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(54) Title: METHODS FOR PROLONGING VIABILITY OF CONE CELLS USING MODULATORS OF THE MAMMALIAN TARGET OF RAPAMYCINE (MTOR) PATHWAY

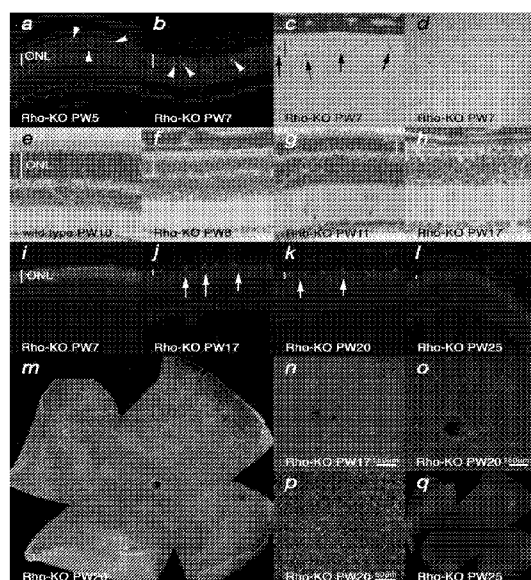


Fig. 1a-1q

(57) Abstract: The present invention is directed to the use of modulators of the mammalian target of rapamycin (mTOR) pathway, glucose and/or glucose enhancers for treating retinal disorders and, in particular, for prolonging the viability of cone cells.



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METHODS FOR PROLONGING VIABILITY OF CONE CELLS USING MODULATORS OF THE MAMMALIAN TARGET OF RAPAMYCINE (mTOR) PATHWAY

RELATED APPLICATIONS

This application claims the benefit of and priority to U.S. Provisional Patent Application Serial No. 61/119,689, filed on December 3, 2008, and U.S. Provisional Application Serial No. 61/120,122, filed on December 5, 2008. This application is also related to U.S. Provisional Patent Application Serial No. 61/169,835, filed on April 16, 2009. The entire contents of each of the foregoing provisional applications are incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with Government support under contract EY014466 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention is directed to the use of modulators of the mammalian target of rapamycin (mTOR) pathway, glucose and/or glucose enhancers for prolonging the viability of cone cells.

BACKGROUND OF THE INVENTION

The retina contains two major types of light-sensitive photoreceptor cells, *i.e.*, rod cells and cone cells. Cone cells are responsible for color vision and require brighter light to function, as compared to rod cells. There are three types of cones, maximally sensitive to long-wavelength, medium-wavelength, and short-wavelength light (often referred to as red, green, and blue, respectively, though the sensitivity peaks are not

actually at these colors). Cones are mostly concentrated in and near the fovea. Only a few are present at the sides of the retina. Objects are seen most sharply in focus when their images fall on this spot, as when one looks at an object directly. Cone cells and rods are connected through intermediate cells in the retina to nerve fibers of the optic nerve. When rods and cones are stimulated by light, the nerves send off impulses through these fibers to the brain.

Reduced viability of cone cells is associated with various retinal disorders, in particular, retinitis pigmentosa. Retinitis pigmentosa is a family of inherited retinal degenerations (RD) that is currently untreatable and frequently leads to blindness. Affecting roughly 1 in 3,000 individuals, it is the most prevalent form of RD caused by a single disease allele (RetNet, www.sph.uth.tmc.edu/Retnet/). The phenotype is characterized by an initial loss of night vision due to the malfunction and death of rod PRs, followed by a progressive loss of cones (Madreperla, S.A., *et al.* (1990) *Arch Ophthalmol* 108, 358-61). Additionally, retinitis pigmentosa is further characterised by the following manifestations: night blindness, progressive loss of peripheral vision, eventually leading to total blindness; ophthalmoscopic changes consist in dark mosaic-like retinal pigmentation, attenuation of the retinal vessels, waxy pallor of the optic disc, and in the advanced forms, macular degeneration. Since cones are responsible for color and high acuity vision, it is their loss that leads to a reduction in the quality of life. In many cases, the disease-causing allele is expressed exclusively in rods; nonetheless, cones die too. Indeed, to date there is no known form of RD in humans or mice where rods die, and cones survive. In contrast, mutations in cone-specific genes result only in cone death.

SUMMARY OF THE INVENTION

The present invention is directed to the use of modulators of the mammalian target of rapamycin (mTOR) pathway for treating retinal disorders and, in particular, for prolonging the viability of cone cells. The present invention is based, at least in part, on the discovery that a modulator of the mTOR pathway can be used to prolong the viability of cone cells by decreasing and/or delaying cone cell death. Accordingly, the present invention provides methods for treating or preventing retinal disorders, in particular retinitis pigmentosa, and for prolonging the viability of cone cells, by contacting cone cells with an mTOR modulator.

In one aspect, the present invention is directed to a method for treating or preventing a retinal disorder in a subject by administering to the subject an mTOR modulator in an amount effective for modulating mTOR activity in the subject, thereby treating or preventing the retinal disorder. In a particular embodiment, the retinal disorder is retinitis pigmentosa. In various embodiments, the retinal disorder is associated with decreased viability of cone and/or rod cells. In another embodiment, the retinal disorder is a genetic disorder. In yet another embodiment, the retinal disorder is not diabetic retinopathy. Alternatively or in addition, the retinal disorder is not associated with blood vessel leakage and/or growth.

In another aspect, the present invention is directed to a method for treating or preventing retinitis pigmentosa in a subject by administering to the subject an mTOR modulator in an amount effective for modulating mTOR activity in the subject, thereby treating or preventing retinitis pigmentosa.

In yet another aspect, the present invention is directed to a method for prolonging the viability of a cone cell, by contacting the cone cell with an mTOR modulator in an amount effective for modulating mTOR activity in the cell, thereby prolonging the viability of the cone cell, *e.g.*, for about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks, about 7 weeks, about 8 weeks, about 3 months, about 4 months, about 5 months, about 6 months, about 7 months, about 8 months, about 9 months, about 10 months, about 11 months, about 12 months, about 2 years, about 3 years, about 4 years, about 5 years, about 10 years, about 15 years, about 20 years, about 25 years, about 30 years, about 40 years, about 50 years, about 60 years, about 70 years, and about 80 years.

In certain embodiments of any of the preceding aspects of the invention, the mTOR modulator is selected from the group consisting of insulin, growth factors, IGF-1, IGF-2, mitogens, serum, phosphatidic acid, amino acids, leucine, and analogues or derivatives thereof. In a particular embodiment, the mTOR modulator is insulin. Alternatively, in another embodiment, the mTOR modulator is not insulin. In other embodiments, the mTOR modulator stimulates mTOR phosphorylation. Alternatively, or in addition, the mTOR modulator activates a receptor and/or a signal transduction cascade upstream of mTOR. In one embodiment, the mTOR modulator is a glucose enhancer.

In another aspect, the present invention is directed to a method for treating or preventing a retinal disorder, for example, retinitis pigmentosa, in a subject by enhancing the intracellular levels of glucose in the subject, thereby treating or preventing the retinal disorder. In certain embodiments, the retinal disorder is associated with decreased viability of cone and/or rod cells. In other embodiments, the retinal disorder is a genetic disorder. In another embodiment the retinal disorder is not diabetic retinopathy. In yet another embodiment, the retinal disorder is not associated with blood vessel leakage and/or growth.

In another aspect, the present invention provides methods for treating or preventing retinitis pigmentosa in a subject by enhancing the intracellular levels of glucose in the subject, thereby treating or preventing retinitis pigmentosa.

In various embodiments of these aspects of the invention, the subject may be administered glucose in an amount effective to enhance the intracellular levels of glucose in the subject. For example, the glucose may be administered to the subject intravenously. In another embodiment, the subject may be administered a composition comprising a glucose enhancer in an amount effective to enhance the intracellular levels of glucose in the subject. In one embodiment, the glucose enhancer modulates biochemical pathways leading to enhanced intracellular glucose levels. In a particular embodiment, the glucose enhancer can serve to increase uptake of glucose into cells, for example rod and/ or cone cells.

In another aspect, the present invention provides methods for prolonging the viability of a cone cell by enhancing the intracellular levels of glucose in the cell, thereby prolonging the viability of the cone cell, *e.g.*, for about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks, about 7 weeks, about 8 weeks, about 3 months, about 4 months, about 5 months, about 6 months, about 7 months, about 8 months, about 9 months, about 10 months, about 11 months, about 12 months, about 2 years, about 3 years, about 4 years, about 5 years, about 10 years, about 15 years, about 20 years, about 25 years, about 30 years, about 40 years, about 50 years, about 60 years, about 70 years, and about 80 years. In a particular embodiment, the cone cell is exposed to glucose in an amount effective to enhance the intracellular levels of glucose in the cone cell. Alternatively, or in addition, the cone cell is exposed to a glucose enhancer in an amount effective to enhance the intracellular levels of glucose in the cone cell. For

example, the glucose enhancer may serve to modulate biochemical pathways leading to enhanced intracellular glucose levels. In a particular embodiment, the glucose enhancer can serve to increase uptake of glucose into cells, for example rod and/ or cone cells.

Other features and advantages of the invention will be apparent from the following detailed description and claims

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1a-1q depict rod death kinetics in the Rho-KO mutant described in Example 1 as follows: (*a-d*) Onset of rod death seen by cleaved nuclear envelope protein LaminA (*a*), Cleaved Caspase3 (*b*) (arrowheads) as well as TUNEL (*c, d*) (arrows) (dark gray in *a, b* shows nuclear DAPI staining). (*d*) Shows a retinal flat mount with view onto the photoreceptor layer. (*e-h*) Progression of rod death determined by the reduction of the ONL as seen by HE staining. (*i-q*) End phase of rod death assessed by section analysis (*i-l*) or by retinal flat mounts (*m-q*). In the Rho-KO the onset of rod death is around PW5 (*a*) and progresses up to PW25 (*l*). By PW17 the ONL is reduced to one row of cells (*h, j*) and in the following 8 weeks the remaining rods die (*j-q*) as seen by immunofluorescence with an antibody directed against guanine nucleotide protein alpha transducin (Gnat1) on sections of progressively older animals (*j-l*). (*m-q*) Retinal flat mounts showing rods visualized by immunofluorescence with an antibody directed against Gnat1. (*m*) Shows entire retina while (*n, o*) show higher magnification around the optic nerve head and (*p*) shows peripheral region. (*q*) Shows no signal at PW25 where on sections rods were also not detected (*l*). Age (in postnatal weeks (PW)) is indicated in the panels. Vertical bar in (*a-c, e-l*) indicates thickness of the ONL.

Figures 2a-2q depict rod death kinetics in the PDE- γ -KO described in Example 1 as follows: (*a-d*) Onset of rod death seen by cleaved caspase 3 (*a, b*). At P12, misplaced and excess cells in the INL were dying as part of developmental cell death, as seen in a wild-type control (*a*) (arrowheads) while in the mutant, cells started to die in the ONL, where photoreceptors reside (*b*) (arrows). The onset of rod death was also seen by immunofluorescence for the cleaved nuclear envelope protein, LaminA (*c*) (arrows) as well as TUNEL (*d*) (arrows; dark gray in *a-d* shows nuclear DAPI staining).

Progression of rod death was determined by the reduction of the thickness of the ONL, as seen by HE staining (*e-h*). (*i-q*) End phase of rod death was assessed by analysis of sections of progressively older animals. (*i-m*) Rods were visualized by immunofluorescence with α -rhodopsin or by *in situ* hybridization for *rhodopsin* (*n-q*). (*i, j*) Retinal section at P16 showing peripheral to central region. (*i*) Same picture as in (*j*) with nuclear DAPI stain. (*k, l*) Higher magnification of section in (*i*) showing peripheral (*k*) and central (*l*) region. As rods die in a central to peripheral manner, more rods were present in the periphery than in the center. By P20, the ONL was reduced to 1 row of cells and rods were found mainly in the periphery (compare arrow (periphery) in (*n*) to arrowhead (central)). The remaining rods in the PDE- γ -KO died over 4 weeks (*n-q*) as seen on sections. By P49 (*q*) all rods had died in this mutant (*o-q*: periphery). Age (in postnatal days (P)) is indicated in the panels. Vertical bar in (*a-h, k, l*) indicates thickness of the ONL. Data for PDE- β -/- are not shown as they are comparable to the PDE- γ -KO and we have presented data on the rod death kinetics of this mutant in an earlier publication (IOVS, 2007, 48 (2): 849-857).

Figures 3a-3o depict cone death kinetics described in Example 1 as follows: (*a*) qRT-PCR analysis for *Opn1sw* during cone degeneration. Changes are indicated as the logarithm of the relative concentration over time on the Y-axis while X-axis indicates postnatal weeks. (*b-h, j, k, m-o*) Show retinal flat mounts. (*k*) Shows a retinal section. Light gray signal shows PNA expression, dark gray signal shows red/green opsin expression (*b, j-n*) or blue opsin expression (*c, d, o*). (*b-d*) Wild type retina at P35. Red/green opsin (*b*) and PNA (*c, d*) expression were detected dorsal and ventral while blue opsin (*c, d*) was detected only ventrally. (*e-g, j-o*) Analysis in the PDE- β mutant. (*e-g*) Central to peripheral gradient of PNA and shortening of cone outer segments (OS). At P20, prior to the major cone death phase, there were fewer elongated OS in the center (*e*) as compared to the periphery. (*f*) High magnification of a central or peripheral (*g*) OS from (*e*). (*h*) Wild type OS (white line in *f-h* marks the OS). (*i*) Quantification of OS length in central and peripheral regions. The data represents an average of 15 measurements on 3 different retinæ of 3 week old mice. With the shortening of OSs during degeneration, red/green opsin was localized throughout the membrane of the cell body and PNA, which detects an extracellular protein(s), was reduced to a small dot attached to the residual OS (*j*) (arrow: yellow shows red/green and PNA overlap). (*k*)

High magnification of a cone showing red/green localization at the membrane of the main cell body (arrow). (l) Cross section showing red/green in cell body (arrows; j-l P70). Red/ green opsin was detected mainly dorsal (l) during degeneration while PNA (m, n) or blue opsin (o) were not altered (m, n: P21, same scale bar; o: P49).

Figures 4a-4g depict rod death kinetics in the P23H mutant described in Example 1 as follows. (a-c) Onset of rod death. As rod death progressed very slowly in this mutant, the upregulation of *glial fibrillary acidic protein* (GFAP) in Muller glia, which has been described as a hallmark of retinal degeneration, was used in conjunction with the other markers to determine the onset of rod degeneration. As seen by antibody staining against GFAP (a, b) degeneration started around PW10 (b). At PW5, GFAP was only found in the ganglion cell layer where it is normally expressed in astrocytes. Consistent with the upregulation of GFAP at PW10, cells positive for cleaved nuclear envelope protein LaminA (c) were also detected (arrow). However, few cells were seen per section due to the slow progression of rod death. (d-f) Progression of rod death determined by the reduction of the ONL as seen by HE staining. (g) End phase of rod death assessed by immunofluorescence with anti-rhodopsin. Although the ONL was reduced to one row of cells by PW35, no end point of rod death was determined. Rods continued to die slowly and even by PW70, many rods were still present (g). Interestingly, most of the rods at that age were confined to the ventral regions of the retina (see also Figure 6). Age (in postnatal weeks (PW)) is indicated in the panels. Vertical bar in (a-f) indicates thickness of the ONL.

Figures 5a-5c depict summaries of the kinetics (a and b) and histological changes (c) that accompany rod and cone death across 4 mouse models. Red/green opsin protein levels were detectable mainly dorsally during cone degeneration (5c).

Figures 6a-6g depict dorsal cone death kinetics seen by the immunofluorescence with anti-red/green opsin as described in Example 1 as follows: (a-c) Loss of dorsal cones in the Rho-KO mutant over time as seen by the reduced expression of red-green opsin. (d, e) Loss of dorsal cones in the P23H mutant over time. (f, g) Higher magnification of a double staining with an antibody against red/green opsin (dark gray signal) and rhodopsin (light gray signal) showing that most rods that survived up to PW80 were in the ventral regions (g) of the retina whereas the red/green expressing cones were mostly dorsal (f).

Figures 7a-7c depict affymetrix microarray analysis as described in Example 1 as follows: (a) Equivalent time points in the 4 different mutants at which the microarray analysis was performed (R: approximately halfway through the major phase of rod death; C0: onset of cone death; C1 & C2 first and second time point during cone death respectively). Time is indicated in postnatal days (P) or postnatal weeks (PW). Cartoons depicting the progression of cone death are shown below the corresponding time points. (b) Distribution in percentage of the 195 genes that were annotated. (c) Distribution in percentage of the 68 genes (34.9%) that are part of metabolism in (b).

Figure 8 depicts that red/green and blue opsin expression was not affected on the RNA level as described in Example 1 as follows: *In situ* hybridization for red/green opsin (first two rows) or blue opsin (third and fourth row) on retinal sections. RNA levels for red/green opsin and blue were comparable between ventral regions of mutant (first column), wild type animals treated with rapamycin (last column) or untreated wild type animals (second column).

Figures 9a-9m depict p*-mTOR in wild type and degenerating retinae as described in Example 1. All panels show immunofluorescence on retinal flat mounts (photoreceptor side up) with the exception of (b, c, g) which show retinal sections. Dark gray shows the nuclear DAPI stain. (a-c) p*-mTOR levels in wild type retinae. (a) Dorsal (up) enrichment of p*-mTOR. Higher magnification of dorsal and ventral region is shown to the right showing p*-mTOR in red and cone segments in light gray as detected by PNA. (b, c) Dorsal retinal sections stained for p*-mTOR (medium gray signal) and PNA (b) (green signal) or α - β -galactosidase (c) (light gray signal). The β -galactosidase is under the control of the human red/green opsin promoter and is expressed in all cones⁴⁸ (see Material & Methods). The insets in (b, c) show higher magnification of the cone segments indicating that the p*-mTOR signal is located in the lower part of the outer segment (OS; IS: inner segment). (d-g) Rapamycin treatment of wild type mice leads to downregulation of red/green opsin ventrally (e) but not dorsally (d) (medium gray signal). Ventral blue opsin (f) (medium gray signal) remains unaffected, as does PNA (d-g) (light gray signal). Rapamycin treatment does also not affect mTOR phosphorylation in wild type (g) (dark gray signal). (h-m) Reduced levels of dorsal p*-mTOR during photoreceptor degeneration (medium gray signal). (h) Wild type control. (i, j) PDE- β mutant. The reduction starts during rod death at P15 (i) as the

OSs (light gray signal: PNA) start to detach from the retinal pigmented epithelium. (i) By P30 only few cones (green signal: α - β -galactosidase) show high levels of p*-mTOR (red signal). (k-l) A similar reduction is seen in dorsal cones of the other three mutants (cones marked in light gray by PNA). (k) PDE- γ -KO P35. (l) Rho-KO PW20. (m) P23H PW70.

Figures 10a-10b depict the dependence of p*-mTOR levels on the presence of glucose as described in Example 1. Different media conditions were tested (a) during 4 hours of retinal explant culture. After culture, retinæ were fixed and stained for p*-mTOR (light gray signal), PNA (medium gray signal) and DAPI (dark gray signal). Retinal flat mounts were imaged (b). Dorsal p*-mTOR was only detected when glucose was present in the media.

Figures 11a-11j depict the upregulation of Hif-1 α and GLUT1 in cones as described in Example 1. All panels show immunofluorescent staining. Left column (a, d, g, h,) shows retinal flat mounts and right column (b, c, e, f, i, j) retinal sections. Dark gray shows nuclear DAPI staining and light gray shows cones marked with PNA. (a-f) Staining for HIF-1 α (medium gray signal). (a) Wild type (PW10) (inset) showing higher magnification. (b, c) Cross sections in wild type (PW10). (c) DAPI overlap of (b). (d-f) During cone degeneration in PDE- β -/- (PW10) increased levels of HIF-1 α are found in cones (d, inset). (e, f) Cross sections show that the increase of Hif-1 α occurs mainly in cones (arrows point to cones that at this stage are located within the top layer of the inner nuclear layer). (f) DAPI overlap of (e). (g) GLUT1 expression in wild type (PW10) (medium gray signal). Most of the signal in between the cones reflects expression in rods. (h-j) Increased expression of GLUT1 in cones during degeneration seen in flat mounts (h) and sections (i-j). (i) Overlap of (j) with PNA.

