

## **CLAIMS:**

1. A method for producing a population of retinal epithelium (RPE) cells, the method comprising:

(i) obtaining cell clusters of PAX6+/MITF+ RPE progenitor cells and dissociating the cell clusters into single cells;

(ii) culturing the single cells in a differentiation medium such that the cells differentiate to RPE cells; and

(iii) harvesting the RPE cells produced in step (ii);

thereby producing a population of RPE cells.

2. A method for producing a population of retinal epithelium (RPE) cells, the method comprising:

(i) obtaining cell clusters of PAX6+/MITF+ RPE progenitor cells,

(ii) culturing the cell clusters in a differentiation medium such that the cells differentiate to RPE cells; and

(iii) harvesting the RPE cells produced in step (ii);

thereby producing a population of RPE cells.

3. The method of claim 1 or 2, further comprising harvesting the RPE cells produced in step (ii) by dissociating the RPE cells, fractionating the RPE cells, collecting RPE cell clusters, dissociating the RPE cell clusters into single RPE cells, and culturing the single RPE cells.

4. The method of claim 1 or 2, further comprising harvesting the RPE cells produced in step (ii) by dissociating the RPE cells, collecting RPE cell clusters, and selectively picking RPE cell clusters.

5. The method of claim 4, further comprising dissociating the selectively picked RPE cell clusters into single RPE cells and culturing the single RPE cells.

6. The method of any one of the preceding claims, wherein the PAX6+/MITF+ RPE progenitor cells are obtained from a population of pluripotent stem cells.
7. The method of claim 6, wherein the pluripotent stem cells are human embryonic stem cells or human induced pluripotent stem cells.
8. The method of any one of the preceding claims, further comprising expanding the RPE cells.
9. The method of claim 8, wherein the RPE cells are expanded by culturing the cells in maintenance media supplemented with FGF.
10. The method of claim 9, wherein the maintenance medium comprises FGF during the first 1, 2, or 3 days of RPE proliferation at each passage, followed by culturing the RPE cells in maintenance media lacking FGF.
11. The method of claim 9 or 10, wherein FGF is added before confluence.
12. The method of any one of the preceding claims, wherein the differentiation medium further comprises heparin and/or ROCK inhibitor.
13. The method of any one of the preceding claims, wherein the RPE cells are passaged up to two times.
14. The method any one of claims 1 and 3-13, wherein any one of the dissociation steps is carried out by treating the cells with a dissociation reagent.
15. The method of claim 14, wherein the dissociation reagent is selected from the group collagenase (such as collagenase I or collagenase IV), accutase, chelator (*e.g.*, EDTA-based dissociation solution), trypsin, dispase, or any combinations thereof.
16. The method of any one of the preceding claims, wherein the RPE cells are cryopreserved following harvesting.
17. The method of claim 16, wherein the cells are cryopreserved in a medium comprising one or more cryopreservative selected from the group DMSO (dimethyl sulfoxide), ethylene glycol, glycerol, 2-methyl-2-4-pentanediol (MPD), propylene glycol, and sucrose.

18. The method of any one of claims 6-17, wherein the population of pluripotent stem cells is embryoid bodies.
19. The method of any one of the preceding claims, wherein the cells are cultured on feeder cells.
20. The method of any one claims 1-18, wherein the cells are cultured under feeder-free conditions.
21. The method of any one of the preceding claims, wherein the cells are cultured in a non-adherent culture.
22. The method of any one of claims 1-20, wherein the cells are cultured in an adherent culture.
23. The method of any one of the preceding claims, wherein the differentiation medium is EBDM.
24. The method of any one of claims 1-22, wherein the differentiation medium comprises one or more differentiation agents selected from the group nicotinamide, a transforming factor- $\beta$  (TGF $\beta$ ) superfamily (*e.g.*, activin A, activin B, and activin AB), nodal, anti-mullerian hormone (AMH), bone morphogenetic proteins (BMP) (*e.g.*, BMP2, BMP3, BMP4, BMP5, BMP6, and BMP7, growth and differentiation factors (GDF)), WNT pathway inhibitor (*e.g.*, CKI-7, DKK1), a TGF pathway inhibitor (*e.g.*, LDN193189, Noggin), a BMP pathway inhibitor (*e.g.*, SB431542), a sonic hedgehog signal inhibitor, a bFGF inhibitor, and a MEK inhibitor (*e.g.*, PD0325901).
25. The method of claim 24, wherein the differentiation medium comprises nicotinamide.
26. The method of claims 24 or 25, wherein the differentiation medium comprises activin.
27. The method of any one of the preceding claims, wherein the cell clusters of PAX6+/MITF+ RPE progenitor cells are between about 40  $\mu$ m and about 200  $\mu$ m in size.
28. The method of any one of the preceding claims, wherein the cell clusters of PAX6+/MITF+ RPE progenitor cells are between about 40  $\mu$ m and about 100  $\mu$ m in size.

