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(54) Title: METHODS OF TREATING RETINAL DISEASES

FIG.2C

(57) Abstract: A method of treating a subject with dry-form age-related macular degeneration (AMD) is disclosed. The method comprises administering into the subretina of the subject a therapeutically effective amount of a pharmaceutical composition comprising human RPE cells, wherein at least 95 % of the cells thereof co-express premelanosome protein (PMEL17) and cellular retinaldehyde binding protein (CRALBP), wherein the trans-epithelial electrical resistance of the cells is greater than 100 ohms to the subject, thereby treating the subject.

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METHODS OF TREATING RETINAL DISEASES

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods of treating retinal diseases and more particularly age-related macular degeneration (AMD).

The derivation of hESCs more than a decade ago has raised immense interest in the potential clinical use of these cells for regeneration by serving as the starting material for therapeutic cells. While hESC-derived cells have not yet been approved for clinical use, significant progress has been made through the years towards fulfilling this goal. Important advancements include better understanding of the biology of hESCs, technological improvements in the ability to differentiate them to various cell types and preclinical proof of their therapeutic effect in disease-specific animal models.

The RPE is a monolayer of pigmented cells which lies between the neural retina and the choriocapillaris. The RPE is characterized by an apical to basolateral structural and functional polarity. On the apical side, the cells make direct contact with the photoreceptors. On their lateral walls they form tight, adherent gap junctions and on their basal side, they contact the underlying Bruch's basal membrane which separates them from the choroidal blood vessels. The RPE cells play crucial roles in the maintenance and function of the retina and its photoreceptors. These include the formation of the blood–retinal barrier, absorption of stray light, supply of nutrients to the neural retina, regeneration of visual pigment and uptake and recycling of shed outer segments of photoreceptors.

Dysfunction, degeneration and loss of RPE cells are prominent features of AMD, Best Disease and subtypes of Retinitis Pigmentosa (RP). AMD is the leading cause of visual disability in the Western world. Among people over 75 years of age, 25-30% are affected by Age-Related Macular Degeneration (AMD), with progressive central visual loss that leads to blindness in 6-8% of the patients. The retinal degeneration primarily involves the macula, the central part of the retina responsible for fine visual detail and color perception. The dry form of AMD is initiated by hyperplasia of the RPE and formation of drusen deposits underneath the RPE or within the Bruch's membrane consisting of metabolic end products. It may gradually progress into the advanced stage of geographic atrophy (GA) with degeneration of RPE cells and

photoreceptors over large areas of the macula, causing central visual loss. Ten percent of dry AMD patients will progress to neovascular (wet) AMD, with blood vessels sprouting through the Bruch's membrane and with subsequent intraocular leakage and/or bleeding, accelerating the loss of central vision. While the complicating neovascularization can be treated with anti-VEGF agents, currently there is no effective treatment to halt RPE and photoreceptor degeneration and many patients will eventually lose their sight.

Transplantation studies both in animals and in humans provide evidence for the potential therapeutic effect of transplanting RPE cells in AMD patients. In humans, macular translocation onto more peripheral RPE, as well as autologous transplantation of peripheral RPE as cell suspensions or patches of RPE and choroid, provide proof-of-principle that positioning the macula above relatively more healthy RPE cells can improve visual function in some AMD patients. Nevertheless, the surgical procedures for autologous grafting are challenging and are associated with significant complications. Typically, RPE cells are delivered through a small retinotomy following a standard 3-port vitrectomy to a subretinal space created in the macular area along the border between areas of GA and better preserved extra-foveal retinal and RPE layer. Success of such cellular replacement strategy in treating AMD is contingent on establishing a safe delivery system that enables survival and function of the transplanted cells and minimizes retinal damage.

Background art includes WO 2013/114360, WO 2013/074681, WO 2008/129554 and WO 2013/184809, US Patent Application No. 62/116,972 and US Patent Application No. 62/116,980.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a method of treating a subject with dry-form age-related macular degeneration (AMD) comprising administering into the subretina of the subject a therapeutically effective amount of a pharmaceutical composition comprising human RPE cells, wherein at least 95 % of the cells thereof co-express premelanosome protein (PMEL17) and cellular retinaldehyde binding protein (CRALBP), wherein the trans-epithelial

electrical resistance of the cells is greater than 100 ohms to the subject, thereby treating the subject.

According to an aspect of some embodiments of the present invention there is provided a method of treating a subject with a retinal disease or condition comprising administering into the retina of the subject a therapeutically effective amount of a pharmaceutical composition comprising human polygonal RPE cells, wherein at least 95 % of the cells thereof co-express premelanosome protein (PMEL17) and cellular retinaldehyde binding protein (CRALBP), wherein the trans-epithelial electrical resistance of the cells is greater than 100 ohms to the subject, wherein the therapeutically effective amount is between 50,000 – 5,000,000 cells per administration, thereby treating the subject.

According to an aspect of some embodiments of the present invention there is provided a method of treating a subject with a retinal disease or condition comprising administering into the retina of the subject a therapeutically effective amount of a pharmaceutical composition comprising human RPE cells using a device, wherein the outer diameter of the device through which the cells are administered is between 90-100 μm, thereby treating the subject.

According to some embodiments of the invention, the device is a cannula.

According to some embodiments of the invention, at least 95 % of the cells thereof co-express premelanosome protein (PMEL17) and cellular retinaldehyde binding protein (CRALBP), wherein the trans-epithelial electrical resistance of the cells is greater than 100 ohms to the subject.

According to some embodiments of the invention, the therapeutically effective amount is between 50,000-1,000,000 cells per administration.

According to some embodiments of the invention, the therapeutically effective amount is selected from the group consisting of 50,000 cells per administration, 200,000 cells per administration, 500,000 cells per administration and 1,000,000 cells per administration.

According to some embodiments of the invention, the cells are administered into the subretinal space of the subject.

According to some embodiments of the invention, the cells are administered in a single administration.

According to some embodiments of the invention, the pharmaceutical composition comprises 500 cells per µl – 10,000 cells per µl.

According to some embodiments of the invention, when the amount is 50,000 cells per administration, the pharmaceutical composition comprises about 500-1000 cells per µl.

According to some embodiments of the invention, when the amount is 200,000 cells per administration, the pharmaceutical composition comprises about 2,000 cells per µl.

According to some embodiments of the invention, when the amount is 500,000 cells per administration, the pharmaceutical composition comprises about 5,000 cells per µl.

According to some embodiments of the invention, when the amount is 1,000,000 cells per administration, the pharmaceutical composition comprises about 10,000 cells per µl.

According to some embodiments of the invention, the retinal disease or condition is selected from the group consisting of retinitis pigmentosa, retinal detachment, retinal dysplasia, retinal atrophy, retinopathy, macular dystrophy, cone dystrophy, cone-rod dystrophy, Malattia Leventinese, Doyne honeycomb dystrophy, Sorsby's dystrophy, pattern/butterfly dystrophies, Best vitelliform dystrophy, North Carolina dystrophy, central areolar choroidal dystrophy, angioid streaks, toxic maculopathy, Stargardt disease, pathologic myopia, retinitis pigmentosa, and macular degeneration.

According to some embodiments of the invention, the disease is age-related macular degeneration.

According to some embodiments of the invention, the age-related macular degeneration is dry-form age-related macular degeneration.

According to some embodiments of the invention, the subject fulfils at least one of the criteria selected from the group consisting of:

(i) is aged 55 or older;

(ii) has funduscopic findings of dry AMD with geographic atrophy in the macula, above 0.5 disc area in at least one eye;

(iii) is able to undergo a vitreoretinal surgical procedure under monitored anesthesia care; and

(iv) does not have an immunodeficiency disease.

According to some embodiments of the invention, the subject does not have a retinal disease other than AMD;

According to some embodiments of the invention, the subject fulfils each of the criteria (i) – (iv).

According to some embodiments of the invention, the outer aperture of the device through which the cells are administered is between 90-100 μm.

According to some embodiments of the invention, the device is a cannula.

According to some embodiments of the invention, the device further comprises a needle.

According to some embodiments of the invention, the gauge of the cannula is 25G.

According to some embodiments of the invention, the number of $Oct4^{+}TRA\text{-}1\text{-}60^{+}$ cells in the population is below 1:250,000.

According to some embodiments of the invention, at least 80 % of the cells express Bestrophin 1, as measured by immunostaining.

According to some embodiments of the invention, at least 80 % of the cells express Microphthalmia-associated transcription factor (MITF), as measured by immunostaining.

According to some embodiments of the invention, more than 80 % of the cells express paired box gene 6 (PAX-6) as measured by FACS.

According to some embodiments of the invention, the cells secrete greater than 500 ng of Pigment epithelium-derived factor (PEDF) per ml per day.

According to some embodiments of the invention, the cells secrete PEDF and vascular endothelial growth factor (VEGF) in a polarized manner.

According to some embodiments of the invention, the ratio of apical secretion of PEDF: basal secretion of PEDF is greater than 1.

According to some embodiments of the invention, the ratio remains greater than 1 following incubation for 8 hours at 2-8 ° C.

According to some embodiments of the invention, the trans-epithelial electrical resistance of the cells remains greater than 100 ohms following incubation for 8 hours at 2-8 ° C.

According to some embodiments of the invention, the ratio of basal secretion of VEGF: apical secretion of VEGF is greater than 1.

According to some embodiments of the invention, the ratio remains greater than 1 following incubation for 8 hours at 2-8 ° C.

According to some embodiments of the invention, the cells are capable of rescuing visual acuity in the RCS rat following subretinal administration.

According to some embodiments of the invention, the cells are capable of rescuing photoreceptors for up to 180 days post-subretinal administration in the RCS rat.

According to some embodiments of the invention, the cells are generated by ex-vivo differentiation of human embryonic stem cells.

According to some embodiments of the invention, the cells are generated by:

(a) culturing human embryonic stem cells or induced pluripotent stem cells in a medium comprising nicotinamide so as to generate differentiating cells, wherein the medium is devoid of activin A;

(b) culturing the differentiating cells in a medium comprising nicotinamide and acitivin A to generate cells which are further differentiated towards the RPE lineage; and

(c) culturing the cells which are further differentiated towards the RPE lineage in a medium comprising nicotinamide, wherein the medium is devoid of activin A.

According to some embodiments of the invention, the embryonic stem cells or induced pluripotent stem cells are propagated in a medium comprising bFGF and TGFβ.

According to some embodiments of the invention, the embryonic stem cells are cultured on human umbilical cord fibroblasts.

According to some embodiments of the invention, steps (a)-(c) are effected under conditions wherein the atmospheric oxygen level is less than about 10 %.

According to some embodiments of the invention, the method further comprises culturing embryonic stem cells or induced pluripotent stem cells in a medium under

conditions wherein the atmospheric oxygen level is greater than about 10 % in the presence of nicotinamide prior to step (a).

According to some embodiments of the invention, the method further comprises culturing the differentiated cells in a medium under conditions wherein the atmospheric oxygen level is greater than about 10 % in the presence of nicotinamide following step (c).

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIG. 1 is a drawing of the subretinal injection scheme. Note that in this drawing, the placement of the surgical ports is not anatomically accurate (Stout and Francis, 2011, Human Gene Ther. 2011 May 22(5): 531-5).

FIGs. 2A-C are a photograph illustrating assembly of the device of delivery and loading of the formulated cells. A. Syringe connected to 18G blunt fill needle. B. Replacement of the 18G blunt fill needle with the extension tube. C. The assembled device of delivery.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods of treating retinal diseases and more particularly age-related macular degeneration (AMD).

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

AMD is a progressive chronic disease of the central retina and a leading cause of vision loss worldwide. Most visual loss occurs in the late stages of the disease due to one of two processes: neovascular ("wet") AMD and geographic atrophy (GA, "dry"). In neovascular age-related macular degeneration, choroidal neovascularisation breaks through to the sub-RPE or subretinal space, leaking fluid, lipid, and blood and leading to fibrous scarring. In GA, progressive atrophy of the retinal pigment epithelium, choriocapillaris, and photoreceptors occurs. The dry form of AMD is more common (85-90% of all cases), but may progress to the "wet" form, which, if left untreated, leads to rapid and severe vision loss.

The estimated prevalence of AMD is 1 in 2,000 people in the US and other developed countries. This prevalence is expected to increase together with the proportion of elderly in the general population. The risk factors for the disease include both environmental and genetic factors.

The pathogenesis of the disease involves abnormalities in four functionally interrelated tissues, i.e., retinal pigment epithelium (RPE), Bruch's membrane, choriocapillaries and photoreceptors. However, impairment of RPE cell function is an early and crucial event in the molecular pathways leading to clinically relevant AMD changes.

There is currently no effective or approved treatment for dry-AMD. Prophylactic measures include vitamin/mineral supplements. These reduce the risk of developing wet AMD but do not affect the development of progression of geographic atrophy.

Currently, there are about twenty therapies in various stages of clinical development. Among these are complement system inhibitors and corticosteroids, visual cycle modulators, anti-oxidants, neuroprotectants, vascular enhancers and cell

and gene therapies – see for example Dugel et al., 2014, Retina Today, pages 70-72; and Patel et al, 2015, Practical Retina, January 2015 · Vol. 46, No. 1, pages 8-13.

Human embryonic stem cells have been proposed as a cellular source for the generation of RPE cells. Two general approaches have been used to obtain RPE cells from hESCs, spontaneous differentiation and directed differentiation. In spontaneous differentiation, hESCs in flat colonies or in embryoid bodies (EBs) are allowed to spontaneously differentiate into a population of cells containing pigmented RPE cells. The directed differentiation method uses a number of factors to drive the differentiation of hESCs to RPE cells see for example WO 2008/129554.

The present inventors have now discovered optimal conditions for clinical treatment with PRE cells including doses and treatment regimens and devices for delivering the cells. The present invention further provides criteria for selection of populations of patients who would gain benefit by treatment with the cells.

Thus, according to one aspect of the present invention there is provided a method of treating a subject with a retinal disease (e.g. dry-form age-related macular degeneration (AMD)) comprising administering into the subretina of the subject a therapeutically effective amount of a pharmaceutical composition comprising human RPE cells, wherein at least 95 % of the cells thereof co-express premelanosome protein (PMEL17) and cellular retinaldehyde binding protein (CRALBP), wherein the trans-epithelial electrical resistance of the cells is greater than 100 ohms to the subject, thereby treating the subject.

Eye conditions for which the pharmaceutical compositions serve as therapeutics include, but are not limited to retinal diseases or disorders generally associated with retinal dysfunction, retinal injury, and/or loss of retinal pigment epithelium. A non-limiting list of conditions which may be treated in accordance with the invention comprises retinitis pigmentosa, lebers congenital amaurosis, hereditary or acquired macular degeneration, age related macular degeneration (AMD), Best disease, retinal detachment, gyrate atrophy, choroideremia, pattern dystrophy as well as other dystrophies of the RPE, Stargardt disease, RPE and retinal damage due to damage caused by any one of photic, laser, inflammatory, infectious, radiation, neo vascular or traumatic injury.

According to a particular embodiment, the disease is dry-form age-related macular degeneration.

Subjects which may be treated include primate (including humans), canine, feline, ungulate (e.g., equine, bovine, swine (e.g., pig)), avian, and other subjects. Humans and non- human animals having commercial importance (e.g., livestock and domesticated animals) are of particular interest. Exemplary mammals which may be treated include, canines; felines; equines; bovines; ovines; rodentia, etc. and primates, particularly humans. Non-human animal models, particularly mammals, e.g. primate, murine, lagomorpha, etc. may be used for experimental investigations.

According to one embodiment, the subject who is treated is aged 55 or older.

According to another embodiment, the subject who is treated has funduscopic findings of dry AMD with geographic atrophy in the macula, above 0.5 disc area ($1.25mm^2$ and up to 17 mm^2) in at least one eye.

According to still another embodiment, the subject who is treated is in a condition such that he is able to undergo a vitreoretinal surgical procedure under monitored anesthesia care;

According to still another embodiment, the subject does not have an immunodeficiency disease.

According to still another embodiment, the subject does not have axial myopia greater than -6 diopters.

According to still another embodiment, the subject does not have a history of retinal detachment repair.

According to still another embodiment, the subject does not have a retinal disease other than AMD.

Preferably, the subject who is treated fulfils at least one, two, three, four, five, six or all of the above criteria.

The RPE cells generated as described herein may be transplanted to various target sites within a subject's eye. In accordance with one embodiment, the transplantation of the RPE cells is to the subretinal space of the eye, which is the normal anatomical location of the RPE (between the photoreceptor outer segments and the choroids). In addition, dependent upon migratory ability and/or positive paracrine effects of the cells, transplantation into additional ocular compartments can be

considered including the vitreal space, inner or outer retina, the retinal periphery and within the choroids.

The number of viable cells that may be administered to the subject are typically between 50,000-5x10^6, between 50,000-4x10^6 between 50,000-3x10^6 between 50,000-2x10^6 between 50,000-1x10^6, between 50,000-500,000 per injection.

The present invention contemplates a single administration or multiple administrations. Preferably, the time between a first administration to an eye and a second administration to the same eye is at least one month.

According to a specific embodiment, the number of viable cells that may be administered per eye of the subject are between 50,000-2x10^6, between 50,000-1x10^6, between 50,000-500,000. Exemplary doses include 50,000 cells per eye, 100,000 cells per eye, 200,000 cells per eye, 300,000 cells per eye, 400,000 cells per eye, 500,000 cells per eye and 1x10^6 cells per eye.

The cells are typically formulated in a carrier (e.g. an isotonic solution and/or a saline) such as BSS plus™. The carrier may optionally comprise additional factors that support RPE engraftment, integration, survival, potency etc.

An exemplary concentration of a pharmaceutical composition comprising 50,000 cells is about 500 cells per µl or 1,000 cells per µl. An exemplary concentration of a pharmaceutical composition comprising 200,000 viable cells is about 500 viable cells per µl or 1,000 viable cells per µl. An exemplary concentration of a pharmaceutical composition comprising 500,000 cells is about 5,000 viable cells per µl or about 10,000 viable cells per µl. An exemplary concentration of a pharmaceutical composition comprising 1x10^6cells is about 10,000 viable cells per µl.

The transplantation may be performed by various techniques known in the art. Methods for performing RPE transplants are described in, for example, U.S. Pat. Nos. 5,962,027, 6,045,791, and 5,941,250 and in Eye Graefes Arch Clin Exp Opthalmol March 1997; 235(3):149-58; Biochem Biophys Res Commun Feb. 24, 2000; 268(3): 842-6; Opthalmic Surg February 1991; 22(2): 102-8. Methods for performing corneal transplants are described in, for example, U.S. Pat. No. 5,755,785, and in Eye 1995; 9 (Pt 6 Su):6-12; Curr Opin Opthalmol August 1992; 3 (4): 473-81; Ophthalmic Surg Lasers April 1998; 29 (4): 305-8; Ophthalmology April 2000; 107 (4): 719-24; and Jpn J Ophthalmol November-December 1999; 43(6): 502-8. If mainly paracrine effects are

to be utilized, cells may also be delivered and maintained in the eye encapsulated within a semi-permeable container, which will also decrease exposure of the cells to the host immune system (Neurotech USA CNTF delivery system; PNAS March 7, 2006 vol. 103(10) 3896-3901).

The step of administering may comprise intraocular administration of the RPE cells into an eye in need thereof. The intraocular administration may comprise injection of the RPE cells into the subretinal space.

In accordance with one embodiment, transplantation is performed via pars plana vitrectomy surgery followed by delivery of the cells through a small retinal opening into the sub-retinal space or by direct injection.

The RPE cells may be transplanted in various forms. For example, the RPE cells may be introduced into the target site in the form of cell suspension, with matrix or adhered onto a matrix or a membrane, extracellular matrix or substrate such as a biodegradable polymer or a combination. The RPE cells may also be transplanted together (co-transplantation) with other retinal cells, such as with photoreceptors.

According to a particular embodiment, an aqueous solution (e.g. an isotonic solution and/or a saline) or air is administered into the subretinal space, thereby forming an initial bleb. Then the RPE cells as a suspension or upon a scaffold are administered into the same subretinal space. The injection may be through a needle or injection cannula.

According to a particular embodiment, the cells are delivered as a cell suspension using a delivery device (e.g. needle or injection cannula) which has an outer diameter between 90-100 μm. According to another embodiment, the cells are delivered as a cell suspension using a delivery device (e.g. needle or injection cannula) which has an inner aperture diameter between 65-75 μm.

According to embodiments of this aspect of the present invention the RPE cells in their final formulation at a concentration of 70,000-1.4 $x10^6$ viable cells/100 μl are loaded into a delivery device (e.g. 1 mL syringe) using a 18G needle (70,000 cells are loaded for the 50,000 dose, 700,000 cells are loaded for the 500,000 dose and 1.4 $x10^6$ cells are loaded for the 1 $x10^6$ dose). The 18G needle may then be replaced with an extension tube (e.g. between 5-10 cm) and air is removed through the extension tube. An injection cannula which has a tip having an outer diameter between 90-100 μm (e.g.

41G) may then be attached to the end of the extension tube. According to another embodiment, the inner diameter of the aperture of the tip is about 65-75 μm (e.g. about 70 μm).

The concentration of the RPE cells upon loading into the needle or injection cannula may be between about 2,000 viable cells/μl and about 14,000 viable cells/μl. The concentration of viable RPE cells to be delivered from the needle or injection cannula may be between about 1,000 viable cells/μl and about 10,000 viable cells/μl.

According to a particular embodiment, the cannula comprises a 41G tip(example as manufactured by Peregrine). According to still another embodiment, the cannula is a 25G cannula.

The present invention provides for an article of manufacture comprising an 18G needle and a 25G/41G cannula. Such a device may be used for the uptake of RPE cells and the subsequent intraocular administration of RPE cells.

The device may further comprise an extension tube (e.g. between about 5 and 15 cm) and a syringe (e.g. 1-2 ml syringe).

According to another aspect there is provided an article of manufacture comprising a 25G/41G cannula, a syringe (e.g. 1-2 ml syringe) and an extension tube (e.g. between about 5 and 15 cm). The article of manufacture may further comprise an 18G needle.

The effectiveness of treatment may be assessed by different measures of visual and ocular function and structure, including, among others, best corrected visual acuity (BCVA), retinal sensitivity to light as measured by perimetry or microperimetry in the dark and light-adapted states, full-field, multi-focal, focal or pattern elecroretinography ERG), contrast sensitivity, reading speed, color vision, clinical biomicroscopic examination, fundus photography, optical coherence tomography (OCT), fundus auto-fluorescence (FAF), infrared and multicolor imaging, fluorescein or ICG angiography, and additional means used to evaluate visual function and ocular structure.

The subject may be administered corticosteroids prior to or concurrently with the administration of the RPE cells, such as prednisolone or methylprednisolone, Predforte.

According to another embodiment, the subject is not administered corticosteroids prior to or concurrently with the administration of the RPE cells, such as prednisolone or methylprednisolone, Predforte.

Immunosuppressive drugs may be administered to the subject prior to, concurrently with and/or following treatment.

The immunosuppressive drug may belong to the following classes:

Glucocorticoids, Cytostatics (e.g. alkylating agent or antimetabolite), antibodies (polyclonal or monoclonal), drugs acting on immunophilins (e.g. ciclosporin, Tacrolimus or Sirolimus). Additional drugs include interferons, opiods, TNF binding proteins, mycophenolate and small biological agents.

