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(54) Title: RETINAL PIGMENT EPITHELIAL STEM CELLS

(57) Abstract: The present invention relates to a retinal pigment epithelial stem cell isolated from a posterior region of the retinal pigment epithelium of an adult mammal. The invention also relates to a method of inducing differentiation of retinal epithelial stem and progenitor cells in vitro, wherein the cells of the invention are highly plastic, multipotential stem cells. The invention also includes methods for the treatment of retinal diseases and vision loss involving the transplantation of retinal pigment epithelial stem cells or cells differentiated from retinal pigment epithelial stem cells to the retina of a patient in need of treatment.



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## **RETINAL PIGMENT EPITHELIAL STEM CELLS**

### **CROSS-REFERENCE TO RELATED APPLICATIONS**

[001] Priority is claimed to U.S. provisional application Serial No. 61/047,102 filed April 22, 2008, the contents of which are hereby incorporated by reference in their entirety.

### **STATEMENT REGARDING GOVERNMENT SPONSORED RESEARCH OR DEVELOPMENT**

[002] This invention was made in part in the course of research sponsored by the Ruth and Milton Steinbach Fund, Inc. This entity may have certain rights in this invention.

### **FIELD OF THE INVENTION**

[003] The present invention relates to retinal pigment epithelial stem cells (RPESCs), isolated from the retinal pigment epithelium (RPE) of adult mammals, which may be differentiated into a wide variety of progeny from each of the major developmental lineages. The invention also includes pharmaceuticals made with RPESCs or retinal cells which may be used to restore vision lost due to diseases, disorders or abnormal physical states of the retina, other neurological, non-neurological diseases, such as cancer, and/or tissues injuries that benefit from stem cell replacement therapy.

### **BACKGROUND OF THE INVENTION**

[004] The therapeutic strategies for treating loss of vision caused by retinal cell damage vary, but they are all directed to controlling the illness causing the damage, rather than reversing the damage caused by an illness by restoring or regenerating retinal cells. As one example, the treatments of uveitis are drawn from the knowledge of changes in the retinal environment when inflammation occurs. Corticosteroids, such as prednisone, are the preferred drug of treatment. However, these drugs are immunosuppressants with numerous side effects. As well, the systemic immunosuppression may have significant negative effects on the development of children as well as on adults in poor health, such as the elderly and patients with chronic disease. These patients must try alternative drugs such as alkylating agents or antimetabolites, which also have side

effects. Clearly, patients with eye diseases remain vulnerable to sustaining permanent damage to the retinal cells, even if drug treatments are available. Thus, successful treatments of retinal cell damage will include approaches that aid in the regeneration of damaged retinal cells without causing the harmful side effects caused by current treatment methods.

[005] A current area of study that will be important for treating diseases of the eye and other tissues involves the use of stem cells to regenerate damaged cells. Stem cells are undifferentiated cells that exist in many tissues of embryos and adult mammals. In embryos, blastocyst stem cells are the source of cells which differentiate to form the specialized tissues and organs of the developing fetus. In adults, specialized stem cells in individual tissues are the source of new cells which replace cells lost through cell death due to natural attrition, disease or injury. No stem cell is common to all tissues in adults. Rather, the term "stem cell" in adults describes different groups of cells in different tissues and organs with common characteristics.

[006] Stem cells are capable of producing either new stem cells or cells called progenitor cells that differentiate to produce the specialized cells found in mammalian organs. Symmetric division occurs where one stem cell divides into two daughter stem cells. Asymmetric division occurs where one stem cell forms one new stem cell and one progenitor cell. A progenitor cell differentiates to produce the mature specialized cells of mammalian organs. In contrast, stem cells never terminally differentiate (*i.e.*, they never differentiate into a specialized tissue cell). Progenitor cells and stem cells are referred to collectively as "precursor cells". This term is used when it is unclear whether a researcher is dealing with stem cells or progenitor cells or both.

[007] Progenitor cells may differentiate in a manner which is unipotential or multipotential. A unipotential progenitor cell is one which can form only one particular type of cell when it is terminally differentiated. A multipotential progenitor cell has the potential to differentiate to form more than one type of tissue cell. Which type of cell it ultimately becomes depends on conditions in the local environment such as the presence or absence of particular peptide growth factors, cell-cell communication, amino acids and steroids. For example, it has been determined that the hematopoietic stem cells of the bone marrow produce all of the mature lymphocytes and erythrocytes present in fetuses and adult mammals. There are several well-

studied progenitor cells produced by these stem cells, including three unipotential and one multipotential tissue cell. The multipotential progenitor cell may divide to form one of several types of differentiated cells depending on which hormones act upon it.

[008] Figure 1 shows a schematic cross-section of the human eye, in which both the retina and the retinal pigment epithelium (RPE) are indicated. The retina is a layer of light sensitive tissue lining the inner surface of the eye. The RPE is a layer of pigmented cells just beneath the retina, which nourishes and supports the overlying retinal cells.

[009] Retinal progenitor cells (RPCs) are multipotent, proliferative, and give rise to the various retinal cell types, while retinal pigment epithelium (RPE) progenitor cells normally generate solely RPE. Reh & Fischer, *Methods Enzymol.* (2006) 419:52-73. The RPE is a monolayer of neuroepithelial cells underlying and supporting the sensory retina. During development the RPE begins as a plastic tissue capable of regenerating lens or sensory retina but then differentiates very early [around E38 in humans and E9.5 in mouse (Bharti *et al.*, *Pigment Cell Res.* (2006) 19(5):380-394)] and remains non-proliferative throughout life. RPE cells form a pigmented, single cell epithelium between the neural retina and the vascular choriocapillaris that has important roles in maintaining photoreceptor function. During development, the optic neuroepithelium evaginates into two outpocketings of the diencephalon. The dorsal aspect of the resulting optic vesicle is specified to generate the RPE, while the ventral aspect becomes neural retina. Bharti *et al.*, *Pigment Cell Res.* (2006) 19(5):380-394. The other types of cells located in the retina include rod cells, cone cells, bipolar cells, amacrine cells, horizontal cells, Müller cells, glial cells, and retinal ganglion cells.

[010] Interestingly, in amphibians, and in embryonic chick, RPE cells can produce other retinal and even lens tissues, indicating an inherent plasticity. Reh & Fischer, *Methods Enzymol.* (2006) 419:52-73. In amphibians, embryonic chick and embryonic rodents, RPE cells can proliferate and differentiate into neural progenitors and retinal cells. *Id.* This can occur *in vivo* and *in vitro* after induction with fibroblast growth factors (FGFs) (Park & Hollenberg, *Dev. Biol.* (1989) 134:201-205; Sakaguchi *et al.*, *Dev. Dyn.* (1997) 209(4):387-398), or after enforced expression of retinal development genes, including Pax6, Ath5, NeuroD and NSCL, or after surgical removal of endogenous neural retina. Ma *et al.*, *Dev. Biol.* (2004) 265(2):320-328;

Azuma *et al.*, *Mol. Genet.* (2005) 14(8):1059-1068; Yan & Wang, *Neurosci. Lett.* (2000) 280(2):83-86. In humans, RPE cells can proliferate *in vivo*, for example following retinal detachment (Machemer & Laqua, *Am. J. Ophthalmol.* (1975) 80:1-23) and *in vitro* they proliferate and differentiate into  $\beta$ -tubulin III<sup>+</sup> neurons, but their capacity to differentiate into a variety of neural cell types has not been demonstrated. Amemiya *et al.*, *Biochem. Biophys. Res. Commun.* (2004) 316:1-5; Viores *et al.*, *Exp. Eye Res.* (1995) 60:385-400.

[011] Retinal stem cells (RSCs) isolated from the ciliary epithelium and iris pigmented epithelium of adult rodents and humans have been described, and are reported to self-renew *in vitro* and differentiate into retinal neurons and glia. Reh & Fischer, *Methods Enzymol.* (2006) 419:52-73; Ohta *et al.*, *Dev. Growth Differ.* (2008) 50:253-259. *See also* U.S. Pat. No. 6,117,675; and Tropepe *et al.*, *Science* (2000) 287:2032-2036. However, these reports have been criticized by others, who have presented data indicating that the putative RSCs are, in fact, differentiated, pigmented ciliary epithelial cells. *See* Cicero *et al.*, "Cells Previously Identified as Retinal Stem Cells Are Pigmented Ciliary Epithelial Cells," *Proc. Nat. Acad. Sci. U.S.A.* (e-publication April 3, 2009) <URL <http://www.pnas.org/cgi/doi/10.1073/pnas.0901596106>> (last accessed April 13, 2009). Moreover, there is as yet no evidence that a stem-like cell exists in the adult RPE. It is particularly important to look for the presence of these cells in humans, because of the relevance to human ocular diseases such as retinitis pigmentosa (RP), cone dystrophy, and age-related macular degeneration. da Cruz *et al.*, *Prog. Retin. Eye Res.* (2007) 26:598-635.

[012] There are no known successful treatments for RP and other retinal dystrophies. There are also no treatments which regenerate new cells endogenously or which transplant healthy tissue to the retina. Even if it were possible to develop some form of transplantation, it would be subject to the same problems that accompany transplants in other organ systems. These include: in many cases, implants provide only temporary relief as the symptoms associated with the disease often return after a number of years, rejection by the patient of foreign tissue, adverse reactions associated with immunosuppression (immunosuppression is needed to try to help the patient accept the foreign tissue), the inability of a sufficient number of cells in the tissue being implanted to survive during and after implantation, transmitting other diseases or disorders may be transmitted to the patient via the implant, and the results may not justify the costs and efforts of a complex procedure.

[013] Thus, there is a need for new treatment options, other than transplantation, for the treatment of diseases of the retina and of many other tissues where cell regeneration would be beneficial. Given the immense potential for stem cells to provide new therapeutic treatments for a broad array of human diseases such as those described above, there remains a need for stem and/or progenitor cells that may be easily isolated from adult tissue, and that are multipotential, thereby having the capacity to differentiate into a broad array of different tissue types. The present invention describes such cells.

[014] The citation and/or discussion of cited references in this section and throughout the specification is provided merely to clarify the description of the present invention and is not an admission that any such reference is "prior art" to the present invention. All cited references are incorporated herein by reference in their entirety.

### SUMMARY OF THE INVENTION

[015] The present invention provides a type of stem cell that is referred to throughout this specification and the claims as a retinal pigment epithelial stem cell or "RPESC". RPESC of the invention are self-renewing; that is to say, they are capable of replicating (typically through mitotic division) to produce progeny cells that are the same RPESC cell type. RPESC of the invention can preferably go through a plurality of cell division cycles while maintaining their undifferentiated state; *i.e.*, while maintaining their identity as RPESC. Even more preferably, RPESC of the invention can go through at least six, more preferably at least ten and still more preferably at least 11 or more passages and still maintain their identity as RPESC. In addition, RPESC of the invention are capable of differentiating into any of a plurality of different progeny cell types which, in turn, can give rise to a plurality of different fully differentiated cell types. For example, RPESC of the invention can be differentiated to obtain retinal progenitor cells; retinal neurons and other neuronal cell lines such as dopaminergic neurons (*i.e.*, neuronal cells that synthesize or are capable of synthesizing dopamine) and neural crest cells; as well as other progenitor and differentiated cell lines, including different mesoderm lineages (for example, myogenic, osteogenic, chondrogenic and/or adipogenic lineages), endoderm lineages such as hepatic lineages, and/or ectoderm lineages. The invention therefore includes methods for differentiating RPESC into any of these different cell types or cell lines.

[016] Preferred RPESC of the invention are characterized by the fact that, in addition to being self renewing and capable of differentiating into any of a plurality of different cell types, they are isolated from the retinal pigment epithelium (“RPE”) of an animal, which is preferably a mammal and even more preferably a human, or they are derived from (*e.g.*, are descended from or are the progeny of) other cells (typically other RPESC) derived from the such RPE. However, RPESC of the invention can be obtained from other tissues and cell types, and are not limited to RPESC isolated or derived from RPE cells or tissue. For example, it is understood that other stem cells, such as embryonic stem cells (“ESC”) from humans and other organisms (preferably other mammals) can be induced to differentiate into RPESC, and such RPESC are also considered part of the invention. For example, it is to be understood that RPESC of the invention may be obtained by culturing a human or other ESC line under conditions described, *e.g.*, by Haruta *et al.*, *Invest. Ophthalmol. Vis. Sci.* (2004) 45:1020-1025; and by Lund *et al.*, *Cloning Stem Cells* (2006) 8:189-199, for differentiating those ESC into retinal cells.

[017] Preferred RPESC of the invention can also be characterized by the presence or absence of certain cellular markers; *i.e.*, the RPESC are said to either express or to not express certain cellular markers. The presence or absence of cellular markers characteristic of RPESC and other cell lines of this invention can be detected using techniques and other procedures that are well known and routine to persons of ordinary skill in the relevant field(s) of this invention. Preferred methods including immuno-detection of proteins corresponding to the different cellular markers in cells or tissue samples of interest, *e.g.*, in a western blot type of analysis, as well as polymerase chain reaction (“PCR”) methods such as reverse-transcriptase polymerase chain reaction (“RT-PCR”) and quantitative real time polymerase chain reaction (“qPCR” or “qRT-PCR”) for detecting the expression of messenger RNA (mRNA) corresponding to the different markers, or cDNA derived therefrom, in cell or tissue samples.

[018] Exemplary methods for practicing these techniques, including preferred antibodies and nucleotide primer for detecting different markers useful to this invention, are described in the examples. Those skilled in the relevant art(s) will recognize, however, that these lists of antibodies and primers are not exclusive, and will be able to readily isolate and/or design other suitable antibodies and/or primers that will be suitable for detecting these markers, including suitable homologs and orthologs of the human genes and proteins listed in this

application (*e.g.*, orthologs and homologs isolated from other species of organism, such as from other mammals). Likewise, the detection of cellular markers useful in this invention is not limited to the techniques described in the examples; any techniques suitable for determining whether a particular marker is or is not expressed by a particular cell or tissue sample can be used. A cellular marker is said to be expressed when it is detected in a cell or tissue sample using one of these techniques. Conversely, a particular marker is said to not be expressed if it is not detected, to within the detectable limits of the relevant technique, in a cell or tissue sample.

[019] Preferred RPESC of the invention express or are capable of expressing one or more markers characteristic of RPE cells and/or tissue; and/or they express or are capable of expressing one or more markers characteristic of embryonic stem cells (“ES cells” or “ESC”) and/or of induced pluripotent stem cells (“iPSC”). Preferred markers characteristic of RPE cells and tissue include the cellular markers RPE65, Mitf, Cralbp, Otx2 and Bestrophin. Preferred markers characteristic of ESC include the cellular markers SSEA-4, Sox2, KLF4 and c-Myc. Hence, in preferred embodiments, RPESC of the invention express or are capable of expressing one or more markers characteristic of RPE cells and/or tissue, which are preferably selected from the group consisting of RPE65, Mitf, Cralbp, Otx2 and Bestrophin. In other preferred embodiments, an RPESC of the invention expresses or is capable of expressing one or more markers characteristic of an ESC, which are preferably selected from the group consisting of SSEA-4, Sox2, KLF4 and c-Myc. In still other embodiments, an RPESC of the invention expresses or is capable of expressing one or more markers characteristic of RPE cells and/or tissue, and may also express one or more markers characteristic of an ESC, such as any one or more of the markers recited in the different Markush groups, *supra*.

[020] A particularly preferred marker characteristic of ES cells is Sox2. Hence, in particularly preferred embodiments, an RPESC of the invention expresses or is capable of expressing Sox2.

[021] In preferred embodiments, the RPESC of this invention do not express the cellular marker CHX10. In still other embodiments, an RPESC of the invention may be transformed or transfected with a heterologous gene, *e.g.*, in an expression construct or other suitable expression system.



[022] The present invention also provides methods that use RPESC of the invention to treat various disease and disorders, including retinal diseases and disorders, and neurodegenerative diseases and disorders. Retinal diseases and disorders that may be treated according to the invention include, but are not limited to, macular degeneration, including age-related macular degeneration (“AMD”), retinitis pigmentosa and Leber’s hereditary optic neuropathy. A particularly preferred retinal disease for treatment using the methods of this invention is age-related macular degeneration. Preferred methods of treating these and other retinal diseases and disorders comprise the administration, to the retina of a patient, an RPESC according to the invention in an amount effective for treating or ameliorating the retinal disease or disorder. In other preferred methods, an RPESC of the invention is differentiated, preferably *in vitro*, to obtain retinal cells, which are then administered to the retina of a patient in an amount effective for treating or ameliorating the disease or disorder. In particularly preferred embodiments, the RPESC used in these methods are RPESC isolated from the patient being treated.

