

CORNEAL ENDOTHELIAL CELL (CEC) CULTURING METHODS AND COMPOSITIONS**RELATED APPLICATIONS**

This application claims priority to U.S. Provisional Appln. No. 63/616,300, filed on
5 December 29, 2023, the contents of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

In humans, the cornea is comprised of five layers, in order from the outside (body
surface), of corneal epithelium, Bowman's membrane (external boundary), Lamina propria,
10 Descemet's membrane (internal boundary), and corneal endothelium. Unless specifically noted
otherwise, parts other than epithelium and endothelium may be collectively called corneal
stroma. The corneal endothelium is composed of a single layer of cells located on the posterior
surface of the cornea, facing the anterior chamber. The endothelium governs fluid and nutrient
transport across the posterior surface of the cornea in a pump-and-barrier function and
15 maintains the cornea in an optimized state required for optical transparency and optimal vision.

Unlike the epithelium, which has a self-renewing capacity, the endothelium is not
capable of regenerating. Corneal endothelial disease is a sight-threatening and debilitating
condition affecting millions of people throughout the world. Corneal transplants can be used to
treat corneal disease, but often have variable results, as well as being limited by the number of
20 available donors in comparison to the number of patients in need. Injectable cell therapies to
treat corneal endothelial disease have recently been developed. In clinical studies, injected
corneal endothelial cells (CECs) have been used to replenish endothelial cells to treat patients
having Fuchs' endothelial corneal dystrophy, pseudophakic bullous keratopathy,
pseudoexfoliation syndrome keratopathy and corneal graft failure (Kinoshita S, Koizumi N, Ueno
25 M, et al. *N Engl. J Med.* 2018;378(11):995-1003).

Human donor corneas are used to obtain CECs suitable for injection into a patient.
CECs are first isolated from donor cornea and subsequently cultured and expanded in media. In
culture, the CECs should maintain their morphology such that they will act as a therapeutic upon
delivery. The culturing process also should increase number of CECs such that there are
30 enough cells for delivery. A study by Peh et al. (2011) *PLoS ONE* 6(12): e28310, found that
certain cultured human CECs lost their characteristic morphology at the third passage of the
cells. Thus, identifying a process by which donor cornea can be used to cultivate and expand

CECs in a manner where cell number is increased and CEC morphology is maintained is a challenge.

SUMMARY OF THE INVENTION

5 Robust proliferation and expansion of cultured human corneal endothelial cells (CECs) is important for CEC therapy. This disclosure provides human corneal endothelial cell (CEC) culturing methods that increase the number of cells that are expanded, e.g., from a single cornea. The compositions and methods disclosed herein provide a greater number of cells per donor cornea that can be used in CEC therapies to treat corneal endothelial diseases.

10 Provided herein is a method of culturing human corneal endothelial cells (CECs), comprising culturing human corneal endothelial cells (CECs) in an initial passage 0 (P0); and expanding the human CECs through at least four additional passages comprising passage 1 (P1), passage 2 (P2), passage 3 (P3), and passage 4 (P4), wherein both P3 and P4 have time periods that are shorter than each of P0, P1, and P2; and wherein at least 1×10^7 cells are obtained by the end of P4. Further, the CECs in each passage are cultured in a culture vessel.

In certain embodiments, at least 1×10^8 cells are obtained by the end of P4.

In other embodiments, at least 1×10^9 cells are obtained by the end of P4.

20 In further embodiments, the methods disclosed herein further comprise expanding the CECs through passage 5 (P5). In some embodiments, P5 is shorter in duration than each of P0, P1, and P2. In some embodiments, at least 1×10^8 cells are obtained by the end of P5. In other embodiments, at least 1×10^9 cells are obtained by the end of P5. In still other embodiments, at least 1×10^{10} cells are obtained by the end of P5.

25 In a further embodiment, the methods disclosed herein comprise culturing the CECs at P0 for at least 35 days, e.g., for 35 to 80 days.

In another embodiment, the methods disclosed herein comprise expanding the CECs at P1 for at least 30 days, e.g., for 30-80 days.

In one embodiment, the methods disclosed herein comprise expanding the CECs at P2 for at least 30 days, e.g., for 30-80 days.

30 In still other embodiments, the methods disclosed herein comprise expanding the CECs at P3 for at least 20 days, e.g., for 20 to 45 days or for 20 to 30 days.

In a further embodiment, the methods disclosed herein comprise expanding the CECs for at least 20 days, e.g., for 20 to 30 days.

In a further embodiment, the methods disclosed herein comprise culturing corneal endothelial cells (CECs) at passage 0 (P0) for 30-45 days; expanding the CECs at passage 1 (P1) for 30-45 days; expanding the CECs at passage 2 (P2) for 30-45 days; expanding the CECs at passage 3 (P3) for 20-30 days; and expanding the CECs at passage 4 (P4) for 20-30 days.

In some embodiments, the culture vessel of the CECs in P3 and/or P4 is a stacked cell culture chamber comprising two or more layers, e.g., 2 to 10 layers.

In some embodiments, the culture vessel has a medium volume of 130 to 8000 mL, e.g., a medium volume of 130 to 2000 mL.

In some embodiments, the culture vessel has a cell growth area of about 600 to about 6400 cm², e.g., a cell growth area of about 1200 to about 6400 cm².

Also provided herein is a method of culturing corneal endothelial cells, the method comprising culturing corneal endothelial cells (CECs) at passage 0 (P0) for at least 30 days; expanding the CECs at passage 1 (P1) for at least 30 days; expanding the CECs at passage 2 (P2) for at least 30 days; expanding the CECs at passage 3 (P3) for less than 30 days; and expanding the CECs at passage 4 (P4) for less than 30 days.

In certain embodiments, at least 1×10^7 cells are obtained by the end of P4. In other embodiments, at least 1×10^8 cells are obtained by the end of P4. In still other embodiments, at least 1×10^9 cells are obtained by the end of P4.

In certain embodiments, the method further comprises expanding the CECs at passage 5 (P5) for less than 30 days.

In some embodiments, at least 1×10^8 cells are obtained by the end of P5. In other embodiments, at least 1×10^9 cells are obtained by the end of P5. In yet other embodiments, at least 1×10^{10} cells are obtained by the end of P5.

In some embodiments, each of the passages is performed at the same temperature.

In some embodiments, each passage is performed at a temperature of at least about 31°C, e.g., at a temperature of about 37°C or at a temperature of about 31°C to 41°C.

In one embodiment, the CECs are derived from a single donor.

In one embodiment, any one of P0, P1, P2, P3, or P4 are performed in a cell culture medium supplemented with ascorbic acid, fetal bovine serum, chondroitin sulfate, calcium chloride, and/or a Rho kinase inhibitor. For example, the Rho kinase inhibitor is Y-27632.

In certain embodiments, at least 70% of the CECs are viable at the end of P4 as determined by a cell viability assay. In some embodiments, at least 90% of the CECs are viable at the end of P4 as determined by a cell viability assay.

5 In some embodiments, at least 70%, optionally at least 75%, of the CECs have a cell surface expression at the end of P4 of a marker selected from the group consisting of CD166 positive, CD44 negative to CD44 weakly positive, CD24 negative to weakly positive, CD44 negative to weakly positive, CD105 negative to weakly positive, CD26 negative to weakly positive, CD200 negative to weakly positive, and CD90 negative to weakly positive phenotypes.

10 In some embodiments, at least 70%, optionally at least 75%, of the CECs have a cell surface expression at the end of P4 of a marker selected from the group consisting of sodium-potassium ATPase, ZO-1, VDAC3, SLC4A4, CLCN3, COL4A2, COL8A1, COL8A2, CDH2, CD98, CD166, CD340, Integrin $\alpha\beta 1$, CD56, Prdx-6, CD248, SLC4A11, and CYYR1.

15 In some embodiments, at the end of P4 at least 70% of the CECs are viable as determined by a cell viability assay; at least 75% of the CECs have a cell surface expression at the end of P4 of a marker selected from the group consisting of CD166 positive, CD44 negative to CD44 weakly positive, CD24 negative to weakly positive, CD44 negative to weakly positive, CD105 negative to weakly positive, CD26 negative to weakly positive, CD200 negative to weakly positive, and CD90 negative to weakly positive phenotypes; and at least 75% of the CECs have a cell surface expression at the end of P4 of a marker selected from the group
20 consisting of sodium-potassium ATPase, ZO-1, VDAC3, SLC4A4, CLCN3, COL4A2, COL8A1, COL8A2, CDH2, CD98, CD166, CD340, Integrin $\alpha\beta 1$, CD56, Prdx-6, CD248, SLC4A11, and CYYR1.

In some embodiments, at least 70% of the CECs at the end of P4 have a hexagonal morphology as detected by microscopy.

25 In one embodiment, the cells are grown on a cell culture surface comprising an extracellular matrix.

In another embodiment, the cells are grown on a cell culture surface comprising an extracellular matrix protein. In certain embodiments, the extracellular matrix protein is collagen, laminin, fibronectin, proteoglycan, SWI/SNF-related matrix-associated actin-dependent regulator
30 of chromatin subfamily A, SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1, insulin-like growth factor-binding protein 5, vitronectin, fibrillin-1, fibrillin-2, tensin, Wnt-5b, citron Rho-interacting kinase, chondroitin sulphate proteoglycan 4, cyclin-dependent kinase 1, cyclin-dependent kinase 4, periostin, thrombospondin-4, Tubulin

alpha chain-like 3, Tubulin alpha-1B chain, Tubulin beta-1 chain, Tubulin beta-4A chain, or versican core protein.

In one embodiment, the size of the culture vessel is increased with each passage.

In another embodiment, the culture vessel is tissue-culture (TC)-treated.