Examples of immunosuppressive drugs include: mesenchymal stem cells, anti-lymphocyte globulin (ALG) polyclonal antibody, anti-thymocyte globulin (ATG) polyclonal antibody, azathioprine, BAS1 L1X1MAB® (anti-I L-2Ra receptor antibody), cyclosporin (cyclosporin A), DACLIZUMAB® (anti-I L-2Ra receptor antibody), everolimus, mycophenolic acid, RITUX1MAB® (anti-CD20 antibody), sirolimus, tacrolimus, Tacrolimus and or Mycophenolate mofetil.

Antibiotics may be administered to the subject prior to, concurrently with and/or following treatment. Examples of antibiotics include Oflox, Gentamicin, Chloramphenicol, Tobrex, Vigamox or any other topical antibiotic preparation authorized for ocular use.

“Retinal pigment epithelium cells”, “RPE cells”, “RPEs”, which may be used interchangeably as the context allows, refers to cells of a cell type functionally similar to that of native RPE cells which form the pigment epithelium cell layer of the retina (e.g. upon transplantation within an eye, they exhibit functional activities similar to those of native RPE cells). Thus, the terms “retinal pigment epithelium cells”, “RPE cells”, or “RPEs” may be used to refer to both native RPE cells of the pigmented layer of the retina and RPE cells directly differentiated from human stem cells (hSCs), in accordance with the present disclosure.

The term "hSC-derived RPE cells" is used herein to denote RPE cells that are obtained by directed differentiation from hSCs. In accordance with a preferred embodiment, the hSC-derived RPE cells are functional RPE cells as exhibited by parameters defined herein below. The term "directed differentiation" is used

interchangeably with the term "RPE induced differentiation" and is to be understood as meaning the process of manipulating hSCs under culture conditions which induce/promote differentiation into the RPE cell type.

According to a particular embodiment, the RPE cells are obtained by directed differentiation of hSCs in the presence of one or more members of the TGFβ superfamily, and exhibit at least one of the following characteristics:

- during differentiation, the cultured cells respond to TGFβ signaling;
- the RPE cells express markers indicative of terminal differentiation, e.g. bestrophin 1, CRALBP and/or RPE65;
- following transplantation (i.e. in situ), the RPE cells exhibit trophic effect supporting photoreceptors adjacent to RPE cells;
- further, in situ the RPE cells are capable of functioning with phagocytosis of shed photoreceptor outer segments as part of the normal renewal process of these photoreceptors;
- further, in situ the RPE cells are capable of generating a retinal barrier and functioning in the visual cycle.

As used herein, the phrase “stem cells” refers to cells which are capable of remaining in an undifferentiated state (*e.g.,* pluripotent or multipotent stem cells) for extended periods of time in culture until induced to differentiate into other cell types having a particular, specialized function (*e.g.,* fully differentiated cells). Preferably, the phrase “stem cells” encompasses embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), adult stem cells, mesenchymal stem cells and hematopoietic stem cells.

According to a particular embodiment, the RPE cells are generated from ESC.

The phrase “embryonic stem cells” refers to embryonic cells which are capable of differentiating into cells of all three embryonic germ layers *(i.e.*, endoderm, ectoderm and mesoderm), or remaining in an undifferentiated state. The phrase “embryonic stem cells” may comprise cells which are obtained from the embryonic tissue formed after gestation (e.g., blastocyst) before implantation of the embryo (*i.e.*, a pre-implantation blastocyst), extended blastocyst cells (EBCs) which are obtained from a post-implantation/pre-gastrulation stage blastocyst (see WO2006/040763) and embryonic germ (EG) cells which are obtained from the genital tissue of a fetus any time during

gestation, preferably before 10 weeks of gestation. The embryonic stem cells of some embodiments of the invention can be obtained using well-known cell-culture methods. For example, human embryonic stem cells can be isolated from human blastocysts. Human blastocysts are typically obtained from human *in vivo* preimplantation embryos or from *in vitro* fertilized (IVF) embryos. Alternatively, a single cell human embryo can be expanded to the blastocyst stage. For the isolation of human ES cells the zona pellucida is removed from the blastocyst and the inner cell mass (ICM) is isolated by a procedure in which the trophectoderm cells are lysed and removed from the intact ICM by gentle pipetting. The ICM is then plated in a tissue culture flask containing the appropriate medium which enables its outgrowth. Following 9 to 15 days, the ICM derived outgrowth is dissociated into clumps either by a mechanical dissociation or by an enzymatic degradation and the cells are then re-plated on a fresh tissue culture medium. Colonies demonstrating undifferentiated morphology are individually selected by micropipette, mechanically dissociated into clumps, and re-plated. Resulting ES cells are then routinely split every 4-7 days. For further details on methods of preparation human ES cells see Reubinoff et al Nat Biotechnol 2000, May: 18(5): 559; Thomson et al., [U.S. Pat. No. 5,843,780; Science 282: 1145, 1998; Curr. Top. Dev. Biol. 38: 133, 1998; Proc. Natl. Acad. Sci. USA 92: 7844, 1995]; Bongso et al., [Hum Reprod 4: 706, 1989]; and Gardner et al., [Fertil. Steril. 69: 84, 1998].

It will be appreciated that commercially available stem cells can also be used according to some embodiments of the invention. Human ES cells can be purchased from the NIH human embryonic stem cells registry [Hypertext Transfer Protocol://grants (dot) nih (dot) gov/stem_cells/registry/current (dot) htm]. Non-limiting examples of commercially available embryonic stem cell lines are HAD-C102, ESI, BG01, BG02, BG03, BG04, CY12, CY30, CY92, CY10, TE03, TE32, CHB-4, CHB-5, CHB-6, CHB-8, CHB-9, CHB-10, CHB-11, CHB-12, HUES 1, HUES 2, HUES 3, HUES 4, HUES 5, HUES 6, HUES 7, HUES 8, HUES 9, HUES 10, HUES 11, HUES 12, HUES 13, HUES 14, HUES 15, HUES 16, HUES 17, HUES 18, HUES 19, HUES 20, HUES 21, HUES 22, HUES 23, HUES 24, HUES 25, HUES 26, HUES 27, HUES 28, CyT49, RUES3, WA01, UCSF4, NYUES1, NYUES2, NYUES3, NYUES4, NYUES5, NYUES6, NYUES7, UCLA 1, UCLA 2, UCLA 3, WA077 (H7), WA09 (H9), WA13 (H13), WA14 (H14), HUES 62, HUES 63, HUES 64, CT1, CT2,

CT3, CT4, MA135, Eneavour-2, WIBR1, WIBR2, WIBR3, WIBR4, WIBR5, WIBR6, HUES 45, Shef 3, Shef 6, BJNhem19, BJNhem20, SA001, SA001.

According to a specific embodiment, the embryonic stem cell line is HAD-C102 or ESI.

In addition, ES cells can be obtained from other species as well, including mouse (Mills and Bradley, 2001), golden hamster [Doetschman et al., 1988, Dev Biol. 127: 224-7], rat [Iannaccone et al., 1994, Dev Biol. 163: 288-92] rabbit [Giles et al. 1993, Mol Reprod Dev. 36: 130-8; Graves & Moreadith, 1993, Mol Reprod Dev. 1993, 36: 424-33], several domestic animal species [Notarianni et al., 1991, J Reprod Fertil Suppl. 43: 255-60; Wheeler 1994, Reprod Fertil Dev. 6: 563-8; Mitalipova et al., 2001, Cloning. 3: 59-67] and non-human primate species (Rhesus monkey and marmoset) [Thomson et al., 1995, Proc Natl Acad Sci U S A. 92: 7844-8; Thomson et al., 1996, Biol Reprod. 55: 254-9].

Extended blastocyst cells (EBCs) can be obtained from a blastocyst of at least nine days post fertilization at a stage prior to gastrulation. Prior to culturing the blastocyst, the zona pellucida is digested [for example by Tyrode's acidic solution (Sigma Aldrich, St Louis, MO, USA)] so as to expose the inner cell mass. The blastocysts are then cultured as whole embryos for at least nine and no more than fourteen days post fertilization (*i.e.*, prior to the gastrulation event) *in vitro* using standard embryonic stem cell culturing methods.

Another method for preparing ES cells is described in Chung et al., Cell Stem Cell, Volume 2, Issue 2, 113-117, 7 February 2008. This method comprises removing a single cell from an embryo during an in vitro fertilization process. The embryo is not destroyed in this process.

Yet another method for preparing ES cells is by parthenogenesis. The embryo is also not destroyed in the process.

Currently practiced ES culturing methods are mainly based on the use of feeder cell layers which secrete factors needed for stem cell proliferation, while at the same time, inhibit their differentiation. Exemplary feeder layers include Human embryonic fibroblasts, adult fallopian epithelial cells, primary mouse embryonic fibroblasts (PMEF), mouse embryonic fibroblasts (MEF), murine fetal fibroblasts (MFF), human embryonic fibroblast (HEF), human fibroblasts obtained from the differentiation of

human embryonic stem cells, human fetal muscle cells (HFM), human fetal skin cells (HFS), human adult skin cells, human foreskin fibroblasts (HFF), human fibroblasts obtained from the umbilical cord or placenta, and human marrow stromal cells (hMSCs). Growth factors may be added to the medium to maintain the ESCs in an undifferentiated state. Such growth factors include bFGF and/or TGFβ.

Feeder cell free systems have also been used in ES cell culturing, such systems utilize matrices supplemented with serum replacement, cytokines, IL6, soluble IL6 receptor chimera and/or growth factors as a replacement for the feeder cell layer. Stem cells can be grown on a solid surface such as an extracellular matrix (e.g., Matrigel^{RTM} or laminin) in the presence of a culture medium. Unlike feeder-based cultures which require the simultaneous growth of feeder cells and stem cells and which may result in mixed cell populations, stem cells grown on feeder-free systems are easily separated from the surface. The culture medium used for growing the stem cells contains factors that effectively inhibit differentiation and promote their growth such as MEF-conditioned medium and bFGF. However, commonly used feeder-free culturing systems utilize an animal-based matrix (e.g., Matrigel^{RTM}) supplemented with mouse or bovine serum, or with MEF conditioned medium [Xu C, et al. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol. 19: 971-4] which present the risk of animal pathogen cross-transfer to the human ES cells, thus compromising future clinical applications.

Numerous methods are known for differentiating ESCs towards the RPE lineage and include both directed differentiation protocols such as those described in WO 2008/129554, 2013/184809 and spontaneous differentiation protocols such as those described in U.S. Patent No. 8,268,303 and U.S. Patent application 20130196369, the contents of each being incorporated by reference.

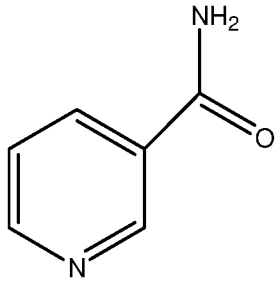
According to a particular embodiment, the RPE cells are generated from ESC cells using a directed differentiation protocol.

In one exemplary differentiation protocol, the embryonic stem cells are differentiated towards the RPE cell lineage using a first differentiating agent and then further differentiated towards RPE cells using a member of the transforming growth factor-ß (TGFß) superfamily, (e.g. TGFβ1, TGFβ2, and TGFβ3 subtypes, as well as homologous ligands including activin (e.g., activin A, activin B, and activin AB), nodal,

anti-mullerian hormone (AMH), some bone morphogenetic proteins (BMP), e.g. BMP2, BMP3, BMP4, BMP5, BMP6, and BMP7, and growth and differentiation factors (GDF)). According to a specific embodiment, the member of the transforming growth factor-ß (TGFß) superfamily is activin A - e.g. between 20-200 ng/ml e.g. 100-180 ng/ml.

According to a particular embodiment, the first differentiating agent is nicotinamide (NA) - e.g. between 1-100 mM, 5-50 mM, 5-20 mM, e.g. 10 mM.

NA, also known as "niacinamide", is the amide derivative form of Vitamin B3 (niacin) which is thought to preserve and improve beta cell function. NA has the chemical formula $C_6H_6N_2O$. NA is essential for growth and the conversion of foods to energy, and it has been used in arthritis treatment and diabetes treatment and prevention.



Nicotinamide (NA)

In the context of the present disclosure, the term NA also denotes derivatives of NA and nicotinamide mimics. The term "derivative of nicotinamide (NA)" as used herein denotes a compound which is a chemically modified derivative of the natural NA. The chemical modification may include, for example, a substitution on the pyridine ring of the basic NA structure (via the carbon or nitrogen member of the ring), via the nitrogen or the oxygen atoms of the amide moiety, as well as deletion or replacement of a group, e.g. to form a thiobenzamide analog of NA, all of which being as appreciated by those versed in organic chemistry. The derivative in the context of the invention also includes the nucleoside derivative of NA (e.g. nicotinamide adenine). A variety of derivatives of NA are described, some also in connection with an inhibitory activity of the PDE4 enzyme (WO03/068233; WO02/060875; GB2327675A), or as VEGF-receptor tyrosine kinase inhibitors (WO01/55114). For example, the process of preparing 4-aryl-nicotinamide derivatives (WO05/014549).

Nicotinamide mimics may be substituted for nicotinamide in the media. Nicotinamide mimics encompass any compound which recapitulates the effects of nicotinamide in the differentiation and maturation of RPE cells from pluripotent cells.

Nicotinamide mimics include modified forms of nicotinamide, and chemical analogs of nicotinamide. Exemplary nicotinamide mimics include benzoic acid, 3-aminobenzoic acid, and 6-aminonicotinamide. Another class of compounds that may act as nicotinamide mimics are inhibitors of poly(ADP-ribose) polymerase (PARP). Exemplary PARP inhibitors include 3-aminobenzamide, Iniparib (BSI 201), Olaparib (AZD-2281), Rucaparib (AG014699, PF- 01367338), Veliparib (ABT-888), CEP 9722, MK 4827, and BMN-673.

According to a particular embodiment, the differentiation is effected as follows:

a) culture of ESCs in a medium comprising a first differentiating agent (e.g. nicotinamide); and

b) culture of cells obtained from step a) in a medium comprising a member of the TGFß superfamily (e.g. activin A) and the first differentiating agent (e.g. nicotinamide).

Preferably step (a) is effected in the absence of the member of the TGFß superfamily.

The above described protocol may be continued by culturing the cells obtained in step b) in a medium comprising the first differentiating agent (e.g. nicotinamide), but devoid of a member of the TGFß superfamily (e.g. activin A). This step is referred to herein as step (c).

The above described protocol is now described in further detail, with additional embodiments.

Step (a): The differentiation process is started once sufficient quantities of ESCs are obtained. They are typically removed from the adherent cell culture (e.g. by using collagenase A, dispase, TrypLE select, EDTA) and plated onto a non-adherent substrate (e.g. cell culture plate) in the presence of nicotinamide (and the absence of activin A). Once the cells are plated onto the non-adherent substrate (e.g. cell culture plate), the cell culture may be referred to as a cell suspension, preferably free floating clusters in a suspension culture, i.e. aggregates of cells derived from human embryonic stem cells (hESCs). Sources of free floating stem cells were previously described in WO

06/070370, which is herein incorporated by reference in its entirety. This stage may be effected for a minimum of 1 day, more preferably two days, three days, 1 week or even 10 days. Preferably, the cells are not cultured for more than 2 weeks in suspension together with the nicotinamide (and in the absence of activin).

According to a preferred embodiment, when the cells are cultured on the non-adherent substrate e.g. cell culture plates, the atmospheric oxygen conditions are manipulated such that the percentage is less than about 20 %, 15 %, 10 %, more preferably less than about 9 %, less than about 8 %, less than about 7 %, less than about 6 % and more preferably about 5 %.

According to a particular embodiment, the cells are cultured on the non-adherent substrate initially under normal atmospheric oxygen conditions and then lowered to less than normal atmospheric oxygen conditions.

Examples of non-adherent substrates include but are not limited to fibronectin, laminin, polyD-lysine and gelatin.

Examples of non-adherent cell culture plates include those manufactured by Hydrocell (e.g. Cat No. 174912), Nunc etc.

Step (b): Following the first stage of directed differentiation, (step a; i.e. culture in the presence of nicotinamide (e.g. 10 mM) under non-adherent culture conditions under low or normal oxygen atmospheric conditions), the semi-differentiated cells are then subjected to a further stage of differentiation on an adherent substrate - culturing in the presence of nicotinamide (e.g. 10 mM) and activin A (e.g. 140 ng/ml, 150 ng/ml, 160 ng/ml or 180 ng/ml). This stage may be effected for at least one day, at least two days, at least three days, at least 5 days, at least one week, at least two weeks, at least three weeks, at least four weeks, at least five weeks, at least six weeks, at least seven weeks, at least eight weeks, at least nine weeks, at least ten weeks. Preferably this stage is effected for about two weeks. This stage of differentiation may be effected at low or normal atmospheric oxygen conditions, as detailed herein above.

Step (c): Following the second stage of directed differentiation (i.e. culture in the presence of nicotinamide and activin A on an adherent substrate; step (b)), the further differentiated cells are optionally subjected to a subsequent stage of differentiation on the adherent substrate - culturing in the presence of nicotinamide (e.g. 10 mM), in the absence of activin A. This stage may be effected for at least one day, 2,

days, 5 days, at least one week, at least two weeks, at least three weeks or even four weeks. Preferably this stage is effected for about one week. This stage of differentiation may also be carried out at low or normal atmospheric oxygen conditions, as detailed herein above.

Following this differentiation step, the atmospheric oxygen conditions may optionally be returned to normal atmospheric conditions and cultured for at least one more day, at least 2 more days, at least 5 more days at least one more week (e.g. up to two weeks) in the presence of nicotinamide (e.g. 10 mM) and in the absence of activin A.

The basic medium in accordance with the invention is any known cell culture medium known in the art for supporting cells growth *in vitro*, typically, a medium comprising a defined base solution, which includes salts, sugars, amino acids and any other nutrients required for the maintenance of the cells in the culture in a viable state. Non-limiting examples of commercially available basic media that may be utilized in accordance with the invention comprise Nutristem (without bFGF and TGFβ for ESC differentiation, with bFGF and TGFβ for ESC expansion) Neurobasal™, KO-DMEM, DMEM, DMEM/F12, Cellgro™ Stem Cell Growth Medium, or X-Vivo™. The basic medium may be supplemented with a variety of agents as known in the art dealing with cell cultures. The following is a non-limiting reference to various supplements that may be included in the culture system to be used in accordance with the present disclosure:

- serum or with a serum replacement containing medium, such as, without being limited thereto, knock out serum replacement (KOSR), Nutridoma-CS, TCH™, N2, N2 derivative, or B27 or a combination;
- an extracellular matrix (ECM) component, such as, without being limited thereto, fibronectin, laminin, collagen and gelatin. The ECM may them be used to carry the one or more members of the TGFß superfamily of growth factors;
- an antibacterial agent, such as, without being limited thereto, penicillin and streptomycin;
- non-essential amino acids (NEAA),

neurotrophins which are known to play a role in promoting the survival of SCs in culture, such as, without being limited thereto, BDNF, NT3, NT4.

According to a preferred embodiment, the medium used for differentiating the ESCs is Nutristem medium (Biological Industries, 05-102-1A or 05-100-1A).

According to a particular embodiment differentiation of ESCs is effected under xeno free conditions.

According to one embodiment, the proliferation/growth medium is devoid of xeno contaminants i.e. free of animal derived components such as serum, animal derived growth factors and albumin. Thus, according to this embodiment, the culturing is performed in the absence of xeno contaminants.

Other methods for culturing ESCs under xeno free conditions are provided in U.S. Patent Application No. 20130196369, the contents of which are incorporated in their entirety.

The preparations comprising RPE cells may be prepared in accordance with Good Manufacturing Practices (GMP) (e.g., the preparations are GMP-compliant) and/or current Good Tissue Practices (GTP) (e.g., the preparations may be GTP-compliant).

During differentiation steps, the embryonic stem cells may be monitored for their differentiation state. Cell differentiation can be determined upon examination of cell or tissue-specific markers which are known to be indicative of differentiation.

Tissue/cell specific markers can be detected using immunological techniques well known in the art [Thomson JA et al., (1998). Science 282: 1145-7]. Examples include, but are not limited to, flow cytometry for membrane-bound or intracellular markers, immunohistochemistry for extracellular and intracellular markers and enzymatic immunoassay, for secreted molecular markers (e.g. PEDF).

Thus, according to another aspect of the present invention there is provided a method of generating retinal epithelial cells comprising:

(a) culturing pluripotent stem cells in a medium comprising a differentiating agent so as to generate differentiating cells, wherein the medium is devoid of a member of the transforming growth factor β (TGF β) superfamily;

(b) culturing the differentiating cells in a medium comprising the member of the transforming growth factor β (TGF β) superfamily and the differentiating agent to generate cells which are further differentiated towards the RPE lineage;

(c) analyzing the secretion of Pigment epithelium-derived factor (PEDF) from the cells which are further differentiated towards the RPE lineage; and

(d) culturing the cells which are further differentiated towards the RPE lineage in a medium comprising a differentiating agent so as to generate RPE cells, wherein the medium is devoid of a member of the transforming growth factor β (TGF β) superfamily, wherein step (d) is effected when the amount of the PEDF is above a predetermined level.

Preferably, step (d) is effected when the level of PEDF is above 100 ng/ml/day, 200 ng/ml/day, 300 ng/ml/day, 400 ng/ml/day, or 500 ng/ml/day.

Another method for determining potency of the cells during or following the differentiation process is by analyzing barrier function and polarized PEDF and VEGF secretion.

Once the cells are promoted into the RPE fate, the RPE cells may be selected and/or expanded.

According to a particular embodiment, the selection is based on a negative selection - i.e. removal of non-RPE cells. This may be done mechanically by removal of non-pigmented cells or by use of surface markers.

According to another embodiment, the selection is based on a positive selection i.e. selection of pigmented cells. This may be done by visual analysis or use of surface markers.

According to still another embodiment, the selection is based first on a negative selection and then on a positive selection.

Expansion of RPE cells may be effected on an extra cellular matrix, e.g. gelatin or collagen, laminin and poly-D-lysine. For expansion, the cells may be cultured in serum-free KOM, serum comprising medium (e.g. DMEM + 20 %) or Nutristem medium (06-5102-01-1A Biological Industries). Under these culture conditions, the pigmented cells reduce pigmentation and acquire a fibroid-like morphology. Following further prolonged culture and proliferation into high-density cultures, the cells re-acquire the characteristic polygonal shape morphology and increase pigmentation of RPE cells.

The RPE cells may be expanded in suspension or in a monolayer. The expansion of the RPE cells in monolayer cultures may be modified to large scale expansion in bioreactors by methods well known to those versed in the art.

The population of RPE cells generated according to the methods described herein may be characterized according to a number of different parameters.

Thus, for example, the RPE cells obtained are polygonal in shape and are pigmented.

According to one embodiment, at least 95 %, at least 96 %, at least 97 %, at least 98 %, at least 99 % or even 100 % of the cells of the RPE cell populations obtained co-express both premelanosome protein (PMEL17) and cellular retinaldehyde binding protein (CRALBP).

According to a particular embodiment, the cells coexpress PMEL17 (SwissProt No. P40967) and at least one polypeptide selected from the group consisting of cellular retinaldehyde binding protein (CRALBP; SwissProt No. P12271), lecithin retinol acyltransferase (LRAT; SwissProt No. 095327) and sex determining region Y-box 9 (SOX 9; P48436).