[023] A preferred neurodegenerative disease that may be treated according to the invention includes, but is not limited to, Parkinson’s disease (“PD”). Preferred methods of treating PD and other neurodegenerative diseases involve administering, to a patient, an RPESC according to the invention in an amount effective for treating or ameliorating the neurodegenerative disease or disorder. In other preferred methods, an RPESC of the invention is differentiated, preferably *in vitro*, to obtain neural cells, preferably dopaminergic neural cells, which are administered to the patient in an amount effective for treating or ameliorating the disease or disorder. In particularly preferred embodiments, the RPESC used in these methods are RPESC isolated from the patient being treated.

[024] It is to be understood that, in these and other treatment regiments described herein, RPESC can be administered to the patient along with one or more exogenous factors (*e.g.*, one or more growth factors) to induce their differentiation *in vivo* to a desired cell type. For example, an RPESC of the invention may be isolated from the retina of the patient and propagated *in vitro* to obtain a number of cells effective for treating the patient. The propagated RPESC can then be administered to the patient along with one or more of the growth factors described in the examples, *infra*, for differentiating RPESC into a desired cell type (*e.g.*, retinal or neuron cells).

One or more other exogenous factors, such as those described in United States patent application Serial No. 12/398,888 filed March 5, 2009 and incorporated herein by reference in its entirety can also be administered in combination with RPESC of the invention. Growth and other exogenous factors can also be administered, *e.g.*, by administering one or more cells that secrete the factors into the eye, such as genetically engineered cells carrying a vector that expresses a desired factor, coupled to a suitable promoter and secretion signal. It is to be understood that references to administering RPESC “in combination” with one or more other elements (*e.g.*, growth or exogenous factors) includes, unless otherwise indicated, administering other elements before or after administration of the RPESC, as well as their administration concurrently with (*i.e.*, at the same time as) the RPESC. In alternative embodiments, one or more growth factors and/or other endogenous factors can be administered to a patient, so as to stimulate the proliferation and differentiation of the patient’s own endogenous RPESCs.

[025] The RPESC, growth and/or exogenous factors can be administered using any delivery system described herein, as well as with other delivery systems known in the art; including delivery systems described in the above-cited application Serial No. 12/398,888.

[026] RPESC of the invention are also useful for cell-based models and drug screening assays for a variety of disease and disorders, including any of the diseases and disorders described in this application. Hence, the invention also provides methods that use RPESC to screen for therapeutic compounds; *i.e.*, for compounds that are or may be useful for treating a disease or disorder of interest.

[027] Preferred screening methods of the invention generally comprise steps of contacting an RPESC, or a cell descended from an RPESC, with a test compound, and determining whether the test compound changes or modulates (*e.g.*, increases or decreases) one or more characteristics associated with the disease or disorder. The RPESC are preferably grown under conditions in which they exhibit one or more of the characteristics of the disease or disorder of interest. For example, the RPESC may be cultured or grown under conditions in which they differentiated into either normal or pathological cells associated with a disease or disorder of interest, or into cells exhibiting one or more normal or pathological conditions associated with the disease or disorder of interest. The one or more characteristics can include,

for example, the expression level of one or more genes or gene products whose abnormal expression is associated with the disease or disorder of interest. A change or modulation of one or more of these characteristics in cells treated with the test compound, compared to cells not treated with the test compound, is indicative that the test compound may be useful for treating the disease or disorder of interest. In certain preferred embodiments a characteristic associated with the disease or disorder of interest may be the elevated expression of a particular gene or gene product, and a decreased expression of that gene or gene product in cells treated with the test compound, relative to cells not treated with the test compound, indicates that the test compound may be useful for treating the disease or disorder of interest. Alternatively, a characteristic associated with the disease or disorder may be decreased or lower expression of a particular gene or gene product, and increased expression of the gene or gene product in cells treated with the test compound, relative to cells not treated with the test compound, can indicate that the test compound may be useful for treating the disease or disorder of interest.

[028] A particularly preferred screening assay of the invention identifies compounds that are or may be useful for treating age-related macular degeneration (AMD). A preferred characteristic associated with AMD is an elevated or increased expression of the gene  $\alpha$ B-crystallin and its gene product. Hence, in preferred embodiments, RPESC or cells derived therefrom are used to screen for a compound to treat AMD by screening for compounds that modulate expression of the  $\alpha$ B-crystallin gene and/or its gene product. Such screening assays involve a step of contacting a test compound to an RPESC of the invention, or to a cell derived therefrom, and determining whether the test compound modulates (*e.g.*, increases or decreases) expression of the  $\alpha$ B-crystallin gene or gene product compared to cells (preferably of the same cell type cultured under the same or substantially the same conditions) that are not treated with the test compound. The RPESC are preferably cultured under conditions for culturing RPE cells that also increase expression of the  $\alpha$ B-crystallin gene or gene product. Alternatively, the RPESC may be induced to differentiate into RPE cells, *e.g.*, as described herein, which may then be cultured under conditions that increase expression of the  $\alpha$ B-crystallin gene or gene product. For example, the cells may be cultured under conditions of oxidative stress, such as exposure to hydrogen peroxide ( $H_2O_2$ ) or light (*e.g.*, blue or UV-light). In preferred embodiment, the test compound is identified as a compound for treating AMD if it decreases expression of the  $\alpha$ B-crystallin gene or gene product in the RPESC or progeny thereof.

[029] RPESC of the invention can also be used in cell based assays and models for other diseases and disorders; including, for example, retinitis pigmentosa, proliferative vitreoretinopathy, epiretinal membrane formation and retinitis, as well as any of the other disease and disorders described herein. Such assays and models are therefore also considered part of the invention.

### BRIEF DESCRIPTION OF THE DRAWINGS

[030] Figure 1 is a schematic representation of a sagittal section of the adult human eye.

[031] Figure 2 demonstrates the experimental procedure used to generate RPE and RPESC cultures.

[032] Figure 3 illustrates the metabolic pathways of eumelanin and dopamine synthesis.

[033] Figure 4 is a schematic representation of neural induction progression *in vitro* in the presence of FGF2 (also referred to as bFGF) and SHH/FGF8.

### DETAILED DESCRIPTION

[034] The present invention relates to a population of retinal pigment epithelial stem and progenitor cells isolated from the adult human retinal pigment epithelium (RPE) that can be expanded many fold *in vitro* and produce a wide variety of progeny from each of the major developmental lineages (endoderm, mesoderm and ectoderm). Self-renewal of these cells occurs, indicating a new kind of stem cell, the RPE stem cell (RPESC). Prior studies have shown that the eye contains progenitor cells in the ciliary margin and the iris epithelium that can produce retinal cell progeny. Reh & Fischer, *Methods Enzymol.* (2006) 419:52-73. RPESCs are distinguished from such retinal stem cells (RSCs) by their location in the main posterior retinal pigment epithelium layer, where RSCs are not found, by their lack of CHX10 staining and by their requirement for growth factors for expansion. Tropepe *et al.*, *Science* (2000) 287:2032-2036.

[035] Notably, while the RPESCs are capable of producing retinal cells like RSCs, they also are capable of producing a much wider repertoire of progeny, including bone, muscle and adipocytes. RPESCs appear to be a more primitive type of stem cell, which upon treatment with

growth factors, express genes associated with early embryonic stem cells including Sox2. Embryonic stem (ES) cells readily produce RPE cells (Klimanskaya *et al.*, *Cloning Stem Cells* (2004) 6:217-245) and in this sense are closely related to RPESCs. This invention establishes the human RPESC as a unique source of multipotent stem cells for the study of cell fate choice, cell replacement therapy and disease modeling.

[036] Vision loss may be caused by disease or damage to the retina of the eye. The retina consists of a specialized layer of cells at the back of the eye where light entering the eye is sensed as an image. These cells normally respond to all aspects of the light emitted from an object and allow perception of color, shape and intensity. When normal retinal function is impaired, it may lead to a loss of color perception, blind spots, reduced peripheral vision, night blindness, photophobia, decreased visual acuity or blindness. For example, acquired immunodeficiency virus ("AIDS") patients may suffer cytomegalovirus retinitis which is caused by spread of the cytomegalovirus to the retina (Bloom *et al.*, *Medicine*, 109(12): 963-968 (1988)). This and other infectious processes can lead to loss of visual field, decreased visual acuity, and blindness.

[037] Uveitis is an inflammation of the eye which can affect the retina and can lead to decreased visual acuity. Its effects on the retina include inflamed or leaking vasculature which may appear as perivascular exudation or hemorrhage, edema of the retina, chorioretinal lesions, neovascularization or inflammatory changes in the peripheral retina. (Anglade *et al.*, *Drugs*, 49(2):213-223 (1995)). Furthermore, cancers of the retina also impair vision. One example is retinoblastoma, which is a childhood type of cancer. Physical damage to retinal cells may also occur through retinal detachment which leads to retinal degeneration and blindness.

[038] Many different genetic diseases lead to retinal damage and blindness. A relatively common example is retinitis pigmentosa ("RP"), which affects one person in four thousand worldwide. Patients with RP have normal vision for one or more decades, and then experience progressive loss of vision due to the premature death of rod or cone cells. Blindness may result. Other types of retinal degenerations (retinal dystrophies) may result from the programmed death of other retinal cell types.

[039] Age-related macular degeneration (AMD) is a progressive degeneration of photoreceptors and their underlying RPE in the macula region of the retina, leading at end-stage to development of a disciform or an atrophic scar (Penfold et al. (2001) Prog. Retinal Eye Res. 20, 385-414; Sarks and Sarks, (1994) Chapter 67 in Retina, volume 2, Ed. S. Ryan. Publ. Mosby). At least two forms of AMD are recognized: "dry" and "wet." It is to be understood that the methods and compositions of this invention can be applied to either the dry or wet forms of AMD, or to both. Hence, the term AMD, as used herein, encompasses both these forms. In the United States, one recent estimate places the incidence of AMD at more than 11 million patients, and 1.2 million of these are legally blind with loss of central vision. Only a limited number of AMD patients are amenable to treatment despite the high incidence and severity of vision impairment (Ciulla et al. (1998) Surv. Ophthalmol. 43, 134-146). To date, there is a great need and effort to develop effective treatments or preventative measures, and to slow down or halt the progression of AMD would be a major achievement.

[040] In certain embodiments, RPESC cultures provide useful assay cultures for toxicity testing or for drug development testing. Toxicity testing is done by culturing stem cells or cells differentiated from stem cells in a suitable medium and introducing a substance, such as a pharmaceutical or chemical, to the culture. The stem cells or differentiated cells are examined to determine if the substance has had an adverse effect on the culture. Drug development testing may be done by developing derivative cell lines, for example a pathogenic retinal cell line, which may be used to test the efficacy of new drugs. Affinity assays for new drugs may also be developed from the RPESCs, RPE progenitor cells, or differentiated cells or cell lines derived from the RPESCs or differentiated cells.

[041] In other embodiments, RPESCs provide a culture system from which genes, proteins and other metabolites involved in cell development can be isolated and identified. The composition of stem cells may be compared with that of progenitor cells and differentiated cells in order to determine the mechanisms and compounds which stimulate production of stem cells, progenitor cells or mature cells. This invention also provides a method for stimulating stem cells of the posterior region of the retinal pigment epithelial layer to proliferate and differentiate *in vivo*. The cells of the present invention have the ability to recreate diverse cell types including

retinal cells, neuronal cells, neural cells, bone cells, muscle cells, ectoderm, mesoderm and endoderm.

[042] The RPESC also has utility in research not directly related to disease such as to reveal biological mechanisms, responses to environmental change or for comparison to other stem cell types. Proliferation is induced by administering one or more growth factors to the retina. Proliferation is also induced by administering genetically engineered cells which secrete growth factors into the eye. RPESCs may also be used in model systems *in vitro* to screen for new drugs for the treatment of diseases, such as AMD or RP.

[043] With respect to the eye, current medical and surgical drug treatments are inadequate for restoring vision lost when retinal cells are damaged, so the potential clinical applications of pharmaceutical compositions containing RPESCs or to stimulate endogenous proliferation of RPESCs are tremendous. RPESCs would have the potential to act as *in vivo* targets for stimulation by growth factors in order to produce healthy tissue. This may be done, for example, by injecting growth factors or genetically engineered cells which secrete growth factors into the eye.

[044] In the case of other neurological sites, such as the central and peripheral nervous system sites, there is a need in the art for the treatment of neurodegenerative disorders, and ideally treatments which do not induce unfavorable reactions, such as graft rejection or graft versus host disease in the patient. Parkinson's disease (PD), for example, is the most common neurodegenerative movement disorder [Forman *et al.*, (2004) *Nat. Med.* 10:1055-1063] and is characterized by degeneration of the dopaminergic neurons in the substantia nigra pars compacta, accompanied by decreases in striatal dopamine (DA) and the appearance of intracytoplasmic Lewy body inclusions. Once striatal DA loss reaches the 80% critical value [Hornykiewicz *et al.*, (2001) *J. Chem. Neuroanat* 22:3-12], a progressive motor impairment develops that is characterized by resting tremor, rigidity, bradykinesia, hypokinesia, and postural instability [Calne *et al.*, (1992) *Ann. Neurol.* 32 *Suppl*:S125-127]. The synthesis of DA involves the enzyme tyrosine hydroxylase (TH), which converts L-Tyrosine into L-3, 4-dihydroxyphenylalanine (L-DOPA), an important intermediate in the DA synthesis pathway [See Fig. 3].

[045] In certain embodiments of the present invention, RPESCs are differentiated into midbrain dopaminergic neurons that express TH and the neural markers Nestin and Tuj1. Thus, RPESCs are useful for the treatment of PD.

[046] Ballism is typically associated with damage to the subthalamic nucleus, often due to acute vascular accident. Also included are neurogenic and myopathic diseases which ultimately affect the somatic division of the peripheral nervous system and are manifest as neuromuscular disorders. Examples include chronic atrophies such as amyotrophic lateral sclerosis, Guillain-Barre syndrome and chronic peripheral neuropathy, as well as other diseases which can be manifest as progressive bulbar palsies or spinal muscular atrophies. Spinal muscular atrophy (SMA) is a term applied to a number of different disorders, all having in common a genetic cause and the manifestation of weakness due to loss of the motor neurons of the spinal cord and brainstem. In certain embodiments, the present invention relates to the treatment of neurological or myopathic disease with an effective amount of the RPESCs of the present invention.

[047] Leber's hereditary optic neuropathy (LHON) or Leber optic atrophy is a mitochondrially inherited (mother to all offspring) degeneration of retinal ganglion cells (RGCs) and their axons that leads to an acute or subacute loss of central vision; this affects predominantly young adult males [Nikoskelainen *et al.*, (1995) *J. Neurol. Neurosurg. Psychiatry* 2:160-164]. The eye pathology is limited to the retinal ganglion cell layer especially the maculopapillary bundle. Degeneration is evident from the retinal ganglion cell bodies to the axonal pathways leading to the lateral geniculate nuclei. Experimental evidence reveals impaired glutamate transport and increased reactive oxygen species (ROS) causing apoptosis of retinal ganglion cells. Also, experiments indicate that normal non LHON affected retinal ganglion cells produce less of the potent superoxide radical than other normal central nervous system neurons [Hoegger *et al.*, (2008) *BMC Neurosci* 8;9:4].

[048] One aspect of the present invention relates to the methods of expanding endothelial cells *in vitro* by co-culturing them with RPESCs. Endothelial cells are important for vascularization and angiogenesis. In yet another aspect of the invention, RPESCs transplanted onto chick chorioallantoic membrane (CAM) develop into teratomas. The CAM model of



teratoma formation, as well as angiogenesis, closely mimics characteristics of these processes in humans and provides a useful tool for studying human disease.

[049] Yet another aspect of the present invention involves progenitor cells derived from RPESCs. RPESCs are closely related to early RPE progenitors. These early progenitors are multipotential cells that may be differentiated into all of the major lineage groups including mesoderm and endoderm. Thus, bone cells and muscle cells may be generated by differentiation of RPESCs and RPE progenitor cells.

