells in cytotoxic lymphocyte media without MSC co-culture maintain the viability and count through the 3-day assay period. This will also suggest that when not activated, cytotoxic lymphocyte cells are more resilient against the sampling procedure, hence maintaining viability, while those that are activated will show fragility to pipetting as shown previously resulting in low viability numbers for the co-cultured cytotoxic lymphocyte cells.

[0306] iPSC derived cytotoxic lymphocyte cells will be assessed on Day 3 of co-culture. The iPSC derived cytotoxic lymphocyte cells are not expected to demonstrate any appreciable killing effect within the time window of this assay and the specific ratio in which they will be seeded on MSCs. It is expected that iPSC derived cytotoxic lymphocyte cells will also demonstrate the killing effect if seeded at a higher ratio such as 1:1 or 5:1 NK to MSC.

[0307] The MSCs on the other hand, significantly decrease in number for Lines D, E, F, and G compared to Line A which shows an increase instead of decrease. This suggests the killing action of iPSC derived cytotoxic lymphocyte cells on MSCs that expresses IL-21, while those which do not express IL-21 (Line A) continue expanding in culture by Day 3.

[0308] After restimulation, a significant increase in cell number will be observed, documented by imaging and after performing cell count on the supernatant. Cells activated on feeder Lines D, E, F, and G will be shown to expand significantly by multiple folds (e.g., 13 to 16 folds) after 3 days of re-stimulation on fresh feeders.

[0309] (IX) Closing Paragraphs. The nucleic acid and amino acid sequences provided herein are shown using letter abbreviations for nucleotide bases and amino acid residues, as defined in 37 C.F.R. §1.831-1.835 and set forth in WIPO Standard ST.26 (implemented on July 1, 2022). Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included in embodiments where it would be appropriate.

[0310] Variants of the sequences disclosed and referenced herein are also included. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing biological activity can be found using computer programs well known in the art, such as DNASTAR™ (Madison, Wisconsin) software. Preferably, amino acid changes in the protein variants disclosed herein are conservative amino acid changes, i.e., substitutions of similarly charged or uncharged amino acids. A conservative amino acid change involves substitution of

one of a family of amino acids which are related in their side chains.

[0311] In a peptide or protein, suitable conservative substitutions of amino acids are known to those of skill in this art and generally can be made without altering a biological activity of a resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, e.g., Watson et al. *Molecular Biology of the Gene*, 4th Edition, 1987, The Benjamin/Cummings Pub. Co., p. 224). Naturally occurring amino acids are generally divided into conservative substitution families as follows: Group 1: Alanine (Ala), Glycine (Gly), Serine (Ser), and Threonine (Thr); Group 2: (acidic): Aspartic acid (Asp), and Glutamic acid (Glu); Group 3: (acidic; also classified as polar, negatively charged residues and their amides): Asparagine (Asn), Glutamine (Gln), Asp, and Glu; Group 4: Gln and Asn; Group 5: (basic; also classified as polar, positively charged residues): Arginine (Arg), Lysine (Lys), and Histidine (His); Group 6 (large aliphatic, nonpolar residues): Isoleucine (Ile), Leucine (Leu), Methionine (Met), Valine (Val) and Cysteine (Cys); Group 7 (uncharged polar): Tyrosine (Tyr), Gly, Asn, Gln, Cys, Ser, and Thr; Group 8 (large aromatic residues): Phenylalanine (Phe), Tryptophan (Trp), and Tyr; Group 9 (non-polar): Proline (Pro), Ala, Val, Leu, Ile, Phe, Met, and Trp; Group 11 (aliphatic): Gly, Ala, Val, Leu, and Ile; Group 10 (small aliphatic, nonpolar or slightly polar residues): Ala, Ser, Thr, Pro, and Gly; and Group 12 (sulfur-containing): Met and Cys. Additional information can be found in Creighton (1984) *Proteins*, W.H. Freeman and Company.

[0312] In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, *J. Mol. Biol.* 157(1), 105-32). Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics (Kyte and Doolittle, 1982). These values are: Ile (+4.5); Val (+4.2); Leu (+3.8); Phe (+2.8); Cys (+2.5); Met (+1.9); Ala (+1.8); Gly (−0.4); Thr (−0.7); Ser (−0.8); Trp (−0.9); Tyr (−1.3); Pro (−1.6); His (−3.2); Glutamate (−3.5); Gln (−3.5); aspartate (−3.5); Asn (−3.5); Lys (−3.9); and Arg (−4.5).

[0313] It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, i.e., still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ± 2 is preferred, those within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred. It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity.

[0314] As detailed in US 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: Arg (+3.0); Lys (+3.0); aspartate (+3.0±1); glutamate (+3.0±1); Ser (+0.3); Asn (+0.2); Gln (+0.2); Gly (0); Thr (-0.4); Pro (-0.5±1); Ala (-0.5); His (-0.5); Cys (-1.0); Met (-1.3); Val (-1.5); Leu (-1.8); Ile (-1.8); Tyr (-2.3); Phe (-2.5); Trp (-3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ± 2 is preferred, those within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

[0315] As outlined above, amino acid substitutions may be based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. As indicated elsewhere, variants of gene sequences can include codon optimized variants, sequence polymorphisms, splice variants, and/or mutations that do not affect the function of an encoded product to a statistically-significant degree.

[0316] Variants of the protein, nucleic acid, and gene sequences disclosed herein also include sequences with at least 70% sequence identity, 80% sequence identity, 85% sequence identity, 90% sequence identity, 95% sequence identity, 96% sequence identity, 97% sequence identity, 98% sequence identity, or 99% sequence identity to the protein, nucleic acid, or gene sequences disclosed herein.