Figures 12a-12h depict the upregulation of Hif-1 α and GLUT1 in cones as described in Example 1. All panels show immunofluorescent signals within retinal sections. Dark gray shows nuclear DAPI staining and light gray shows cones marked with PNA. (a-d) Staining for HIF-1 α (medium gray signal). (a) Wild-type at PW10 (see also Figure 11a-c). (b) PDE- γ -KO at PW5. (c) Rho-KO at PW20. (d) P23H at PW70. (e-h) Staining for GLUT1 (red signal). (e) Wild-type at PW10. (f) PDE- γ -KO at PW5 with PNA overlap. (f') Same image as (f) without PNA. (g) Rho-KO at PW20. (g') Same

image as (g) without PNA. (h) P23H at PW70. (h') Same image as (h) without PNA. White dotted line marks border between the ONL and INL.

Figures 13a-13d depict the increased levels of LAMP-2 at the lysosomal membrane as described in Example 1 as follows: (a-c) Immunofluorescence on retinal flat mounts where LAMP-2 is shown in light gray, red/green opsin in medium gray and dark gray signal shows nuclear DAPI stain. Insets in upper right corner (with box) show enlarged cells (arrow). (a) Wild type retinae at PW5 showing lysosome (small light gray dots) with normal LAMP-2 distribution. Weak red/green opsin signal is detected at the level of the PR nuclei since in wild type it is mainly found in the OSs. (b, c) PDE- β mutant at PW5. (b) Enlarged lysosomes (dots) due to accumulation of LAMP-2 at the lysosomal membrane are seen specifically in cones. (c) Confocal section of same field as in (b) taken at the level of the inner nuclear layer showing levels of LAMP-2 similar to those in wild type (a). (d) qRT-PCR for the 3 different LAMP-2 splice forms showing the relative concentration and the ratios between the PDE- β mutant and wild type.

Figures 14a-14m depict a retroviral vector, as described in Example 1, encoding a fusion protein between GFP and LC3 as used to infect the retinae of wild type (a-c) and PDE- β ^{-/-} (d-f) mice. Light gray signal shows expression of the fusion protein, medium gray signal shows red/green opsin expression, and dark gray signal shows nuclear DAPI staining. (a-f) Retinal flat mounts at PW10 showed uniform expression of the GFP fusion protein in cones without the formation of vesicular structures in wild type and mutant retinae. (a) DAPI overlap of (b). (c) 3D reconstruction of (b). Cone outer segments, as shown by red/green opsin signal (arrow), were attached to the cone inner segments (arrowhead), as shown by GFP signal. (d) DAPI overlap of (e). (f) Single confocal section showing cytoplasmic GFP and membrane bound red/green opsin (see also Fig. 3). Figure 14 further depicts the levels of phosphorylated S6 (p*-S6) (medium gray signal, light gray signal marks cones with PNA, dark gray nuclear DAPI stain) in wild type (g, h) and mutant (i-m) cones. (g) Low levels of p*-S6 were seen in wild type cones but not in cone OSs. (h) DAPI overlap of (g). (i, j) Strong uniform expression in cones was seen the PDE- β mutant shortly after the end of the major rod death phase (PW3). Area in lower right corner shows a region where cones had started to die. (j) DAPI overlap of (i). (k-l) Higher magnification at PW5 showing same field at three different confocal depths. (k) Within the plane of the cone outer segments, high levels of

p*-S6 were seen in cones when compared to segments of wild type cones. (l) Strong staining was also seen in the plane of the cone nuclei, indicating a uniform cytoplasmic distribution. (m) Within the plane of the INL, levels of p*-S6 were much lower than in cones.

Figures 15a-15h depict the effect of insulin levels on cone survival as set forth in Example 1 as follows: (a-c) Retinal flat mounts of PDE- β mutants at PW7 stained for lacZ (Wang, Y. *et al.* (1992) *Neuron* 9, 429-40; Punzo, C. & Cepko, C. (2007) *Invest Ophthalmol Vis Sci* 48, 849-57) to detect cones (see Material & Methods and Figure 16). (a) Example of untreated control. (b) Example of mouse injected with streptozotocin. (c) Example of mouse injected daily with insulin. (d) Quantification of cone survival after 4 weeks of treatment. Data represents an average of at least 8 retinæ and indicates on the y-axis percentage of cone surface area *versus* surface area of entire retina (see Figures 17 and 18). (e) Measurements of blood glucose levels and body weight (f) performed weekly over the time span of the experiment. (g, h) Immunofluorescent staining on retinal flat mounts for HIF-1 α (medium gray signal) and PNA (light gray signal) in untreated control PDE- $\beta^{-/-}$ (g) and PDE- $\beta^{-/-}$ mice treated for 4 weeks with insulin (h). Dark gray shows nuclear DAPI.

Figures 16a-16f depict the cone-lacZ transgene in the PDE- β mutant at 7 weeks of age as described in Example 1 as follows: (a, b) Double labeling of cones with PNA (dark gray signal) and lacZ staining (light gray signal). More cones were labeled by lacZ than by PNA. Since PNA marks an extracellular matrix protein of the OS, once the OSs were reduced, PNA became a less reliable marker. (c, d) Double labeling of cones by α -red/green opsin (dark gray signal) and lacZ staining (X-gal; light gray signal) in the dorsal (c) and ventral (d) retina. Red/green opsin levels decreased ventrally during degeneration which made this marker not suitable for detection of cones across the retina. (e, f) Sections of retina stained for lacZ showing the signal in cones on top of the INL.

Figures 17a-17e depict a method to calculate cone survival as described in Example 1 as follows: (a-c) Show retinal flat mounts stained for lacZ (see Fig. 15). (a) Untreated control PDE- $\beta^{-/-}$ mouse at PW7. (b) PDE- $\beta^{-/-}$ mouse at PW7 treated with one injection of Streptozotocin at PW3. (c) PDE- $\beta^{-/-}$ mouse at PW7 treated for 4 weeks with daily injections of insulin starting at PW3. (a'-c') Show inverted color images of

corresponding panels (a-c). (a''-c'') Show only the green channels whereas (a'''-c''') show only the red channels of the inverted color images (a'-c'). The red channel served as a proxy for the lacZ stain whereas the green channel served as a proxy for the retina. (d) Quantification of cone survival by calculating the surface area of red that co-localizes with green. Two different methods were employed, a fixed threshold and an adjusted threshold. The fixed threshold was determined by adjusting the lower intensity of the red channel in the image with the most intense lacZ staining (most intense red channel) to reflect the pattern of the lacZ staining. The same threshold for the red channel was then applied to all other images. As this method would under represent cone survival in mice that were not treated with insulin due to the less intense lacZ staining a second method was employed. For each image the lower intensity of the red channel was adjusted individually to match the blue pattern of the lacZ staining avoiding the problem of the difference in lacZ intensity. The increased intensity of lacZ in the insulin treated mice could be due to healthier cones that either have an increased transcription/translation or decreased protein degradation. (e) Shows the actual calculated values in percentage of cone survival for all retinæ. Values are shown for the untreated mice, the Streptozotocin treated mice and the insulin treated mice. Values for both types of calculations are shown, for the fixed threshold and adjusted threshold.

Figures 18a-18c depict the assessment of cone survival after prolonged Insulin treatment as described in Example 1 as follows: (a) Composite of retinæ after lacZ staining. First column shows untreated PDE- $\beta^{-/-}$ mice at PW10. Second column shows retinæ of PDE- $\beta^{-/-}$ mice at PW10 that received a single injection of streptozotocin at PW3. Third column shows retinæ of PDE- $\beta^{-/-}$ mice at PW10 that received daily injections of insulin starting at PW3. (b) Shows quantification of cone survival by the two methods described in Figure 17. There was no significant difference in cone survival between treated and untreated mice at PW10. (c) Shows comparison between the 4 and 7 weeks treatment.

Figure 19 depicts an exemplary mTOR pathway.

Figure 20 depicts a table of 230 genes that had statistically significant changes in all 4 mouse models and had fold changes >2 at the onset of cone death, when compared to the other three time points. The fold change is indicated as $\log_2$. (AVG: Average of

fold change from the 4 mutants; C0: Onset of cone death; R: peak of rod death; C1 & C2: first and second time point during cone death respectively, see also Figure 7a).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to the use of modulators of the mammalian target of rapamycin (mTOR) pathway for treating retinal disorders and, in particular, for prolonging the viability of a cone cell. The present invention is based, at least in part, on the discovery that a modulator of the mTOR pathway can be used to prolong the viability of a cone cell by decreasing and/or delaying cone cell death. Accordingly, the present invention provides methods for treating or preventing retinal disorders, *e.g.*, retinitis pigmentosa, and for prolonging the viability of a cone cell, by contacting the cone cell with an mTOR modulator.

In addition, the present invention is directed to methods which involve increasing intracellular levels of glucose in a subject in order to treat retinal disorders, such as retinitis pigmentosa, in a subject and, further, to prolong the viability of a cone cell in a subject. In this regard, the present invention is based on the discovery that enhanced intracellular glucose levels can serve to prolong the viability of a cone cell by decreasing and/or delaying cone cell death. In various embodiments, a subject or isolated cell may be exposed to either glucose itself or glucose enhancers which serve to enhance the levels of intracellular glucose in the subject or the cell in order to achieve the desired therapeutic effect.

As used herein, the term “retinal disorders” refers generally to disorders of the retina. In one embodiment, the retinal disorder is associated with decreased viability, for example, death, of cone cells, and/ or rod cells. Moreover, in a particular embodiment, the retinal disorders of the present invention are not associated with blood vessel leakage and/or growth, for example, as is the case with diabetic retinopathy, but, instead are characterized primarily by reduced viability of cone cells and/ or rod cells. In certain embodiments, the retinal disorders are genetic disorders. In a particular embodiment, the retinal disorder is retinitis pigmentosa.

As used herein, the term “retinitis pigmentosa” is art known and encompasses a disparate group of genetic disorders of rods and cones. Retinal pigmentosa generally refers to retinal degeneration often characterized by the following manifestations: night blindness, progressive loss of peripheral vision, eventually leading to total blindness; ophthalmoscopic changes consist in dark mosaic-like retinal pigmentation, attenuation of the retinal vessels, waxy pallor of the optic disc, and in the advanced forms, macular degeneration. In some cases there can be a lack of pigmentation. Retinitis pigmentosa can be associated to degenerative opacity of the vitreous body, and cataract. Family history is prominent in retinitis pigmentosa; the pattern of inheritance may be autosomal recessive, autosomal dominant, or X-linked; the autosomal recessive form is the most common and can occur sporadically.

As used herein, the term “mTOR” or “mammalian target of rapamycin” refers to the art recognized serine/ threonine protein kinase involved in the regulation of cell growth, cell proliferation, cell motility, protein synthesis and transcription. mTOR further serves as a sensor of cellular nutrient status, energy status and redox status. As well known in the art, mTOR integrates input from multiple upstream pathways, including, but not limited to, insulin, growth factors (IGF-1 and IGF-2) and mitogens. Moreover, mTOR is known to operate as the catalytic subunit of two distinct molecular complexes in cells, *i.e.*, mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2).

Specifically, mTORC1 is composed of mTOR, regulatory associated protein of mTOR (Raptor), and mammalian LST8/G-protein β -subunit like protein (mLST8/G β L) and functions as a nutrient/energy/redox sensor and to control protein synthesis. The activity of this complex is stimulated by insulin, growth factors, serum, phosphatidic acid, amino acids (particularly leucine), and oxidative stress.

mTOR Complex 2 (mTORC2) is composed of mTOR, rapamycin-insensitive companion of mTOR (Rictor), G β L, and mammalian stress-activated protein kinase interacting protein 1 (mSIN1) and functions as an important regulator of the cytoskeleton through its stimulation of F-actin stress fibers, paxillin, RhoA, Rac1, Cdc42, and protein kinase C α (PKC α). In addition, mTORC2 phosphorylates the serine/threonine protein kinase Akt/PKB at a serine residue, thereby ultimately leading to full Akt activation.

As used herein, the term “mTOR pathway” refers to biochemical pathways involving mTOR or mTOR complexes, for example, in the regulation of cell growth, cell proliferation, cell motility, protein synthesis and transcription and, further, the sensing of cellular nutrient status, energy status and redox status. In a particular embodiment, the mTOR pathway is as depicted in Figure 19.

As used herein, the terms “mTOR modulator,” “modulator of mTOR,” “mTOR pathway modulator” or modulator of the mTOR pathway” refer to any moiety that modulates, for example, upregulates or downregulates, the activity, viability, presence, transcription, translation, and/or post-transcriptional or post-translational modification of mTOR and/or mTOR complex and/or that modify the activity and/or metabolic flux through the mTOR pathway and/or pathways upstream or downstream of the mTOR pathway. For example, such moieties include small molecules, proteins, amino acids, nucleic acid molecules, siRNA, aptamers, adnectins, antibodies or fragments thereof, growth factors, or hormones. Any of the art known mTOR modulators (*e.g.*, one or more of the mTOR modulators described in PCT Publication No. WO 2008/027855, the contents of which are hereby incorporated herein by reference) may be used in the methods of the present invention.

In various embodiments, the mTOR modulator is selected from the group consisting of insulin, growth factors, IGF-1, IGF-2, mitogens, serum, phosphatidic acid, caffeic acid phenethyl ester (CAPE), amino acids, leucine, zinc and analogues or derivatives thereof. In a particular embodiment, the mTOR modulator is insulin. In certain embodiments, the mTOR modulator stimulates mTOR phosphorylation, *e.g.*, Lysophosphatidic acid acyltransferase (LPAAT), *e.g.*, LPAAT-theta. Alternatively or in addition, the mTOR modulator activates a receptor and/or a signal transduction cascade upstream of mTOR. In one embodiment of the invention, an mTOR modulator is a glucose enhancer. In one embodiment, an mTOR modulator acts through the insulin receptor.

As used herein, the term “glucose enhancer” refers to any moiety that increases the level of intracellular glucose. For example, such moieties include small molecules, proteins, amino acids, nucleic acid molecules, siRNA, aptamers, adnectins, antibodies or fragments thereof, growth factors, or hormones. In various embodiments, the glucose enhancers may serve to modulate biochemical pathways leading to enhanced

intracellular glucose levels. In one embodiment, the glucose enhancers may serve to increase glucose uptake into cells by, for example, enhancing the activity or levels of glucose transporters such as GLUT-1, GLUT-2, GLUT-3, GLUT-4 and/or GLUT-5 glucose transporters. In one embodiment, a glucose enhancer is a nucleic acid molecule encoding a glucose transporter protein cloned into a recombinant expression vector, *e.g.*, a viral vector, for use in gene therapy.

As used herein, the term "subject" includes warm-blooded animals, preferably mammals, including humans. In a preferred embodiment, the subject is a primate. In an even more preferred embodiment, the primate is a human.

As used herein, the various forms of the term "modulate" are intended to include stimulation (*e.g.*, increasing or upregulating a particular response or activity) and inhibition (*e.g.*, decreasing or downregulating a particular response or activity).

As used herein, the term "contacting" (*e.g.*, contacting a cell with an agent, such as an mTOR modulator, glucose, and/or glucose enhancer) is intended to include incubating the agent and the cell together *in vitro* (*e.g.*, adding the agent to cells in culture) or administering the agent to a subject such that the agent and cells of the subject are contacted *in vivo*. The term "contacting" is not intended to include exposure of cells to an agent that may occur naturally in a subject (*i.e.*, exposure that may occur as a result of a natural physiological process).

As used herein, the term "administering" to a subject includes dispensing, delivering or applying an mTOR modulator, glucose, and/or a glucose enhancer to a subject by any suitable route for delivery of the mTOR modulator, glucose, and/or a glucose enhancer to the desired location in the subject, including delivery by intraocular administration or intravenous administration. Alternatively or in combination, delivery is by the topical, parenteral or oral route, intracerebral injection, intramuscular injection, subcutaneous/intradermal injection, buccal administration, transdermal delivery and administration by the rectal, colonic, vaginal, intranasal or respiratory tract route.

As used herein, the term "effective amount" includes an amount effective, at dosages and for periods of time necessary, to achieve the desired result, *e.g.*, sufficient to treat a subject suffering from a retinal disorder, for example, retinitis pigmentosa; sufficient to prevent a retinal disorder, for example, in a subject likely to develop the

retinal disorder; or sufficient to prolong the viability of a cone cell. An effective amount of an mTOR modulator, glucose, and/or glucose enhancer, as defined herein may vary according to factors such as the state, severity and extent of the condition, *e.g.*, abnormal cone cell death or a retinal disorder such as retinitis pigmentosa, age, and weight of the subject, and the ability of the compound to elicit a desired response in the subject. Dosage regimens may be adjusted to provide the optimum therapeutic response. An effective amount is also one in which any toxic or detrimental effects (*e.g.*, side effects) of the mTOR modulator, glucose, and/or glucose enhancer are outweighed by the therapeutically beneficial effects.

Various additional aspects of the methods of the invention are described in further detail in the following subsections.

I. Methods of the Invention

The present invention provides methods for treating or preventing a retinal disorder in a subject. The methods include administering to the subject an mTOR modulator in an amount effective for modulating mTOR activity in the subject, thereby treating or preventing a retinal disorder in the subject.

The present invention also provides methods for treating or preventing retinitis pigmentosa in a subject. The methods generally comprise administering to the subject an mTOR modulator in an amount effective for modulating mTOR activity in the subject, thereby treating or preventing retinitis pigmentosa in the subject.

The present invention further provides methods for prolonging the viability of a cone cell. The methods generally comprise contacting the cell with an mTOR modulator in an amount effective for modulating mTOR activity in the cone cell, thereby prolonging the viability of the cone cell.

In one embodiment, the viability or survival of a cone cell is prolonged for *e.g.*, about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks, about 7 weeks, about 8 weeks, about 3 months, about 6 months, about 1 year, about 2 years, about 3 years, about 4 years, about 5 years, about 10 years, about 15, years, about 20 years, about 25 years, about 30 years, about 40 years, about 50 years,

about 60 years, about 70 years, or about 80 years. Times intermediate to the above-recited times are also contemplated by the invention.

In another aspect, the present invention provides methods for treating or preventing a retinal disorder in a subject. Such methods generally comprise administering to the subject an agent which enhances the intracellular levels of glucose, thereby treating or preventing a retinal disorder in a subject.

The present invention also provides methods for treating or preventing retinitis pigmentosa in a subject by administering to the subject an agent which enhances the intracellular levels of glucose, thereby treating or preventing retinitis pigmentosa in the subject.

The present invention further provides methods for prolonging the viability of a cone cell by contacting the cell with an agent which enhances the intracellular levels of glucose in the cone cell, thereby prolonging the viability of the cone cell.

The viability or survival of a cone cell may be prolonged for *e.g.*, about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks, about 7 weeks, about 8 weeks, about 3 months, about 6 months, about 1 year, about 2 years, about 3 years, about 4 years, about 5 years, about 10 years, about 15 years, about 20 years, about 25 years, about 30 years, about 40 years, about 50 years, about 60 years, about 70 years, or about 80 years. Times intermediate to the above-recited times are also contemplated by the invention.

