

CLAIMS

1. A method for generating a universal donor cell, the method comprising delivering to a cell:

(a) a ~~first first site directed nuclease~~ CRISPR nuclease and a first guide RNA (gRNA) targeting a target site within or near a gene that encodes thioredoxin interacting protein (TXNIP) ~~a survival factor~~; and

(b) a first nucleic acid comprising a nucleotide sequence encoding ~~a~~ HLA class I histocompatibility antigen, alpha chain E (HLA-E) ~~first tolerogenic factor~~ that is flanked by (i) a nucleotide sequence homologous with a region located left of the target site of (a) and (ii) a nucleotide sequence homologous with a region located right of the target site of (a),

wherein the first site-directed nuclease cleaves the target site of (a) and the first nucleic acid of (b) is inserted at a site that partially overlaps, completely overlaps, or is contained within, the site of (a), thereby generating a universal donor cell, wherein the universal donor cell has increased cell survival compared to a cell in which the nucleic acid of (b) has not been inserted.

2. The method of claim 1, wherein the nucleotide sequence encoding HLA-E comprises a sequence encoding a HLA-E trimer comprising a B2M signal peptide fused to an HLA-G presentation peptide fused to a B2M membrane protein fused to HLA-E without its signal peptide, optionally wherein the sequence encoding the HLA-E trimer consists essentially of SEQ ID NO: 55 ~~survival factor is TXNIP, ZNF143, FOXO1, JNK, or MANF.~~

3. ~~The method of claim 1 or 2, wherein the first tolerogenic factor is PD-L1, HLA-E, HLA-G, CTLA-4, or CD47.~~

4. ~~The method of any one of claims 1 to 3, wherein the survival factor is TXNIP.~~

5. ~~The method of claim 4, wherein the first tolerogenic factor is HLA-E.~~

6. ~~The method of any one of claims 1 to 5, wherein the first site directed nuclease is a CRISPR system comprising a CRISPR nuclease and a guide RNA (gRNA).~~

~~7. The method of claim 6, wherein the CRISPR nuclease is a Type II Cas9 nuclease or a Type V Cpf1 nuclease, and the CRISPR nuclease is linked to at least one nuclear localization signal.~~

~~3.~~ The method of claim ~~6 or 7~~ 1 or 2, wherein the first gRNA comprises a spacer sequence corresponding to a target sequence consisting of SEQ ID NOS: 15-24.

~~8.~~

~~9.4.~~ The method of any one of claims ~~6 to 8~~ 1 to 3, wherein the nucleotide sequence of (b)(i) consists essentially of SEQ ID NO: 25, and the nucleotide sequence of (b)(ii) consists essentially of SEQ ID NO: 32.

~~10.5.~~ The method of any one of claims 1 to ~~94~~, wherein the method further comprises delivering to the cell:

(c) a second CRISPR nuclease and a second guide RNA (gRNA) site-directed ~~nuclease-targeting~~ a site within or near a gene that encodes beta-2-microglobulin (B2M) ~~one or more of a MHC I or MHC II human leukocyte antigen or a component or a transcriptional regulator of a MHC I or MHC II complex; and~~

(d) a second nucleic acid comprising a nucleotide sequence encoding programmed death ligand 1 (PD-L1) ~~a second tolerogenic factor~~ that is flanked by (iii) a nucleotide sequence homologous with a region located left of the target site of (c) and a (iv) nucleotide sequence homologous with a region located right of the target site of (c), ~~wherein the second tolerogenic factor of (d) differs from the first tolerogenic factor of (b),~~

wherein the second CRISPR nuclease site-directed nuclease ~~cleaves~~ the target site of (c) and the second nucleic acid of (d) is inserted at a site that partially overlaps, completely overlaps, or is contained within, the site of (c), wherein the universal donor cell has increased immune evasion and/or cell survival compared to a cell in which the second nucleic acid of (d) has not been inserted.