According to a particular embodiment, at least 80 % of the cells of the population express detectable levels of PMEL17 and one of the above mentioned polypeptides (e.g. CRALBP), more preferably at least 85 % of the cells of the population express detectable levels of PMEL17 and one of the above mentioned polypeptides (e.g. CRALBP), more preferably at least 90 % of the cells of the population express detectable levels of PMEL17 and one of the above mentioned polypeptides (e.g. CRALBP), more preferably at least 95 % of the cells of the population express detectable levels of PMEL17 and one of the above mentioned polypeptides (e.g. CRALBP), more preferably 100 % of the cells of the population express detectable levels of PMEL17 and one of the above mentioned polypeptides (e.g. CRALBP as assayed by a method known to those of skill in the art (e.g. FACS).

According to another embodiment, the level of CRALBP and one of the above mentioned polypeptides (e.g. PMEL17) coexpression (e.g. as measured by the mean fluorescent intensity) is increased by at least two fold, more preferably at least 3 fold, more preferably at least 4 fold and even more preferably by at least 5 fold, at least 10

fold, at least 20 fold, at least 30 fold, at least 40 fold, at least 50 as compared to non-differentiated ESCs.

In one embodiment, the RPE are terminally differentiated) and do not express Pax6.

In another embodiment, the RPE cells are terminally differentiated and express Pax6.

The RPE cells described herein may also act as functional RPE cells after transplantation where the RPE cells form a monolayer between the neurosensory retina and the choroid in the patient receiving the transplanted cells. The RPE cells may also supply nutrients to adjacent photoreceptors and dispose of shed photoreceptor outer segments by phagocytosis.

According to one embodiment, the trans-epithelial electrical resistance of the cells in a monolayer is greater than 100 ohms.

Preferably, the trans-epithelial electrical resistance of the cells is greater than 150, 200, 250, 300, 300, 400, 500, 600, 700, 800 or even greater than 900 ohms.

Devices for measuring trans-epithelial electrical resistance (TEER) are known in the art and include for example EVOM2 Epithelial Voltohmmeter, (World Precision Instruments).

It will be appreciated that the cell populations disclosed herein are devoid of undifferentiated human embryonic stem cells. According to one embodiment, less than 1:250,000 cells are $Oct4^{+}TRA\text{-}1\text{-}60^{+}$ cells, as measured for example by FACS. The cells also have down regulated (by more than 5,000 fold) expression of GDF3 or TDGF as measured by PCR.

The RPE cells of this aspect of the present invention do not express embryonic stem cell markers. Said one or more embryonic stem cell markers may comprise OCT-4, NANOG, Rex- 1, alkaline phosphatase, Sox2, TDGF- beta, SSEA-3, SSEA-4, TRA-1 -60, and/or TRA- 1 -81.

The RPE preparations may be substantially purified, with respect to non-RPE cells, comprising at least about 75%, 80%, 85%, 90%, 91 %, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% RPE cells. The RPE cell preparation may be essentially free of non-RPE cells or consist of RPE cells. For example, the substantially purified preparation of RPE cells may comprise less than about 25%, 20%, 15%, 10%, 9%, 8%,

7%, 6%, 5%, 4%, 3%, 2%, or 1% non-RPE cell type. For example, the RPE cell preparation may comprise less than about 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, 0.09%, 0.08%, 0.07%, 0.06%, 0.05%, 0.04%, 0.03%, 0.02%, 0.01%, 0.009%, 0.008%, 0.007%, 0.006%, 0.005%, 0.004%, 0.003%, 0.002%, 0.001%, 0.0009%, 0.0008%, 0.0007%, 0.0006%, 0.0005%, 0.0004%, 0.0003%, 0.0002%, or 0.0001% non-RPE cells.

The RPE cell preparations may be substantially pure, both with respect to non-RPE cells and with respect to RPE cells of other levels of maturity. The preparations may be substantially purified, with respect to non-RPE cells, and enriched for mature RPE cells. For example, in RPE cell preparations enriched for mature RPE cells, at least about 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99%, or 100% of the RPE cells are mature RPE cells. The preparations may be substantially purified, with respect to non-RPE cells, and enriched for differentiated RPE cells rather than mature RPE cells. For example, at least about 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of the RPE cells may be differentiated RPE cells rather than mature RPE cells.

The preparations described herein may be substantially free of bacterial, viral, or fungal contamination or infection, including but not limited to the presence of HIV I, HIV 2, HBV, HCV, HAV, CMV, HTLV 1, HTLV 2, parvovirus B19, Epstein-Barr virus, or herpesvirus 1 and 2, SV40, HHV5, 6, 7, 8, CMV, polyoma virus, HPV, Enterovirus. The preparations described herein may be substantially free of mycoplasma contamination or infection.

Another way of characterizing the cell populations disclosed herein is by marker expression. Thus, for example, at least 80 %, 85 %, 90 %, 95 % or 100 % of the cells express Bestrophin 1, as measured by immunostaining. According to one embodiment, between 85-100 % of the cells express bestrophin.

According to another embodiment, at least 80 %, 85 %, 87 %, 89 %, 90 %, 95%, 97 % or 100 % of the cells express Microphthalmia-associated transcription factor (MITF), as measured by immunostaining. For example, between 85-100% of the cells express MITF.

According to another embodiment, at least 80 % 85 %, 87 %, 89 %, 90 %, 95%, 97 % or 100 % of the cells express paired box gene 6 (PAX-6) as measured by FACS.

The cells described herein can also be characterized according to the quantity and/or type of factors that they secrete. Thus, according to one embodiment, the cells preferably secrete more than 500, 1000, 2000, 3000 or even 4000 ng of Pigment epithelium-derived factor (PEDF) per ml per day, as measured by ELISA. According to a particular embodiment, the cells secrete between about 1250-4000 ng of Pigment epithelium-derived factor (PEDF) per ml per day.

It will be appreciated that the RPE cells generated herein secrete PEDF and vascular endothelial growth factor (VEGF) in a polarized manner. According to particular embodiments, the ratio of apical secretion of PEDF: basal secretion of PEDF is greater than 1. According to particular embodiments, the ratio of apical secretion of PEDF: basal secretion of PEDF is greater than 2. According to particular embodiments, the ratio of apical secretion of PEDF: basal secretion of PEDF is greater than 3, greater than 4, greater than 5, greater than 6, greater than 7 or even greater than 8. In addition, the ratio of basal secretion of VEGF: apical secretion of VEGF is greater than 1. According to particular embodiments, the ratio of basal secretion of VEGF: apical secretion of VEGF is greater than 1.5, 2 or 2.5.

The stability of the cells is another characterizing feature. Thus, for example the amount of PEDF secretion remains stable in the cells following a 6 hour, 8 hour, 10 hour, 12 hour or even 24 hour incubation at 2-8 ºC (when the cells are formulated in BSS + buffer). Further, the polarized secretion of PEDF and VEGF remains stable following a 6 hour, 8 hour, 10 hour, 12 hour or even 24 hour incubation at 2-8 ºC (when the cells are formulated in BSS + buffer). Further, the TEER of the cells remains stable in the cells following a 6 hour, 8 hour, 10 hour, 12 hour or even 24 hour incubation at 2-8 ºC (when the cells are formulated in BSS + buffer).

In another embodiment, the cells are characterized by their therapeutic effect. Thus, for example the present inventors have shown that the cell populations are capable of rescuing visual acuity in the RCS rat following subretinal administration. In addition, the cell populations are capable of rescuing photoreceptors (e.g. cone photoreceptors) for up to 180 days post-subretinal administration in the RCS rat.

It would be well appreciated by those versed in the art that the derivation of RPE cells is of great benefit. They may be used as an *in vitro* model for the development of new drugs to promote their survival, regeneration and function. RPE cells may serve for high throughput screening for compounds that have a toxic or regenerative effect on RPE cells. They may be used to uncover mechanisms, new genes, soluble or membrane-bound factors that are important for the development, differentiation, maintenance, survival and function of photoreceptor cells.

The RPE cells may also serve as an unlimited source of RPE cells for transplantation, replenishment and support of malfunctioning or degenerated RPE cells in retinal degenerations. Furthermore, genetically modified RPE cells may serve as a vector to carry and express genes in the eye and retina after transplantation.

The RPE cells produced by the method of the present disclosure may be used for large scale and/or long term cultivation of such cells. To this end, the method of the invention is to be performed in bioreactors suitable for large scale production of cells, and in which undifferentiated hSCs are to be cultivated in accordance with the invention. General requirements for cultivation of cells in bioreactors are well known to those versed in the art.

Harvesting of the cells may be performed by various methods known in the art. Non-limiting examples include mechanical dissection and dissociation with papain or trypsin (e.g. TrypLE select). Other methods known in the art are also applicable.

"Effective amount," as used herein, refers broadly to the amount of a compound or cells that, when administered to a patient for treating a disease, is sufficient to effect such treatment for the disease. The effective amount may be an amount effective for prophylaxis, and/or an amount effective for prevention. The effective amount may be an amount effective to reduce, an amount effective to prevent the incidence of signs/symptoms, to reduce the severity of the incidence of signs/symptoms, to eliminate the incidence of signs/symptoms, to slow the development of the incidence of signs/symptoms, to prevent the development of the incidence of signs/symptoms, and/or effect prophylaxis of the incidence of signs/symptoms. The "effective amount" may vary depending on the disease and its severity and the age, weight, medical history, susceptibility, and preexisting conditions, of the patient to be treated. The term

"effective amount" is synonymous with "therapeutically effective amount" for purposes of this disclosure.

It is expected that during the life of a patent maturing from this application many relevant technologies will be developed for the generation of RPE cells, and the term RPE cells is intended to include all such new technologies *a priori*.

As used herein the term “about” refers to ± 10 %.

The terms "comprises", "comprising", "includes", "including", “having” and their conjugates means "including but not limited to".

The term “consisting of” means “including and limited to”.

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

As used herein, the term "treating" includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

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

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed.

(1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXAMPLE 1

Device of delivery

Study objective: to compare the performance of two retinal cannulas: MedOne PolyTip 25 gauge (G) needles with 38G flexible cannula to Peregrine 25 (G) needle with 41 G flexible cannula.

Experimental Design: Cryopreserved vials of RPE cells (1.5 x10^6 cells/vial/1 ml frozen cell suspension) were thawed, contents were transferred gently to DMEM containing 20 % human serum, filtered, washed again with DMEM containing 20 % human serum, and then washed with BSS Plus. The cell pellet was then resuspended in 0.5-1 ml BSS Plus and viable cell enumeration by Trypan Blue exclusion was carried out. The cells were resuspended at different cell densities that following injection

through the device of delivery would yield the intended Phase I/IIa clinical doses of 50 $x10^3$, $200x10^3$ or 500 $x10^3$ viable cells per 100-150 µl BSS Plus. Prior to loading into the device of delivery, the appearance of formulated RPE cells was tested to verify absence of visible foreign particles. RPE cells in their final formulation were loaded into a sterile 1 mL syringe using a sterile 18G needle (Figure 2A). The 18G needle was then replaced with a sterile 10 cm extension tube (Figure 2B), air was removed through the extension tube, and a sterile 25G/38G cannula (MedOne) or a 25G/41G cannula (Peregrine) was attached to the end of the extension tube (Figure 2C). Air was then removed from the cannula until a drop of cells appeared at the tip of the cannula. According to one option, the plunger is pushed back and 10-20 µl of air is inserted into the end of the cannula.

The RPE cells were delivered in fractions of 100-150 µl at an approximate rate of 50 µl/minute. Cell concentration, volume, viability post-delivery and delivery rate (volume/min) were determined in each study and compared.

Vitality and functional activity of the RPE cells prior and post-delivery through the device were determined.

Reproducibility of the actual cell dose delivered per injected was assessed for each of the intended clinical doses (i.e. 50 $x10^3$, $200x10^3$ or 500 $x10^3$ viable cells per 100-150 µl BSS Plus) by 3 testers.

RESULTS

The inner and outer diameters of the MedOne cannula are 99 µm and 120 µm respectively. The retinal cannul Peregrine 25 (G) needle with 41G flexible cannula has a smaller inner and outer diameter of 71 µm and 96.5 µm respectively. Similar cell recovery was seen with the MedOne and the Peregrine cannulas (see Table 1 herein below).

Table 1

Cannula / *Parameter*	*MedOne 25G/38G* (*Mean* ± SD, n=3)	*Peregrine 25G/41G* (*Mean* ± SD, n=3)
Initial Viability (%)	84 ± 2.4	84 ± 3.7
Viability post Delivery (%)	79.3 ± 6.8	85 ± 3.5
Initial Dose (# cells/100 µL)	500,000	500,000
Dose Post Delivery (# cells/100 µL)	380,417 ± 110,894	394,167 ± 73,126
Dose Recovery (%)	76.1 ± 22.2	78.8 ± 14.6
Delivery Rate (µL/min)	67.3 ± 12.7	85.7 ± 35.3
Delivery Volume (µL)	107.7 ± 9.3	117.3 ± 27.2

Determination of the Initial cell dose for recovery of the high, medium and low clinical doses using Peregrine 25G/41G:

According to the data above, the recovery of the high clinical cell dose (500,000 cells/100µl) with Peregrine 25G/41G was 78.8 +/- 14.6 % (mean +/- SD). To yield the intended high clinical cell dose of 500,000 cells /100µl, several initial cell doses were tested of which an initial cell dose of 700,000 cells/100µl that yielded 507,296 +/- 81,803 cells/100µl (mean +/- SD, n=6, 3 testers) was selected for use. Table 2 below summarizes the data obtained by 3 different testers.

Table 2

Cannula / *Parameter*	*Tester 1* *(Mean ± SD, n=3)*	*Tester 2* *(Mean ± SD, n=2)*	*Tester 3* *(n=1)*	*Testers 1-3* *(Mean ± SD, n=6)*
Initial Viability (%)	90 ± 1.5	94 ± 1.6	91	92 ± 2
Viability post Delivery (%)	83 ± 7	89 ± 2.8	89	86 ± 5.7
Initial Dose (# cells/100 µL)	700,000	700,000	700,000	700,000
Dose Post Delivery (# cells/100 µL)	460,508 ± 70,313	592,375 ± 40,481	477,500	**507,296 ± 81,803**
Dose Recovery (%)	67.7 ± 12.9	77.2 ± 4.7	68.2	70.9 ± 9.7
Delivery Rate (µL/min)	64 ± 7.1	80 ± 4.7	110.8	77 ± 19
Delivery Volume (µL)	115 ± 20	120 ± 0	122	118 ± 13

To yield the intended mid clinical cell dose of 200,000 cells /100µl, an initial cell dose of 270,000 cells/100µl (range 247,000-292,000 cells/100µl) that yielded 191,250 +/- 67,511 cells/100µl (mean +/- SD, n=6, 3 testers) was selected for use. Table 3 below summarizes the data obtained by 3 different testers.

Table 3

Cannula / *Parameter*	*Tester 1* *(Mean ± SD, n=3)*	*Tester 2* *(n=1)*	*Tester 3* *(Mean ± SD, n=2)*	*Testers 1-3* *(Mean ± SD, n=6)*
Initial Viability (%)	92 ± 3	94	94 ± 1.5	93 ± 2.2
Viability post Delivery (%)	89 ± 0	92	88 ± 4.2	89 ± 2.4
Initial Dose (# cells/100 µL)	260,333 ± 12,220	292,000	262,000 ± 8,485	266,167 ± 15,327
Dose Post Delivery (# cells/100 µL)	178,333 ± 12,829	250,000	161,875 ± 62,755	**191,250 ± 67,511**
Dose Recovery (%)	68.7 ± 8.3	85.6	61.4 ± 21.9	84.9 ± 28.7
Delivery Rate (µL/min)	67 ± 2.3	140	84.1 ± 11.4	81.5 ± 32.5
Delivery Volume (µL)	100 ± 4	140	81 ± 16	105 ± 23

To yield the intended low clinical cell dose of 50,000 cells /100µl, an initial cell dose of 70,000 cells/100µl that yielded 50,688 +/- 6,533 cells/100µl (mean +/- SD, n=5, 3 testers) was selected for use. Table 4 below summarizes the data obtained by 3 different testers.

Table 4

Cannula / *Parameter*	Tester 1 *(Mean ± SD, n=3)*	*Tester 2 (n=1)*	*Tester 3 (n=1)*	Testers 1-3 *(Mean ± SD, n=5)*
Initial Viability (%)	91 ± 3	91	94	91 ± 2.7
Viability post Delivery (%)	83 ± 6	93	91	87 ± 6.5
Initial Dose (# cells/100 µL)	70,000	70,000	70,000	70,000
Dose Post Delivery (# cells/100 µL)	51,042 ± 5,610	57,500	42,813	**50,688 ± 6,553**
Dose Recovery (%)	72.9 ± 8	82.1	61.1	72.4 ± 9.3
Delivery Rate (µL/min)	64.7 ± 1.9	95.2	118.4	82.6 ± 23.5
Delivery Volume µL)	115 ± 5	108	80	107 ± 16

RPE Potency Post Delivery through Peregrine 25G/41G:

To qualify the Peregrine 26G/41G cannula, the potency of the RPE cells formulated at the high initial clinical dose of 700,000 cells/100µl was tested post-delivery. Potency was assessed using the polarization assay in which the barrier function of the RPE cells as well as the ability to secrete Pigment Epithelium Derived Factor (PEDF) and Vascular Endothelial Derived Factor (VEGF) in a polarized manner were tested using the transwell system. As can be seen in Table 5A, barrier function/trans-epithelial electrical resistance (TEER) and polarized secretion of PEDF and VEGF were similar prior and post delivery. Viability and cell concentration post delivery were within the expected range (Table 5B).

Table 5A

Experiment # 47	*PEDF day 14 (ng/mL/day)*	*TEER (Ω)*	*PEDF Apical/Basal*	*VEGF Basal/Apical*
Prior to Delivery	1,501	384	4.74	2.58
Post Deliver	1,812	492	4.85	2.99
OpRegen 5C Control	1,858	314	4.3	2.61

Table 5B

Outcome Measure	*Test Results*
Initial Viability (%)	95
Viability post Delivery (%)	91
Initial Dose (# cells/100 µL)	700,000
Dose Post Delivery (# cells/100 µL)	621,000
Dose Recovery (%)	73.9
Delivery Rate (µL/min)	76.6
Delivery Volume (µL)	120

In addition to maintained polarization ability, the RPE cell vitality post-delivery was preserved (data not shown).

The data presented herein above support the use of the Peregrine 25G/41G cannula at the intended clinical doses of 50,000, 200,000 and 500,000 cells per 100 µl. To reach these final clinical doses, RPE cells in a final concentration of 70,000, 270,000 and 700,000 viable cells per 100 µl, respectively may be prepared.

Determination of the Initial RPE Cell Dose for Recovery of the Low Clinical Dose of 50,000 Cells /50 µl:

A cell dose of 70,000 RPE cells/50 µL was initially tested for the ability to yield the intended low clinical cell dose of 50,000 cells/50 µL. As shown in Table 6, an initial cell dose of 70,000 cells/50 µL yielded 38,697 ± 5,505 cells/50 µL (mean ± SD, n=3, 3 testers) was prepared. Since the average recovery was 55% ± 7.8% (mean ± SD, n=3, 3 testers), and since the intended dose of 50,000 cells/50 µL was not reached, an initial cell density of 100,000 cells/50 µL was tested in the second set of experiments. As shown in Table 7, an initial cell dose of 100,000 cells/50 µL yielded 62,517 ± 4,625 cells/50 µL (mean ± SD, n=3, 3 testers; OpRegen® Batch 5D). The average recovery was 61% ± 4.5% (mean ± SD, n=3, 3 testers).

Table 6

Cannula / *Parameter*	*Tester 1*	*Tester 2*	*Tester 3*	Testers 1-3 *(Mean ± SD, n=3)*
Initial Viability (%)	95	95	92	94 ± 1.7
Viability post Delivery (%)	88	85	90	88 ± 2.5
Initial Dose (# cells/50 µL)	70,000	70,000	70,000	70,000
Dose Post Delivery (# cells/50 µL)	44,842	34,218	37,030	**38,697 ± 5,505**
Dose Recovery (%)	64	49	53	55 ± 7.8
Delivery Rate (µL/min)	97	85	94	92 ± 6.2
Delivery Volume (µL)	35	61	60	52 ± 14.7

Table 7

Cannula / *Parameter*	*Tester 1*	*Tester 2*	*Tester 3*	Testers 1-3 *(Mean ± SD, n=3)*
Initial Viability (%)	88	91	96	92 ± 4
Viability post Delivery (%)	88	88	90	89 ± 1.2
Initial Dose (# cells/50 µL)	100,000	100,000	100,000	100,000
Dose Post Delivery (# cells/50 µL)	57,343	66,250	63,958	**62,517 ± 4,625**
Dose Recovery (%)	57	66	64	61 ± 4.5
Delivery Rate (µL/min)	87	111	110	103 ± 14
Delivery Volume (µL)	53	65	60	59 ± 6

EXAMPLE 2

Clinical Experiment

Study Design: Single center Phase I/IIa study of 15 patients with advanced dry form AMD and geographic atrophy (GA) divided into four cohorts: the first 3 cohorts, each consisting of 3 legally blind patients with best corrected visual acuity of 20/200 or less, will receive a single subretinal injection of RPE cells, using sequentially escalating dosages of $50x10^3$, $200x10^3$, and $500x10^3$ cells per cohort, respectively. The fourth cohort will include 6 patients with best corrected visual acuity of 20/100 or less, who will receive a single subretinal injection of 500,000 RPE cells. Staggering intervals within and between cohorts will be applied.

Following a vitrectomy, cells will be delivered into the subretinal space in the macular area via a cannula through a small retinotomy. A total volume of up to 50-150 µl cell suspension will be injected in areas at risk for GA expansion.

Along with the surgical procedure, patients will receive light immunosuppression and antibiotic treatment, consisting of the following:

1. Topical steroidal and antibiotic treatment as customary following vitrectomy: A course of topical steroid therapy (Predforte drops 4-8 times daily, with gradual taper) and topical antibiotic drops (Oflox or equivalent 4 times daily) over the course of 6 weeks.

2. Systemic (PO) Tacrolimus 0.01 mg/kg daily (dose will be adjusted to reach blood concentration of 3-7 ng/ml), from a week before transplantation and continued until 6 weeks post transplantation.

3. Systemic (PO) Mycophenolate mofetil, total 2 gr/day, given from 2 weeks before transplantation and continued for one year post transplantation.

Patients will be assessed at pre-scheduled intervals throughout the 12 months following the administration of the cells. Post study follow-up will occur at 15 months, 2, 3, 4 and 5 years post-surgery. In patients who develop side effects related to the immunosuppressive treatment, an attempt will be made to control these side effects (for example, improve blood pressure control if hypertension develops). In the case of uncontrollable side effects, treatment with the causative immunosuppressive agent will be modified in consultation with the study internist.