Without intending to be bound by theory, it is believed that the mTOR modulators, glucose and/or glucose enhancers serve to increase nutrient levels, for example, intracellular glucose, to increase glucose uptake by cells, to increase membrane synthesis or the rate thereof, to increase the synthesis of phospholipids, to increase metabolic flux through the pentose phosphate cycle and/ or to increase intracellular generation of NADPH. By doing so, the methods of the present invention serve to increase the viability of cone cells and to treat and/or prevent retinal disorders such as retinitis pigmentosa.

mTOR modulators suitable for use in the methods of the invention include, for example, insulin, growth factors, IGF-1, IGF-2, mitogens, serum, phosphatidic acid, caffeic acid phenethyl ester (CAPE), amino acids, leucine, zinc and analogues or

derivatives thereof. In a particular embodiment, the mTOR modulator is insulin. In certain embodiments, the mTOR modulator stimulates mTOR phosphorylation, *e.g.*, Lysophosphatidic acid acyltransferase (LPAAT), *e.g.*, LPAAT-theta. Alternatively or in addition, the mTOR modulator activates a receptor and/or a signal transduction cascade upstream of mTOR. In one embodiment, the mTOR modulator is a glucose enhancer.

In various embodiments, an agent that enhances glucose levels may serve to modulate biochemical pathways leading to enhanced intracellular glucose levels. In one embodiment, the agent that enhances the activity or level of intracellular glucose is a glucose transporter. In one embodiment, the agent is a nucleic acid molecule. For example, glucose transporters for use in the present invention may belong to the GLUT family of transporters (including at least one of GLUT1, GLUT2, GLUT3, GLUT4, GLUT5, GLUT6, GLUT7, GLUT8, GLUT9, GLUT10, GLUT11, GLUT12 and GLUT13), encoded by the SLC2 family of genes (including at least one of SLC2A1, SLC2A2, SLC2A3, SLC2A4, SLC2A5, SLC2A6, SLC2A7, SLC2A8, SLC2A9, SLC2A10, SLC2A11, SLC2A12 and SLC2A13).

In one embodiment of the invention, a glucose enhancer for use in the methods of the invention is a nucleic acid molecule encoding a glucose transporter. For example, a cDNA (full length or partial cDNA sequence) may be cloned into a recombinant expression vector used as a gene therapy vector, and the vector may be transfected into cells using standard molecular biology techniques. The cDNA can be obtained, for example, by amplification using the polymerase chain reaction (PCR) or by screening an appropriate cDNA library.

The nucleic acids for use in the methods of the invention can also be prepared, *e.g.*, by standard recombinant DNA techniques. A nucleic acid of the invention can also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-phase synthesis which has been automated in commercially available DNA synthesizers (See *e.g.*, Itakura et al. U.S. Patent No. 4,598,049; Caruthers et al. U.S. Patent No. 4,458,066; and Itakura U.S. Patent Nos. 4,401,796 and 4,373,071, incorporated by reference herein).

In one embodiment, a nucleic acid molecule encoding an mTOR modulator or a glucose enhancer, *e.g.*, a glucose transporter, may be present in an inducible construct. In

another embodiment, a nucleic acid molecule encoding an mTOR modulator or a glucose enhancer may be present in a construct which leads to constitutive expression. In one embodiment, a nucleic acid molecule encoding an mTOR modulator or a glucose enhancer may be delivered to cells, or to subjects, in the absence of a vector.

A nucleic acid molecule encoding an mTOR modulator or a glucose enhancer may be delivered to cells or to subjects using a viral vector, preferably one whose use for gene therapy is well known in the art. Techniques for the formation of vectors or virions are generally described in "Working Toward Human Gene Therapy," Chapter 28 in Recombinant DNA, 2nd Ed., Watson, J. D. et al., eds., New York: Scientific American Books, pp. 567-581 (1992). An overview of suitable viral vectors or virions is provided in Wilson, J. M., Clin. Exp. Immunol. 107(Suppl. 1):31-32 (1997), as well as Nakanishi, M., Crit. Rev. Therapeu. Drug Carrier Systems 12:263-310 (1995); Robbins, P. D., et al., Trends Biotechnol. 16:35-40 (1998); Zhang, J., et al., Cancer Metastasis Rev. 15:385-401(1996); and Kramm, C. M., et al., Brain Pathology 5:345-381 (1995). Such vectors may be derived from viruses that contain RNA (Vile, R. G., et al., Br. Med Bull. 51:12-30 (1995)) or DNA (Ali M., et al., Gene Ther. 1:367-384 (1994)).

Examples of viral vector systems utilized in the gene therapy art and, thus, suitable for use in the present invention, include the following: retroviruses (Vile, R. G., supra; U.S. Pat. Nos. 5,741,486 and 5,763,242); adenoviruses (Brody, S. L., et al., Ann. N.Y. Acad. Sci. 716: 90-101 (1994); Heise, C. et al., Nat. Med. 3:639-645 (1997)); adenoviral/retroviral chimeras (Bilbao, G., et al., FASEB J. 11:624-634 (1997); Feng, M., et al., Nat. Biotechnol. 15:866-870 (1997)); adeno-associated viruses (Flotte, T. R. and Carter, B. J., Gene Ther. 2:357-362 (1995); U.S. Patent No. 5,756,283); herpes simplex virus I or II (Latchman, D. S., Mol. Biotechnol. 2:179-195 (1994); U.S. Patent No. 5,763,217; Chase, M., et al., Nature Biotechnol. 16:444-448 (1998)); parvovirus (Shaughnessy, E., et al., Semin Oncol. 23:159-171 (1996)); reticuloendotheliosis virus (Donburg, R., Gene Therap. 2:301-310 (1995)). Extrachromosomal replicating vectors may also be used in the gene therapy methods of the present invention. Such vectors are described in, for example, Calos, M. P. (1996) Trends Genet. 12:463-466, the entire contents of which are incorporated herein by reference. Other viruses that can be used as vectors for gene delivery include poliovirus, papillomavirus, vaccinia virus, lentivirus, as well as hybrid or chimeric vectors incorporating favorable aspects of two or more viruses

(Nakanishi, M. (1995) Crit. Rev. Therapeu. Drug Carrier Systems 12:263-310; Zhang, J., et al. (1996) Cancer Metastasis Rev. 15:385-401; Jacoby, D. R., et al. (1997) Gene Therapy 4:1281-1283).

The term "AAV vector" refers to a vector derived from an adeno-associated virus serotype, including without limitation, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, or AAVX7. "rAAV vector" refers to a vector that includes AAV nucleotide sequences as well as heterologous nucleotide sequences. rAAV vectors require only the 145 base terminal repeats in cis to generate virus. All other viral sequences are dispensable and may be supplied in trans (Muzyczka (1992) Curr. Topics Microbiol. Immunol. 158:97). Typically, the rAAV vector genome will only retain the inverted terminal repeat (ITR) sequences so as to maximize the size of the transgene that can be efficiently packaged by the vector. The ITRs need not be the wild-type nucleotide sequences, and may be altered, *e.g.*, by the insertion, deletion or substitution of nucleotides, as long as the sequences provide for functional rescue, replication and packaging. In particular embodiments, the AAV vector is an AAV2/5 or AAV2/8 vector. Suitable AAV vectors are described in, for example, U.S. Patent No. 7,056,502 and Yan et al. (2002) J. Virology 76(5):2043-2053, the entire contents of which are incorporated herein by reference.

As used herein, the term "lentivirus" refers to a group (or genus) of retroviruses that give rise to slowly developing disease. Viruses included within this group include HIV (human immunodeficiency virus; including but not limited to HIV type 1 and HIV type 2), the etiologic agent of the human acquired immunodeficiency syndrome (AIDS); visna-maedi, which causes encephalitis (visna) or pneumonia (maedi) in sheep; the caprine arthritis-encephalitis virus, which causes immune deficiency, arthritis, and encephalopathy in goats; equine infectious anemia virus (EIAV), which causes autoimmune hemolytic anemia, and encephalopathy in horses; feline immunodeficiency virus (FIV), which causes immune deficiency in cats; bovine immune deficiency virus (BIV), which causes lymphadenopathy, lymphocytosis, and possibly central nervous system infection in cattle; and simian immunodeficiency virus (SIV), which cause immune deficiency and encephalopathy in sub-human primates. Diseases caused by these viruses are characterized by a long incubation period and protracted course. Usually, the viruses latently infect monocytes and macrophages, from which they spread

to other cells. HIV, FIV, and SIV also readily infect T lymphocytes (i.e., T-cells). In one embodiment of the invention, the lentivirus is not HIV.

As used herein, the term “adenovirus” (“Ad”) refers to a group of double-stranded DNA viruses with a linear genome of about 36 kb. See, *e.g.*, Berkner et al., Curr. Top. Microbiol. Immunol., 158: 39-61 (1992). In some embodiments, the adenovirus-based vector is an Ad-2 or Ad-5 based vector. See, *e.g.*, Muzyczka, Curr. Top. Microbiol. Immunol., 158: 97-123, 1992; Ali et al., 1994 Gene Therapy 1: 367-384; U.S. Pat. Nos. 4,797,368, and 5,399,346. Suitable adenovirus vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (*e.g.*, Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses are advantageous in that they do not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types. Additionally, introduced adenovirus DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (*e.g.*, retroviral DNA). Moreover, the carrying capacity of the adenovirus genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Haj-Ahmand et al. J. Virol. 57, 267-273 [1986]).

In one embodiment, an adenovirus is a replication defective adenovirus. Most replication-defective adenoviral vectors currently in use have all or parts of the viral E1 and E3 genes deleted but retain as much as 80% of the adenovirus genetic material. Adenovirus vectors deleted for all viral coding regions are also described by Kochanek et al. and Chamberlain et al. (U.S. Pat. No. 5,985,846 and U.S. Pat. No. 6,083,750). Such viruses are unable to replicate as viruses in the absence of viral products provided by a second virus, referred to as a “helper” virus.

In one embodiment, an adenoviral vector is a “gutless” vector. Such vectors contain a minimal amount of adenovirus DNA and are incapable of expressing any adenovirus antigens (hence the term “gutless”). The gutless replication defective Ad vectors provide the significant advantage of accommodating large inserts of foreign DNA while completely eliminating the problem of expressing adenoviral genes that result in an immunological response to viral proteins when a gutless replication defective Ad vector is used in gene therapy. Methods for producing gutless replication defective

Ad vectors have been described, for example, in U.S. Pat. No. 5,981,225 to Kochanek et al., and U.S. Pat. Nos. 6,063,622 and 6,451,596 to Chamberlain et al; Parks et al., PNAS 93:13565 (1996) and Lieber et al., J. Virol. 70:8944-8960 (1996).

In another embodiment, an adenoviral vector is a “conditionally replicative adenovirus” (“CRAds”). CRAds are genetically modified to preferentially replicate in specific cells by either (i) replacing viral promoters with tissue specific promoters or (ii) deletion of viral genes important for replication that are compensated for by the target cells only. The skilled artisan would be able to identify epithelial cell specific promoters.

Other art known adenoviral vectors may be used in the methods of the invention. Examples include Ad vectors with recombinant fiber proteins for modified tropism (as described in, *e.g.*, van Beusechem et al., 2000 Gene Ther. 7: 1940-1946), protease pre-treated viral vectors (as described in, *e.g.*, Kuriyama et al., 2000 Hum. Gene Ther. 11: 2219-2230), E2a temperature sensitive mutant Ad vectors (as described in, *e.g.*, Engelhardt et al., 1994 Hum. Gene Ther. 5: 1217-1229), and “gutless” Ad vectors (as described in, *e.g.*, Armentano et al., 1997 J. Virol. 71: 2408-2416; Chen et al., 1997 Proc. Nat. Acad. Sci. USA 94: 1645-1650; Schieder et al., 1998 Nature Genetics 18: 180-183).

In a particular embodiment, the viral vector for use in the methods of the present invention is an AAV vector. In particular embodiments, the viral vector is an AAV2/5 or AAV2/8 vector. Such vectors are described in, for example, U.S. Patent No. 7,056,502, the entire contents of which are incorporated herein by reference.

In one embodiment, an LIA retrovirus may be used to deliver nucleic acids encoding an mTOR modulator or a glucose enhancer (Cepko et al. (1998) Curr. Top. Dev. Biol. 36:51; Dyer and Cepko (2001) J. Neurosci. 21:4259). The viral titer may be varied to alter the expression levels. The viral titer may be in any suitable range. For example, the viral titer may range from about 10^6 cfu/ml to 10^8 cfu/ml. The amount of virus to be added may also be varied. The volume of virus, or other nucleic acid and reagent, added can be in any suitable range.

The vector will include one or more promoters or enhancers, the selection of which will be known to those skilled in the art. Suitable promoters include, but are not limited to, the retroviral long terminal repeat (LTR), the SV40 promoter, the human

cytomegalovirus (CMV) promoter, and other viral and eukaryotic cellular promoters known to the skilled artisan.

Guidance in the construction of gene therapy vectors and the introduction thereof into affected subjects for therapeutic purposes may be obtained in the above-referenced publications, as well as in U.S. Patent Nos. 5,631,236, 5,688,773, 5,691,177, 5,670,488, 5,529,774, 5,601,818, and PCT Publication No. WO 95/06486, the entire contents of which are incorporated herein by reference.

Generally, methods are known in the art for viral infection of the cells of interest. The virus can be placed in contact with the cell of interest or alternatively, can be injected into a subject suffering from a retinal disorder, for example, as described in United States Provisional Patent Application No. 61/169,835 and PCT Application No. PCT/US09/053730, the contents of each of which are incorporated by reference.

Gene therapy vectors comprising, an mTOR modulator or a glucose enhancer, *e.g.*, a glucose transporter, can be delivered to a subject or a cell by any suitable method in the art, for example, intravenous injection (*e.g.*, intravitreal or subretinal injection), local administration, *e.g.*, application of the nucleic acid in a gel, oil, or cream, (see, *e.g.*, U.S. Patent No. 5,328,470), stereotactic injection (see, *e.g.*, Chen et al. (1994) Proc. Natl. Acad. Sci. U.S.A. 91:3054), gene gun, or by electroporation (see, *e.g.*, Matsuda and Cepko (2007) Proc. Natl. Acad. Sci. U.S.A. 104:1027), using lipid-based transfection reagents, or by any other suitable transfection method.

As used herein, the terms “transformation” and “transfection” are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection (*e.g.*, using commercially available reagents such as, for example, LIPOFECTIN® (Invitrogen Corp., San Diego, CA), LIPOFECTAMINE® (Invitrogen), FUGENE® (Roche Applied Science, Basel, Switzerland), JETPEI™ (Polyplus-transfection Inc., New York, NY), EFFECTENE® (Qiagen, Valencia, CA), DREAMFECT™ (OZ Biosciences, France) and the like), or electroporation (*e.g.*, in vivo electroporation). Suitable methods for transforming or transfecting host cells can be found in Sambrook, et al. (Molecular Cloning: A

Laboratory Manual. 2nd, ed., Cold Spring harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), and other laboratory manuals.

In one embodiment, an mTOR modulator or glucose enhancer is delivered to a subject or cells in the form of a peptide or protein. In order to produce such peptides or proteins, recombinant expression vectors of the invention can be designed for expression of one or more mTOR modulator proteins and/or glucose enhancer proteins, *e.g.*, glucose transporter proteins, and/or portion(s) thereof in prokaryotic or eukaryotic cells. For example, one or more glucose transporter proteins and/or portion(s) thereof can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, Calif. (1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

In one embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include retinal cell-type-specific promoters (*e.g.*, rhodopsin regulatory sequences, Cabp5, Cralbp, Nrl, Crx, Ndr4, clusterin, Rax, Hes1 and the like (Matsuda and Cepko, *supra*)), the albumin promoter (liver-specific, Pinkert et al. (1987) *Genes Dev.* 1:268), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:5473). Developmentally-regulated promoters are also encompassed, for example the α -fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537).

In certain embodiments, the methods of the present invention involve co-administration of multiple mTOR modulators or a pharmaceutically acceptable salt thereof, for example, at least two of mTOR modulators selected from the group consisting of insulin, growth factors, IGF-1, IGF-2, mitogens, serum, phosphatidic acid, amino acids, leucine and analogues or derivatives thereof. Alternatively, mTOR modulators may be administered in combination with glucose and/or glucose enhancers.

The methods described herein can be performed *in vitro*. For example, mTOR activity and/or intracellular glucose levels can be modulated in a cell *in vitro* and then the treated cells can be administered or re-administered to a subject. For practicing the methods *in vitro*, cells (*e.g.*, mammalian cells, such as human cells) can be obtained from a subject by standard methods and incubated (*e.g.*, cultured) *in vitro* with an agent which modulates mTOR and/or enhances intracellular glucose levels. Methods for isolating cells are well known in the art. The cells can be re-administered to the same subject, or another subject which is compatible with the donor of the cells.

For administration of cells to a subject, it may be preferable to first remove residual agents in the culture from the cells before administering them to the subject. This can be done, for example, by gradient centrifugation of the cells or by washing of the tissue. Methods for the *ex vivo* genetic modification of cells followed by re-administration to a subject are well known in the art and described in, for example, U.S. Patent No. 5,399,346 the entire contents of which are incorporated herein by reference.

The claimed methods of modulation are not meant to include naturally occurring events. For example, the term "agent" or "modulator" is not meant to embrace endogenous mediators produced by the cells of a subject.

Application of the methods of the invention for the treatment and/or prevention of a retinal disorder can result in curing the disorder, decreasing at least one symptom associated with the disorder, either in the long term or short term or simply a transient beneficial effect to the subject. Accordingly, as used herein, the terms "treat," "treatment" and "treating" include the application or administration of agents, as described herein, to a subject who is suffering from a retinal disorder, or who is susceptible to such conditions with the purpose of curing, healing, alleviating, relieving, altering, remedying, ameliorating, improving or affecting such conditions or at least one symptom of such conditions. As used herein, the condition is also "treated" if recurrence of the condition is reduced, slowed, delayed or prevented.

Subjects suitable for treatment using the regimens of the present invention should have or are susceptible to developing a retinal disorder. For example, subjects may be genetically predisposed to development of a retinal disorder. Alternatively, abnormal progression of the following factors including, but not limited to visual acuity, the rate of

death of cone and/ or rod cells, night vision, peripheral vision, attenuation of the retinal vessels, and other ophthalmoscopic factors associated with retinal disorders such as retinitis pigmentosa may indicate the existence of or a predisposition to a retinal disorder. Other art recognized symptoms or risk factors may be monitored using methods well known in the art.

In one embodiment of the invention, the retinal disorder is retinitis pigmentosa. In another embodiment, the retinal disorder is associated with decreased viability of cone cells. In yet another embodiment, the retinal disorder is associated with decreased viability of rod cells. In one embodiment, the retinal disorder is not diabetic retinopathy. In another embodiment, the retinal disorder is not associated with blood vessel leakage and/or blood vessel growth.

The mTOR modulators, glucose, and/or glucose enhancers, as described herein, may be administered as necessary to achieve the desired effect and depend on a variety of factors including, but not limited to, the severity of the condition, age and history of the subject and the nature of the composition, for example, the identity of the genes or the affected biochemical pathway. In various embodiments, the mTOR modulators, glucose, and/or glucose enhancers may be administered at least two, three, four, five or six times a day. Additionally, the therapeutic or preventative regimens may cover a period of at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 weeks, 3 months, 6 months, 1 year, 2 years, 3 years, 4 years, 5 years, 10 years, 15 years, 20 years, 30 years, 40 years, 50 years, 60 years, 70 years, or 80 years. Times intermediate to the above-recited times are also contemplated by the invention.

The ability of an agent to modulate the activity of mTOR and/or intracellular levels of glucose can be determined as described herein, *e.g.*, by determining the ability of the agent to modulate: cell viability (*e.g.*, modulation of apoptosis), cleavage of LaminA or Caspase 3; expression of *Opn1sw*, *Opn1mw*, *LAMP-2A*, *LAMP-2B*, or *LAMP-2C*; protein production of LAMP-2A, LAMP-2B, LAMP-2C, HIF1- α , or GLUT1; phosphorylation of mTOR, S6K1, AMPK, PTEN, or Akt; phospholipid production; production of reactive oxygen species; and/or the expression and protein synthesis of photoreceptor specific opsins. The assays described in the Examples section below may also be used to determine whether an agent modulates the activity of mTOR and/or intracellular levels of glucose.

In various embodiments, the methods of the present invention further comprise monitoring the effectiveness of treatment. For example, visual acuity, the rate of death of cone and/ or rod cells, night vision, peripheral vision, attenuation of the retinal vessels, and other ophthalmoscopic changes associated with retinal disorders such as retinitis pigmentosa may be monitored to assess the effectiveness of treatment. The assays described in the Examples section below may also be used to monitor the effectiveness of treatment.

