

CORNEAL ENDOTHELIAL CELL COMPOSITIONS AND DELIVERY METHODS THEREOF

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 63/616,104, filed on
5 Dec. 29, 2023, the contents of which are incorporated herein by reference in their entirety.

BACKGROUND

Corneal endothelial disease is a sight-threatening and debilitating condition affecting
millions of people throughout the world. When corneal endothelial cells die or degrade, they do
10 not regenerate. If left untreated, corneal endothelial cell loss can cause corneal edema and loss
of vision. Although topical therapy can relieve symptoms of early-stage disease, the only
treatments for more severe corneal endothelial disease are full- or partial-thickness corneal
transplantation, referred to as penetrating (PK) or endothelial (DSAEK, DMEK) keratoplasty,
respectively. Although corneal transplants are effective, there are disadvantages with these
15 procedures, including limited donor organ supply and the risk of donor rejection. In addition,
post-operative recovery for corneal transplant patients requires that the patients lie flat on their
backs for up to three days for the transplant to adhere to the corneal stroma.

To address these limitations, injectable cell therapies to treat corneal endothelial disease
have been developed. In early clinical studies, injected corneal endothelial cells (CECs) have
20 been used to replenish endothelial cells in Fuchs' endothelial corneal dystrophy, pseudophakic
bullous keratopathy, pseudoexfoliation syndrome keratopathy and corneal graft failure
(Kinoshita S, Koizumi N, Ueno M, et al. *N Engl. J Med.* 2018;378(11):995-1003). For example,
AURN001 is a combination cell therapy including corneal endothelial cells (i.e., neltependocel
(allogeneic human corneal endothelial cells) and a Rho kinase (ROCK) inhibitor compound (i.e.,
25 Y-27632). Adhesion of the CECs is promoted by ROCK inhibition through the suppression of the
Rho/ROCK/MLC (myosin light chain)-signaling cascade. Without the ROCK inhibitor, the
dissociated cells contract due to actin cytoskeleton activation, which leads to a significant
decrease in the cell surface area and a decrease in cell adhesion (Okumura N, Sakamoto Y,
Fujii K, et al. *Sci Rep.* 2016;6:1-11.) CEC therapy presents an exciting treatment for those
30 having corneal endothelial diseases. As such, a need exists for improved compositions and
methods of delivering CEC therapy.

SUMMARY OF THE INVENTION

Injectable corneal endothelial cell (CEC) therapies that rely on CEC suspensions enable the cells to restore corneal thickness and vision in patients affected by corneal diseases. However, cell delivery methodologies often require patients to remain immobile in a prone position for hours to promote cell adhesion, limiting the patient and the medical provider.

Provided herein are methods and related compositions for treating corneal endothelial disease with corneal endothelial cell (CEC) aggregates. The provided CEC aggregate compositions and methods enable faster CEC delivery relative to methods that rely on CEC suspensions, thereby promoting cell adhesion and viability and reducing the amount of time that patients spend in a prone position following injection. The present methods and compositions also have the advantage of potentially eliminating a requirement for a combination therapy involving a Rho kinase inhibitor.

In one aspect, the methods described herein relate to a corneal endothelial cell (CEC) composition including a plurality of aggregates of human corneal endothelial cells (CEC), wherein an aggregate includes at least two cells.

In some embodiments, at least 1% (e.g., 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more) of the CECs are in aggregate form relative to single-cell form. In some embodiments, at least 50% of the CECs are in aggregate form relative to single-cell form. In some embodiments, at least 80% of the CECs are in aggregate form relative to single-cell form. In some embodiments, at least 90% of the CECs are in aggregate form relative to single-cell form.

In some embodiments, at least 50% of the CECs are in aggregate form in aggregates comprising at least 50 cells relative to single-cell form. In some embodiments, at least 80% of the CECs are in aggregate form in aggregates comprising at least 50 cells relative to single-cell form. In some embodiments, at least 90% of the CECs are in aggregate form in aggregates comprising at least 50 cells relative to single-cell form.

In some embodiments, the plurality of aggregates have a diameter of at least about 20 microns (e.g. 20 microns, 30 microns, 40 microns, 50 microns, 60 microns, 70 microns, 80 microns, 90 microns, 100 microns, 150 microns, 200 microns, 250 microns, 300 microns, 350 microns, 400 microns, 450 microns, 500 microns, 550 microns, 600 microns, 650 microns, 700 microns, 750 microns, 800 microns, 850 microns, 900 microns, 950 microns, 1 mm, 1.1 mm, 1.2 mm, 1.3 mm, 1.4 mm, 1.5 mm, 1.6 mm, 1.7 mm, 1.8 mm, 1.9 mm, or more than about 2 mm). In some embodiment, the plurality of aggregates have a diameter of at least about 50 microns. In

some embodiment, the plurality of aggregates have a diameter of at least about 100 microns. In some embodiment, the plurality of aggregates, e.g., at least 60%, 70%, or 80% of aggregates, have a diameter of about 50-300 microns. In some embodiment, the plurality of aggregates, e.g., at least 60%, 70%, or 80% of aggregates, have a diameter of about 50-250 microns. In some embodiment, the plurality of aggregates, e.g., at least 60%, 70%, or 80% of aggregates, have a diameter of about 50-200 microns. In some embodiment, the plurality of aggregates, e.g., at least 60%, 70%, or 80% of aggregates, have a diameter of about 100-300 microns. In some embodiment, the plurality of aggregates, e.g., at least 60%, 70%, or 80% of aggregates, have a diameter of about 100-250 microns. In some embodiment, the plurality of aggregates, e.g., at least 60%, 70%, or 80% of aggregates, have a diameter of about 100-200 microns.

In some embodiments, the plurality of aggregates is at a density of at least about one aggregate per 300 microliters. In some embodiments, the plurality of aggregates is at a density of at least about one aggregate to one million aggregates per 300 microliters. In some embodiments, the plurality of aggregates is at a density of about 1×10^4 to about 1×10^5 aggregates per 300 microliters. In some embodiments, the plurality of aggregates is at a density of about 1×10^4 to about 1×10^6 aggregates per 300 microliters.

In some embodiments, the CEC composition further includes a cell substrate. In some embodiments, the cell substrate is selected from the group consisting of collagen, gelatin, cellulose, polystyrene, polyester, polycarbonate, poly(N-isopropylacrylamide), polylactic acid, polyglycolic acid, hydroxyapatite, and amniotic membrane.

In some embodiments, the composition does not include a rho kinase inhibitor.

In another aspect, the present disclosure provides a pharmaceutical composition including the CEC composition described herein and a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition includes an effective dose of corneal endothelial cells (CECs) for the treatment of a corneal disorder.