[0317] "% sequence identity" refers to a relationship between two or more sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between protein, nucleic acid, or gene sequences as determined by the match between strings of such sequences. "Identity" (often referred to as "similarity") can be readily calculated by known methods, including those described in: Computational Molecular Biology (Lesk, A. M., ed.) Oxford University Press, NY (1988); Biocomputing: Informatics and Genome Projects (Smith, D. W., ed.) Academic Press, NY (1994); Computer Analysis of Sequence Data, Part I (Griffin, A. M., and Griffin, H. G., eds.) Humana Press, NJ (1994); Sequence Analysis in Molecular Biology (Von Heijne, G., ed.) Academic Press (1987); and Sequence Analysis Primer (Gribkov, M. and Devereux, J., eds.) Oxford University Press, NY (1992). Methods to determine identity are designed to give the best match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Sequence alignments and percent identity calculations may be performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR, Inc., Madison, Wisconsin). Multiple alignment of the sequences can also be performed using the Clustal method of alignment (Higgins

and Sharp CABIOS, 5, 151-153 (1989) with default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Relevant programs also include the GCG suite of programs (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, Wisconsin); BLASTP, BLASTN, BLASTX (Altschul, et al., J. Mol. Biol. 215:403-410 (1990); DNASTAR (DNASTAR, Inc., Madison, Wisconsin); and the FASTA program incorporating the Smith-Waterman algorithm (Pearson, Comput. Methods Genome Res., [Proc. Int. Symp.] (1994), Meeting Date 1992, 111-20. Editor(s): Suhai, Sandor. Publisher: Plenum, New York, N.Y.). Within the context of this disclosure it will be understood that where sequence analysis software is used for analysis, the results of the analysis are based on the "default values" of the program referenced. As used herein "default values" will mean any set of values or parameters, which originally load with the software when first initialized.

[0318] Variants also include nucleic acid molecules that hybridize under stringent hybridization conditions to a sequence disclosed herein and provide the same function as the reference sequence. Exemplary stringent hybridization conditions include an overnight incubation at 42 °C in a solution including 50% formamide, 5XSSC (750 mM NaCl, 75 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5XDenhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1XSSC at 50 °C. Changes in the stringency of hybridization and signal detection are primarily accomplished through the manipulation of formamide concentration (lower percentages of formamide result in lowered stringency); salt conditions, or temperature. For example, moderately high stringency conditions include an overnight incubation at 37°C in a solution including 6XSSPE (20XSSPE=3M NaCl; 0.2M NaH₂PO₄; 0.02M EDTA, pH 7.4), 0.5% SDS, 30% formamide, 100 µg/ml salmon sperm blocking DNA; followed by washes at 50 °C with 1XSSPE, 0.1% SDS. In addition, to achieve even lower stringency, washes performed following stringent hybridization can be done at higher salt concentrations (e.g. 5XSSC). Variations in the above conditions may be accomplished through the inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. Typical blocking reagents include Denhardt's reagent, BLOTTO, heparin, denatured salmon sperm DNA, and commercially available proprietary formulations. The inclusion of specific blocking reagents may require modification of the hybridization conditions described above, due to problems with compatibility.

[0319] "Specifically binds" refers to an association of a binding molecule to its cognate binding molecule with an affinity or K_a (i.e., an equilibrium association constant of a particular binding interaction with units of 1/M) equal to or greater than 10^5 M^{-1} , while not significantly associating with any other molecules or components in a relevant environment sample. Binding molecules

may be classified as "high affinity" or "low affinity". In particular embodiments, "high affinity" binding molecules refer to those binding molecules with a K_a of at least 10^7 M^{-1} , at least 10^8 M^{-1} , at least 10^9 M^{-1} , at least 10^{10} M^{-1} , at least 10^{11} M^{-1} , at least 10^{12} M^{-1} , or at least 10^{13} M^{-1} . In particular embodiments, "low affinity" binding molecules refer to those binding molecules with a K_a of up to 10^7 M^{-1} , up to 10^6 M^{-1} , up to 10^5 M^{-1} . Alternatively, affinity may be defined as an equilibrium dissociation constant (K_d) of a particular binding interaction with units of M (e.g., 10^{-5} M to 10^{-13} M). In certain embodiments, a binding molecule may have "enhanced affinity," which refers to a selected or engineered (i.e., genetically modified) binding molecules with stronger binding to a cognate binding molecule than a wild type (or parent) binding molecule. For example, enhanced affinity may be due to a K_a (equilibrium association constant) for the cognate binding molecule that is higher than the reference binding molecule or due to a K_d (dissociation constant) for the cognate binding molecule that is less than that of the reference binding molecule, or due to an off-rate (K_{off}) for the cognate binding molecule that is less than that of the reference binding molecule. A variety of assays are known for detecting binding molecules that specifically bind a particular cognate binding molecule as well as determining binding affinities, such as Western blot, ELISA, and BIACORE® analysis (see also, e.g., Scatchard, *et al.*, 1949, *Ann. N.Y. Acad. Sci.* 51:660; and U.S. Patent Nos. 5,283,173, 5,468,614, or the equivalent).

[0320] Unless otherwise indicated, the practice of the present disclosure can employ conventional techniques of immunology, molecular biology, microbiology, cell biology and recombinant DNA. These methods are described in the following publications. See, e.g., Sambrook, *et al.* Molecular Cloning: A Laboratory Manual, 2nd Edition (1989); F. M. Ausubel, *et al.* eds., Current Protocols in Molecular Biology, (1987); the series Methods IN Enzymology (Academic Press, Inc.); M. MacPherson, *et al.*, PCR: A Practical Approach, IRL Press at Oxford University Press (1991); MacPherson *et al.*, eds. PCR 2: Practical Approach, (1995); Harlow and Lane, eds. Antibodies, A Laboratory Manual, (1988); and R. I. Freshney, ed. Animal Cell Culture (1987).