29. The method of any one of the preceding claims, wherein in step (ii), the cells are cultured on an extracellular matrix selected from the group laminin or a fragment thereof, fibronectin, vitronectin, Matrigel, CellStart, collagen, and gelatin.
30. The method of claim 29, wherein the extracellular matrix is laminin or a fragment thereof.
31. The method of claim 30, wherein the laminin is selected from laminin-521 and laminin-511.
32. The method of claim 31, wherein the laminin is iMatrix511.
33. The method of any one of the preceding claims, wherein the duration of culturing in step (ii) is about 1 week to about 8 weeks.
34. The method of any one of the preceding claims, wherein the duration of culturing in step (ii) is at least about 3 weeks.
35. The method of any one of the preceding claims, wherein the duration of culturing in step (ii) is about 6 weeks.
36. The method of any one of claims 3-35, wherein the RPE cell clusters are between about 40  $\mu\text{m}$  and 200  $\mu\text{m}$  in size.
37. The method of claim 36, wherein the RPE cell clusters are between about 40  $\mu\text{m}$  and 100  $\mu\text{m}$  in size.
38. The method of any one of claims 3-37, wherein the single RPE cells are cultured in a medium that supports RPE growth or differentiation.
39. The method of claim 38, wherein the single RPE cells are cultured on an extracellular matrix selected from the group laminin or a fragment thereof, fibronectin, vitronectin, Matrigel, CellStart, collagen, and gelatin.
40. The method of claim 39, wherein the extracellular matrix is gelatin.
41. The method of claim 39, wherein the extracellular matrix is laminin or a fragment thereof.

42. The method of any one of the preceding claims, wherein the population of RPE cells are at least 75% pure, at least 80% pure, at least 90% pure, at least 95% pure, at least 96% pure, at least 97% pure, at least 98% pure, or at least 99% pure.

43. The method of any one of the preceding claims, wherein the RPE cells are human RPE cells.

44. A method for producing a population of retinal epithelium (RPE) cells, the method comprising:

(i) culturing a population of pluripotent stem cells in a first differentiation medium, such that the cells differentiate into RPE progenitor cells;

(ii) dissociating the RPE progenitor cells, fractionating the cells to collect cell clusters, dissociating the cell clusters into single cells, and subculturing the single cells in a second differentiation medium such that the cells differentiate to RPE cells; and

(iii) harvesting the RPE cells produced in step (ii)

thereby producing a population of RPE cells.

45. A method for producing a population of retinal epithelium (RPE) cells, the method comprising:

(i) culturing a population of pluripotent stem cells in a first differentiation medium, such that the cells differentiate into RPE progenitor cells;

(ii) dissociating the RPE progenitor cells, fractionating the cells to collect cell clusters, and subculturing the collected cell clusters in a second differentiation medium such that the cells differentiate to RPE cells; and

(iii) harvesting the RPE cells produced in step (ii)

thereby producing a population of RPE cells.

46. The method of claim 44 or 45, further comprising harvesting the RPE cells produced in step (ii) by dissociating the RPE cells, fractionating the RPE cells to collect RPE cell clusters, dissociating the RPE cell clusters into single RPE cells, and culturing the single RPE cells.

47. The method of claim 44 or 45, further comprising harvesting the RPE cells produced in step (ii) by dissociating the RPE cells, collecting RPE cell clusters, and selectively picking RPE cell clusters.
48. The method of claim 47, further comprising dissociating the selectively picked RPE cell clusters into single RPE cells and culturing the single RPE cells.
49. The method of any one of claims 44-48, wherein the RPE progenitor cells are positive for PAX6/MITF.
50. The method of any one of claims 44-49, further comprising expanding the RPE cells.
51. The method of claim 50, wherein the RPE cells are expanded by culturing the cells in maintenance media supplemented with FGF.
52. The method of claim 51, wherein the maintenance medium comprises FGF during the first 1, 2, or 3 days of RPE proliferation at each passage, followed by culturing the RPE cells in maintenance media lacking FGF.
53. The method of claim 51 or 52, wherein FGF is added before confluence.
54. The method of any one of claims 44-53, wherein the first and/or second differentiation medium further comprises heparin and/or ROCK inhibitor.
55. The method of any one of claims 44-54, wherein the RPE cells are passaged up to two times.
56. The method any one of claims 44-55, wherein any one of the dissociation steps is carried out by treating the cells with a dissociation reagent.
57. The method of claim 56, wherein the dissociation reagent is selected from the group collagenase (such as collagenase I or collagenase IV), accutase, chelator (*e.g.*, EDTA-based dissociation solution), trypsin, dispase, or any combinations thereof.
58. The method of any one of claims 44-57, wherein the RPE cells are cryopreserved following harvesting.

