[0012] In the more severe wet form, newly formed blood vessels from the choroid infiltrate the space behind the macula; the newly formed blood vessels are fragile and often leak blood—thereby causing the death of photoreceptors and their supporting cells.

[0013] While diseases that cause damage to specific cells or tissues are clear candidates for cellular therapy, there remains a need in the art for improved methods of cellular therapy, which include methods, substrates, and tools to improve the efficacy of cellular therapy, as well as methods and compositions allowing for long term storage of functional and viable cells to be used in such therapies.

SUMMARY

[0014] In various examples, the present disclosure relates generally to methods and compositions for the cryopreservation of cells grown on a substrate. In particular, methods and compositions for the cryopreservation of cells seeded and/or grown on a polymeric substrate. In certain applications, the cells are retinal pigment epithelial (RPE) cells, photoreceptor cells, stem cells, pre-differentiated cells, differentiations of such cells, or a combination thereof, regardless of the differentiation, derivation or culture history of the cells.

[0015] To address the need for improved long term storage of cell-containing compositions for use in cell therapy, there is provided in some examples, a method of cryopreserving cells on a substrate comprising exposing a substrate seeded with a composition of cells to a temperature ramp-down phase having a desired temperature reduction rate, transferring the cell-seeded substrate to a desired intermediate temperature range for a first period of time, and maintaining the cell-seeded substrate at a desired storage temperature range for a second period of time, resulting in cryopreserved cells on a substrate that are suitable for long term storage and later use in cell therapy after thawing.

[0015a] In one aspect, the present disclosure refers to a method of cryopreserving cells on a substrate, the method comprising: providing a biocompatible polymer substrate seeded with a monolayer of immature retinal pigment epithelium (RPE) cells, the polymer substrate providing a cell seeding surface; identifying when i) the monolayer of immature RPE cells reaches between 90% and 99% confluence on the substrate and ii) most of the immature RPE cells are not fully pigmented; exposing, upon the identifying, the substrate seeded with cells

to a controlled temperature reduction rate between about -1 ℃ per minute to about -30 ℃ per minute until a first temperature below -20 ℃ is reached.

[0015b] In another aspect, the present disclosure refers to a method of generating an implantable cell seeded substrate , the method comprising: providing a biocompatible polymer substrate seeded with a monolayer of immature retinal pigment epithelium (RPE) cells, the polymer substrate providing a cell seeding surface; cryopreserving cells on the substrate by exposing the substrate seeded with cells to a controlled temperature reduction rate between about -1 C° per minute to about -30 C° per minute, transferring the cell seeded substrate to a temperature below 4 °C, thereby obtaining cryopreserved or hibernated cells; thawing the cryopreserved cells on said substrate by warming the cell seeded substrate to a target temperature using a temperature ramp-up heating rate to obtain thawed cells seeded on the substrate, wherein the thawed cells retain viability and/or functionality post-thaw; culturing the seeded cells for an additional period of time to reach a mature state prior to implantation.

[0016] The present disclosure provides, in various examples, specific cryopreservation methods and variations of procedural steps to accommodate a variety of cell characteristics. Additionally, specific cell characteristics and substrate characteristics are selected at various stages of the cryopreservation process to improve the cell viability and implant success of the cell seeded substrate for efficacious treatment.

[0017] The present disclosures introduce new methods and apparatus that provide many advantages including beneficial outcomes for cryopreservation of substrates including higher

cell survival, minimal damage to substrates, and an overall simplified process for generating cell seeded substrates suitable for cell growth and direct implantation.

[0018] In another example, the disclosure introduces methods of intentional suppression of melanogenesis. Such methods may be enabled by specific items included in a kit.

[0019] The present disclosure also introduces new methods of forming cell specific culture media to improve cell growth, cell growth rate, viability, and differentiation. In certain examples, such cell specific culture mediums may be extracted and further be adapted for direct injections such as intravitreal (IVT) delivery to a region of the specific cells or an already implanted cell seeded substrate.