5 In some embodiments, the CEC are cultured under conditions for use in a therapeutic composition for use in humans.

Also included herein is a corneal endothelial cell (CEC) composition comprising CECs prepared by any method disclosed herein.

Further included herein is a pharmaceutical composition comprising the CEC
10 composition comprising CECs prepared by any method disclosed herein.

The disclosure also includes a tissue-culture treated (TC-treated) cell culture vessel comprising corneal endothelial cells (CECs).

BRIEF DESCRIPTION OF THE DRAWINGS

15 **Fig. 1** depicts a schematic of the cell culture and expansion process described in Example 1. **Fig. 1** shows a series of containers used for each passage in the expansion process described in Example 1.

Fig. 2 depicts the results of an experiment evaluating the number of doses after different expansion. **Fig. 2** shows the number of doses as a function of days for each assessed
20 expansion run until the end of passage 4 (P4).

DETAILED DESCRIPTION

I. Definitions

25 As used herein, the term “corneal endothelial cell” or “CEC” refers to a cell from a corneal endothelium layer. Preferably, CECs are human CECs. Included in the term are CECs obtained from a donor corneal endothelium layer.

As used herein, the term “corneal endothelial disease” refers to diseases affecting corneal endothelial cells. Non-limiting examples of corneal endothelial diseases include bullous
30 keratopathy, corneal endothelial dystrophies (e.g., cornea guttata, Fuchs endothelial corneal dystrophy, posterior polymorphous corneal dystrophy, and congenital hereditary corneal endothelial dystrophy), iridocorneal endothelial syndrome, viral diseases (e.g., cytomegalovirus endotheliitis and herpetic endotheliitis), exfoliation syndrome, and corneal endothelial graft

rejection; as well as inflammation or physical damage associated with external factors, such as keratouveitis, interstitial keratitis, corneal endotheliitis, corneal endothelial cell loss after corneal transplantation, corneal injury after intraocular surgery (e.g., cataract surgery, vitreous surgery, glaucoma surgery), corneal injury induced by glaucomatous attack, corneal injury caused by long-term contact lens use, corneal trauma, corneal edema, and intrapartum corneal trauma.

As used herein, the term “expanding” refers to a cell culture process that includes transferring (i.e., passaging) cells into a new culture vessel at a lower cell density in order to permit further growth of the cells.

As used herein, the term “passage”, as used herein in the context of cell culture, refers to the removal of medium and transfer of cells from a previous culture into fresh medium. Passaging allows for a lower cell density that can stimulate further propagation. Passaging of cells is also referred to as subculturing. When used in reference to a number, a passage number, e.g., P1, indicates how many times the cell population has been passaged. P0 indicates an initial cell culture.

II. Methods of Culturing Corneal Endothelial Cells (CECs)

A cornea is one of the lamellar tissues constituting an eye. In humans, the cornea is composed of five layers, corneal epithelium, Bowman's membrane (external boundary), Lamina propria, Descemet's membrane (internal boundary), and corneal endothelium, in order from the outside (body surface). The corneal endothelium is a single layer of cells that covers the posterior cornea. Markers for characterizing CECs and methods of identifying CECs are known in the art (See e.g., Hamuro J, *et al. Invest Ophthalmol Vis Sci.* 2016 Aug 1;57(10):4385-92; Wongvisavavit, R., *et al* (2021). *Regenerative medicine*, 16(09), 871-891).

Robust proliferation and expansion of cultured human CECs is important for CEC therapy. The compositions and methods disclosed herein provide a greater number of cells per donor cornea that can be used in CEC therapy to treat corneal endothelial diseases.

In one embodiment, provided herein are culturing methods that increase the number of human corneal endothelial cells (CECs) that are expanded from a single cornea. In one aspect, provided herein is a method of culturing human corneal endothelial cells (CECs). The method includes culturing human CECs in an initial culture (Passage 0; P0) followed by at least four additional passages (e.g., passage 1 (P1), passage 2 (P2), passage 3 (P3), and passage 4 (P4)). In certain embodiments, the method provides enough CECs that a thousand or more doses of CECs can be used for CEC-based therapies.

Collection of Corneal Endothelial Cells and Culture Thereof in a Culture Vessel In Vitro

Corneal endothelial cells (e.g., human CECs) may be collected by any conventional methods known in the art from the cornea of a suitable corneal donor. In brief, the CECs may be isolated by stripping Descemet's membrane, followed by enzyme treatment to remove the collagen matrix. These cells may undergo further analysis to confirm their biological characteristics and to verify criteria for therapeutic use. Markers for characterizing CECs and methods of identifying CECs are known in the art (See, e.g., Hamuro J, Ueno M, Toda M, Sotozono C, Montoya M, Kinoshita S. *Invest Ophthalmol Vis Sci*. 2016 Aug 1;57(10):4385-92; and Wongvisavavit, R., *et al* (2021). *Regenerative medicine*, 16(09), 871-891).

In some embodiments, the CECs are from a human CEC primary cell line. Homogeneous corneal endothelial cells may be prepared using methods known in the art. For example, the Descemet's membrane and the endothelial cell layer of a corneal tissue may be detached from the corneal stroma, transferred into a culture vessel (e.g., a culture dish), and treated with an enzyme, such as collagenase A. In some embodiments, the CECs with Descemet's membrane and the endothelial cell layer are digested in a basal growth medium (e.g., OPTI-MEM® I Reduced Serum Media (Thermo Fisher Scientific, Inc., e.g., free of ammonium meta vanadate combined with fetal bovine serum (e.g., 8%)), which may be supplemented with additional components such as calcium chloride (e.g., 200 mg/L), chondroitin sulfate (e.g., 0.08%), and/or an antibiotic (e.g., gentamicin). The CECs may be digested at 37°C for two to 24 hours. As a result, the corneal endothelial cells are detached from the Descemet's membrane. The corneal endothelial cells remaining in the Descemet's membrane can be further detached by mechanical methods, such as pipetting. This step may additionally include one or more washing steps (e.g., using the basal growth medium without an enzyme).

After removal of the Descemet's membrane, the corneal endothelial cells may then be cultivated in a suitable culture medium that permits growth of CECs (e.g., in an initial culture at passage 0). For example, in some embodiments, the CECs are cultured in a basal growth medium (e.g., OPTI-MEM® I Reduced Serum Media (Thermo Fisher Scientific, Inc., e.g., free of ammonium meta vanadate and combined with fetal bovine serum (e.g., 8%)). In some embodiments, the basal growth medium is further supplemented with an epidermal growth factor (EGF) and/or ascorbic acid (e.g., 20 µg/mL). In one embodiment, the basal growth medium further comprises a Rho-associated protein kinase (ROCK)-inhibitor, such as Y-27632. As a further example, commercially available DMEM (Dulbecco's Modified Eagle's Medium)

appropriately supplemented with FBS (fetal bovine serum), b-FGF (basic-fibroblast growth factor) and antibiotics such as penicillin, streptomycin and the like can be used.

5 In two-dimensional culture systems, adherent cells are grown in a monolayer system on a flat surface, e.g. in a culture dish, plate, or flask. In some embodiments, the culture vessel has a surface coated with Type I collagen, Type IV collagen, fibronectin, laminin or an extracellular matrix of bovine corneal endothelial cells and the like. Alternatively, a conventional culture vessel treated with a commercially available coating agent, such as an FNC coating mix may be used.

10 The temperature for cultivating corneal endothelial cells is not limited as long as the cells proliferate. For example, in some embodiments, the cells are cultured at a temperature of about 31°C to about 41°C (e.g., about 32°C to about 41°C, about 35°C to about 41°C). In certain embodiments, the cells are cultured at a temperature of at least about 31° C (e.g., about 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C). In certain embodiments, the cells are cultured at a temperature of about 37° C. In certain embodiments, the cells are
15 cultured at a temperature of about 32° C. In certain embodiments, the cells are cultured at a temperature of about 41° C. The cells may be incubated in a conventional incubator for cell culture under humidification in an environment of about 5-10% CO₂.

Passaging

20 Following the initial culture (passage 0), the cultured corneal endothelial cells (CECs) are passaged into fresh growth medium. The methods herein provide a subculturing method that increases the number of cells that may be expanded from a single donor (e.g., a human corneal donor).

The passage or subculture may include one or more of the following steps. First, spent
25 media may be removed and the cells may be detached from the surface of the culture vessel (e.g., by a treatment with trypsin-EDTA, TrypLE enzyme, by scraping, and/or by shaking etc.) and recovered. At the time the cells are detached, the cells may be sub-confluent or confluent. A culture medium may then be added to the recovered cells to generate a cell suspension. In some embodiments, centrifugation may be performed during or after recovery of the cells (e.g.,
30 500 rpm (30 G)-1000 rpm (70 G), 1 to 10 minutes). Such centrifugal treatment generates a cell suspension with a high cell density.

The cell suspension may then be seeded and cultured in a culture vessel. The dilution ratio during passage may vary depending on the condition of the cells. In some embodiments,

the dilution ratio during passage is about 1:2-1:4. In some embodiments, the dilution ratio is about 1:3. The culture time may vary depending on the condition of the cells to be used.

The passages after the initial culture (P0) are alternatively referred to herein as Passage 1 (P1), Passage 2 (P2), Passage (P3), Passage (P4), and so on. In some embodiments, the cultured CECs are passaged at least four times (e.g., prior to collecting the CECs for incorporation into an injectable therapeutic composition). In certain embodiments, the cultured CECs are passaged at least five times (e.g., prior to collecting the CECs for incorporation into an injectable therapeutic composition). For example, in certain embodiments, the method involves initially culturing the cell (P0) and expanding the cells via four additional passages (e.g., P1, P2, P3, and P4. In some embodiments, the method involves initially culturing the cell (P0) and expanding the cells via five additional passages (e.g., P1, P2, P3, P4, and P5). In some embodiments, the method involves initially culturing the cell (P0) and expanding the cells via more than five additional passages (e.g., P1, P2, P3, P4, P5, P6, or more).