II. Pharmaceutical Compositions for Use in the Methods of the Invention

The mTOR modulators, glucose and/or glucose enhancers used in the methods of the present invention may be incorporated into pharmaceutical compositions suitable for administration to a subject, which may, for example, allow for sustained delivery of the active agent for a period of at least several weeks to a month or more. Preferably, the mTOR modulator, glucose and/or glucose enhancer is the only active ingredient(s) formulated into the pharmaceutical composition, although in certain embodiments the mTOR modulator, glucose and/or glucose enhancer may be combined with one or more other active ingredients including, for example, modulators of pathways upstream or downstream of the mTOR pathway. In addition, at least two of the mTOR modulator, glucose and/or glucose enhancer may be present in the composition. Other pharmaceutically active compounds that may be used can be found in Harrison's Principles of Internal Medicine, Thirteenth Edition, Eds. T.R. Harrison *et al.* McGraw-Hill N.Y., NY; and the Physicians Desk Reference 50th Edition 1997, Oradell New Jersey, Medical Economics Co., the complete contents of which are expressly incorporated herein by reference.

In one embodiment, the pharmaceutical composition comprises an mTOR modulator, glucose and/or glucose enhancer and a pharmaceutically acceptable carrier. As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. In one embodiment, the carrier is suitable for intraocular, parenteral, intravenous, intraperitoneal, topical, or intramuscular administration. In another embodiment, the carrier is suitable for oral

administration. Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

The pharmaceutical compositions of the present invention may be administered in the form of injectable compositions which can be prepared either as liquid solutions or suspensions. The pharmaceutical compositions may also be emulsified. Suitable excipients for use in such compositions are, for example, water, saline, dextrose, glycerol, or ethanol, and combinations thereof. In addition, if desired, the pharmaceutical compositions may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH-buffering agents, adjuvants or immunopotentiators.

In a particular embodiment, the mTOR modulator, glucose and/or glucose enhancer is incorporated in a composition suitable for intraocular administration. For example, the compositions may be designed for intravitreal, subconjunctival, sub-tenon, periocular, retrobulbar, suprachoroidal, and/or intrascleral administration, for example, by injection, to effectively treat the retinal disorder. Additionally, a sutured or refillable dome can be placed over the administration site to prevent or to reduce "wash out", leaching and/or diffusion of the active agent in a non-preferred direction.

Relatively high viscosity compositions, as described herein, may be used to provide effective, and preferably substantially long-lasting delivery of an mTOR modulator, glucose and/or glucose enhancer, for example, by injection to the posterior segment of the eye. A viscosity inducing agent can serve to maintain the mTOR modulator, glucose and/or glucose enhancer in a desirable suspension form, thereby preventing deposition of the composition and the mTOR modulator in the bottom surface of the eye. Such compositions can be prepared as described in U.S. Patent No. 5,292,724, the contents of which are hereby incorporated herein by reference.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution,

microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, monostearate salts and gelatin. Moreover, the compounds of the invention can be administered in a time release formulation, for example in a composition which includes a slow release polymer. The mTOR modulator, glucose and/or glucose enhancer compositions can be prepared with carriers that will protect the active agent against rapid release, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, polylactic acid and polylactic, polyglycolic copolymers (PLG). Many methods for the preparation of such formulations are patented or generally known to those skilled in the art.

Sterile injectable solutions can be prepared by incorporating the mTOR modulator, glucose and/or glucose enhancer in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The pharmaceutical compositions of the invention can be formulated with one or more additional compounds that enhance the solubility of the mTOR modulator, glucose and/or glucose enhancer. Preferred compounds to be added to formulations to enhance

the solubility of the mTOR modulator, glucose and/or glucose enhancer are cyclodextrin derivatives, preferably hydroxypropyl- γ -cyclodextrin. For example, inclusion in the formulation of hydroxypropyl- γ -cyclodextrin at a concentration 50-200 mM may increase the aqueous solubility of the active agent.

Another formulation for the mTOR modulator, glucose and/or glucose enhancer comprises the detergent Tween-80, polyethylene glycol (PEG) and ethanol in a saline solution. A non-limiting example of such a preferred formulation is 0.16% Tween-80, 1.3% PEG-3000 and 2% ethanol in saline.

In one embodiment, the mTOR modulator, glucose and/or glucose enhancer composition is administered to the subject as a sustained-release formulation using a pharmaceutical composition comprising a solid ionic complex of mTOR modulator, glucose and/or glucose enhancer and a carrier macromolecule, wherein the carrier and the active agent used to form the complex are combined at a weight ratio of carrier: active agent of, for example, 0.5:1 to 0.1:1. In other embodiments, the carrier and active agent used to form the complex are combined at a weight ratio of carrier: active agent of 0.8:1, 0.7:1, 0.6:1, 0.5:1, 0.4:1, 0.3:1, 0.25:1, 0.2:1, 0.15:1, or 0.1:1. Ranges intermediate to the above recited values, *e.g.*, 0.8:1 to 0.4:1, 0.6:1 to 0.2:1, or 0.5:1 to 0.1:1 are also intended to be part of this invention. For example, ranges of values using a combination of any of the above recited values as upper and/or lower limits are intended to be included.

In another embodiment, the mTOR modulator, glucose and/or glucose enhancer is administered to the subject using a pharmaceutical composition comprising a solid ionic complex of the mTOR modulator, glucose and/or glucose enhancer and a carrier macromolecule, wherein the mTOR modulator, glucose and/or glucose enhancer content of the complex is at least 0.05% by weight, preferably at least 0.05%, 0.10%, 0.15%, 0.20%, 0.25%, 0.30%, 0.35%, 0.40%, 0.45%, 0.50%, 0.55%, 0.60%, 0.65%, 0.70%, 0.75%, 0.80%, 0.85%, 0.90%, 0.95% or 1.00% w/w. Ranges intermediate to the above recited amounts, *e.g.*, about 0.08% to about 0.73% w/w, are also intended to be part of this invention. For example, ranges of values using a combination of any of the above recited values as upper and/or lower limits are intended to be included.

As used herein, the term "sustained delivery" or "sustained release" is intended to refer to continual delivery of mTOR modulator, glucose and/or glucose enhancer *in vivo* over a period of time following administration, preferably at least several days, a week or several weeks and up to a month or more. In a preferred embodiment, a formulation of the invention achieves sustained delivery for at least about 7, 14, 21 or 28 days, at which point the sustained release formulation can be re-administered to achieve sustained delivery for another 28 day period (which re-administration can be repeated every 7, 14, 21 or 28 days to achieve sustained delivery for several months to years). Sustained delivery of the mTOR modulator, glucose and/or glucose enhancer can be demonstrated by, for example, the continued therapeutic effect of the active agent over time. Alternatively, sustained delivery of the mTOR modulator, glucose and/or glucose enhancer may be demonstrated by detecting the presence of the active agent *in vivo* over time.

In another embodiment, the mTOR modulator, glucose and/or glucose enhancer is incorporated into a composition suitable for oral administration. Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: A binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant: such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

In one embodiment, mTOR modulators, glucose and/or glucose enhancers described herein are prepared with carriers that will protect the compounds against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will

be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

Systemic administration of an mTOR modulator, glucose, and/or glucose enhancer may also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The pharmaceutical formulation contains an effective amount of the mTOR modulator, glucose and/or glucose enhancer. An effective amount of an mTOR modulator, glucose and/or glucose enhancer, as defined herein may vary according to factors such as the state, severity and extent of the condition, *e.g.*, abnormal cone cell death or a retinal disorder such as retinitis pigmentosa, age, and weight of the subject, and the ability of the compound to elicit a desired response in the subject. Dosage regimens may be adjusted to provide the optimum therapeutic response. An effective amount is also one in which any toxic or detrimental effects of the mTOR modulator, glucose and/or glucose enhancer are outweighed by the therapeutically or prophylactically beneficial effects.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures and Sequence Listing, are hereby incorporated by reference.

EXAMPLES

MATERIAL AND METHODS

Animals: Wild type (wt) mice (C57Bl/6N) and PDE- β ^{-/-} mice (normally referred as rd1 or FVB/N) were purchased from Taconic Farms. The PDE- β ^{-/-} mice have a mutation in the β -subunit of cGMP *phosphodiesterase* (Bowes, C. *et al.* (1990) *Nature* 347, 677-80) (PDE). The PDE- γ knock-out (PDE- γ -KO) lacks the γ -subunit of PDE and was provided by Steve Tsang (Tsang, S.H. *et al.* (1996) *Science* 272, 1026-9) (UCLA). The rhodopsin knock-out (Rho-KO) lacks the rod-specific opsin gene and was provided by Janis Lem (Tsang, S.H. *et al.* (1996) *Science* 272, 1026-9; Lem, J. *et al.* (1999) *Proc Natl Acad Sci U S A* 96, 736-41) (Tufts Medical School). The P23H mouse has a proline-23 to histidine mutation in the rhodopsin gene and was provided by Muna Naash (Naash, M.I., *et al.* (1990) *Proc Natl Acad Sci U S A* 90, 5499-503) (University of Oklahoma). As this mouse carries a transgene the strain was always crossed back to C57Bl/6N to ensure that none of the progeny would carry two alleles of the transgene. The transgene is specifically expressed in rods (Gouras, P., *et al.* (1994) *Vis Neurosci* 11, 1227-31; Woodford, B.J., *et al.* (1994) *Exp Eye Res* 58, 631-5; al-Ubaidi, M.R. *et al.* (1990) *J Biol Chem* 265, 20563-9) and carries 3 mutations in the rhodopsin gene (Val-20 to Gly, Pro-23 to His, Pro-27 to Leu). In this study it is referred as the P23H mutant. The cone-lacZ strain was provided by Jeremy Nathans (Wang, Y. *et al.* (1992) *Neuron* 9, 429-40) (Johns Hopkins School of Medicine). All procedures involving animals were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Affymetrix array analysis: RNA was extracted as described previously (Punzo, C. & Cepko, C. (2007) *Invest Ophthalmol Vis Sci* 48, 849-57). Three to 4 retinae were used per extraction. A minimum of two arrays were analyzed per time point. The statistical significance of each gene expression profile was determined by a Jonckheere-Terpstra test of the hypothesized cone-death patterned alternative, using exact p-values calculated by the Harding algorithm (Harding, E.F. (1984) *Applied Statistics* 33, 1-6).

qRT-PCR was performed as described previously with the same primers and conditions for *Opn1sw* and *gapdh* (Punzo, C. & Cepko, C. (2007) *Invest Ophthalmol Vis Sci* 48, 849-57). The following primers and conditions were used for the three *LAMP-2*

splice forms: *LAMP-2* forw. ctgaaggaagtgaatgtctacatg; *LAMP-2A* rev. gctcatatccagtatgatggc; *LAMP-2B* rev. cagagtctgatatccagcatag; *LAMP-2C* rev. gacagactgataaccagtacg. Conditions for all three PCRs: 95° for 3 sec, 52° for 15 sec, 72° for 25 sec. The data in figure 3a and figure 13d represent an average of 3 measurements corrected for *gapdh*.

Retinal explant cultures: The retina was dissected free from other ocular tissues in DMEM, and then incubated in conditions according to the chart in Figure 10a. Regular DMEM was at 4.5g/L glucose, low glucose was at 1g/L, leucine was added at 200μM and FCS at 10%. Incubation was performed for 4h and the retinæ were fixed and processed for antibody staining as described below.

TUNEL, X-gal histochemistry and *In Situ* Hybridizations were performed as described previously (Punzo, C. & Cepko, C. (2007) *Invest Ophthalmol Vis Sci* 48, 849-57). For the double labeling of cones (see Figure 16), retinæ were first fixed in 2% PFA for 15min. then processed for the X-GAL reaction and then post fixed in 4% PFA for 15min. A biotin-PNA was used in an antibody staining procedure (see below) and detected with Streptavidin-POD (1:500, Roche) by a DAB stain (Sigma) according to the manufacture's instructions. The following ESTs were used for the red/green opsin and blue opsin probes respectively: red/green opsin (BE950633); blue opsin (BI202577). Probe for *rhodopsin* was generated by sub-cloning the coding sequence of the gene into pGEM-T Easy (Promega). The following primers were used for amplification of the coding sequence: forw. agccatgaacggcacagagg (SEQ ID NO:1); rev. cttagctggagccacctggct (SEQ ID NO:2). The antisense RNA was generated with T7 RNA polymerase.

Viral injections were performed as described previously (Punzo, C. & Cepko, C.L. (2008) *Dev Dyn* 237, 1034-42). Mice were injected at embryonic day 10 and harvested at postnatal week 10. The fusion protein was generated with a NotI site at the 5' end followed by GFP, then LC3, and then an XhoI site at the 3'end and cloned into pQCXIX (Clontech: cat.# 631515). The following primers were used for the fusion protein: 5'NotI-GFP atcggggccgcccaccatggtgagcaagggcgaggagc (SEQ ID NO:3), 3'GFP-LC3 aggtcttctcggacggcatctgtacagctcgtccatgccgag (SEQ ID NO:4), 5'LC3 atgccgtccgagaagaccttaagc (SEQ ID NO:5), 3'LC3-XhoI atctcgagttacagccattgtgtccgaatg (SEQ ID NO:6).

Rapamycin, Streptozotocin and Insulin treatments were performed as follows. Rapamycin was diluted to 10mg/ml in ethanol. The stock was diluted to 0.015mg/ml in drinking water over a period of 2 weeks. A single intraperitoneal injection of 150 μ l (12mg/ml in 0.1M citric acid, pH4.5) of Streptozotocin was injected at postnatal day (P) 21. Insulin was injected intraperitoneally daily starting at P21. The concentration was increased weekly such that the first week, 10U/kg body weight, the second 15U/kg, the third 20U/kg and fourth 30U/kg body weight, were injected. In the treatment that lasted 7 weeks 30U/kg body weight were injected for the remaining 3 weeks. Blood glucose levels were measured by collecting a drop of blood from the tail directly onto a test strip from TrueTrack smart system (CVS pharmacy). Eye bleeds were avoided due to the fact that cell survival in the retina was being assayed.

Quantification of cone survival was performed as follows. The colors of the bright light image were inverted and processed with Imaris software (Bitplane Inc) to calculate the percentage of blue surface area versus the total retinal surface area (see also Figure 17). A minimum of 8 retinæ per treatment, and for the control, were analyzed. P-values were calculated by the student's t-test. The cone lacZ transgene was chosen over PNA as a cone marker since the transgene labels cones more persistently, since, due to the shortening of the cone OS, PNA was found to stain less reliably than lacZ (see Figure 16).

Whole mount and section antibody staining were performed as previously (Punzo, C. & Cepko, C.L. (2008) *Dev Dyn* 237, 1034-42) described with the following modifications. Antibody staining for LAMP-2: Triton was replaced with 0.01% Saponin. Antibody staining for p*-mTOR and p*-S6: PBS was replaced by TBS in every step of the procedure. Primary antibody dilutions: mouse α -rhodopsin Rho4D2 1:20051; goat α - β -Galactosidase (Serotec) 1:400; rabbit α -blue opsin (Jeremy Nathans) 1:1000; rabbit α -Gnat1 1:200 (Santa Cruz); rabbit α -Cleaved Caspase-3 (Cell Signaling) 1:100; rabbit α -Cleaved Lamin A (Cell Signaling) 1:100; rabbit α -GLUT-1 (Alpha Diagnostics) 1:100; rabbit α -p*-mTOR (Ser2448) (Cell Signaling) 1:300; rabbit α -p*-S6 (Ser235/236) (Cell Signaling) 1:100; rabbit α -HIF-1 α (R&D Systems) 1:300; rat α -LAMP-2 (clone: GL2A7, from DSHB) 1:200. Time points analyzed for the rod and cone death kinetics (P: postnatal day; PW: postnatal week): PDE- β -/-: P10-P20 daily, PW3-10 weekly, PW 12, PW15, PW18, PW45; PDE- γ -KO: P10-P20 daily, PW3-PW10 weekly, PW15, PW25,

PW45; Rho-KO: PW4-PW8 weekly, PW10, PW11, PW17, PW20, PW25, PW27, PW31, PW34, PW37, PW45, PW55, PW80; P23H: PW5, PW10, PW16, PW25, PW30, PW35, PW40, PW65, PW70, PW75, PW80, PW85.

EXAMPLE 1: Rod and cone death kinetics

To establish a framework for comparing gene expression in 4 different models of RP, the equivalent stages of disease pathology were established through examination of the kinetics of rod (Figure 1) (see also Figures 2 and 4) and cone (Fig. 3) (see also Fig. 6) death. Rod death kinetics were established by determining the onset, progression and end phase of rod death (Fig. 1). The time from the onset of rod death to the time when the outer nuclear layer (ONL) was reduced to 1 row of cells will be referred to as the major rod death phase. The time thereafter until rod death was complete will be referred to as the end phase of rod death. To determine the beginning of the major phase of rod death, cleavage of the nuclear envelope protein LaminA (Fig. 1a), and of the apoptotic protease Caspase3 (Fig. 1b), as well as TUNEL (Fig. 1c, d) were used. The continuation of the major rod death phase was monitored by these assays, as well as inspection of histological sections (Fig. 1e-h), as rods account for more than 95% of all PRs. Once the ONL reached one row of cells, the major phase of rod death was over. The end phase of rod death was determined using rod-specific markers to perform either *in situ* hybridization (see Figure 2) or immunohistochemistry (Fig. 1i-l) on retinal sections. However, unless every section of a single retina is collected it is difficult to determine if any rods remain. Thus, retinal flat mounts also were used to allow a comprehensive analysis of the end phase of rod death (Fig. 1m-q). Interestingly, while in the two PDE mutants and in the Rho-KO mutant the end phase of rod death was clearly defined, in the P23H mutant, rods died so slowly that even 50 weeks (latest time point analyzed) after the end of the major phase of rod death, some rods were still present (see Fig. 4).

Two methods were used to determine the onset and progression of cone death. First, the overall time frame of cone demise was determined by quantitative real-time polymerase chain reaction (qRT-PCR) (Fig. 3a) for the ventral (Applebury, M.L. *et al.* (2000) *Neuron* 27, 513-23) cone specific transcript *Opn1sw* (opsin1 short-wave-sensitive: blue cone opsin). This allowed for an initial quantitative comparison among

different strains, but was not adequate to determine the number of cones as transcript levels could vary prior to cell death. Next, whole mount immunohistochemistry for red/green opsin (*Opn1mw*: opsin1 medium-wave-sensitive) and peanut agglutinin lectin (PNA) were used (Fig. 3*b-n*). Both markers are expressed throughout the murine retina allowing for the visualization of cones (Fig. 3*b-d*). Interestingly, the onset of cone death always occurred at the equivalent stage of rod death, namely after the major rod death phase, when the thickness of the ONL was reduced to only a single row of cells. Cone death was found to proceed from the center to the periphery in all 4 models, as seen by staining with PNA (Fig. 3*e*). It was preceded by a gradual reduction of the outer segment (OS) length (Fig. 3*f-i*) and by opsin localization from the OS to the entire cell membrane (Fig. 3*j-l*). In addition, red/green opsin (*Opn1mw*) protein, which is normally detected throughout the mouse retina (Fig. 3*b*), was detected mainly dorsally during cone degeneration (Fig. 3*m, n*). However, PNA staining showed no appreciable difference across the dorsal/ventral axis (Fig. 3*m, n*). Similarly, blue opsin expression, which is normally detected only ventrally (Applebury, M.L. *et al.* (2000) *Neuron* 27, 513-23) (Fig. 3*c, d*), was not affected during degeneration (Fig. 3*o*). Shortening of cone OSs and loss of cone-specific markers has also been described in human cases of RP (John, S.K., *et al.* (2000) *Mol Vis* 6, 204-15).