[050] One aspect of the present invention concerns the therapeutic use of the pharmaceutical compositions of this invention to treat patients having degenerative diseases, such as age-related macular degeneration, or disorders or abnormal physical states of the eye, which includes an acceptable carrier, auxiliary or excipient. The compositions can be for topical, parenteral, local, intraocular or intraretinal use.

[051] The pharmaceutical composition can be administered to humans or animals. Dosages to be administered depend on patient needs, on the desired effect and on the chosen route of administration.

[052] The pharmaceutical compositions can be prepared by known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an effective quantity of the cells is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985).

[053] On this basis, the pharmaceutical compositions could include an active compound or substance, such as growth factors, genetically engineered stem cells or retinal cells which secrete growth factor or other substances, in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids. The methods of combining growth factor or cells with the vehicles or combining them with diluents are well known to those skilled in the art. The

composition could include a targeting agent for the transport of the active compound or cells to specified sites within the eye, such as specific cells, tissues or organs.

[054] In preferred embodiments, pharmaceutical compositions of the invention can include a sustained delivery composition comprising one or more exogenous factors, for administration in combination with an RPESC of the invention or in combination with a differentiated RPESC of the invention. The exogenous factors can include, for example, at least one growth factor such as Nerve Growth Factor (NGF), Glial Cell-Line Derived Growth Factor (GDNF), Neurotrophin (NT) 3, NT4/5, NT6, Ciliary Neurotrophic Factor (CNTF), Interleukin 6 (IL6), Interleukin 11 (IL11), Cardiotrophin 1, a growth factor hormone, hyaluronidase, chondroitinase ABC (CABC), basic fibroblast growth factor (BDNF), epidermal growth factor (EGF), sonic hedgehog (Shh) or another other growth factor or other exogenous factor described throughout this specification or in U.S. patent application Serial No. 12/398,888 filed March 5, 2009 and incorporated herein by reference in its entirety.

[055] Sustained delivery compositions comprising one or more exogenous factors can be administered concurrently with, before or after the administration of either an RPESC or a differentiated RPESC. For example, U.S. patent application Serial No. 12/398,888 filed March 5, 2009 and incorporated herein by reference describes the administration of sustained release biodegradable microspheres loaded with one or more exogenous factors such as sonic hedgehog (Shh) and/or retinoic acid in combination with neural stem cells. The use of such compositions is also contemplated in the presented invention, for administration in combination with an RPESC or a differentiated RPESC, such as an RPESC that has been differentiated into a dopaminergic or other neuron.

[056] The present invention also relates to the use of the stem cells and progenitor cells of this invention to introduce recombinant proteins into the diseased or damaged retina. The cells act as a vector to transport a recombinant molecule, for example, or to transport a sense or antisense sequence of a nucleic acid molecule. In the case of a recombinant molecule, the molecule would contain suitable transcriptional or translational regulatory elements.

[057] Suitable regulatory elements may be derived from a variety of sources, and they may be readily selected by one of ordinary skill in the art. Examples of regulatory elements

include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the vector employed, other genetic elements, such as selectable markers, may be incorporated into the recombinant molecule.

[058] The recombinant molecule may be introduced into stem cells or retinal cells differentiated from stem cells of a patient using *in vitro* delivery vehicles such as retroviral vectors, adenoviral vectors, DNA virus vectors, amplicons and liposomes. They may also be introduced into these cells using physical techniques such as microinjection and electroporation or chemical methods such as coprecipitation and incorporation of DNA into liposomes.

[059] Suitable regulatory elements may be derived from a variety of sources, and they may be readily selected by one of ordinary skill in the art. If one were to upregulate the expression of the gene, one would insert the sense sequence and the appropriate promoter into the vehicle. If one were to downregulate the expression of the gene, one would insert the antisense sequence and the appropriate promoter into the vehicle. These techniques are known to those skilled in the art.

[060] The pharmaceutical compositions of the present invention could also include the active compound or substance, such as the RPESCs of this invention, or retinal progenitor cells or differentiated cells derived from those stem cells, in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids. The methods of combining cells with the vehicles or combining them with diluents is well known to those skilled in the art. The composition could include a targeting agent for the transport of the active compound to specified sites within the eye, such as specific cells, tissues or organs.

## **Definitions**

[061] The following definitions are provided for clarity and illustrative purposes only, and are not intended to limit the scope of the invention.

[062] As used herein, the term "stem cell" refers to a cell that retains the ability to renew itself through mitotic cell division and can differentiate into a diverse range of specialized cell types.

[063] As used herein, the term "plasticity" refers to the ability of an adult stem cell to adopt a variety of cell fates.

[064] As used herein, the term "neotony" is the retention by adult cells of traits previously only seen in embryonic cells.

[065] The term "growth factor" can be a naturally occurring, endogenous or exogenous protein, or recombinant protein, capable of stimulating cellular proliferation and/or cellular differentiation.

[066] As used herein, the term "morphogenic factor" refers to a substance governing the pattern of tissue development and, in particular, the positions of the various specialized cell types within a tissue.

[067] As used herein, "central nervous system" includes brain and/or the spinal cord of a mammal. The term may also include the eye, retina, and optic nerve in some instances.

[068] As used herein, the term "neuron" as used herein describes a nerve cell capable of receiving and conducting electrical impulses from the central nervous system. A nerve cell or "neuron" may typically include a cell body, an axon, axon terminals, and dendrites.

[069] As used herein, the term "exogenous factor" describes those compounds capable of inducing differentiation of a stem cell into another cell type. These compounds include, but are not limited to antioxidants, trophic factors, morphogenic factors, and growth factors.

[070] As used herein, the term "agent" refers to a chemical or recombinant compound that may be used to treat a condition or disease.

[071] As used herein, the term "autologous" refers to cells, tissues or even proteins that are isolated and reimplanted into the same individual.

[072] As used herein, the term “non-autologous” refers to cells, tissues, or even proteins that are transplanted from one individual to another individual.

### **Expression Construct**

[073] By “expression construct” is meant a nucleic acid sequence comprising a target nucleic acid sequence or sequences whose expression is desired, operatively associated with expression control sequence elements which provide for the proper transcription and translation of the target nucleic acid sequence(s) within the chosen host cells. Such sequence elements may include a promoter and a polyadenylation signal. The “expression construct” may further comprise “vector sequences”. By “vector sequences” is meant any of several nucleic acid sequences established in the art which have utility in the recombinant DNA technologies of the invention to facilitate the cloning and propagation of the expression constructs including (but not limited to) plasmids, cosmids, phage vectors, viral vectors, and yeast artificial chromosomes.

[074] Expression constructs of the present invention may comprise vector sequences that facilitate the cloning and propagation of the expression constructs. A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and prokaryotic host cells. Standard vectors useful in the current invention are well known in the art and include (but are not limited to) plasmids, cosmids, phage vectors, viral vectors, and yeast artificial chromosomes. The vector sequences may contain a replication origin for propagation in *E. coli*; the SV40 origin of replication; an ampicillin, neomycin, or puromycin resistance gene for selection in host cells; and/or genes (*e.g.*, dihydrofolate reductase gene) that amplify the dominant selectable marker plus the gene of interest.

### **Express and Expression**

[075] The terms “express” and “expression” mean allowing or causing the information in a gene or DNA sequence to become manifest, for example producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene or DNA sequence. A DNA sequence is expressed in or by a cell to form an “expression product” such as a protein. The expression product itself, *e.g.*, the resulting protein, may also be said to be

“expressed” by the cell. An expression product can be characterized as intracellular, extracellular or secreted. The term “intracellular” means something that is inside a cell. The term “extracellular” means something that is outside a cell. A substance is “secreted” by a cell if it appears in significant measure outside the cell, from somewhere on or inside the cell.

[076] The term "transfection" means the introduction of a foreign nucleic acid into a cell. The term “transformation” means the introduction of a “foreign” (*i.e.*, extrinsic or extracellular) gene, DNA or RNA sequence to a cell, so that the host cell will express the introduced gene or sequence to produce a desired substance, typically a protein or enzyme coded by the introduced gene or sequence. The introduced gene or sequence may also be called a “cloned” or “foreign” gene or sequence, may include regulatory or control sequences, such as start, stop, promoter, signal, secretion, or other sequences used by a cells genetic machinery. The gene or sequence may include nonfunctional sequences or sequences with no known function. A host cell that receives and expresses introduced DNA or RNA has been “transformed” and is a "transformant" or a "clone". The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell, or cells of a different genus or species.

### **Expression System**

[077] The term "expression system" means a host cell and compatible vector under suitable conditions, *e.g.*, for the expression of a protein coded for by foreign DNA carried by the vector and introduced to the host cell.

### **Gene or Structural Gene**

[078] The term "gene", also called a "structural gene" means a DNA sequence that codes for or corresponds to a particular sequence of amino acids which comprise all or part of one or more proteins or enzymes, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed. Some genes, which are not structural genes, may be transcribed from DNA to RNA, but are not translated into an amino acid sequence. Other genes may function as regulators of structural genes or as regulators of DNA transcription.

[079] A coding sequence is "under the control of" or "operatively associated with" expression control sequences in a cell when RNA polymerase transcribes the coding sequence into RNA, particularly mRNA, which is then trans-RNA spliced (if it contains introns) and translated into the protein encoded by the coding sequence.

[080] The term "expression control sequence" refers to a promoter and any enhancer or suppression elements that combine to regulate the transcription of a coding sequence. In a preferred embodiment, the element is an origin of replication.

### **Heterologous**

[081] The term "heterologous" refers to a combination of elements not naturally occurring. For example, heterologous DNA refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. Preferably, the heterologous DNA includes a gene foreign to the cell. For example, the present invention includes chimeric DNA molecules that comprise a DNA sequence and a heterologous DNA sequence which is not part of the DNA sequence. A heterologous expression regulatory element is such an element that is operatively associated with a different gene than the one it is operatively associated with in nature. In the context of the present invention, a gene encoding a protein of interest is heterologous to the vector DNA in which it is inserted for cloning or expression, and it is heterologous to a host cell containing such a vector, in which it is expressed.

### **Homologous**

[082] The term "homologous" as used in the art commonly refers to the relationship between nucleic acid molecules or proteins that possess a "common evolutionary origin," including nucleic acid molecules or proteins within superfamilies (*e.g.*, the immunoglobulin superfamily) and nucleic acid molecules or proteins from different species (Reeck et al., *Cell* 1987; 50: 667). Such nucleic acid molecules or proteins have sequence homology, as reflected by their sequence similarity, whether in terms of substantial percent similarity or the presence of specific residues or motifs at conserved positions.

**Host Cell**

[083] The term "host cell" means any cell of any organism that is selected, modified, transformed, grown or used or manipulated in any way for the production of a substance by the cell. For example, a host cell may be one that is manipulated to express a particular gene, a DNA or RNA sequence, a protein or an enzyme. Host cells can further be used for screening or other assays that are described *infra*. Host cells may be cultured *in vitro* or one or more cells in a non-human animal (*e.g.*, a transgenic animal or a transiently transfected animal). Suitable host cells include but are not limited to *Streptomyces* species and *E. coli*.

**Treating or Treatment**

[084] "Treating" or "treatment" of a state, disorder or condition includes:

[085] (1) preventing or delaying the appearance of clinical or sub-clinical symptoms of the state, disorder or condition developing in a mammal that may be afflicted with or predisposed to the state, disorder or condition but does not yet experience or display clinical or subclinical symptoms of the state, disorder or condition; or

[086] (2) inhibiting the state, disorder or condition, *i.e.*, arresting, reducing or delaying the development of the disease or a relapse thereof (in case of maintenance treatment) or at least one clinical or sub-clinical symptom thereof; or

[087] (3) relieving the disease, *i.e.*, causing regression of the state, disorder or condition or at least one of its clinical or sub-clinical symptoms.

[088] The benefit to a subject to be treated is either statistically significant or at least perceptible to the patient or to the physician.

**Patient or Subject**

[089] "Patient" or "subject" refers to mammals and includes human and veterinary subjects.



**Therapeutically Effective Amount**

[090] A "therapeutically effective amount" means the amount of a compound that, when administered to a mammal for treating a state, disorder or condition, is sufficient to effect such treatment. The "therapeutically effective amount" will vary depending on the compound, the disease and its severity and the age, weight, physical condition and responsiveness of the mammal to be treated.

**About or Approximately**

[091] The term "about" or "approximately" means within an acceptable range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *e.g.*, the limitations of the measurement system. For example, "about" can mean a range of up to 20 %, preferably up to 10 %, more preferably up to 5 %, and more preferably still up to 1 % of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value. Unless otherwise stated, the term 'about' means within an acceptable error range for the particular value.

**Dosage**

[092] The dosage of the therapeutic formulation will vary widely, depending upon the nature of the disease, the patient's medical history, the frequency of administration, the manner of administration, the clearance of the agent from the host, and the like. The initial dose may be larger, followed by smaller maintenance doses. The dose may be administered as infrequently as weekly or biweekly, or fractionated into smaller doses and administered daily, semi-weekly, etc., to maintain an effective dosage level.

**Carrier**

[093] The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and

aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Alternatively, the carrier can be a solid dosage form carrier, including but not limited to one or more of a binder (for compressed pills), a glidant, an encapsulating agent, a flavorant, and a colorant. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

### **Isolated**

[094] As used herein, the term "isolated" means that the referenced material is removed from the environment in which it is normally found. Thus, an isolated biological material can be free of cellular components, *i.e.*, components of the cells in which the material is found or produced. Isolated nucleic acid molecules include, for example, a PCR product, an isolated mRNA, a cDNA, or a restriction fragment. Isolated nucleic acid molecules also include, for example, sequences inserted into plasmids, cosmids, artificial chromosomes, and the like. An isolated nucleic acid molecule is preferably excised from the genome in which it may be found, and more preferably is no longer joined to non-regulatory sequences, non-coding sequences, or to other genes located upstream or downstream of the nucleic acid molecule when found within the genome. An isolated protein may be associated with other proteins or nucleic acids, or both, with which it associates in the cell, or with cellular membranes if it is a membrane-associated protein.

### **Mutant**

[095] As used herein, the terms "mutant" and "mutation" refer to any detectable change in genetic material (*e.g.*, DNA) or any process, mechanism, or result of such a change. This includes gene mutations, in which the structure (*e.g.*, DNA sequence) of a gene is altered, any gene or DNA arising from any mutation process, and any expression product (*e.g.*, protein or enzyme) expressed by a modified gene or DNA sequence. As used herein, the term "mutating" refers to a process of creating a mutant or mutation.

## Nucleic Acid Hybridization

[096] The term “nucleic acid hybridization” refers to anti-parallel hydrogen bonding between two single-stranded nucleic acids, in which A pairs with T (or U if an RNA nucleic acid) and C pairs with G. Nucleic acid molecules are “hybridizable” to each other when at least one strand of one nucleic acid molecule can form hydrogen bonds with the complementary bases of another nucleic acid molecule under defined stringency conditions. Stringency of hybridization is determined, *e.g.*, by (i) the temperature at which hybridization and/or washing is performed, and (ii) the ionic strength and (iii) concentration of denaturants such as formamide of the hybridization and washing solutions, as well as other parameters. Hybridization requires that the two strands contain substantially complementary sequences. Depending on the stringency of hybridization, however, some degree of mismatches may be tolerated. Under “low stringency” conditions, a greater percentage of mismatches are tolerable (*i.e.*, will not prevent formation of an anti-parallel hybrid). *See* Molecular Biology of the Cell, Alberts et al., 3rd ed., New York and London: Garland Publ., 1994, Ch. 7.

[097] Typically, hybridization of two strands at high stringency requires that the sequences exhibit a high degree of complementarity over an extended portion of their length. Examples of high stringency conditions include: hybridization to filter-bound DNA in 0.5 M NaHPO<sub>4</sub>, 7 % SDS, 1 mM EDTA at 65°C, followed by washing in 0.1x SSC/0.1 % SDS at 68°C (where 1x SSC is 0.15M NaCl, 0.15M Na citrate) or for oligonucleotide molecules washing in 6xSSC/0.5 % sodium pyrophosphate at about 37°C (for 14 nucleotide-long oligos), at about 48°C (for about 17 nucleotide-long oligos), at about 55°C (for 20 nucleotide-long oligos), and at about 60°C (for 23 nucleotide-long oligos). Accordingly, the term “high stringency hybridization” refers to a combination of solvent and temperature where two strands will pair to form a “hybrid” helix only if their nucleotide sequences are almost perfectly complementary (*see* Molecular Biology of the Cell, Alberts et al., 3rd ed., New York and London: Garland Publ., 1994, Ch. 7).