As described in the Examples, the passages must be of an appropriate length to achieve a high number of viable CECs by later passages. For instance, in some embodiments, P0, P1, or P2 are longer in duration than later passages (e.g., P3, P4 and/or P5). In some embodiments, P3, P4, and/or P5 are shorter in duration than earlier passages (e.g., P0, P1, and P2). In certain embodiments, P3 is shorter in duration than each of P0, P1, and P2. In certain embodiments, P4 is shorter in duration than each of P0, P1, and P2. In certain embodiments, P5 is shorter in duration than each of P0, P1, and P2. In certain embodiments, P3 and P4 are shorter in duration than each of P0, P1, and P2. In certain embodiments, P4 and P5 are shorter in duration than each of P0, P1, and P2. In certain embodiments, P3, P4, and P5 are shorter in duration than each of P0, P1, and P2.

In some embodiments, the method involves culturing the CECs at P0 for at least 25 days (e.g., at least 25 days, at least 26 days, at least 27 days, at least 28 days, at least 29 days, at least 30 days, at least 31 days, at least 32 days, at least 33 days, at least 34 days, at least 35 days, at least 36 days, at least 37 days, at least 38 days, at least 39 days, at least 40 days, at least 41 days, at least 42 days, at least 43 days, at least 44 days, at least 45 days, and so forth). In certain embodiments, the method involves culturing the CECs at P0 for at least 35 days.

In some embodiments, the CECs are cultured at P0 for 25 to 80 days (e.g., 25 to 80 days, 25 to 70 days, 25 to 60 days, 25 to 50 days, 25 to 40 days, 25 to 35 days, 30 to 70 days, 30 to 60 days, 30 to 50 days, 30 to 45 days, 30 to 40 days, 30 to 35 days, 35 to 80 days, 35 to

70 days, 35 to 60 days, 35 to 50 days, 35 to 45 days, 40 to 80 days, 40 to 70 days, 40 to 60 days, 40 to 50 days). In certain embodiments, the CECs are cultured at P0 for 35 to 45 days.

In some embodiments, the method involves culturing the CECs at P1 for at least 25 days (e.g., at least 25 days, at least 26 days, at least 27 days, at least 28 days, at least 29 days, at least 30 days, at least 31 days, at least 32 days, at least 33 days, at least 34 days, at least 35 days, at least 36 days, at least 37 days, at least 38 days, at least 39 days, at least 40 days, at least 41 days, at least 42 days, at least 43 days, at least 44 days, at least 45 days, and so forth). In certain embodiments, the method involves culturing the CECs at P1 for at least 35 days.

In some embodiments, the CECs are cultured at P1 for 25 to 80 days (e.g., 25 to 80 days, 25 to 70 days, 25 to 60 days, 25 to 50 days, 25 to 40 days, 25 to 35 days, 30 to 70 days, 30 to 60 days, 30 to 50 days, 30 to 45 days, 30 to 40 days, 30 to 35 days, 35 to 80 days, 35 to 70 days, 35 to 60 days, 35 to 50 days, 35 to 45 days, 40 to 80 days, 40 to 70 days, 40 to 60 days, 40 to 50 days). In certain embodiments, the CECs are expanded at P1 for 30 to 45 days.

In some embodiments, the method involves culturing the CECs at P2 for at least 25 days (e.g., at least 25 days, at least 26 days, at least 27 days, at least 28 days, at least 29 days, at least 30 days, at least 31 days, at least 32 days, at least 33 days, at least 34 days, at least 35 days, at least 36 days, at least 37 days, at least 38 days, at least 39 days, at least 40 days, at least 41 days, at least 42 days, at least 43 days, at least 44 days, at least 45 days, and so forth). In certain embodiments, the method involves culturing the CECs at P2 for at least 35 days.

In some embodiments, the CECs are cultured at P2 for 25 to 80 days (e.g., 25 to 80 days, 25 to 70 days, 25 to 60 days, 25 to 50 days, 25 to 40 days, 25 to 35 days, 30 to 70 days, 30 to 60 days, 30 to 50 days, 30 to 45 days, 30 to 40 days, 30 to 35 days, 35 to 80 days, 35 to 70 days, 35 to 60 days, 35 to 50 days, 35 to 45 days, 40 to 80 days, 40 to 70 days, 40 to 60 days, 40 to 50 days). In certain embodiments, the CECs are expanded at P2 for 30 to 45 days.

In some embodiments, the method involves expanding the CECs at P3 for at least 20 days (e.g., at least 20 days, at least 21 days, at least 22 days, at least 23 days, at least 24 days, at least 25 days, at least 26 days, at least 27 days, at least 28 days, at least 29 days, at least 30 days, at least 31 days, at least 32 days, at least 33 days, at least 34 days, at least 35 days, at least 36 days, at least 37 days, at least 38 days, at least 39 days, at least 40 days, at least 41 days, at least 42 days, at least 43 days, at least 44 days, at least 45 days, and so forth).

In some embodiments, the CECs are expanded at P3 for 20 to 45 days (e.g., 20 to 45 days, 20 to 40 days, 20 to 35 days, 20 to 30 days, 20 to 25 days, 25 to 45 days, 25 to 40 days,

25 to 35 days, 35 to 30 days, 30 to 45 days, 30 to 44 days, 30 to 42 days, 30 to 40 days, 30 to 38 days, 30 to 36 days, 30 to 34 days, 30 to 32 days, 32 to 44 days, 32 to 42 days, 32 to 40 days, 32 to 38 days, 32 to 36 days, 32 to 34 days, 35 to 45 days, 35 to 44 days, 35 to 42 days, 35 to 40 days, 35 to 38 days, 36 to 45 days, 36 to 44 days, 36 to 42 days, 36 to 40 days, 36 to 38 days, 38 to 45 days, 38 to 44 days, 38 to 42 days, 38 to 40 days, 40 to 45 days, 40 to 44 days, 40 to 42 days, 42 to 45 days).

In some embodiments, the CECs are expanded at P3 for 20 to 30 days (e.g., 20 to 30 days, 20 to 28 days, 20 to 26 days, 20 to 24 days, 20 to 22 days, 22 to 30 days, 22 to 28 days, 22 to 26 days, 22 to 24 days, 24 to 30 days, 24 to 28 days, 24 to 26 days, 25 to 30 days, 25 to 28 days, 26 to 30 days, 26 to 28 days). In certain embodiments, the CECs are expanded at P3 for 21 to 45 days. In some embodiments, the CECs are expanded at P3 for 20 to 30 days.

In some embodiments, the CECs are expanded at P3 for less than 31 days (e.g., less than 30 days, less than 29 days, less than 28 days, less than 27 days, less than 26 days, less than 25 days, less than 24 days, less than 23 days, less than 22 days, less than 21 days). In certain embodiments, the CECs are expanded at P3 for 20 to 30 days.

In some embodiments, the method involves expanding the CECs at P4 for at least 20 days (e.g., at least 20 days, at least 21 days, at least 22 days, at least 23 days, at least 24 days, at least 25 days, at least 26 days, at least 27 days, at least 28 days, at least 29 days, at least 30 days). In some embodiments, the CECs are expanded at P4 for 20 to 30 days (e.g., 20 to 30 days, 20 to 28 days, 20 to 26 days, 20 to 24 days, 20 to 22 days, 22 to 30 days, 22 to 28 days, 22 to 26 days, 22 to 24 days, 24 to 30 days, 24 to 28 days, 24 to 26 days, 25 to 30 days, 25 to 28 days, 26 to 30 days, 26 to 28 days). In certain embodiments, the CECs are expanded at P3 for 20 to 30 days.

In some embodiments, the CECs are expanded at P4 for less than 31 days (e.g., less than 30 days, less than 29 days, less than 28 days, less than 27 days, less than 26 days, less than 25 days, less than 24 days, less than 23 days, less than 22 days, less than 21 days).

In some embodiments, the method includes expanding the CECs through passage 5 (P5). In some embodiments, P5 is shorter in duration than each of P0, P1, and P2. In some embodiments, the method involves expanding the CECs at P5 for at least 20 days (e.g., at least 20 days, at least 21 days, at least 22 days, at least 23 days, at least 24 days, at least 25 days, at least 26 days, at least 27 days, at least 28 days, at least 29 days, at least 30 days). In some embodiments, the CECs are expanded at P5 for 20 to 30 days (e.g., 20 to 30 days, 20 to 28 days, 20 to 26 days, 20 to 24 days, 20 to 22 days, 22 to 30 days, 22 to 28 days, 22 to 26 days,

22 to 24 days, 24 to 30 days, 24 to 28 days, 24 to 26 days, 25 to 30 days, 25 to 28 days, 26 to 30 days, 26 to 28 days). In certain embodiments, the CECs are expanded at P5 for 20 to 30 days.

5 In some embodiments, the method includes expanding the CECs through one or more additional passages beyond P5 (e.g., P6, P7, and so forth)

In some embodiments, the CECs are expanded at P5 (or later passages) for less than 31 days (e.g., less than 30 days, less than 29 days, less than 28 days, less than 27 days, less than 26 days, less than 25 days, less than 24 days, less than 23 days, less than 22 days, less than 21 days).

10 In a further aspect, provided herein is a method of culturing corneal endothelial cells, the method involving culturing corneal endothelial cells (CECs) at passage 0 (P0) for at least 35 days; expanding the CECs at passage 1 (P1) for at least 30 days; expanding the CECs at passage 2 (P2) for at least 30 days; expanding the CECs at passage 3 (P3) for less than 30 days (e.g., 20-30 days); and expanding the CECs at passage 4 (P4) for less than 30 days (e.g.,
15 20-30 days). The method may further involve expanding the cells at passage (P5) for less than 30 days (e.g., 20-30 days). In some embodiments, each of the passages is performed at a temperature of about 37°C.