In summary, the kinetics and histological changes that accompanied rod and cone death shared several features across the 4 models. First, cone degeneration always started after the major rod death phase (Fig. 5*a, b*). This point was reached at very different ages in three of the 4 mutants, as the overall kinetics of rod death were quite different. Second, cone death was always central to peripheral and was preceded by a reduction in OS length. Third, in all 4 mutants, red/green opsin protein levels were detectable mainly dorsally during cone degeneration (Fig. 5*c*). These common features evidence a common mechanism(s) of cone death. Moreover, gene expression changes that were common across the 4 models at the onset of cone death serve to elucidate this common mechanism.

EXAMPLE 2: Microarray analysis

To determine common gene expression changes, RNA samples from all 4 models were collected halfway through the major phase of rod death, at the onset of cone death, and from two time points during the cone death phase (Fig. 7a). The RNA was then hybridized to an Affymetrix 430 2.0 mouse array. Gene expression changes were compared within the same strain across the 4 time points. Two criteria had to be fulfilled to select a gene for cross comparison among the 4 strains. First, the change over time had to be statistically significant (see Material & Methods). Second, a gene had to be upregulated at least 2 fold at the onset of cone death compared to the other three time points. This second criterion removed rod-specific changes that were still occurring at the onset of cone death while at the same time enriched for changes at the onset of cone death. A total of 240 Affymetrix IDs were found that satisfied both criteria within each of the 4 strains. The 240 IDs matched to 230 genes (see Figure 20). Of the 195 genes that could be annotated, 34.9% (68 genes) were genes involved in cellular metabolism (Fig. 7b, c). The signaling pathway with the highest number of hits (12 genes) was the insulin/mTOR (mammalian target of rapamycin) signaling pathway (Fig. 7b), a key pathway in regulating many aspects of cellular metabolism. Thus, the data evidences that events at the onset of cone death coincided with changes in cellular metabolism likely to be regulated by the insulin/mTOR pathway.

EXAMPLE 3: mTOR in wild type and degenerating retinae

Based on the findings of the microarray analysis, the insulin/mTOR signaling pathway was examined during the period of cone death. The kinase, mTOR, is a key regulator of protein synthesis and ribosome biogenesis (Reiling, J.H. & Sabatini, D.M. (2006) *Oncogene* 25, 6373-83). When cellular energy levels are high, mTOR allows energy consuming processes, such as translation, and prevents autophagy, while nutrient poor conditions have the reverse effect. Therefore, glucose, which increases cellular ATP levels, and amino acid availability, especially that of leucine, positively affect mTOR activity. To understand if cellular energy levels or amino acid availability might be compromised in cones during degeneration, levels of phosphorylated mTOR (p*-mTOR) were examined by immunofluorescence. Phosphorylation of mTOR increases

kinase activity, and therefore levels of p*-mTOR can serve as an indicator of its activity level. Since every eukaryotic cell expresses mTOR, a certain level of p*-mTOR is likely to be found in every cell. Surprisingly, high levels of p*-mTOR were detected only in dorsal cones of wild type retinae (Fig. 9*a-c*). This phosphorylation pattern was reminiscent of the red/green opsin pattern seen during cone degeneration (Fig. 5*c*). Since mTOR is a key regulator of translation, we investigated whether the ventral red/green opsin downregulation that occurred during cone degeneration could be mimicked by a reduction in mTOR activity. To this end, wild type mice were treated with rapamycin, an mTOR inhibitor¹⁸. This treatment resulted in ventral downregulation of red/green opsin, without affecting blue opsin or PNA staining or the dorsal phosphorylation of mTOR itself (Fig. 9*d-g*). Thus, inhibition of mTOR in wild type recapitulated the expression of red/green opsin and blue opsin, as well as the pattern of PNA staining, in the mutants during degeneration, indicating that the ventral downregulation of red/green opsin seen during degeneration might be due to reduced mTOR activity. As expected for mTOR function, the downregulation of red/green opsin did not occur at the RNA level, but at the protein level, in untreated mutant mice, as well as in wild type mice treated with rapamycin (see Figure 8). Finally, analysis of mutant retinae showed a decrease of p*-mTOR levels in dorsal cones during cone degeneration (Fig. 9*h-m*). To test whether the high level of p*-mTOR found in dorsal wild type cones was glucose-dependent, retinal explants of wild type mice were cultured in media for 4 hours in the presence or absence of glucose. Dorsal p*-mTOR was abolished in the absence of glucose even when leucine concentrations were increased in the medium (see Figure 10). Thus, the data on mTOR establish a link between mTOR activity, the expression changes of red/green opsin seen during degeneration, and the microarray data, which indicated metabolic changes at the onset of cone death. Those changes may be caused by compromised glucose uptake in cones.

EXAMPLE 4: Responses of cones to nutritional imbalance

The data on mTOR evidenced a nutritional imbalance in cones during cone degeneration, possibly caused by reduced glucose levels in cones. To test this idea, the level of the heterodimeric transcription factor, Hypoxia inducible factor 1 (HIF-1 α/β), which improves glycolysis under stress conditions such as low oxygen, was examined.

HIF-1 and mTOR are tightly linked as low oxygen results in low energy due to reduced oxidative phosphorylation, and therefore in reduced mTOR activity (Reiling, J.H. & Sabatini, D.M. (2006) *Oncogene* 25, 6373-83; Dekanty, A., *et al.* (2005) *J Cell Sci* 118, 5431-41; Hudson, C.C. *et al.* (2002) *Mol Cell Biol* 22, 7004-14; Treins, C., *et al.* (2002) *J Biol Chem* 277, 27975-81; Zhong, H. *et al.* (2000) *Cancer Res* 60, 1541-5; Thomas, G.V. *et al.* (2006) *Nat Med* 12, 122-7). An upregulation of the regulated subunit HIF-1 α would likely reflect low glucose levels in cones, and not hypoxic conditions, as oxygen levels are increased due to the loss of rods (Yu, D.Y. & Cringle, S.J. (2005) *Exp Eye Res* 80, 745-51). Immunofluorescence analysis of HIF-1 α during cone degeneration revealed an upregulation of the protein in cones in all 4 mouse models (Figure 11a-f and 12a-d). Consistent with the upregulation of HIF-1 α , glucose transporter 1 (GLUT1), a HIF-1 α target gene (Wang, G.L., *et al.* (1995) *Proc Natl Acad Sci U S A* 92, 5510-4; Ebert, B.L., *et al.* (1995) *J Biol Chem* 270, 29083-9) also was found to be upregulated in cones, again in all 4 mouse models (Figure 11g-j and Figure 12e-h). Thus HIF-1 α and GLUT1 upregulation are consistent with a response in cones to overcome a shortage of glucose. It also provides a link to the decreased p*-mTOR levels found during degeneration as well as the sensitivity of p*-mTOR to glucose.

To ascertain if cones are nutritionally deprived, autophagy within cones was assessed. Two types of autophagy are inducible by various degrees of nutrient deprivation: macroautophagy and chaperone mediated autophagy (CMA) (Massey, A., *et al.* (2004) *Int J Biochem Cell Biol* 36, 2420-34; Finn, P.F. & Dice, J.F. (2006) *Nutrition* 22, 830-44; Codogno, P. & Meijer, A.J. (2005) *Cell Death Differ* 12 Suppl 2, 1509-18; Dice, J.F. (2007) *Autophagy* 3, 295-9). Macroautophagy is non-selective, targets proteins or entire organelles, and is marked by *de novo* formation of membranes that form intermediate vesicles (autophagosomes) that fuse with the lysosomes. The machinery required for macroautophagy has been shown to be present in PRs (Kunchithapautham, K. & Rohrer, B. (2007) *Autophagy* 3, 433-41). In contrast, CMA is selective and targets individual proteins for transport to the lysosomes. The presence of macroautophagy was assessed by infection with a viral vector encoding a fusion protein of green fluorescent protein (GFP) and light chain 3 (LC3), an autophagosomal membrane marker (Kabeya, Y. *et al.* (2000) *Embo J* 19, 5720-8; Mizushima, N., *et al.* (2004) *Mol Biol Cell* 15, 1101-11; Punzo, C. & Cepko, C.L. (2008) *Dev Dyn* 237, 1034-

42). No difference was observed in GFP distribution in cones of wild type and mutant mice, indicating that formation of autophagosomes was absent during cone death (see Figure 14a-f). Additionally, high levels of phosphorylated ribosomal protein S6 were found in all, or most, cones (see Figure 14g-h) reflecting an increased activity of ribosomal S6 kinase 1 (S6K1), an inhibitor of macroautophagy (Codogno, P. & Meijer, A.J. (2005) *Cell Death Differ* 12 Suppl 2, 1509-18). Consistent with these findings is the fact that macroautophagy reflects an acute short-term response to nutrient deprivation or cellular stress conditions (Massey, A., *et al.* (2004) *Int J Biochem Cell Biol* 36, 2420-34; Finn, P.F. & Dice, J.F. (2006) *Nutrition* 22, 830-44). Prolonged non-selective degradation of newly synthesized proteins to overcome the stress condition would not be favorable to cells and would likely result in the relatively rapid death of most cones, rather than the slow death seen in RP.

CMA is normally activated over extended periods of starvation and results in increased levels of lysosomal-associated membrane protein (LAMP) type 2A at the lysosomal membrane (Massey, A., *et al.* (2004) *Int J Biochem Cell Biol* 36, 2420-34; Finn, P.F. & Dice, J.F. (2006) *Nutrition* 22, 830-44; Cuervo, A.M. & Dice, J.F. (2000) *Traffic* 1, 570-83). Both starvation and oxidative stress can induce CMA Massey, A., *et al.* (2004) *Int J Biochem Cell Biol* 36, 2420-34). Starvation increases LAMP-2A by preventing its degradation while oxidative stress results in *de novo* synthesis of LAMP-2A (Kiffin, R., *et al.* (2004) *Mol Biol Cell* 15, 4829-40). A LAMP-2 antibody that recognizes the proteins resulting from all 3 splice isoforms (Cuervo, A.M. & Dice, J.F. (2000) *J Cell Sci* 113 Pt 24, 4441-50) (A, B, C) showed high levels of LAMP-2 at the lysosomal membrane in all 4 mutants during cone degeneration (Fig. 13a-c; data only shown for PDE- β -/-). The high levels were specific to cones and were not seen in cells of the inner nuclear layer (Fig. 13b, c), which might reflect the possibility that cones are the only starving cells in the RP retina. qRT-PCR for the three splice isoforms showed only a minor increase in mRNA levels of LAMP-2A (1.2x) and a decrease in LAMP-2C (Fig. 13d) indicating that the increase seen in protein at the membrane is mainly due to nutritional deprivation and only to a lesser extent to oxidative stress (Komeima, K., *et al.* (2006) *Proc Natl Acad Sci U S A* 103, 11300-5; Komeima, K., *et al.* (2007) *J Cell Physiol.* 275, 28139-28143; Kiffin, R., *et al.* (2004) *Mol Biol Cell* 15, 4829-40). Taken

together, the data demonstrates that nutritional imbalance in cones leads to the activation of CMA, a process that is consistent with prolonged starvation.

EXAMPLE 5: Stimulation of the insulin receptor pathway prolongs cone survival

The data on mTOR, HIF-1 α , GLUT1 and the induction of CMA demonstrated that a shortage of glucose in cones resulting in starvation and further demonstrated that the insulin/mTOR pathway plays an important role during cone death. To determine if the insulin/mTOR pathway can influence cone survival, we stimulated the pathway by systemic treatment of PDE- β ^{-/-} mice with insulin. The PDE- β mutant was chosen over the other three mutants due to its faster cone death kinetics, allowing for a better read-out of cone survival. Mice were treated with daily intraperitoneal injections of insulin over a 4 week period, starting at the onset of cone death. To reduce insulin, a single injection of streptozotocin, a drug that kills the insulin-producing beta cells of the pancreas, also was examined. Systemic administration of insulin results in a desensitized insulin receptor due to a feedback loop in the pathway, which causes an increase in blood glucose levels. Injection of streptozotocin, which also results in increased blood glucose levels, served as a control for the effect of elevated blood glucose, and also provided animals with reduced levels of insulin. PDE- β ^{-/-} mice injected with insulin showed improved cone survival compared to uninjected control mice. PDE- β ^{-/-} mice injected with Streptozotocin showed a decrease in cone survival (Fig. 15*a-d*). Improved cone survival was therefore due to insulin and not to the increased blood glucose levels (Fig. 15*e*). Additionally, cones in mutant mice treated with insulin did not show the upregulation of HIF-1 α seen normally in cones during degeneration, consistent with the notion that cones were responding to insulin directly (Fig 15*g, h*).

DISCUSSION

The results presented herein show that cones exhibit signs of nutritional imbalance during the period of cone degeneration in RP mice. The microarray analysis demonstrates that there are changes in cellular metabolism involving the insulin/mTOR pathway at the onset of cone death. It was demonstrated that inhibition of mTOR in wild type mice resulted in the same pattern of loss of red/green opsin as seen during

degeneration. In accord with changes in p*-mTOR, and its sensitivity to glucose, an upregulation of HIF-1 α and GLUT1 was observed, demonstrating that glucose uptake, and/or the intracellular levels of glucose, may be compromised in cones of RP mice. Additionally, systemic administration of insulin prolonged cone survival, whereas depletion of endogenous insulin had the reverse effect. The systemic treatment with insulin prevented the upregulation of HIF-1 α in cones seen normally during cone degeneration, demonstrating that insulin was directly acting on cones. Interestingly, a prolonged treatment of insulin during a time span of 7 weeks instead of 4 weeks did not show any significant improvement of cone survival (see Figure 18). This may reflect the feedback loop of the pathway in which S6K1 acts directly onto the insulin-receptor substrate (IRS). The results indicate that nutrient availability in cones may be altered during the period of cone degeneration and that the insulin/mTOR pathway plays a crucial role. A recent report showed that constitutive expression of proinsulin in the rd10 mouse model of RP delays photoreceptor death, both of rods and cones (Corrochano, S. *et al.* (2008) *Invest Ophthalmol Vis Sci* 49, 4188-94). However, proinsulin seems not to act through the insulin receptor as mice treated with proinsulin did not develop hyperglycemia. Proinsulin blocks developmental cell death and thus may interfere with the apoptotic pathway in the postnatal retina. Macroautophagy, which is controlled by mTOR through its downstream target S6K1, was not detected during cone degeneration, while CMA appeared to be activated. Increased LAMP-2A levels at the lysosomal membrane indicated activation of CMA. In addition, the observations concerning mTOR, HIF-1 α , and GLUT1 are consistent with starvation and CMA. The lack of detectable macroautophagy does not rule out the possibility that macroautophagy might occur for a short period of time (*e.g.*, 24 hours) prior to the activation of CMA. The data only show that macroautophagy is not the main form of autophagy over an extended period of time, which is consistent with the notion that macroautophagy is a short-term response. The prolonged inhibition of macroautophagy is likely due to increased S6K1 activity as seen by increased p*-S6 levels. S6K1 is positively regulated by mTOR and AMP-activated protein kinase (AMPK) (Codogno, P. & Meijer, A.J. (2005) *Cell Death Differ* 12 Suppl 2, 1509-18), which reads out cellular ATP levels. Therefore, while mTOR may report metabolic problems with respect to glucose uptake, and reduce energy consuming processes and improve glycolysis through HIF-1 α , AMPK may report normal cellular ATP levels and inhibit macroautophagy. This represents a specific response to

the energy requirements of cones. Most of the glucose taken up by PRs never enters the Krebs cycle (Poitry-Yamate, C.L., *et al.* (1995) *J Neurosci* 15, 5179-91). Thus the shortage of glucose may not cause a shortage of ATP. Lactate, provided by Muller glia, can generate ATP via the Krebs cycle (Poitry-Yamate, C.L., *et al.* (1995) *J Neurosci* 15, 5179-91). However, glucose is needed to generate NADPH in the pentose phosphate cycle, and NADPH is required for synthesis of phospholipids, the building blocks of cell membranes. PRs constantly shed their membranes at the tip of the OSs. Since reduced levels of glucose would result in reduction of membrane synthesis, the rate of OS phagocytosis by the RPE may be higher than the rate of membrane synthesis by cones. Consistent with this, OS shortening preceded cell death in these 4 models, as is also observed in human cases of RP17. Additionally, changes that affect lipid metabolism were also seen by the microarray analysis.

These studies described herein were designed to determine why the loss of rods result in cone death in RP. The previous hypotheses attributing cone death either to a toxin released by rod cells or to the lack of a trophic factor produced by rod cells and necessary for cone survival each fail to explain the pathology found in humans. The rod and cone death kinetics shown here clearly argue against a toxin produced by dying rods as a cause for cone death since the onset of cone death always occurred after the major rod death period. If a rod toxin caused cone death, then the onset of cone death should have either coincided with the onset of rod death or should have started shortly thereafter, since this would be the period of peak toxin production. Interestingly, the lack of a trophic factor produced by healthy rods and required for cone survival would agree with the onset of cone death seen in all four models as one would expect the onset of cone death during the end stages of rod death. However, the progression of cone death and the end phase of rod death make this unlikely hypothesis as the sole reason for cone death. In the two PDE mutants and in the Rho-KO mutant, cones were dying for many weeks after the end phase of rod death, indicating that they could survive quite awhile in the absence of rods. In addition, in the P23H model, rods died so slowly during the end phase of rod death, that during the entire period of cone death, rods were still present. The hypothesis that a lack of a rod trophic factor being the main cause for cone death seems unlikely given these discrepancies.

The observations described herein of nutritionally deprived cones demonstrate the dependence of cones on rods. The OS-RPE interactions are vital since the RPE shuttles nutrition and oxygen from the choroidal vasculature to PRs. Roughly 95% of all PRs in mouse and human are rods and approximately 20-30 OSs contact one RPE cell (Snodderly, D.M., *et al.* (2002) *Invest Ophthalmol Vis Sci* 43, 2815-8; Young, R.W. (1971) *Journal of Cell Biology* 49, 303-318). Thus, only 1-2 of those RPE-OS contacts are via cones. During the collapse of the ONL, the remaining cone:RPE interactions are likely perturbed. If these interactions drop below a threshold required for the proper flow of nutrients, the loss of rods results in a reduced flow of nutrients to cones. In all 4 mouse models, the onset of cone death occurred when the ONL reached one row of cells. This cell density therefore represents the critical threshold. Then, while the remaining rods die due to a mutation in a rod-specific gene, cone death begins due to nutrient deprivation. In accord with this notion, cone death progressed more slowly when the remaining rods died slowly. This mechanism would also explain why the loss of cones does not lead to rod death (Biel, M. *et al.* (1999) *Proc Natl Acad Sci U S A* 96, 7553-7; Yang, R.B. *et al.* (1999) *J Neurosci* 19, 5889-97). Since in humans and mouse, cones are less than 5% of all PRs, the critical threshold that perturbs OS-RPE interactions would not be reached. Further support for this idea is provided by studies in zebrafish where the overall ratio of rods to cones is reversed (1:8). Additionally, the distribution of rods and cones in zebrafish is uneven such that certain regions are cone-rich whereas other regions are rod-rich. A recently isolated mutation in a cone-specific gene resulted in rod death, but only in regions of high cone density (Stearns, G., *et al.* (2007) *J Neurosci* 27, 13866-74), leading Stearns and co-workers to conclude that cell density is the crucial determinant. We determined that once a critical threshold of cell density is breached, improper OS-RPE interactions result in reduced flow of nutrients (*e.g.*, glucose). This results in reduced OS membrane synthesis, which in turn further contributes to a reduced uptake of nutrients from the RPE. Ultimately, prolonged starvation, as indicated by the activation of CMA, leads to cell death. Since starvation can occur slowly over extended periods of time, and because the rate may fluctuate due to fluctuations in nutrient uptake, the slow and irregular demise of cones observed in humans results therefrom. Therefore, the results presented herein not only provide a new mechanism of cone death in RP that should direct future therapeutic approaches, but also consolidate the data from the

literature with respect to the death kinetics of rods and cones seen in mice and patients with different RP mutations.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

We claim:

1. A method for treating or preventing a retinal disorder in a subject comprising administering to said subject an mTOR modulator in an amount effective for modulating mTOR activity in the subject, thereby treating or preventing the retinal disorder in the subject.
2. The method of claim 1, wherein the retinal disorder is retinitis pigmentosa.
3. The method of claim 1, wherein the retinal disorder is associated with decreased viability of cone cells.
4. The method of claim 1, wherein the retinal disorder is associated with decreased viability of rod cells.
5. The method of claim 1, wherein the retinal disorder is a genetic disorder.
6. The method of claim 1, wherein the retinal disorder is not diabetic retinopathy.
7. The method of claim 1, wherein the retinal disorder is not associated with blood vessel leakage and/or growth.
8. A method for treating or preventing retinitis pigmentosa in a subject comprising administering to said subject an mTOR modulator in an amount effective for modulating mTOR activity in the subject, thereby treating or preventing retinitis pigmentosa in the subject.