~~11. The method of claim 10, wherein the MHC I or MHC II human leukocyte antigen or the component or the transcriptional regulator of the MHC I or MHC II complex is HLA A, HLA B, HLA C, HLA DP, HLA DM, HLA DOA, HLA DOB, HLA DQ, HLA DR, B2M, NLRC5, CHTA, RFX5, RFXAP, or RFXANK.~~

~~12. The method of claim 10 or 11, wherein the second tolerogenic factor is PD-L1, HLA-E, HLA-G, CTLA-4, or CD47.~~

~~13. The method of any one of claims 10 to 12, wherein the MHC I or MHC II human leukocyte antigen or the component or the transcriptional regulator of the MHC I or MHC II complex is B2M.~~

~~14. The method of claim 13, wherein the second tolerogenic factor is PD-L1.~~

~~15. The method of any one of claims 10 to 14, wherein the second site-directed nuclease is CRISPR system comprising a CRISPR nuclease and a gRNA.~~

~~16. The method of claim 15, wherein the CRISPR nuclease is a Type II Cas9 nuclease or a Type V Cpf1 nuclease, and the CRISPR nuclease is linked to at least one nuclear localization signal.~~

~~17.6. The method of claim 15 or 16, wherein the second gRNA comprises a spacer sequence corresponding to a target sequence consisting of SEQ ID NOS: 1-3 or 35-44.~~

~~18.7. The method of any one of claims 15 to 17 claim 5 or 6, wherein the nucleotide sequence of (d)(iii) consists essentially of SEQ ID NO: 7, and the nucleotide sequence of (d)(iv) consists essentially of SEQ ID NO: 13.~~

8. The method of any one of claims 1 to 7, wherein the first CRISPR nuclease is a Type II Cas9 nuclease or a Type V Cpf1 nuclease and the first CRISPR nuclease is linked to at least one nuclear localization signal; and/or the second CRISPR nuclease is a Type II Cas9 nuclease or a Type V Cpf1 nuclease and the second CRISPR nuclease is linked to at least one nuclear localization signal.

19.9. The method of any one of claims 6 to 9 or claims 15 to 18 1 to 8, wherein the first CRISPR nuclease and the first gRNA are present at a molar ratio of 1:3 and/or the second CRISPR nuclease and the second gRNA are present at a molar ratio of 1:3.

20.10. The method of any one of claims 1 to 19, wherein the nucleotide sequence encoding the first tolerogenic factor HLA-E is operably linked to an exogenous promoter, and/or the nucleotide sequence encoding the second tolerogenic factor PD-L1 is operably linked to an exogenous promoter.

~~21.11.~~ The method of claim ~~20~~10, wherein the exogenous promoter is a constitutive, inducible, temporal-, tissue-, or cell type-specific promoter, optionally wherein the exogenous promoter is a CMV, EF1 α , PGK, CAG, or UBC promoter.

~~22.~~—A method for generating a universal donor cell, the method comprising delivering to a cell:

(a) a first site-directed nuclease targeting a site within or near a gene that encodes a survival factor;

(b) a first nucleic acid comprising a nucleotide sequence encoding a first tolerogenic factor that is flanked by (i) a nucleotide sequence homologous with a region located left of the target site of (a) and (ii) a nucleotide sequence homologous with a region located right of the target site of (a), wherein the first site-directed nuclease cleaves the target site of (a) and, through a process of homologous recombination, the first nucleic acid of (b) is utilized as a template for inserting the nucleotide sequence encoding the first tolerogenic factor into a site that partially overlaps, completely overlaps, or is contained within, the site of (a), thereby disrupting the gene of (a);

(c) a second site-directed nuclease targeting a site within or near a gene that encodes one or more of a MHC I or MHC II human leukocyte antigen or a component or a transcriptional regulator of a MHC I or MHC II complex; and

(d) a second nucleic acid comprising a nucleotide sequence encoding a second tolerogenic factor that is flanked by (iii) a nucleotide sequence homologous with a region located left of the target site of (c) and a (iv) nucleotide sequence homologous with a region located right of the target site of (c), wherein the tolerogenic factor of (d) differs from the tolerogenic factor (b), wherein the second site-directed nuclease cleaves the target site of (c) and, through a process of homologous recombination, the second nucleic acid of (d) is utilized as a template for inserting the nucleotide sequence encoding the second tolerogenic factor into a site that partially overlaps, completely overlaps, or is contained within, the site of (c), thereby disrupting the gene of (c), thereby generating a universal donor cell, wherein the universal donor cell has increased cell survival compared to a cell in which the first nucleic acid of (b) and the second nucleic acid of (d) has not been inserted.