[098] Conditions of intermediate or moderate stringency (such as, for example, an aqueous solution of 2XSSC at 65°C; alternatively, for example, hybridization to filter-bound DNA in 0.5 M NaHPO<sub>4</sub>, 7 % SDS, 1 mM EDTA at 65°C, and washing in 0.2 x SSC/0.1 % SDS at 42°C) and low stringency (such as, for example, an aqueous solution of 2XSSC at 55°C), require correspondingly less overall complementarity for hybridization to occur between two

sequences. Specific temperature and salt conditions for any given stringency hybridization reaction depend on the concentration of the target DNA and length and base composition of the probe, and are normally determined empirically in preliminary experiments, which are routine (*see* Southern, *J. Mol. Biol.* 1975; 98: 503; Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 2, ch. 9.50, CSH Laboratory Press, 1989; Ausubel et al. (eds.), 1989, *Current Protocols in Molecular Biology*, Vol. I, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at p. 2.10.3).

[099] As used herein, the term "standard hybridization conditions" refers to hybridization conditions that allow hybridization of sequences having at least 75 % sequence identity. According to a specific embodiment, hybridization conditions of higher stringency may be used to allow hybridization of only sequences having at least 80 % sequence identity, at least 90 % sequence identity, at least 95 % sequence identity, or at least 99 % sequence identity.

[0100] Nucleic acid molecules that "hybridize" to any desired nucleic acids of the present invention may be of any length. In one embodiment, such nucleic acid molecules are at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, and at least 70 nucleotides in length. In another embodiment, nucleic acid molecules that hybridize are of about the same length as the particular desired nucleic acid.

### **Nucleic Acid Molecule**

[0101] A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear (*e.g.*, restriction fragments) or circular DNA molecules, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only

the sequence in the 5' to 3' direction along the non-transcribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

### **Orthologs**

[0102] As used herein, the term "orthologs" refers to genes in different species that apparently evolved from a common ancestral gene by speciation. Normally, orthologs retain the same function through the course of evolution. Identification of orthologs can provide reliable prediction of gene function in newly sequenced genomes. Sequence comparison algorithms that can be used to identify orthologs include without limitation BLAST, FASTA, DNA Strider, and the GCG pileup program. Orthologs often have high sequence similarity. The present invention encompasses all orthologs of the desired protein.

### **Operatively Associated**

[0103] By "operatively associated with" is meant that a target nucleic acid sequence and one or more expression control sequences (*e.g.*, promoters) are physically linked so as to permit expression of the polypeptide encoded by the target nucleic acid sequence within a host cell.

### **Percent Sequence Similarity or Percent Sequence Identity**

[0104] The terms "percent (%) sequence similarity", "percent (%) sequence identity", and the like, generally refer to the degree of identity or correspondence between different nucleotide sequences of nucleic acid molecules or amino acid sequences of proteins that may or may not share a common evolutionary origin (*see* Reeck et al., *supra*). Sequence identity can be determined using any of a number of publicly available sequence comparison algorithms, such as BLAST, FASTA, DNA Strider, GCG (Genetics Computer Group, Program Manual for the GCG Package, Version 7, Madison, Wisconsin), etc.

[0105] To determine the percent identity between two amino acid sequences or two nucleic acid molecules, the sequences are aligned for optimal comparison purposes. The percent

identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, percent identity = number of identical positions/total number of positions (*e.g.*, overlapping positions) x 100). In one embodiment, the two sequences are, or are about, of the same length. The percent identity between two sequences can be determined using techniques similar to those described below, with or without allowing gaps. In calculating percent sequence identity, typically exact matches are counted.

[0106] The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. A non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, Proc. Natl. Acad. Sci. USA 1990, 87:2264, modified as in Karlin and Altschul, Proc. Natl. Acad. Sci. USA 1993, 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al., J. Mol. Biol. 1990; 215: 403. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to sequences of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to protein sequences of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., Nucleic Acids Res. 1997, 25:3389. Alternatively, PSI-Blast can be used to perform an iterated search that detects distant relationship between molecules. *See* Altschul et al. (1997) *supra*. When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. *See* [ncbi.nlm.nih.gov/BLAST/](http://ncbi.nlm.nih.gov/BLAST/) on the WorldWideWeb. Another non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS 1988; 4: 11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0), which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

[0107] In a preferred embodiment, the percent identity between two amino acid sequences is determined using the algorithm of Needleman and Wunsch (J. Mol. Biol. 1970, 48:444-453), which has been incorporated into the GAP program in the GCG software package

(Accelrys, Burlington, MA; available at accelrys.com on the WorldWideWeb), using either a Blossum 62 matrix or a PAM250 matrix, a gap weight of 16, 14, 12, 10, 8, 6, or 4, and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package using a NWSgapdna.CMP matrix, a gap weight of 40, 50, 60, 70, or 80, and a length weight of 1, 2, 3, 4, 5, or 6. A particularly preferred set of parameters (and the one that can be used if the practitioner is uncertain about what parameters should be applied to determine if a molecule is a sequence identity or homology limitation of the invention) is using a Blossum 62 scoring matrix with a gap open penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

[0108] In addition to the cDNA sequences encoding various desired proteins, the present invention further provides polynucleotide molecules comprising nucleotide sequences having certain percentage sequence identities to any of the aforementioned sequences. Such sequences preferably hybridize under conditions of moderate or high stringency as described above, and may include species orthologs.

### **Variant**

[0109] The term "variant" may also be used to indicate a modified or altered gene, DNA sequence, enzyme, cell, etc., *i.e.*, any kind of mutant.

### **Pharmaceutically Acceptable**

[0110] When formulated in a pharmaceutical composition, a therapeutic compound of the present invention can be admixed with a pharmaceutically acceptable carrier or excipient. As used herein, the phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are generally believed to be physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human.

### **Pharmaceutically Acceptable Derivative**

[0111] The term “pharmaceutically acceptable derivative” as used herein means any pharmaceutically acceptable salt, solvate or prodrug, *e.g.*, ester, of a compound of the invention, which upon administration to the recipient is capable of providing (directly or indirectly) a compound of the invention, or an active metabolite or residue thereof. Such derivatives are recognizable to those skilled in the art, without undue experimentation. Nevertheless, reference is made to the teaching of Burger’s Medicinal Chemistry and Drug Discovery, 5th Edition, Vol 1: Principles and Practice, which is incorporated herein by reference to the extent of teaching such derivatives. Preferred pharmaceutically acceptable derivatives are salts, solvates, esters, carbamates, and phosphate esters. Particularly preferred pharmaceutically acceptable derivatives are salts, solvates, and esters. Most preferred pharmaceutically acceptable derivatives are salts and esters.

### **Pharmaceutical Compositions and Administration**

[0112] While it is possible to use a composition provided by the present invention for therapy as is, it may be preferable to administer it in a pharmaceutical formulation, *e.g.*, in admixture with a suitable pharmaceutical excipient, diluent, or carrier selected with regard to the intended route of administration and standard pharmaceutical practice. Accordingly, in one aspect, the present invention provides a pharmaceutical composition or formulation comprising at least one active composition, or a pharmaceutically acceptable derivative thereof, in association with a pharmaceutically acceptable excipient, diluent, and/or carrier. The excipient, diluent and/or carrier must be “acceptable” in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

[0113] The compositions of the invention can be formulated for administration in any convenient way for use in human or veterinary medicine.

### **Kits**

[0114] In one embodiment, the invention relates to a kit comprising RPESCs useful for modeling a disease or condition *in vitro*. This kit further comprises a means for detecting improvement in the disease or condition following treatment with an agent.



[0115] The effective amounts of compounds of the present invention include doses that partially or completely achieve the desired therapeutic, prophylactic, and/or biological effect. The actual amount effective for a particular application depends on the condition being treated and the route of administration. The effective amount for use in humans can be determined from animal models. For example, a dose for humans can be formulated to achieve circulating and/or gastrointestinal concentrations that have been found to be effective in animals.

[0116] The abbreviations in the specification correspond to units of measure, techniques, properties or compounds as follows: "min" means minutes, "h" means hour(s), "μL" means microliter(s), "mL" means milliliter(s), "mM" means millimolar, "M" means molar, "μl" means microliter(s); "mmole" means millimole(s), "kb" means kilobase, "bp" means base pair(s), and "IU" means International Units. "Polymerase chain reaction" is abbreviated PCR; "Reverse transcriptase polymerase chain reaction" is abbreviated RT-PCR; "Estrogen receptor" is abbreviated ER; "DNA binding domain" is abbreviated DBD; "Untranslated region" is abbreviated UTR; "Sodium dodecyl sulfate" is abbreviated SDS; and "High Pressure Liquid Chromatography" is abbreviated HPLC.

## EXAMPLES

[0117] The following examples are included to demonstrate certain embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

## Materials and Methods

[0118] The following describes the materials and methods employed in the examples.

*Isolation and Culture of Human RPE cells.*

[0119] Human ocular tissues from 60-100 year old donors were obtained from The Eye-Bank for Sight Restoration, Inc, New York, NY, and the National Disease Research Interchange, Philadelphia, Pa. RPE dissection and single cell dissociation was performed essentially as described. Burke *et al.*, *Exp. Eye Res.* (1996) 62:63-73; Maminishkis *et al.*, *Invest. Ophthalmol. Vis. Sci.* (2006) 47:3612-3624. RPE cells were transferred in non-coated 60 mm plate and cultured in Minimum Essential Medium Eagle, 2mM L-glutamine, 1 % Penicillin/Streptomycin, 1 % Na-Pyruvate, 50% fetal bovine serum (FBS), 10 ng/ml FGF2 and 1 ng/ml EGF (Gibco/Invitrogen). Cells began to form spheres within 3-4 days in culture and continued to grow in mass and number. Half of the medium was changed every 4 days. For passaging of cells, the culture medium containing the floating spheres was collected in a 15-ml centrifuge tube and centrifuged at 800 to 12,400 rpm for 5 min. The pellet was resuspended in 200 to 500  $\mu$ l of Accutase (Innovative Cell Technologies), triturated 5 times, placed at 37°C for 10 min, triturated again and resuspended in medium. In some experiments, cells were grown in adherent cell culture conditions in 6-well plates coated with fibronectin (5  $\mu$ g/ml)/laminin (1  $\mu$ g/ml) (Gibco/Invitrogen). ARPE-19 cells, available from the American Type Culture Collection (ATCC), Manassas, Virginia (Accession No. CRL-2302) were cultured in the same culture medium as described above and passed when they were confluent. The cells were incubated in a 37°C humidified incubator under 95 % air and 5 % CO<sub>2</sub> and the medium was replaced every 2-3 days.

*In vitro-Induced Differentiation*

[0120] RPE cells were cultured in medium designed for ES cell maintenance. They were seeded on uncoated 60 mm plates and cultured as RPESC spheres in KSR medium (Gibco) supplemented with FGF2 (20 ng/ml, Sigma-Aldrich) and incubated at 37°C in humidified 5 % CO<sub>2</sub> in air for 7 days. The medium was changed every 3 days.

[0121] For experiments in which dopaminergic neurons were generated, the following methods were used: The RPESCs were grown on gelatin-coated tissue culture plates in ES cell medium consisting of knockout DMEM supplemented with 15% FBS, 0.1 mM MEM nonessential amino acids, 0.1 mM 2-mercaptoethanol (2-ME), 2mM L-glutamine, 1%

Penicillin/Streptomycin (Gibco-Invitrogen). RPESC cells were dissociated (0.25% trypsin-EDTA) to single cells and transferred to an uncoated plate at a density of  $2-2.5 \times 10^4$  cells  $\text{cm}^2$  in knockout DMEM supplemented with 10% knockout serum replacement, 0.1 mM MEM nonessential amino acids, 0.1 mM 2-mercaptoethanol (2-ME), 2 mM L-glutamine, 1% Penicillin/Streptomycin (Gibco-Invitrogen) (KSR medium) supplemented with FGF2 (20 ng/ml, Sigma-Aldrich). KSR medium was replaced every 3 days. RPESCs were cultured under the same conditions as used for the maintenance of mouse embryonic stem cells. They were seeded on uncoated 60 mm plates and cultured in KSR medium supplemented with FGF2 (20ng/ml, Sigma-Aldrich) and incubated at 37°C in humidified 5% CO<sub>2</sub> in air for 7 days. The medium was changed every 3 days.

[0122] The following conditions were used to differentiate the cultured RPE into different lineage pathways, using combinations of published protocols modified as described:

[0123] **Neurogenic Lineage:** RPESC spheres were seeded on uncoated plates and cultured in neural differentiation medium (N2/B27): ES-Cult™ Basal Medium-A, B-27, N2, ITS (insulin, transferrin, selenium) Supplement-B (Stemcell Technologies Inc), 1% Penicillin/Streptomycin (Gibco-Invitrogen) and supplemented with all trans retinoic acid (RA, 2 μM, Sigma-Aldrich), FGF2 (20 ng/ml, Sigma-Aldrich), FGF8b (100 ng/ml, R&D Systems), Shh (100 ng/ml, R&D Systems) for 4 days. RPE spheres were transferred into 24 well plates coated with fibronectin (5 μg/ml)/laminin (1 μg/ml) and incubated in neural differentiation medium supplemented with all growth factors except FGF2 (RA, FGF8, and Shh). The cells were maintained in neural differentiation medium for >4 weeks, with medium and growth factors changed every third day.

[0124] **Osteogenic Lineage:** RPE spheres were seeded on uncoated plates and cultured in osteogenic differentiation medium: IMDM medium with 20 % FBS, 0.1 mM MEM nonessential amino acids, 0.1 mM 2-ME, 2 mM L-glutamine, 1 % Penicillin/Streptomycin (Gibco-Invitrogen), B-27, N2 supplement (Stemcell Technologies Inc.) , and osteogenic supplements 1 nM dexamethasone, 10 mM beta-glycerophosphate and 50 μM ascorbic acid-2-phosphate (Sigma-Aldrich) for 4 days. Duplomb *et al.*, *Stem Cells* (2007) 25:544-552; Karner *et al.*, *Stem Cells Dev.* (2007) 16:39-52. RPE spheres were transferred onto 24 well plate coated

and incubated in osteogenic differentiation medium supplemented with all osteogenic supplements. The cells were maintained in osteogenic differentiation medium for >4 weeks, with medium and osteogenic supplements changed every third day.

[0125] **Myogenic Lineage:** RPE spheres were seeded on uncoated plates and cultured in myogenic differentiation medium: ES-Cult™ Basal Medium-A with 20 % KO serum replacement, 0.1 mM MEM nonessential amino acids, 0.1 mM 2-ME, 2 mM L-glutamine, 1 % Penicillin/Streptomycin (Gibco-Invitrogen), B-27, N2 supplement (Stemcell Technologies Inc.) and supplemented with FGF2 (20 ng/ml, Sigma-Aldrich), BMP4 (50 ng/ml, R&D Systems), BMP2 (50 ng/ml, R&D Systems) for 4 days. Hosseinkhani *et al.*, *Stem Cells* (2007) 25:571-580; Yao *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* (2006) 103:6907-6912. The cells were maintained in myogenic differentiation medium for >4 weeks, with medium and osteogenic supplements changed every second day.

[0126] **Adipogenic Lineage:** Human RPE spheres were seeded on uncoated plates and grown to confluence, followed by exposure of 0.5 mM isobutylxanthine, 1mM dexamethasone and 10µg/ml insulin (all from Sigma-Aldrich) in Mesencult® MSC Human Basal Medium (Stemcell Technologies Inc) containing 10% FBS for >3 weeks.

[0127] **Endoderm Lineage:** Human RPE spheres were seeded on uncoated plates and cultured in endoderm differentiation medium: IMDM medium with 2 mM L-glutamine, 1 % Penicillin/Streptomycin (Gibco-Invitrogen), B-27, N2, ITS Supplement-B (Stemcell Technologies Inc.), and supplemented with FGF2 (20 ng/ml, Sigma-Aldrich), BMP4 (50 ng/ml, R&D Systems), Activin (50 ng/ml, R&D Systems), 50 µM ascorbic acid (Stemcell Technologies Inc.) for 4 days. Gouon-Evans *et al.*, *Nat. Biotechnol.* (2006) 24:1402-1411; Kubo *et al.*, *Development* (2004) 131:1651-1662. The cells were maintained in endoderm differentiation medium for >4 weeks, with medium and supplements changed every third day.