9. A method for prolonging the viability of cone cells, comprising contacting the cone cells with an mTOR modulator in an amount effective for modulating mTOR activity, thereby prolonging the viability of the cone cells.
10. The methods of any one of claim 1, 8, or 9, wherein the mTOR modulator is a glucose enhancer.
11. The method of any one of claims 1, 8 or 9, wherein the mTOR modulator is selected from the group consisting of insulin, growth factors, IGF-1, IGF-2, mitogens, serum, phosphatidic acid, amino acids, leucine, and analogues or derivatives thereof.
12. The method of claim 10 or 11, wherein the mTOR modulator is insulin.
13. The method of claim 10 or 11, wherein the mTOR modulator is not insulin.
14. The method of any one of claims 1, 8 or 9, wherein the mTOR modulator stimulates mTOR phosphorylation.
15. The method of any one of claims 1, 8 or 9, wherein the mTOR modulator activates a receptor and/ or a signal transduction cascade upstream of mTOR.
16. A method for treating or preventing a retinal disorder in a subject comprising enhancing the intracellular levels of glucose in said subject, thereby treating or preventing the retinal disorder.
17. The method of claim 16, wherein the retinal disorder is retinitis pigmentosa.

18. The method of claim 16, wherein the retinal disorder is associated with decreased viability of cone cells.
19. The method of claim 16, wherein the retinal disorder is associated with decreased viability of rod cells.
20. The method of claim 16, wherein the retinal disorder is a genetic disorder.
21. The method of claim 16, wherein the retinal disorder is not diabetic retinopathy.
22. The method of claim 16, wherein the retinal disorder is not associated with blood vessel leakage and/or growth.
23. A method for treating or preventing retinitis pigmentosa in a subject comprising enhancing the intracellular levels of glucose in said subject, thereby treating or preventing retinitis pigmentosa.
24. The method of claim 16 or 23, wherein the subject is administered glucose in an amount effective to enhance the intracellular levels of glucose in the subject.
25. The method of claim 23, wherein glucose is administered intravenously.
26. The method of claim 16 or 23, wherein the subject is administered a glucose enhancer in an amount effective to enhance the intracellular levels of glucose in the subject.

27. The method of claim 26, wherein the glucose enhancer modulates biochemical pathways leading to enhanced intracellular glucose levels.
28. The method of claim 26, wherein the glucose enhancer increases the uptake of glucose into rod and/or cone cells.
29. A method for prolonging the viability of cone cells, comprising enhancing the intracellular levels of glucose in said cell, thereby prolonging the viability of the cone cells.
30. The method of claim 29, comprising exposing the cone cells to glucose in an amount effective to enhance the intracellular levels of glucose in the cone cells.
31. The method of claim 29, comprising exposing the cone cells to a glucose enhancer in an amount effective to enhance the intracellular levels of glucose in the cone cells.
32. The method of claim 31, wherein the glucose enhancer modulates biochemical pathways leading to enhanced intracellular glucose levels.
33. The method of claim 29, wherein the glucose enhancer increases the uptake of glucose into rod and/or cone cells.

