Claims

1. A method for modifying primary islet cells, the method comprising:

i) dissociating a primary islet cluster into a suspension of primary islet cells;

ii) contacting the suspension of primary islet cells with one or more reagents to modify gene expression; and

iii) incubating the modified islet cells under conditions for re-clustering the cells into an islet, wherein at least a portion of the incubating is carried out with motion.

2. A method of modification of a population of cells, the method comprising:

i) contacting a population of cells with one or more reagents to modify gene expression in cells of the population; and

ii) subjecting the population of cells to motion after contact with the one or more reagents.

3. The method of claim 2, wherein the population of cells are:

(i) primary cells;

(ii) cells derived from stem cells, optionally wherein:

(a) the stem cells are selected from the group consisting of a pluripotent stem cell (PSC), an induced pluripotent stem cell, an embryonic stem cell, a hematopoietic stem cell, a mesenchymal stem cell, an endothelial stem cell, an epithelial stem cell, an adipose stem cell, a germline stem cell, a lung stem cell, a cord blood stem cell, and a multipotent stem cell;

(b) the stem cells are pluripotent stem cells; or

(c) the stem cells are induced pluripotent stem cells, mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), or embryonic stem cells (ESCs); or

(iii) selected from the group consisting of islet cells, immune cells, B cells, T cells, natural killer (NK) cells, natural killer T (NKT) cells, macrophages, endothelial cells, muscle cells, cardiac muscle cells, smooth muscle cells, skeletal muscle cells, hepatocytes, a glial progenitor cells, dopaminergic neurons, retinal pigment epithelial cells, thyroid cells, skin cells, glial progenitor cells, neural cells, cardiac cells, and blood cells.

4. The method of claim 2 or claim 3, wherein the population of cells are:

(a) naturally present in a 3D network;

(b) in suspension;

(c) in a vessel that has a low-attachment surface; and/or

(d) produced by dissociating cells from an adherent culture or a cell cluster prior to the contacting.

5. The method of any of claims 2-4, wherein the population of cells are islet cells, optionally wherein the population of cells comprise beta islet cells.

6. The method of claim 5, wherein population of cells comprising beta islet cells are produced by dissociating a primary islet cluster into a suspension of cells comprising primary beta islet cells.

7. The method of any of claims 1-6, wherein the contacting is carried out for 30 seconds to 24 hours prior to subjecting the cells to motion.

8. The method of any of claims 1-7, wherein the contacting is carried out for 1 minute to 60 minutes, 2 minutes to 30 minutes, 5 minutes to 15 minutes prior to subjecting the cells to motion.

9. The method of claim 1, wherein the primary islet cluster is a human primary cadaveric islet cluster.

10. The method of claim 1 or claim 9, wherein at least a portion of the incubating is carried out under static conditions, wherein the incubating comprises a first incubation under static conditions followed by the incubating with motion or the incubating comprises the incubating with motion followed by a second incubation under static conditions.

11. The method of any of claims 1 and 7-10, wherein steps i)-iii) are repeated.

12. The method of claim 11, wherein the one or more reagents in the first iteration of the method are different from the one or more reagents in the repeated iteration of the method.

13. The method of any of claims 1 and 7-12, wherein the one or more reagents are one or more first reagents and the re-clustered islet cells are a first modified primary islet cluster and wherein the method further comprises:

iv) dissociating the first modified primary islet cluster into a suspension of modified primary islet cells;

v) further contacting the modified primary islet cells of the suspension with one or more further reagents to modify gene expression; and

vi) incubating the further modified primary islet cells after the further contacting under conditions for re-clustering into a second modified primary islet cluster, wherein at least a portion of the incubating is carried out with motion.

14. The method of any of claims 1 and 7-13, wherein prior to the incubating in iii), the method comprises selecting for islet cells that have modified gene expression, optionally relative to the primary islet cells before the contacting.

15. The method of claim 13 or claim 14, wherein prior to v), the method comprises selecting, from the dissociated islet cells in iv), islet cells that have modified gene expression, optionally modified relative to primary islet cells before the contacting, and optionally repeating steps iii) and iv) on the selected islet cells.