[0128] **Chondrocyte Lineage:** Chondrogenic differentiation of human RPESCs was induced with high glucose Gibco® DMEM medium (available from Invitrogen Corporation, Carlsbad, California) supplemented with ITS (6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 ng/ml selenium) (available from Stem Cell Technologies, Vancouver, British Columbia), 1.25 mg/ml bovine serum albumin, 5.35 µg/ml linoleic acid (available from BD Biosciences, San

Jose, California), 40 µg/ml L-proline (available from Sigma-Aldrich Corporation, St. Louis, Missouri), 50 µg/ml ascorbic acid 2-phosphate (available from Sigma, a subsidiary of Sigma-Aldrich), 1% sodium pyruvate (Sigma), 1% nonessential amino acids (Gibco-Invitrogen), 10<sup>-7</sup>M dexamethason (Sigma-Aldrich) and 1% penicillin/streptomycin (Gibco-Invitrogen). After incubation for 24 hours to allow cell attachment (day 1 of differentiation), 100 ng/ml recombinant human BMP2 (available from R&D Systems, Inc., Minneapolis, Minnesota) was added into the medium for a period of 21 days with media change every alternate day.

[0129] **Neural Crest Lineage:** Neural crest differentiation of human RPESC was induced with DMEM/F12 medium with N2 basal medium supplemented with 2mM L-glutamine, 1% penicillin/streptomycin (Gibco-Invitrogen), 10 ng/ml recombinant human FGF2 (Invitrogen) and 20 ng/ml recombinant human BMP2 (R&D Systems) for a period of 8 days with media change every alternate day.

[0130] **Dopaminergic Neurons:** RPESCs were plated in non-adherent dishes in serum-free KSR medium supplemented with FGF2. After 2 days the medium was replaced and SHH/FGF8 was added for an additional 5 to 7 days (*See Fig. 4 for a time line of neural induction*).

### *Immunostaining*

[0131] Cultured RPE cell monolayers, RPESC spheres, or differentiated RPESCs were fixed for 10 min at room temperature in 4 % (w/v) paraformaldehyde in PBS. RPESC spheres were embedded in OTC (Sakura Finetek) and sectioned (12-16 µm) on a cryostat (Leica CM-3050). Cell cultures were permeabilized and blocked in PBS with 0.1 % (v/v) Triton and 10 % (v/v) normal goat serum for 1 hr at room temperature prior to staining with a primary antibody for 1 hr at room temperature or overnight at 4°C (*See Table 1 for description of each primary antibody and its usage*). Cells were washed three times (for 15 min/wash) with PBS with 0.05 % (v/v) Triton, followed by incubation with the secondary antibody for 45 min at room temperature. Nuclei were counter-stained with Hoechst 33342 (Molecular Probes). For immunohistochemical analysis of RPE adult human eyes, frozen sections were fixed and processed as described. De *et al.*, *Arch. Ophthalmol.* (2007) 125:641-645. Secondary antibody staining was done using the corresponding goat anti-rabbit IgG Alexa Fluor 488, goat anti-mouse IgG Alexa Fluor 546, goat

anti-mouse IgM Alexa Fluor 546 (Molecular Probes) and Rhodamine (Jackson ImmunoResearch). Phase and fluorescent images were taken with a Zeiss Axiovert 200 inverted microscope and a Zeiss AxioCam MRm digital camera with AxioVision 4 software.

**Table 1: Antibodies for Immunocytochemistry Analysis**

Cell Type	Antibody	Dilution	Isotype	Company
Stem cell Marker	SSEA-4	1:20	Mouse IgG3	Chemicon <sup>(1)</sup>
Stem cell Marker	Nanog	1:20	Goat IgG	R&D System <sup>(2)</sup>
Stem cell Marker	Oct-4	1:100	Rabbit IgG	Santa Cruz Lab <sup>(3)</sup>
RPE Marker	Cralbp (UW55)	1:1000	Rabbit IgG1	See Stecher <i>et al.</i> <sup>(4)</sup>
RPE Marker	RPE65	1:200	Rabbit IgG1	See Hamel <i>et al.</i> <sup>(5)</sup>
RPE Marker	Mitf	1:50	Mouse IgG1	NeoMarkers <sup>(6)</sup>
RPE Marker	PDEF	1:50	Mouse IgG2a	Chemicon
RPE, Epithelial Marker	ZO-1	1:100	Rabbit IgG1	Zymed <sup>(7)</sup>
RPE, Epithelial Marker	Cytokeratin	1:800	Mouse IgG1	Sigma <sup>(8)</sup>
RPE Marker	Bestrophin	1:100	Mouse IgG1	Novus Biologicals <sup>(9)</sup>
RPE, Retina Progenitor, Amacrine cells	Pax6	1:25	Mouse IgG1	Hybridoma Bank <sup>(10)</sup>
RPE, Retina Progenitor Marker	Sox2	1:1000	Rabbit IgG1	Chemicon
Pre-Neural Marker	Nestin	1:200	Mouse IgG1	Chemicon
Neural Progenitor	Olig2	1:50	Rabbit IgG1	See Ligon <i>et al.</i> <sup>(21)</sup>
Neural Progenitor	Otx2	1:1000	Rabbit IgG1	Chemicon
Neural Marker	$\beta$ -Tubullin III (Tuj1)	1:1000	Mouse IgG2b	Covance <sup>(11)</sup>
Neural Marker	Neurofilament 150DK	1:200	Rabbit IgG1	Chemicon
Rod Photoreceptor Marker	Rhodopsin Rho 1D4	1:100	Mouse IgG1	Chemicon
Rod Photoreceptor Marker	Rom1C	1:300	Rabbit IgG1	See Mata <i>et al.</i> <sup>(12)</sup>
Rod Photoreceptor Marker	Math5	1:500	Rabbit IgG1	Chemicon
Rod Photoreceptor, Bipolar cells Marker	Recoverin	1:500	Rabbit IgG1	Chemicon
Amacrine cell Marker	NeuroD	1:1000	Rabbit IgG1	Chemicon
Amacrine cell Marker	Syntaxin	1:200	Mouse IgG1	MBL <sup>(13)</sup>
Amacrine cell, Horizontal cell Marker	Calbindin 28KD	1:2500	Rabbit IgG1	Swant <sup>(14)</sup>
Horizontal cell Marker	Lim1	1:200	Rabbit IgG1	Novus Biologicals
Horizontal cell Marker	NF150 KD	1:200	Rabbit IgG1	Chemicon
Horizontal cell, Bipolar cell, Amacrine cell Marker	Prox1	1:5000	Rabbit IgG1	Covance
Bipolar cell Marker	PKC $\alpha$	1:50	Rabbit IgG1	Cell Signaling <sup>(15)</sup>
Ganglion cell Marker	Math5	1:1000	Rabbit IgG1	Chemicon
Glia cell Marker	GFAP	1:1000	Rabbit IgG1	Dako <sup>(16)</sup>
Hepatic cell Marker	$\alpha$ 1 fetoprotein (AFP)	1:500	Rabbit IgG1	Dako
Smooth muscle cell Marker	$\alpha$ smooth muscle actin	1:500	Mouse IgG2	Dako
Skeletal muscle cell Marker	$\alpha$ -actinin (sarcomeric)	1:800	Mouse IgG1	Sigma
Striated muscle cell Marker	Light Meromyosin (MF20)	1:20	Mouse IgG2b	Hybridoma Bank

**Table 1: Antibodies for Immunocytochemistry Analysis**

Cell Type	Antibody	Dilution	Isotype	Company
Bone cell Marker	BMP-4	1:100	Mouse IgG2b	Chemicon
Nuclear Marker	Human Nuclei (HuNu)	1:300	Mouse IgG1	Chemicon
Nuclear Marker	DAPI	1:1000	N/A	Molecular Probes <sup>(17)</sup>
Dopaminergic Neuron Marker	anti-Tyrosine hydroxylase	1:100		Pel-Freez Biologicals <sup>(18)</sup>
	$\beta$ -Tubulin I	1:1000		Covance
Cardiac cell marker	Cardiac Troponin	1:20	Mouse IgG2a	Hybridoma Bank
Bone cell marker	Osteopontin	1:50	Mouse IgG2b	Hybridoma Bank
Cartilage marker	Collagen II	1:40	Mouse IgG1	Chemicon
Neural crest marker	Sox10	1:200	Rabbit IgG	Affinity BioReagents <sup>(19)</sup>
Neural crest marker	Sox9	1:50	Rabbit IgG	Chemicon
Mesenchymal marker, endothelial marker	Vimentin	1:100	Mouse IgG2a	Dako
Adipocyte marker	FABP4	1:100	Rabbit IgG	Cayman Chemicals <sup>(20)</sup>

Abbreviations: <sup>(1)</sup> Chemicon® a subsidiary of Millipore, Billerica, Maryland; <sup>(2)</sup> R&D Systems, Inc., Minneapolis, Minnesota; <sup>(3)</sup> Santa Cruz Biotechnology, Inc., Santa Cruz, California; <sup>(4)</sup> Stecher *et al.*, *J. Biol. Chem.* (1999) 247:8577-8585; <sup>(5)</sup> Hamel *et al.*, *J. Biol. Chem.* (1993) 268:15751-15757; <sup>(6)</sup> NeoMarkers, Inc., Fremont, California; <sup>(7)</sup> Zymed Laboratories, Inc., South San Francisco, California; <sup>(8)</sup> Sigma is a subsidiary of Sigma-Aldrich Corporation, St. Louis, Missouri; <sup>(9)</sup> Novus Biologicals LLC, Littleton, Colorado; <sup>(10)</sup> Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa; <sup>(11)</sup> Covance, Inc., Princeton, New Jersey; <sup>(12)</sup> Mata *et al.*, *Neuron* (2002) 36:69-80; <sup>(13)</sup> MBL International Corporation, Woodburn, Massachusetts; <sup>(14)</sup> Swant, Bellinzona, Switzerland; <sup>(15)</sup> Cell Signaling Technology, Danvers, Massachusetts; <sup>(16)</sup> Dako Denmark A/S, Glostrup, Denmark; <sup>(17)</sup> Molecular Probes, Inc., Eugene, Oregon; <sup>(18)</sup> Pel-Freez® Biologicals, Rogers, Arkansas; <sup>(19)</sup> Affinity Bioreagents, Inc. is a subsidiary of Thermo-Fisher Scientific, Rockford, Illinois; <sup>(20)</sup> Cayman Chemical Company, Ann Arbor, Michigan; <sup>(21)</sup> Ligon *et al.*, *J. Neuropathol. Exp. Neurol.* (2004) 63:499-509.

### *In vivo transplantation*

[0132] Human RPE cells were injected ( $1 \times 10^6$  cells/CAM in 50  $\mu$ l of PBS plus  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) onto the CAMs of 9–10 day old White Leghorn chick embryos. Eggs were incubated for 7 days post-transplantation. CAMs with human RPE cells were fixed in 4 % paraformaldehyde and cryosectioned for immunohistochemical analysis.

### *Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)*

[0133] Total RNA was extracted using an RNeasy mini kit (QIAGEN) from acutely isolated RPE and retina tissue derived from adult human donors; cultured RPE spheres, cultured RPE monolayers, and differentiated RPESC cells. cDNAs were synthesized from 50 ng of total RNA by SuperScript II Reverse Transcriptase (RT) (Invitrogen) primed with oligo (dT)12–18 according to the manufacturer's instructions. The amount of cDNA was normalized to actin

mRNA. The primer sequences, annealing temperatures, and the number of cycles are shown in Table 2.

[0134] For more sensitive, quantitative, real-time RT-PCR, RNA was reverse-transcribed using a SuperScript™ First-Strand Synthesis System (Invitrogen) to synthesize cDNA, following the manufacturer's instructions. Real-time RT-PCR was carried out on an Applied Biosystems 7900 instrument in 20 µl reaction volumes containing 10 µl of SYBR® Green PCR mix (available from Applied Biosystems, Foster City, California), 0.375 µM of each primer, and diluted cDNA. All primer pairs used were confirmed to approximately double the amount of product within one PCR cycle and to yield a single product of the predicted size. Primer sequences used are provided in Table 3, below. Relative mRNA levels were calculated using the comparative CT method described in the Applied Biosystems instruction manual, and presented either as percentage of housekeeping gene expression or as a percentage of biological controls. In all cases, results were essentially independent of the gene used for normalization (Gapdh).

**Table 2: Primer Sequences Used for Reverse Transcription-Polymerase Chain Reaction Analysis**

Gene Target (GenBank No)	Primer Sequences (5'→3')	SEQ ID NO.	Product Size (bp)	Annealing Temp (°C)	Cycles
h NANOG (NM_024865)	CAGAAGGCCTCAGCACCTAC	1	298	62°C	40x
	GTCAGTGGCAGGAGAATTTGG	2			
h Oct4 (NM_002701)	CTTGCTGCAGAAGTGGGTGGAGGAA	3	187	65°C	35x
	CTGCAGTGTGGGTTTCGGGCA	4			
h c-MYC (NM_002467)	ACTCTGAGGAGGAACAAGAA	5	158	60°C	35x
	TGGAGACGTGGCACCTCTT	6			
h KLF4 (NM_004235)	TCTCAAGGCACACCTGCGAA	7	104	60°C	35x
	TAGTGCCTGGTCAGTTCATC	8			
h NESTIN (NM_006617)	AGGATGTGGAGGTAGTGAGA	9	266	60°C	40x
	TGGAGATCTCAGTGGCTCTT	10			
h MUSASHI (NM_002442)	GGCAGACTACGCAGGAAGG	11	291	58°C	40x
	TTCACGTCCTCCACCGTG	12			
h TUJ1 (NM_006086)	CATGGACAGTGTCCGCTCAG	13	175	60°C	35x
	CAGGCAGTCGCAGTTTTCA	14			
h SOX2 (NM_003106)	GGCAGCTACAGCATGATGCAG	15	396	66°C	35x
	GCTCTGGTAGTGCTGGGACATG	16			
h Pax6 (NM_001604)	TCCATCAGTTCCAACGGAGAAG	17	337	62°C	35x
	GTGGAATTGGTTGGTAGACACTG	18			
h Otx2 (NM_021728)	CCATGACCTATACTCAGGCTTCAGG	19	211	62°C	35x
	GAAGCTCCATATCCCTGGGTGGAAAG	20			
h NEUROD1 (NM_002500)	CGCTGGAGCCCTTCTTTG	21	118	58°C	35x
	GCGGACGGTTCGTGTTTG	22			
h CRX	ATGATGGCGTATATGAACCC	23	263	60°C	35x