59. The method of claim 58, wherein the cells are cryopreserved in a medium comprising one or more cryopreservative selected from the group DMSO (dimethyl sulfoxide), ethylene glycol, glycerol, 2-methyl-2-4-pentanediol (MPD), propylene glycol, and sucrose.
60. The method of any one of claims 44-59, wherein the pluripotent stem cells are human embryonic stem cells.
61. The method of any one of claims 44-59, wherein the pluripotent stem cells are human induced pluripotent stem cells.
62. The method of any one claims 44-61, wherein the population of pluripotent stem cells is embryoid bodies.
63. The method of any one of claims 44-62, wherein prior to step (i), the pluripotent stem cells are cultured on feeder cells in a medium that supports pluripotency.
64. The method of any one of claims 44-62, wherein prior to step (i), the pluripotent stem cells are cultured feeder-free in a medium that supports pluripotency.
65. The method of claim 63 or 64, wherein the medium that supports pluripotency is supplemented with bFGF.
66. The method of any one of claims 44-65, wherein step (i), (ii), and/or (iii) is performed in a non-adherent culture.
67. The method of any one of claims 44-65, wherein step (i), (ii), and/or (iii) is performed in an adherent culture.
68. The method of any one of claims 44-67, wherein the first and second differentiation medium are the same.
69. The method of any one of claims 44-67, wherein the first and second differentiation medium are different.
70. The method of any one of claims 44-68, wherein the first and second differentiation medium is EBDM.
71. The method of any one of claims 44-69, wherein the first differentiation medium comprises one or more differentiation agents selected from the group nicotinamide, a

transforming factor- $\beta$  (TGF $\beta$ ) superfamily (*e.g.*, activin A, activin B, and activin AB), nodal, anti-mullerian hormone (AMH), bone morphogenetic proteins (BMP) (*e.g.*, BMP2, BMP3, BMP4, BMP5, BMP6, and BMP7, growth and differentiation factors (GDF)), WNT pathway inhibitor (*e.g.*, CKI-7, DKK1), a TGF pathway inhibitor (*e.g.*, LDN193189, Noggin), a BMP pathway inhibitor (*e.g.*, SB431542), a sonic hedgehog signal inhibitor, a bFGF inhibitor, and a MEK inhibitor (*e.g.*, PD0325901).

72. The method of any one of claims 44-69, wherein the second differentiation medium comprises one or more differentiation agents selected from the group nicotinamide, a transforming factor- $\beta$  (TGF $\beta$ ) superfamily (*e.g.*, activin A, activin B, and activin AB), nodal, anti-mullerian hormone (AMH), bone morphogenetic proteins (BMP) (*e.g.*, BMP2, BMP3, BMP4, BMP5, BMP6, and BMP7, growth and differentiation factors (GDF)), WNT pathway inhibitor (*e.g.*, CKI-7, DKK1), a TGF pathway inhibitor (*e.g.*, LDN193189, Noggin), a BMP pathway inhibitor (*e.g.*, SB431542), a sonic hedgehog signal inhibitor, a bFGF inhibitor, and a MEK inhibitor (*e.g.*, PD0325901).

73. The method of claim 71 or 72, wherein the first differentiation medium comprises nicotinamide.

74. The method of any one of claims 71-73, wherein the second differentiation medium comprises activin.

75. The method of any one claims 44-74, wherein the duration of culturing in step (i) is about 1 weeks to about 12 weeks.

76. The method of any one of claims 44-75, wherein the duration of culturing in step (i) is at least about 3 weeks.

77. The method of any one of claims 44-76, wherein the duration of culturing in step (i) is about 6 to about 10 weeks.

78. The method of any one of claims 44-77, wherein the cell clusters collected in step (ii) are between about 40  $\mu\text{m}$  and about 200  $\mu\text{m}$  in size.