16. The method of any of claims 13-15, wherein after the incubating in vi), the method comprises dissociating the second modified primary islet cluster into a suspension comprising modified primary islet cells and selecting for islet cells that have modified gene expression, optionally modified gene expression relative to the primary islet before the contacting or the further contacting.

17. The method of claim 15 or claim 16, wherein the method comprises incubating the selected modified islet cells under conditions for re-clustering into a modified primary islet cluster, wherein at least a portion of the incubating is carried out with motion.

18. The method of any of claims 1 and 7-17, wherein the suspension of primary islet cells is present in a vessel that has a low-attachment surface.

19. The method of any of claims 1-18, wherein:

(i) the motion is at a speed of between about 20 rpm and about180 rpm, between about 40 rpm and about 125 rpm or between about 60 rpm and about 100 rpm, each inclusive;

(ii) the motion is at a speed of between about 85 rpm and about 95 rpm, inclusive;

(iii) the motion is shaking, optionally wherein the shaking comprises orbital motion;

(iv) the motion is an undulating motion, optionally wherein the undulating motion is with a shaker device that combines orbital movement and rocking movement;

(v) the motion comprises bidirectional linear movement;

(vi) the motion is with an orbital shaker; or

(vii) the motion is with a tilt angle, optionally wherein the tilt angle is between 1° and 8°.

20. The method of any of claims 1-19, wherein the one or more reagents or the further one or more reagents reduce expression of one or more genes encoding an endogenous protein in the cell or increase expression of one or more heterologous proteins in the cell.

21. The method of any of claims 1-20, wherein the one or more first reagents, the one or more further reagents, or both, comprise a genome-modifying protein for gene editing a target gene encoding an endogenous protein and/or an agent comprising an exogenous polynucleotide encoding an exogenous protein.

22. The method of claim 21, wherein the genome-modifying protein is associated with gene editing by a sequence-specific nuclease, a CRISPR-associated transposase (CAST), prime editing, or Programmable Addition via Site-specific Targeting Elements (PASTE).

23. The method of claim 22, wherein the sequence specific nuclease is selected from the group consisting of an RNA-guided DNA endonuclease, a meganuclease, a transcription activator-like effector nuclease (TALEN), and a zinc-finger nuclease (ZFN), optionally wherein the RNA-guided nuclease comprises a Cas nuclease and a guide RNA (CRISPR-Cas combination), more optionally wherein the CRISPR-Cas combination is a ribonucleoprotein (RNP) complex comprising the gRNA and the Cas nuclease, more optionally wherein the Cas nuclease is a Type II or Type V Cas protein.

24. The method of claim 23, wherein the genome-modifying protein is selected from the group consisting of Cas3, Cas4, Cas5, Cas8a, Cas8b, Cas8c, Cas9, Cas10, Cas12, Cas12a (Cpf1), Cas12b (C2c1), Cas12c (C2c3), Cas12d (CasY), Cas12e (CasX), Cas12f (C2c10), Cas12g, Cas12h, Cas12i, Cas12k (C2c5), Cas13, Cas13a (C2c2), Cas13b, Cas13c, Cas13d, C2c4, C2c8, C2c9, Cmr5, Cse1, Cse2, Csf1, Csm2, Csn2, Csx10, Csx11, Csy1, Csy2, Csy3, Mad7, a zinc finger nuclease (ZFN), a transcription activator-like effector nuclease (TALEN), a meganuclease, and a CRISPR-associated transposase, or a homologue of any of the foregoing.

25. The method of any of claims 1-24, wherein the one or more reagents are for reducing expression of one or more major histocompatibility complex (MHC) class I molecules, optionally wherein the one or more MHC class I molecule is an HLA-A protein, an HLA-B protein, or HLA-C protein and/or for reducing expression of one or more MHC class II molecules, optionally wherein the one or more MHC class II molecule is an HLA-DP protein, an HLA-DR protein, or an HLA-DQ protein.