Inclusion Criteria:

1. Age 55 and older;
2. Diagnosis of dry (non-neovascular) age related macular degeneration in both eyes;
3. Funduscopic findings of dry AMD with geographic atrophy in the macula, above 0.5 disc area ($1.25mm^2$ and up to 17 mm^2) in size in the study eye and above 0.5 disc area in the fellow eye;
4. Best corrected central visual acuity equal or less than 20/200 in cohorts 1-3 and equal or less than 20/100 in cohort 4 in the study eye by ETDRS vision testing;
5. Vision in the non-operated eye must be better than or equal to that in the operated eye;
6. Patients with sufficiently good health to allow participation in all study-related procedures and complete the study (medical records);
7. Ability to undergo a vitreoretinal surgical procedure under monitored anesthesia care;

8. Normal blood counts, blood chemistry, coagulation and urinalysis;
9. Negative for HIV, HBC, and HCV, negative for CMV IgM and EBV IgM;
10. Patients with no current or history of malignancy (with the exception of successfully treated basal/squamous cell carcinoma of the skin) based on age matched screening exam (at discretion of the study physician);
11. Patients allowed to discontinue taking aspirin, aspirin-containing products and any other coagulation-modifying drugs, 7 days prior to surgery;
12. Willing to defer all future blood and tissue donation;
13. Able to understand and willing to sign informed consent.

Exclusion Criteria:

1. Evidence of neovascular AMD by history, as well as by clinical exam, fluorescein angiography (FA), or ocular coherence tomography (OCT) at baseline in either eye;
2. History or presence of diabetic retinopathy, vascular occlusions, uveitis, Coat's disease, glaucoma, cataract or media opacity preventing posterior pole visualization or any significant ocular disease other than AMD that has compromised or could compromise vision in the study eye and confound analysis of the primary outcome;
3. History of retinal detachment repair in the study eye;
4. Axial myopia greater than -6 diopters;
5. Ocular surgery in the study eye in the past 3 months;
6. History of cognitive impairments or dementia;
7. Contraindication for systemic immunosuppression;
8. History of any condition other than AMD associated with choroidal neovascularization in the study eye (e.g. pathologic myopia or presumed ocular histoplasmosis);
9. Active or history for the following diseases: cancer, renal disease, diabetes, myocardial infraction in previous 12 months, immunodeficiency;
10. Female; pregnancy or lactation;
11. Current participation in another clinical study. Past participation (within 6 months) in any clinical study of a drug administered systemically or to the eye.

The safety and tolerability of the surgical procedure and the safety of the cell graft will be assessed separately. Assessment of surgical safety will include the following measures:

1. Unhealing retinal detachment
2. Proliferative vitreo-retinopathy (PVR)
3. Subretinal, retinal or intravitreal hemorrhage
4. Injury to relatively still healthy retina at the site of surgery

The safety and tolerability of the cell graft will be evaluated using the following adverse events that will be graded according to the National Cancer Institute (NCI) grading system:

1. Teratoma and/or tumor and/or ectopic tissue formation
2. Infection
3. Uveitis, Vasculitis or PVR
4. Accelerated progression of GA
5. Progression to neovascular AMD in the study eye
6. Serious inflammatory reaction against the allotransplanted cells

The secondary exploratory efficacy endpoints will be measured by duration of graft survival and by the examination of the following:

1. Rate of GA progression
2. Retinal sensitivity in engrafted regions, extent and depth of central scotomata
3. Changes in visual acuity

Surgical Procedure:

The eye chosen for RPE administration will be the eye with worse visual function. The surgery will be performed by retro-bulbar or peri-bulbar anesthetic block accompanied by monitored intravenous sedation or by general anesthesia, at the discretion of the surgeon and in discussion with the patient. The eye undergoing surgery will be prepped and draped in sterile fashion according to the institution protocol. After the placement of a lid speculum, a standard 3-port vitrectomy will be performed. This will include the placement of a 23G infusion cannula and two 23G ports. After visual inspection of the infusion cannula in the vitreous cavity, the infusion line will be opened to ensure that structure of the eye globe is maintained throughout the surgery. A careful core vitrectomy will then be performed with standard 23G instruments, followed by detachment of the posterior vitreous face. This will allow unobstructed access to the posterior pole.

RPE will be introduced into the subretinal space at a predetermined site within the posterior pole, preferably penetrating the retina in an area that is still relatively preserved close to the border of GA. Blood vessels will be avoided. The cells will be delivered to the subretinal space via formation of a small bleb, with a volume of 50-150 µl.

The delivery system is composed of a 1 mL syringe that through a 10 cm extension tube is connected to a Peregrine 25G/41G flexible retinal cannula.

Any cells that refluxed into the vitreal space will be removed and fluid-air exchange will be performed. Prior to removal of the infusion cannula, careful examination will be performed to ensure that no iatrogenic retinal tears or breaks were created. The infusion cannula will then be removed. Subconjunctival antibiotics and steroids will be administered. The eye will be covered with a patch and plastic shield. The surgical administration procedure will be recorded.

Dose: A low dose of 50,000 cells/50 µL or 50,000 cells/100 µL, medium dose of 200,000 cells/100 µL and a high dose of 500,000 cells/100 µL will be used. Dose selection was based on the safety of the maximal feasible dose tested in preclinical studies and the human equivalent dose calculated based on eye and bleb size.

End-point parameters:

1. Safety of Surgical Procedure:

Persistent/recurrent retinal detachment

Proliferative vitreo-retinopathy (PVR)

Hemorrhage

Injury to relatively still healthy retina at the site of surgery

2. Product Safety:

Teratoma, tumor, and/or ectopic tissue development

Bulky graft of proliferating cells

Infection

Serious inflammatory immune reaction to the graft

Accelerated progression of GA

Progression to neovascular AMD in the study eye

3. Efficacy:

Duration of graft survival

Decreased rate of GA progression

Retinal sensitivity to light in engrafted regions and depth of scotomata

Visual acuity

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

WHAT IS CLAIMED IS:

1. A method of treating a subject with dry-form age-related macular degeneration (AMD) comprising administering into the subretina of the subject a therapeutically effective amount of a pharmaceutical composition comprising human RPE cells, wherein at least 95 % of the cells thereof co-express premelanosome protein (PMEL17) and **cellular retinaldehyde binding protein** (CRALBP), wherein the trans-epithelial electrical resistance of the cells is greater than 100 ohms to the subject, thereby treating the subject.

2. A method of treating a subject with a retinal disease or condition comprising administering into the retina of the subject a therapeutically effective amount of a pharmaceutical composition comprising human polygonal RPE cells, wherein at least 95 % of the cells thereof co-express premelanosome protein (PMEL17) and **cellular retinaldehyde binding protein** (CRALBP), wherein the trans-epithelial electrical resistance of the cells is greater than 100 ohms to the subject, wherein the therapeutically effective amount is between 50,000 – 5,000,000 cells per administration, thereby treating the subject.

3. A method of treating a subject with a retinal disease or condition comprising administering into the retina of the subject a therapeutically effective amount of a pharmaceutical composition comprising human RPE cells using a device, wherein the outer diameter of the device through which the cells are administered is between 90-100 μm, thereby treating the subject.

4. The method of claim 3, wherein said device is a cannula.

5. The method of claim 3, wherein at least 95 % of the cells thereof co-express premelanosome protein (PMEL17) and **cellular retinaldehyde binding protein** (CRALBP), wherein the trans-epithelial electrical resistance of the cells is greater than 100 ohms to the subject.

6. The method of claims 1 or 3, wherein the therapeutically effective amount is between50,000-1,000,000 cells per administration.

7. The method of claims 2 or 6, wherein the therapeutically effective amount is selected from the group consisting of 50,000 cells per administration, 200,000 cells per administration, 500,000 cells per administration and 1,000,000 cells per administration.

8. The method of any one of claims 1-7, wherein the cells are administered into the subretinal space of the subject.

9. The method of any one of claims 1-7, wherein the cells are administered in a single administration.

10. The method of claims 2 or 7, wherein the pharmaceutical composition comprises 500 cells per µl – 10,000 cells per µl.

11. The method of claims 2 or 7, wherein when said amount is 50,000 cells per administration, the pharmaceutical composition comprises about 500-1000 cells per µl.

12. The method of claims 2 or 7, wherein when said amount is 200,000 cells per administration, the pharmaceutical composition comprises about 2,000 cells per µl.

13. The method of claims 2 or 7, wherein when said amount is 500,000 cells per administration, the pharmaceutical composition comprises about 5,000 cells per µl.

14. The method of claims 2 or 7, wherein when said amount is 1,000,000 cells per administration, the pharmaceutical composition comprises about 10,000 cells per µl.

15. The method of claims 2 or 3, wherein said retinal disease or condition is selected from the group consisting of retinitis pigmentosa, retinal detachment, retinal dysplasia, retinal atrophy, retinopathy, macular dystrophy, cone dystrophy, cone-rod dystrophy, Malattia Leventinese, Doyne honeycomb dystrophy, Sorsby's dystrophy, pattern/butterfly dystrophies, Best vitelliform dystrophy, North Carolina dystrophy, central areolar choroidal dystrophy, angioid streaks, toxic maculopathy, Stargardt disease, pathologic myopia, retinitis pigmentosa, and macular degeneration.

16. The method of claim 15, wherein said disease is age-related macular degeneration.

17. The method of claim 16, wherein said age-related macular degeneration is dry-form age-related macular degeneration.

18. The method of claims 1 or 17, wherein the subject fulfils at least one of the criteria selected from the group consisting of:

(i) is aged 55 or older;

(ii) has funduscopic findings of dry AMD with geographic atrophy in the macula, above 0.5 disc area in at least one eye;

(iii) is able to undergo a vitreoretinal surgical procedure under monitored anesthesia care; and

(iv) does not have an immunodeficiency disease.

19. The method of claim 18, wherein the subject does not have a retinal disease other than AMD.

20. The method of any one of claims 18 or 19, wherein the subject fulfils each of the criteria (i) – (iv).

21. The method of claims 1 or 2, wherein the outer aperture of the device through which the cells are administered is between 90-100 μm.

22. The method of claim 21, wherein said device is a cannula.

23. The method of claims 4 or 22, wherein said device further comprises a needle.

24. The method of claims 4 or 22, wherein the gauge of said cannula is 25G.

25. The method of any one of claims 1-24, wherein the number of $Oct4^{+}TRA\text{-}1\text{-}60^{+}$ cells in the population is below 1:250,000.

26. The method of any one of claims 1-25, wherein at least 80 % of the cells express Bestrophin 1, as measured by immunostaining.

27. The method of any one of claims 1-26, wherein at least 80 % of the cells express Microphthalmia-associated transcription factor (MITF), as measured by immunostaining.

28. The method of any one of claims 1-27, wherein more than 80 % of the cells express paired box gene 6 (PAX-6) as measured by FACS.

29. The method of any one of claim 1-28, wherein the cells secrete greater than 500 ng of Pigment epithelium-derived factor (PEDF) per ml per day.

30. The method of any one of claim 1-29, wherein the cells secrete PEDF and vascular endothelial growth factor (VEGF) in a polarized manner.

31. The method of claim 30, wherein the ratio of apical secretion of PEDF: basal secretion of PEDF is greater than 1.

32. The method of claim 31, wherein said ratio remains greater than 1 following incubation for 8 hours at 2-8 ° C.

33. The method of any one of claims 1, 2 or 5 wherein said trans-epithelial electrical resistance of the cells remains greater than 100 ohms following incubation for 8 hours at 2-8 ° C.

34. The method of claims 30 or 31, wherein the ratio of basal secretion of VEGF: apical secretion of VEGF is greater than 1.

35. The method of claim 34, wherein said ratio remains greater than 1 following incubation for 8 hours at 2-8 ° C.

36. The method of any one of claims 1-35, wherein the cells are capable of rescuing visual acuity in the RCS rat following subretinal administration.

37. The method of any one of claims 1-35, wherein the cells are capable of rescuing photoreceptors for up to 180 days post-subretinal administration in the RCS rat.

38. The method of any one of claims 1-37, wherein the cells are generated by ex-vivo differentiation of human embryonic stem cells.

39. The method of any one of claims 1-38, wherein the cells are generated by:

(a) culturing human embryonic stem cells or induced pluripotent stem cells in a medium comprising nicotinamide so as to generate differentiating cells, wherein said medium is devoid of activin A;

(b) culturing said differentiating cells in a medium comprising nicotinamide and acitivin A to generate cells which are further differentiated towards the RPE lineage; and

(c) culturing said cells which are further differentiated towards the RPE lineage in a medium comprising nicotinamide, wherein said medium is devoid of activin A.

40. The method of claim 39, wherein said embryonic stem cells or induced pluripotent stem cells are propagated in a medium comprising bFGF and TGFβ.

41. The method of claim 39, wherein said embryonic stem cells are cultured on human umbilical cord fibroblasts.

42. The method of any one of claims 39-41, wherein steps (a)-(c) are effected under conditions wherein the atmospheric oxygen level is less than about 10 %.

43. The method of claim 42, wherein the method further comprises culturing embryonic stem cells or induced pluripotent stem cells in a medium under conditions wherein the atmospheric oxygen level is greater than about 10 % in the presence of nicotinamide prior to step (a).

44. The method of claims 42 or 43, wherein the method further comprises culturing said differentiated cells in a medium under conditions wherein the atmospheric oxygen level is greater than about 10 % in the presence of nicotinamide following step (c).

FIG. 1

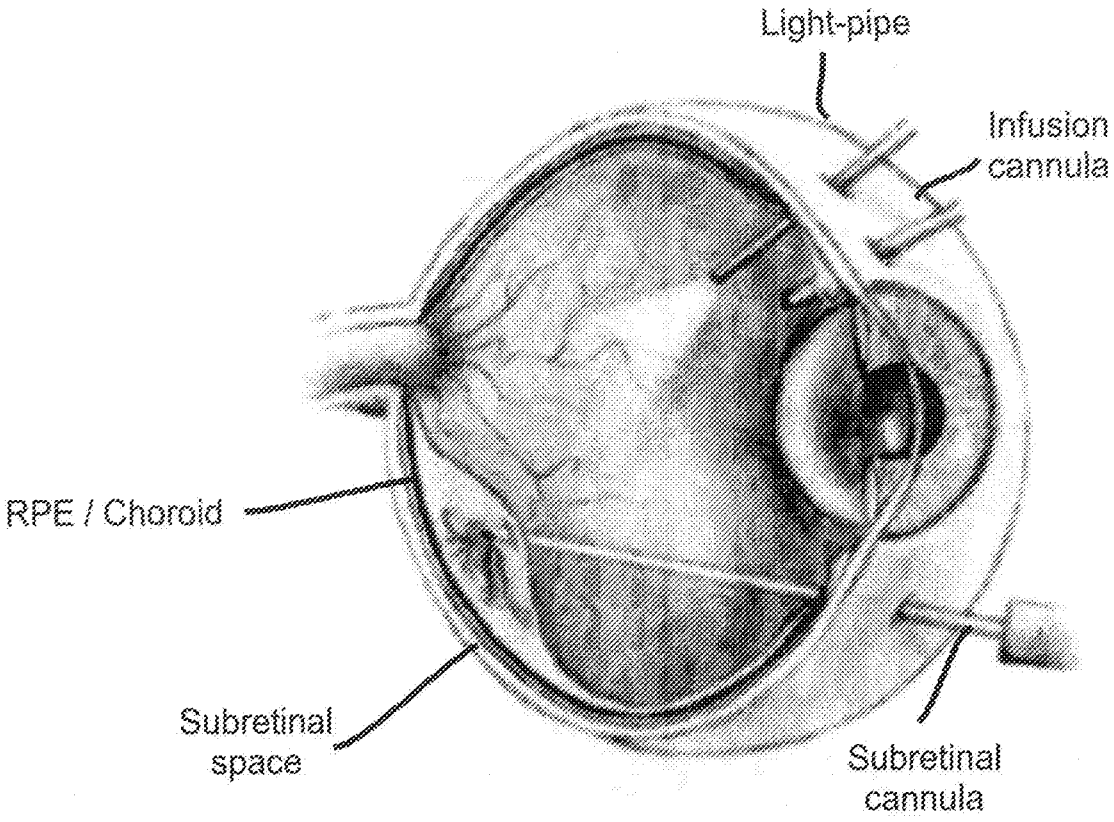

Light-pipe
Infusion cannula
RPE / Choroid
Subretinal space
Subretinal cannula

FIG.2C

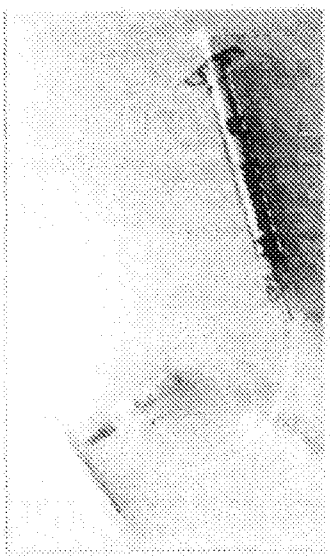


FIG. 2B

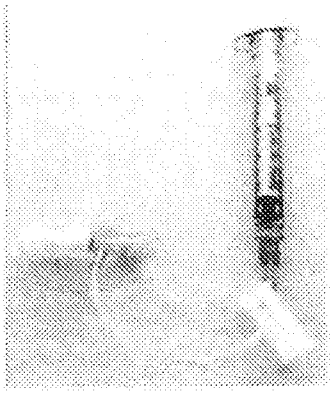
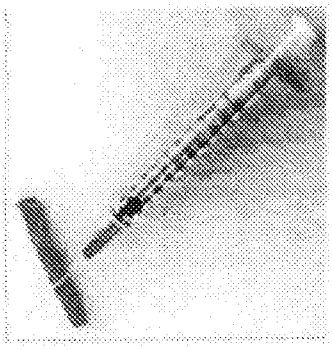


FIG. 2A



INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL2015/050456

A. CLASSIFICATION OF SUBJECT MATTER
IPC (2015.01) A61P 27/00, A61M 35/00, A61F 9/007, A61K 35/30

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC (2015.01) A61M, A61P, A61K, A61F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See extra sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013074681 A1 ADVANCED CELL TECH INC [US]; GAY ROGER [US]; KLIMANSKAYA IRINA [US]; LANZA ROBERT [US]Cited in the document 23 May 2013 (2013/05/23) paragraphs 0024, 0068, 00259, 0271-0273, Examples 1-3, 6 and 8, tables 6,9-12 figure 15, claims 209-213	3,4,6-17
Y	paragraphs 0032, 0063, 00114, 00155-00157,00175-00190, 00200, 00216, Example 4, 8, claims	1,2,5,18-44
Y	VUGLER, Anthony, et al. Elucidating the phenomenon of HESC-derived RPE: anatomy of cell genesis, expansion and retinal transplantation. Experimental neurology, 2008, 214.2: 347-361. [online], [retrieved on 2015-07-06]. Retrieved from the Internet <URL: http://www.researchgate.net/publication/23389123_Elucidating_the_phenomenon_of_HESC-derived_RPE_anatomy_of_cell_genesis_expansion_and_retinal_transplantation._Exp_Neurol >< DOI: 10.1016/j.expneurol.2008.09.007> 27 Sep 2008 (2008/09/27) Figures 3 and 5, page 359 right column paragraph 7	1,2,5,18-44

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 Aug 2015	12 Aug 2015
Name and mailing address of the ISA: Israel Patent Office Technology Park, Bldg.5, Malcha, Jerusalem, 9695101, Israel Facsimile No. 972-2-5651616	Authorized officer RON-COHEN Yael Telephone No. 972-2-5651737

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL2015/050456

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KAMAO, Hiroyuki, et al. Characterization of human induced pluripotent stem cell-derived retinal pigment epithelium cell sheets aiming for clinical application. Stem cell reports, 2014, 2.2: 205-21.[online], [retrieved on 2015-07-06]. Retrieved from the Internet <URL: http://www.sciencedirect.com/science/article/pii/S2213671113001756><DOI: 10.1016/j.stemcr.2013.12.007> 23 Jan 2014 (2014/01/23) the whole document, Figures 2B, 2C, last paragraph on page 214 right column	1,2,5,18-44
Y	LUND, Raymond D., et al. Human embryonic stem cell–derived cells rescue visual function in dystrophic RCS rats. Cloning and stem cells, 2006, 8.3: 189-199. [online], [retrieved on 2015-07-06]. Retrieved from the Internet <URL: http://online.liebertpub.com/doi/abs/10.1089/clo.2006.8.189> 29 Sep 2006 (2006/09/29) the whole document, especially FIG 3, table 1	36-38
Y	IDELSON, Maria, et al. Directed differentiation of human embryonic stem cells into functional retinal pigment epithelium cells. Cell stem cell, 2009, 5.4: 396-408. [online], [retrieved on 2015-07-06]. Retrieved from the Internet <URL: http://www.sciencedirect.com/science/article/pii/S1934590909003361> <DOI 10.1016/j.stem.2009.07.002> 02 Oct 2009 (2009/10/02) the whole document, especially abstract, Figures 1-5	39-44
A	CLEGG, Dennis O., et al. Derivation of retinal pigmented epithelial cells for the treatment of ocular disease. In: Stem Cells Handbook. Springer New York, 2013. p. 411-418. [online], [retrieved on 2015-07-06]. Retrieved from the Internet <URL: http://link.springer.com/chapter/10.1007/978-1-4614-7696-2_29> 03 Jul 2013 (2013/07/03) the whole document	1-44

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IL2015/050456

Patent document cited search report	Publication date	Patent family member(s)	Publication Date
WO 2013074681 A1	23 May 2013	WO 2013074681 A1	23 May 2013
		WO 2013074681 A9	28 Nov 2013
		AU 2012340020 A1	03 Jul 2014
		CA 2855941 A1	23 May 2013
		CN 104080464 A	01 Oct 2014
		EP 2780022 A1	24 Sep 2014
		EP 2780022 A4	05 Aug 2015
		GB 201220511 D0	26 Dec 2012
		GB 2496969 A	29 May 2013
		IL 232600 D0	30 Jun 2014
		JP 2014533289 A	11 Dec 2014
		KR 20140096368 A	05 Aug 2014
		TW 201333200 A	16 Aug 2013
		US 2013195806 A1	01 Aug 2013

B. FIELDS SEARCHED:

* Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Databases consulted: NCBI, THOMSON INNOVATION, Esp@cenet, Google Patents, CAPLUS, MEDLINE, WPI Data, PubMed, Google Scholar
Search terms used: age-related macular degeneration, AMD, retinal pigment epithelium cells, RPE cells, premelanosome protein, PMEL, PMEL17, cellular retinaldehyde binding protein ,CRALBP, transepithelial electric resistance, TEER, device, cannula, VEGF, PEDF, MITF, bestrophin, PAX-6, Oct4, TRA-1-60, RCS rat, Nicotinamide, Activin A, applicant

(19)中华人民共和国国家知识产权局

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(51)Int.Cl.
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A61P 27/02(2006.01)
A61P 9/10(2006.01)
A61M 35/00(2006.01)
A61F 9/007(2006.01)

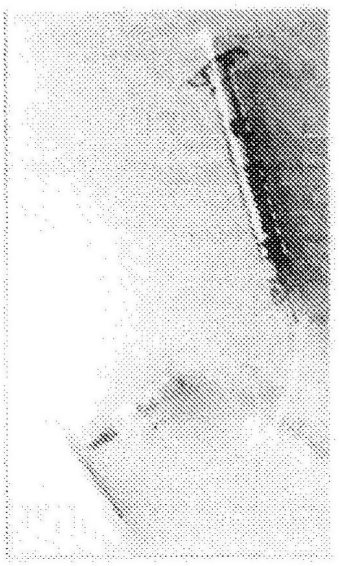
权利要求书3页 说明书24页 附图2页

(54)发明名称

治疗视网膜疾病的方法

(57)摘要

本发明公开了一种治疗具有干性年龄相关性黄斑变性(AMD)的受试者的方法。该方法包括向受试者的视网膜下施用治疗有效量的包含人RPE细胞的药物组合物,其中至少95%的细胞共表达前黑素体蛋白(PMEL17)和细胞视黄醛结合蛋白(CRALBP),其中该细胞的跨上皮细胞电阻大于100欧姆,由此治疗受试者。