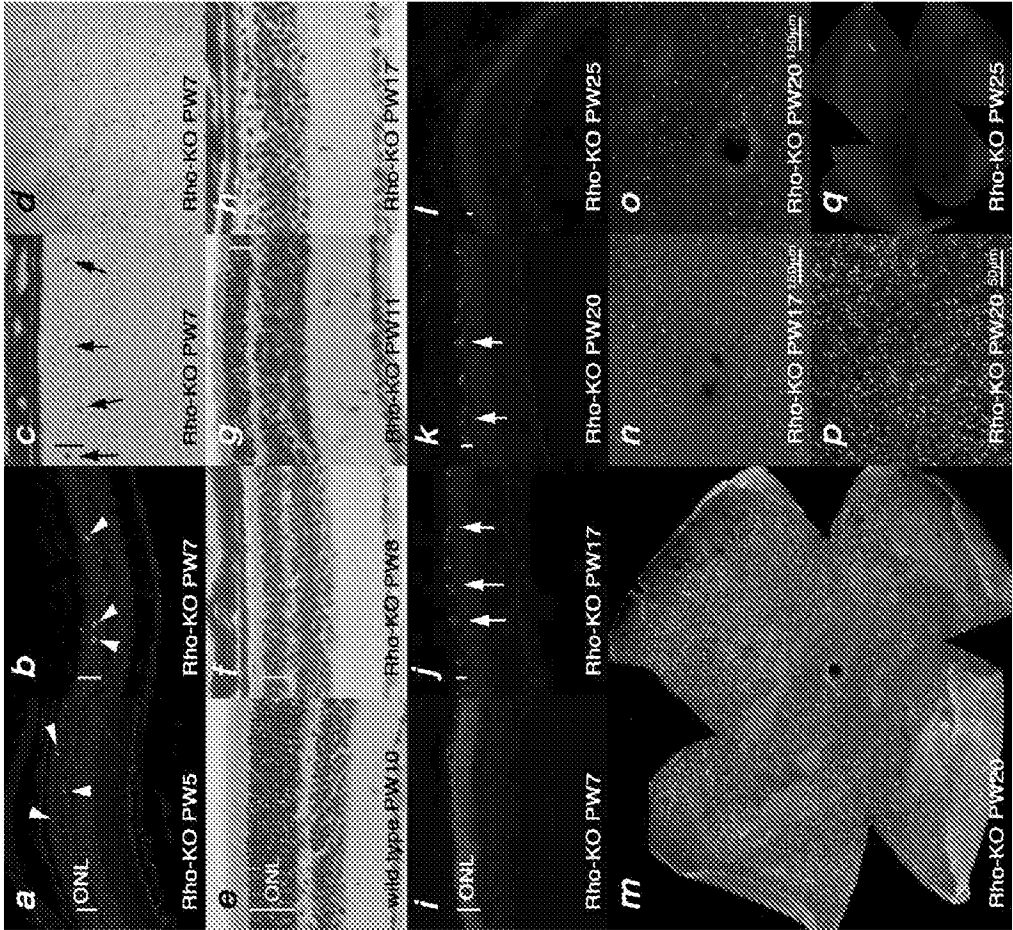


Fig. 1a-1q

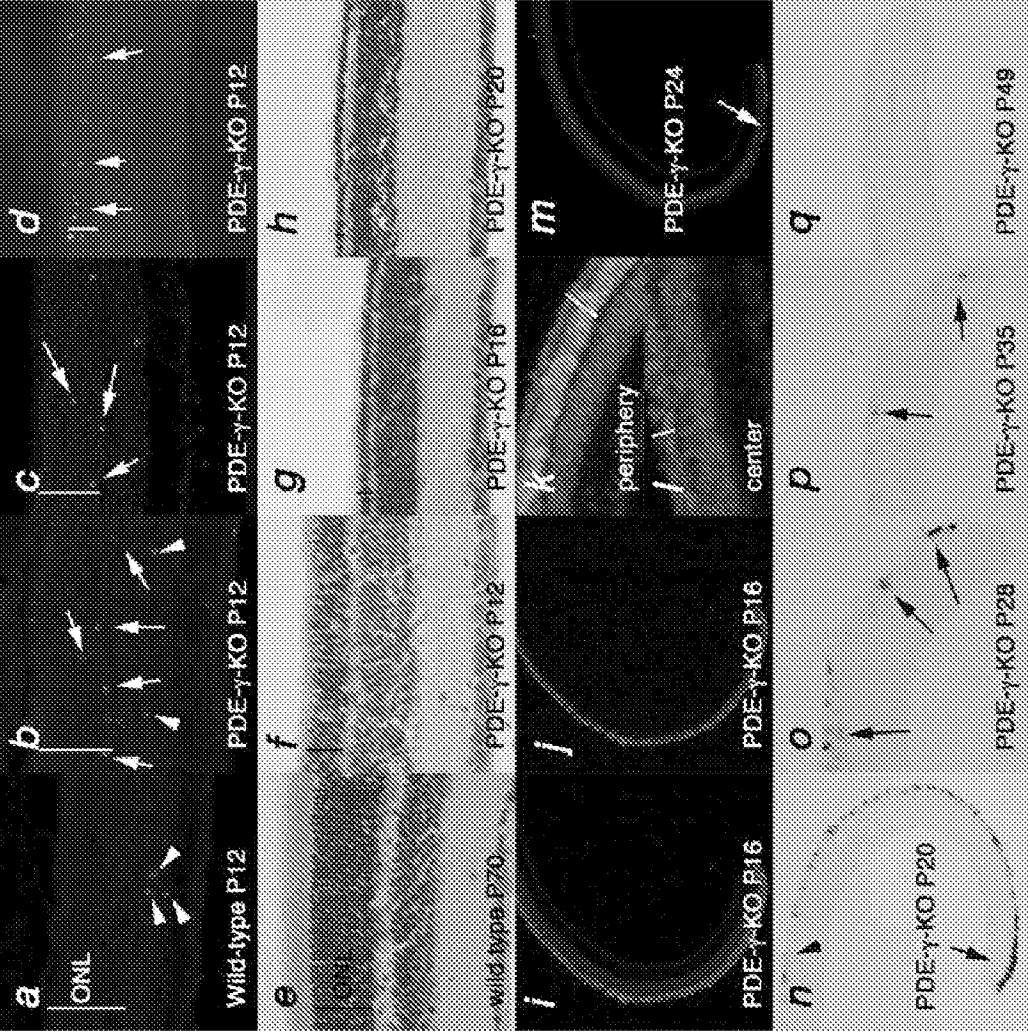


Fig. 2a-2q

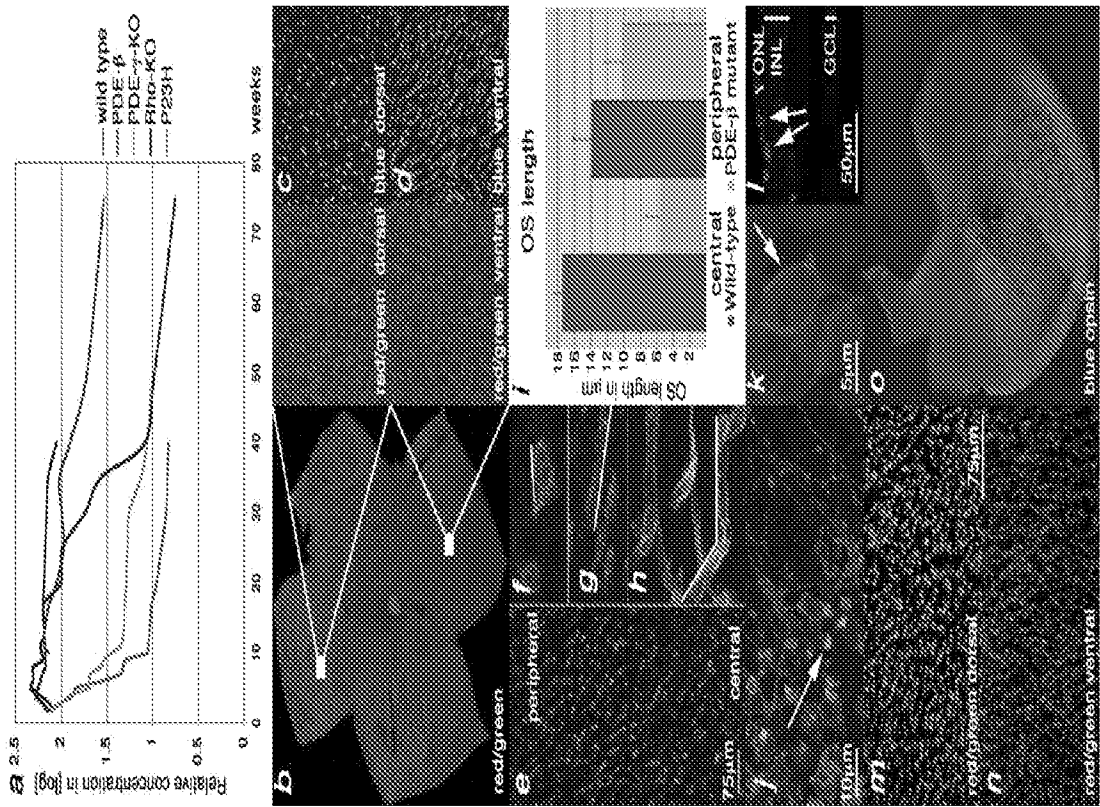


Fig. 3a-30

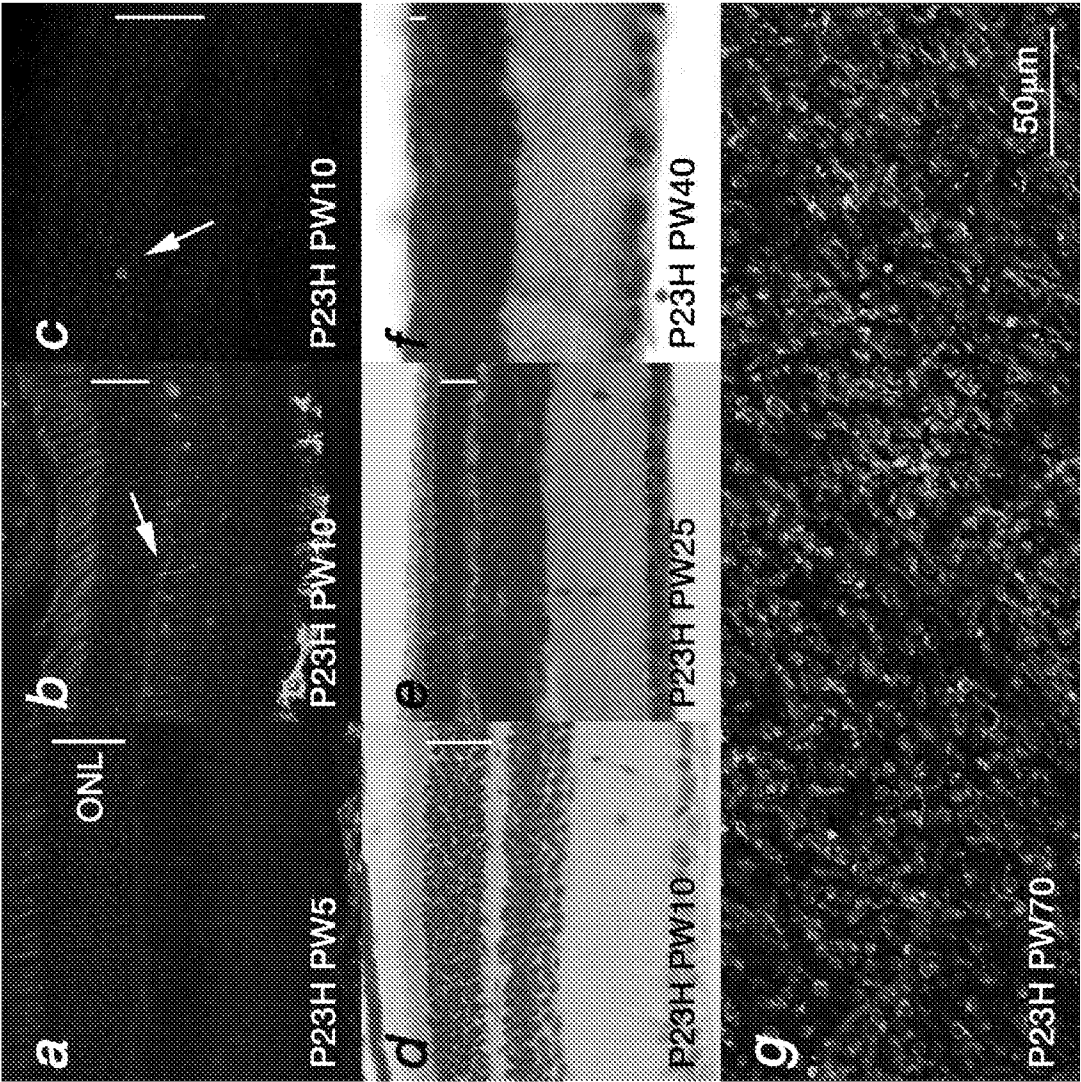


Fig. 4a-4g

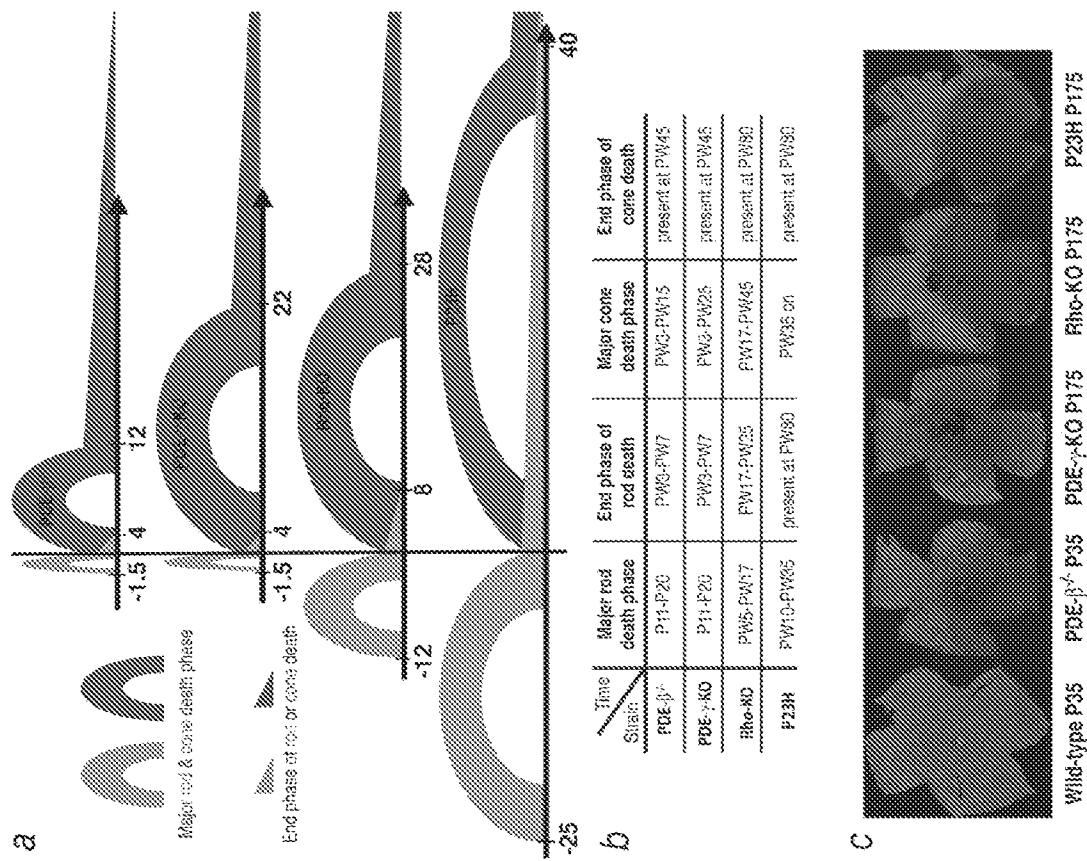


Fig. 5a-5c

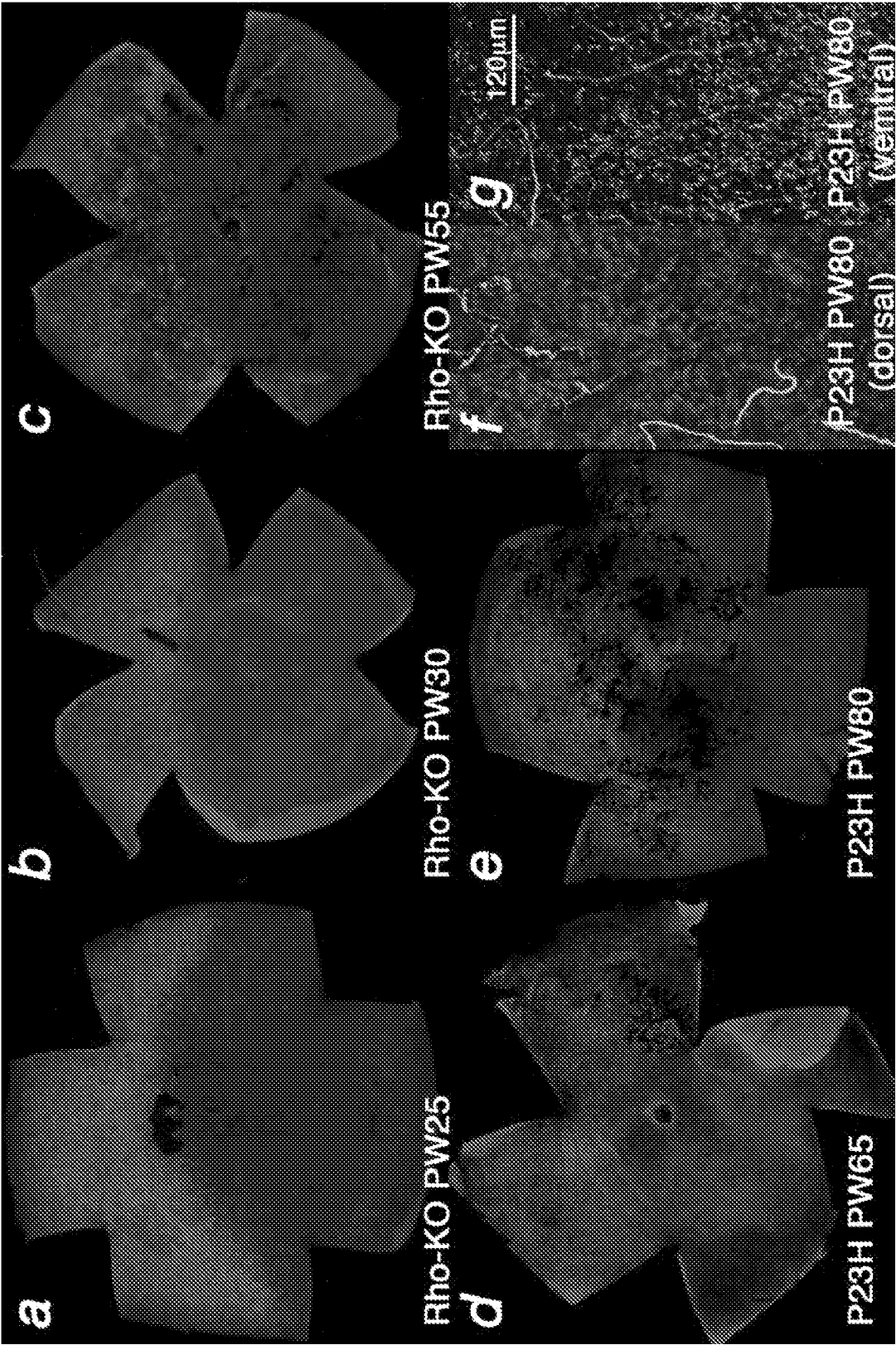


Fig. 6a-6g

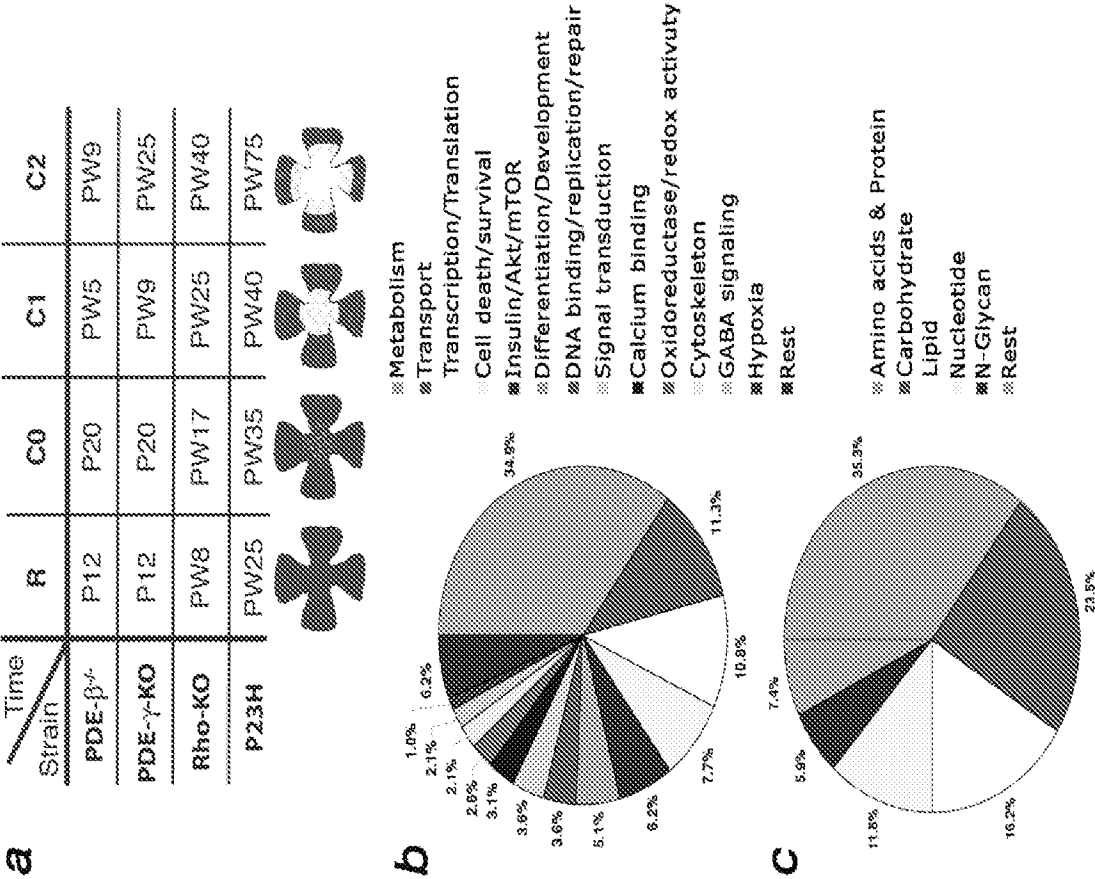


Fig. 7a-7c

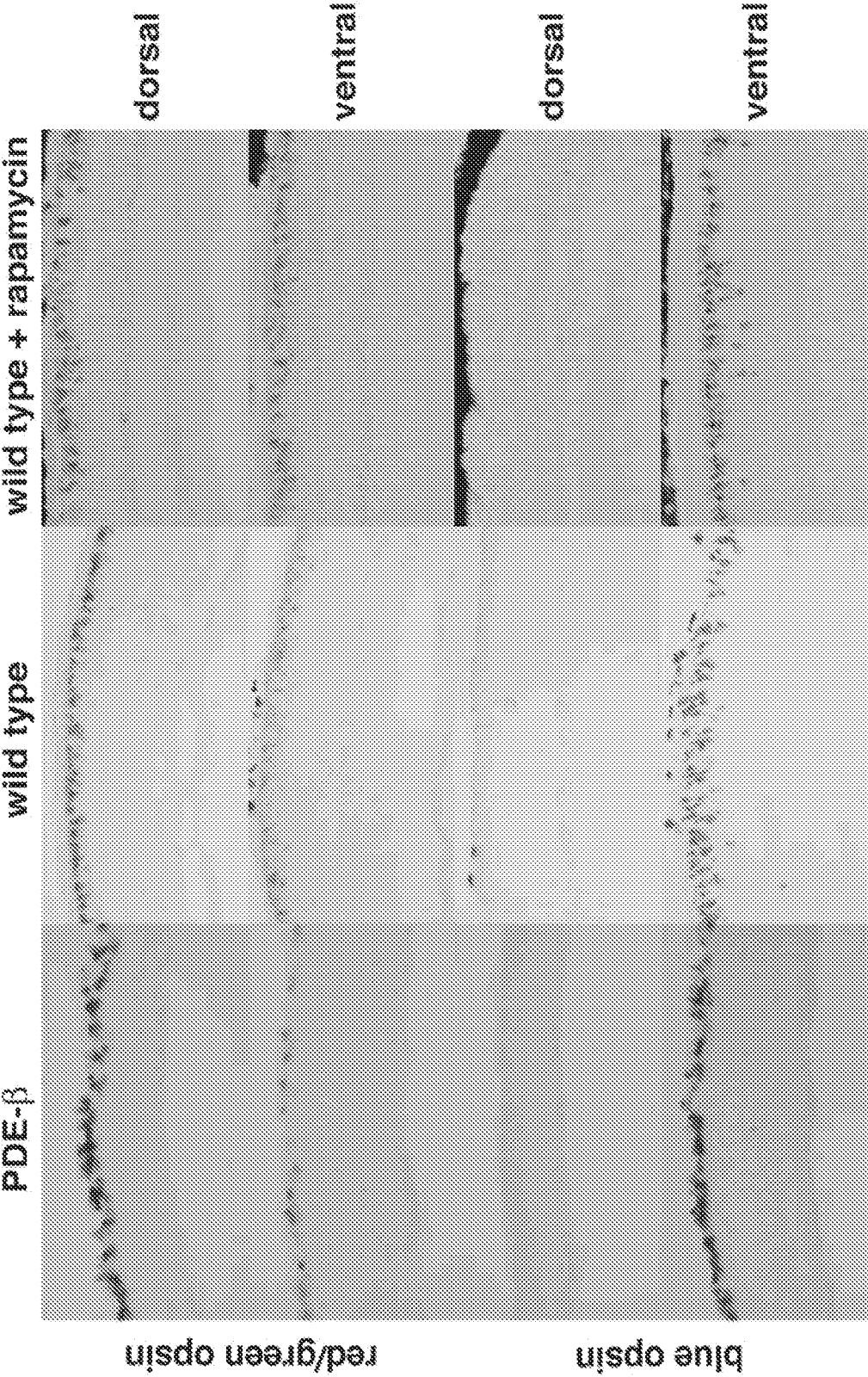


Fig. 8

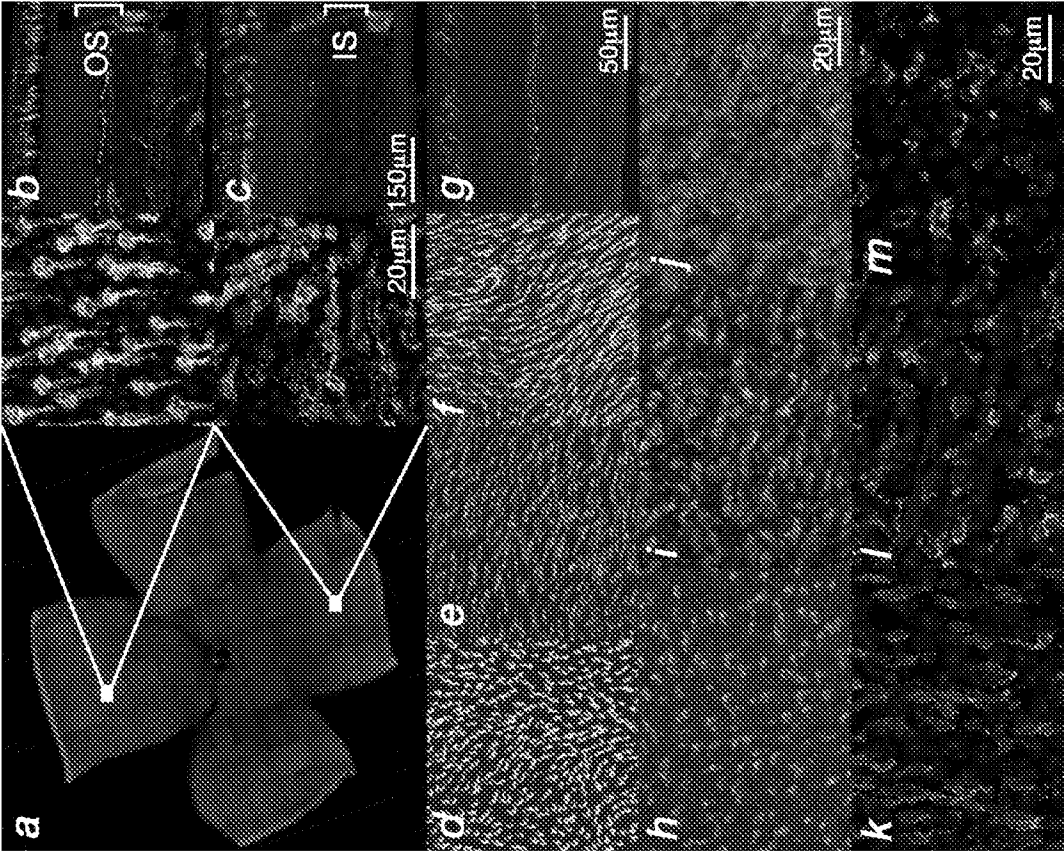