In a further aspect, provided herein is a method of culturing corneal endothelial cells, the method involving culturing corneal endothelial cells (CECs) at passage 0 (P0) for at least 35
20 days; expanding the CECs at passage 1 (P1) for at least 30 days; expanding the CECs at passage 2 (P2) for at least 30 days; expanding the CECs at passage 3 (P3) for at least 20 days; and expanding the CECs at passage 4 (P4) for at least 20 days. The method may further involve expanding the cells at passage (P5) for at least 20 days. In some embodiments, each of the passages is performed at a temperature of about 37°C.

25 In certain embodiments, the method involves culturing corneal endothelial cells (CECs) at passage 0 (P0) for 30-45 days; expanding the CECs at passage 1 (P1) for 30-45 days; expanding the CECs at passage 2 (P2) for 30-45 days; expanding the CECs at passage 3 (P3) for 20-30 days; and expanding the CECs at passage 4 (P4) for 20-30 days. The method may further involve expanding the cells at passage (P5) for 20-30 days. In some embodiments, each
30 of the passages is performed at a temperature of about 37°C.

In some embodiments, the total time to reach the desired number of cells (e.g., at least 1×10^7 cells, at least 1×10^8 cells, at least 1×10^9) by end of P4 is less than 200 days, less than 195 days, less than 190 days, less than 185 days, less than 180 days, less than 175 days, less

than 170 days, less than 165 days, less than 160 days, less than 155 days, less than 150 days, less than 145 days, less than 140 days, or less than 135 days.

In some embodiments, the total time to reach the desired number of cells (e.g., at least 1×10^7 cells, at least 1×10^8 cells, at least 1×10^9 , at least 1×10^{10}) by end of P5 is less than 225 days, less than 220 days, less than 215 days, less than 210 days, less than 205 days, less than 200 days, less than 195 days, less than 190 days, less than 185 days, less than 180 days, less than 175 days, less than 170 days, less than 165 days, or less than 160 days.

The compositions and methods disclosed herein provide a greater number of cells per donor cornea that can be used in CEC therapy to treat corneal endothelial diseases. This, in turn, increases the number of doses that can be obtained per corneal donor, which improves scalability of the CEC production process. For example, in certain embodiments, the method involves expanding the culture to at least 1×10^7 cells, at least 1×10^8 cells, or at least 1×10^9 by the end of P4. In instances where the method involves a fifth passage, the method may involve expanding the culture to at least 1×10^8 cells, at least 1×10^9 , or at least 1×10^{10} by the end of P5.

In some embodiments, at least 1×10^7 cells, at least 2×10^7 cells, at least 3×10^7 cells, at least 4×10^7 cells, at least 5×10^7 cells, at least 6×10^7 cells, at least 7×10^7 cells, at least 8×10^7 cells, at least 9×10^7 cells, at least 1×10^8 cells, at least 2×10^8 cells, at least 3×10^8 cells, at least 4×10^8 cells, at least 5×10^8 cells, at least 6×10^8 cells, at least 7×10^8 cells, at least 8×10^8 cells, at least 9×10^8 cells, or at least 1×10^9 cells are obtained by the end of P4. In some embodiments, at least 1×10^7 cells are obtained by the end of P4. In some embodiments, at least 1×10^8 cells are obtained by the end of P4. In some embodiments, at least 1×10^9 cells are obtained by the end of P4.

In some embodiments, at least 1×10^7 cells, at least 2×10^7 cells, at least 3×10^7 cells, at least 4×10^7 cells, at least 5×10^7 cells, at least 6×10^7 cells, at least 7×10^7 cells, at least 8×10^7 cells, at least 9×10^7 cells, at least 1×10^8 cells, at least 2×10^8 cells, at least 3×10^8 cells, at least 4×10^8 cells, at least 5×10^8 cells, at least 6×10^8 cells, at least 7×10^8 cells, at least 8×10^8 cells, at least 9×10^8 cells, at least 1×10^9 cells, at least 2×10^9 cells, at least 3×10^9 cells, at least 4×10^9 cells, at least 5×10^9 cells, at least 6×10^9 cells, at least 7×10^9 cells, at least 8×10^9 cells, at least 9×10^9 cells, or at least 1×10^{10} cells are obtained by the end of P5. In some embodiments, at least 1×10^7 cells are obtained by the end of P5. In some embodiments, at least 1×10^8 cells are obtained by the end of P5. In some embodiments, at least 1×10^9 cells

are obtained by the end of P5. In some embodiments, at least 1×10^{10} cells are obtained by the end of P5.

The temperature for cultivating corneal endothelial cells is not limited as long as the cells proliferate. For example, in some embodiments, the cells are passaged at a temperature of about 31°C to about 41°C (e.g., about 32°C to about 41°C, about 35°C to about 41°C). In certain embodiments, the cells are passaged at a temperature of at least about 31° C (e.g., about 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C). In certain embodiments, the cells are passaged at a temperature of about 37° C. In certain embodiments, the cells are passaged at a temperature of about 32° C. In certain embodiments, the cells are passaged at a temperature of about 41° C. The cells may be incubated in a conventional incubator for cell culture under humidification in an environment of about 5-10% CO₂.

In some embodiments, each of the passages are performed at the same temperature. In certain embodiments, each passage (i.e., P0, P1, P2, P3, P4, and optionally, P5 or later passages) is performed at about 37°C. In some embodiments, one or more of the passages is performed at a different temperature relative to the other passages.

The CECs may be cultured in a variety of cell culture vessels. Clinical studies involving CECs have generally used polystyrene T-flasks for expanding the CECs. However, the present method can alternatively be performed in other suitable culture vessels for later passages, such as layered or stacked culture chambers (e.g., Polystyrene CellSTACK™ chambers. Accordingly, in some embodiments, the cells are expanded in commercially available T-flasks (e.g., T-25, T-75, T-150, T-225) for P1, P2, P3, P4, and/or P5 (or passages beyond P5). In some embodiments, the CECs in P3 and/or P4 are expanded in a stacked cell culture chamber includes two or more layers (e.g., 2-10 layers). See Fig. 1 for examples of suitable vessels for each passage.

Cell culture vessels having different volumes or surface areas may be utilized in the present method (e.g., see Figure 1). For example, in some embodiments, the cell culture chamber has a medium volume of 130 to 8000 mL. In some embodiments, the cell culture chamber has a medium volume of 130 to 2000 mL. In some embodiments, the cell culture chamber has a medium volume of 5 to 1000 mL. In some embodiments, the cell culture chamber has a cell culture area of at least about 25 cm². In some embodiments, the cell culture chamber has a cell culture area of at least about 75 cm². In some embodiments, the cell culture chamber has a cell culture area of at least about 100 cm². In some embodiments, the cell culture chamber has a cell culture area of at least about 150 cm². In some embodiments, the

cell culture chamber has a cell culture area of at least about 175 cm². In some embodiments, the cell culture chamber has a cell culture area of at least about 200 cm². In some embodiments, the cell culture chamber has a cell culture area of at least about 1000 cm². In some embodiments, the cell culture chamber has a cell culture area of at least about 2000 cm².

5 In some embodiments, the cell culture chamber has a cell culture area of at least about 3000 cm². In some embodiments, the cell culture chamber has a cell culture area of at least about 5000 cm². In some embodiments, the cell culture chamber has about 600 to about 6400 cm² culture area. In some embodiments, the cell culture chamber has about 1200 to about 6400 cm² culture area.

10 Different types or volumes of cell culture vessels are used during the passaging process. In some embodiments, the volume and/or surface area of the cell culture chamber may be increased with each passage. For example, in certain embodiments, P0 is performed in a T25 flask, P1 is performed in a T75 flask, P2 is performed in a T150 flask, P3 is performed in a T225 flask, and P4 is performed in a CS1, CS2, or CS5 vessel.

15 In some embodiments, the surface of the cell culture vessel is coated with a cell substrate. Examples of the aforementioned substrate include polymer materials derived from naturally-occurring substances such as collagen, gelatin, cellulose and the like, synthesized polymer materials such as polystyrene, polyester, polycarbonate, poly(N-isopropylacrylamide) and the like, biodegradable polymer materials such as polylactic acid, polyglycolic acid and the like, hydroxyapatite, amniotic membrane and the like. In one embodiment, the cell substrate is collagen (e.g., a collagen-coated plate).

CECs may be cultured in culture vessels coated with an extracellular matrix, or proteins therefrom, to promote cellular proliferation (see, e.g., San Choi, Jin, et al. *Biomedical materials* 8.1 (2013): 014108.; Okumura, Naoki, et al. *Investigative Ophthalmology & Visual Science* 56.5 (2015): 2933-2942.; Parekh, Mohit, et al. *Acta Ophthalmologica* 99.4 (2021): e512-e522; Blake, Diane A., et al. *Investigative Ophthalmology & Visual Science* 38.6 (1997): 1119-1129, which are hereby incorporated by reference). Accordingly, in some embodiments, the cells are grown on a cell culture surface comprising an extracellular matrix. For example, the extracellular matrix may be one derived from CECs (e.g., human CECs or bovine CECs). See, e.g., Blake *et al* (1997) and Parekh *et al* (2021).

30 In another embodiment, the cells are grown on a cell culture surface comprising an extracellular matrix protein. In some embodiment, the extracellular matrix protein is selected from the group consisting of collagen (e.g., collagen type I, collagen type IV), laminin,

fibronectin, proteoglycan, SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A, SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1, insulin-like growth factor-binding protein 5, vitronectin, fibrillin-1, fibrillin-2, tensin, Wnt-5b, citron Rho-interacting kinase, chondroitin sulphate proteoglycan 4, cyclin-dependent kinase 1, cyclin-dependent kinase 4, periostin, thrombospondin-4, Tubulin alpha chain-like 3, Tubulin alpha-1B chain, Tubulin beta-1 chain, Tubulin beta-4A chain, and versican core protein. In certain embodiments, the extracellular matrix protein is collagen, laminin, or fibronectin. In one embodiment, the extracellular matrix protein is collagen.