CN 107427534 A

1.一种治疗具有干性年龄相关性黄斑变性(AMD)的受试者的方法,其包括向所述受试者的视网膜下施用治疗有效量的包含人RPE细胞的药物组合物,其中至少95%的所述细胞共表达前黑素体蛋白(PMEL17)和细胞视黄醛结合蛋白(CRALBP),其中所述细胞的跨上皮细胞电阻大于100欧姆,由此治疗所述受试者。

2.一种治疗具有视网膜疾病或病症的受试者的方法,其包括向所述受试者的视网膜中施用治疗有效量的包含人多角形RPE细胞的药物组合物,其中至少95%的所述细胞共表达前黑素体蛋白(PMEL17)和细胞视黄醛结合蛋白(CRALBP),其中所述细胞的跨上皮细胞电阻大于100欧姆,其中所述治疗有效量为每次施用50,000-5,000,000个细胞,由此治疗所述受试者。

3.一种治疗具有视网膜疾病或病症的受试者的方法,其包括使用装置向所述受试者的视网膜中施用治疗有效量的包含人RPE细胞的药物组合物,其中通过其施用所述细胞的所述装置的外直径在90-100μm之间,由此治疗所述受试者。

4.权利要求3所述的方法,其中所述装置是套管。

5.权利要求3所述的方法,其中至少95%的所述细胞共表达前黑素体蛋白(PMEL17)和细胞视黄醛结合蛋白(CRALBP),其中所述细胞的跨上皮细胞电阻大于100欧姆。

6.权利要求1或3所述的方法,其中所述治疗有效量为每次施用50,000-1,000,000个细胞。

7.权利要求2或6所述的方法,其中所述治疗有效量选自每次施用50,000个细胞、每次施用200,000个细胞、每次施用500,000个细胞和每次施用1,000,000个细胞。

8.权利要求1-7中任一项所述的方法,其中将所述细胞施用到所述受试者的视网膜下腔中。

9.权利要求1-7中任一项所述的方法,其中所述细胞在单次施用中施用。

10.权利要求2或7所述的方法,其中所述药物组合物包含500个细胞/μl-10,000个细胞/μl。

11.权利要求2或7所述的方法,其中当所述量为每次施用50,000个细胞时,所述药物组合物包含约500-1000个细胞/μl。

12.权利要求2或7所述的方法,其中当所述量为每次施用200,000个细胞时,所述药物组合物包含约2,000个细胞/μl。

13.权利要求2或7所述的方法,其中当所述量为每次施用500,000个细胞时,所述药物组合物包含约5,000个细胞/μl。

14.权利要求2或7所述的方法,其中当所述量为每次施用1,000,000个细胞时,所述药物组合物包含约10,000个细胞/μl。

15.权利要求2或3所述的方法,其中所述视网膜疾病或病症选自色素性视网膜炎、视网膜脱离、视网膜发育不良、视网膜萎缩、视网膜病变、黄斑营养不良、视锥细胞营养不良、视锥-视杆细胞营养不良、Malattia Leventinese、多英蜂窝状营养不良、眼底营养不良、模式/蝶状营养不良、Best卵黄样营养不良、北卡罗来纳营养不良、中央晕轮状脉络膜营养不良、血管样条纹、中毒性黄斑病变、斯特格氏病、病理性近视、色素性视网膜炎和黄斑变性。

16.权利要求15所述的方法,其中所述疾病是年龄相关性黄斑变性。

17.权利要求16所述的方法,其中所述年龄相关性黄斑变性是干性年龄相关性黄斑变

性。

18.权利要求1或17所述的方法,其中所述受试者符合选自以下的标准中的至少一项:

(i)55岁或以上;

(ii)眼底镜检查发现在黄斑中具有地图样萎缩的干性AMD,在至少一只眼睛中大于0.5视盘面积;

(iii)能够在受监测的麻醉下进行玻璃体视网膜外科手术;和

(iv)不具有免疫缺陷疾病。

19.权利要求18所述的方法,其中所述受试者不具有AMD以外的视网膜疾病。

20.权利要求18或19中任一项所述的方法,其中所述受试者符合标准(i)-(iv)中的每一项。

21.权利要求1或2所述的方法,其中通过其施用所述细胞的所述装置的外孔径在90-100μm之间。

22.权利要求21所述的方法,其中所述装置是套管。

23.权利要求4或22所述的方法,其中所述装置还包括针。

24.权利要求4或22所述的方法,其中所述套管的规格为25G。

25.权利要求1-24中任一项所述的方法,其中群体中$Oct4^{+}TRA\text{-}1\text{-}60^{+}$细胞的数量低于1:250,000。

26.权利要求1-25中任一项所述的方法,其中如通过免疫染色测量的,至少80%的所述细胞表达卵黄状黄斑病蛋白1。

27.权利要求1-26中任一项所述的方法,其中如通过免疫染色测量的,至少80%的所述细胞表达小眼畸形相关转录因子(MITF)。

28.权利要求1-27中任一项所述的方法,其中如通过FACS测量的,超过80%的所述细胞表达配对盒基因6(PAX-6)。

29.权利要求1-28中任一项所述的方法,其中所述细胞分泌大于500ng的色素上皮衍生因子(PEDF)/ml/天。

30.权利要求1-29中任一项所述的方法,其中所述细胞以极化方式分泌PEDF和血管内皮生长因子(VEGF)。

31.权利要求30所述的方法,其中PEDF的顶部分泌:PEDF的基底部分泌的比率大于1。

32.权利要求31所述的方法,其中所述比率在2-8℃下孵育8小时后保持大于1。

33.权利要求1、2或5中任一项所述的方法,其中所述细胞的所述跨上皮细胞电阻在2-8℃下孵育8小时后保持大于100欧姆。

34.权利要求30或31所述的方法,其中VEGF的基底部分泌:VEGF的顶部分泌的比率大于1。

35.权利要求34的方法,其中所述比率在2-8℃下孵育8小时后保持大于1。

36.权利要求1-35中任一项所述的方法,其中所述细胞在视网膜下施用后能够挽救RCS大鼠的视敏度。

37.权利要求1-35中任一项所述的方法,其中所述细胞在视网膜下施用后能够挽救RCS大鼠的光感受器至多180天。

38.权利要求1-37中任一项所述的方法,其中所述细胞通过人胚胎干细胞的离体分化

产生。

39.权利要求1-38中任一项所述的方法,其中所述细胞通过以下产生:

(a)在包含烟酰胺的培养基中培养人胚胎干细胞或诱导多能干细胞以产生分化细胞,其中所述培养基不含激活素A;

(b)在包含烟酰胺和激活素A的培养基中培养所述分化细胞以产生进一步朝向RPE谱系分化的细胞;和

(c)在包含烟酰胺的培养基中培养进一步朝向RPE谱系分化的所述细胞,其中所述培养基不含激活素A。

40.权利要求39所述的方法,其中所述胚胎干细胞或诱导多能干细胞在包含bFGF和TGFβ的培养基中繁殖。

41.权利要求39所述的方法,其中所述胚胎干细胞在人脐带成纤维细胞上培养。

42.权利要求39-41中任一项所述的方法,其中步骤(a)-(c)在其中环境氧水平低于约10%的条件下进行。

43.权利要求42所述的方法,其中所述方法还包括在步骤(a)之前,在存在烟酰胺的情况下,在其中环境氧水平大于约10%的条件下,在培养基中培养胚胎干细胞或诱导多能干细胞。

44.权利要求42或43所述的方法,其中所述方法还包括在步骤(c)之后,在存在烟酰胺的情况下,在其中环境氧水平大于约10%的条件下,在培养基中培养所述分化细胞。

治疗视网膜疾病的方法

技术领域

[0001] 本发明在其一些实施方式中涉及治疗视网膜疾病，更特别地是年龄相关性黄斑变性(AMD)的方法。

背景技术

[0002] 十多年前，hESC的获得已经引起对于通过将这些细胞用作治疗性细胞的起始材料而用于再生的潜在临床用途的极大兴趣。虽然尚未批准hESC衍生的细胞用于临床应用，但多年来朝着实现这一目标已经取得显著进展。重要的进展包括对hESC的生物学的更好理解、在使其分化为各种细胞类型的能力方面的技术改进以及其在疾病特异性动物模型中的治疗效果的临床前证据。

[0003] RPE是位于神经视网膜与脉络膜毛细血管层之间的单层色素细胞。RPE特征在于顶部至基底侧的结构和功能的极性。在顶部侧，细胞与光感受器直接接触。在它们的侧壁上，它们形成紧密的、粘附的间隙连接，并且在它们的基底侧面上，它们接触下面的布鲁赫氏基底膜，该基底膜将它们与脉络膜血管分开。RPE细胞在视网膜及其光感受器的维持和功能中发挥关键作用。这些包括血-视网膜屏障的形成、杂散光的吸收、对神经视网膜的营养供应、视觉色素的再生以及脱落的光感受器外段的摄取和再循环。

[0004] RPE细胞的功能障碍、退化和丧失是AMD、Best病和色素性视网膜炎(RP)亚型的突出特征。AMD是西方世界中的视觉障碍的主要原因。在75岁以上年龄的人群中，25-30％受到年龄相关性黄斑变性(AMD)的影响，伴随进行性中心视力丧失，这导致6-8％的患者失明。视网膜变性主要涉及黄斑，其为负责精细视觉细节和色觉的视网膜中心部分。干性AMD由RPE的增生和RPE下方或布鲁赫氏膜内由代谢终产物构成的玻璃膜疣沉积的形成所引起。干性AMD可能逐渐进展到地图样萎缩(GA)的晚期阶段，具有在黄斑的大面积上RPE细胞和光感受器的退化，导致中心视力丧失。10％的干性AMD患者将进展到新生血管(湿性)AMD，其中血管通过布鲁赫氏膜生长且随后具有出现眼内渗漏和/或出血，从而加速中心视力的丧失。虽然可以用抗VEGF剂治疗并发的新生血管形成，但目前没有有效的治疗以停止RPE和光感受器退化，因而许多患者将最终失去其视力。

[0005] 在动物和人类中的移植研究都提供了将RPE细胞移植到AMD患者中的潜在治疗效果的证据。在人类中，黄斑移位到更加外周的RPE上，以及外周RPE作为RPE和脉络膜的细胞悬浮液或补片的自体移植，提供了在相对更加健康的RPE细胞上设置黄斑可以在一些AMD患者中改善视觉功能的原理论证。然而，自体移植的外科手术程序具有挑战性，并且与严重的并发症相关。通常，RPE细胞通过标准3口玻璃体切除术后的小型视网膜切开术递送到在黄斑区域中沿着在GA和更好地保持的中央凹外视网膜的区域与RPE层之间的边界产生的视网膜下腔(subretinal space)。这样的细胞置换策略在治疗AMD方面的成功取决于建立能够使移植细胞存活和发挥功能并使视网膜损伤最小化的安全递送系统。

[0006] 背景技术包括WO2013/114360、WO2013/074681、WO2008/129554和WO2013/184809、美国专利申请号62/116,972和美国专利申请号62/116,980。

发明内容

[0007] 根据本发明的一些实施方式的一个方面，提供了治疗具有干性年龄相关性黄斑变性(AMD)的受试者的方法，其包括向受试者的视网膜下(subretina)施用治疗有效量的包含人RPE细胞的药物组合物，其中至少95％的该细胞共表达前黑素体蛋白(PMEL17)和细胞视黄醛结合蛋白(CRALBP)，其中细胞的跨上皮细胞电阻(trans-epithelial electrical resistance)大于100欧姆，由此治疗受试者。

[0008] 根据本发明的一些实施方式的一个方面，提供了治疗具有视网膜疾病或病症的受试者的方法，其包括向受试者的视网膜中施用治疗有效量的包含人多角形RPE细胞的药物组合物，其中至少95％的该细胞共表达前黑素体蛋白(PMEL17)和细胞视黄醛结合蛋白(CRALBP)，其中细胞的跨上皮细胞电阻大于100欧姆，其中治疗有效量为每次施用50,000-5,000,000个细胞，由此治疗受试者。

[0009] 根据本发明的一些实施方式的一个方面，提供了治疗具有视网膜疾病或病症的受试者的方法，其包括使用装置向受试者的视网膜中施用治疗有效量的包含人RPE细胞的药物组合物，其中细胞通过其施用的装置的外直径在90-100μm之间，由此治疗受试者。

[0010] 根据本发明的一些实施方式，所述装置是套管。

[0011] 根据本发明的一些实施方式，至少95％的该细胞共表达前黑素体蛋白(PMEL17)和细胞视黄醛结合蛋白(CRALBP)，其中细胞的跨上皮细胞电阻大于100欧姆。

[0012] 根据本发明的一些实施方式，治疗有效量为每次施用50,000-1,000,000个细胞。

[0013] 根据本发明的一些实施方式，治疗有效量选自每次施用50,000个细胞、每次施用200,000个细胞、每次施用500,000个细胞和每次施用1,000,000个细胞。

[0014] 根据本发明的一些实施方式，将细胞施用到受试者的视网膜下腔中。

[0015] 根据本发明的一些实施方式，细胞在单次施用中施用。

[0016] 根据本发明的一些实施方式，药物组合物包含500个细胞/μl-10,000个细胞/μl。

[0017] 根据本发明的一些实施方式，当所述量为每次施用50,000个细胞时，药物组合物包含约500-1000个细胞/μl。

[0018] 根据本发明的一些实施方式，当所述量为每次施用200,000个细胞时，药物组合物包含约2,000个细胞/μl。

[0019] 根据本发明的一些实施方式，当所述量为每次施用500,000个细胞时，药物组合物包含约5,000个细胞/μl。

[0020] 根据本发明的一些实施方式，当所述量为每次施用1,000,000个细胞时，药物组合物包含约10,000个细胞/μl。

[0021] 根据本发明的一些实施方式，视网膜疾病或病症选自色素性视网膜炎、视网膜脱离、视网膜发育不良、视网膜萎缩、视网膜病变、黄斑营养不良、视锥细胞营养不良、视锥-视杆细胞营养不良、Malattia Leventinese、多英蜂窝状营养不良、眼底营养不良(Sorsby's dystrophy)、模式/蝶状营养不良(pattern/butterfly dystrophies)、Best卵黄样营养不良、北卡罗来纳营养不良、中央晕轮状脉络膜营养不良、血管样条纹、中毒性黄斑病变、斯特格氏病、病理性近视、色素性视网膜炎和黄斑变性。

[0022] 根据本发明的一些实施方式，疾病是年龄相关性黄斑变性。

[0023] 根据本发明的一些实施方式，年龄相关性黄斑变性是干性年龄相关性黄斑变性。

[0024] 根据本发明的一些实施方式，受试者符合选自以下的标准中的至少一项：

[0025] (i)55岁或以上；

[0026] (ii)眼底镜检查发现在黄斑中具有地图样萎缩的干性AMD，在至少一只眼睛中大于0.5视盘面积(disc area)；

[0027] (iii)能够在受监测的麻醉下进行玻璃体视网膜外科手术；和

[0028] (iv)不具有免疫缺陷疾病。

[0029] 根据本发明的一些实施方式，受试者不具有AMD以外的视网膜疾病。

[0030] 根据本发明的一些实施方式，受试者符合标准(i)-(iv)中的每一项。

[0031] 根据本发明的一些实施方式，细胞通过其施用的装置的外孔在90-100μm之间。

[0032] 根据本发明的一些实施方式，装置是套管。

[0033] 根据本发明的一些实施方式，装置还包括针。

[0034] 根据本发明的一些实施方式，套管的规格为25G。

[0035] 根据本发明的一些实施方式，群体中$Oct4^{+}TRA\text{-}1\text{-}60^{+}$细胞的数量低于1:250,000。

[0036] 根据本发明的一些实施方式，如通过免疫染色测量的，至少80％的细胞表达卵黄状黄斑病蛋白(Bestrophin)1。

[0037] 根据本发明的一些实施方式，如通过免疫染色测量的，至少80％的细胞表达小眼畸形(Microphthalmia)相关转录因子(MITF)。

[0038] 根据本发明的一些实施方式，如通过FACS测量的，超过80％的细胞表达配对盒基因6(PAX-6)。

[0039] 根据本发明的一些实施方式，细胞分泌大于500ng色素上皮衍生因子(PEDF)/ml/天。

[0040] 根据本发明的一些实施方式，细胞以极化方式分泌PEDF和血管内皮生长因子(VEGF)。

[0041] 根据本发明的一些实施方式，PEDF的顶部分泌(apical secretion):PEDF的基底部分泌(basal secretion)的比率大于1。

[0042] 根据本发明的一些实施方式，该比率在2-8℃下孵育8小时后保持大于1。

[0043] 根据本发明的一些实施方式，细胞的跨上皮细胞电阻在2-8℃下孵育8小时后保持大于100欧姆。

[0044] 根据本发明的一些实施方式，VEGF的基底部分泌:VEGF的顶部分泌的比率大于1。

[0045] 根据本发明的一些实施方式，该比率在2-8℃下孵育8小时后保持大于1。

[0046] 根据本发明的一些实施方式，细胞在RCS大鼠中视网膜下施用后能够挽救视敏度。

[0047] 根据本发明的一些实施方式，细胞在RCS大鼠中视网膜下施用后能够挽救光感受器至多180天。

[0048] 根据本发明的一些实施方式，细胞通过人胚胎干细胞的离体分化产生。

[0049] 根据本发明的一些实施方式，细胞通过以下产生：

[0050] (a)在包含烟酰胺的培养基中培养人胚胎干细胞或诱导多能干细胞以产生分化细胞，其中培养基不含激活素A；

[0051] (b)在包含烟酰胺和激活素A的培养基中培养分化细胞以产生进一步朝向RPE谱系

分化的细胞;和

[0052] (c)在包含烟酰胺的培养基中培养进一步朝向RPE谱系分化的细胞,其中培养基不含激活素A。

[0053] 根据本发明的一些实施方式,胚胎干细胞或诱导多能干细胞在包含bFGF和TGFβ的培养基中繁殖。

[0054] 根据本发明的一些实施方式,胚胎干细胞在人脐带成纤维细胞上培养。

[0055] 根据本发明的一些实施方式,步骤(a)-(c)在其中环境氧水平低于约10%的条件下进行。

[0056] 根据本发明的一些实施方式,该方法还包括在步骤(a)之前,在存在烟酰胺的情况下,在其中环境氧水平大于约10%的条件下,在培养基中培养胚胎干细胞或诱导多能干细胞。

[0057] 根据本发明的一些实施方式,该方法还包括在步骤(c)之后,在存在烟酰胺的情况下,在其中环境氧水平大于约10%的条件下,在培养基中培养分化细胞。

[0058] 除非另有规定,否则本文使用的所有技术和/或科学术语都具有与本发明所属领域的普通技术人员通常理解的相同的含义。尽管类似于或等同于本文所述的那些的方法和材料可以用于本发明的实施方式的实践或测试中,但下文描述了示例性方法和/或材料。在冲突的情况下,将以本专利说明书(包括定义)为准。此外,材料、方法和实施例仅是说明性的,而非旨在必定是限制性的。

附图说明

[0059] 本文仅通过示例的方式参照附图描述本发明的一些实施方式。现在具体参考附图,应当强调的是,所示细节是作为示例并且是出于说明性地讨论本发明的实施方式的目的。在这方面,结合附图的描述使得对于本领域技术人员而言可以如何实施本发明的实施方式是显而易见的。

[0060] 在附图中:

[0061] 图1是视网膜下注射方案的示意图。注意在该图中,手术口的设置在解剖学上不是精确的(Stout和Francis,2011,Human Gene Ther.2011年5月,22(5):531-5)。

[0062] 图2A-C是说明递送和加载配制细胞的装置的组装的照片。A.连接至18G钝头填充针的注射器。B.用延长管代替18G钝头填充针。C.组装的递送装置。

具体实施方式

[0063] 本发明在其一些实施方式中涉及治疗视网膜疾病,更特别地是年龄相关性黄斑变性(AMD)的方法。

[0064] 在详细说明本发明的至少一个实施方式之前,应当理解,本发明在其应用中不一定限于下文说明书中阐述的或实施例中例示的细节。本发明能够具有其他实施方式或以各种方式实施或执行。

[0065] AMD是中心视网膜的进行性慢性疾病,并且是世界范围内的视力丧失的主要原因。由于以下两种进程之一,大多数视力丧失发生在该疾病的晚期阶段:新生血管(“湿性”)AMD和地图样萎缩(GA,“干性”)。在新生血管性年龄相关性黄斑变性中,脉络膜新血管生成突破

至RPE下或视网膜下腔，从而渗漏液体、脂质和血液并导致纤维性瘢痕形成。在GA中，出现了视网膜色素上皮细胞、脉络膜毛细血管层和光感受器的进行性萎缩。干性AMD更为常见(占所有病例的85-90％)，但可能进展到“湿”性，其如果不经治疗，则导致快速和严重的视力丧失。

[0066] AMD在美国和其他发达国家中的估计患病率为2000人中1人。该流行率预期与普通人群中的老年人的比例一起增加。该疾病的风险因素包括环境和遗传因素二者。

[0067] 该疾病的发病机制涉及四种功能相关组织(即视网膜色素上皮(RPE)、布鲁赫氏膜、脉络膜毛细血管层和光感受器)的异常。然而，RPE细胞功能的受损是导致临床相关的AMD变化的分子途径中的早期和关键事件。

[0068] 目前对于干性AMD没有有效或获批的疗法。预防措施包括维生素/矿物质补充剂。这些降低了发生湿性AMD的风险，但不影响出现地图样萎缩的进展。

[0069] 目前，具有约二十种处于临床开发的各个阶段中的疗法。其中包括补体系统抑制剂和皮质类固醇、视觉周期调节剂、抗氧化剂、神经保护剂、血管增强剂以及细胞和基因疗法-参见例如Dugel等，2014，Retina Today，第70-72页；和Patel等，2015，Practical Retina，2015年1月，Vol.46，No.1，第8-13页。

[0070] 已经提议人胚胎干细胞作为产生RPE细胞的细胞来源。已经使用两种一般途径从hESC获得RPE细胞：自发分化和定向分化。在自发分化中，允许扁平集落或类胚体(EB)中的hESC自发分化成含有色素性RPE细胞的细胞群。定向分化方法使用许多因素来驱动hESC到RPE细胞的分化，参见例如WO2008/129554。

[0071] 本发明人现在已经发现用PRE细胞进行临床治疗的最佳条件，包括剂量和治疗方案以及用于递送细胞的装置。本发明还提供了将通过用细胞进行治疗而获得益处的患者群体的选择标准。

[0072] 因此，根据本发明的一个方面，提供了治疗具有视网膜疾病(例如干性年龄相关性黄斑变性(AMD))的受试者的方法，其包括向受试者的视网膜下施用治疗有效量的包含人RPE细胞的药物组合物，其中至少95％的该细胞共表达前黑素体蛋白(PMEL17)和细胞视黄醛结合蛋白(CRALBP)，其中细胞的跨上皮细胞电阻大于100欧姆，由此治疗受试者。