In a further aspect, the present disclosure provides a method of manufacturing corneal endothelial cell (CEC) aggregates, said method including seeding CECs into a three-dimensional culture; and incubating the CECs in the three-dimensional culture to form CEC aggregates.

In some embodiments, the CECs are seeded onto a two-dimensional culture prior to seeding the CECs into the three-dimensional culture. In some embodiments, the three-dimensional culture includes a scaffold. In some embodiments, the three-dimensional culture

includes a scaffold-free suspension. In some embodiments, the method further includes subjecting CEC to fluid dynamics that result in aggregate formation.

In some embodiments, the three-dimensional culture is maintained for less than one minute. In some embodiments, the three-dimensional culture is maintained for at least about 1
5 hour. In some embodiments, the three-dimensional culture is maintained for at least about 10 hours. In some embodiments, the three-dimensional culture is maintained for at least about 18 hours.

In some embodiments, the method further includes plating the CEC aggregates onto a cell substrate. In some embodiments, the cell substrate is selected from the group consisting of
10 collagen, gelatin, cellulose, polystyrene, polyester, polycarbonate, poly(N-isopropylacrylamide), polylactic acid, polyglycolic acid, hydroxyapatite, and amniotic membrane.

In some embodiments, the method further includes harvesting the CEC aggregates.

In some embodiments, the method includes incorporating the CEC aggregates into a pharmaceutical composition. In some embodiments, the pharmaceutical composition lacks a
15 Rho kinase inhibitor.

In some embodiments, the CECs are human CECs (hCECs).

In a further aspect, provided is a method for treating or preventing a corneal endothelial disease in a subject in need thereof, including administering an effective amount of any CEC aggregate composition provided herein. In some embodiments, the method includes
20 administering an effective amount a pharmaceutical composition provided herein.

In another aspect, provided herein is a method for treating or preventing a corneal endothelial disease in a human subject in need thereof, including administering an effective amount of a composition including corneal endothelial cell (CEC) aggregates to an eye of a subject.

25 In some embodiments, the subject lies face down in a prone position for less than three hours after administration of the composition including the CEC aggregates.

In some embodiments, the method involves administering the composition including the CEC aggregates to the subject in the absence of a rho kinase inhibitor.

In some embodiments, the method involves administering the composition including the
30 CEC aggregates to an anterior chamber of the eye of the subject.

In some embodiments, the effective amount of the CEC aggregates includes at least about 1×10^3 CECs. In some embodiments, the effective amount of the CEC aggregates

includes about 1×10^3 to about 2×10^6 CECs. In some embodiments, the effective amount of the CEC aggregates includes about 1×10^5 to about 2×10^6 CECs.

In some embodiments, the corneal endothelial disease is a bullous keratopathy, a corneal edema, a corneal leukoma, a corneal endothelial inflammation, or a corneal dystrophy.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A-1E depict the results of an assay evaluating the kinetics of corneal endothelial cell (CEC) aggregate adhesion. **Fig. 1A** depicts images of plates (AGGREWELL™) shortly after seeding a CEC suspension into each well (left panel) and 24 hours after incubation to permit the formation of aggregates (right panel). **Figs. 1B** and **1C** depicts micrographs at 5x magnification (**Fig. 1B**) and 10x magnification (**Fig. 1C**) of CEC aggregates plated on collagen-coated plate after incubation at each of the indicated time points (i.e., 15 min, 60 min, and 180 min). **Fig. 1D** depicts micrographs at 10x magnification of a cell monolayer formed by CEC aggregates plated on a collagen-coated plate after 14 days of incubation. **Fig. 1E** depicts micrographs at 10x magnification of a cell monolayer formed by CEC aggregates (left panel) and a CEC suspension (right panel) plated on a collagen-coated plate after 25 days of incubations.

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Fig. 2 graphically depicts the results of an assay evaluating the adhesion of CEC aggregates to a collagen-coated plate in the presence (+Y) and absence (-Y) of Y-27632 (an inhibitor of Rho-associated, coiled-coil containing protein kinase [ROCK]).

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DETAILED DESCRIPTION OF INVENTION

I. Definitions

As used herein, the term “corneal endothelial disease” refers to a disease affecting corneal endothelial cells. 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, 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,

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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 “corneal endothelial cell” or “CEC” refers to a cell derived from a corneal endothelium layer or a cell that otherwise has functional and biochemical characteristics of cells in the corneal endothelium layer, including but not limited to primary culture cells, cultured or subcultured cells, and cells induced to differentiate from undifferentiated cells such as stem cells (e.g., embryonic stem cells or induced pluripotent stem cells (iPSCs)). 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. doi: 10.1167/iov.16-19771. PMID: 27564520; Wongvisavavit, R., *et al* (2021). *Regenerative medicine*, 16(09), 871-891).

As used herein, the term “cell aggregate” or “aggregate form” refers to a plurality of corneal endothelial cells (e.g., at least two CECs) clustered together into a three-dimensional structure.

As used herein, the term “treating” in the context of a corneal endothelial disease, refers to therapeutic treatment in order to alleviate one or more symptoms of a corneal endothelial disease. A subject who is treated according to the methods described herein, has a corneal endothelial disease, such that treatment alleviates or slows progression of one or more symptoms of the disease. Exemplary symptoms of the corneal endothelial disease include, but are not limited to, loss of vision, blindness, mechanical disruption of the visual axis, opacification and decreased vision, or an otherwise impairment of visual function. In some embodiments, the present disclosure provides a method of reducing or ameliorating these symptoms. That is, in some embodiments, the present disclosure provides a method of increasing vision by administering CEC aggregates to a subject having a corneal endothelial disease.

The term “preventing” as used herein in the context of a corneal endothelial disease refers to a prophylactic measure whereby symptoms of a corneal endothelial disease are inhibited in a subject who may be at risk of developing a corneal endothelial disease or who has been diagnosed with a corneal endothelial disease but is not yet showing certain symptoms, e.g., is not yet showing vision loss. In some embodiments, the disclosure provides a method of

preventing vision loss in a patient who is at risk of a corneal endothelial disease or who has been diagnosed with a corneal endothelial disease who is at risk of vision loss.

The terms "patient" and "subject" are used interchangeably herein. Preferably, the patient is a human patient.

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II. Corneal Endothelial Cell Aggregate Compositions

Provided herein are methods and related compositions involving corneal endothelial cell aggregates for use in treating or preventing corneal endothelial disease. The corneal endothelial cell (CEC) aggregates of the provided compositions have properties that improve delivery times and help mediate cell viability and adhesion for proliferation *in vivo* (e.g., following
10 transplantation into a cornea of a subject). Cell aggregates have several advantages over cells in suspension, including the ability to sink and attach to the posterior side of the cornea faster than current CEC cell suspensions. CEC aggregates also have improved viability relative to CECs in suspension, which are more prone to apoptosis and cell death.