~~23.— The method of claim 22, wherein the survival factor is TXNIP, the first tolerogenic factor is HLA E, the MHC I or MHC II human leukocyte antigen or a component or a transcriptional regulator of a MHC I or MHC II complex is B2M, and the second tolerogenic factor is PD-L1.~~

~~24.12.~~ The method of any one of claims 1 to ~~23~~11, wherein the cell is a mammalian cell, optionally wherein the cell is a human cell.

~~25.— The method of any one of claims 1 to 24, wherein the cell is a stem cell.~~

~~26.13.~~ The method of any one of claims 1 to ~~24~~12, wherein the cell is a pluripotent stem cell, an embryonic stem cell, an adult stem cell, an induced pluripotent stem cell, or a hematopoietic stem cell.

~~27.—~~ The method of any one of claims 1 to ~~24~~13, wherein the cell is a differentiated cell, or a somatic cell and/or -

~~28.14.~~ The method of any one of claims 1 to ~~24~~, wherein the universal donor cell is capable of being differentiated into lineage-restricted progenitor cells or fully differentiated somatic cells.

~~29.—~~ The method of claim ~~28~~14, wherein the lineage-restricted progenitor cells are pancreatic endoderm progenitors, pancreatic endocrine progenitors, mesenchymal progenitor cells, muscle progenitor cells, blast cells, hematopoietic progenitor cells, or neural progenitor cells and/or -

~~30.15.~~ The method of claim ~~28~~, wherein the fully differentiated somatic cells are pancreatic beta cells, epithelial cells, endodermal cells, macrophages, hepatocytes, adipocytes, kidney cells, blood cells, cardiomyocytes, or immune system cells.

~~31.— A plurality of universal donor cells generated by the method of any one of claims 1 to 27.~~

~~32.— The plurality of universal donor cells of claim 31, maintained for a time and under conditions sufficient for the cells to undergo differentiation.~~

~~33.— The plurality of cells of claims 31 or 32 for use in treating a subject in need thereof.~~

~~34.— The plurality of cells for use of claim 33, wherein the subject is a human who has, is suspected of having, or is at risk for a disease.~~

~~35.— A method comprising administering to a subject the plurality of universal donor cells of claim 31 or 32.~~

~~36. A method for treating of a subject in need thereof, the method comprising:~~

~~(a) obtaining or having obtained the plurality of universal donor cells of claim 31 following differentiation into lineage restricted progenitor cells or fully differentiated somatic cells; and~~

~~(b) administering the lineage restricted progenitor cells or fully differentiated somatic cells to the subject.~~

~~37. A method of obtaining cells for administration to a subject in need thereof, the method comprising:~~

~~(a) obtaining or having obtained the universal donor cells of claim 31; and~~

~~(b) maintaining the universal donor cells for a time and under conditions sufficient for the cells to differentiate into lineage restricted progenitor cells or fully differentiated somatic cells.~~

~~38. The method of claim 36 or 37, wherein the lineage restricted progenitor cells are pancreatic endoderm progenitors, pancreatic endocrine progenitors, mesenchymal progenitor cells, muscle progenitor cells, blast cells, hematopoietic progenitor cells, or neural progenitor cells.~~

~~39. The method of claim 36 or 37, wherein the fully differentiated somatic cells are pancreatic beta cells, epithelial cells, endodermal cells, macrophages, hepatocytes, adipocytes, kidney cells, blood cells, cardiomyocytes, or immune system cells.~~

~~40. The method of any one of claims 35 to 39, wherein the subject is a human who has, is suspected of having, or is at risk for a disease.~~

~~41. The method of claim 40, wherein the disease is a genetically inheritable disease.~~

~~42. A guide RNA comprising a spacer sequence corresponding to a target sequence consisting of SEQ ID NO: 15-24.~~

~~43.16. A process for generating universal donor cells, the process comprising:~~

~~(a) modifying stem cells by inserting a nucleotide sequence encoding a first tolerogenic factor programmed death ligand 1 (PD-L1) within or near a gene encoding a MHC-I or MHC-II human leukocyte antigen or a component or a transcriptional regulator of a MHC-I or MHC-II complex beta-2 microglobulin (B2M), thereby generating first tolerogenic factor PD-L1 positive cells;~~