[0020] A system of one or more computers can be configured to perform particular operations or actions by virtue of having software, firmware, hardware, or a combination of them installed on the system that in operation causes or cause the system to perform the actions. One or more computer programs can be configured to perform particular operations or actions by virtue of including instructions that, when executed by data processing apparatus, cause the apparatus to perform the actions. One general example includes a method of cryopreserving cells on a substrate, the method including: providing a biocompatible polymer substrate seeded with a monolayer of immature retinal pigment epithelium (rpe) cells, the polymer substrate providing a cell seeding surface. The method of cryopreserving cells also includes identifying when i) the monolayer of immature rpe cells reaches between 90% and 99% confluence on the substrate and ii) most of the immature rpe cells are not fully pigmented. The method of cryopreserving cells also includes exposing, upon the identifying, the substrate seeded with cells to a controlled temperature reduction rate between about -1 c per minute to about -30 c per minute until a first temperature below -20 c is reached; Other examples of this example include corresponding computer systems, apparatus, and computer programs recorded on one or more computer storage devices, each configured to perform the actions of the methods.

[0021] Implementations may include one or more of the following features. The method where the cell-seeded substrate reaches a temperature below that which delineates a latent heat release of the seeded cells. The method where a surface of the is substantially parallel to the monolayer of the immature rpe cells seeded on the substrate, sufficient to induce nucleation and efficient temperature compensation in response to the latent heat release of the seeded cells. The method further including: maintaining the cell-seeded substrate at the first

(bdnf), complement factor h (cfh), ciliary neurotrophic factor (cntf), fibulin 3/5, fibroblast growth factor (fgf) 2, heparin binding-epidermal growth factor (hb-egf), hepatocyte growth factor (hgf), insulin-like growth factor (igf)-i, leukemia inhibitory factor (lif), matrix metalloproteinase (mmp)-9, nerve growth factor (ngf), tropoelastin. The method the identifying includes determining gene expression of immature rpe cells of at least one or more of rpe65, rex1, eif2b2, serf2, ube2r2 to be below that of mature rpe cells. The method where the substrate includes thin regions configured to allow the cells to diffuse nutrients therethrough. Implementations of the described techniques may include hardware, a method or process, or computer software on a computer-accessible medium.

[0022] One general example includes a method of generating an implantable cell seeded substrate the method including: providing a biocompatible polymer substrate seeded with a monolayer of immature retinal pigment epithelium (rpe) cells, the polymer substrate providing a cell seeding surface. The method also includes cryopreserving cells on the substrate by exposing the substrate seeded with cells to a controlled temperature reduction rate between about -1 c per minute to about -30 c per minute. The method also includes transferring the cell seeded substrate to a temperature below 4 c, thereby obtaining cryopreserved or hibernated cells. The method also includes thawing the cryopreserved cells on said substrate by warming the cell seeded substrate to a target temperature using a temperature ramp-up heating rate to obtain thawed cells seeded on the substrate, where the thawed cells retain viability and/or functionality post-thaw. The method also includes culturing the seeded cells for an additional period of time to reach a mature state prior to implantation. Other examples of this example include corresponding computer systems, apparatus, and computer programs recorded on one or more computer storage devices, each configured to perform the actions of the methods.

[0023] Implementations may include one or more of the following features. The method further including: culturing the post-thaw substrate seeded cells in a first medium including a basal medium supplemented with a combination of including of at least one or more of bovine serum albumin (bsa), activin a, hepatocyte growth factor (fgf), insulin-like growth factor (igf) 1, dickkopf-related protein 1 (dkk1), and noggin. The method further including: culturing the post-thaw substrate seeded cells in a first medium including a basal medium supplemented by supernatant derived from previously cultured rpe cells and subsequently culturing the cells in a second medium including a basal medium supplemented with insulin-like growth factor (ifg) 1, dickkopf-related protein 1 (dkk1), and noggin. The method where

the supernatant in the first medium is derived from the culturing of immature rpe cells that includes rpe cells exhibiting optimal cryopreservation viability characteristics selected from: (i) non-pigmented or partially pigmented rpe cells, (ii) non-polarized or partially polarized rpe cells, (iii) rpe cells with or withouat least 50%t mature cobblestone morphology, (iv) rpe cells with gene expression levels below that of mature cells or lacking specific gene expression, and (v) rpe cells in a subconfluent or confluent monolayer configuration. The method where the supernatant in the first medium is derived from the culturing of rpe cells exhibiting mature characteristics selected from: (i) pigmented rpe cells, (ii) polarized rpe cells, (iii) rpe cells with mature cobblestone morphology, (iv) rpe cells with gene expression levels comparable to mature rpe cells, and (v) rpe cells in a confluent monolayer configuration. Implementations of the described techniques may include hardware, a method or process, or computer software on a computer-accessible medium.