[0321] As will be understood by one of ordinary skill in the art, each embodiment disclosed herein can comprise, consist essentially of or consist of its particular stated element, step, ingredient or component. Thus, the terms "include" or "including" should be interpreted to recite: "comprise, consist of, or consist essentially of." As used herein, the transition term "comprise" or "comprises" means has, but is not limited to, and allows for the inclusion of unspecified elements, steps, ingredients, or components, even in major amounts. The transitional phrase "consisting of" excludes any element, step, ingredient or component not specified. The transitional phrase "consisting essentially of" limits the scope of the embodiment to the specified elements, steps, ingredients or components and to those that do not materially affect the embodiment.

[0322] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about.” Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. When further clarity is required, the term “about” has the meaning reasonably ascribed to it by a person skilled in the art when used in conjunction with a stated numerical value or range, i.e. denoting somewhat more or somewhat less than the stated value or range, to within a range of $\pm 20\%$ of the stated value; $\pm 19\%$ of the stated value; $\pm 18\%$ of the stated value; $\pm 17\%$ of the stated value; $\pm 16\%$ of the stated value; $\pm 15\%$ of the stated value; $\pm 14\%$ of the stated value; $\pm 13\%$ of the stated value; $\pm 12\%$ of the stated value; $\pm 11\%$ of the stated value; $\pm 10\%$ of the stated value; $\pm 9\%$ of the stated value; $\pm 8\%$ of the stated value; $\pm 7\%$ of the stated value; $\pm 6\%$ of the stated value; $\pm 5\%$ of the stated value; $\pm 4\%$ of the stated value; $\pm 3\%$ of the stated value; $\pm 2\%$ of the stated value; or $\pm 1\%$ of the stated value.

[0323] Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

[0324] The terms “a,” “an,” “the” and similar referents used in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

[0325] Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member may be referred to and claimed individually or in any combination with other members of the group or other elements found herein. It is anticipated that one or more members of a group may be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

[0326] Certain embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Of course, variations on these described embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventor expects skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[0327] Furthermore, numerous references have been made to patents, printed publications, journal articles and other written text throughout this specification (referenced materials herein). Each of the referenced materials are individually incorporated herein by reference in their entirety for their referenced teaching.

[0328] In closing, it is to be understood that the embodiments of the invention disclosed herein are illustrative of the principles of the present invention. Other modifications that may be employed are within the scope of the invention. Thus, by way of example, but not of limitation, alternative configurations of the present invention may be utilized in accordance with the teachings herein. Accordingly, the present invention is not limited to that precisely as shown and described.

[0329] The particulars shown herein are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of various embodiments of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for the fundamental understanding of the invention, the description taken with the drawings and/or examples making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

[0330] Definitions and explanations used in the present disclosure are meant and intended to be

controlling in any future construction unless clearly and unambiguously modified in the examples or when application of the meaning renders any construction meaningless or essentially meaningless. In cases where the construction of the term would render it meaningless or essentially meaningless, the definition should be taken from Webster's Dictionary, 3rd Edition or a dictionary known to those of ordinary skill in the art, such as the Oxford Dictionary of Biochemistry and Molecular Biology (Eds. Attwood T et al., Oxford University Press, Oxford, 2006).

CLAIMS

What is claimed is:

1. A stem cell comprising a conditional immortalization gene.
2. The stem cell of claim 1, wherein the conditional immortalization gene encodes TERT.
3. The stem cell of claim 1, wherein the conditional immortalization gene encodes SV40 large T antigen.
4. The stem cell of claim 1, wherein the conditional immortalization gene comprises TERT and SV40 large T antigen.
5. The stem cell of claim 1, wherein the conditional immortalization gene is induced by a drug.
6. The stem cell of claim 5, wherein the drug comprises tetracycline or doxycycline.
7. The stem cell of claim 1, wherein the stem cell is a totipotent stem cell, a pluripotent stem cell, a multipotent stem cell, or a unipotent stem cell.
8. The stem cell of claim 1, wherein the pluripotent stem cell is an embryonic stem cell, a cord blood stem cell, or an induced pluripotent stem cells (iPSC).
9. The stem cell of claim 1, wherein the multipotent stem cell is a hematopoietic stem cell, a mesenchymal stem cell, or a neuronal stem cell.
10. The stem cell of claim 1, further comprising an exogenous sequence that encodes an expression product.
11. The stem cell of claim 10, wherein the expression product is a protein.
12. The stem cell of claim 11, wherein the protein comprises a recombinant receptor, a detectable label, an antigen, an antibody, or an enzyme.
13. The stem cell of claim 11, wherein the protein comprises CD70, α CD3, CD28, 4-1BB, MICA, 4-1BBL, membrane-bound interleukin (IL)-15, and/or membrane-bound IL21.
14. The stem cell of claim 11, wherein the protein comprises membrane-bound IL21.
15. The stem cell of claim 11, wherein the protein comprises a cancer antigen.
16. The stem cell of claim 15, wherein the cancer comprises multiple myeloma, lymphoma, acute lymphocytic leukemia (ALL), acute myelocytic leukemia (AML), chronic lymphocytic leukemia (CLL), breast cancer, colorectal cancer, ovarian cancer, renal cell carcinoma (RCC), glioblastoma, prostate cancer, neuroblastoma, melanoma, Ewing sarcoma, or hepatocellular cancer (HCC).
17. The stem cell of claim 15, wherein the cancer antigen comprises BCMA, CD4, CD5, CD7, CD19, CD20, CD22, CD33, CD73, CD123, CD133, CD138, CD244, CD276, CS1, EGFR, EGFRVIII, EpCAM, FLT3, GD2, GPA7, GPC3, HER2, Mesothelin, MUC1, NKG2D, PSMA, PSCA, or TF.