Accordingly, provided herein is a CEC composition comprising a plurality of aggregates of corneal endothelial cells (CEC), such as human CECs (hCECs). Preferably, each cell aggregate comprises a plurality of viable CECs. Although the exact number of cells per aggregate may vary, one skilled in the art will recognize that the size of each aggregate and the number of cells per aggregate is limited by the capacity of nutrients to diffuse to the central
15 cells, and that this number may vary within the composition. Cell aggregates may comprise a minimal number of cells (e.g., two or three cells) per aggregate, or may comprise hundreds of cells per aggregate. Accordingly, an individual composition may have aggregates with a range of sizes and cell numbers.

In some embodiments, at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%,
25 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or more than 90% of the cells in the composition are in aggregate form. In some embodiments, the cells in the present compositions are primarily in aggregate form in contrast to cell suspensions, which primarily include single cells in non-aggregate form. For example, in some embodiments, at least about 50%, 60%, 70%, 80%, 90%, or more than 90% of the cells in the composition are
30 in aggregates. In certain embodiments, at least about 50% of the cells are in aggregate form in the composition.

In some embodiments, the plurality of aggregates are at a density of at least about one aggregate per 300 microliters (e.g., 1 aggregate, 10 aggregates, 50 aggregates, 100

aggregates, 500 aggregates or more per 300 microliter). In some embodiments, the plurality of aggregates are at a density of at least about 100 aggregates per 300 microliters (e.g., 100 aggregates, 250 aggregates, 500 aggregates, 750 aggregates, 1000 aggregates or more per 300 microliter). In some embodiments, the plurality of aggregates is at a density of at least
5 about 1000 aggregates per 300 microliters (e.g., 1000 aggregates, 2500 aggregates, 5000 aggregates, 7500 aggregates, 10,000 aggregates or more per 300 microliters). In some embodiments, the plurality of aggregates is at a density of at least about 10,000 aggregates per 300 microliters (e.g., 10,000 aggregates, 25,000 aggregates, 50,000 aggregates, 75,000 aggregates, 100,000 aggregates or more per 300 microliters). In some embodiments, the
10 plurality of aggregates is at a density of at least about 100,000 aggregates per 300 microliters (e.g., 100,000 aggregates, 250,000 aggregates, 500,000 aggregates, 750,000 aggregates, 1,000,000 aggregates or more per 300 microliters). In some embodiments, the plurality of aggregates are at a density of about 1 to about 1×10^6 aggregates per 300 microliters (e.g., about 1 to about 1×10^2 aggregates, about 1 to about 1×10^3 aggregates, about 1 to about $1 \times$
15 10^4 aggregates, about 1 to about 1×10^5 aggregates, about 1×10^2 to about 1×10^3 aggregates, about 1×10^2 to about 1×10^4 aggregates, about 1×10^2 to about 1×10^5 aggregates, about 1×10^2 to about 1×10^6 aggregates, about 1×10^3 to about 1×10^4 aggregates, about 1×10^3 to about 1×10^5 aggregates, about 1×10^3 to about 1×10^6 aggregates, about 1×10^4 to about $1 \times$
20 10^5 aggregates, about 1×10^4 to about 1×10^6 aggregates, about 1×10^5 to about 1×10^6 aggregates, or about 2×10^4 aggregates per 300 microliters).

In some embodiments, the plurality of cell aggregates includes from about two to 1000 or more cells per aggregate. In some embodiments, the plurality of aggregates have an average of at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, or more than 1000 cells per aggregate. In certain
25 embodiments, the plurality of aggregates has an average of at least about 50 cells per aggregate.

In some embodiments, a subset of at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, and 95% of the aggregates in the population have a cell number above a given threshold. For example, in some embodiments, at least about 50% of the aggregates have at
30 least 2, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, or more than 100 cells. In certain embodiments, at least about 50% of the aggregates have at least about 50 cells.

In some embodiments, the cell aggregates are from about 20 microns (i.e., about two cells) to about 1 mm or more in size (e.g., diameter). For example, in some embodiments, the plurality of cell aggregates have an average diameter of at least about 20 microns, 30 microns, 40 microns, 50 microns, 60 microns, 70 microns, 80 microns, 90 microns, 100 microns, 150
5 microns, 200 microns, 250 microns, 300 microns, 350 microns, 400 microns, 450 microns, 500 microns, 550 microns, 600 microns, 650 microns, 700 microns, 750 microns, 800 microns, 850 microns, 900 microns, 950 microns, 1 mm, 1.1 mm, 1.2 mm, 1.3 mm, 1.4 mm, 1.5 mm, 1.6 mm, 1.7 mm, 1.8 mm, 1.9 mm, or more than about 2 mm. In some embodiment, the plurality of aggregates have a diameter of at least about 50 microns or 100 microns. In some embodiment,
10 the plurality of aggregates, e.g., at least 60%, 70%, or 80% of aggregates, have a diameter of about 50-300 microns, 50-250 microns, 50-200 microns, 100-300 microns, 100-250 microns, or 100-200 microns.

The size of the cell aggregates may vary within a composition (e.g., non-uniform in size). However, in some embodiments, the size of the aggregates present may be substantially
15 uniform in size. By “substantially uniform in size” it is meant that the aggregates' size distribution has a spread not larger than about 10%. In one embodiment, the aggregates' size distribution has a spread not larger than about 5%.

The cell aggregates used herein can be of various shapes, such as, for example, a sphere, a cylinder, rod-like, or cuboidal (i.e., cubes), among others. In certain embodiments, the
20 aggregates are spheroidal in shape. By “spheroidal cell aggregates” it is meant that while the aggregate is generally shaped like a sphere or ellipsoid, the radii of curvature of the aggregate may not be substantially equal for all points on the surface of the aggregate (i.e., vary by substantially more than 10% over all points on the surface of the aggregate).

In some embodiments, the plurality of aggregates is non-uniform in shape. In some
25 embodiments, the plurality of aggregates is substantially uniform in shape. By “substantially uniform in shape” it is meant that the spread in uniformity of the aggregates is not more than about 10%. In another embodiment, the spread in uniformity of the aggregates is not more than about 5%.

In some embodiments, the CEC aggregates of the composition are in a monolayer
30 structure. In further embodiments, the CEC aggregates are layered onto a cell substrate. In addition, the substrate may act as a scaffold for cultivating the corneal endothelial cells or may only carry the corneal endothelial cell layer after culture. In certain embodiments, the substrate

is used for culturing the corneal endothelial cells and also acts as a scaffold that can be transplanted after completion of the culture.

Examples of the 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 some embodiments, the aforementioned substrate is collagen.