~~(b) enriching for first tolerogenic factor PD-L1 positive cells;~~

(c) modifying the ~~first tolerogenic factor~~PD-L1 positive cells from (b) by inserting a nucleotide sequence encoding HLA class I histocompatibility antigen, alpha chain E (HLA-E) ~~a second tolerogenic factor~~ within or near a gene encoding thioredoxin interacting protein (TXNIP)~~a survival factor~~, thereby generating ~~first tolerogenic factor~~PD-L1 positive/~~second tolerogenic factor~~HLA-E positive cells;

(d) enriching for ~~first tolerogenic factor~~PD-L1 positive/~~second tolerogenic factor~~HLA-E positive cells;

(e) single cell sorting to select for ~~first tolerogenic factor~~PD-L1 positive/~~second tolerogenic factor~~HLA-E positive cells;

(f) characterizing the cells from (e) as universal donor cells; and

(g) freezing the universal donor cells for long term storage.

44. — The process of claim 43, wherein the enriching for first tolerogenic factor positive cells at (b) ~~comprises magnetic assisted cell sorting (MACS), single cell cloning, expanding said first tolerogenic factor positive cells, or a combination thereof.~~

45. — The process of claim 43 or 44, wherein the enriching for first tolerogenic factor positive/~~second tolerogenic factor~~ positive cells at (d) ~~comprises magnetic assisted cell sorting, single cell cloning, expanding said first tolerogenic factor positive/~~second tolerogenic factor~~ positive cells, or a combination thereof.~~

46. — The process of any one of claims 43 to 45, further comprising in (a) ~~expanding the generated first tolerogenic factor positive cells, in (c) expanding the generated first tolerogenic factor positive/~~second tolerogenic factor~~ positive cells, in (e) expanding the selected first tolerogenic factor positive/~~second tolerogenic factor~~ positive cells, or a combination thereof.~~

47.17. The process of ~~any one of claims 43 to 46~~claim 16, wherein the modifying at (a) comprises delivering to the stem cells (1) a first RNA-guided nuclease and a first guide RNA (gRNA) targeting a target site in a ~~MHC-I or MHC-II human leukocyte antigen or a component or a transcriptional regulator of a MHC-I or MHC-II complex~~a beta-2 microglobulin (B2M) gene locus and (2) a first vector comprising a first nucleic acid, the first nucleic acid comprising (i) a nucleotide sequence homologous with a region located left of the target site in the ~~MHC-I or MHC-II human leukocyte antigens or a component or a transcriptional regulator of a MHC-I or MHC-II complex~~B2M gene locus, (ii) the nucleotide sequence encoding the ~~first tolerogenic factor~~PD-L1,

and (iii) a nucleotide sequence homologous with a region located right of the target site in the ~~MHC I or MHC II human leukocyte antigen or a component or a transcriptional regulator of a MHC I or MHC II complex~~B2M gene locus, wherein the ~~MHC I or MHC II human leukocyte antigen or a component or a transcriptional regulator of a MHC I or MHC II complex~~B2M gene locus is cleaved at the target site and the first nucleic acid comprising the nucleotide sequence encoding ~~first tolerogenic factor~~PD-L1 is inserted into the ~~MHC I or MHC II human leukocyte antigen or a component or a transcriptional regulator of a MHC I or MHC II complex~~B2M gene locus, thereby disrupting the ~~MHC I or MHC II human leukocyte antigen or a component or a transcriptional regulator of a MHC I or MHC II complex~~B2M gene.

~~48.~~ The process of ~~claim 47~~, wherein the first RNA-guided nuclease and the first gRNA form a first ribonucleoprotein (RNP) complex.