26. The method of claim 25, wherein:

a. the one or more reagents or the further one or more reagents for reducing expression of one or more MHC class I molecule reduces expression of B-2 microglobulin (B2M); and/or

b. the one or more reagents or the further one or more reagents for reducing expression of one or more MHC class II molecule reduces expression of CIITA.

27. The method of any of claims 20-26, wherein the one or more reagents for increasing expression of one or more exogenous proteins in the cell comprises an agent comprising an exogenous polynucleotide.

28. The method of claim 27, wherein the one or more exogenous proteins is one or more tolerogenic factors.

29. The method of claim 27 or claim 28, wherein the one or more reagents comprise an agent for increasing expression of one or more tolerogenic factors.

30. The method of claim 29 or claim 30, wherein the one or more tolerogenic factors is selected from the group consisting of CD47, A20/TNFAIP3, C1-Inhibitor, CCL21, CCL22, CD16, CD16 Fc receptor, CD24, CD27, CD35, CD39, CD46, CD52, CD55, CD59, CD200, CR1, CTLA4-Ig, DUX4, FasL, H2-M3, HLA-C, HLA-E, HLA-E heavy chain, HLA-G, PD-L1, IDO1, IL-10, IL15-RF, IL-35, MANF, Mfge8, PD-1, PD-L1 or Serpinb9 , and any combination thereof.

31. The method of any of claims 27-30, wherein the exogenous polynucleotide is integrated into the genome of the cell, optionally wherein the integration is by non-targeted insertion into the genome of the cell, optionally by introduction of the exogenous polynucleotide into the cell using a lentiviral vector.

32. The method of claim 31, wherein the integration is by targeted insertion into a target genomic locus of the cell.

33. The method of any of claims 1 and 5-33, wherein the islet cell is a beta islet cell.

34. The method of any of claims 1-33, wherein the viability of the cells produced by the method is greater than about 30%, 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or more.

35. The method of any of claims 1-33, wherein the percentage of cells of the population modified by the method is greater than about 30%, 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or more.

36. A method for modifying primary islet cells, the method comprising:

i) dissociating a primary islet cluster into a suspension of primary islet cells;

ii) contacting the suspension of primary islet cells with one or more first reagents to modify gene expression to produce modified islet cells, wherein the one or more first reagents comprise (1) a gene editing system comprising a genome-modifying protein for gene editing of a target gene encoding an endogenous protein and/or (2) an agent comprising an exogenous polynucleotide encoding an exogenous protein;

iii) after the contacting, incubating the modified islet cells under conditions for re-clustering the cells into a first modified primary islet cluster, wherein at least a portion of the incubating is carried out with shaking comprising orbital motion;

iv) dissociating the first modified primary islet cluster into a suspension of modified primary islet cells;

v) further contacting the modified primary islet cells of the suspension with one or more second reagents, wherein the one or more second reagents comprise (i) a gene editing system comprising a genome-modifying protein for gene editing a target gene encoding an endogenous protein and/or (ii) an agent comprising an exogenous polynucleotide encoding an exogenous protein; and

vi) incubating the further modified primary islet cells after the further contacting under conditions for re-clustering into a second modified primary islet cluster, wherein at least a portion of the incubating is carried out with shaking comprising orbital motion.

37. A population of engineered cells produced by the method of any of claims 1-36.

38. The population of claim 37, wherein at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, 99.9%, or 99.99% of cells in the population comprise:

(i) the modifications;

(ii) an exogenous polynucleotide encoding CD47;

(iii) reduced expression of one or more MHC class I molecules and/or one or more MHC class II molecules relative to a cell of the same cell type that does not comprises the modification(s);

(iv) reduced expression of B2M and/or CIITA relative to a cell of the same cell type that does not comprises the modification(s);

(v) reduced expression of B2M relative to a cell of the same cell type that does not comprises the modification(s);

(vi) reduced expression of B2M and CIITA relative to a cell of the same cell type that does not comprises the modification(s);

(vii) one or more alterations that inactivate both alleles of an endogenous *B2M* gene; and/or

(viii) one or more alterations that inactivate both alleles of an endogenous *CIITA* gene.