[0073] 药物组合物用作治疗剂的眼睛病症包括但不限于总体与视网膜功能障碍、视网膜损伤和/或视网膜色素上皮的损失相关的视网膜疾病或紊乱。根据本发明可以治疗的病症的非限制性列表包括色素性视网膜炎、利伯先天性黑朦、遗传性或获得性黄斑变性、年龄相关性黄斑变性(AMD)、Best病、视网膜脱离、回旋状萎缩、无脉络膜症、模式营养不良以及其它RPE的营养不良、斯特格氏病，由光性、激光、炎症、感染、辐射、新生血管或创伤性损伤中的任一种引起的伤害所导致的RPE和视网膜损害。

[0074] 根据一个特别实施方式，疾病是干性年龄相关性黄斑变性。

[0075] 可以治疗的受试者包括灵长类(包括人类)、犬科动物、猫科动物、有蹄类动物(例如马科动物、牛科动物、猪类动物(swine)(例如猪))、鸟类和其他受试者。人类和具有商业重要性的非人类动物(例如，牲畜和家养动物)特别令人感兴趣。可以治疗的示例性哺乳动物包括犬科动物；猫科动物；马科动物；牛科动物；绵羊类动物；啮齿动物等及灵长类，特别是人类。非人动物模型，特别是哺乳动物，例如，灵长类、鼠科动物、兔形目动物等可用于实验研究。

[0076] 根据一个实施方式，被治疗的受试者年龄为55岁或以上。

[0077] 根据另一个实施方式，被治疗的受试者的眼底镜检查发现在黄斑中具有地图样萎缩的干性AMD，在至少一只眼睛中大于0.5视盘面积(1.25mm^2，并且至多17mm^2)。

[0078] 根据又一个实施方式，被治疗的受试者处于使得他能够在受监测的麻醉下进行玻璃体视网膜外科手术的状态。

[0079] 根据又一个实施方式，受试者不具有免疫缺陷疾病。

[0080] 根据又一个实施方式，受试者不具有大于-6屈光度的轴向近视。

[0081] 根据又一个实施方式，受试者不具有视网膜脱离修复的历史。

[0082] 根据又一个实施方式，受试者不具有AMD以外的视网膜疾病。

[0083] 优选地，被治疗的受试者符合上述标准中的至少一项、两项、三项、四项、五项、六项或所有。

[0084] 如本文所述产生的RPE细胞可以移植到受试者眼睛内的各种靶位点。根据一个实施方式，RPE细胞是移植到眼睛的视网膜下腔，其是RPE的正常解剖学位置(在光感受器外段与脉络膜之间)。此外，取决于细胞的迁移能力和/或积极旁分泌作用，可以考虑移植到另外的眼部区室中，包括玻璃体腔、内部或外部视网膜、视网膜周边和脉络膜内。

[0085] 可以施用于受试者的活细胞的数量通常在每次注射50,000-5×10^{6}之间、50,000-4×10^{6}之间、50,000-3×10^{6}之间、50,000-2×10^{6}之间、50,000-1×10^{6}之间、50,000-500,000之间。

[0086] 本发明考虑单次施用或多次施用。优选地，第一次施用于眼睛和第二次施用于同一眼睛之间的时间为至少一个月。

[0087] 根据一个具体实施方式，受试者的每只眼睛可以施用的活细胞的数量在50,000-2×10^{6}之间、50,000-1×10^{6}之间、50,000-500,000之间。示例性剂量包括每只眼睛50,000个细胞、每只眼睛100,000个细胞、每只眼睛200,000个细胞、每只眼睛300,000个细胞、每只眼睛400,000个细胞、每只眼睛500,000个细胞和每只眼睛1×10^{6}个细胞。

[0088] 细胞通常配制在载体(例如等渗溶液和/或盐水)如BSS plus^{TM}中。载体可以任选地包含支持RPE植入、整合、存活、效力等的另外的因子。

[0089] 包含50,000个细胞的药物组合物的示例性浓度为约500个细胞/μl或1,000个细胞/μl。包含200,000个活细胞的药物组合物的示例性浓度为约500个活细胞/μl或1,000个活细胞/μl。包含500,000个细胞的药物组合物的示例性浓度为约5,000个活细胞/μl或约10,000个活细胞/μl。包含1×10^{6}个细胞的药物组合物的示例性浓度为约10,000个活细胞/μl。

[0090] 移植可以通过本领域已知的各种技术进行。用于进行RPE移植的方法描述于例如美国专利号5,962,027、6,045,791和5,941,250中以及Eye Graefes Arch Clin Exp Opthalmol，1997年3月；235(3):149-58；Biochem Biophys Res Commun，2000年2月24日；268(3):842-6；Opthalmic Surg，1991年2月；22(2):102-8中。用于进行角膜移植的方法描述于例如美国专利号5,755,785中以及Eye 1995；9(Pt 6Su)；6-12；Curr Opin Opthalmol，1992年8月；3(4):473-81；Ophthalmic Surg Lasers，1998年4月；29(4):305-8；Ophthalmology，2000年4月；107(4):719-24；和Jpn J Ophthalmol，1999年11-12月；43(6):502-8中。如果主要利用旁分泌作用，则细胞也可以包封在半渗透性容器内被递送并保持在

眼睛中，这也将减少细胞暴露于宿主免疫系统(Neurotech USA CNTF delivery system；PNAS，2006年3月7日，vol.103(10)3896-3901)。

[0091] 施用步骤可以包括将RPE细胞眼内施用到有需要的眼睛中。眼内施用可以包括将RPE细胞注射到视网膜下腔中。

[0092] 根据一个实施方式，移植通过扁平部玻璃体切除手术，然后将细胞经小的视网膜开口递送到视网膜下腔中进行，或者通过直接注射进行。

[0093] RPE细胞可以以各种形式移植。例如，RPE细胞可以以细胞悬浮液的形式引入到靶位点中，细胞悬浮液具有基质或粘附到基质或膜、细胞外基质或基底如可生物降解聚合物或组合上。RPE细胞也可以与其他视网膜细胞(如与光感受器)一起移植(共移植)。

[0094] 根据一个特别实施方式，将水性溶液(例如等渗溶液和/或盐水)或空气施用到视网膜下腔中，由此形成初始的泡(bleb)。然后将作为悬浮液或在支架上的RPE细胞施用到同一视网膜下腔中。注射可以是通过针或注射套管。

[0095] 根据一个特别实施方式，使用外直径在90-100μm之间的递送装置(例如针或注射套管)递送作为细胞悬浮液的细胞。根据另一个实施方式，使用内孔直径在65-75μm之间的递送装置(例如针或注射套管)递送作为细胞悬浮液的细胞。

[0096] 根据本发明的这个方面的实施方式，使用18G针将在70,000-1.4×10^6个活细胞/100μl浓度的最终制剂中的RPE细胞装载到递送装置(例如1mL注射器)中(70,000个细胞是装载用于50,000的剂量，700,000个细胞是装载用于500,000的剂量，且1.4×10^6个细胞是装载用于1×10^6的剂量)。然后可以用延长管(例如5-10cm之间)替换18G针，并且通过延长管去除空气。然后可以将具有外直径在90-100μm之间的尖端(例如41G)的注射套管附接到延长管的末端。根据另一个实施方式，尖端的孔的内直径为约65-75μm(例如约70μm)。

[0097] 装载到针或注射套管中时，RPE细胞的浓度可以在约2,000个活细胞/μl和约14,000个活细胞/μl之间。从针或注射套管递送的活RPE细胞的浓度可以在约1,000个活细胞/μl和约10,000个活细胞/μl之间。

[0098] 根据一个特别实施方式，套管包括41G尖端(如由Peregrine制造的实例)。根据又一个实施方式，套管是25G套管。

[0099] 本发明提供包括18G针和25G/41G套管的制品。这样的装置可用于RPE细胞的摄取和RPE细胞的后续眼内施用。

[0100] 该装置还可包括延长管(例如约5和15cm之间)和注射器(例如1-2ml注射器)。

[0101] 根据另一个方面，提供了包括25G/41G套管、注射器(例如1-2ml注射器)和延长管(例如约5和15cm之间)的制品。该制品还可包括18G针。

[0102] 治疗的有效性可以通过视觉和眼部功能和结构的不同测量来评估，包括尤其是最佳矫正视力(BCVA)，如通过在暗和光适应状态下的视野测量或微视野检查所测量的视网膜对光的敏感性，全视野、多焦点、焦点或图形视网膜电图(ERG)，对比敏感度，阅读速度，色觉，临床生物显微镜检查，眼底照相，光学相干断层扫描(OCT)，眼底自发荧光(FAF)，红外和多色成像，荧光素或ICG血管造影术，以及用于评估视力功能和眼部结构的另外的手段。

[0103] 受试者可以在施用RPE细胞之前或同时施用皮质类固醇，例如泼尼松龙或甲泼尼龙、百力特(Predforte)。

[0104] 根据另一个实施方式，受试者在施用RPE细胞之前或同时不施用皮质类固醇，例如

泼尼松龙或甲泼尼龙、百力特。

[0105] 免疫抑制药物可以在治疗之前、同时和/或之后施用于受试者。

[0106] 免疫抑制药物可以属于以下类别：

[0107] 糖皮质激素、细胞生长抑制剂(例如烷化剂或抗代谢物)、抗体(多克隆或单克隆)、作用于免疫亲和素的药物(例如环孢素、他克莫司或西罗莫司)。另外的药物包括干扰素、阿片类、TNF结合蛋白、麦考酚酯和小的生物药剂。

[0108] 免疫抑制药物的实例包括：间充质干细胞、抗淋巴细胞球蛋白(ALG)多克隆抗体、抗胸腺细胞球蛋白(ATG)多克隆抗体、硫唑嘌呤、BAS1L1X1MAB®(抗-IL-2Ra受体抗体)、环孢菌素(环孢菌素A)、DACLIZUMAB®(抗-IL-2Ra受体抗体)、依维莫司、霉酚酸、RITUX1MAB®(抗CD20抗体)、西罗莫司、他克莫司、大环哌喃和/或麦考酚酯。

[0109] 抗生素可以在治疗之前、同时和/或之后施用于受试者。抗生素的实例包括Oflox、庆大霉素、氯霉素、Tobrex、Vigamox或任何其他批准用于眼部使用的局部抗生素制剂。

[0110] 当上下文允许时可以互换使用的“视网膜色素上皮细胞”、“RPE细胞”、“RPE”是指功能上类似于形成视网膜的色素上皮细胞层的天然RPE细胞的细胞类型的细胞(例如在移植到在眼内时，它们表现出与天然RPE细胞相似的功能活性)。因此，根据本公开，术语“视网膜色素上皮细胞”、“RPE细胞”或“RPE”可以用于指视网膜色素层的天然RPE细胞和直接从人干细胞(hSC)分化的RPE细胞二者。

[0111] 术语“hSC衍生的RPE细胞”在本文中用于表示通过从hSC定向分化获得的RPE细胞。根据一个优选实施方式，hSC衍生的RPE细胞是如通过下文定义的参数所表现的功能性RPE细胞。术语“定向分化”与术语“RPE诱导分化”可互换使用并且被理解为意指在诱导/促进分化为RPE细胞类型的培养条件下操纵hSC的过程。

[0112] 根据一个特别实施方式，RPE细胞通过在TGFβ超家族的一个或多个成员的存在下hSC的定向分化而获得，并且表现出以下特征中的至少一个：

[0113] -在分化期间，培养的细胞响应TGFβ信号传导；

[0114] -RPE细胞表达指示终末分化的标志物，例如卵黄状黄斑病蛋白1、CRALBP和/或RPE65；

[0115] -在移植后(即原位)，RPE细胞表现出支持与RPE细胞相邻的光感受器的营养作用；

[0116] -另外，作为这些光感受器的正常更新过程的部分，RPE细胞能够原位地通过吞噬脱落的光感受器外段发挥作用；

[0117] 另外，RPE细胞能够原位地产生视网膜屏障并且在视觉周期中起作用。

[0118] 如本文所用的，短语“干细胞”是指能够在培养物中延长的时间段内保持未分化状态(例如多能或多潜能干细胞)直至被诱导分化为具有特定、专门功能的其它细胞类型(例如，完全分化的细胞)的细胞。优选地，短语“干细胞”包括胚胎干细胞(ESC)、诱导多能干细胞(iPSC)、成体干细胞、间充质干细胞和造血干细胞。

[0119] 根据一个特别实施方式，从ESC产生RPE细胞。

[0120] 短语“胚胎干细胞”是指能够分化为所有三个胚胎胚层(即内胚层、外胚层和中胚层)的细胞或保持未分化状态的胚胎细胞。短语“胚胎干细胞”可以包括从在怀孕之后(例如胚泡)、植入胚胎之前(即，植入前胚泡)形成的胚胎组织获得的细胞，从植入后/原肠胚形成前阶段胚泡获得的扩展胚泡细胞(EBC)(参见WO2006/040763)，以及在妊娠期间的任何时

间，优选在妊娠第10周之前从胎儿的生殖器组织获得的胚胎生殖(EG)细胞。本发明的一些实施方式的胚胎干细胞可以使用公知的细胞培养方法获得。例如，人胚胎干细胞可以从人胚泡分离。人胚泡通常从人体内植入前胚胎或从体外受精(IVF)胚胎获得。或者，单细胞人胚胎可以扩展到胚泡阶段。为了分离人ES细胞，从胚泡中去除透明带，并通过其中将滋养外胚层细胞裂解并通过温和吸打从完整内细胞团(ICM)除去的程序分离ICM。然后将ICM接种在含有使其能够长出的适当培养基的组织培养瓶中。在9至15天之后，通过机械解离或通过酶促降解将ICM衍生的生长物解离成团块，然后将细胞再接种在新鲜组织培养基上。通过微量移液管单独选择显示未分化形态的集落，将其机械解离成团并再接种。然后每4-7天常规分割所得ES细胞。关于制备人ES细胞的方法的进一步细节参见Reubinoff等，Nat Biotechnol，2000年5月:18(5):559;Thomson等，[美国专利号5,843,780;Science 282:1145,1998;Curr.Top.Dev.Biol.38:133,1998;Proc.Natl.Acad.Sci.USA 92:7844,1995];Bongso等，[Hum Reprod 4:706,1989];和Gardner等，[Fertil.Steril.69:84,1998]。

[0121] 将理解，根据本发明的一些实施方式，市售干细胞也可以使用。人ES细胞可以从NIH人胚胎干细胞注册处购买[Hypertext Transfer Protocol://grants(dot)nih(dot)gov/stem_cells/registry/current(dot)htm]。市售胚胎干细胞系的非限制性实例是HAD-C102、ESI、BG01、BG02、BG03、BG04、CY12、CY30、CY92、CY10、TE03、TE32、CHB-4、CHB-5、CHB-6、CHB-8、CHB-9、CHB-10、CHB-11、CHB-12、HUES 1、HUES 2、HUES 3、HUES 4、HUES 5、HUES 6、HUES 7、HUES 8、HUES 9、HUES 10、HUES 11、HUES 12、HUES 13、HUES 14、HUES 15、HUES 16、HUES 17、HUES 18、HUES 19、HUES 20、HUES 21、HUES 22、HUES 23、HUES 24、HUES 25、HUES 26、HUES 27、HUES 28、CyT49、RUES3、WA01、UCSF4、NYUES1、NYUES2、NYUES3、NYUES4、NYUES5、NYUES6、NYUES7、UCLA 1、UCLA 2、UCLA 3、WA077(H7)、WA09(H9)、WA13(H13)、WA14(H14)、HUES 62、HUES 63、HUES 64、CT1、CT2、CT3、CT4、MA135、Eneavour-2、WIBR1、WIBR2、WIBR3、WIBR4、WIBR5、WIBR6、HUES 45、Shef 3、Shef 6、BJNhem19、BJNhem20、SA001、SA001。

[0122] 根据一个具体实施方式，胚胎干细胞系是HAD-C102或ESI。

[0123] 此外，ES细胞也可以从其他物种获得，包括小鼠(Mills和Bradley，2001)、金仓鼠[Doetschman等，1988，Dev Biol.127:224-7]、大鼠[Iannaccone等，1994，Dev Biol.163:288-92]、兔[Giles等，1993，Mol Reprod Dev.36:130-8;Graves&Moreadith，1993，Mol Reprod Dev.1993，36:424-33]、多种家畜物种[Notarianni等，1991，J Reprod Fertil Suppl.43:255-60;Wheeler，1994，Reprod Fertil Dev.6:563-8;Mitalipova等，2001，Cloning.3:59-67]和非人类灵长类物种(猕猴和狨猴)[Thomson等，1995，Proc Natl Acad Sci U S A.92:7844-8;Thomson等，1996，Biol Reprod.55:254-9]。

[0124] 扩展胚泡细胞(EBC)可以在原肠胚形成之前的阶段，从受精后至少9天的胚泡获得。在培养胚泡之前，透明带被消化[例如通过Tyrode酸性溶液(Sigma Aldrich，St Louis，MO，USA)]以暴露内细胞团。然后在体外使用标准胚胎干细胞培养方法将胚泡作为全胚胎培养受精后至少9天并且不超过14天(即，在原肠胚形成事件之前)。

[0125] Chung等，Cell Stem Cell，Volume 2，Issue 2，113-117，2008年2月7日中描述了另一种用于制备ES细胞的方法。该方法包括在体外受精过程中从胚胎移除单个细胞。胚胎在该过程中不被破坏。

[0126] 又一种用于制备ES细胞的方法是通过单性生殖。胚胎在该过程中也没有被破坏。

[0127] 目前实践的ES培养方法主要基于使用分泌干细胞增殖所需要的因子而同时抑制其分化的饲养细胞层。示例性的饲养层包括人胚胎成纤维细胞、成体输卵管上皮细胞、原代小鼠胚胎成纤维细胞(PMEF)、小鼠胚胎成纤维细胞(MEF)、小鼠胎儿成纤维细胞(MFF)、人胚胎成纤维细胞(HEF)、从人胚胎干细胞的分化获得的人成纤维细胞、人胎儿肌细胞(HFM)、人胎儿皮肤细胞(HFS)、人成体皮肤细胞、人包皮成纤维细胞(HFF)、从脐带或胎盘获得的人成纤维细胞,以及人骨髓基质细胞(hMSC)。可以将生长因子添加到培养基中以使ESC维持在未分化状态。这样的生长因子包括bFGF和/或TGFβ。

[0128] 无饲养细胞系统也已经用于ES细胞培养,这样的系统利用补充有血清替代物、细胞因子、IL6、可溶性IL6受体嵌合体和/或生长因子的基质作为饲养细胞层的替代物。干细胞可以在培养基的存在下在固体表面例如细胞外基质(例如,Matrigel^{RTM}或层粘连蛋白)上生长。不同于需要饲养细胞和干细胞的同时生长并且可能导致混合细胞群体的基于饲养细胞的培养物,生长在无饲养细胞的系统上的干细胞容易从表面分离。用于生长干细胞的培养基含有有效抑制分化并促进其生长的因子,例如MEF条件培养基和bFGF。然而,常用的无饲养细胞培养系统利用补充有小鼠或牛血清,或MEF条件培养基的基于动物的基质(例如Matrigel^{RTM})[Xu C等(2001).Feeder-free growth of undifferentiated human embryonic stem cells.Nat Biotechnol.19:971-4],其呈现动物病原体交叉转移到人ES细胞的风险,因此危及未来的临床应用。

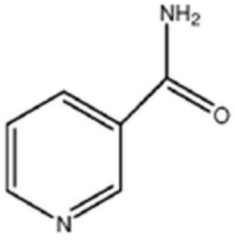
[0129] 已知有许多方法使ESC朝向RPE谱系分化,并且包括诸如WO2008/129554、2013/184809中描述的那些的定向分化方案和诸如美国专利号8,268,303和美国专利申请20130196369中描述的那些的自发分化方案二者,其各自内容通过引用并入。

[0130] 根据一个特别实施方式,使用定向分化方案从ESC细胞产生RPE细胞。

[0131] 在一个示例性的分化方案中,使用第一分化剂使胚胎干细胞朝向RPE细胞谱系分化,然后使用转化生长因子-β(TGFβ)超家族的成员(例如,TGFβ1、TGFβ2和TGFβ3亚型、以及同源配体,包括激活素(例如激活素A、激活素B和激活素AB)、结节、抗缪勒氏管激素(AMH)、一些骨形态发生蛋白(BMP),例如BMP2、BMP3、BMP4、BMP5、BMP6和BMP7,以及生长和分化因子(GDF))使其进一步朝向RPE细胞分化。根据一个具体实施方式,转化生长因子-β(TGFβ)超家族的成员是激活素A,例如20-200ng/ml之间,例如100-180ng/ml。

[0132] 根据一个特别实施方式,第一分化剂是烟酰胺(NA),例如1-100mM、5-50mM、5-20mM之间,例如10mM。

[0133] NA,也称为“尼克酰胺”,是被认为保留和改善β细胞功能的维生素B3(烟酸)的酰胺衍生物形式。NA具有化学式$C_6H_6N_2O$。NA对于生长和将食物转化为能量是必不可少的,并且已经用于关节炎治疗以及糖尿病治疗和预防。

[0134] 

烟酰胺(NA)

[0135] 在本公开的上下文中,术语NA还表示NA的衍生物和烟酰胺模拟物。如本文所用的

术语“烟酰胺(NA)的衍生物”表示作为天然NA的化学修饰衍生物的化合物。化学修饰可以包括例如基本NA结构的吡啶环上的取代(经由环的碳或氮成员),经由酰胺部分的氮或氧原子,以及删除或替换基团,例如以形成NA的硫代苯甲酰胺类似物,所有这些都是有机化学中的熟练人员所理解的。本发明的情况中的衍生物还包括NA的核苷衍生物(例如烟酰胺腺嘌呤)。描述了NA的各种衍生物,一些也与PDE4酶的抑制活性有关(WO03/068233;WO02/060875;GB2327675A),或作为VEGF-受体酪氨酸激酶抑制剂(WO01/55114)。例如,制备4-芳基-烟酰胺衍生物的方法(WO05/014549)。

[0136] 烟酰胺模拟物可以在培养基中代替烟酰胺。烟酰胺模拟物包括重现烟酰胺在RPE细胞从多能细胞分化和成熟的方面的作用的任何化合物。

[0137] 烟酰胺模拟物包括烟酰胺的修饰形式和烟酰胺的化学类似物。示例性烟酰胺模拟物包括苯甲酸、3-氨基苯甲酸和6-氨基烟酰胺。可以用作烟酰胺模拟物的另一类化合物是聚(ADP-核糖)聚合酶(PARP)的抑制剂。示例性PARP抑制剂包括3-氨基苯甲酰胺、依尼帕尼(Iniparib)(BSI 201)、奥拉帕尼(AZD-2281)、Rucaparib(AG014699,PF-01367338)、维利帕尼(Veliparib)(ABT-888)、CEP 9722、MK 4827和BMN-673。

[0138] 根据一个特别实施方式,分化如下进行:

[0139] a)在包含第一分化剂(例如烟酰胺)的培养基中培养ESC;和

[0140] b)在包含TGFβ超家族成员(例如激活素A)和第一分化剂(例如烟酰胺)的培养基中培养从步骤a)获得的细胞。

[0141] 优选地,在不存在TGFβ超家族成员的情况下进行步骤(a)。

[0142] 可以通过在包含第一分化剂(例如烟酰胺),但不含TGFβ超家族成员(例如激活素A)的培养基中培养步骤b)中获得的细胞而继续上述方案。该步骤在本文中称为步骤(c)。