[0024] One general example includes a method of producing a cell line specific culture medium or acellular therapy, the method including: culturing immature rpe cells on a growth support structure; the rpe cells exhibiting optimal cryopreservation viability characteristics selected from: (i) non-pigmented rpe cells, (ii) non-polarized or partially polarized rpe cells, (iii) rpe cells without mature cobblestone morphology, (iv) rpe cells with gene expression levels below that of mature cells or lacking specific gene expression, and (v) rpe cells in a subconfluent monolayer configuration. The method also includes collecting media containing secreted factors. The method also includes purifying the media to create the culture medium or acellular therapy. Other examples of this example include corresponding computer systems, apparatus, and computer programs recorded on one or more computer storage devices, each configured to perform the actions of the methods.

[0025] Implementations may include one or more of the following features. The method further including: culturing the rpe cells until the cells exhibit mature characteristics selected from: (i) pigmented rpe cells, (ii) polarized rpe cells, (iii) rpe cells with mature cobblestone morphology, (iv) rpe cells with gene expression levels similar to mature cells, and (v) rpe cells in a confluent monolayer configuration, prior to purifying the media. The method where purifying is accomplished by centrifugal filtration, fractionation, size exclusion chromatography, affinity chromatography, size-exclusion filtration, or precipitation. The method further including: packaging the supernatant media in a syringe. The method where the supernatant is supplemented by a drug vehicle or diluent. The method further including: culturing photoreceptor cells or photoreceptor progenitor cells. The method where the growth

support structure includes a substrate, a lattice structure, agar, or a hydrogel. The method where the cells are grown on, in, or encapsulated in a biocompatible substrate or container. Implementations of the described techniques may include hardware, a method or process, or computer software on a computer-accessible medium.

[0026] One general example includes the method for treating a disease or disorder, including: administering to a subject an effective amount of rpe cell specific culture medium or acellular therapy obtained by a method including:. The method also includes culturing rpe cells on a growth support structure, the rpe cells exhibiting mature characteristics as characterized by rpe65 level detection. The method also includes collecting media containing secreted factors. The method also includes purifying the media to create the culture medium or acellular therapy. Other examples of this example include corresponding computer systems, apparatus, and computer programs recorded on one or more computer storage devices, each configured to perform the actions of the methods.

[0027] Implementations may include one or more of the following features. The method further including: transplanting the rpe cells. The method further including: transplanting rpe cells seeded onto a substrate. The method further including: transplantating photoreceptor cells. The method further including: transplantating the rpe cells and photoreceptor cells onto a substrate. The method where purifying is accomplished by centrifugal filtration, fractionation, size exclusion chromatography, affinity chromatography, size-exclusion filtration, or precipitation. The method where the growth support structure may be a substrate, a lattice structure, agar, or a hydrogel. The method where the cells are grown on, in, or encapsulated in a biocompatible substrate or container. Implementations of the described techniques may include hardware, a method or process, or computer software on a computer-accessible medium.

[0028] One general example includes a substrate for cellular therapy to generate an external limiting membrane of the retinal anatomy including: a substrate with a normal state or flexible state curvature and size substantially similar to that of a diseased portion of functional external limiting membrane of a patient. The substrate also includes rpe cells and photoreceptor cells seeded on the substrate as independent individual layers which post-implantation mature to generate an external limiting membrane. Other examples of this example include corresponding computer systems, apparatus, and computer programs recorded

on one or more computer storage devices, each configured to perform the actions of the methods.

[0029] Implementations may include one or more of the following features. The substrate where the substrate is coated with media to attract the patient's precursor cells post-implantation to generate the external limiting membrane. The substrate further including a supernatant media manufactured by: culturing rpe cells on a growth support structure. The substrate may also include the rpe cells exhibiting mature characteristics as characterized by rpe65 level detection. The substrate may also include collecting media containing secreted factors. The substrate may also include purifying the media to create the culture medium or acellular therapy. Implementations of the described techniques may include hardware, a method or process, or computer software on a computer-accessible medium.