18. The stem cell of claim 15, wherein the cancer antigen comprises BCMA, CD19, CD20, CD33, CD133, CD138, CS1, EGFR, EGFRVII, EpCAM, GD2, GPA7, HER2, NKG2D, MUC1, or PSCA.
19. The stem cell of claim 11, wherein the protein comprises a viral, bacterial, fungal, or parasitic antigen.
20. The stem cell of claim 11, wherein the protein comprises insulin, factor VIII, factor IX, factor XI, alpha-1 antitrypsin (A1AT), glucocerebrosidase (GC), acid sphingomyelinase, mucopolysaccharides, acid alpha-glucosidase, aspartylglucosaminidase, alpha-galactosidase A, palmitoyl protein thioesterase, tripeptidyl peptidase, lysosomal transmembrane protein, cysteine transporter, acid ceramidase, acid alpha-L-fucosidase, cathepsin A, acid beta-glucosidase, acid beta-galactosidase, iduronate-2-sulfatase, alpha-L-iduronidase, galactocerebrosidase, acid alpha-mannosidase, acid beta-mannosidase, arylsulfatase B, arylsulfatase A, N-acetylgalactosamine-6-sulfate, N-acetylglucosamine-1-phosphotransferase, acid sphingomyelinase, NPC-1, alpha-glucosidase, beta-hexosaminidase B, heparan N-sulfatase, alpha-N-acetylglucosaminidase, acetyl-CoA: alpha-glucosaminide, N-acetylglucosamine-6-sulfate, alpha-N-acetylglactosaminidase, alpha-neuramidase, beta-glucuronidase, beta-hexosaminidase A, or acid lipase.
21. The stem cell of claim 12, wherein the recombinant receptor comprises an extracellular component comprising a binding domain; an intracellular component comprising an effector domain; and a transmembrane domain linking the extracellular component to the intracellular component.
22. The stem cell of claim 12, wherein the recombinant receptor comprises a chimeric antigen receptor or an engineered T cell receptor.
23. The stem cell of claim 21, wherein the binding domain of the recombinant receptor binds a cancer antigen, a viral antigen, a bacterial antigen, or a fungal antigen.
24. The stem cell of claim 23, wherein the cancer antigen comprises BCMA, CD4, CD5, CD7, CD19, CD20, CD22, CD33, CD73, CD123, CD133, CD138, CD244, CD276, CS1, EGFR, EGFRVIII, EpCAM, FLT3, GD2, GPA7, GPC3, HER2, Mesothelin, MUC1, NKG2D, PSMA, PSCA, or TF.
25. The stem cell of claim 23, wherein the cancer antigen comprises BCMA, CD19, CD20, CD33, CD133, CD138, CS1, EGFR, EGFRVII, EpCAM, GD2, GPA7, HER2, NKG2D, MUC1, or PSCA.
26. The stem cell of claim 21, wherein the effector domain comprises all or a portion of the signaling domain of CD3 ζ and/or 4-1BB.

27. The stem cell of claim 21, wherein the transmembrane domain comprises a CD28 transmembrane domain.
28. The stem cell of claim 12, wherein the recombinant receptor comprises a CD19 binding domain.
29. The stem cell of claim 12, wherein the recombinant receptor comprises a BCMA binding domain.
30. The stem cell of claim 12, wherein the detectable label comprises a fluorescent protein, a radioisotope, an enzyme label, or a fluorescent label.
31. The stem cell of claim 30, wherein the fluorescent protein comprises luciferase.
32. The stem cell of claim 1, wherein the stem cell is genetically modified to knockout a major histocompatibility complex (MHC).
33. The stem cell of claim 1, wherein the stem cell is genetically modified to knockout β 2-microglobulin (B2M).
34. The stem cell of claim 1, wherein the stem cell is genetically modified to knockout Class I Major Histocompatibility Complex Transactivator and/or Class II Major Histocompatibility Complex Transactivator.
35. The stem cell of claim 1, wherein the stem cell is genetically modified to knockout B2M, CIITA, or B2M and CIITA.
36. The stem cell of claims 33 or 35, wherein B2M is knocked out with the gRNA sequence as set forth in SEQ ID NOs: 34-42.
37. The stem cell of claims 34 or 35, wherein CIITA is knocked out with the gRNA sequence as set forth in SEQ ID NOs: 25-33.
38. The stem cell of claim 1, wherein the stem cell further comprises a suicide gene.
39. The stem cell of claim 38, wherein the suicide gene comprises CDK1 linked Herpes simplex virus-thymidine kinase/ganciclovir (CDK1/HSV-TK/GCV), TOP2A/HSV-TK/GCV, or inducible Casp9.
40. The stem cell of claim 38, wherein the suicide gene comprises CDK1/HSV-TK/GCV.
41. The stem cell of claim 1, wherein the stem cell further comprises a sequence encoding a tag cassette, a transduction marker, selection cassette, or a skipping element.
42. A cell line differentiated from the stem cell of claim 1.
43. The cell line of claim 42, wherein the cell line comprises more differentiated stem cells than the stem cell of claim 1.
44. The cell line of claim 43, wherein the more differentiated stem cells are CD34+ hematopoietic stem cells or mesenchymal stem cells.