[0143] 现在采用另外的实施方式更详细地描述上述方案。

[0144] 步骤(a):一旦获得足够数量的ESC,分化过程就开始。它们通常被从粘附细胞培养物除去(例如通过使用胶原酶A、分散酶、TrypLE选择、EDTA)并在存在烟酰胺的情况下(并且在不存在激活素A的情况下)接种到非粘附基质(例如细胞培养板)上。一旦将细胞铺板到非粘附基质(例如细胞培养板)上,细胞培养物可被称为细胞悬浮液,优选悬浮培养物中的自由漂浮簇,即源自人胚胎干细胞(hESC)的细胞的聚集体。自由漂浮干细胞的来源先前在WO06/070370中描述,其全部内容通过引用并入本文。该阶段可以进行最少1天,更优选2天、3天、1周或甚至10天。优选地,细胞与烟酰胺一起(并且在不存在激活素的情况下)在悬浮液中培养不超过2周。

[0145] 根据一个优选实施方式,当细胞在非粘附基质例如细胞培养板上培养时,操控环境氧条件使得百分比小于约20%、15%、10%,更优选小于约9%、小于约8%、小于约7%、小于约6%且更优选约5%。

[0146] 根据一个特别实施方式,细胞最初在正常环境氧条件下在非粘附基质上培养,然后降低至低于正常环境氧条件。

[0147] 非粘附基质的实例包括但不限于纤连蛋白、层粘连蛋白、聚D-赖氨酸和明胶。

[0148] 非粘附细胞培养板的实例包括由Hydrocell(例如产品编号174912)、Nunc等制造的那些。

[0149] 步骤(b):在定向分化的第一阶段(步骤a;即,在低或正常环境氧条件下,在非粘附

培养条件下，在存在烟酰胺的情况下(例如10mM)培养)之后，接着使半分化细胞在粘附基质上经历进一步的分化阶段——在存在烟酰胺(例如10mM)和激活素A(例如140ng/ml、150ng/ml、160ng/ml或180ng/ml)的情况下培养。该阶段可以进行至少一天、至少两天、至少三天、至少5天、至少一周、至少两周、至少三周、至少四周、至少五周、至少六周、至少七周、至少八周、至少九周、至少十周。优选地，该阶段进行约两周。如上文详述的，该分化阶段可以在低或正常环境氧条件下进行。

[0150] 步骤(c)：在定向分化的第二阶段(即，在粘附基质上，在存在烟酰胺和激活素A的情况下培养；步骤(b))之后，任选地使进一步分化的细胞在粘附基质上经历后续分化阶段——在不存在激活素A的情况下，在存在烟酰胺(例如10mM)的情况下培养。该阶段可以进行至少一天、两天、五天、至少一周、至少两周、至少三周或甚至四周。优选地，该阶段进行约一周。如上文详述的，该分化阶段也可以在低或正常环境氧条件下进行。

[0151] 在该分化步骤之后，环境氧气条件可以任选地返回到正常大气条件，并且在存在烟酰胺(例如10mM)和不存在激活素A的情况下再培养至少一天、至少2天、至少5天、至少一周(例如至多两周)。

[0152] 根据本发明的基础培养基是本领域已知用于体外支持细胞生长的任何已知细胞培养基，通常是包含规定基础溶液的培养基，其包含盐、糖、氨基酸和使培养物中的细胞维持在具有生活力的状态所需的任何其它营养物。可以根据本发明使用的市售基础培养基的非限制性实例包括Nutristem(不具有用于ESC分化的bFGF和TGFβ，具有用于ESC扩增的bFGF和TGFβ)、Neurobasal™、KO-DMEM、DMEM、DMEM/F12、Cellgro™干细胞生长培养基或X-Vivo™。基础培养基可以补充有本领域已知处理细胞培养物的各种试剂。以下是可以包含在根据本公开使用的培养系统中的各种补充剂的非限制性参考：

[0153] -血清或含血清替代物培养基，例如但不限于：敲除血清替代物(KOSR)、Nutridoma-CS、TCH™、N2、N2衍生物或B27，或组合；

[0154] -细胞外基质(ECM)组分，例如但不限于纤连蛋白、层粘连蛋白、胶原蛋白和明胶。ECM然后可用于携带生长因子的TGFβ超家族的一个或多个成员；

[0155] -抗菌剂，例如但不限于青霉素和链霉素；

[0156] -非必需氨基酸(NEAA)，

[0157] -已知在促进培养物中的SC的存活中发挥作用的神经营养因子例如但不限于BDNF、NT3、NT4。

[0158] 根据一个优选实施方式，用于分化ESC的培养基是Nutristem培养基(Biological Industries，05-102-1A或05-100-1A)。

[0159] 根据一个特别实施方式，在无异种(xeno-free)条件下进行ESC的分化。

[0160] 根据一个实施方式，增殖/生长培养基没有异种污染物，即不含动物源组分，如血清、动物源生长因子和白蛋白。因此，根据该实施方式，在不存在异种污染物的情况下进行培养。

[0161] 美国专利申请号20130196369中提供了用于在无异种条件下培养ESC的其它方法，其全部内容并入本文。

[0162] 包含RPE细胞的制备物可以根据良好生产规范(GMP)(例如，制剂符合GMP)和/或当前的良好组织规范(current Good Tissue Practices)(GTP)(例如，制剂符合GTP)制备。

[0163] 在分化步骤期间，可以监测胚胎干细胞的分化状态。细胞分化可以通过检查已知指示分化的细胞或组织特异性标志物而确定。

[0164] 可以使用本领域公知的免疫学技术检测组织/细胞特异性标志物[Thomson JA等，(1998).Science 282:1145-7]。实例包括但不限于用于膜结合或细胞内标志物的流式细胞术、用于细胞外和细胞内标志物的免疫组织化学和用于分泌的分子标志物(例如PEDF)的酶免疫测定。

[0165] 因此，根据本发明的另一个方面，提供了产生视网膜上皮细胞的方法，其包括：

[0166] (a)在包含分化剂的培养基中培养多能干细胞以产生分化细胞，其中该培养基不含转化生长因子β(TGFβ)超家族的成员；

[0167] (b)在包含转化生长因子β(TGFβ)超家族的成员和分化剂的培养基中培养分化细胞以产生进一步朝向RPE谱系分化的细胞；

[0168] (c)分析进一步朝向RPE谱系分化的细胞的色素上皮衍生因子(PEDF)分泌；和

[0169] (d)在包含分化剂的培养基中培养进一步朝向RPE谱系分化的细胞以产生RPE细胞，其中该培养基不含转化生长因子β(TGFβ)超家族的成员，其中步骤(d)在PEDF的量高于预定水平时进行。

[0170] 优选地，当PEDF的水平高于100ng/ml/天、200ng/ml/天、300ng/ml/天、400ng/ml/天或500ng/ml/天时进行步骤(d)。

[0171] 在分化过程期间或之后测定细胞效能的另一种方法是通过分析屏障功能及极化的PEDF和VEGF分泌。

[0172] 一旦细胞被推动到RPE命运中，则可以选择和/或扩增RPE细胞。

[0173] 根据一个特别实施方式，选择是基于负向选择——即除去非RPE细胞。这可以通过除去非色素细胞或通过利用表面标志物而机械地完成。

[0174] 根据另一个实施方式，选择是基于正向选择，即选择色素细胞。这可以通过视觉分析或利用表面标志物而完成。

[0175] 根据又一个实施方式，选择首先是基于负向选择，然后是基于正向选择。

[0176] RPE细胞的扩增可以在额外的细胞基质上进行，例如，明胶或胶原蛋白、层粘连蛋白和聚-D-赖氨酸。为了扩增，可以在无血清KOM、含血清培养基(例如DMEM+20％)或Nutristem培养基(06-5102-01-1A Biological Industries)中培养细胞。在这些培养条件下，色素细胞减少色素沉着并获得纤维样形态。在进一步延长的培养和增殖成高密度培养物之后，细胞重新获得特征性的多角形形态并增加RPE细胞的色素沉着。

[0177] RPE细胞可以在悬浮液中或单层中扩增。RPE细胞在单层培养物中的扩增可以通过本领域技术人员熟知的方法改变成在生物反应器中的大规模扩增。

[0178] 根据本文所述的方法生成的RPE细胞群体可以根据多个不同参数表征。

[0179] 因此，例如，获得的RPE细胞的形状为多角形并且发生色素沉着。

[0180] 根据一个实施方式，所获RPE细胞群体的至少95％、至少96％、至少97％、至少98％、至少99％或甚至100％的细胞共表达前黑素体蛋白(PMEL17)和细胞视黄醛结合蛋白(CRALBP)二者。

[0181] 根据一个特别实施方式，细胞共表达PMEL17(SwissProt No.P40967)和选自以下的至少一种多肽：细胞视黄醛结合蛋白(CRALBP;SwissProt No.P12271)，卵磷脂视黄醇酰

基转移酶(LRAT;SwissProt No.095327)和性别决定区Y盒9(SOX 9;P48436)。

[0182] 根据一个特别实施方式,如通过本领域技术人员已知的方法(例如FACS)测定的,群体的至少80%的细胞表达可检测水平的PMEL17和上述多肽之一(例如CRALBP),更优选地,群体的至少85%的细胞表达可检测水平的PMEL17和上述多肽之一(例如CRALBP),更优选地,群体的至少90%的细胞表达可检测水平的PMEL17和上述多肽之一(例如CRALBP),更优选地,群体的至少95%的细胞表达可检测水平的PMEL17和上述多肽之一(例如CRALBP),更优选地,群体的100%的细胞表达可检测水平的PMEL17和上述多肽之一(例如CRALBP)。

[0183] 根据另一个实施方式,CRALBP和上述多肽之一(例如PMEL17)共表达的水平(例如如通过平均荧光强度测量的)与未分化ESC相比增加至少2倍、更优选至少3倍、更优选至少4倍,且甚至更优选至少5倍、至少10倍、至少20倍、至少30倍、至少40倍、至少50倍。

[0184] 在一个实施方式中,RPE是终末分化的,并且不表达Pax6。

[0185] 在另一个实施方式中,RPE细胞是终末分化的,并且表达Pax6。

[0186] 本文所述的RPE细胞也可以在移植后作为功能性RPE细胞发挥作用,其中RPE细胞在接受移植细胞的患者的感觉神经视网膜与脉络膜之间形成单层。RPE细胞还可以向相邻的光感受器提供营养物质,并通过吞噬作用处理脱落的光感受器外段。

[0187] 根据一个实施方式,单层中细胞的跨上皮细胞电阻大于100欧姆。

[0188] 优选地,细胞的跨上皮细胞电阻大于150、200、250、300、300、400、500、600、700、800或甚至大于900欧姆。

[0189] 用于测量跨上皮细胞电阻(TEER)的装置是本领域已知的,并且包括例如EVOM2上皮伏欧计(World Precision Instruments)。

[0190] 将理解,本文公开的细胞群体没有未分化的人胚胎干细胞。根据一个实施方式,如例如通过FACS测量的,小于1:250,000的细胞是$Oct4^{+}TRA\text{-}1\text{-}60^{+}$细胞。如通过PCR测量的,细胞还具有下调(超过5,000倍)的GDF3或TDGF的表达。

[0191] 本发明的这个方面的RPE细胞不表达胚胎干细胞标志物。所述的一种或多种胚胎干细胞标志物可以包括OCT-4、NANOG、Rex-1、碱性磷酸酶、Sox2、TDGF-β、SSEA-3、SSEA-4、TRA-1-60和/或TRA-1-81。

[0192] 相对于非RPE细胞,RPE制备物可以为基本上纯化的,其包含至少约75%、80%、85%、90%、91%、92%、93%、94%、95%、96%、97%、98%、99%或100%的RPE细胞。RPE细胞制备物可以基本上不含非RPE细胞或由RPE细胞组成。例如,基本上纯化的RPE细胞制备物可以包含少于约25%、20%、15%、10%、9%、8%、7%、6%、5%、4%、3%、2%或1%的非RPE细胞类型。例如,RPE细胞制备物可以包含少于约25%、20%、15%、10%、9%、8%、7%、6%、5%、4%、3%、2%、1%、0.9%、0.8%、0.7%、0.6%、0.5%、0.4%、0.3%、0.2%、0.1%、0.09%、0.08%、0.07%、0.06%、0.05%、0.04%、0.03%、0.02%、0.01%、0.009%、0.008%、0.007%、0.006%、0.005%、0.004%、0.003%、0.002%、0.001%、0.0009%、0.0008%、0.0007%、0.0006%、0.0005%、0.0004%、0.0003%、0.0002%或0.0001%的非RPE细胞。

[0193] 相对于非RPE细胞和相对于具有其他成熟水平的RPE细胞二者,RPE细胞制备物可以是基本上纯的。相对于非RPE细胞,制备物可以是基本上纯化的,并是成熟RPE细胞富集的。例如,在针对成熟RPE细胞富集的RPE细胞制备物中,至少约30%、40%、45%、50%、

55％、60％、65％、70％、75％、80％、85％、90％、91％、92％、93％、94％、95％、96％、97％、98％、99％、99％或100％的RPE细胞是成熟RPE细胞。相对于非RPE细胞，制备物可以是基本上纯化的，并且针对分化RPE细胞而非成熟RPE细胞富集的。例如，至少约30％、40％、45％、50％、55％、60％、65％、70％、75％、80％、85％、90％、91％、92％、94％、95％、96％、97％、98％、99％或100％的RPE细胞可以是分化RPE细胞而非成熟RPE细胞。

[0194] 本文所述的制备物可以基本上不含细菌、病毒或真菌污染或感染，包括但不限于HIV I、HIV 2、HBV、HCV、HAV、CMV、HTLV 1、HTLV 2、细小病毒B 19、爱泼斯坦-巴尔病毒、或疱疹病毒1和2、SV40、HHV5、HHV6、HHV7、HHV8、CMV、多瘤病毒、HPV、肠病毒的存在。本文所述的制备物可以基本上不含支原体污染或感染。

[0195] 表征本文公开的细胞群体的另一种方式是通过标志物表达。因此，例如，如通过免疫染色测量的，至少80％、85％、90％、95％或100％的细胞表达卵黄状黄斑病蛋白1。根据一个实施方式，85-100％的细胞表达卵黄状黄斑病蛋白。

[0196] 根据另一个实施方式，如通过免疫染色测量的，至少80％、85％、87％、89％、90％、95％、97％或100％的细胞表达小眼畸形相关转录因子(MITF)。例如，85-100％的细胞表达MITF。

[0197] 根据另一个实施方式，如通过FACS测量的，至少80％、85％、87％、89％、90％、95％、97％或100％的细胞表达配对盒基因6(PAX-6)。

[0198] 本文所述的细胞也可以根据它们分泌的因子的数量和/或类型进行表征。因此，根据一个实施方式，如通过ELISA测量的，细胞优选分泌超过500、1000、2000、3000或甚至4000ng/ml/天的色素上皮衍生因子(PEDF)。根据一个特别实施方式，细胞分泌约1250-4000ng/ml/天的色素上皮衍生因子(PEDF)。

[0199] 将理解，本文产生的RPE细胞以极化方式分泌PEDF和血管内皮生长因子(VEGF)。根据特别的实施方式，PEDF的顶部分泌:PEDF的基底部分泌的比率大于1。根据特别的实施方式，PEDF的顶部分泌:PEDF的基底部分泌的比率大于2。根据特别的实施方式，PEDF的顶部分泌:PEDF的基底部分泌的比率大于3，大于4，大于5，大于6，大于7或甚至大于8。此外，VEGF的基底部分泌:VEGF的顶部分泌的比率大于1。根据特别的实施方式，VEGF的基底部分泌:VEGF的顶部分泌的比率大于1.5、2或2.5。

[0200] 细胞的稳定性是另一个表征特征。因此，例如，在2-8℃下孵育6小时、8小时、10小时、12小时或甚至24小时(当将细胞配制在BSS+缓冲液中时)后，PEDF分泌量在细胞中保持稳定。另外，在2-8℃下孵育6小时、8小时、10小时、12小时或甚至24小时(当将细胞配制在BSS+缓冲液中时)后，PEDF和VEGF的极化分泌保持稳定。另外，在2-8℃下孵育6小时、8小时、10小时、12小时或甚至24小时(当将细胞配制在BSS+缓冲液中时)后，细胞的TEER在细胞中保持稳定。

[0201] 在另一个实施方式中，细胞的特征在于其治疗效果。因此，例如，本发明人已经证明，细胞群体在视网膜下施用后能够挽救RCS大鼠的视敏度。此外，细胞群体在视网膜下施用后能够挽救RCS大鼠的光感受器(例如视锥细胞光感受器)至多180天。

[0202] 本领域技术人员将很好理解，RPE细胞的获得具有很大的益处。它们可用作开发新药物以促进其生存、再生和功能的体外模型。RPE细胞可用于高通量筛选对RPE细胞具有毒性或再生作用的化合物。它们可用于揭示对光感受器细胞的发育、分化、维持、存活和功能

来说重要的机制、新基因、可溶性的或膜结合的因子。

[0203] RPE细胞还可以用作用于视网膜退化中功能异常或退化的RPE细胞的移植、补充和支持的RPE细胞的无限制来源。此外，遗传修饰的RPE细胞可以在移植后用作在眼睛和视网膜中携带和表达基因的载体。

[0204] 通过本公开的方法产生的RPE细胞可以用于这样的细胞的大规模和/或长期培养。为此，本发明的方法要在适合于大规模细胞生产的生物反应器中进行，且其中未分化hSC根据本发明培养。在生物反应器中培养细胞的一般要求是本领域技术人员所熟知的。

[0205] 细胞的收获可以通过本领域已知的各种方法进行。非限制性实例包括机械解离和用木瓜蛋白酶或胰蛋白酶(例如TrypLE选择)解离。本领域已知的其它方法也适用。

[0206] 如本文所用的“有效量”广泛地指当施用于患者以治疗疾病时足以实现疾病的这种治疗的化合物或细胞的量。有效量可以是对预防(prophylaxis)有效的量和/或对防止(prevention)有效的量。有效量可以是有效减少体征/症状发生、有效预防体征/症状发生、有效减少体征/症状发生的严重度、有效消除体征/症状的发生、有效减缓出现体征/症状的发生、有效预防出现体征/症状的发生、和/或有效预防体征/症状的发生的量。“有效量”可以根据疾病及其严重度以及待治疗的患者的年龄、体重、病史、易感性和预先存在的状况而变化。出于本公开的目的，术语“有效量”与“治疗有效量”是同义的。

[0207] 预期在从本申请获得的专利的有效期内，将开发许多相关技术用于产生RPE细胞，并且术语RPE细胞旨在假定包括所有这样的新技术。

[0208] 如本文所用的术语“约”是指±10％。

[0209] 术语“包含”、“含有”、“包括”、“包括有”、“具有”及其同根词意思是“包括但不限于”。

[0210] 术语“由……组成”是指“包括且限于”。

[0211] 术语“基本上由……组成”是指组合物、方法或结构可以包括另外的成分、步骤和/或部件，但前提是所述另外的成分、步骤和/或部件不实质性改变所要求保护的组合物、方法或结构的基本和新颖特性。

[0212] 如本文所用的，单数形式“一”，“一个”和“该”包括复数引用，除非上下文另有明确规定。例如，术语“一种化合物”或“至少一种化合物”可以包括多种化合物，包括其混合物。

[0213] 在本申请全文中，本发明的各种实施方式可以以范围形式呈现。应当理解，范围形式的描述仅仅是出于方便和简洁，而不应解释为对本发明范围的刻板限制。因此，范围的描述应看做是已经具体公开了所有可能子范围以及该范围内的单个数值。例如，范围描述如1至6应看做是具体公开了子范围如1至3、1至4、1至5、2至4、2至6、3到6等，以及该范围内的单个数值，例如1、2、3、4、5和6。这适用任何范围的宽度。

[0214] 如本文所用的术语“方法”是指用于完成给定任务的方式、手段、技术和程序，包括但不限于化学、药理学、生物学、生物化学和医学领域的从业者已知的或者容易从已知方式、手段、技术和程序开发的那些方式、手段、技术和程序。

[0215] 如本文所用的术语“治疗”包括消除、基本上抑制、减缓或逆转病症的进展，基本上改善病症的临床或美学症状或基本上预防病症的临床或美学症状的出现。

[0216] 应当理解，出于清楚的目的在单独的实施方式的情况中描述的本发明的某些特征也可以在单个实施方式中组合地提供。相反地，出于简洁的目的而在单一实施方式的情况

中描述的本发明的各种特征也可以单独地提供或以任何合适的子组合提供或在合适的情况下在本发明的任何其它所述实施方式中提供。在各种实施方式的上下文中描述的某些特征不被认为是那些实施方式的必要特征，除非该实施方式在没有那些要素的情况下不起作用。

[0217] 如上所述的和如下权利要求书部分中请求保护的本发明的各种实施方式和方面在以下实施例中找到实验支持。

[0218] 实施例

[0219] 现在参考以下实施例，其与以上描述一起以非限制性方式说明本发明的一些实施方式。

[0220] 通常，本文使用的命名法和本发明中使用的实验室程序包括分子、生化、微生物和重组DNA技术。这样的技术在文献中详细解释。参见，例如，"Molecular Cloning:A laboratory Manual"Sambrook等，(1989)；"Current Protocols in Molecular Biology"，第I-III卷，Ausubel，R.M.编(1994)；Ausubel等，"Current Protocols in Molecular Biology"，John Wiley and Sons，Baltimore，Maryland(1989)；Perbal，"A Practical Guide to Molecular Cloning"，John Wiley&Sons，New York(1988)；Watson等，"Recombinant DNA"，Scientific American Books，New York；Birren等(编)"Genome Analysis:A Laboratory Manual Series"，第1-4卷，Cold Spring Harbor Laboratory Press，New York(1998)；美国专利号4,666,828；4,683,202；4,801,531；5,192,659和5,272,057中所述的方法；"Cell Biology:A Laboratory Handbook"，第I-III卷，Cellis，J.E.编(1994)；"Culture of Animal Cells-A Manual of Basic Technique"，Freshney，Wiley-Liss，N.Y.(1994)，第3版；"Current Protocols in Immunology"，第I-III卷，Coligan J.E.编(1994)；Stites等(编)，"Basic and Clinical Immunology"(第8版)，Appleton&La nge，Norwalk，CT(1994)；Mishell和Shiigi(编)，"Selected Methods in Cellular Immunology"，W.H.Freeman and Co.，New York(1980)；可用的免疫测定法在专利和科学文献中广泛描述，参见，例如，美国专利号3,791,932；3,839,153；3,850,752；3,850,578；3,853,987；3,867,517；3,879,262；3,901,654；3,935,074；3,984,533；3,996,345；4,034,074；4,098,876；4,879,219；5,011,771和5,281,521；"Oligonucleotide Synthesis"，Gait，M.J.编(1984)；"Nucleic Acid Hybridization"，Hames，B.D.和Higgins S.J.编(1985)；"Transcription and Translation"，Hames，B.D.和Higgins S.J.编(1984)；"Animal Cell Culture"，Freshney，R.I.编(1986)；"Immobilized Cells and Enzymes"，IRL Press，(1986)；"A Practical Guide to Molecular Cloning"，Perbal，B.，(1984)和"Methods in Enzymology"，第1-317卷，Academic Press；"PCR Protocols:A Guide To Methods And Applications"，Academic Press，San Diego，CA(1990)；Marshak等，"Strategies for Protein Purification and Characterization-A Laboratory Course Manual"，CSHL Press(1996)；所有这些如其完全在本文中阐述那样通过引用并入。其他一般参考文献在本文全文中提供。据信其中的程序在本领域中是众所周知的，并且为了读者的便利而提供。其中包含的所有信息都通过引用并入本文。