In one embodiment, a cell culture vessel used to grow CECs as described herein is a tissue-culture treated (TC-treated) cell culture vessel.

The CECs may be cultured and expanded in a suitable culture medium that permits growth of CECs. For example, in some embodiments, the CECs are cultured in a basal growth medium (e.g., OPTI-MEM® I Reduced Serum Media (Thermo Fisher Scientific, Inc., e.g., free of ammonium meta vanadate and combined with fetal bovine serum (e.g., 8%)). In some embodiments, the basal growth medium is further supplemented with an epidermal growth factor (EGF) and/or ascorbic acid (e.g., 20 µg/mL). In one embodiment, the basal growth medium further comprises a Rho-associated protein kinase (ROCK)-inhibitor, such as Y-27632. In some embodiments, any one of P0, P1, P2, P3, or P4 are performed in a cell culture medium supplemented with ascorbic acid, fetal bovine serum, chondroitin sulfate, calcium chloride, and/or a Rho kinase inhibitor. In some embodiments, each of P0, P1, P2, P3, and P4 are performed in a cell culture medium supplemented with ascorbic acid, fetal bovine serum, chondroitin sulfate, calcium chloride, and/or a Rho kinase inhibitor.

After harvesting the cells at the desired passage, the cells can be formulated in a suitable medium, such as commercially available DMEM (Dulbecco's Modified Eagle's Medium). In some embodiments, the DMEM is supplemented with Human Serum Albumin (e.g., 2% HSA) and/or a Rho-associated protein kinase (ROCK)-inhibitor (e.g., Y-27632).

The provided methods enable expansion of a larger number of viable CECs that exhibit functional properties similar to CECs native to the corneal endothelium. Markers for characterizing or identifying CECs are known in the art (See e.g., Hamuro J, Ueno M, Toda M, Sotozono C, Montoya M, Kinoshita S. *Invest Ophthalmol Vis Sci.* 2016 Aug 1;57(10):4385-92; and Wongvisavavit, R., *et al* (2021). *Regenerative medicine*, 16(09), 871-891). In some embodiments, the cells can be identified or characterized at any stage in the culturing or expansion process based on presence or absence of certain surface markers. For example,

CECs have been characterized as being CD166 positive, CD44 negative to CD44 weakly positive, CD24 negative to weakly positive, CD44 negative to weakly positive, CD105 negative to weakly positive, CD26 negative to weakly positive, CD200 negative to weakly positive, and CD90 negative to weakly positive phenotypes. Additionally, markers that may be found in CECs include, but are not limited to, ATPase, ZO-1, VDAC3, SLC4A4, CLCN3, COL4A2, COL8A1, COL8A2, CDH2, CD98, CD166, CD340, Integrin $\alpha 3\beta 1$, CD56, Prdx-6, CD248, SLC4A11, or CYYR1. Any methods known in the art to assess cell markers can be used to characterize the CECs, such as flow cytometry, western blot, immunocytochemistry, immunohistochemistry, immunoprecipitation, quantitative PCR; and reverse transcription PCR. CECs additionally have a hexagonal morphology that is observable under a microscope.

The method results in a large number of viable CECs for use in therapeutic compositions. In some embodiments, at least 70% (e.g., at least 70%, 75%, 80%, 85%, 90%, 95% or more) of the CECs are viable at the end of P4 as determined by a cell viability assay. In some embodiments, at least 90% of the CECs are viable at the end of P4 as determined by cell viability assay.

In some embodiments, at least 70% (e.g., at least 70%, 75%, 80%, 85%, 90%, 95% or more) of the CECs have a cell surface expression at the end of P4 selected from the group consisting of CD166 positive, CD44 negative to CD44 weakly positive, CD24 negative to weakly positive, CD44 negative to weakly positive, CD105 negative to weakly positive, CD26 negative to weakly positive, CD200 negative to weakly positive, and CD90 negative to weakly positive phenotypes.

In some embodiments, at least 70% (e.g., at least 70%, 75%, 80%, 85%, 90%, 95% or more) of the CECs have a cell surface expression at the end of P5 of a marker selected from the group consisting of CD166 positive, CD44 negative to CD44 weakly positive, CD24 negative to weakly positive, CD44 negative to weakly positive, CD105 negative to weakly positive, CD26 negative to weakly positive, CD200 negative to weakly positive, and CD90 negative to weakly positive phenotypes.

In some embodiments, at least 70% (e.g., at least 70%, 75%, 80%, 85%, 90%, 95% or more) of the CECs have a cell surface expression at the end of P4 of a marker selected from the group consisting of sodium-potassium ATPase, ZO-1, VDAC3, SLC4A4, CLCN3, COL4A2, COL8A1, COL8A2, CDH2, CD98, CD166, CD340, Integrin $\alpha 3\beta 1$, CD56, Prdx-6, CD248, SLC4A11, and CYYR1.

In some embodiments, at least 70% (e.g., at least 70%, 75%, 80%, 85%, 90%, 95% or more) of the CECs have a cell surface expression at the end of P5 of a marker selected from the group consisting of sodium-potassium ATPase, ZO-1, VDAC3, SLC4A4, CLCN3, COL4A2, COL8A1, COL8A2, CDH2, CD98, CD166, CD340, Integrin $\alpha 3 \beta 1$, CD56, Prdx-6, CD248, SLC4A11, and CYYR1.

In some embodiments, at least 70% (e.g., at least 70%, 75%, 80%, 85%, 90%, 95% or more) of the CECs at the end of P4 have a hexagonal morphology as determined by microscopy.

In some embodiments, at least 70% (e.g., at least 70%, 75%, 80%, 85%, 90%, 95% or more) of the CECs at the end of P5 have a hexagonal morphology as determined by microscopy.

The resulting cell composition can be used to formulate CECs for injection into an eye of a subject for the treatment of a corneal endothelial disease, as further described herein.

III. Corneal Endothelial Cell (CEC) Compositions

Further provided herein are compositions including corneal endothelial cells (e.g., for use in treating corneal endothelial disease). The CECs may be cultured, expanded, and harvested in accordance with the disclosed methods to produce a composition having a greater number of cells per donor cornea relative to previously known CEC preparations. The resulting cells can be used in injectable CEC therapies to treat corneal endothelial diseases.

In some embodiments, the compositions herein include a CEC composition capable of cell proliferation *in vivo*, comprising a substrate and a cultured CEC layer, wherein the cultured CECs are cultured *in vitro*. In some embodiments, CECs are used that have been (1) cultured at least in a culture vessel (e.g., culture dish, culture tube, culture tank etc.), (2) such cells passage-cultured further (e.g., 3-10 passages), or (3) such passage-cultured cells that are further cultured on a substrate.

In some embodiments, the cultured corneal endothelial cell layer contained in the composition herein may have at least one, at least two, or all of the following characteristics.

(1) The cell layer may have a monolayer structure. This is one of the characteristics of the corneal endothelial cell layer of living organisms.

(2) The cell density of the cell layer may be about 1,000-about 4,000 cells/mm². Particularly, when the recipient (transplant) is an adult, the density may be about 2,000-about 3,000 cells/mm².

(3) The visual flat plane shape of the cell constituting the cell layer may be approximately hexagonal. This is one of the characteristics of the cell constituting the corneal endothelial cell layer in living organisms.

(4) In the cell layer, cells are regularly aligned. In the corneal endothelial cell layer in living organisms, the cells constituting the layer are regularly aligned, by which it is considered that the corneal endothelial cells maintain normal function and high transparency and the cornea appropriately controls the water content. Having such morphological characteristics (e.g., hexagonal shape), the composition herein may have functions similar to those of the corneal endothelial cell layer in living organisms.

In addition to the cellular composition, the present composition may use other agents in combination such as a steroid agent, antibiotic agent, or a ROCK inhibitor. The additional agent (e.g., antibiotic or ROCK inhibitor) can be prepared as a neutral or salt form or other prodrug (e.g., ester or the like). Pharmaceutically acceptable salts include salts formed with a free carboxyl group, derived from hydrochloric acid, phosphoric acid, acetic acid, oxalic acid, tartaric acid or the like, salts formed with a free amine group, derived from isopropylamine, triethylamine, 2-ethylaminoethanol, histidine, procaine or the like, and salts derived from sodium, potassium, ammonium, calcium, ferric hydroxide or the like.

The CEC compositions provided herein may be formulated with or without a rho kinase inhibitor. In the present disclosure, "Rho kinase" or "ROCK" (Rho-associated coiled-coil forming kinase: Rho-bound kinase) refers to serine/threonine kinase which is activated with activation of Rho. Examples thereof include ROKalpha (ROCK-II: Leung, T. et al., J. Biol. Chem., 270, 29051-29054, 1995), p160ROCK (ROKbeta, ROCK-I: Ishizaki, T. et al., The EMBO J., 15(8), 1885-1893, 1996) and other proteins having serine/threonine kinase activity.

Examples of ROCK inhibitors used as a combined agent include compounds disclosed in US Patent No. 4678783, Japanese Patent No. 3421217, WO 95/28387, WO 99/20620, WO 99/61403, WO 02/076976, WO 02/076977, WO 2002/083175, WO 02/100833, WO 03/059913, WO 03/062227, WO 2004/009555, WO 2004/022541, WO 2004/108724, WO 2005/003101, WO 2005/039564, WO 2005/034866, WO 2005/037197, WO 2005/037198, WO 2005/035501, WO 2005/035503, WO 2005/035506, WO 2005/080394, WO 2005/103050, WO 2006/057270, WO 2007/026664, and the like. Such compounds can be manufactured by the method described in each disclosed document. Examples thereof include 1-(5-isoquinolinesulfonyl)homopiperazine or a salt thereof (e.g., fasudil or fasudil hydrochloride), (+)-trans-4-(1-aminoethyl)-1-(4-pyridylcarbonyl)cyclohexanecarboxamide or a salt thereof (e.g., Y-

27632 ((R)-(+)-trans-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide dihydrochloride monohydrate), and the like), and preferably comprising Y-27632.