45. The cell line of claim 42, wherein the cell line comprises pancreatic cells, epithelial cells, cardiac cells, endothelial cells, liver cells, endocrine cells, connective tissue cells, muscle cells, brain cells, bone cells, kidney cells, cartilage cells, or immune cells.
46. The cell line of claim 45, wherein the pancreatic cells comprise alpha cells, beta cells, or delta cells.
47. The cell line of claim 45, wherein the cardiac cells comprise cardiomyocytes.
48. The cell line of claim 45, wherein the liver cells comprise hepatocytes, hepatic stellate cells (HSCs), Kupffer cells (KCs), or liver sinusoidal endothelial cells (LSECs).
49. The cell line of claim 45, wherein the connective tissue cells comprise fibroblasts.
50. The cell line of claim 45, wherein the muscle cells comprise myoblasts.
51. The cell line of claim 45, wherein the brain cells comprise neurons.
52. The cell line of claim 45, wherein the bone cells comprise osteoblasts or osteoclasts.
53. The cell line of claim 45, wherein the cartilage cells comprise chondrocytes.
54. The cell line of claim 45, wherein the immune cells comprise T-cells, NK cells, or macrophages.
55. The cell line of claim 42, wherein cells within the cell line are genetically modified to express an expression product.
56. The cell line of claim 55, wherein the expression product is a protein.
57. The cell line of claim 56, wherein the protein comprises a recombinant receptor, a detectable label, an antigen, an antibody, or an enzyme.
58. The cell line of claim 56, wherein the protein comprises CD70, α CD3, CD28, 4-1BB, MICA, 4-1BBL, membrane-bound interleukin (IL)-15, and/or membrane-bound IL21.
59. The cell line of claim 56, wherein the protein comprises membrane-bound IL21.
60. The cell line of claim 56, wherein the protein comprises a cancer antigen.
61. The cell line of claim 60, wherein the cancer comprises multiple myeloma, lymphoma, acute lymphocytic leukemia (ALL), acute myelocytic leukemia (AML), chronic lymphocytic leukemia (CLL), breast cancer, colorectal cancer, ovarian cancer, renal cell carcinoma (RCC), glioblastoma, prostate cancer, neuroblastoma, melanoma, Ewing sarcoma, or hepatocellular cancer (HCC).
62. The cell line of claim 60, wherein the cancer antigen comprises BCMA, CD4, CD5, CD7, CD19, CD20, CD22, CD33, CD73, CD123, CD133, CD138, CD244, CD276, CS1, EGFR, EGFRVIII, EpCAM, FLT3, GD2, GPA7, GPC3, HER2, Mesothelin, MUC1, NKG2D, PSMA, PSCA, or TF.
63. The cell line of claim 60, wherein the cancer antigen comprises BCMA, CD19, CD20, CD33,

CD133, CD138, CS1, EGFR, EGFRVII, EpCAM, GD2, GPA7, HER2, NKG2D, MUC1, or PSCA.

64. The cell line of claim 56, wherein the protein comprises a viral, bacterial, fungal, or parasitic antigen.
65. The cell line of claim 56, wherein the protein comprises insulin, factor VIII, factor IX, factor XI, alpha-1 antitrypsin (A1AT), glucocerebrosidase (GC), acid sphingomyelinase, mucopolysaccharides, acid alpha-glucosidase, aspartylglucosaminidase, alpha-galactosidase A, palmitoyl protein thioesterase, tripeptidyl peptidase, lysosomal transmembrane protein, cysteine transporter, acid ceramidase, acid alpha-L-fucosidase, cathepsin A, acid beta-glucosidase, acid beta-galactosidase, iduronate-2-sulfatase, alpha-L-iduronidase, galactocerebrosidase, acid alpha-mannosidase, acid beta-mannosidase, arylsulfatase B, arylsulfatase A, N-acetylgalactosamine-6-sulfate, N-acetylglucosamine-1-phosphotransferase, acid sphingomyelinase, NPC-1, alpha-glucosidase, beta-hexosaminidase B, heparan N-sulfatase, alpha-N-acetylglucosaminidase, acetyl-CoA: alpha-glucosaminide, N-acetylglucosamine-6-sulfate, alpha-N-acetylgalactosaminidase, alpha-neuramidase, beta-glucuronidase, beta-hexosaminidase A, or acid lipase.
66. The cell line of claim 57, wherein the recombinant receptor comprises an extracellular component comprising a binding domain; an intracellular component comprising an effector domain; and a transmembrane domain linking the extracellular component to the intracellular component.
67. The cell line of claim 57, wherein the recombinant receptor comprises a chimeric antigen receptor or an engineered T cell receptor.
68. The cell line of claim 66, wherein the binding domain of the recombinant receptor binds a cancer antigen, a viral antigen, a bacterial antigen, or a fungal antigen.
69. The cell line of claim 68, wherein the cancer antigen comprises BCMA, CD4, CD5, CD7, CD19, CD20, CD22, CD33, CD73, CD123, CD133, CD138, CD244, CD276, CS1, EGFR, EGFRVIII, EpCAM, FLT3, GD2, GPA7, GPC3, HER2, Mesothelin, MUC1, NKG2D, PSMA, PSCA, or TF.
70. The cell line of claim 68, wherein the cancer antigen comprises BCMA, CD19, CD20, CD33, CD133, CD138, CS1, EGFR, EGFRVII, EpCAM, GD2, GPA7, HER2, NKG2D, MUC1, or PSCA.
71. The cell line of claim 66, wherein the effector domain comprises all or a portion of the signaling domain of CD3 ζ and/or 4-1BB.
72. The cell line of claim 66, wherein the transmembrane domain comprises a CD28

transmembrane domain.