[0221] 实施例1

[0222] 递送装置

[0223] 研究目的:比较两种视网膜套管的性能:具有38G柔性套管的MedOne PolyTip 25号(G)针头与具有41G柔性套管的Peregrine 25(G)针。

[0224] 实验设计:将冷冻保存的RPE细胞小瓶(1.5×10^6个细胞/小瓶/1ml冷冻细胞悬浮液)解冻,将内容物温和地转移到含有20%人血清的DMEM中,过滤,用含有20%人血清的DMEM再次洗涤,然后用BSS Plus洗涤。然后将细胞沉淀重悬于0.5-1ml BSS Plus中,并通过台盼蓝拒染法进行活细胞计数。将细胞以不同细胞密度重悬,其随后通过递送装置注射将产生50×10^3、200×10^3或500x10^3个活细胞/100-150μl BSS Plus的预期I/IIa期临床剂量。在装载到递送装置中之前,测试配制的RPE细胞的外观以证实不存在可见外来颗粒。使用无菌18G针将在其最终制剂中的RPE细胞加载到无菌1mL注射器中(图2A)。然后将18G针替换为无菌10cm延长管(图2B),通过延长管除去空气,并将无菌25G/38G套管(MedOne)或25G/41G套管(Peregrine)附接到延长管的末端(图2C)。然后从套管中除去空气,直至在套管尖端出现细胞滴。根据一种选择,将柱塞推回并使10-20μl的空气插入套管的末端中。

[0225] 以50μl/分钟的近似速率递送100-150μl部分的RPE细胞。在每次研究中测定细胞浓度、体积、递送后存活力和递送速率(体积/分钟)并进行比较。

[0226] 测定RPE细胞在通过装置递送前和递送后的活力和功能活性。

[0227] 由3个测试者对每个预期临床剂量(即50×10^3、200×10^3或500×10^3个活细胞/100-150μl BSS Plus)评估每次注射递送的实际细胞剂量的重现性。

[0228] 结果

[0229] MedOne套管的内直径和外直径分别为99μm和120μm。具有41G柔性套管的视网膜套管Peregrine 25(G)针具有更小的内直径和外直径,分别为71μm和96.5μm。采用MedOne和Peregrine套管观察到相似的细胞回收(参见下文表1)。

[0230] 表1

[0231]

套管 / 参数	*MedOne 25G/38G* (均值 ± SD, n=3)	*Peregrine 25G/41G* (均值 ± SD, n=3)
初始存活力(%)	84 ± 2.4	84 ± 3.7
递送后存活力(%)	79.3 ± 6.8	85 ± 3.5
初始剂量(#细胞/100 μL)	500,000	500,000
递送后剂量(#细胞/100 μL)	380,417 ± 110,894	394,167 ± 73,126
剂量回收率(%)	76.1 ± 22.2	78.8 ± 14.6
递送速率(μL/分钟)	67.3 ± 12.7	85.7 ± 35.3
递送体积(μL)	107.7 ± 9.3	117.3 ± 27.2

[0232] 测定使用Peregrine 25G/41G的高、中等和低临床剂量回收的初始细胞剂量:

[0233] 根据以上数据,采用Peregrine 25G/41G的高临床细胞剂量(500,000个细胞/100μl)的回收率为78.8+/-14.6%(均值+/-SD)。为得到500,000个细胞/100μl的预期高临床细胞剂量,测试了多种初始细胞剂量,其中选择使用得到507,296+/-81,803个细胞/100μl(均值+/-SD,n=6,3名测试者)的700,000个细胞/100μl的初始细胞剂量。下表2汇总了由3名不同测试者获得的数据。

[0234] 表2

[0235]

套管 / 参数	测试者1 (均值 ±SD, n=3)	测试者2 (均值 ±SD, n=2)	测试者3 (n=1)	测试者1-3 (均值 ±SD, n=6)
初始存活力(%)	90 ± 1.5	94 ± 1.6	91	92 ± 2
递送后存活力(%)	83 ± 7	89 ± 2.8	89	86 ± 5.7
初始剂量（#细胞/100 μL）	700,000	700,000	700,000	700,000
递送后剂量（#细胞/100 μL）	460,508 ± 70,313	592,375 ± 40,481	477,500	**507,296 ± 81,803**
剂量回收率(%)	67.7 ± 12.9	77.2 ± 4.7	68.2	70.9 ± 9.7
递送速率（μL/分钟）	64 ± 7.1	80 ± 4.7	110.8	77 ± 19
递送体积（μL）	115 ± 20	120 ± 0	122	118 ± 13

[0236] 为得到200,000个细胞/100μl的预期中等临床细胞剂量，选择使用得到191,250+/-67,511个细胞/100μl(均值+/-SD，n＝6，3名测试者)的270,000个细胞/100μl(范围为247,000-292,000个细胞/100μl)的初始细胞剂量。下表3汇总了由3名不同测试者获得的数据。

[0237] 表3

[0238]

套管 / 参数	测试者1 (均值 ±SD, n=3)	测试者2 (n=1)	测试者3 (均值 ±SD, n=2)	测试者1-3 (均值 ±SD, n=6)
初始存活力(%)	92 ± 3	94	94 ± 1.5	93 ± 2.2
递送后存活力(%)	89 ± 0	92	88 ± 4.2	89 ± 2.4
初始剂量（#细胞/100 μL）	260,333 ± 12,220	292,000	262,000 ± 8,485	266,167 ± 15,327
递送后剂量（#细胞/100 μL）	178,333 ± 12,829	250,000	161,875 ± 62,755	**191,250 ± 67,511**
剂量回收率(%)	68.7 ± 8.3	85.6	61.4 ± 21.9	84.9 ± 28.7
递送速率（μL/分钟）	67 ± 2.3	140	84.1 ± 11.4	81.5 ± 32.5
递送体积（μL）	100 ± 4	140	81 ± 16	105 ± 23

[0239] 为得到50,000个细胞/100μl的预期低临床细胞剂量，选择使用得到50,688+/-6,533个细胞/100μl(均值+/-SD，n＝5，3名测试者)的70,000个细胞/100μl的初始细胞剂量。下表4汇总了由3名不同测试者获得的数据。

[0240] 表4

[0241]

套管 / 参数	测试者1 (均值 ±SD, n=3)	测试者2 (n=1)	测试者3 (n=1)	测试者1-3 (均值 ±SD, n=5)
初始存活力(%)	91 ± 3	91	94	91 ± 2.7
递送后存活力(%)	83 ± 6	93	91	87 ± 6.5
初始剂量（#细胞/100 μL）	70,000	70,000	70,000	70,000
递送后剂量（#细胞/100 μL）	51,042 ± 5,610	57,500	42,813	**50,688 ± 6,553**
剂量回收率(%)	72.9 ± 8	82.1	61.1	72.4 ± 9.3
递送速率（μL/分钟）	64.7 ± 1.9	95.2	118.4	82.6 ± 23.5
递送体积（μL）	115 ± 5	108	80	107 ± 16

[0242] 通过Peregrine 25G/41G递送后的RPE效力：

[0243] 为了证明Peregrine 26G/41G套管合格，在递送后测试在700,000个细胞/μl的高

初始临床剂量下配制的RPE细胞的效力。使用极化分析评估效力，其中使用跨室(transwell)系统测试RPE细胞的屏障功能以及以极化方式分泌色素上皮衍生因子(PEDF)和血管内皮衍生因子(VEGF)的能力。从表5A可以看出。递送前和递送后的屏障功能/跨上皮细胞电阻(TEER)以及PEDF和VEGF的极化分泌是相似的。递送后的存活力和细胞浓度在预期范围内(表5B)。

[0244] 表5A

[0245]

实验#47	第14天的PEDF (ng/mL/天)	TEER (Ω)	PEDF 顶部/基底部	VEGF 基底部/顶部
递送前	1,501	384	4.74	2.58
递送后	1,812	492	4.85	2.99
OpRegen 5C对照	1,858	314	4.3	2.61

[0246] 表5B

[0247]

结果测量	测试结果
初始存活力(%)	95
递送后存活力(%)	91
初始剂量(#细胞/100μL)	700,000
递送后剂量(#细胞/100μL)	621,000
剂量回收率(%)	73.9
递送速率(μL/分钟)	76.6
递送体积(μL)	120

[0248] 除了维持极化能力外，还保留了递送后的RPE细胞生活力(数据未显示)。

[0249] 上文提供的数据支持以50,000、200,000和500,000个细胞/100μl的预期临床剂量使用Peregrine 25G/41G套管。为达到这些最终临床剂量，可以分别制备最终浓度为70,000、270,000和700,000个活细胞/100μl的RPE细胞。

[0250] 测定用于50,000细胞/50μl的低临床剂量回收的初始RPE细胞剂量：

[0251] 初始测试70,000个RPE细胞/50μl的细胞剂量得到50,000个细胞/50μl的预期低临床细胞剂量的能力。如表6所示，制备了得到38,697±5,505个细胞/50μl(均值±SD，n＝3，3名测试者)的70,000个细胞/50μl的初始细胞剂量。由于平均回收率为55％±7.8％(均值±SD，n＝3，3名测试者)且由于未达到50,000个细胞/50μl的预期剂量，因此第二组实验中测试100,000个细胞/50μl的初始细胞密度。如表7所示，100,000个细胞/50μl的初始细胞剂量得到62,517±4,625个细胞/50μl(均值±SD，n＝3，3名测试者；OpRegen®Batch 5D)。平均回收率为61％±4.5％(均值±SD，n＝3，3名测试者)。

[0252] 表6

[0253]

套管 / 参数	测试者1	测试者2	测试者3	测试者 1-3 (均值 ±SD, n=3)
初始存活力(%)	95	95	92	94 ± 1.7
递送后存活力(%)	88	85	90	88 ± 2.5
初始剂量（#细胞/50 μL）	70,000	70,000	70,000	70,000
递送后剂量（#细胞/50 μL）	44,842	34,218	37,030	**38,697 ± 5,505**
剂量回收率(%)	64	49	53	55 ± 7.8
递送速率（μL/分钟）	97	85	94	92 ± 6.2
递送体积（μL）	35	61	60	52 ± 14.7

[0254] 表7

[0255]

套管 / 参数	测试者1	测试者2	测试者3	测试者 1-3 (均值 ±SD, n=3)
初始存活力(%)	88	91	96	92 ± 4
递送后存活力(%)	88	88	90	89 ± 1.2
初始剂量（#细胞/50 μL）	100,000	100,000	100,000	100,000
递送后剂量（#细胞/50 μL）	57,343	66,250	63,958	**62,517 ± 4,625**
剂量回收率(%)	57	66	64	61 ± 4.5
递送速率（μL/分钟）	87	111	110	103 ± 14
递送体积（μL）	53	65	60	59 ± 6

[0256] 实施例2

[0257] 临床实验

[0258] 研究设计:单中心I/IIa期研究,将15名具有晚期干性AMD和地图样萎缩(GA)的患者分为4个同龄组:每组由最佳矫正视力为20/200或更低的3名法定失明的患者组成的前3个同龄组将分别使用每同龄组50×10^3、200×10^3和500×10^3个细胞的连续递增剂量接受单次视网膜下RPE细胞注射。第四同龄组将包括最佳矫正视力为20/100或更低的6名患者,其将接受500,000个RPE细胞的单次视网膜下注射。将采用组内和组间的交错间隔(staggering intervals)。

[0259] 在玻璃体切除术后,通过小型视网膜切开术经由套管将细胞递送到黄斑区域中的视网膜下腔中。将至多50-150μl细胞悬浮液的总体积注射到具有GA扩张风险的区域中。

[0260] 伴随外科手术,患者将接受轻度免疫抑制和抗生素治疗,其由以下组成:

[0261] 1.在玻璃体切除术后按照惯例进行局部类固醇和抗生素治疗:在6周过程中的局部类固醇疗程(每天4-8次Predforte滴剂,逐渐减量)和局部抗生素滴剂(每天4次Oflox或等同物)的过程。

[0262] 2.每天0.01mg/kg全身性(PO)他克莫司(将调整剂量以达到3-7ng/ml血药浓度),

从移植前一周开始持续至移植后6周。

[0263] 3.全身性(PO)麦考酚酯，总共2克/天，从移植前2周开始给予并在移植后持续1年。

[0264] 在施用细胞后12个月的过程中将以预定间隔对患者进行评估。研究后随访将在术后15个月，2、3、4和5年时发生。在出现与免疫抑制治疗相关的副作用的患者中，将尝试控制这些副作用(例如，如果出现高血压，则改善血压控制)。在无法控制的副作用的情况下，经与研究内科医师协商，成为病因的免疫抑制剂的治疗将作出改变。

[0265] 入选标准：

[0266] 1.年龄为55岁及以上；

[0267] 2.在两只眼睛中都诊断出干性(非新生血管性)年龄相关性黄斑变性；

[0268] 3.眼底镜检查发现在黄斑中具有地图样萎缩的干性AMD，在研究眼中的尺寸大于0.5视盘面积(1.25mm^2，且至多17mm^2)，且在对侧眼中大于0.5视盘面积；

[0269] 4.通过ETDRS视力测试，同龄组1-3中的最佳矫正中心视力等于或小于20/200，且同龄组4中的最佳矫正中心视力等于或小于20/100；

[0270] 5.非手术眼的视力必须好于或等于手术眼的视力；

[0271] 6.患者健康状况足够良好以允许参与所有研究相关程序并完成研究(医疗记录)；

[0272] 7.在受监测的麻醉下进行玻璃体视网膜外科手术程序的能力；

[0273] 8.正常血细胞计数、血液化学、凝血和尿分析；

[0274] 9.HIV、HBC和HCV阴性，CMV IgM和EBV IgM阴性；

[0275] 10.基于年龄匹配的筛选检查(由研究医师自行决定)，患者没有当前的恶性肿瘤或恶性肿瘤史(除了成功治疗的皮肤基底/鳞状细胞癌)；

[0276] 11.手术前7天，允许患者中止服用阿司匹林、含阿司匹林产品和任何其他凝血改变药物；

[0277] 12.愿意推迟所有未来的血液和组织捐献；

[0278] 13.能够理解并愿意签署知情同意书。

[0279] 排除标准：

[0280] 1.根据历史，以及根据临床检查、荧光素血管造影(FA)或眼相干断层扫描(OCT)在任一眼睛中基线处的新生血管性AMD的证据；

[0281] 2.糖尿病性视网膜病变、血管阻塞、葡萄膜炎、Coat病、青光眼、白内障或防止后极可视化的间质混浊或已经危害或可能危害研究眼的视力并使主要结果的分析混乱的AMD以外的任何重要眼部疾病的历史或存在；

[0282] 3.研究眼中的视网膜脱离修复的历史；

[0283] 4.轴向近视大于-6屈光度；

[0284] 5.过去3个月中研究眼的眼睛手术；

[0285] 6.认知障碍或痴呆的历史；

[0286] 7.全身性免疫抑制的禁忌症；

[0287] 8.与研究眼中的脉络膜新血管形成相关的AMD以外的任何病症的历史(例如病理性近视或推测的眼睛组织胞浆菌病)；

[0288] 9.以下疾病的活动或历史：癌症、肾脏疾病、糖尿病、前12个月中的心肌梗塞、免疫缺陷；

[0289] 10.女性;怀孕或哺乳;

[0290] 11.目前参与另一项临床研究。过去(在6个月内)参与全身性施用或施用至眼的药物的任何临床研究。

[0291] 将分别评估外科手术程序的安全性和耐受性以及细胞移植的安全性。手术安全性评估将包括以下测量:

[0292] 1.未愈合的视网膜脱离;

[0293] 2.增殖性玻璃体视网膜病变(PVR);

[0294] 3.视网膜下、视网膜或玻璃体内出血;

[0295] 4.对手术部位处仍相对健康的视网膜的损伤。

[0296] 将使用以下不良事件评估细胞移植的安全性和耐受性,所述不良事件将根据国家癌症研究所(NCI)分级系统进行分级:

[0297] 1.畸胎瘤和/或肿瘤和/或异位组织形成;

[0298] 2.感染;

[0299] 3.葡萄膜炎、血管炎或PVR;

[0300] 4.GA的加速进展;

[0301] 5.研究眼中向着新生血管性AMD的进展;

[0302] 6.针对同种异体移植细胞的严重炎症反应。

[0303] 次要探索性功效终点将通过移植物存活的持续时间和通过以下检查而测量:

[0304] 1.GA进展速率;

[0305] 2.移植区域的视网膜敏感性,中心盲点的程度和深度;

[0306] 3.视敏度的变化。

[0307] 外科手术程序:

[0308] 选择用于RPE施用的眼睛是具有较差视觉功能的眼睛。手术将通过眼球后或眼球周麻醉阻滞,伴随受监测的静脉内镇静或全身麻醉进行,这由外科医师判断并与患者商议。根据机构方案,进行手术的眼睛将以无菌方式进行准备并用布帘遮盖。在放置开睑器(lid speculum)后,进行标准的3口玻璃体切除术。这包括放置23G输液套管和两个23G口。在目视检查玻璃体腔中的输液套管后,打开输液管线以确保在整个手术过程中保持眼球的结构。然后用标准23G仪器进行细心的核心玻璃体切割术,随后脱离后玻璃体面。这将允许无阻碍地达到后极。

[0309] RPE将被引入到后极中的预定位置处的视网膜下腔中,优选在仍然相对保持靠近GA边界的区域中穿透视网膜。避开血管。细胞将经由形成小泡而递送到视网膜下腔,其体积为50-150μl。

[0310] 递送系统由1mL注射器组成,其通过10cm延长管连接到Peregrine 25G/41G柔性视网膜套管。

[0311] 将除去任何回流到玻璃体腔中的细胞并将进行流体-空气交换。在移除输液套管之前,将进行仔细检查以确保没有产生医源性视网膜撕裂或断裂。然后将移除输液套管。施用结膜下抗生素和类固醇。将在眼睛上覆盖眼罩和塑料防护物。将记录手术进行程序。

[0312] 剂量:将使用50,000个细胞/50μl或50,000个细胞/100μl的低剂量、200,000个细胞/100μl的中剂量和500,000个细胞/100μl的高剂量。剂量选择是基于在临床前研究中测

试的最大可行剂量的安全性以及基于眼睛和泡尺寸计算的人体等效剂量。

[0313] 终点参数：

[0314] 1.外科手术的安全性；

[0315] 持续性/复发性视网膜脱离

[0316] 增殖性玻璃体视网膜病变(PVR)

[0317] 出血

[0318] 对手术部位处仍相对健康的视网膜的损伤

[0319] 2.产品安全性：

[0320] 畸胎瘤、肿瘤和/或异位组织生长

[0321] 增殖细胞的大块移植

[0322] 感染

[0323] 对移植物的严重炎症免疫反应

[0324] GA的加速进展

[0325] 研究眼中向着新生血管性AMD的进展

[0326] 3.功效：

[0327] 移植物存活的持续时间

[0328] 减小的GA进展速率

[0329] 移植区域中视网膜对光的敏感度和盲点的深度

[0330] 视敏度

[0331] 虽然已经结合本发明的具体实施方式描述本发明，但明显的是，许多替代、修改和变化对于本领域技术人员将是显而易见的。因此，旨在包括落入所附权利要求的精神和广泛范围内的所有这样的替代，修改和变化。

[0332] 本说明书中提及的所有出版物、专利和专利申请都以与具体和单独指出每个个体出版物、专利或专利申请都通过引用并入本文的相同程度以其整体通过引用并入本说明书。此外，本申请中对任何参考文献的引用或识别不应被解释为承认这样的参考文献可用作本发明的现有技术。在章节标题被使用的程度上，其不应被解释为必然的限制。

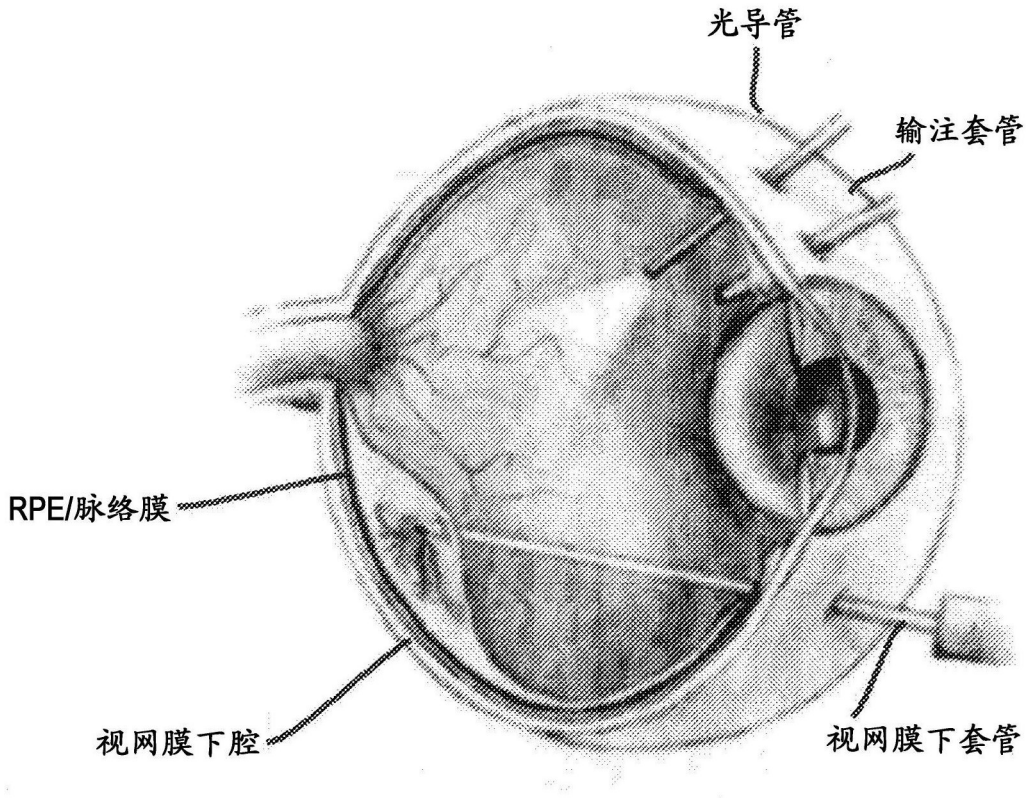


图1

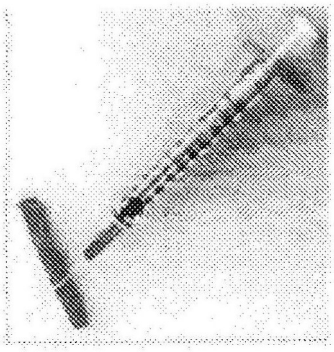


图2A

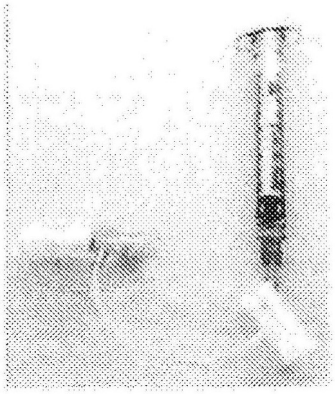


图2B

图2C