[0030] One general example includes a method of producing a cell specific culture medium including: culturing a first cell line on a selectively permeable substrate in a first culturing medium, where said first cell line processes the first culture medium and produces cell secretions. The method also includes collecting the cell secretions. The method also includes adding the cell secretions to a second culturing medium to form a cell specific culture medium. Other examples of this example include corresponding computer systems, apparatus, and computer programs recorded on one or more computer storage devices, each configured to perform the actions of the methods.

[0031] Implementations may include one or more of the following features. The method where the cell secretions include a combination of proteins, hormones, enzymes, byproducts and waste. The method where the cell secretions include apical secretion from rpe cells including at least one of: αb crystallin, hyaluronan, matrix metalloproteinase (mmp)-9, pigment epithelium-derived factor (pedf), transforming growth factor (tgf)-β, tissue inhibitors of metalloproteinases (timp)-i. The method where the cell secretions include basal secretion from rpe cells including at least one of: cystatin c, endothelin i, fibroblast growth factor (fgf) 5, vascular endothelial growth factor (vegf). The method where the cell secretions include non-polar specific secretion from rpe cells including at least one of: bdnf, cfh, cntf, fibulin 3/5, fgf 2, hb-egf, hgf, igf-i, lif, mmp-9, ngf, tropoelastin. The substrate induces polarity of seeded cells to allow for apical, basal, and non-polar specific secretions to be secreted. The cell specific culture medium formed is for controlled growth of non-mature rpe cells. The cell specific culture medium formed is for controlled maturation of non-mature rpe cells into

mature rpe cells. The cell specific culture medium formed is formulated for intravitreal (ivt) injection. Implementations of the described techniques may include hardware, a method or process, or computer software on a computer-accessible medium.

[0032] One general example includes a method of forming a therapeutic composition including: culturing a first cell line on a selectively permeable substrate in a first culturing medium, where said first cell line processes the first culture medium and produces cell secretions. The method also includes collecting cell secretions. The method also includes purifying cell secretions by selecting specific cell secretions. Other examples of this example include corresponding computer systems, apparatus, and computer programs recorded on one or more computer storage devices, each configured to perform the actions of the methods.

[0033] Implementations may include one or more of the following features. The method further including: centrifuging one or more times at different speeds and reconstituting to create one or more pellets of varying compositions. The method further including fractionation, size exclusion chromatography, affinity chromatography, size-exclusion filtration, or precipitation. The method further including: reconstituting into different concentrations. The method where the formed therapeutic composition does not contain cells. Implementations of the described techniques may include hardware, a method or process, or computer software on a computer-accessible medium.

[0034] One general example includes a method of reversibly adhering a polymeric membrane onto another polymeric surface, including: placing the a membrane substantially surrounded by a volume of media on the another polymeric surface. The method also includes incrementally withdrawing a volume of media until the membrane's flat surface contacts a flat surface of another polymeric surface. The method also includes drying to reversibly adhere the membrane's flat surface to the contacting flat surface of another polymeric surface. Other examples of this example include corresponding computer systems, apparatus, and computer programs recorded on one or more computer storage devices, each configured to perform the actions of the methods.

[0035] Implementations may include one or more of the following features. The method where the polymeric surface is the bottom surface of a cell culture plate or well. The method where the media is Dulbecco's phosphate-buffered saline (dpbs). The method where the drying is conducted by a desiccator, temperature/humidity controlled chamber, baking in a

WHAT IS CLAIMED IS:

1. A method of cryopreserving cells on a substrate, the method comprising:

providing a biocompatible polymer substrate seeded with a monolayer of immature retinal pigment epithelium (RPE) cells, the polymer substrate providing a cell seeding surface;

identifying when i) the monolayer of immature RPE cells reaches between 90% and 99% confluence on the substrate and ii) most of the immature RPE cells are not fully pigmented;

exposing, upon the identifying, the substrate seeded with cells to a controlled temperature reduction rate between about -1 °C per minute to about -30 °C per minute until a first temperature below -20 °C is reached;

2. The method of claim 1, wherein the cell-seeded substrate reaches a temperature below that which delineates a latent heat release of the seeded cells.

3. The method of claim 2, wherein a surface of the is substantially parallel to the monolayer of the immature RPE cells seeded on the substrate, sufficient to induce nucleation and efficient temperature compensation in response to the latent heat release of the seeded cells.