Accordingly, in some embodiments, the compositions herein include a substrate (e.g., a collagen substrate) and a cultured CEC aggregate layer. In some embodiments, CECs are (1) cultured in a two-dimensional culture vessel (e.g., culture dish, culture tube, culture tank etc.), (2) the cells are passaged via further sub-cultures (e.g., 1-100 passages), (3) the cells are cultured in three-dimensional culture to form cell aggregates; and/or (4) the aggregates are further plated on a cell substrate (e.g., collagen substrate).

In some embodiments, the cultured corneal endothelial cells may have at least one, at least two, or all of the following characteristics (e.g., characteristics similar to CECs found *in vivo*).

- (1) The cells may have a monolayer structure.
- (2) The cell density of the cells may be about 10 to -about 10,000 cells/mm².
- (3) The visual flat plane shape of the cells may be approximately hexagonal.
- (4) In the cell layer, cells may be regularly aligned.
- (5) the cells may express markers characteristic of CECs found *in vivo*. For example, the CECs have a cell surface expression 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, the CECs have a cell surface expression at the end of P4 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 CYR1.

Having such morphological characteristics, the composition herein may have functions similar to those of the corneal endothelial cells 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 another prodrug (e.g., ester or the like). Pharmaceutically acceptable salts include salts formed with a free
5 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 aggregate compositions provided herein may be formulated with or without a
10 rho kinase inhibitor. Previously, ROCK inhibition was considered necessary to promote adhesion of corneal endothelial cell in cell culture (see, e.g. U.S. Patent No. US11633404B2). However, as shown in the present Examples (e.g., see Example 3), CEC aggregates may achieve adhesion in the presence or absence of a Rho kinase inhibitor. In the present disclosure, "Rho kinase" or "ROCK" (Rho-associated coiled-coil forming kinase: Rho-bound
15 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
20 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
25 , 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
30 monohydrate), and the like), and preferably comprising Y-27632.

In some embodiments, the CEC aggregate composition includes a Rho kinase inhibitor. For example, a Rho kinase inhibitor may be added when culturing, proliferating, differentiating or maturing aggregates 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.

5 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 aggregates of corneal endothelial cells.

 Further provided are pharmaceutical compositions including the CEC aggregate compositions described herein and a pharmaceutically acceptable carrier or excipient. As used
10 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. As used herein "carrier" refers to a culture, infusion vehicle,
15 irrigating solution, diluent, adjuvant, excipient, or vehicle administered in conjunction with a medicament, such as a cellular composition provided herein.

 Such a composition contains a therapeutically effective amount of cellular agent together with a suitable amount of carrier, such that the composition is provided in a form suitable for administration to a patient. In some embodiments, the pharmaceutically acceptable carrier is a
20 cell infusion vehicle. The cell infusion vehicle can be any solution in which a cell can be maintained. Cell infusion vehicles include those which can be used as an intraocular irrigating solution or the like. Examples of solutions used as a cell infusion vehicle include Opti-MEM (e.g., with or without additional supplements), Opeguard-MA, Opeguard-F, and the like.

 The cell infusion vehicle may further comprise additional components, such as at least
25 one of albumin, ascorbic acid (or ascorbate), and lactic acid (or lactate). Addition of these components may facilitate cell maintenance. In some embodiments, albumin, ascorbic acid (or ascorbate), and lactic acid (or lactate) are added to a cell infusion vehicle. In one embodiment, a solution using Opeguard-MA® or Opti-MEM and at least one, two or all three of albumin, ascorbic acid, and lactic acid is used.

30 The composition can be prepared as a pharmaceutical composition adapted to administration to humans in accordance with a known method. Such a composition can be administered by injection or infusion. When a composition is to be administered by injection, the

composition can be distributed by using an injection bottle containing cell infusion solution, aseptic agent-grade water or saline.

III. Methods of Manufacturing Corneal Endothelial Cell Aggregate Compositions

5 Further provided herein are methods of manufacturing corneal endothelial cell (CEC) aggregate compositions. Methods for harvesting and culturing CEC cells in suspension are generally known in the art (See, for example, Kinoshita, S., *et al.* (2018). *New England Journal of Medicine*, 378(11), 995-1003). However, such methods form CEC cell suspensions that reduce the formation of CEC aggregates. In contrast, the manufacturing methods provided
10 herein form compositions enriched in CEC aggregates for improved delivery and adhesion in therapeutic contexts.

The methods of manufacturing provided herein include manufacturing of CEC as a component of a pharmaceutical composition, e.g., a pharmaceutical composition for intraocular administration. In certain embodiments, at least one step of culturing is performed in a closed
15 culture system.

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
20 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, *et al.* *Invest Ophthalmol Vis Sci.* 2016 Aug 1;57(10):4385-92. doi: 10.1167/iops.16-19771. PMID: 27564520; and
25 Wongvisavavit, R., *et al* (2021). *Regenerative medicine*, 16(09), 871-891, which are each hereby incorporated by reference).

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
30 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.

In some embodiments, the CECs are initially cultured in two-dimensional cell culture before generation of cell aggregates. 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 a FNC coating mix may be used.

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 25°C to about 45°C or about 30°C to about 40°C. In certain embodiments, the cells are cultured at a temperature of about 37° C. As a cultivation method, the cells may be cultured in a conventional incubator for cell culture under humidification in an environment of about 5-10% CO₂.

Subculture

In some embodiments, prior to generating the cell aggregates, the cultured corneal endothelial cells can be subjected to a subculture via the passaging of cells into fresh growth medium. In some embodiments, sub-confluent or confluent cells are subjected to the subculture.

- 5 In some embodiments, the subculture includes one or more of the following steps. First, the cells may be detached from the surface of the culture vessel (e.g., by a treatment with trypsin-EDTA, TrypLE enzyme, etc.) and recovered. 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., 500 rpm (30 G)-1000 rpm (70 G), 1 to 10
- 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 in the same manner as in the above-mentioned initial culture. 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
- 15 depending on the condition of the cells to be used. In some embodiments, the culture time is 7-30 days. This subculture can be performed multiple times where necessary. In some embodiments, the growth medium is exchanged every three to four days.

- In some embodiments, the cultured CECs are passaged at least one time prior to generation of CEC aggregates. In certain embodiments, the cultured CECs are passaged at
- 20 least two times prior to generation of CEC aggregates. In some embodiments, the CECs are collected after passage two. In some embodiments, the CECs are collected after passage three.

Generation of Corneal Endothelial Cell Aggregates

- To generate corneal endothelial cell (CEC) aggregates, the CECs collected from the
- 25 aforementioned steps may be seeded into a three-dimensional culture using a three-dimensional culturing method. Three dimensional cultures may be grown with scaffold or scaffold-free techniques to promote the formation of aggregates. Examples of three-dimensional cell culturing systems are described, for example, in Edmondson, Rasheena et al. *Assay and drug development technologies*, vol. 12,4 (2014): 207-18; Jensen, Caleb, and Yong Teng. *Frontiers in molecular biosciences*, 7 (2020): 33. Additionally or alternatively, the CECs may be
- 30 subjected to CEC to fluid dynamics that result in aggregate formation.