In some embodiments, the CEC composition includes a Rho kinase inhibitor. For example, a Rho kinase inhibitor may be added when culturing, proliferating, differentiating or maturing of corneal endothelial cells. Such an agent may be included in a cell composition for administration to a subject, or provided in a separately administered form. In a separately provided or administered form, the additional agent may be provided as a kit or combined agent. When used as a kit or combined agent, a package insert that describes the usage method thereof may also be combined.

Alternatively, in some embodiments, the compositions lack a Rho kinase inhibitor. For example, a Rho kinase inhibitor may be omitted when culturing, proliferating, differentiating or maturing of corneal endothelial cells.

Further provided are pharmaceutical compositions including the CEC compositions described herein and a pharmaceutically acceptable carrier or excipient. As used herein, "pharmaceutically acceptable" refers to a material, such as a carrier or diluent, which does not abrogate the biological activity or properties of the compound, and is relatively non-toxic, i.e., the material may be administered to an individual without causing undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained. Such a composition contains a therapeutically effective amount of CECs together with a suitable amount of carrier, such that the composition is provided in a form suitable for administration to a patient

Further provided herein are methods of treating a corneal endothelial disease in a subject in need thereof by administering an effective amount of a corneal endothelial cell composition to an eye of the subject. The CECs may be initially cultured, expanded, and harvested in accordance with the disclosed methods to produce a composition having a greater number of cells per donor cornea, which in turn increases the number of doses arising from a single donor cornea.

Corneal endothelial disease may occur, for example, when endothelial cells degrade and/or are lost due to conditions such as bullous keratopathy, Fuchs' dystrophy, congenital corneal dystrophies or ocular surgical trauma. Corneal endothelial disease leads to symptoms that impact vision, including blurred vision, vision loss, corneal hydration, increased glare or discomfort, or severe ocular pain.

Accordingly, in some embodiments, the CEC composition of the present disclosure is for use in treating a corneal endothelial disease. Non-limiting examples of corneal endothelial diseases include bullous keratopathy, corneal endothelial dystrophies (e.g., cornea guttata, Fuchs endothelial corneal dystrophy, posterior polymorphous corneal dystrophy, iridocorneal endothelial syndrome, and congenital hereditary corneal endothelial dystrophy), viral diseases (e.g., cytomegalovirus endotheliitis and herpetic endotheliitis), exfoliation syndrome, and corneal endothelial graft rejection; as well as inflammation or physical damage associated with external factors, such as keratouveitis, interstitial keratitis, corneal endotheliitis, corneal endothelial cell loss after corneal transplantation, corneal injury after intraocular surgery (e.g., cataract surgery, vitreous surgery, glaucoma surgery), corneal injury induced by glaucomatous attack, corneal injury caused by long-term contact lens use, corneal trauma, corneal edema, and intrapartum corneal trauma.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting.

Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

Various embodiments and aspects of the present invention as delineated herein above and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

The following examples describe exemplary methods for culturing and expanding corneal endothelial cells (CECs) to produce high numbers of cells to provide doses of CECs for the maximum number of patients in need thereof.

Example 1: Improved Corneal Endothelial Cell Culturing Method

This example provides a method for achieving high levels of CECs by culturing and expanding CECs through Passage 4. The process described in this example is outlined in **Fig. 1**.

Materials

Cell Source: For these experiments, functional human corneal cells (effector cells) were used because they share functions of mature differentiated human corneal endothelial cells (see, e.g., Hamuro J, *et al. Invest Ophthalmol Vis Sci.* 2016 Aug 1;57(10):4385-92). These functional corneal cells were characterized by flow cytometry as having the following surface markers: CD166 positive, CD44 negative to CD44 weakly positive, CD24 negative to weakly positive, CD44 negative to weakly positive, CD105 negative to weakly positive, CD26 negative to weakly positive, CD200 negative to weakly positive, CD90 negative to weakly positive phenotypes.

Flask Type (Base Material): The base material is subject to change from passage to passage. In this Example, the following flasks were used for each passage and are also described in **Fig. 1**:

Passage 0 (P0) was performed in Corning 6 well plate (CAT# 3506) or VWR 6 well plate (CAT#10861-696).

Passage 1 (P1) was performed in Corning T25 (CAT#430639) or Corning T75 (CAT#430641U) or Corning 150 (CAT#430825) or Corning T25 CELLBIND (CAT#3289) or Corning T75 CELLBIND (CAT#3290) or Corning T150 CELL BIND (CAT#3291).

Passage 2 (P2) was performed in Corning T25 (CAT#430639) or Corning T75 (CAT#430641U) or Corning T150 (CAT#430825) or Corning T225 (CAT#431082) or Corning CellSTACK Culture Chamber, 1 chamber (CAT#3268) or Corning T25 CELLBIND (CAT#3289) or Corning T75 CELLBIND (CAT#3290) or Corning T150 CELL BIND (CAT#3291) or Corning T225 CELL BIND (CAT#3293) or Corning CellSTACK Culture Chamber CELLBIND, 1 chamber (CAT#3330).

Passage 3 (P3) was performed in Corning T25 (CAT#430639) or Corning T75 (CAT#430641U) or Corning T150 (CAT#430825) or Corning T225 (CAT#431082) or Corning CellSTACK Culture Chamber, 1 chamber (CAT#3268) or Corning CellSTACK Culture Chamber, 2 chamber (CAT#3269) or Corning CellSTACK Culture Chamber, 5 chamber (CAT#3313) or Corning T25 CELLBIND (CAT#3289) or Corning T75 CELLBIND (CAT#3290) or Corning T150 CELL BIND (CAT#3291) or Corning T225 CELL BIND (CAT#3293) or Corning CellSTACK Culture Chamber CELLBIND, 1 chamber (CAT#3330) or Corning CellSTACK Culture Chamber CELLBIND, 2 chamber (CAT#3310) or Corning CellSTACK Culture Chamber CELL BIND, 5 chamber (CAT#3311).

Passage 4 (P4) was performed in Corning T25 (CAT#430639) or Corning T75 (CAT#430641U) or Corning T150 (CAT#430825) or Corning T225 (CAT#431082) or Corning CellSTACK Culture Chamber, 1 chamber (CAT#3268) or Corning CellSTACK Culture Chamber, 2 chamber (CAT#3269) or Corning CellSTACK Culture Chamber, 5 chamber (CAT#3313) or
 5 Corning CellSTACK Culture Chamber, 10 chamber (CAT#3271) or Corning T25 CELLBIND (CAT#3289) or Corning T75 CELLBIND (CAT#3290) or Corning T150 CELL BIND (CAT#3291) or Corning T225 CELL BIND (CAT#3293) or Corning CellSTACK Culture Chamber CELLBIND, 1 chamber (CAT#3330) or Corning CellSTACK Culture Chamber CELLBIND, 2 chamber (CAT#3310) or Corning CellSTACK Culture Chamber CELL BIND, 5 chamber (CAT#3311) or
 10 Corning CellSTACK Culture Chamber CELL BIND, 10 chamber (3312).

Passage 5 (P5) was performed in Corning T25 (CAT#430639) or Corning T75 (CAT#430641U) or Corning T150 (CAT#430825) or Corning T225 (CAT#431082) or Corning CellSTACK Culture Chamber, 1 chamber (CAT#3268) or Corning CellSTACK Culture Chamber, 2 chamber (CAT#3269) or Corning CellSTACK Culture Chamber, 5 chamber (CAT#3313) or
 15 Corning CellSTACK Culture Chamber, 10 chamber (CAT#3271) or Corning T25 CELLBIND (CAT#3289) or Corning T75 CELLBIND (CAT#3290) or Corning T150 CELL BIND (CAT#3291) or Corning T225 CELL BIND (CAT#3293) or Corning CellSTACK Culture Chamber CELLBIND, 1 chamber (CAT#3330) or Corning CellSTACK Culture Chamber CELLBIND, 2 chamber (CAT#3310) or Corning CellSTACK Culture Chamber CELL BIND, 5 chamber (CAT#3311) or
 20 Corning CellSTACK Culture Chamber CELL BIND, 10 chamber (3312).

Culture Medium:

L-ascorbic Acid Prep: L-ascorbic acid was added to a phosphate buffer solution (PBS), which was then mixed, filtered, and stored at -20°C in cryovials.

25 *Y-27632 Prep:* PBS was added to Y-27632, which was then mixed, filtered, and stored at -20°C in cryovials.

N-Media Prep: Calcium chloride was added to CTS™ Opti-MEM™, which was then mixed and filtered. Separately, chondroitin sulfate was added to CTS™ Opti-MEM™, which was also mixed and filtered. Next, the calcium chloride solution, chondroitin sulfate solution, and fetal
 30 bovine serum were added to CTS™ Opti-MEM™, mixed and stored in a 2-8°C refrigerator. The fetal bovine serum concentration in the prepared media was 8% (v/v). NY-Media was then prepared by removing the N-Media from the refrigerator and warming it to room temperature.

Aliquots of L-ascorbic Acid Prep and Y-27632 Prep were added to the N-Media Prep and thoroughly mixed to supplement the NY-Media.

Methods

Human functional human corneal cells were cultured starting at Passage 0 (P0) every 7 days, every 14 days, every 17 days, every 21 days, or every 30 days. The cell culture and expansion method described herein is outlined in **Table 1** and in **Table 2**.