4. The method of claim 1, further comprising:

maintaining the cell-seeded substrate at the first temperature, the first temperature being between -20 °C to about -100 °C after the controlled temperature reduction rate for a first period of time to obtain uniformity of temperature; and

maintaining the cells at a storage temperature lower than the first temperature for a second period of time within 50°C of the first temperature, thereby obtaining cryopreserved cells.

5. The method of claim 1, further comprising:

maintaining the cell-seeded substrate at the first temperature, the first temperature being between -20 °C to about -100 °C after the controlled temperature reduction rate for a first period of time; and

conducting a second controlled temperature reduction rate to finally maintain the cells at a storage temperature below -196 ℃ for a second period of time, thereby obtaining cryopreserved cells.

6. The method of claim 5, wherein the second period of time is between 24 hours and 60 months.

7. The method of claim 5, wherein the second controlled temperature reduction rate is between about -1 °C per minute to about -30 °C per minute.

8. The method of claim 1, wherein the monolayer of cells have a cell seeding density between 200,000 and 700,000 cells per milliliter of cell suspension, or between 100,000 and 350,000 cells per square centimeter of substrate surface.

9. The method of claim 1, wherein over 50% of the RPE cells have a cobblestone morphology.

10. The method of claim 1, wherein the RPE cells have no pigmentation or partial pigmentation.

11. The method of claim 1, wherein the cells are RPE cells exhibiting cryopreservation viability characteristics selected from: (i) non-pigmented or partially pigmented RPE cells, (ii) adhered but non-polarized, partially polarized, or fully polarized RPE cells, (iii) RPE cells with or without mature cobblestone morphology, (iv) RPE cells with gene expression levels below that of mature cells or lacking specific gene expression, and (v) RPE cells in a confluent or subconfluent over 90% monolayer configuration.

12. The method of claim 1, wherein the substrate has one or more characteristics selected from: (i) a coefficient of thermal expansion of the substrate, (ii) a substrate elasticity parameter, (iii) a substrate thickness, (iv) surface modification, (v) shear force resistance, said characteristics helping to enhance viability of the seeded cells and functionality of the substrate during cryopreservation and thawing.

13. The method of claim 1, wherein the biocompatible polymer comprises parylene.

14. The method of claim 1, wherein the identifying includes determining when at least one or more apical secretions, basal secretions, or non-polar specific secretions of the immature RPE cells at levels below that of mature RPE cells.

15. The method of claim 14, wherein the apical secretions comprise αB crystallin, hyaluronan, matrix metallopeptidase (MMP)-9, pigment epithelium-derived factor (PEDF), transforming growth factor (TGF)-β, tissue inhibitors of metalloproteinases (TIMP)-I, or mechano growth factor (MGF)-E8.

16. The method of claim 14, wherein the basal secretions comprise cystatin C, endothelin I, fibroblast growth factor (FGF) 5, or vascular endothelial growth factor (VEGF).

17. The method of claim 14, wherein the non-polar specific secretions comprise brain-derived neurotrophic factor (BDNF), complement factor H (CFH), ciliary neurotrophic factor (CNTF), fibulin 3/5, fibroblast growth factor (FGF) 2, heparin binding-epidermal growth factor (HB-EGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF)-I, leukemia inhibitory factor (LIF), matrix metalloproteinase (MMP)-9, nerve growth factor (NGF), Tropoelastin.

18. The method of claim 1, the identifying includes determining gene expression of immature RPE cells of at least one or more of RPE65, REX1, EIF2B2, SERF2, UBE2R2 to be below that of mature RPE cells.

19. The method of claim 1, wherein the substrate comprises thin regions configured to allow the cells to diffuse nutrients therethrough.

20. A method of generating an implantable cell seeded substrate , the method comprising:

providing a biocompatible polymer substrate seeded with a monolayer of immature retinal pigment epithelium (RPE) cells, the polymer substrate providing a cell seeding surface;

cryopreserving cells on the substrate by exposing the substrate seeded with cells to a controlled temperature reduction rate between about -1 C° per minute to about -30 C° per minute,

transferring the cell seeded substrate to a temperature below 4 °C, thereby obtaining cryopreserved or hibernated cells;

thawing the cryopreserved cells on said substrate by warming the cell seeded substrate to a target temperature using a temperature ramp-up heating rate to obtain thawed cells seeded on the substrate, wherein the thawed cells retain viability and/or functionality post-thaw;

culturing the seeded cells for an additional period of time to reach a mature state prior to implantation.