79. The method of any one of claims 44-78, wherein the cell clusters collected in step (ii) are between about 40  $\mu\text{m}$  and about 100  $\mu\text{m}$  in size.

80. The method of any one of claims 44-79, wherein in step (ii), the cells are subcultured on an extracellular matrix selected from the group laminin, fibronectin, vitronectin, Matrigel, CellStart, collagen, and gelatin.

81. The method of claim 80, wherein the extracellular matrix comprises laminin or a fragment thereof.

82. The method of claim 81, wherein the laminin or fragment thereof is selected from laminin-521 and laminin-511.

83. The method of any one of claims 44-82, wherein the duration of subculturing in step (ii) is about 1 week to about 8 weeks.

84. The method of any one claims 44-83, wherein the duration of subculturing in step (ii) is at least about 3 weeks.

85. The method of any one of claims 44-84, wherein the duration of subculturing in step (ii) is about 6 weeks.

86. The method of any one of claims 46 and 48-85, wherein the RPE cell clusters are between about 40  $\mu\text{m}$  and 200  $\mu\text{m}$  in size.

87. The method of claim 86, wherein the RPE cell clusters are between about 40  $\mu\text{m}$  and 100  $\mu\text{m}$  in size.

88. The method of any one of claims 46 and 48-87, wherein the single RPE cells are cultured in a medium that supports RPE growth or differentiation.

89. The method of claim 88, wherein the single RPE cells are cultured on an extracellular matrix selected from the group laminin or a fragment thereof, fibronectin, vitronectin, Matrigel, CellStart, collagen, and gelatin.

90. The method of claim 89, wherein the extracellular matrix is gelatin.

91. The method of claim 89, wherein the extracellular matrix is laminin or a fragment thereof.

92. The method of any one of claims 44-91, wherein the population of RPE cells are at least 75% pure, at least 80% pure, at least 90% pure, at least 95% pure, at least 96% pure, at least 97% pure, at least 98% pure, or at least 99% pure.
93. The method of any one of claims 44-92, wherein the RPE cells are human RPE cells.
94. The method of any of the preceding claims, wherein the RPE cells express one or more of markers selected from the group RPE65, CRALBP, PEDF, Bestrophin, MITF, OTX2, PAX2, PAX6, premelanosome protein (PMEL or gp-100), tyrosinase, and ZO1.
95. The method of any one of the preceding claims, wherein the RPE cells express Bestrophin, PMEL, CRALBP, MITF, PAX6, and ZO1.
96. The method of any one of claims 1-94, wherein the RPE cells express Bestrophin, PAX6, MITF, and RPE65.
97. The method of any one of claims 1-94, wherein the RPE cells express MITF and at least one marker selected from Bestrophin and PAX6.
98. The method of any one of the preceding claims, wherein the RPE cells lack substantial expression of one or more stem cell markers selected from the group OCT4, NANOG, Rex- 1, alkaline phosphatase, SOX2, TDGF- 1, DPPA-2, DPPA-4, stage specific embryonic antigen (SSEA)-3 and SSEA-4, tumor rejection antigen (TRA)-1 -60 and TRA-1-80.
99. The method of any one of the preceding claims, wherein the RPE cells lack substantial expression of OCT4, SSEA4, TRA-1-81, and alkaline phosphatase.
100. The method of any one of claims 1-98, wherein the RPE cells lack substantial expression of OCT4, NANOG, and SOX2.
101. A composition comprising a population of RPE cells produced by the method of any one of the preceding claims.
102. A pharmaceutical composition comprising a population of RPE cells produced by the method of any one of claims 1-100 and a pharmaceutically acceptable carrier.

103. ~~Use of A method of treating a patient with or at risk of a retinal disease, the method comprising administering an effective amount of~~ the composition of claim 101 or the pharmaceutical composition of claim 102 in the manufacture of a medicament for the treatment of a patient with or at risk of a retinal disease, wherein an effective amount of the composition of claim 101 or the pharmaceutical composition of claim 102 is to be administered to the patient.

104. The ~~use~~method of claim 103, wherein the retinal disease is selected from the group retinal degeneration, choroideremia, diabetic retinopathy, age-related macular degeneration (dry or wet), retinal detachment, retinitis pigmentosa, Stargardt's Disease, Angioid streaks, Myopic Macular Degeneration, and glaucoma.

105. The composition of claim 101 or the pharmaceutical composition of claim 102 for use in therapy.