Table 1: Overview of order of operations

Unit Operation
Receive and wash starting material
Digestion and Wash
P0 Culture (35-45 days in culture)
P1 Culture (30-45 days in culture)
P2 Culture (30-45 days in culture)
P3 Culture (21-45 days in culture)
P4 Culture (21 days in culture)
Harvest
Formulate, Fill, and Finish

Each passage (P0+) was performed at 37°C and a relative humidity of about 95 percent. The CO₂ concentration, about 5 percent, was controlled to match physiologic conditions and to maintain a constant pH. The passages (P0 +) were performed in media with a pH of 7.2 to 7.5. O₂ was not monitored or controlled.

A P0 of 7 days, 14 days, or 21 days in duration did not allow cell expansion comparable to the control process. It was determined that a P0 of 35 to 45 days was necessary to enable expansion. A prolonged 35-45 day P0 process (35-45 days) was assessed in conjunction with (i) 7 day passages for P1, P2, P3, and P4; (ii) 14 day passages for P1, P2, P3, and P4; or (iii) 21 day passages for P1, P2, P3, and P4. However, each of these expansion processes did not generate sufficient numbers of cells to provide ~1000 doses per donor cornea. Finally, a prolonged 30-45 day passage for P1 and P2 (30-45 days) was assessed in conjunction with a prolonged passage 0 (35-45 days in length). Passages 3 and 4 were maintained at 21 days

each. This process provided cell numbers that supported 1000 doses per donor at the end of passage 4.

P0 Culture: The OD (oculus dexter; right eye) tube and OS (oculus sinister; left eye) tube were removed from the incubator and centrifuged. The supernatant was then removed, NY-Media was added, and the wash was repeated. The cell starting material was resuspended and added to the first well in a 6-well plate. The cell starting material was mixed, as necessary, prior to adding additional cell starting material to the first well of the 6-well plate. The above procedure was repeated with the other cell starting material tube and added to the second well of the 6-well plate. The well plate was removed from the incubator with the spent media removed from each well and replaced with NY-Media. The spent media was saved for sterility testing. The well plate was then returned to the incubator. Passage took place when cells were in culture for at least 35 days, not to exceed 45 days.

P1 Culture: NY-Media was added to the collection tube and the contents were mixed. The cell solution was then resuspended, dispensed into separate flasks, and the cells were evenly dispersed in the flasks. The flasks were placed in the incubator. The P1 flasks were removed from the incubator and the spent media was removed from the flasks. Fresh NY-Media was added back to the flasks and the flasks were returned to the incubator. This media change can be performed eleven additional times. The P1 Maintenance stage may take 30 days to 45 days. The NY-Media, PBS, and TrypLE (10x) were warmed to 37°C. The P1 flasks were removed from the incubator and inspected under a microscope. The spent media was sampled for endotoxins, gram stain, and mycoplasma testing. PBS was added to each flask and the flasks were agitated. The PBS was aspirated, additional PBS was added, and the flasks were placed in the incubator. After incubation, the flasks were removed, PBS was removed from each flask, and TrypLE (10x) was added, and the flasks were incubated. The flasks were removed from the incubator and the flasks were tapped to aid in the disassociation of the cells from the flask. NY-Media was added to each flask, each flask was rinsed with the cell solution, and the cell solution from the flask was then added to the Collection Tube. The flask was then rinsed with NY-Media, and this rinse was added to the Collection Tube. The Collection Tube was centrifuged, and the supernatant was aspirated and discarded. The cell pellet was resuspended, and NY-Media was added to the Collection Tube. The contents in the Collection Tube were mixed, and a portion of the cell suspension was removed (termed Cell Suspension Aliquot_P1). Two aliquots were removed from Cell Suspension Aliquot_P1 and a cell count was performed

on the two aliquots. After the completion of the cell count, the contents of the Cell Suspension Aliquot_P1 were returned to the Collection Tube and the volume of the cell suspension recorded.

P2 Culture: NY-Media was added to the Collection Tube and the contents were mixed.

- 5 The cell solution was then resuspended, dispensed into separate flasks, and the cells were evenly dispersed in the flasks. The flasks were placed in the incubator. The P2 flasks were removed from the incubator and the spent media was removed from the flasks. Fresh NY-Media was added back to the flasks and the flasks were returned to the incubator. This media change may be performed eleven additional times. The P2 Maintenance stage may take 30 to 45 days.
- 10 The NY-Media, PBS, and TrypLE (10x) were warmed to 37°C. The P1 flasks were removed from the incubator and inspected under a microscope. The spent media was sampled for endotoxins, gram stain, and mycoplasma testing. PBS was added to each flask and the flasks were agitated. The PBS is aspirated, additional PBS was added, and the flasks were placed in the incubator. After incubation, the flasks were removed, PBS was removed from each flask, and
- 15 TrypLE (10x) was added, and the flasks were incubated. The flasks were removed from the incubator and the flasks were tapped to aid in the disassociation of the cells from the flask. NY-Media was added to each flask, each flask was rinsed with the cell solution, and the cell solution from the flask was then added to the Collection Tube. The flask was then rinsed with NY-Media, and this rinse was added to the Collection Tube. The Collection Tube was centrifuged, and the
- 20 supernatant was aspirated and discarded. The cell pellet was resuspended, and NY-Media was added to the Collection Tube. The contents in the Collection Tube were mixed, and a portion of the cell suspension was removed (termed Cell Suspension Aliquot_P2). Two aliquots were removed from Cell Suspension Aliquot_P2 and a cell count was performed on the two aliquots. After the completion of the cell count, the contents of the Cell Suspension Aliquot_P2 were
- 25 returned to the Collection Tube and the volume of the cell suspension recorded.

P3 Culture: NY-Media was added to the Collection Tube and the contents were mixed.

- The cell solution was then resuspended, dispensed into separate flasks, and the cells were evenly dispersed in the flasks. The flasks were placed in the incubator. The P3 flasks were removed from the incubator and the spent media was removed from the flasks. Fresh NY-Media
- 30 was added back to the flasks and the flasks were returned to the incubator. This media change may be performed eleven additional times. The P3 Maintenance may take 21 to 45 days. The NY-Media, PBS, and TrypLE (10x) were warmed to 37°C. The P1 flasks were removed from the

incubator and inspected under a microscope. The spent media was sampled for endotoxins, gram stain, and mycoplasma testing. PBS was added to each flask and the flasks were agitated. The PBS was aspirated, additional PBS was added, and the flasks were placed in the incubator. After incubation, the flasks were removed, PBS was removed from each flask, and

5 TrypLE (10x) was added, and the flasks were incubated. The flasks were removed from the incubator and the flasks were tapped to aid in the disassociation of the cells from the flask. NY-Media was added to each flask, each flask was rinsed with the cell solution, and the cell solution from the flask was then added to the Collection Tube. The flask was then rinsed with NY-Media, and this rinse was added to the Collection Tube. The Collection Tube was centrifuged, and the

10 supernatant was aspirated and discarded. The cell pellet was resuspended, and NY-Media was added to the Collection Tube. The contents in the Collection Tube were mixed, and a portion of the cell suspension was removed (termed Cell Suspension Aliquot_P3). Two aliquots were removed from Cell Suspension Aliquot_P3 and a cell count was performed on the two aliquots. After the completion of the cell count, the contents of the Cell Suspension Aliquot_P3 were

15 returned to the Collection Tube and the volume of the cell suspension recorded.

P4 culture/harvest: After completion of the cell culture and expansion, the next steps of the methodology incorporate additional culturing (e.g., a P5 passage or later passage) and harvest steps.

Table 2 provides a summary of the various passages and the time for each as described

20 above.

Table 2. Passage timeline

Passage no.	Time per passage (days)	Total time (days)
0	35-45	35-45
1	30-45	65-90
2	30-45	95-135
3	20-30	115-165
4	20-30	135-195
5	20-30	155-225

Results

As shown in **Fig. 2 and Table 3**, the modified expansion process outlined above and in **Fig. 1 and Table 2** resulted in a cell count that supports at least 1000 doses by P4 (see data enclosed by Circle “B” in Fig. 2).

- 5 **Table 3** summarizes the number of doses obtained in different cell culture formats at the indicated passages (Passage 3, Passage 4, or Passage 5) after passaging the cells in accordance with the timeline in **Table 2**.

Table 3.

	Passage 3		Passage 4		Passage 5	
Vessel	# of vessels	Doses	# of vessels	Doses	# of vessels	Doses
T25	133	567	851	4204	6307	26575
T75	44	414	207	2670	1335	10816
T225	14	583	97	3698	616	11022
CS2	2	534	16	2276	68	5965
CS5	N/A	N/A	6	2218	26	3971

- 10 T25: cell culture polystyrene flask; 25 cm² culture area (Thermo Scientific)
 T75: cell culture polystyrene flask; 75 cm² culture area (Thermo Scientific)
 T225: cell culture polystyrene flask; 225 cm² culture area (Thermo Scientific)
 CS2: Polystyrene CellSTACK; 2 chambers with 1272 cm² cell growth area (Corning)
 CS5: Polystyrene CellSTACK; 5 chambers with 3180 cm² cell growth area (Corning)

- 15 As shown in **Table 3**, all culture formats assessed yielded cell counts that support over 1000 doses by P4. As cells continued to expand in P5, some culture vessels additionally provided the potential for larger scale cultures by P5 (e.g., >10,000 doses). These results suggest a reproducible process that can be adapted to different culture vessels.