39. A population of engineered primary islet cells comprising a plurality of engineered primary islet cells, wherein the engineered primary islet cell comprises modifications comprising:

(i) a transgene comprising an exogenous polynucleotide encoding CD47,

(ii) inactivation or disruption of both alleles of a *B2M* gene, and

(iii) inactivation or disruption of both alleles of a *CIITA* gene,

wherein:

at least 90% of cells in the population comprise reduced expression of one or more MHC class I molecules relative to a primary islet cell that does not comprise the modifications and at least 90% of cells in the population comprise the exogenous polynucleotide encoding CD47.

40. A composition comprising the population of any of claims 37-39.

41. A composition comprising a population of engineered primary cells, wherein the engineered primary cells comprise: (i) a transgene comprising an exogenous polynucleotide encoding CD47 and (ii) inactivation or disruption of both alleles of a *B2M* gene, and wherein the primary cells comprise primary islet cells, T cells, thyroid cells, skin cells, endothelial cells, or retinal pigmented cells.

42. The composition of claim 40 or claim 41, wherein engineered primary cells of the population of engineered primary cells comprise:

(i) an indel in both alleles of the *B2M* gene;

(ii) inactivation or disruption of both alleles of a *CIITA* gene, optionally wherein engineered primary cells of the population of engineered primary cells comprise an indel in both alleles of the *CIITA* gene; and/or

(iii) the phenotype B2M$^{indel/indel}$; CIITA$^{indel/indel}$; CD47*tg*.

43. The composition of claim 41, wherein the engineered primary cell is an engineered primary islet cell and the engineered primary islet cell comprises modifications comprising a transgene comprising an exogenous polynucleotide encoding CD47.

44. The composition of claim 41 or claim 42, wherein the engineered primary cell is an engineered primary islet cell and the engineered primary islet cell comprises modifications comprising inactivation or disruption of both alleles of a *B2M* gene.

45. The composition of any of claims 41, 42 and 44, wherein the engineered primary cell is an engineered primary islet cell and the engineered primary islet cell comprises modifications comprising inactivation or disruption of both alleles of a *CIITA* gene.

46. The composition of any of claims 41 and 43-45, at least 90% of cells in the population comprise reduced expression of one or more MHC class I molecules relative to a primary islet cell that does not comprise the modifications and at least 90% of cells in the population comprise the exogenous polynucleotide encoding CD47.

47. The composition of any of claims 40-46, wherein the composition is a pharmaceutical composition comprising a pharmaceutically acceptable excipient.

48. Use of the population of claim 37 or claim 38, the composition of any of claims 40-46, or the pharmaceutical composition of claim 47, in the manufacture of a medicament for treating a disease, condition, or cellular deficiency in a patient in need thereof.

49. Use of a population of islet cells of claim 37 or claim 38, the composition of any of claims 40-46, or the pharmaceutical composition of claim 47, in the manufacture of a medicament for treating diabetes in a patient in need thereof, optionally wherein the population of cells is to be administered as a cluster of islet cells.

50. The use of claim 49, wherein the cluster of islet cells is a cluster of beta islet cells.

51. The use of claim 49 or claim 50, wherein the beta islet cells improve glucose tolerance in the subject.

52. The use of claim 51, wherein the subject is a diabetic patient, optionally wherein the diabetic patient has type I diabetes or type II diabetes.

53. The use of any of claims 49-52, wherein glucose tolerance is improved relative to the subject's glucose tolerance prior to administration of the islet cells or as measured by HbA1c levels.

54. The use of any of claims 49 and 51-53, wherein the beta islet cells reduce exogenous insulin usage in the subject.

55. The use of claim 53 or claim 54, wherein the subject is fasting.

56. The use of any one of claims 49 and 51-55, wherein the islet cells improve insulin secretion in the subject, optionally wherein insulin secretion is improved relative to the subject's insulin secretion prior to administration of the islet cells.