~~49.~~18. The process of ~~any one of claims 43 to 46~~claim 16, wherein the modifying at (a) comprises delivering to the stem cells (1) a first ribonucleoprotein (RNP) complex comprising a first RNA-guided nuclease and a first guide RNA (gRNA) targeting a target site in a ~~MHC I or MHC II human leukocyte antigen or a component or a transcriptional regulator of a MHC I or MHC II complex~~beta-2 microglobulin (B2M) gene locus and (2) a first vector comprising a first nucleic acid, the first nucleic acid comprising (i) a nucleotide sequence homologous with a region located left of the target site in the ~~MHC I or MHC II human leukocyte antigen or a component or a transcriptional regulator of a MHC I or MHC II complex~~B2M gene locus, (ii) the nucleotide sequence encoding the ~~first tolerogenic factor~~PD-L1, and (iii) a nucleotide sequence homologous with a region located right of the target site in the ~~MHC I or MHC II human leukocyte antigen or a component or a transcriptional regulator of a MHC I or MHC II complex~~B2M gene locus, wherein the ~~MHC I or MHC II human leukocyte antigen or a component or a transcriptional regulator of a MHC I or MHC II complex~~B2M gene locus is cleaved at the target site and the first nucleic acid comprising the nucleotide sequence encoding ~~first tolerogenic factor~~PD-L1 is inserted into the ~~MHC I or MHC II human leukocyte antigen or a component or a transcriptional regulator of a MHC I or MHC II complex~~B2M gene locus, thereby disrupting the ~~MHC I or MHC II human leukocyte antigen or a component or a transcriptional regulator of a MHC I or MHC II complex~~B2M gene.

~~50.— The process of any one of claims 43 to 49, wherein the MHC I or MHC II human leukocyte antigen or a component or a transcriptional regulator of a MHC I or MHC II complex gene is HLA A, HLA B, HLA C, HLA DP, HLA DM, HLA DOA, HLA DOB, HLA DQ, HLA DR, B2M, NLRC5, CIITA, RFX5, RFXAP, or RFXANK.~~

~~51.— The process of claim 50, wherein the MHC I or MHC II human leukocyte antigen or a component or a transcriptional regulator of a MHC I or MHC II complex gene is B2M.~~

~~52.— The process of claim 51, wherein the nucleotide sequence of (a)(2)(i) consists essentially of SEQ ID NO: 7, and the nucleotide sequence of (a)(2)(iii) consists essentially of SEQ ID NO: 13.~~

~~53.— The process of claims 51 or 52, wherein the first gRNA comprises a spacer sequence corresponding to a target sequence consisting of SEQ ID NO: 2.~~

~~54.— The process of any one of claims 47 to 53, wherein the first RNA guided nuclease is a Cas9 nuclease.~~

~~55.— The process of claim 54, wherein the Cas9 nuclease is linked to at least one nuclear localization signal.~~

~~56.— The process of any one of claims 47 to 55, wherein the first RNP comprises a molar ratio of first gRNA: first RNA guided nuclease of 3:1.~~

~~57.— The process of any one of claims 43 to 56, wherein the first tolerogenic factor is PD L1, HLA E, HLA G, CTLA 4, or CD47.~~

~~58.— The process of any one of claims 43 to 57, wherein the nucleotide sequence encoding the first tolerogenic factor is operably linked to an exogenous promoter.~~

~~59.— The process of claim 58, wherein the exogenous promoter is a CMV, EF1 α , PGK, CAG, or UBC promoter.~~

~~60.— The process of any one of claims 43 to 59, wherein the first tolerogenic factor is PD L1.~~

~~61.— The process of claim 60, wherein the nucleotide sequence encoding PD L1 consists essentially of SEQ ID NO: 11.~~

~~62.— The process of claim 61, wherein the nucleotide sequence encoding PD L1 is operably linked to a CAG promoter.~~

~~63.— The process of any one of claims 60 to 62, wherein the first vector comprises a nucleotide sequence consisting of SEQ ID NO: 33.~~

~~64.19. The process of any one of claims 43 to 63~~16 to 18, wherein the modifying at (c) comprises delivering to the stem cells (1) a second RNA-guided nuclease and a second guide RNA (gRNA) targeting a target site in a thioredoxin interacting protein (TXNIP) survival factor gene locus and (2) a second vector comprising a second nucleic acid, the second nucleic acid comprising (i) a nucleotide sequence homologous with a region located left of the target site in the TXNIP survival factor gene locus, (ii) the nucleotide sequence encoding the ~~second tolerogenic factor~~HLA-E, and (iii) a nucleotide sequence homologous with a region located right of the target site in the ~~survival factor~~TXNIP gene locus, wherein the ~~survival factor~~TXNIP gene locus is cleaved at the target site and the second nucleic acid comprising the nucleotide sequence encoding the ~~second tolerogenic factor~~HLA-E is inserted into the ~~survival factor~~TXNIP gene locus, thereby disrupting the ~~survival factor~~TXNIP gene.