**Table 2: Primer Sequences Used for Reverse Transcription-Polymerase Chain Reaction Analysis**

Gene Target (GenBank No)	Primer Sequences (5'→3')	SEQ ID NO.	Product Size (bp)	Annealing	
				Temp (°C)	Cycles
(NM_000554)	TCTTGAACCAAACCTGAACC	24			
h ATHO7 (Math5) (NM_145178)	TCGCATCATCAGACCTATGG CCGAACAGGACAAACTCACA	25 26	245	60°C	40x
h PEDF (NM_002615)	GGA CGC TGG ATT AGA AGG CAG TTG TAT GCA TTG AAA CCT TAC AGG	27 28	1472	65°C	35x
h RECOVERIN (NM_002903)	CCAGAGCATCTACGCCAAGT CACGTCGTAGAGGGAGAAGG	29 30	186	60°C	40x
h RHODOPSIN (NM_000539)	TCATCATGGTCATCGCTTTC CATGAAGATGGGACCGAAGT	31 32	100	60°C	40x
h S-OPSIN (NM_001708)	GATGAATCCGACACATGCAG CTGTTGCAAACAGGCCAATA	33 34	103	55°C	40x
h CHX10 (NM_182894.1)	AGCTAGAGGAGCTGGAGAAG CATGATGCCATCCTTGGCTG	35 36	259	57°C	35x
h MASH1 (NM_004316)	CGGCCAACAAGAAGATGAGT GCCATGGAGTTCAAGTCGTT	37 38	168	60°C	35x
h RPE65 (NM_000329)	CCTTCTTCATGGAGTCTTTG ATTGCAGTGGCAGTTGTATTG	39 40	390	52°C	35x
h VEGF (NM_001025366)	TTGCCTTGCTGCTCTACCTC AAATGCTTCTCCGCTCTGA	41 42	547	65°C	35x
h CRALBP (NM_000326)	ATGTCAGAAGGGGTGGG TCAGAAGGCTGTGTTCTCA	43 44	953	60°C	40x
h BESTROPHIN (NM_004183)	GGCCAGATCTATGTACTGGAATAAGCCCGAGC GGCCCTCGAGTTAGGAATGTGCTTCATCCCTG	45 46	773	65°C	40x
h SIX3 (NM_005413)	GGAATGTGATGTATGATAGCC TGATTTCGTTTGTCTGG	47 48	139	52°C	35x
h AFP (NM_001134)	TTGCTGCAAAGCTGAAAATG GCAGCATTCTGTTATTTTGTGTTGAC	49 50	337	54°C	35x
h BRACHYURY (NM_003181)	TGCTTCCCTGAGACCCAGTT GATCACTTCTTTCCTTTGCATCAAG	51 52	120	54°C	35x
h MESP1 (NM_018670)	CTCGTCTCGTCCCAGACTCAT AGTTTCTCCCGCTCACTGGC	53 54	172	60°C	35x
h BMP-4 (NM_130851)	CATGCTAGTTTGATACCTGAGACG CTGAGGTTAAAGAGGAAAACGAAAAG	55 56	376	54°C	35x
h RUNX2 (NM_004348)	TCTGGCCTTCCACTCTCAGT GACTGGCGGGGTGTAAGTAA	57 58	161	54°C	35x
h BSP (NM_004967)	CGGAGGAGACAATGGAGAAG GACGCCCGTGTATTCGTA	59 60	226	54°C	35x
h OPN (BC022844)	TGAATCTGATGAACTGGTCACTGA GGTGATGTCCTCGTCTGTAGC	61 62	190	54°C	35x
h COL I (NM000088)	AGACACTGGTGCTAAGGGAGAG GACCAGCAACACCATCTGCG	63 64	182	54°C	35x
h αSMA (NM_001102)	GTGCAGGAGAAGTGCCAGCT GAGGGTGGCGGTCTCATAGT	65 66	300	62°C	35x
h PPARγ (NM_015869)	CTCCTATTGACCCAGAAAGC GTAGAGCTGAGTCTTCTCAG	67 68	350	57°C	35x
h GAPDH (NM_002046)	CCCCTTCATTGACCTCAACTACA TTGCTGATGATCTTGAGGCTGT	69 70	342	60°C	35x

**Table 2: Primer Sequences Used for Reverse Transcription-Polymerase Chain Reaction Analysis**

Gene Target (GenBank No)	Primer Sequences (5'→3')	SEQ ID NO.	Product Size (bp)	Annealing	
				Temp (°C)	Cycles
h TFAP2A (NM_001032280)	TCCCTGTCCAAGTCCAACAGCAAT	71	396	52°C	35x
	AAATTCGGTTTCGCACACGTACCC	72			
h ERBB3 (NM_001982)	GGTGCTGGGCTTGCTTTT	73	365	57°C	35x
	CGTGGCTGGAGTTGGTGTTA	74			
h PAX3 (NM_181459)	GCACTGTACACCAAAGCACG	75	349	57°C	35x
	TAGGTGGGTGGACAGTAGGA	76			
h SNAI2 (NM_003068)	AGCGAACTGGACACACATAC	77	410	57°C	35x
	TCTAGACTGGGCATCGCAG	78			
h SNAI1 (NM_005985)	CTCCTCTACTTCAGCCTCTT	79	611	52°C	35x
	CTTCATCAAAGTCCTGTGGG	80			
h SOX10 (NM_006941)	ATACGACACTGTCCCGGCCCTAAA	81	250	62°C	35x
	TTCTCCTCTGTCCAGCCTGTTCTC	82			
h PAX7 (NM_013945)	CAGGAGACCGGGTCCATC	83	216	58°C	35x
	CGAACTTGATTCTGAGCAGC	84			
h Actin	TGCGTGACATTAAGGAGAAG	85	258	52°C	35x
	TGAAGGTAGTTTCGTGGATG	86			
h EN1	CCGCACCACCAACTTTTTCAT	87	171	60°C	35x
	TGGACAGGGTCTCTACCTGC	88			
m EN1	TCAAGACTGACTCACAGCAACCCC	89	376	60°C	35x
	CTTTGTCCTGAACCGTGGTGGTAG	90			
h EN2	ATCCCCTAAGCTCCAT	91	431	57°C	35x
	AGGAGGGAGTTAGGTG	92			
m EN2	CTTCTTCAGGTCCCAGGTCC	93	136	58°C	35x
	CTCTGTCAGGTACCTGTTGG	94			
h TH	GAGTACACCGCCGAGGAGATTG	95	278	62°C	35x
	GCGGATATACTGGGTGCACTGG	96			
m TH	TCCTGCACTCCCTGTCAGAG	97	423	60°C	35x
	CCAAGAGCAGCCCATCAAAGG	98			
h Nurr1	TTCTCCTTTAAGCAATCGCCC	99	332	60°C	35x
	AAGCCTTTGCAGCCCTCACAG	100			
m Nurr1	CTGGCTATGGTCACAGAGAG	101	132	58°C	35x
	ACAGGTAGTTGGGTCCGGTTC	102			
h Pax2	ATGTTCCGCTGGGAGATTCCG	103	429	58°C	35x
	GCAAGTGCTTCCGCAAAGT	104			
m Pax2	CCAAAGTGGTGGACAAGATTGCC	105	544	60°C	35x
	GGGATAGGAAGGACGCTCAAAGAC	106			
h Sox1	CAATGCGGGGAGGAGAAGTC	107	464	60°C	35x
	CTCTGGACCAAAGTGTGGCG	108			
m Sox1	CCTCGGATCTCTGGTCAAGT	109	593	58°C	35x
	TACAGAGCCGGCAGTCATAC	110			
h Ptx3	ACTAGGCCCTACACAC	111	160	55°C	35x
	TTTTTTTGACAGTCCGC	112			
m Ptx3	AGGACGGCTCTCTGAAGAA	113	372	60°C	35x
	TTGACCGAGTTGAAGGCGAA	114			
m Tuj1	TCAGCGATGAGCACGGCATA	115	300	58°C	35x
	CACTCTTCCGCACGACATC	116			
m Nextin	GGAGAGTCGCTTAGAGGTGC	117	326	58°C	35x

**Table 2: Primer Sequences Used for Reverse Transcription-Polymerase Chain Reaction Analysis**

Gene Target (GenBank No)	Primer Sequences (5'→3')	SEQ ID NO.	Product Size (bp)	Annealing Temp (°C)	Cycles
	TCAGGAAAGCCAAGAGAAGC	118			

Abbreviations: Tuj1,  $\beta$ -tubulin III; CRALBP, cellular retinaldehyde binding protein; PEDF, pigment epithelium derived factor; VEGF, vascular endothelial growth factor; PPAR $\gamma$ , peroxisome proliferative activated receptor gamma; GAPDH, glyceraldehyd-3-phosphate dehydrogenase;  $\alpha$ SMA, alpha smooth muscle actinin; BSP, bone sialoprotein; AFP,  $\alpha$ -fetoprotein; bp, base pair; h, human; m, murine.

**Table 3: Primer Sequences Used for Quantitative Real-Time RT-PCR Analysis**

Gene Target (GenBank No)	Primer Sequences (5'→3')	SEQ ID NO.	Product Size (bp)	Annealing Temp	Cycles
Human qOct4 (NM_002701)	AGCGAACCAGTATCGAGAAC	119	187	60.6°C	40x
	TTACAGAACCACACTCGGAC	120			
Human qSox2 (NM_003106)	AGCTACAGCATGATGCAGGA	121	130	60.0°C	40x
	GGTCATGGAGTTGTAAGCA	122			
Human qNanog (NM_024865)	TGAACCTCAGCTACAAACAG	123	124	60.7°C	40x
	TGGTGGTAGGAAGAGTAAAG	124			
Human qMYC NM_002467)	ACTCTGAGGAGGAACAAGAA	125	158	60.3°C	40x
	TGGAGACGTGGCACCTCTT	126			
Human qKLF4 (NM_004235)	TCTCAAGGCACACCTGCGAA	127	104	60.1°C	40x
	TAGTGCCTGGTCAGTTCATC	128			
Human qLIN28 (NM_024674)	AGGCGGTGGAGTTCACCTTTAAGA	129	189	60.1°C	40x
	AGCTTGCATTCCCTTGGCATGATGG	130			
Human qGAPDH (NM_002046)	CCCCTTCATTGACCTCAACTACA	131	154	60.1°C	40x
	TTGCTGATGATCTTGAGGCTGT	132			

### (A) Isolation and Characterization of Retinal Pigment Epithelial Stem Cells (RPESC)

[0135] To determine whether human RPE contains stem/progenitor cells, RPE was isolated from human adult eye donors, dissociated to single cells and cultured in non-adherent conditions permissive for floating, multi-cell neurosphere formation (Fig. 2). Reynolds & Rietze, *Nat. Methods* (2005) 2:333-336. About 5-10 % of cells proliferated to form small sphere colonies composed of darkly pigmented cells which after 7 days were larger and included nonpigmented cells.

[0136] To demonstrate that these floating spheres were RPE progenitor cells, they were plated in conditions typically used to culture adult human RPE cells. The floating sphere colonies were collected and transferred to fibronectin/laminin coated plates where they attached and proliferated further. After 1-2 weeks they formed contact-inhibited monolayers of cells with

an epithelial or fusiform morphology that were lightly pigmented, typical of cultured RPE. Burke *et al.*, *Exp. Eye Res.* (1996) 62:63-73. They expressed an array of characteristic RPE markers, including RPE65, Mitf, Pax6, Cralbp, Otx2 and Bestrophin. Martínez-Morales *et al.*, *Bioessays* (2004) 26:766-777; Ohno-Matsui *et al.*, *Mol. Vis.* (2005) 11:1-10. ARPE-19 cells, a cell line derived from RPE cells [Dunn *et al.*, *Exp. Eye Res.* (1996) 62:155-169], were used as a positive control.

### **(B) Plasticity**

[0137] The plasticity of the cultured human RPESCs was tested. Starting with either adherent or sphere-cultured RPE cells, the cells were dissociated to single cells and the resulting cell suspension was replated into KSR medium, which is formulated to grow embryonic stem (ES) cells, in non-adherent conditions. These passaged RPE cells created growing sphere colonies (henceforth called RPESC spheres) that were fixed at 7 days and sectioned for immunohistochemistry. The RPESC sphere cells expressed the neural progenitor marker Nestin which RPE cells do not normally express *in vivo*, implying that they had acquired a neural progenitor fate. Some of the cells expressed Pax6, which is a master regulator of eye development. Pax6 is downregulated early during RPE generation and is not normally expressed in adult human RPE; however, some Pax6<sup>+</sup> cells in RPE cultures were observed.

### **(C) Markers and Phenotypes**

[0138] It was also found that RPESCs showed immunoexpression of markers characteristic of ES cells (Wang *et al.*, *Nature* (2006) 444:364-368; Carpenter *et al.*, *Cloning Stem Cells* (2003) 5:79-88): SSEA-4 and Sox2, which is expressed by both ES cells and neural progenitor cells. Sox2 expression was predominantly cytoplasmic in some cells, though it may be noted that Sox2 can be cytoplasmic in preimplantation embryos and in developing lens fiber cells. Avilion *et al.*, *Cloning Stem Cells* (2003) 5:79-88; Hever *et al.*, *Clin. Genet.* (2006) 69:459-470. Western blot analysis of normal RPE tissue samples showed that the RPE tissue expresses c-Myc, but does not express Oct4, Nanog, Sox2 or KLF4. RPESCs, however, do express Sox2, KLF4 and c-Myc, but do not express significant amounts of either Oct4 or Nanog.

[0139] Quantitative PCR analysis confirmed that Oct4 is not expressed in RPESCs, in RPE primary cells, in RPE tissue, or in retina tissue. However, a very low expression of both Nanog and Lin28 was detected in retina tissue. Low expression of Sox2 was detected in RPESCs, in RPE primary cells, and in RPE tissue. Slightly higher Sox2 expression levels were detected in retina tissue. Higher expression levels of c-Myc and KLF4 were detected in RPESCs, in RPE primary cells and in RPE tissue compared to the expression of those markers in ES cells. However, no expression of those genes was detected in retina tissue. The marker CHX10 was detected in retina tissue by PCR, but was not detected in either RPESC or in normal RPE cells.

[0140] Hence, RPESC cells can express three out of five stem cell genes shown to be necessary for cell reprogramming. Takahashi *et al.*, *Cell* (2007) 131:861-872; Maherali *et al.*, *Cell Stem Cell* (2007) 1:55-70. The fact that RPE cells can be readily differentiated from huES cells (Klimanskaya *et al.*, *Cloning Stem Cells* (2004) 6:217-245) demonstrates that there is a close relationship between these two cell types, and may reflect the very early ontogeny of RPE *in vivo*.

#### **(D) Tests for differentiation potential**

[0141] Human RPESCs were tested for their retinal differentiation potential. RPESC spheres were transferred into fresh neural differentiation medium supplemented with FGF2, FGF8, SHH and RA, conditions shown to promote retinal differentiation from ES cells (Osakada *et al.*, *Nat. Biotechnol.* (2008) 26:215-224), then plated onto fibronectin/laminin coated plates in the same medium. After 3-4 days, spheres had begun to flatten and the majority of the cells expressed Nestin, Sox2 and Olig2, a neural retina progenitor marker. RT-PCR at this stage also showed expression of mRNA for neural progenitor markers Nestin, Musashi, Sox2 and Pax6, but no expression of markers of postmitotic retinal cells, including Math5, Recoverin, Rhodopsin, Crx, PKC- $\alpha$ , NeuroD and Prox1.

#### **(E) Marker Expression**

[0142] After a further 2-3 weeks of differentiation, the cells had acquired a variety of morphologies that, while not being typical of neurons, were clearly no longer RPE-like. The cells now abundantly expressed the neuronal marker TuJ1 and cell-type specific markers for a variety

of postmitotic retinal neurons, including Recoverin, Rom1c, Rho1D4, PKC $\alpha$ , Pax6, Syntaxin, Prox1, Lim1, Calbindin, Neurofilament-M (NF150 $\alpha$ ) and Math5. Only rare cells (<2 %) expressed GFAP, a retinal Müller glial cell marker. GFAP, Tuj1, and these other neural retinal markers are not normally expressed in RPE cells *in vivo*, demonstrating the plasticity of these cells after this culture regime. RT-PCR confirmed expression of markers for postmitotic retinal neurons. Notably, expression of the retinal marker CHX10 was induced after culturing the RPESC in retinal differentiation conditions as described here, even though CHX10 was not expressed in the original RPESC.

#### **(F) Test for Multipotency**

[0143] RPESC cells were further tested to determine if they are more broadly multipotent. Spheres were grown in culture conditions that are typically used to push ES cells into different lineages, corresponding to the mesoderm (myogenic, osteogenic, chondrogenic and adipogenic) endoderm (hepatic) lineages, and neural crest cells. Trounson, *Endocrine Reviews* (2006) 27:208-219. RPESC spheres were cultured in non-adherent conditions in each of the differentiation media tested, then after 4-5 days they were seeded onto gelatin coated plates in the same medium to promote differentiation for one to three weeks.

[0144] RPESCs that had been exposed to endoderm conditions, in medium supplemented with FGF2, BMP4, activin and ascorbic acid, after three weeks expressed the hepatic marker  $\alpha$ -fetoprotein (AFP). Expression of mRNA coding for AFP marker was also detected.

#### **(G) Myogenic Lineages**

[0145] RPESCs were induced to differentiate into myogenic lineages using medium supplemented with FGF2, BMP2 and BMP4. After one week of differentiation, early myogenic markers were observed by RT-PCR, including Brachyury and Mesp1. After 3 weeks of differentiation, RPE cells adopted myofibroblast morphologies. Cells in these cultures stained positive for the smooth muscle marker  $\alpha$ -SMA, which is not expressed in RPE cultures, and for the skeletal muscle marker sarcomeric  $\alpha$ -actinin, and cells were occasionally positive for cardiac troponin marker cardiomyocyte marker MHC (MF20) but there was no detectable expression of NKx2.5, Myh7, Troponin 1 and Myf5.