57. The use of any of claims 48-56, wherein one or more immunosuppressive agents is to be administered to the patient.

58. The use of any of claims 48-56, where the patient has been administered one or more immunosuppressive agents.

59. The use of claim 57 or 58, wherein the one or more immunosuppressive agents are a small molecule or an antibody, optionally wherein the one or more immunosuppressive agents are selected from the group consisting of cyclosporine, azathioprine, mycophenolic acid, mycophenolate mofetil, a corticosteroids, prednisone, methotrexate, gold salts, sulfasalazine, antimalarials, brequinar, leflunomide, mizoribine, 15-deoxyspergualine, 6-mercaptopurine, cyclophosphamide, rapamycin, tacrolimus (FK-

506), OKT3, anti-thymocyte globulin, thymopentin (thymosin-α), and an immunosuppressive antibody.

60. The use of any of claims 57-59, wherein the one or more immunosuppressive agents are one or more immunomodulatory agents, optionally wherein the one or more immunomodulatory agents comprise a small molecule or an antibody, more optionally wherein the antibody binds to one or more of receptors or ligands selected from the group consisting of p75 of the IL-2 receptor, MHC, CD2, CD3, CD4, CD7, CD28, B7, CD40, CD45, IFN-gamma, TNF-alpha, IL-4, IL-5, IL-6R, IL-6, IGF, IGFR1, IL-7, IL-8, IL-10, CD11a, CD58, and antibodies binding to any of their ligands.

61. The use of any of claims 57-60, wherein the one or more immunosuppressive agents:

(i) are to be or have been administered to the patient prior to administration of the engineered cells;

(ii) are to be or have been administered to the patient at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days prior to administration of the engineered cells;

(iii) are to be or have been administered to the patient at least 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks or more prior to administration of the engineered cells;

(iv) are to be or have been administered to the patient at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days after administration of the engineered cells;

(v) are to be or have been administered to the patient at least 1 week after administration of the engineered cells;

(vi) are to be or have been administered to the patient at least 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, or more, after administration of the engineered cells;

(vii) are to be or have been administered to the patient on the same day as the first administration of the engineered cells;

(viii) are to be or have been administered to the patient after administration of the engineered cells;

(ix) are to be or have been administered to the patient after administration of the engineered cells;

(x) are to be or have been administered to the patient after administration of a first and/or second administration of the engineered cells;

(xi) are to be or have been administered to the patient prior to administration of a first and/or second administration of the engineered cells;

(xii) are to be or have been administered to the patient at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days prior to administration of a first and/or second administration of the engineered cells;

(xiii) are to be or have been administered to the patient at least 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks or more prior to administration of a first and/or second administration of the engineered cells;

(xiv) are to be or have been administered to the patient at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days after administration of a first and/or second administration of the engineered cells;

(xv) are to be or have been administered to the patient at least 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, or more, after administration of a first and/or second administration of the engineered cells; or

(xvi) are to be administered at a lower dosage compared to the dosage of one or more immunosuppressive agents to be administered to reduce immune rejection of immunogenic cells that do not comprise the modifications of the engineered cells.

62. The use of any of claims 57-61, wherein the engineered cell is capable of controlled killing of the engineered cell.

63. The use of any of claims 57-62, wherein the engineered cell comprises a suicide gene or a suicide switch, optionally wherein the suicide gene or the suicide switch induces controlled cell death in the presence of a drug or prodrug, or upon activation by a selective exogenous compound, more optionally wherein the suicide gene or the suicide switch is an inducible protein capable of inducing apoptosis of the engineered cell.

64. The use of any of claims 57-63, wherein an agent that allows for depletion of an engineered cell of the population of engineered cells is to be administered, optionally wherein the agent that allows for depletion of the engineered cell is an antibody that recognizes a protein expressed on the surface of the engineered cell.

65. The use of any of claims 48-56 and 64, wherein an agent that recognizes the one or more tolerogenic factors on the surface of the engineered cell is to be administered, optionally wherein engineered cell is engineered to express the one or more tolerogenic factors, more optionally wherein the one or more tolerogenic factors is CD47.

66. The method or use or cells of any of claims 1-65, wherein the cell is an allogeneic cell.