~~65.— The process of claim 64, wherein the second RNA-guided nuclease and the second gRNA form a second ribonucleoprotein (RNP) complex.~~

~~66.20. The process of any one of claims 43 to 63~~16 to 18, wherein the modifying at (c) comprises delivering to the ~~first tolerogenic factor~~PD-L1 positive cells (1) a second ribonucleoprotein (RNP) complex comprising a second RNA-guided nuclease and a second guide RNA (gRNA) targeting a target site in a thioredoxin interacting protein (TXNIP) survival factor gene locus and (2) a second vector comprising a second nucleic acid, the second nucleic acid comprising (i) a nucleotide sequence homologous with a region located left of the target site in the ~~survival factor~~TXNIP gene locus, (ii) the nucleotide sequence encoding the ~~second tolerogenic factor~~HLA-E, and (iii) a nucleotide sequence homologous with a region located right of the target site in the ~~second survival factor~~TXNIP gene locus, wherein the ~~survival factor~~TXNIP gene locus is cleaved at the target site and the second nucleic acid comprising the nucleotide sequence encoding the ~~second tolerogenic factor~~HLA-E is inserted into the ~~survival factor~~TXNIP gene locus, thereby disrupting the ~~survival factor~~TXNIP gene.

~~67.— The process of any one of claims 43 to 66, wherein the survival gene is TXNIP, ZNF143, FOXO1, JNK, or MANF.~~

~~68.— The process of claim 67, wherein the survival gene is TXNIP.~~

~~69.— The process of claim 68, wherein the second gRNA comprises a spacer sequence corresponding to a target sequence consisting of SEQ ID NO: 20.~~

~~70.— The process of claims 68 or 69, wherein the nucleotide sequence of (c)(2)(i) consists essentially of SEQ ID NO: 25, and the nucleotide sequence of (c)(2)(iii) consists essentially of SEQ ID NO: 32.~~

~~71.— The process of any one of claims 64 to 70, wherein the second RNA-guided nuclease is a Cas9 nuclease.~~

~~72.— The process of claim 71, wherein the Cas9 nuclease is linked to at least one nuclear localization signal.~~

~~73.— The process of any one of claims 64 to 72, wherein the second RNP comprises a molar ratio of second gRNA:second RNA-guided nuclease of 3:1.~~

~~74.— The process of any one of claims 43 to 73, wherein the second tolerogenic factor is PD-L1, HLA-E, HLA-G, CTLA-4, or CD47.~~

~~75.— The process of any one of claims 43 to 74, wherein the nucleotide sequence encoding the second tolerogenic factor is operably linked to an exogenous promoter.~~

~~76.— The process of claim 75, wherein the exogenous promoter is a CMV, EF1 α , PGK, CAG, or UBC promoter.~~

~~77.— The process of any one of claims 43 to 76, wherein the second tolerogenic factor is HLA-E.~~

~~78.— The process of claim 77, wherein the nucleotide sequence encoding HLA-E comprises a sequence encoding a HLA-E trimer comprising a B2M signal peptide fused to an HLA-G presentation peptide fused to a B2M membrane protein fused to HLA-E without its signal peptide.~~

~~79.— The process of claim 78, wherein the sequence encoding the HLA-E trimer consists essentially of SEQ ID NO: 55.~~

~~80.— The process of claims 78 or 79, wherein the nucleotide sequence encoding HLA-E is operably linked to a CAG promoter.~~

~~81.— The process of any one of claims 77 to 80, wherein the second vector comprises a nucleotide sequence consisting of SEQ ID NO: 34 or 56.~~

~~82.— The process of any one of claims 43 to 81, wherein the single cell sorting at (e) comprises fluorescence activated cell sorting (FACS), single cell cloning, expanding said single cell sorted cells, or a combination thereof.~~

~~83.— The process of any one of claims 43 to 82, wherein the characterizing at (f) comprises DNA analyses for zygosity and/or indel profile.~~

~~84.— The process of any one of claims 43 to 83, wherein the characterizing at (f) comprises cell analyses for morphology, viability, karyotyping, endotoxin levels, mycoplasma levels, on/off target analysis, random vector insertion, residual Cas9, residual vector, pluripotency status, differentiation capacity, or a combination thereof.~~

~~85.— The process of any one of claims 43 to 84, wherein the process further comprises freezing prior to the characterizing at (f).~~