In some embodiments, the three-dimensional culture comprises a scaffold, such as a hydrogel, bioceramic, metallic, or polymer scaffold. In some embodiments, the scaffold is a solid

surface (e.g., a plate or well) having a plurality of microwells or cavities (see, e.g., International Publication Nos. WO2008106771A1, which is hereby incorporated by reference). In one embodiment, the solid scaffold comprising microwells is an AGGREWELL™ plate. The microwells may be of a variety of sizes, depending on the particular embodiment and the intended use of plate. For example, wells may have a dimension of about 100 microns, 200 microns, 400 microns, or about 800 microns. In one embodiment, the microwell is about 400 microns in diameter. The surface may include a plurality of microwells (e.g., at least 1000 microwells).

In some embodiments an anti-adherent coating (e.g., pluronic acid or commercially available anti-adherent solutions, e.g., StemCell Technologies, cat#421254) may be applied to sidewalls of the scaffold surface. However, in some embodiments, due to the characteristics of the surface, such a coating may not be necessary to promote aggregation. In yet other embodiments, sidewalls may be Matrigel coated. In some embodiments, the surface is washed with the anti-adherence solution prior to plating the CEC cells on the surface. In some embodiments, the surface is further washed with a cell culture medium.

Once a scaffold is prepared, cells can be seeded onto the surface to generate cell aggregates. For example, the number of cells in a suspension of corneal endothelial cells can be counted and a desired number of cells can be added to the surface in culture medium. For example, in the case of surfaces having microwells, a desired number of cells per microwell can be added (e.g., 10, 20, 30, 40, 50, or more cells per microwell). The cells may be centrifuged (e.g., at 100xg for 3 minutes) to ensure the cells are sedimented to the bottom of the scaffold surface.

In some embodiments, the three-dimensional culture comprises a scaffold-free culture that promotes formation of CEC aggregates, e.g., forced-floating method, hanging drop method, or agitation-based method. In the forced-floating method, a cell suspension is loaded into the wells of a low adhesion polymer-coated microplate. The microplate is then centrifuged to force the cells to form aggregates. In the hanging drop method, a cell suspension is loaded into the wells of a hanging drop plate. The suspension will hang from the plate in droplets. The cells aggregate in the tips of these drops and form aggregates. In the agitation-based method, a cell suspension is placed in a rotating bioreactor. The cells do not adhere to the walls due to the continuous stirring, resulting in the formation of aggregates.

Once the cells have been seeded into an appropriate three-dimensional culture, the cells may be incubated for a period of time to permit aggregate formation. For example, in some

embodiments, the cells are incubated for at least about 1 hour (e.g., at least about 1 hour, 2 hours, 5 hours, 10 hours, 15 hours, 18 hours, 20 hours, 24 hours, or 48 hours or any value therebetween) during which the cells form aggregates ("first incubation"). In some embodiments, the cells are incubated for about 1 hour to 48 hours, 1 to 2 hours, 2 to 5 hours, 5
5 to 10 hours, 10 to 15 hours, 16 to 24 hours, or 18 to 20 hours. Alternatively, the cells may be incubated for shorter periods of time (e.g., in instances where additional methodologies, such as centrifugation, are used to facilitate aggregate formation). Accordingly, in some embodiments, the cells are incubated for less than one hour (e.g., less than 1 hour, 45 minutes, 30 minutes, 15 minutes, 10 minutes, 5 minutes, 1 minute, 30 seconds, 20 seconds, 10 seconds, 10 minutes to
10 less than an hour, 10-50 minutes, 10-40 minutes, 10-30 minutes, 10-20 minutes), during which the cells form aggregates.

Following the period of aggregate formation, the aggregates may be recovered (e.g., by either pipetting or by spinning out the aggregates). Optionally, following this procedure, the aggregates may be maintained in suspension for a period ranging from 1 to 6 days ("second
15 incubation"). The aggregates may then be harvested for analysis or further processing. Using this protocol, aggregates may self-organize over time. This may occur in the original well plate during the first incubation or after recovery during the second incubation.

After collection of aggregates, in some embodiments, the CEC aggregates may be plated onto a cell substrate to form a cell layer. In certain embodiments, the substrate is used
20 for culturing the corneal endothelial cells and also acts as a scaffold that can be transplanted after completion of the culture. 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,
25 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).

The number of cells to be seeded may be adjusted to form a cell layer having a desired cell density in the finally-produced corneal endothelial preparation. In some embodiments, the cells are seeded to form a cell layer having a cell density of about 1,000-about 5,000 cells/mm².
30 The aggregates may be incubated at any suitable temperature and time to permit adhesion to the cell substrate. For example, in some embodiments, the plate is incubated at a temperature of about 25°C to about 45°C or about 30°C to about 40°C. In certain embodiments, the cells are cultured at a temperature of about 37° C.

The culture time may vary depending on the condition of the cells to be used. For example, in some embodiments, the culture time is 3-30 days. In some embodiments, the CEC aggregates are incubated on the cell substrate for e.g., at least 3 days, at least one week, at least two weeks, at least three weeks or more than three weeks. The CEC aggregates may then be collected for incorporation into a composition, e.g., a pharmaceutical composition for therapeutic uses.

To form a composition suitable for administration to a subject, the aggregates may be mixed with a suitable carrier to maintain viability of the corneal endothelial cells before transplantation. Examples of such carriers include an OPTIM-MEM I medium, corneoscleral graft presentation solution OPTISOL-GS™, an eye preservation solution for corneal transplantation EPII™, saline, phosphate buffered saline (PBS) and the like.

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

IV. Therapeutic Uses

Further provided herein are methods of treating or preventing a corneal endothelial disease in a subject in need thereof by administering an effective amount of a composition having aggregates of corneal endothelial cells to an eye of the subject.

Corneal endothelial disease

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, 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 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 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 aggregate composition of the present disclosure is for use in treating or preventing a corneal endothelial disease. Non-limiting
5 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
10 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.

15 Corneal endothelial diseases include those having different grades, such as corneal endothelial disorder Grade 3 (e.g., CEC density below 500 cells/mm² but no corneal edema) or corneal endothelial disorder Grade 4 (e.g., bullous keratopathy). This grade system was previously established based upon the severity of the corneal endothelial disorders (e.g., see Japanese Journal of Ophthalmology 118: 81-83, 2014).

20 In some embodiments, the corneal endothelial disease is a bullous keratopathy, a corneal edema (e.g., Corneal Edema Secondary to Corneal Endothelial Dysfunction), a corneal leukoma, a corneal endothelial inflammation, or a corneal dystrophy.