- 20 The foregoing studies also suggest that in order to obtain high numbers of cells (translating into 1000+ doses), the CECs should be kept in culture for about 30 to about 45 days at P0, P1, and P2. At a passage time of 30 to 45 days, cells go through a growth phase and then undergo contact inhibition, exit the growth phase and regain a non-proliferative state similar to their native state *in vivo*. So keeping the cells in culture for 30-45 days may help the
 25 cells transition from an *in vivo* non proliferative state (their native state in the cornea that was used as starting material) to *in vitro* culture conditions. Following P0-P2, P3 and P4 are

shortened passages that keep the cells in an active growth state. This enables increased cell expansion scale. Another part of the process that increases cell expansion is to increase flask size progressively, i.e. P0: T25 > P1: T75 > P2: T150 > P3: T225 > P4: CS1 or CS2 or CS5.

5

INCORPORATION BY REFERENCE

The contents of all references, patents and published patent applications cited throughout this application are incorporated herein by reference.

CLAIMS

What is claimed is:

1. A method of culturing human corneal endothelial cells (CECs), said method comprising:
 - 5 culturing human corneal endothelial cells (CECs) in an initial passage 0 (P0); and
 - expanding the human CECs through at least four additional passages comprising passage 1 (P1), passage 2 (P2), passage 3 (P3), and passage 4 (P4),
 - wherein both P3 and P4 have time periods that are shorter than each of P0, P1, and P2;
 - 10 wherein CECs in each passage are cultured in a culture vessel; and
 - wherein at least 1×10^7 cells are obtained by the end of P4.
2. The method of claim 1, wherein at least 1×10^8 cells are obtained by the end of P4.
- 15 3. The method of claim 2, wherein at least 1×10^9 cells are obtained by the end of P4.
4. The method of any one of claims 1-3, further comprising expanding the CECs through passage 5 (P5).
- 20 5. The method of claim 4, wherein P5 is shorter in duration than each of P0, P1, and P2.
6. The method of claim 5, wherein at least 1×10^8 cells are obtained by the end of P5.
7. The method of claim 6, wherein at least 1×10^9 cells are obtained by the end of P5.
- 25 8. The method of claim 7, wherein at least 1×10^{10} cells are obtained by the end of P5.
9. The method of any one of the preceding claims, comprising culturing the CECs at P0 for at least 35 days.
- 30 10. The method of claim 9, comprising culturing the CECs at P0 for 35 to 80 days.

11. The method of any one of the preceding claims, comprising expanding the CECs at P1 for at least 30 days.
12. The method of claim 11, comprising expanding the CECs at P1 for 30-80 days.
- 5 13. The method of any one of the preceding claims, comprising expanding the CECs at P2 for at least 30 days.
14. The method of claim 13, comprising expanding the CECs at P2 for 30-80 days.
- 10 15. The method of any one of the preceding claims, comprising expanding the CECs at P3 for at least 20 days.
16. The method of claim 15, comprising expanding the CECs at P3 for 20 to 45 days.
- 15 17. The method of claim 16, comprising expanding the CECs at P3 for 20 to 30 days.
18. The method of any one of the preceding claims, wherein P4 comprises expanding the CECs for at least 20 days.
- 20 19. The method of claim 18, wherein P4 comprises expanding the CECs at P4 for 20 to 30 days.
20. The method of any one of the preceding claims, wherein the culture vessel of the CECs in
25 P3 and/or P4 is a stacked cell culture chamber comprising two or more layers.
21. The method of claim 20, wherein the stacked cell culture chamber comprises 2-10 layers.
22. The method of any one of the preceding claims, wherein the cell culture vessel has a
30 medium volume of 130 to 8000 mL.
23. The method of claim 22, wherein the cell culture vessel has a medium volume of 130 to 2000 mL.

24. The method of any one of claims 1-21, wherein the cell culture vessel has a cell growth area of about 600 to about 6400 cm².
- 5 25. The method of any one of claims 1-21, wherein the cell culture vessel has a cell growth area of about 1200 to about 6400 cm².
26. A method of culturing corneal endothelial cells, the method comprising:
culturing corneal endothelial cells (CECs) at passage 0 (P0) for at least 30 days;
10 expanding the CECs at passage 1 (P1) for at least 30 days;
expanding the CECs at passage 2 (P2) for at least 30 days;
expanding the CECs at passage 3 (P3) for less than 30 days; and
expanding the CECs at passage 4 (P4) for less than 30 days.
- 15 27. A method of culturing corneal endothelial cells, the method comprising:
culturing corneal endothelial cells (CECs) at passage 0 (P0) for 30-45 days;
expanding the CECs at passage 1 (P1) for 30-45 days;
expanding the CECs at passage 2 (P2) for 30-45 days;
expanding the CECs at passage 3 (P3) for 20- 30 days; and
20 expanding the CECs at passage 4 (P4) for 20-30 days.
28. The method of claim 26 or 27, wherein at least 1×10^7 cells are obtained by the end of P4.
29. The method of claim 26 or 27, wherein at least 1×10^8 cells are obtained by the end of P4.
25
30. The method of claim 26 or 27, wherein the at least 1×10^9 cells are obtained by the end of P4.
31. The method of any one of claims 26-30, further comprising expanding the CECs at passage
30 5 (P5) for less than 30 days.
32. The method of any one of claims 26-31, further comprising expanding the CECs at passage
5 (P5) for 20-30 days.

33. The method of claim 31 or 32, wherein at least 1×10^8 cells are obtained by the end of P5.
34. The method of claim 31 or 32, wherein at least 1×10^9 cells are obtained by the end of P5.
- 5 35. The method of claim 31 or 32, wherein at least 1×10^{10} cells are obtained by the end of P5.
36. The method of any one of claims 1-35, wherein each of the passages is performed at the same temperature.
- 10 37. The method of any one of claims 1-36, wherein each passage is performed at a temperature of at least about 31°C.
38. The method of claim 37, wherein each passage is performed at a temperature of about 37°C.
- 15 39. The method of claim 37, wherein each passage is performed at a temperature of about 31°C to 41°C.
- 20 40. The method of any one of claims 1-39, wherein the CECs are derived from a single donor.
41. The method of any one of claims 1-40, wherein any one of P0, P1, P2, P3, or P4 are performed in a cell culture medium supplemented with ascorbic acid, fetal bovine serum, chondroitin sulfate, calcium chloride, and/or a Rho kinase inhibitor.
- 25 42. The method of claim 41, wherein the Rho kinase inhibitor is Y-27632.
43. The method of any one of claims 1-42, wherein at least 70% of the CECs are viable at the end of P4 as determined by a cell viability assay.
- 30 44. The method of claim 43, wherein at least 90% of the CECs are viable at the end of P4 as determined by a cell viability assay.

45. The method of any one of claims 1-44, wherein at least 70%, optionally at least 75%, of the CECs have a cell surface expression at the end of P4 of a marker selected from the group consisting of CD166 positive, CD44 negative to CD44 weakly positive, CD24 negative to weakly positive, CD44 negative to weakly positive, CD105 negative to weakly positive, CD26
5 negative to weakly positive, CD200 negative to weakly positive, and CD90 negative to weakly positive phenotypes.

46. The method of any one of claims 1-45, wherein at least 70%, optionally at least 75%, of the CECs have a cell surface expression at the end of P4 of a marker selected from the group
10 consisting of sodium-potassium ATPase, ZO-1, VDAC3, SLC4A4, CLCN3, COL4A2, COL8A1, COL8A2, CDH2, CD98, CD166, CD340, Integrin $\alpha\beta 1$, CD56, Prdx-6, CD248, SLC4A11, and CYYR1.

47. The method of any one of claims 1-46, wherein at the end of P4 at least 70% of the
15 CECs are viable as determined by a cell viability assay;
at least 75% of the CECs have a cell surface expression at the end of P4 of a marker selected from the group consisting of CD166 positive, CD44 negative to CD44 weakly positive, CD24 negative to weakly positive, CD44 negative to weakly positive, CD105 negative to weakly positive, CD26 negative to weakly positive, CD200 negative to weakly positive, and CD90
20 negative to weakly positive phenotypes; and

at least 75% of the CECs have a cell surface expression at the end of P4 of a marker selected from the group consisting of sodium-potassium ATPase, ZO-1, VDAC3, SLC4A4, CLCN3, COL4A2, COL8A1, COL8A2, CDH2, CD98, CD166, CD340, Integrin $\alpha\beta 1$, CD56, Prdx-6, CD248, SLC4A11, and CYYR1.

25

48. The method of any one of claims 1-47, wherein at least 70% of the CECs at the end of P4 have a hexagonal morphology as detected by microscopy.

49. The method of any one of claims 1-48, wherein the cells are grown on a cell culture surface
30 comprising an extracellular matrix.

50. The method of any one of claims 1-49, wherein the cells are grown on a cell culture surface comprising an extracellular matrix protein.

51. The method of claim 50, wherein the extracellular matrix protein is selected from the group consisting of collagen, laminin, fibronectin, proteoglycan, SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A, SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1, insulin-like growth factor-binding protein 5, vitronectin, fibrillin-1, fibrillin-2, tensin, Wnt-5b, citron Rho-interacting kinase, chondroitin sulphate proteoglycan 4, cyclin-dependent kinase 1, cyclin-dependent kinase 4, periostin, thrombospondin-4, Tubulin alpha chain-like 3, Tubulin alpha-1B chain, Tubulin beta-1 chain, Tubulin beta-4A chain, and versican core protein.
52. The method of any one of claims 1-51, wherein the size of the culture vessel is increased with each passage.
53. The method of any one of claims 1-52, wherein the culture vessel is tissue-culture (TC)-treated.
54. The method of any one of claims 1-53, wherein the CECs are cultured for use in a therapeutic composition for use in humans.
55. A corneal endothelial cell (CEC) composition comprising CECs prepared by the method of any one of claims 1-54.
56. A pharmaceutical composition comprising the CEC composition of claim 55.
57. A tissue-culture treated (TC-treated) cell culture vessel comprising corneal endothelial cells (CECs).