**(H) Adipocytic, Osteogenic and Chondrogenic Lineages**

[0146] RPESC were also shown to be capable of adipocytic differentiation. After culturing in medium supplemented with isobutylxanthine, dexamethason and insulin the cells accumulated lipid droplets, which were detected by staining with Oil Red fluorescent dye. Cells in these cultures also stained positive with the lipid fluorescence marker HCS LipidTox™ (available from Molecular Probes, Inc., Eugene, Oregon), and for fatty acid binding protein 4 (FABP4), an adipocyte marker. Osteogenic differentiation was induced with medium supplemented with dexamethasone,  $\beta$ -glicerophosphate and ascorbic acid-2-phosphate. After 3 weeks of differentiation, RPESCs expressed the osteogenic markers BMP4 and osteopontin. Mineral deposition was also detected in these cells by Alizarin red staining. Expression of mRNA coding for osteogenic markers BMP4, BSP, Rnx2, Opn and Collagenase 1 was detected. Finally, after chondrogenic differentiation RPESC showed glycosaminoglycan accumulation, detected by staining with Alcian Blue dye, and also expressed the chondrogenic marker Collagen II. The meso, endo or osteo markers were not found in RPE cells cultured in serum-containing medium.

**(I) Neural Crest Lineage**

[0147] RPESC were also induced to differentiate in mesenchymal cell-like and neural crest cells using medium supplemented with FGF2 and BMP2. After one week of differentiation, neural crest markers were observed by RT-PCT, including Pax7, Pax3, Sox10, ErbB3 and TFAP2A, as well as SNAI1 and SNAI2, which regulate changes in gene expression patterns underlying epithelial-mesenchymal transition (EMT). Cells were stained for the mesenchymal marker vimentin and for neural crest markers Sox9 and Sox10.

**(J) Dopaminergic Neurons**

[0148] In human, melanin pigment is biosynthesized in the neuroectodermic RPE and neural crest-derived melanocytes. Melanins in RPE are mainly eumelanin. Melanin-synthesizing cells contain specific organelles, the premelanosomes, in which glycoproteinic transmembrane tyrosinase catalyzes melanin biosynthesis. Enzymes involved in eumelanin biosynthesis include tyrosinase (TYR), tyrosinase-related-protein-1 (TRP1) and tyrosinase-related-protein-2 (TRP2)

[Lu *et al.*, (2007) *Mol. Vis.* 13:2066-2072]. Tyrosinase is the rate limiting enzyme of melanin biosynthesis and catalyses the first two steps of melanin synthesis: hydroxylation of L-tyrosine to L-3, 4-dihydroxyphenylalanine (L-DOPA), and the oxidation of L-DOPA to DOPAquinone and the oxidation of 5,6-dihydroxyindole to 5,6dihydroxyquinone (Fig. 3) [Ando *et al.*, (2007) *J. Invest. Dermatol.* 127:751-761]. It is assumed that RPE melanogenesis is only found prenatally. However, tyrosinase activity has been found in adult cultured human RPE cells. TYR gene can be upregulated in response to different growth factors such as pigment epithelium-derived factor (PEDF), FGF2,  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH), L-tyrosine, verapamil, cholera toxin or phagocytosis of rod outer segments (ROS) by RPE cells *in vitro* [Julien *et al.*, (2007) *Graefes Arch. Clin. Exp. Ophthalmol.* 245:1495-1505]. Little is known about the expression of TYR, TPR1 and TPR2 genes involved in melanin biosynthesis in cultured RPE cells.

[0149] The concept of cell replacement to compensate for cell loss and restore functionality has entered several disease entities including neurodegenerative disorders. Parkinson's Disease (PD) is the most common neurodegenerative movement disorder [Forman *et al.*, (2004) *Nat. Med.* 10:1055-1063] and is characterized by degeneration of the dopaminergic neurons in the substantia nigra pars compacta, accompanied by decreases in striatal dopamine (DA) and the appearance of intracytoplasmic Lewy body inclusions. Once striatal DA loss reaches the 80% critical value [Hornykiewicz *et al.*, (2001) *J. Chem. Neuroanat.* 22:3-12], a progressive motor impairment develops that is characterized by resting tremor, rigidity, bradykinesia, hypokinesia, and postural instability [Calne *et al.*, (1992) *Ann. Neurol.* 32 *Suppl*:S125-127]. L-DOPA is produced as an intermediate in the eumelanin synthesis pathway, from the metabolism of tyrosine through the action of tyrosinase enzyme (Fig. 3) [Dryja *et al.*, (1978) *Invest. Ophthalmol. Vis. Sci.* 17:511-514; Smith *et al.*, (1998) *Exp. Eye. Res.* 66:403-410]. L-DOPA can be excreted by the cell or further metabolized into dopaquinone, the next step in melanin synthesis. RPE can differentiate into different cell types depending on the combination of growth factors. The data demonstrate that RPESC can differentiate into dopaminergic neurons and represent a model for cell replacement therapy for PD.

[0150] The RPESCs of the current invention can provide a constant source of dopamine replacement via the melanin synthetic pathway enzyme tyrosinase. The transplantation of RPE cells, from which RPESCs are isolated, in rat and monkey models of PD showed an alleviation



of symptoms; however, several months later the symptoms returned because the RPE cells eventually died in the striatum. In one experiment, RPESCs are optimized for the production of levodopa and neurotrophic factors in order to survive in the striatum while providing a neuroprotective effect.

[0151] The data demonstrate that RPESCs differentiate into dopaminergic neurons when cultured in the presence of the appropriate factors. These dopaminergic neurons have the capacity to provide a continuous source of dopamine and to provide neurotrophic support that can modify PD progression. The generation of ventral midbrain and hindbrain type neurons requires the ventralizing signal sonic hedgehog (SHH) in conjunction with factors that define anterior patterning (such as FGF8 or FGF4).

[0152] To differentiate RPESC, a time course strategy for neural induction was developed in order to monitor the expression of specific markers (Fig. 4). RPESC and murine embryonic stem ("mES") cells were plated separately in non-adherent dishes to form spheres in KSR medium supplemented with FGF2. After 2 days the medium was replaced and SHH/FGF8 was added for an additional 5 to 7 days.

[0153] The RPESC spheres and mES cells were then used for immunocytochemistry and RT-PCR analysis. RPESC spheres and mES cells demonstrated an increase in the expression of neuronal marker class III  $\beta$ -Tubulin (Tuj1) and Nestin as compared to controls after 5 days incubation with SHH/FGF8. TH was expressed in the control RPESC spheres and its expression was increased after 5 days of SHH/FGF8 treatment. However, TH expression was not observed in either control or SHH/FGF8 treated mES cells.

[0154] After 12 days in culture with SHH/FGF8, more than 90% of the RPESC and mES cells expressed the neural progenitor markers Sox2, Olig2 and Tuj1, indicating that a majority of the cells were neuronal. However, less than 2% of the RPESC and mES cells were positive for TH expression, indicating a longer incubation with SHH/FGF8 was necessary.

[0155] Gene expression analysis from day 0 to day 7 of differentiation showed that midbrain markers in both RPESC and mES cells were not expressed in control conditions (N2 medium without growth factors). However, some TH expression was observed in RPESC. After

7 days in the presence of SHH/FGF8, some differences in the expression of midbrain progenitor markers was observed. Pax2 expression was not found in RPE cells, En1 expression was not found in mES cells, and dopaminergic markers (Nurr1, TH and Ptx3) were found in RPE cells but only Nurr1 was found in mES cells. These data indicate that RPESCs have the capacity to express markers of midbrain dopaminergic neuron development after 7 days in the presence of SHH/FGF8. These markers were readily detected in the hRPE cells during this period but were not expressed in the mES cell.

[0156] The data further indicate that RPESCs have the capacity to express midbrain dopaminergic markers after 7-12 days of culture in the presence of SHH/FGF8.

[0157] The ability to generate dopaminergic neurons that continuously release dopamine is an important step toward exploring the potential of human RPESCs in preclinical models of Parkinson's disease.

[0158] In another experiment, the yield of cultured midbrain dopaminergic neurons is improved. The RPESCs are co-cultured with stromal cells (MS5) derived from bone marrow. These cells have been shown to promote neural differentiation in mouse, monkey and human ES cells. Cells in these structures express markers compatible with a neural plate identity and show extensive capacity for self-renewal.

[0159] In another experiment, RPESCs are differentiated into dopaminergic neurons using a protocol adapted from Perrier *et al.*, (2004) *Proc. Natl. Acad. Sci. USA* 101:12543-12548, in which the addition of factors such as ascorbic acid, BDNF, TGF  $\beta$ 3, and cAMP are added to the culture to improve the yield of dopaminergic neurons.

[0160] In yet another experiment, the expression of TYR, TPR-1 and TPR2 genes, which are involved in the eumelanin biosynthesis pathway, and the expression of key transcription factors such as Pax2, Pax5, and En1, during neural induction and in committed dopaminergic precursors is determined by qPCR and immunofluorescence microscopy. Furthermore, the markers for dopaminergic neurons such as MAP2, AADC, VMAT2 are used to characterize the cells during the different stages of midbrain differentiation.

**(K) *In vivo* Characteristics**

[0161] The *in vivo* potential of human RPESCs was tested after transplantation into the chick chorioallantoic membrane (CAM), a highly vascularized tissue that supports the growth of teratomas. Auerbach *et al.*, *Int. J. Cancer* (1975) 15:241-245. The CAM is widely accepted to be an advantageous *in vivo* model for studying cancer, development, ophthalmology, and other research related to the retina [See Leng *et al.* 2004 24: (3); 427-434]. RPESC spheres were grafted into the CAM of H&H stage 22-34 chick embryos. After 7 days the RPE cells had formed a mass that was clearly vascularized. Immunostaining using human-specific antibodies show cells within the mass expressing Nestin, AFP, and  $\alpha$ SMA, indicating multi-lineage differentiation.

[0162] RPESC were also transplanted under the skin of nude mice. Notably, these transplanted RPESC did not form tumors, and no evidence of tumor formation was observed. Other stem cells, including embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC), readily form tumors in such transplantation experiments. Hence, RPESC of the invention may have a reduced capacity to form tumors *in vivo*, *e.g.*, in cell transplant therapies. As such, the RPESC provide a significant advantage over ESC, iPSC and other stem cells, since they may be less likely to form tumors in cell transplant therapies.

**(L) Screening Assays Using RPESC**

[0163] As explained above, RPESC of the invention are useful, *inter alia*, as models for a variety of diseases and disorders, including as *in vitro* (*e.g.*, cell culture based) models. The invention therefore encompasses the use of RPESC in such models, including cell cultures comprising RPESC and/or cells derived from RPESC. The invention also encompasses uses of such models, including cell-based screening assays that are based on these models.

[0164] Generally speaking, such assays involve culturing RPESC of the invention under conditions such that the cells exhibit one or more characteristics of a disease or disorder of interest. For example, RPESC may be cultured or grown under conditions in which they differentiate into either normal or pathological cells associated with a disease or disorder of interest, or into cells exhibiting one or more normal or pathological characteristics associated

with that disease or disorder. The RPESC, or the cells derived therefrom, may then be contacted with one or more test compounds, *e.g.*, by incubating or culturing the cells in the presence of the test compound. The cells should be contacted with the test compound under conditions and for a period of time sufficient to permit the test compound to modulate the characteristic or characteristics associated with the disease or disorder. Preferably, another preparation or culture of the RPESC (or of the cells derived therefrom) should be cultured under identical conditions but either in the absence of the test compound or, alternatively, in the presence of a suitable “placebo” compound that does not effect the characteristic or characteristics associated with the disease or disorder. The test compound’s effect on the characteristic or characteristics associated with the disease or disorder is then ascertained, typically by determining the status of the characteristic or characteristics in cells treated with the test compound, and comparing them to the characteristic or characteristics in cells that either were not treated with a compound or were treated with the placebo. A change of the characteristic or characteristics in cells treated with the test compound, compared to cells that have not been treated with the test compound (*e.g.*, in cells treated only with a placebo) indicates that the test compound may be useful, *e.g.*, as a therapeutic agent for treating or modulating one or more effects of the disease or disorder. Preferably the change or changes observed are ones associated with amelioration of the disease or disorder, indicating that the test compound may be useful as a therapeutic agent for treating or ameliorating the disease or disorder.

[0165] For example, in many preferred embodiments the characteristic or characteristics observed in such screening assays will be one or more genes or gene products, whose abnormal expression is associated with the disease or disorder of interest. Screening assays of the invention can then be used to determine whether a test compound increases, decreases or otherwise modulates expression of one or more of these genes or gene products. Preferably, the change will be a change whose nature or direction is associated with amelioration of the disease or disorder of interest; or it may be a change whose nature or direction is opposite to the pathological change associated with the disease or disorder. Thus, for example, in embodiments where the characteristic(s) include a gene or gene product whose expression is elevated in pathological cells compared to its expression in normal cells, a test compound identified in a screening assay may decrease expression of that gene or gene product in the cells treated with that compound. Conversely, in embodiments where the characteristic(s) include a gene or gene

product whose expression is decreased in pathological compared to normal cells, a test compound identified in the screening assay may increase expression of that gene or gene product in the treated cells.

[0166] As an example, and not by way of limitation, age-related macular degeneration (AMD) is associated with abnormally high accumulation of “drusen,” deposits of extracellular material, on the Bruch’s membrane beneath the RPE. Proteolytic analysis of drusen has indicated that certain proteins, in particular the protein  $\alpha$ B-crystallin, are present at high levels in drusen from the eyes of patients with AMD then in drusen from eyes of normal (non-AMD affected) donors. De *et al.*, *Arch. Ophthalmol.* (2007) 125:641-646. Hence, elevated expression of the  $\alpha$ B-crystallin gene and/or its gene product is one characteristic of AMD.

[0167] In certain preferred embodiments, therefore, RPESC, or cells derived therefrom, can be used to screen for compound to treat AMD by screening for compounds that modulate expression of the  $\alpha$ B-crystallin gene, or its gene product, in those cells. For example, RPESC of the invention may be cultured under suitable conditions, such as the conditions described by De *et al.*, *supra*, for culturing RPE cells. Alternatively, RPESC of the invention may be cultured under conditions that promote their differentiation into RPE or RPE-like cells (*see, e.g., supra* in these examples), which may then be cultured under conditions for culturing RPE cells. Preferably, the cultured cells are exposed to conditions that induce a pathological state or states associated with AMD and, in particular, increase expression of the  $\alpha$ B-crystallin gene or gene product. For example, the cells may be cultured or exposed to conditions of oxidative stress, such as exposing them to hydrogen peroxide ( $H_2O_2$ ), light (*e.g.*, blue or UV-light) and/or other agents of oxidative stress.

[0168] These cell cultures can then be contacted with one or more test compounds to determine if any of those compounds may be useful for treating AMD. In particular, a test compound that modulates expression of  $\alpha$ B-crystallin gene or gene product in these cells can be identified as a compound that is useful (or potentially useful) for treating AMD. In preferred embodiments, a test compound is identified as useful (or as potentially useful) if it decreases expression of the  $\alpha$ B-crystallin gene or gene product compared to expression in untreated or in placebo treated cells.

## Conclusion

[0169] The present invention describes a previously unappreciated plasticity in an adult neural cell that can be extracted from even aged human RPE. Thus, the RPESC represents a type of 'neotonous' progenitor cell, which because of its early ontogeny and arrested development retains remarkable plasticity. Removing this cell from its native environment into culture releases it from dormancy and it actively proliferates to produce a variety of multi-lineage progeny. Because RPE cultures can be greatly expanded, they offer a long term source of RPESCs. In contrast to induced pluripotent stem cells, RPESC cell plasticity does not require exogenous gene transduction. Park *et al.*, *Nature* (2008) 451:141-146; Nakagawa *et al.*, *Nat. Biotechnol.* (2008) 26:101-111; Yu *et al.*, *Science* (2007) 318:1917-1920. The ability to harvest RPE from patients indicates the possibility of generating patient-matched RPESC lines. In sum, the RPESC is a novel source of plastic adult human stem cells.