In some embodiments, the disease is a bullous keratopathy. In some embodiments, the corneal endothelial disease is Fuchs endothelial corneal dystrophy, PEX-BK (pseudoexfoliation
25 bullous keratopathy; bullous keratopathy involving pseudoexfoliation syndrome), post-laser iridotomy bullous keratopathy, post-cataract surgery bullous keratopathy (including pseudophakic or aphakic bullous keratopathy), post-glaucoma surgery bullous keratopathy, intraocular surgery-related bullous keratopathy, post-trauma bullous keratopathy, bullous keratopathy of an unknown cause after multiple surgeries, post-corneal transplantation graft
30 failure, congenital corneal endothelial dystrophy, or congenital anterior chamber angle hypoplasia syndrome.

In some embodiments, the corneal endothelial disease is one characterized by low endothelial cell count. In some embodiments, the corneal endothelial disease is one

characterized by visual impairment, blurred vision, eye discomfort, eye pain, or corneal edema. In one embodiment, the disease is a corneal edema (e.g., Corneal Edema Secondary to Corneal Endothelial Dysfunction).

The CEC aggregates may be prepared for administration by any suitable method, including the presently disclosed manufacturing methods (e.g., see Example 1 and Section III). For example, donor cells may be prepared by isolating CECs with multiple steps of cultivation to ensure desirable cell characteristics. 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 analysis to confirm their biological characteristics and to verify criteria for therapeutic use. Aggregates are then formed and may be stored in media that maintain the cells' biochemical characteristics. Prior to injection the cell aggregates may optionally be supplemented with additional agents, such as a Rho-associated protein kinase (ROCK) inhibitor. However, in some embodiments, the method involves administering the CEC aggregates to the subject in the absence of a rho kinase inhibitor.

In some embodiments, the eye of the recipient is prepared by mechanically removing abnormal extracellular material and degenerated CECs on Descemet's membrane (e.g., in the central 8 mm diameter area) of the cornea. This may be performed with a silicone cannula through a 1.6 mm incision at the corneal limbus under local anesthesia. A desired number of cells (e.g., a total of at least 1×10^5 , at least 1×10^6 CECs, etc.) can then be injected with a syringe into the anterior chamber of the eye. Postoperative care may include topical steroids and/or prophylactic antibiotics.

Unlike traditional delivery methods using CEC suspensions, the patient is not required to lay face-down for three hours, as the CEC aggregates rapidly settle on the inner cornea and allow for cell adhesion. Accordingly, in some embodiments, the subject lies face down for less than three hours (e.g., less than 180 min, 170 min, less than 160 min, less than 150 min, less than 140 min, less than 130 min, less than 120 min, less than 110 min, less than 100 min, less than 90 min, less than 80 min, less than 70 min, less than 60 min, less than 50 min, less than 40 min, less than 30 min, less than 20 min, less than 10 min) after administration of the composition comprising the CEC aggregates. In some embodiments, the subject lies face down for less than three hours, e.g., less than 120 minutes, less than 60 minutes, less than 30 minutes, about 30-60 minutes.

In addition to a cell aggregate, the composition of the present disclosure may be administered in conjunction with an additional agent. Agents that are generally used in

ophthalmic therapy (e.g., steroid agent, antimicrobial, antibacterial or NSAID) may be used. Such an addition agent may be administered together with the cell composition or provided in a separately administered form.

5 Various delivery systems are known, and such systems can be used to administer the composition to a suitable site (e.g., ocular anterior chamber). A typical dosage form of the cellular composition is injected into the anterior chamber. In such a case, cell aggregates can be suspended in an infusion vehicle and injected with a needle (e.g., 26-gauge needle) into the anterior chamber.

10 In one embodiment, the composition can be prepared as a pharmaceutical composition adapted to administration to humans in accordance with a known method. Such a composition can be administered by injection or infusion. When a composition is to be administered by injection, the composition can be distributed by using an injection bottle containing a pharmaceutically acceptable carrier, such as a cell infusion solution, aseptic agent-grade water or saline.

15 The dosage of the composition that is effective in therapy of a specific disorder or condition may vary depending on the properties of the disorder or condition. However, such an amount can be determined by those skilled in the art by a standard clinical technique based on the descriptions herein. Furthermore, an in vitro assay can be used in some cases to assist the identification of the optimal dosing range. The precise dose to be used in a preparation may
20 also vary depending on the administration pathway or the severity of the disease or disorder.

The dosage of the CEC aggregate composition is not particularly limited, but any cell density and amount described herein or within a range between any two values thereof can be used. For example, in some embodiments, the dosage is at least about 1×10^3 cells (e.g., at least about 1×10^3 cells, 2×10^3 cells, 3×10^3 cells, 4×10^3 cells, 5×10^3 , 6×10^3 cells, 7×10^3 cells, 8×10^3
25 cells, 9×10^3 cells or more or any value therebetween). In some embodiments, the dosage is at least about 1×10^4 cells (e.g., at least about 1×10^4 cells, 2×10^4 cells, 3×10^4 cells, 4×10^4 cells, 5×10^4 , 6×10^4 cells, 7×10^4 cells, 8×10^4 cells, 9×10^4 cells or more or any value therebetween). In some embodiments, the dosage is at least about 1×10^5 cells (e.g., at least about 1×10^5 cells, 2×10^5 cells, 3×10^5 cells, 4×10^5 cells, 5×10^5 cells, 6×10^5 cells, 7×10^5 cells, 8×10^5 cells, 9×10^5 cells
30 or more or any value therebetween). In some embodiments, the dosage is at least about 1×10^6 about cells (e.g., at least about 1×10^6 about cells or 2×10^6 cells or any value therebetween). In some embodiments, the dosage is about 1×10^3 cells to about 2×10^6 cells, about 1×10^3 cells to about 1×10^6 cells, about 1×10^3 cells to about 1×10^5 cells, about 1×10^3 cells to about 1×10^4

cells, about 1×10^4 cells to about 2×10^6 cells, about 1×10^4 cells to about 1×10^6 cells, about 1×10^4 cells to about 1×10^5 cells, about 1×10^5 cells to about 2×10^6 cells, about 1×10^5 cells to about 1×10^6 cells, about 2×10^5 cells to about 2×10^6 cells, or any value at or therebetween. In one embodiment, the dose is at least about 1×10^6 cells. In one embodiment, the dose is about 2×10^5 cells to about 2×10^6 cells.

The dosing interval may vary depending on the application. For example, in some embodiments, the CEC aggregate composition is administered via a single dose. In other embodiments, the CEC aggregate composition is administered in multiple doses at an interval of every 1, 7, 14, 21, or 28 days or in the range of any period there between. In some
10 embodiments, the CEC aggregate composition is administered in multiple doses at an interval of one or more years after the first dose (e.g., 1 year, 2 years, 5 years, 10 years, 15 years, 20 years, 25 years, 30 years, or 35 years after the first dose). The dosage, dosing interval, and dosing method may be appropriately selected depending on the age or weight of the patient, symptom, target disease or the like.