73. The cell line of claim 57, wherein the recombinant receptor comprises a CD19 binding domain.
74. The cell line of claim 57, wherein the recombinant receptor comprises a BCMA binding domain.
75. The cell line of claim 57, wherein the detectable label comprises a fluorescent protein, a radioisotope, an enzyme label, or a fluorescent label.
76. The cell line of claim 75, wherein the fluorescent protein comprises luciferase.
77. The cell line of claim 42, wherein cells within the cell line are genetically modified to knockout a major histocompatibility complex (MHC).
78. The cell line of claim 42, wherein cells within the cell line are genetically modified to knockout β 2-microglobulin (B2M).
79. The cell line of claim 42, wherein cells within the cell line are genetically modified to knockout Class I Major Histocompatibility Complex Transactivator and/or Class II Major Histocompatibility Complex Transactivator.
80. The cell line of claim 42, wherein cells within the cell line are genetically modified to knockout B2M, CIITA, or B2M and CIITA.
81. The cell line of claims 78 or 80, wherein B2M is knocked out with the gRNA sequence as set forth in SEQ ID NOs: 34-42.
82. The cell line of claims 79 or 80, wherein CIITA is knocked out with the gRNA sequence as set forth in SEQ ID NOs: 25-33.
83. The cell line of claim 42, wherein cells within the cell line further comprise a suicide gene.
84. The cell line of claim 83, wherein the suicide gene comprises CDK1 linked Herpes simplex virus-thymidine kinase/ganciclovir (CDK1/HSV-TK/GCV), TOP2A/HSV-TK/GCV, or inducible Casp9.
85. The cell line of claim 83, wherein the suicide gene comprises CDK1/HSV-TK/GCV.
86. A method comprising genetically modifying a stem cell to comprise a conditional immortalization gene.
87. The method of claim 86, wherein the genetically modifying comprises transfecting a stem cell with the conditional immortalization gene using the Tet inducible system.
88. The method of claim 86, wherein the conditional immortalization gene encodes TERT.
89. The method of claim 86, wherein the conditional immortalization gene encodes SV40 large T antigen.
90. The method of claim 86, wherein the conditional immortalization gene comprises TERT and SV40 large T antigen.

91. The method of claim 86, wherein the conditional immortalization gene is induced by a drug.
92. The method of claim 91, wherein the drug comprises tetracycline or doxycycline.
93. The method of claim 86, wherein the stem cell is a totipotent stem cell, a pluripotent stem cell, a multipotent stem cell, or a unipotent stem cell.
94. The method of claim 93, wherein the pluripotent stem cell is an embryonic stem cell, a cord blood stem cell, or an induced pluripotent stem cells (iPSC).
95. The method of claim 93, wherein the multipotent stem cell is a hematopoietic stem cell, a mesenchymal stem cell, or a neuronal stem cell.
96. The method of claim 86, further genetically modifying the stem cell to comprise an exogenous sequence that encodes an expression product.
97. The method of claim 96, wherein the genetically modifying the stem cell to comprise an exogenous sequence comprises transfecting the stem cell with an expression construct using a transposon-based system or a lentivirus system.
98. The method of claim 96, wherein the genetically modifying the stem cell to comprise an exogenous sequence comprises transfecting the stem cell with an expression construct using a transposon-based system.
99. The method of claim 96, wherein the expression product is a protein.
100. The method of claim 99, wherein the protein comprises a recombinant receptor, a detectable label, an antigen, an antibody, or an enzyme.
101. The method of claim 99, wherein the protein comprises CD70, α CD3, CD28, 4-1BB, MICA, 4-1BBL, membrane-bound interleukin (IL)-15, and/or membrane-bound IL21
102. The method of claim 99, wherein the protein comprises membrane-bound IL21.
103. The method of claim 99, wherein the protein comprises a cancer antigen.
104. The method of claim 103, wherein the cancer comprises multiple myeloma, lymphoma, acute lymphocytic leukemia (ALL), acute myelocytic leukemia (AML), chronic lymphocytic leukemia (CLL), breast cancer, colorectal cancer, ovarian cancer, renal cell carcinoma (RCC), glioblastoma, prostate cancer, neuroblastoma, melanoma, Ewing sarcoma, or hepatocellular cancer (HCC).
105. The method of claim 103, wherein the cancer antigen comprises BCMA, CD4, CD5, CD7, CD19, CD20, CD22, CD33, CD73, CD123, CD133, CD138, CD244, CD276, CS1, EGFR, EGFRVIII, EpCAM, FLT3, GD2, GPA7, GPC3, HER2, Mesothelin, MUC1, NKG2D, PSMA, PSCA, or TF.
106. The method of claim 103, wherein the cancer antigen comprises BCMA, CD19, CD20, CD33, CD133, CD138, CS1, EGFR, EGFRVII, EpCAM, GD2, GPA7, HER2, NKG2D, MUC1,

or PSCA.