[0170] The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

[0171] While the compositions and methods of this invention have been described in terms of specific embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the scope of the invention as defined by the appended claims.

[0172] It is further to be understood that all values are approximate, and are provided for description.

[0173] Patents, patent applications, publications, product descriptions, GenBank<sup>®</sup> reference numbers, and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

**WHAT IS CLAIMED IS:**

1. A retinal pigment epithelial stem cell (RPESC) isolated from the retinal pigment epithelium (RPE) of a mammal, or an RPESC descended therefrom, that is self renewing and capable of differentiating into a plurality of cell types.
2. An RPESC according to claim 1 that expresses one or more markers characteristic of RPE.
3. An RPESC according to claim 2, wherein the markers characteristic of RPE are selected from the group consisting of RPE65, Mitf, Pax6, Cralbp, Otx2 and Bestrophin.
4. An RPESC according to claim 1 that expresses one or more markers characteristic of embryonic stem (ES) cells.
5. An RPESC according to claim 4, wherein the markers characteristic of ES cells are selected from the group consisting of SSEA-4, Sox2, KLF4 and c-Myc.
6. An RPESC according to claim 5 that expresses Sox2.
7. An RPESC according to claim 1 that does not express CHX10.
8. An RPESC according to claim 1 that is isolated from the RPE of a human.
9. An RPESC according to claim 1 that is transformed or transfected with a heterologous gene.
10. A retinal pigment epithelial stem cell (RPESC) that is self renewing and capable of differentiating into a plurality of cell types and expresses one or more markers selected from the group consisting of SSEA-4, Sox2, KLF4 and c-Myc.
11. An RPESC according to claim 10 that additionally expresses one or more markers characteristic of RPE.



12. An RPESC according to claim 11, wherein the markers characteristic of RPE are selected from the group consisting of RPE65, Mitf, Pax6, Cralbp, Otx2 and Bestrophin.
13. An RPESC according to claim 10 that does not express CHX10.
14. An RPESC according to claim 10 that is a human RPESC.
15. An RPESC according to claim 10 that is transformed or transfected with a heterologous gene.
16. An RPESC according to claim 1 or 10 that is capable of differentiating into a retinal progenitor cell, which retinal progenitor cell expresses one or more marker selected from the group consisting of Pax6, Nestin Musashi and Olig2.
17. An RPESC according to claim 16, which differentiates into the retinal progenitor cell in the presence of FGF2, FGF8 and RA.
18. An RPESC according to claim 1 or 10 that is capable of differentiating into a retinal neuron, which retinal neuron expresses one or more markers selected from the group consisting of Tuji, Recoverin, Rom1c, Rho1D4, PKC $\alpha$ , Pax6, Syntaxin, Prox1, Lim1, Calbindin, Neurofilament-M and Math5.
19. An RPESC according to claim 18, which retinal neuron expresses Tuj1.
20. An RPESC according to claim 1 or 10 that is capable of differentiating into a mesoderm lineage, an endoderm lineage or a neural crest cell.
21. An RPESC according to claim 20 that is capable of differentiating into a mesoderm lineage selected from the group consisting of a myogenic lineage, an osteogenic lineage, a chondrogenic lineage and an adipogenic lineage.
22. An RPESC according to claim 20 that is capable of differentiating into an endoderm lineage that is a hepatic lineage.

23. An RPESC according to claim 1 or 10 that is capable of differentiating into a dopaminergic neuron.
24. An RPESC according to claim 23, wherein the dopaminergic neuron expresses one or more markers selected from the group consisting of tyrosine hydroxylase (TH), Nestin and Tuj1.
25. An RPESC according to claim 24, wherein the dopaminergic neuron expresses TH.
26. A method for treating retinal disease, which method comprises administering, to the retina of a patient, an amount of a retinal pigment epithelial stem cells (RPESCs) effective for treating the retinal disease.
27. A method for treating retinal disease, which method comprises differentiating one or more retinal pigment epithelial stem cells (RPESCs) to obtain retinal cells.
28. A method according to claim 27, in which the RPESCs are differentiated by administering one or more exogenous factors suitable to differentiate endogenous RPESC of the patient into retinal cells.
29. A method according to claim 27, in which the RPESCs are differentiated *in vitro* to obtain retinal cells, and an amount of the retinal cells effective for treating the retinal disease is administered to the retina of the patient.
30. A method according to claim 29, in which the RPESCs are isolated from retinal pigment epithelium (RPE) of the patient.
31. A method according to claim 26 or 27, in which the RPESCs are isolated from the retinal pigment epithelium (RPE) of a mammal, or are descended from an RPESC isolated from the RPE of the mammal.

32. A method according to claim 26 or 27 in which the retinal disease is selected from the group consisting of age-related macular degeneration, retinitis pigmentosa and Leber's hereditary optic neuropathy.

33. A method for treating a neurodegenerative disease or disorder, which method comprises administering, to a patient, an amount of retinal pigment epithelial stem cells (RPESCs) effective for treating the neurodegenerative disease or disorder.

34. A method for treating a neurodegenerative disease or disorder, which method comprises steps of:

- (a) differentiating one or more retinal pigment epithelial stem cells (RPESCs) to obtain neuron cells; and
- (b) administering, to a patient, an amount of the neuron cells effective for treating the neurodegenerative disease or disorder.

35. A method according to claim 34, in which the neuronal cells are dopaminergic neuron cells.

36. A method according to claim 33 or 34, in which the neurodegenerative disease is Parkinson's disease.

37. A method according to claim 33 or 34, in which the RPESCs are isolated from the retinal pigment epithelium (RPE) of a mammal, or are descended from an RPESC isolated from the RPE of the mammal.

38. A method according to claim 37, in which the RPESCs are isolated from the RPE of the patient.

39. A method for identifying a compound to treat a disease or disorder, which method comprises steps of:

- (a) contacting a retinal pigment epithelial stem cell (RPESC), or a cell derived therefrom, with a test compound, the RPESC or cell derived therefrom exhibiting a characteristic of the disease or disorder;

- (b) determining whether said characteristic is modulated in the RPESC or cell derived therefrom that is contacted with the test compound,

wherein modulation of the characteristic in the RPESC or cell derived therefrom identifies the test compound as a compound for treating the disease or disorder.

40. A method according to claim 39, in which the RPESC is isolated from the retinal pigment epithelium of a mammal, or is an RPESC descended therefrom.

41. A method according to claim 39, in which the RPESC expresses one or more markers selected from the group consisting of SSEA-4, Sox2, KLF4 and c-Myc.

42. A method according to claim 39, in which the disease or disorder is age-related macular degeneration (AMD).

43. A method according to claim 42, in which the characteristic is expression of and  $\alpha$ B-crystallin gene or gene product.

44. A method for identifying a compound for treating age-related macular degeneration (AMD), which method comprises steps of:

- (a) contacting a retinal pigment epithelial stems cell (RPESC) with a test compound, which RPESC expresses at least one gene or gene product associated with AMD;
- (b) determining whether expression of the gene or gene product is modulated in the RPESC contacted with the test compound;

wherein modulation of the gene or gene product in the RPESC contacted with the test compound identifies the test compound as a compound for treating AMD.

45. A method according to claim 44, in which the gene or gene product associated with AMD is an  $\alpha$ B-crystallin gene or gene product.

46. A method for identifying a compound for treating age-related macular degeneration (AMD), which method comprises steps of:

- (a) contacting a retinal pigment epithelium (RPE) cell with a test compound, which RPE cell expresses at least one gene or gene product associated with AMD, and is obtained by differentiating an isolated retinal pigment epithelial stem cell (RPESC);
- (b) determining whether expression of the gene or gene product is modulated in the RPE cell contacted with the test compound;

wherein modulation of the gene or gene product in the RPE cell contacted with the test compound identifies the test compound as a compound for treating AMD.

47. A method according to claim 46, in which the gene or gene product associated with AMD is an  $\alpha$ B-crystallin gene or gene product.

\* \* \* \* \*

FIGURE 1

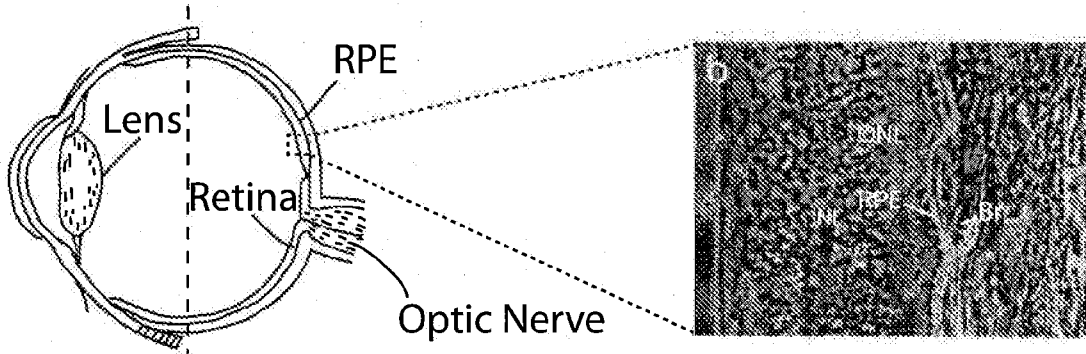
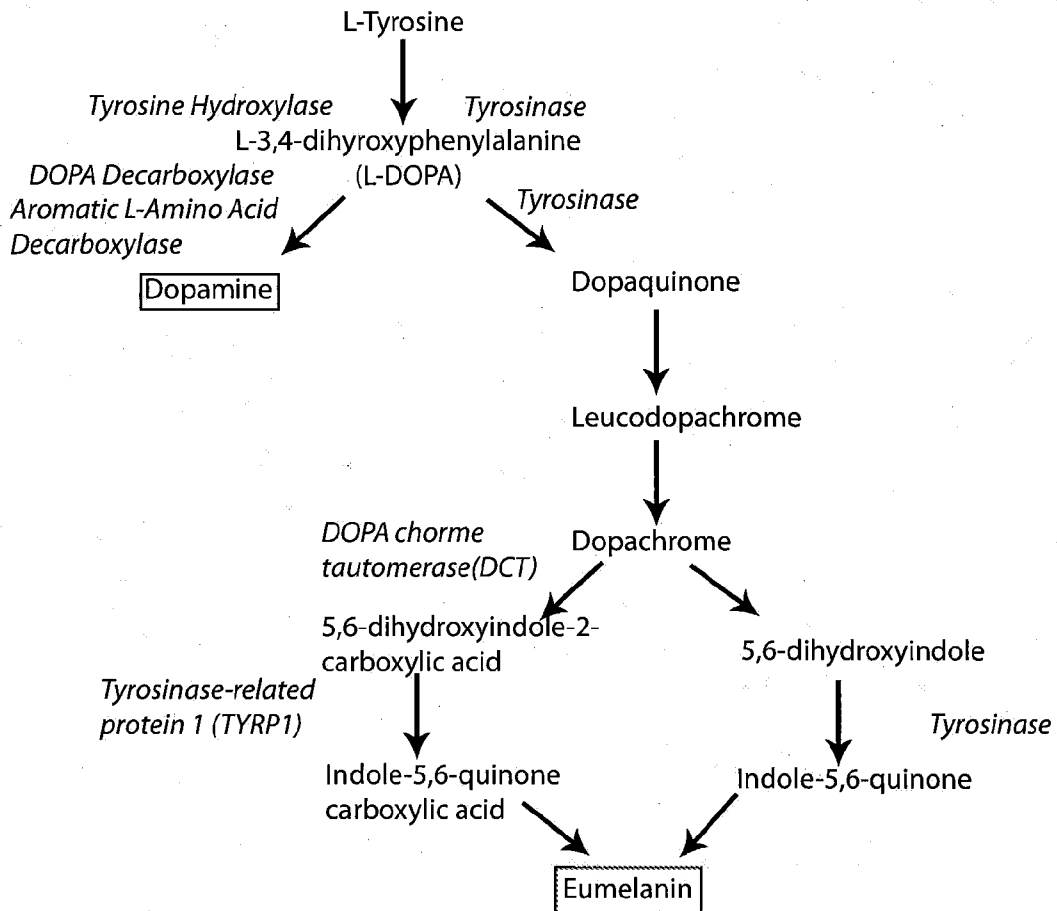


Figure 3



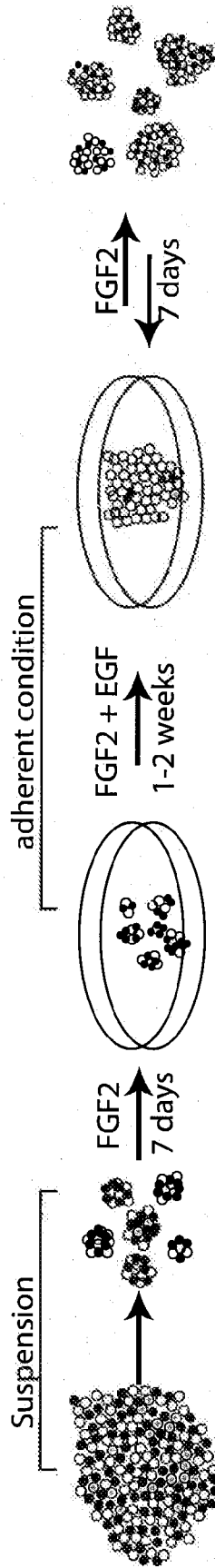
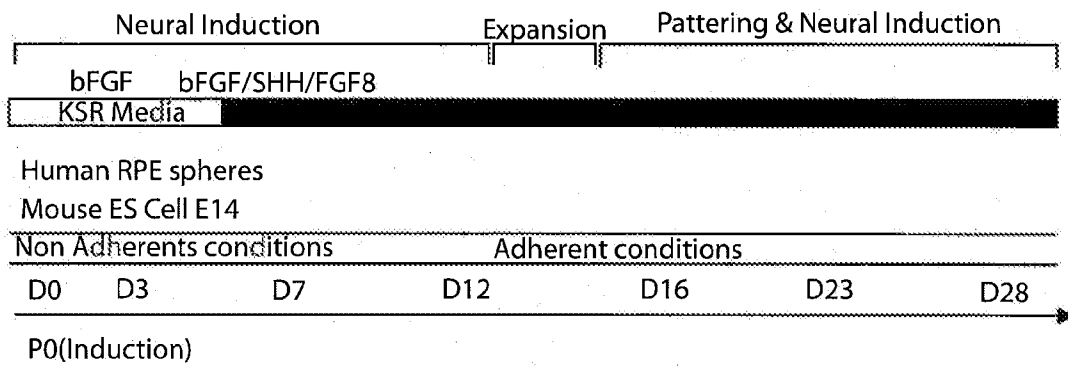


FIGURE 2

# FIGURE 4





## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 09/41472

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 5/10;C12N 5/00; A61K 48/00 (2009.01)

USPC - 435/371, 435/377, 424/93.21

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)

USPC: 435/371, 435/377, 424/93.21

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

USPC: 435/371, 366, 363, 325, 377, 375, 424/93.21, 93.2, 93.1

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

Electronic Databases Searched: (PGPB, EPAB, JPAB, UPST); Google Scholar

Search Terms Used: retinal pigment epithelial stem cells (RPE stem cells or RPESC), embryonic stem cells (ES cells), age related macular degeneration (AMD), Sox2, RPE65,Pax6, Mitf, Otx2, Sox2, transdifferentiation FGF2, FGF8, dopaminergic neuron

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	GU et al. Isolation of retinal progenitor and stem cells from the porcine eye. IN: Mol Vis 29 June 2007 Vol 13 No 1 Pages 1045-1057. Especially abstract, pg 1049 right col para 2	1-3, 8 ---- 4-7, 9-47
Y	US 2007/0196919 A1 (REH et al). 23 August 2007 (23.08.2007). Especially para [0012], [0016],[0068], [0103], [0111], [0112], [	4-6, 9-25, 39-47
Y	US 2007/0031386 A1 (KLIMANSKAYA). 8 February 2007 (08.02.2007). Especially para [0015], [0016], [0075], [0092].	7, 13, 21-38
Y	ALGE et al. Retinal Pigment Epithelium Is Protected Against Apoptosis by alpha B-Crystallin. IN: IOVS November 2002 Vol 43 No 11 Pages 3575-3582. Especially abstract.	43, 45, 47

 Further documents are listed in the continuation of Box C.


\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"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

"&amp;" document member of the same patent family

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6 July 2009 (06.07.2009)

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