15 In some embodiments, the composition includes a therapeutically effective amount of CEC aggregates, or an amount effective for exerting a desired effect. When a marker indicating a pathological condition significantly decreases after administration, the presence of a therapeutic effect may be acknowledged. The effective dose can be estimated from a dose-reaction curve obtained from an in vitro or animal model testing system. In some embodiments,
20 the subject is a mammal. In one embodiment, the subject is a human.

In some embodiments, the method is effective to achieve a desired level of adhesion of the injected cells to the corneal endothelial layer of the subject's eyes. For example, in some embodiments, the method effective to achieve adhesion of at least about 5% (e.g., at least 5%, 10%, 13%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or more) of the CECs to a corneal
25 endothelial layer of the subject within a given time (e.g., 20 minutes). In some embodiments, therapeutic efficacy is measured based on the restoration of corneal transparency with a threshold level of CEC density (e.g., 500 cells per square millimeter at the central cornea) after cell injection into a subject. In a further embodiment, therapeutic efficacy is measured based on a corneal thickness after cell injection (e.g., a corneal thickness of less than 630 μm after cell
30 injection), with a decrease in corneal thickness from the preoperative baseline measurement. In another embodiment, therapeutic efficacy is measured based on an improvement in best corrected visual acuity of two lines or more on a measure of visual acuity after cell injection

(e.g., as measured via Snellen's eye chart, Jaeger eye chart, Landolt C eye chart, or other measures known in the art).

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

Example 1: Materials and methods – Spheroid generation for faster cell human Corneal Endothelial Cell (HCEC) attachment to the substrate for rapid cell delivery

2-D HCEC culture conditions

HCEC primary cell line was cultured in tissue culture flasks in a proprietary Opti-MEM growth medium to passage 2 prior to cell harvest for spheroid generation. The medium was exchanged every 3-4 days and the cells were used for experiments between passages 2 and 3. The cells were cultured in 2D before spheroid generation.

3D spheroid culture generation and culture conditions

The CEC HCEC spheroids were generated using AGGREWELL 400™ plates (Stemcell Technologies, Cat. No. 34411/34415). 24-well AGGREWELL™ plates were used, where each well contained ~1,200 microwells with each microwell measuring 400 μm in diameter.

Before spheroid generation, AGGREWELL™ plates were washed with anti-adherence rinsing solution (StemCell Technologies, cat#421254), 500 μL of solution was used per well. The plates were centrifuged at 1300xg for 5 minutes; the second centrifugation was performed to remove air bubbles trapped in microwells. After aspirating of anti-adherence solution, each well was rinsed with 2 ml of basal medium; the medium was aspirated and replaced by 1 ml complete medium per well. After single cell suspension was prepared and the cells were counted, 6×10^4 cells were added to each well in 1 ml of culture medium-this resulted in 50 cells per microwell. AGGREWELL™ plates containing cells were centrifuged at 100xg for 3 minutes

and observed under inverted microscope to ensure all cells sedimented to the bottom of a microwell.

18-20 hours after spheroid generation, spheroids were harvested from wells by adding culture medium with 1 ml micropipette, rinsing spheroids out of microwells, and passing them through a 37 μ m reversible strainer into a 15 ml Falcon tube. Spheroids were combined from two wells and about 2400 spheroids were plated into collagen-coated clear flat bottom tissue culture plates (Corning, cat#354407). After plating, spheroids were placed into a 37°C incubator and allowed to attach for 20 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, and 3 hours. Spheroids were plated in culture media in either presence or absence of 100 μ M Y-27632 (Eurofins CDMO Alphore, Y-27632 Dichloride, Item code: 70F089P1).

Single cells were plated as controls

Single cells were generated from passage 2 and passage 3 and plated at a concentration of 637,000 cells per well of a collagen-coated clear flat bottom tissue culture plates (Corning, cat#354407) and allowed to attach for the period of 3hrs.

Example 2: CEC aggregate generation and study of adhesion kinetics

This Example evaluated the adhesion of CEC aggregates. CEC aggregates (i.e., spheroids) were generated using the method described in Example 1. A cell suspension including approximately 1.80×10^6 viable cells was utilized for spheroid creation in this Example. A 1x6 well plate (AGGREWELL™) was seeded for 24 hours, with 50 cells per microwell (~6000 spheres for each well of a six well plate). The number of cells in the aggregates was assessed 24 hours after seeding. As shown in **Fig. 1A** (right panel), within 24 hours, CEC aggregates of 50+ cells were formed.

Spheroids were removed from each well were seeded into 6x wells of a collagen-coated 24-well plate to measure and image the kinetics of aggregate attachment. After incubation for 15, 60 or 180 minutes, medium and unattached aggregates in each well were removed and the remaining attached aggregates were subject to analyses. For the analyses, images and timelapse videos were acquired, the number of cells in the attached aggregates were counted, and the number of viable cells in the attached aggregates were counted. Images of the aggregates at each time point are shown at 5x and 10x magnification in **Figs. 1B and 1C**, respectively.

The kinetics of CEC aggregate sedimentation was additionally assessed by video imaging (data not shown). The video showed that the majority of aggregates reached the bottom of the plate within approximately 30 seconds, with smaller aggregates tending to sediment more slowly. This suggests that the sedimentation speed is correlated to CEC aggregate size. Further, timelapse imaging indicated that CEC aggregates formed a cell layer within 24 hours. Within approximately two weeks, the CEC aggregates formed a cell monolayer, as shown in Fig. 1D. Morphologically, the CEC monolayers obtained from CEC aggregates appeared similar to CEC monolayers obtained from CEC suspension.

In summary, the results suggested that CEC aggregates mediate fast CEC adhesion while retaining CEC viability. Further, CEC aggregates appeared reform a CEC monolayer that is similar to a CEC monolayer obtained from a CEC suspension. Altogether, this data suggests that CEC aggregates could recreate an endothelial cell layer able to restore vision.

Example 3: CEC aggregate adhesion kinetics in the presence and absence of a Rho kinase inhibitor (Y-27632)

This Example evaluated the impact of Y-27632 (an inhibitor of Rho-associated, coiled-coil containing protein kinase [ROCK]) on adhesion of CEC aggregates. Previously, Rho kinase inhibitors were considered necessary to promote adhesion of corneal endothelial cell in cell culture (see, e.g., U.S. Patent No. US11633404B2).