107. The method of claim 99, wherein the protein comprises a viral, bacterial, fungal, or parasitic antigen.
108. The method of claim 99, wherein the protein comprises insulin, factor VIII, factor IX, factor XI, alpha-1 antitrypsin (A1AT), glucocerebrosidase (GC), acid sphingomyelinase, mucopolysaccharides, acid alpha-glucosidase, aspartylglucosaminidase, alpha-galactosidase A, palmitoyl protein thioesterase, tripeptidyl peptidase, lysosomal transmembrane protein, cysteine transporter, acid ceramidase, acid alpha-L-fucosidase, cathepsin A, acid beta-glucosidase, acid beta-galactosidase, iduronate-2-sulfatase, alpha-L-iduronidase, galactocerebrosidase, acid alpha-mannosidase, acid beta-mannosidase, arylsulfatase B, arylsulfatase A, N-acetylgalactosamine-6-sulfate, N-acetylglucosamine-1-phosphotransferase, acid sphingomyelinase, NPC-1, alpha-glucosidase, beta-hexosaminidase B, heparan N-sulfatase, alpha-N-acetylglucosaminidase, acetyl-CoA: alpha-glucosaminide, N-acetylglucosamine-6-sulfate, alpha-N-acetylgalactosaminidase, alpha-neuramidase, beta-glucuronidase, beta-hexosaminidase A, or acid lipase.
109. The method of claim 100, wherein the recombinant receptor comprises an extracellular component comprising a binding domain; an intracellular component comprising an effector domain; and a transmembrane domain linking the extracellular component to the intracellular component.
110. The method of claim 100, wherein the recombinant receptor comprises a chimeric antigen receptor or an engineered T cell receptor.
111. The method of claim 109, wherein the binding domain of the recombinant receptor binds a cancer antigen, a viral antigen, a bacterial antigen, or a fungal antigen.
112. The method of claim 111, wherein the cancer antigen comprises BCMA, CD4, CD5, CD7, CD19, CD20, CD22, CD33, CD73, CD123, CD133, CD138, CD244, CD276, CS1, EGFR, EGFRVIII, EpCAM, FLT3, GD2, GPA7, GPC3, HER2, Mesothelin, MUC1, NKG2D, PSMA, PSCA, or TF.
113. The method of claim 111, wherein the cancer antigen comprises BCMA, CD19, CD20, CD33, CD133, CD138, CS1, EGFR, EGFRVII, EpCAM, GD2, GPA7, HER2, NKG2D, MUC1, or PSCA.
114. The method of claim 109, wherein the effector domain comprises all or a portion of the signaling domain of CD3 ζ and/or 4-1BB.
115. The method of claim 109, wherein the transmembrane domain comprises a CD28 transmembrane domain.

116. The method of claim 100, wherein the recombinant receptor comprises a CD19 binding domain.
117. The method of claim 100, wherein the recombinant receptor comprises a BCMA binding domain.
118. The method of claim 100, wherein the detectable label comprises a fluorescent protein, a radioisotope, an enzyme label, or a fluorescent label.
119. The method of claim 118, wherein the fluorescent protein comprises luciferase.
120. The method of claim 86, wherein the stem cells are genetically modified to knockout a major histocompatibility complex (MHC).
121. The method of claim 86, wherein the stem cells are genetically modified to knockout β 2-microglobulin (B2M).
122. The method of claim 86, wherein the stem cells are genetically modified to knockout Class I Major Histocompatibility Complex Transactivator and/or Class II Major Histocompatibility Complex Transactivator.
123. The method of claim 86, wherein the stem cells are genetically modified to knockout B2M, CIITA, or B2M and CIITA.
124. The method of claims 121 or 123, wherein B2M is knocked out with the gRNA sequence as set forth in SEQ ID NOs: 34-42.
125. The method of claims 122 or 123, wherein CIITA is knocked out with the gRNA sequence as set forth in SEQ ID NOs: 25-33.
126. The method of claim 86, wherein the stem cells are further genetically modified to comprise a suicide gene.
127. The method of claim 126, wherein the suicide gene comprises CDK1 linked Herpes simplex virus-thymidine kinase/ganciclovir (CDK1/HSV-TK/GCV), TOP2A/HSV-TK/GCV, or inducible Casp9.
128. The method of claim 126, wherein the suicide gene comprises CDK1/HSV-TK/GCV.
129. A method comprising differentiating a stem cell of claim 1 into a more differentiated cell type.
130. The method of claim 129, wherein the more differentiated stem cells comprise CD34+ hematopoietic stem cells, mesenchymal stem cells, or neural stem cells.
131. The method of claim 129, wherein the more differentiated cell type comprises more differentiated stem cells, pancreatic cells, epithelial cells, cardiac cells, endothelial cells, liver cells, endocrine cells, connective tissue cells, muscle cells, brain cells, bone cells, kidney cells, cartilage cells, cancer cells, or immune cells.

132. The method of claim 131, wherein the pancreatic cells comprise alpha cells, beta cells, or delta cells.
133. The method of claim 131, wherein the cardiac cells comprise cardiomyocytes
134. The method of claim 131, wherein the liver cells comprise hepatocytes, hepatic stellate cells (HSCs), Kupffer cells (KCs), and liver sinusoidal endothelial cells (LSECs).
135. The method of claim 131, wherein the connective tissue cells comprise fibroblasts.
136. The method of claim 131, wherein the muscle cells comprise myoblasts.
137. The method of claim 131, wherein the brain cells comprise neurons.
138. The method of claim 131, wherein the bone cells comprise osteoblasts and osteoclasts.
139. The method of claim 131, wherein the cartilage cells comprise chondrocytes.
140. The method of claim 131, wherein the immune cells comprise T-cells, NK cells, or macrophages.
141. The method of claim 129, further comprising genetically modifying the more differentiated cell type to comprise an exogenous sequence that encodes an expression product.
142. The method of claim 141, wherein the expression product is a protein.
143. The method of claim 142, wherein the protein comprises a recombinant receptor, a detectable label, an antigen, an antibody, or an enzyme.
144. The method of claim 142, wherein the protein comprises CD70, α CD3, CD28, 4-1BB, MICA, 4-1BBL, membrane-bound interleukin (IL)-15, and/or membrane-bound IL21.
145. The method of claim 142, wherein the protein comprises membrane-bound IL21.
146. The method of claim 142, wherein the protein comprises a cancer antigen.
147. The method of claim 146, wherein the cancer comprises multiple myeloma, lymphoma, acute lymphocytic leukemia (ALL), acute myelocytic leukemia (AML), chronic lymphocytic leukemia (CLL), breast cancer, colorectal cancer, ovarian cancer, renal cell carcinoma (RCC), glioblastoma, prostate cancer, neuroblastoma, melanoma, Ewing sarcoma, or hepatocellular cancer (HCC).
148. The method of claim 146, wherein the cancer antigen comprises BCMA, CD4, CD5, CD7, CD19, CD20, CD22, CD33, CD73, CD123, CD133, CD138, CD244, CD276, CS1, EGFR, EGFRVIII, EpCAM, FLT3, GD2, GPA7, GPC3, HER2, Mesothelin, MUC1, NKG2D, PSMA, PSCA, or TF.
149. The method of claim 146, wherein the cancer antigen comprises BCMA, CD19, CD20, CD33, CD133, CD138, CS1, EGFR, EGFRVII, EpCAM, GD2, GPA7, HER2, NKG2D, MUC1, or PSCA.
150. The method of claim 142, wherein the protein comprises a viral, bacterial, fungal, or

parasitic antigen.

151. The method of claim 142, wherein the protein comprises insulin, factor VIII, factor IX, factor XI, alpha-1 antitrypsin (A1AT), glucocerebrosidase (GC), acid sphingomyelinase, mucopolysaccharides, acid alpha-glucosidase, aspartylglucosaminidase, alpha-galactosidase A, palmitoyl protein thioesterase, tripeptidyl peptidase, lysosomal transmembrane protein, cysteine transporter, acid ceramidase, acid alpha-L-fucosidase, cathepsin A, acid beta-glucosidase, acid beta-galactosidase, iduronate-2-sulfatase, alpha-L-iduronidase, galactocerebrosidase, acid alpha-mannosidase, acid beta-mannosidase, arylsulfatase B, arylsulfatase A, N-acetylgalactosamine-6-sulfate, N-acetylglucosamine-1-phosphotransferase, acid sphingomyelinase, NPC-1, alpha-glucosidase, beta-hexosaminidase B, heparan N-sulfatase, alpha-N-acetylglucosaminidase, acetyl-CoA: alpha-glucosaminide, N-acetylglucosamine-6-sulfate, alpha-N-acetylgalactosaminidase, alpha-neuramidase, beta-glucuronidase, beta-hexosaminidase A, or acid lipase.
152. The method of claim 143, wherein the recombinant receptor comprises an extracellular component comprising a binding domain; an intracellular component comprising an effector domain; and a transmembrane domain linking the extracellular component to the intracellular component.
153. The method of claim 143, wherein the recombinant receptor comprises a chimeric antigen receptor or an engineered T cell receptor.
154. The method of claim 152, wherein the binding domain of the recombinant receptor binds a cancer antigen, a viral antigen, a bacterial antigen, or a fungal antigen.
155. The method of claim 154, wherein the cancer antigen comprises BCMA, CD4, CD5, CD7, CD19, CD20, CD22, CD33, CD73, CD123, CD133, CD138, CD244, CD276, CS1, EGFR, EGFRV8, EpCAM, FLT3, GD2, GPA7, GPC3, HER2, Mesothelin, MUC1, NKG2D, PSMA, PSCA, or TF.
156. The method of claim 154, wherein the cancer antigen comprises BCMA, CD19, CD20, CD33, CD133, CD138, CS1, EGFR, EGFRV7, EpCAM, GD2, GPA7, HER2, NKG2D, MUC1, or PSCA.
157. The method of claim 152, wherein the effector domain comprises all or a portion of the signaling domain of CD3 ζ and/or 4-1BB.
158. The method of claim 152, wherein the transmembrane domain comprises a CD28 transmembrane domain.
159. The method of claim 143, wherein the recombinant receptor comprises a CD19 binding domain.

160. The method of claim 143, wherein the recombinant receptor comprises a BCMA binding domain.
161. The method of claim 143, wherein the detectable label comprises a fluorescent protein, a radioisotope, an enzyme label, or a fluorescent label.
162. The method of claim 161, wherein the fluorescent protein comprises luciferase.
163. The method of claim 129, further comprising genetically modifying the more differentiated cell type to knockout major histocompatibility complex (MHC).
164. The method of claim 163, wherein the stem cells are genetically modified to knockout Class I Major Histocompatibility Complex Transactivator and/or Class II Major Histocompatibility Complex Transactivator.
165. The method of claim 163, wherein the genetically modifying the more differentiated cell type to knockout MHC comprises knocking out B2M; B2M and CITA; B2M and CIITA; or B2M, CITA, and CIITA.
166. The method of claim 165, wherein the knocking out B2M and CIITA comprises delivering the Cas9 nuclease, B2M gRNA, and CIITA gRNA to feeder cells.
167. The method of claim 166, wherein the B2M gRNA comprises SEQ ID NOs: 34-42.
168. The method of claim 166, wherein the CIITA gRNA comprises SEQ ID NOs: 25-33.
169. The method of claim 129, further comprising genetically modifying the more differentiated cell type to comprise a suicide gene.
170. The method of claim 169, wherein the suicide gene comprises CDK1 linked Herpes simplex virus-thymidine kinase/ganciclovir (CDK1/HSV-TK/GCV) or inducible Casp9.