CEC aggregates (i.e., spheroids) were generated using the method described in Example 1. 18-20 hours after aggregate formation, the aggregates were harvested and plated into collagen-coated clear flat bottom tissue culture plates (Corning, cat#354407). After plating, spheroids were placed into a 37°C incubator and allowed to attach for 20 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, and 3 hours. Spheroids were plated in culture media in either presence or absence of 100uM Y-27632 (Eurofins CDMO Alphore, Y-27632 Dichloride, Item code: 70F089P1). The total viable cells attached, the percent of viable cells and the percent adhesion are summarized in Table 1.

Table 1. Kinetics of CEC adhesion with and without Y-27632

Condition	Total viable cells attached (n=2)	Viability%	Adhesion%
20 min (+Y)	5.70 x 10 ⁴	99.6	47.5
20 min (-Y)	4.99 x 10 ⁴	100	41.6
30 min (+Y)	5.90 x 10 ⁴	100	49.2
30 min (-Y)	6.30 x 10 ⁴	98.3	52.5
45 min (+Y)	7.20 x 10 ⁴	100	60.0
45 min (-Y)	7.97 x 10 ⁴	100	66.4
60 min (+Y)	1.06 x 10 ⁵	100	88.4
60 min (-Y)	9.67 x 10 ⁴	99.8	81.0
180 min (+Y)	1.07 x 10 ⁵	100	88.8
180 min (-Y)	1.15 x 10 ⁵	100	96.0

With a 1 x 10⁶ cell dose, ~13% adhesion is typically sufficient for efficacy of a cell therapy including a combination of neltependocel (allogeneic human corneal endothelial cells (CECs)) and Y-27632 (an inhibitor of Rho-associated, coiled-coil containing protein kinase (ROCK)). As shown in Table 1 and **Fig. 2**, CEC aggregates achieved greater than 40% adhesion within 20 minutes with or without Y-27632.

These results suggest that CEC delivery using CEC aggregates appears to eliminate the need for a Rho kinase inhibitor, such as Y-27632.

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 corneal endothelial cell (CEC) composition comprising a plurality of aggregates of human corneal endothelial cells (CEC), wherein an aggregate comprises at least two cells.
2. The CEC composition of claim 1, wherein an aggregate comprises at least 50 cells.
3. The CEC composition of claim 1 or 2, wherein at least 1% of the CECs are in aggregate form relative to single-cell form.
4. The CEC composition of claim 1 or 2, wherein at least 50% of the CECs are in aggregate form relative to single-cell form.
5. The CEC composition of any one of claims 1-4, wherein the plurality of aggregates have a diameter of at least about 20 microns.
6. The CEC composition of any one of claims 1-4, wherein at least 60%, 70%, or 80% of the aggregates have a diameter of about 50-250 microns.
7. The CEC composition of any one of claims 1-6, wherein the aggregates have a substantially uniform size.
8. The CEC composition of any one of claims 1-7, wherein the plurality of aggregates is at a density of at least about one aggregate per 300 microliters.
9. The CEC composition of any one of claims 1-8, further comprising a cell substrate.
10. The CEC composition of claim 9, wherein the cell substrate is selected from the group consisting of collagen, gelatin, cellulose, polystyrene, polyester, polycarbonate, poly(N-isopropylacrylamide), polylactic acid, polyglycolic acid, hydroxyapatite, and amniotic membrane.

11. The CEC composition of any one of claims 1-10, wherein the composition does not comprise a rho kinase inhibitor.
12. A pharmaceutical composition comprising the CEC composition of any one of claims 1-11, and a pharmaceutically acceptable carrier.
13. The pharmaceutical composition of claim 12, comprising an effective dose of corneal endothelial cells (CECs) for the treatment of a corneal disorder.
14. A method of manufacturing corneal endothelial cell (CEC) aggregates, said method comprising
 - seeding CECs into a three-dimensional culture; and
 - incubating the CECs in the three-dimensional culture to form CEC aggregates.
15. The method of claim 14, wherein the CECs are seeded onto a two-dimensional culture prior to seeding the CECs into the three-dimensional culture.
16. The method of claim 14 or 15, wherein the three-dimensional culture comprises a scaffold.
17. The method of claim 14 or 15, wherein the three-dimensional culture comprises a scaffold-free suspension.
18. The method of any one of claims 14-17, further comprising subjecting CEC to fluid dynamics that result in aggregate formation.
19. The method of any one of claims 14-18, wherein the three-dimensional culture is maintained for less than one minute.
20. The method of any one of claims 14-19, wherein the three-dimensional culture is maintained for at least about 1 hour.
21. The method of any one of claims 14-20, wherein the three-dimensional culture is maintained for at least about 10 hours.

22. The method of any one of claims 14-20, wherein the three-dimensional culture is maintained for at least about 18 hours.
23. The method of any one of claims 14-22, further comprising plating the CEC aggregates onto a cell substrate.
24. The method of claim 23, wherein the cell substrate is selected from the group consisting of collagen, gelatin, cellulose, polystyrene, polyester, polycarbonate, poly(N-isopropylacrylamide), polylactic acid, polyglycolic acid, hydroxyapatite, and amniotic membrane.
25. The method of any one of claims 14-24, further comprising harvesting the CEC aggregates.
26. The method of any one of claims 14-25, further comprising incorporating the CEC aggregates into a pharmaceutical composition.
27. The method of claim 26, wherein the pharmaceutical composition lacks a Rho kinase inhibitor.
28. The method of any one of claims 14-27, wherein the CECs are human CECs (hCECs).
29. A method for treating or preventing a corneal endothelial disease in a subject in need thereof, comprising administering an effective amount of the corneal endothelial cell composition of any one of claims 1-13.
30. A method for treating or preventing a corneal endothelial disease in a human subject in need thereof, comprising administering an effective amount of the pharmaceutical composition of claim 12 or 13.
31. A method for treating or preventing a corneal endothelial disease in a human subject in need thereof, comprising administering an effective amount of a composition comprising corneal endothelial cell (CEC) aggregates to an eye of a subject.

32. The method of claim 31, wherein the subject lies face down in a prone position for less than three hours after administration of the composition comprising the CEC aggregates.

33. The method of claim 31 or 32, comprising administering the composition comprising the CEC aggregates to the subject in the absence of a rho kinase inhibitor.

34. The method of any one of claims 31-33, comprising administering the composition comprising the CEC aggregates to an anterior chamber of the eye of the subject.

35. The method of any one of claims 31-34, wherein the effective amount of the CEC aggregates comprises at least about 1×10^3 CECs.

36. The method of any one of claims 31-34, wherein the effective amount of the CEC aggregates comprises about 1×10^3 to about 2×10^6 CECs.

37. The method of any one of claims 31-36, wherein the corneal endothelial disease is a bullous keratopathy, a corneal edema, a corneal leukoma, a corneal endothelial inflammation, or a corneal dystrophy.