Fig. 9a-9m

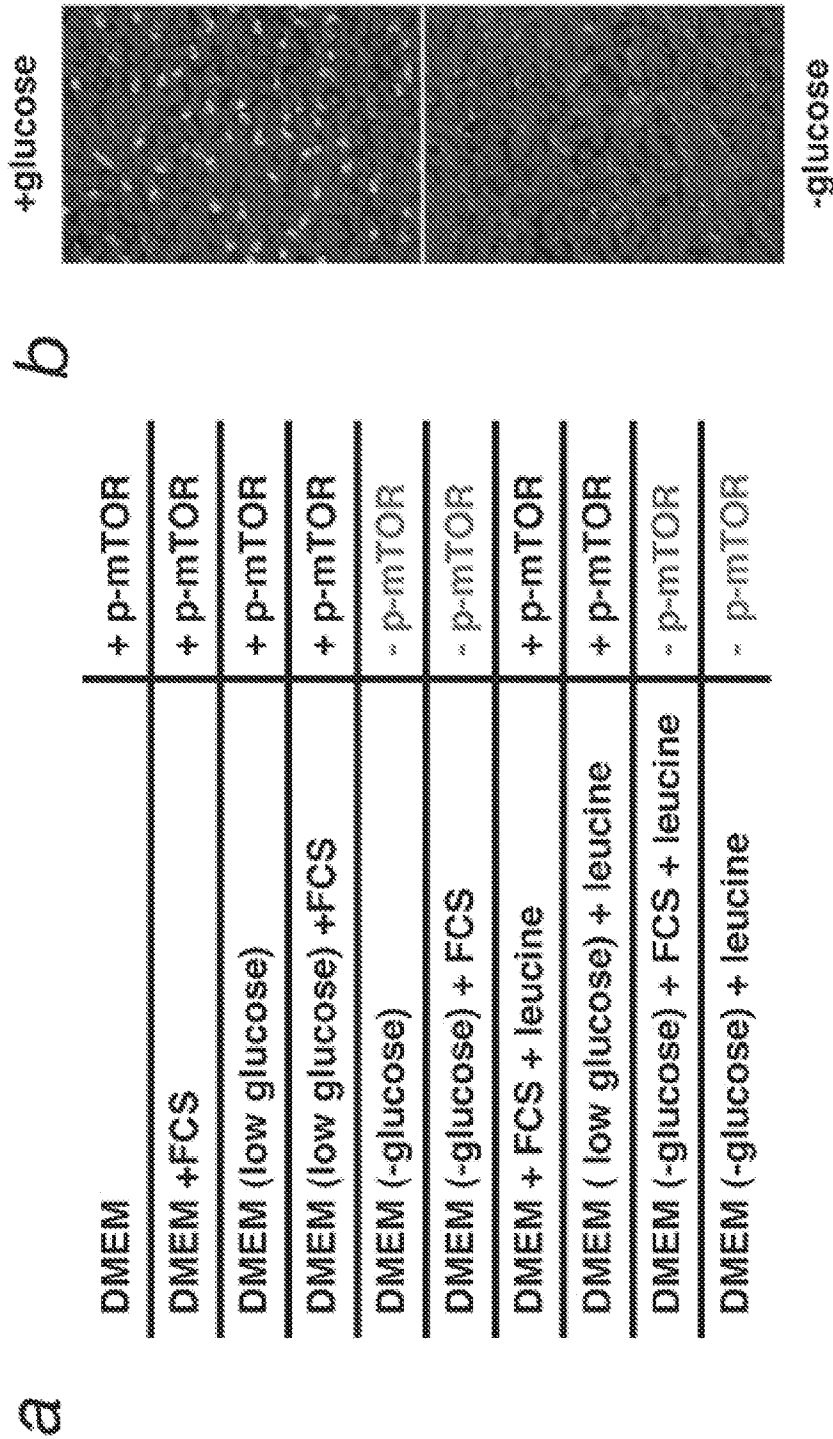


Fig. 10a-10b

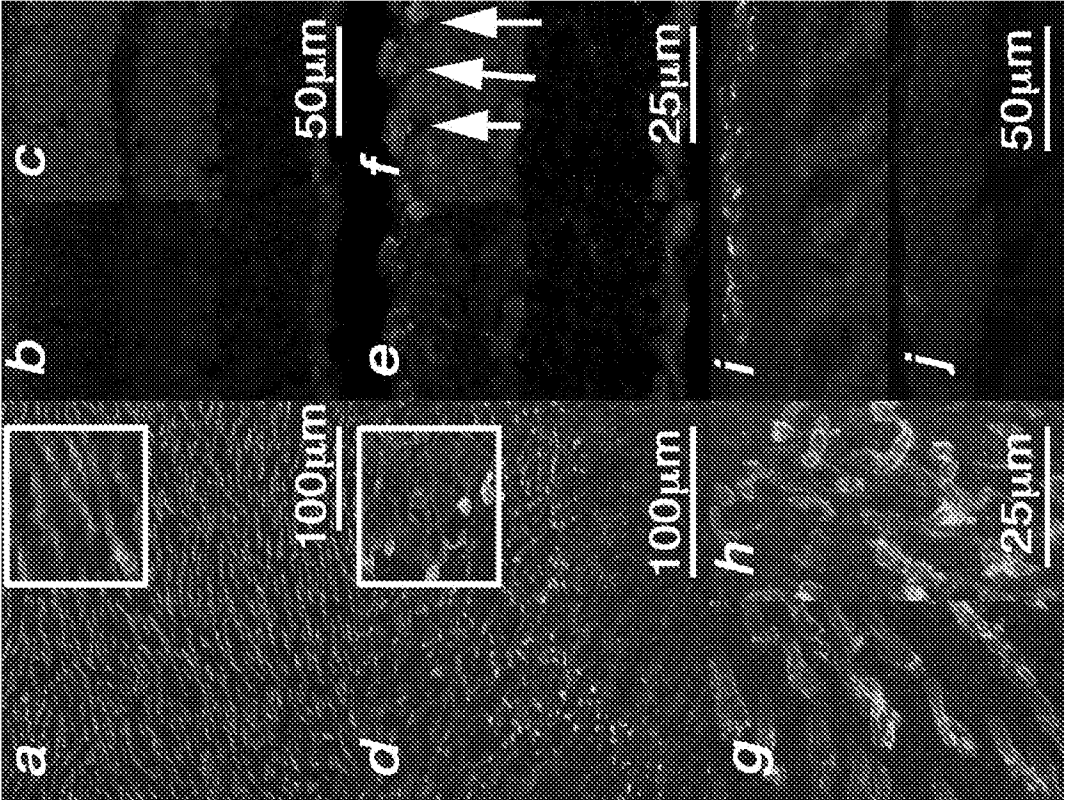


Fig. 11a-11j

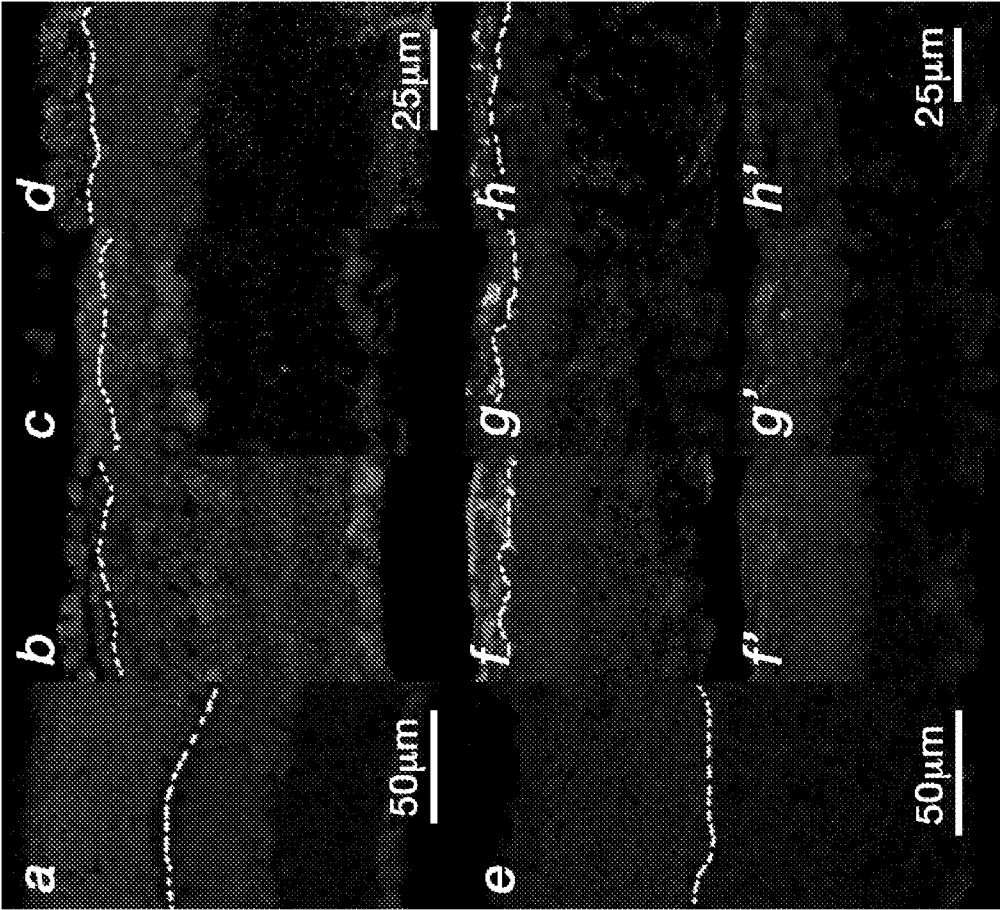


Fig. 12a-12h

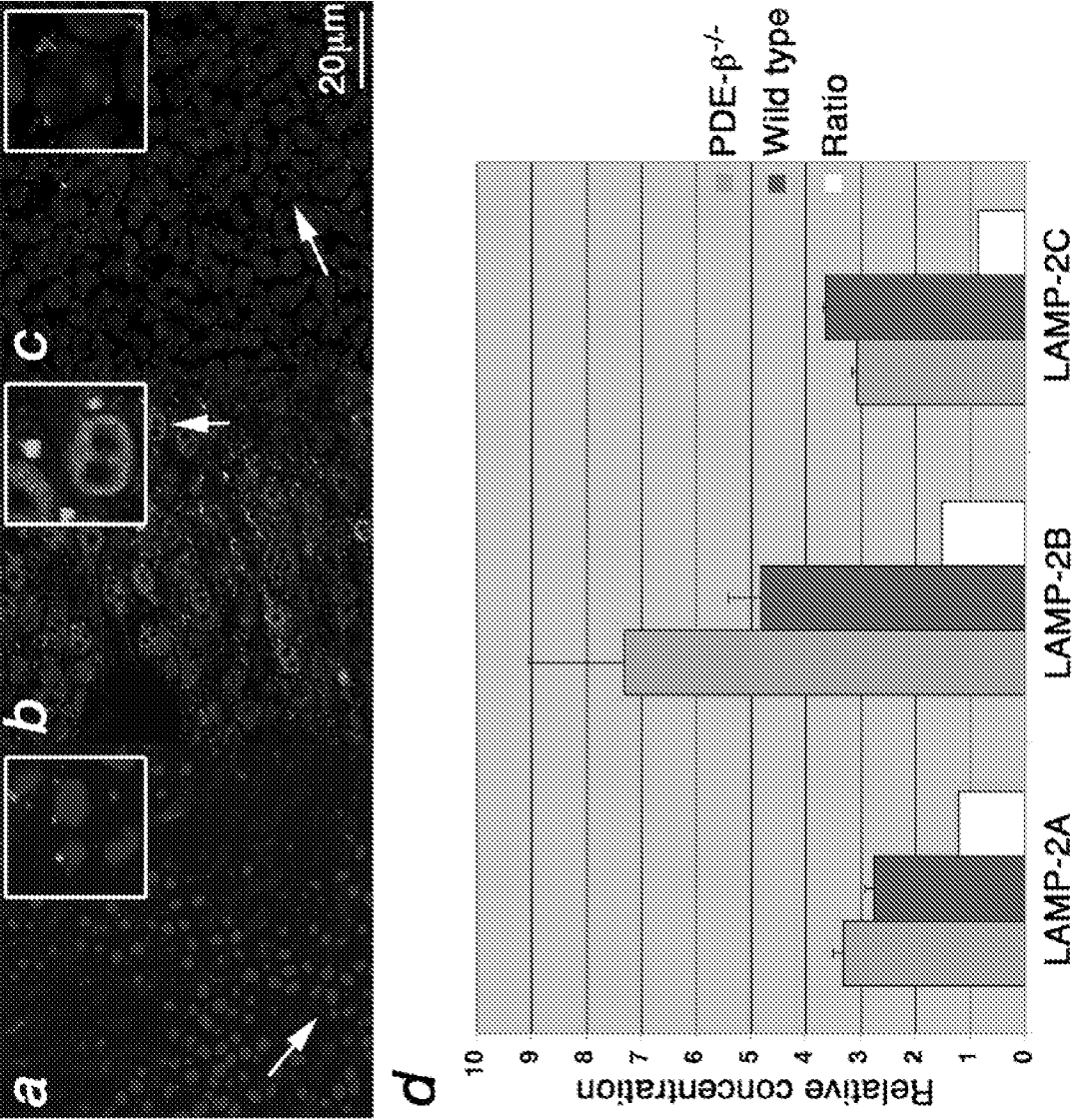


Fig. 13a-13d

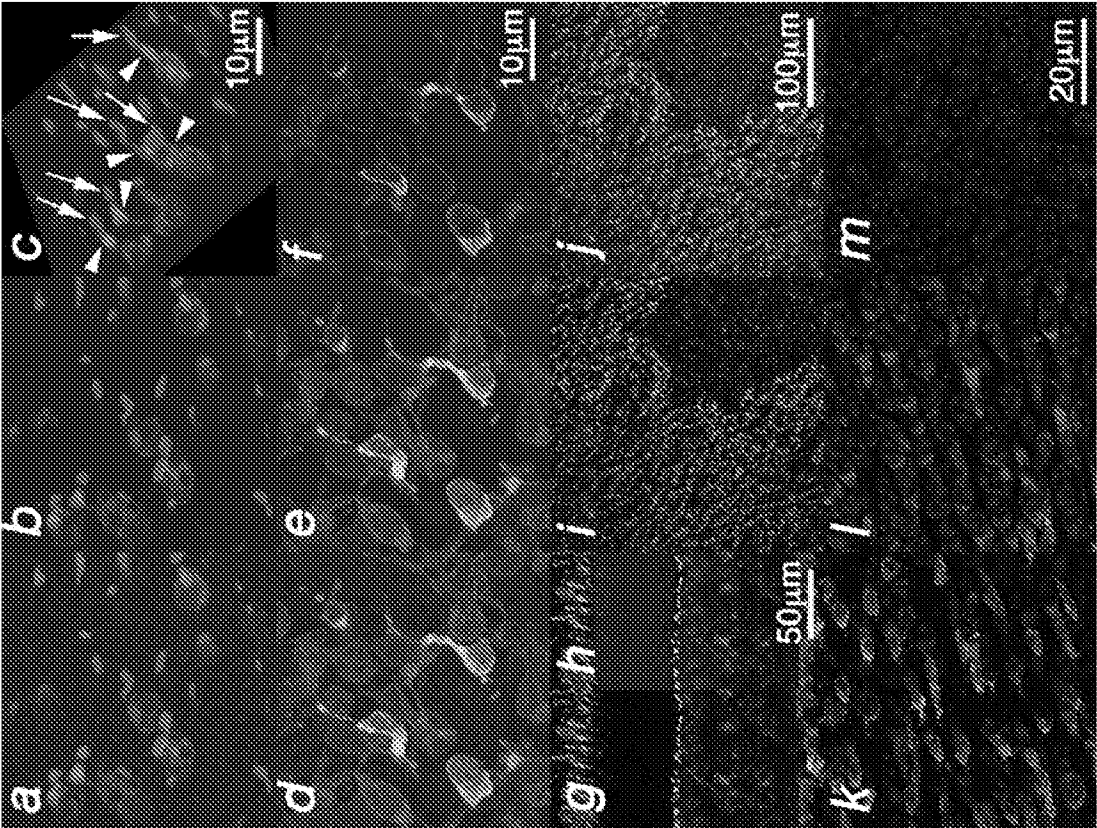


Fig. 14a-14m

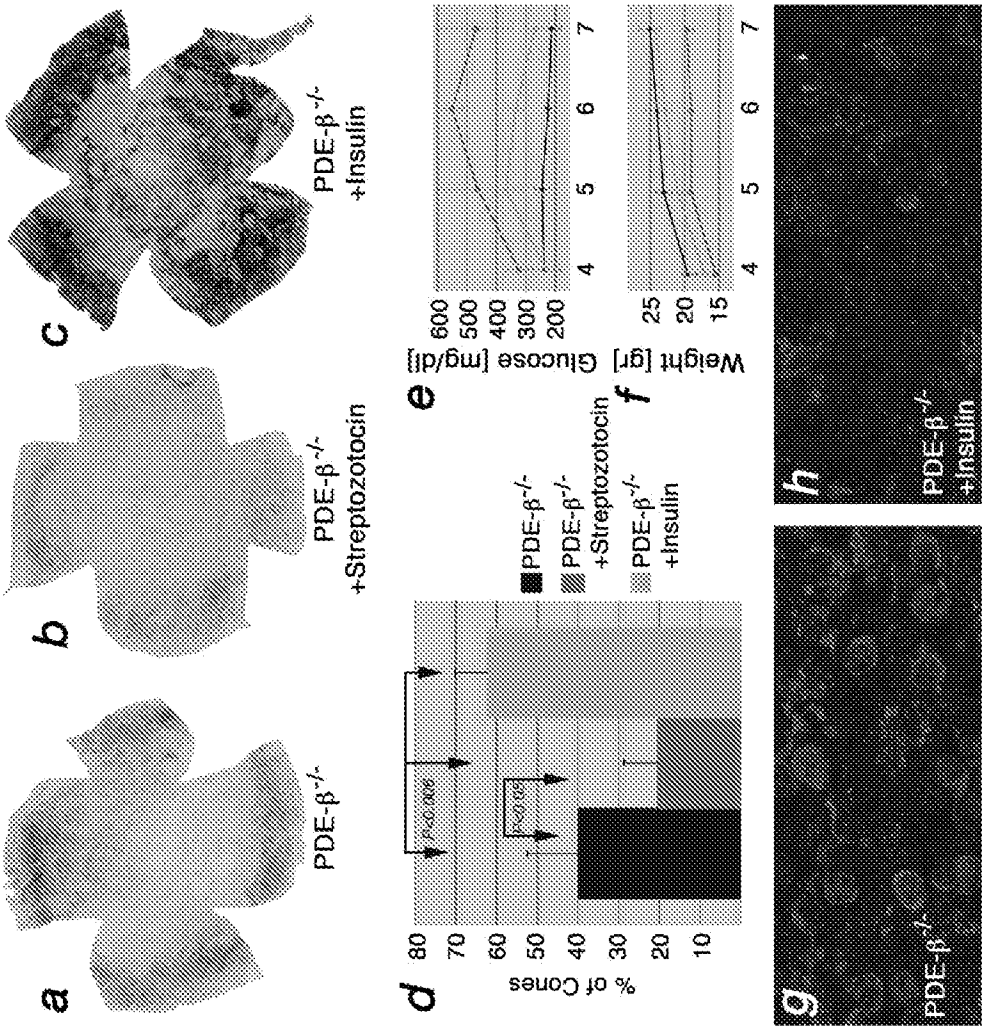


Fig. 15a-15h

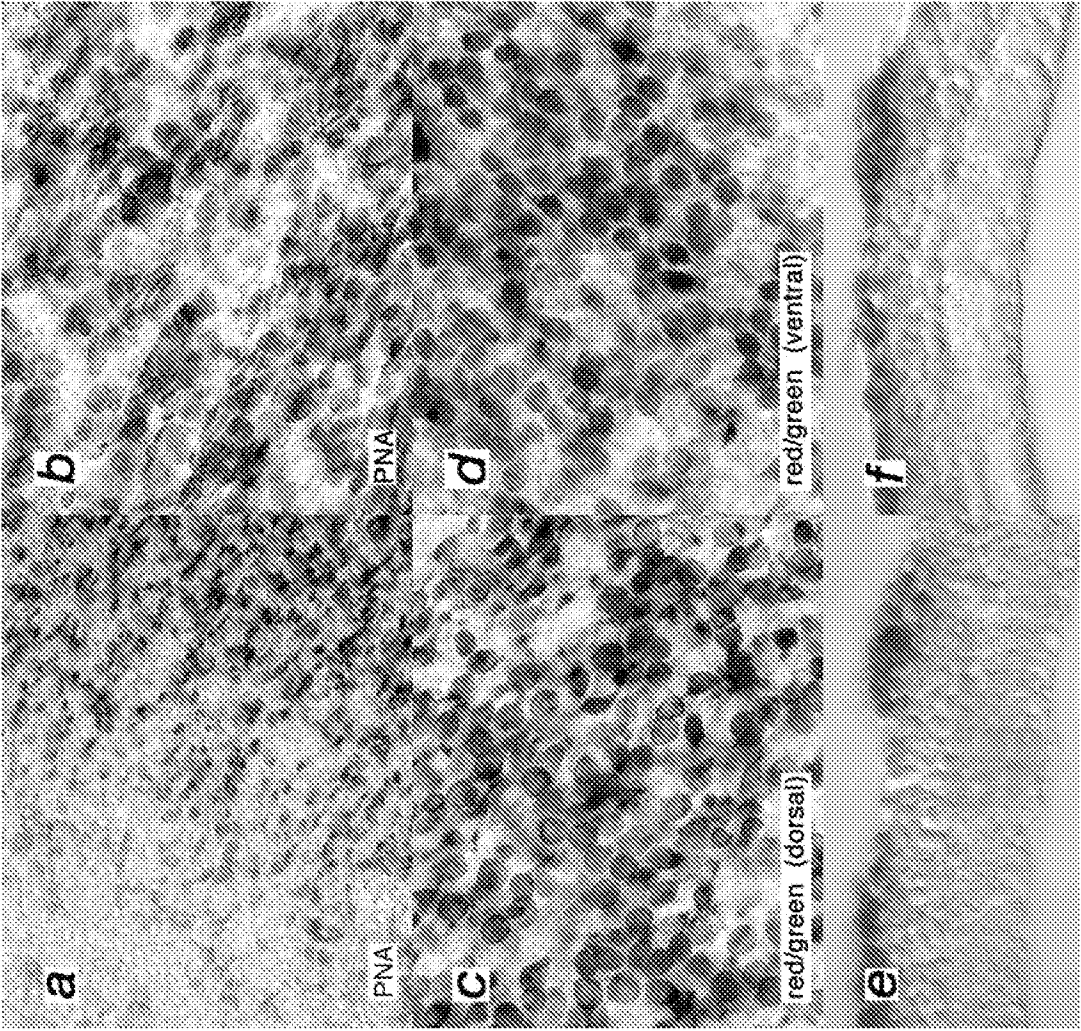


Fig. 16a-16f

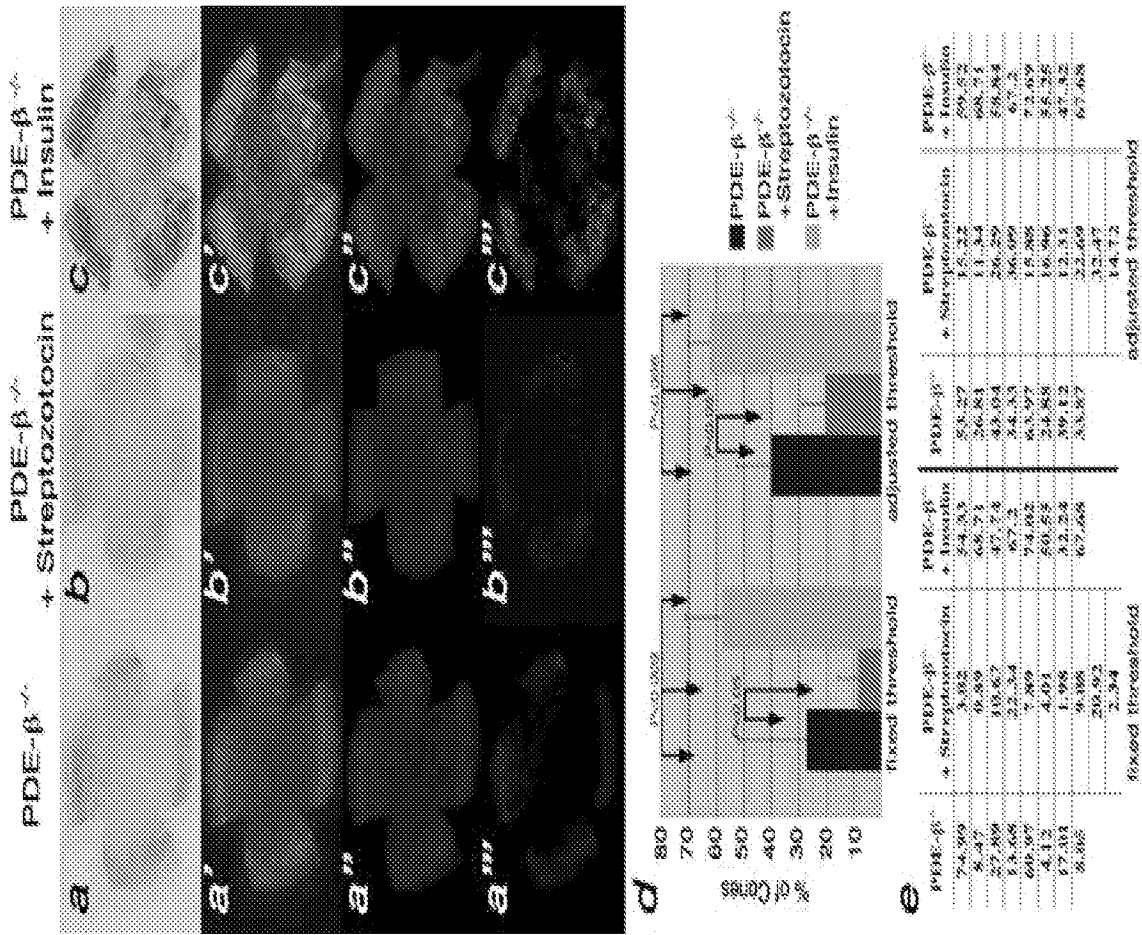


Fig. 17a-17e

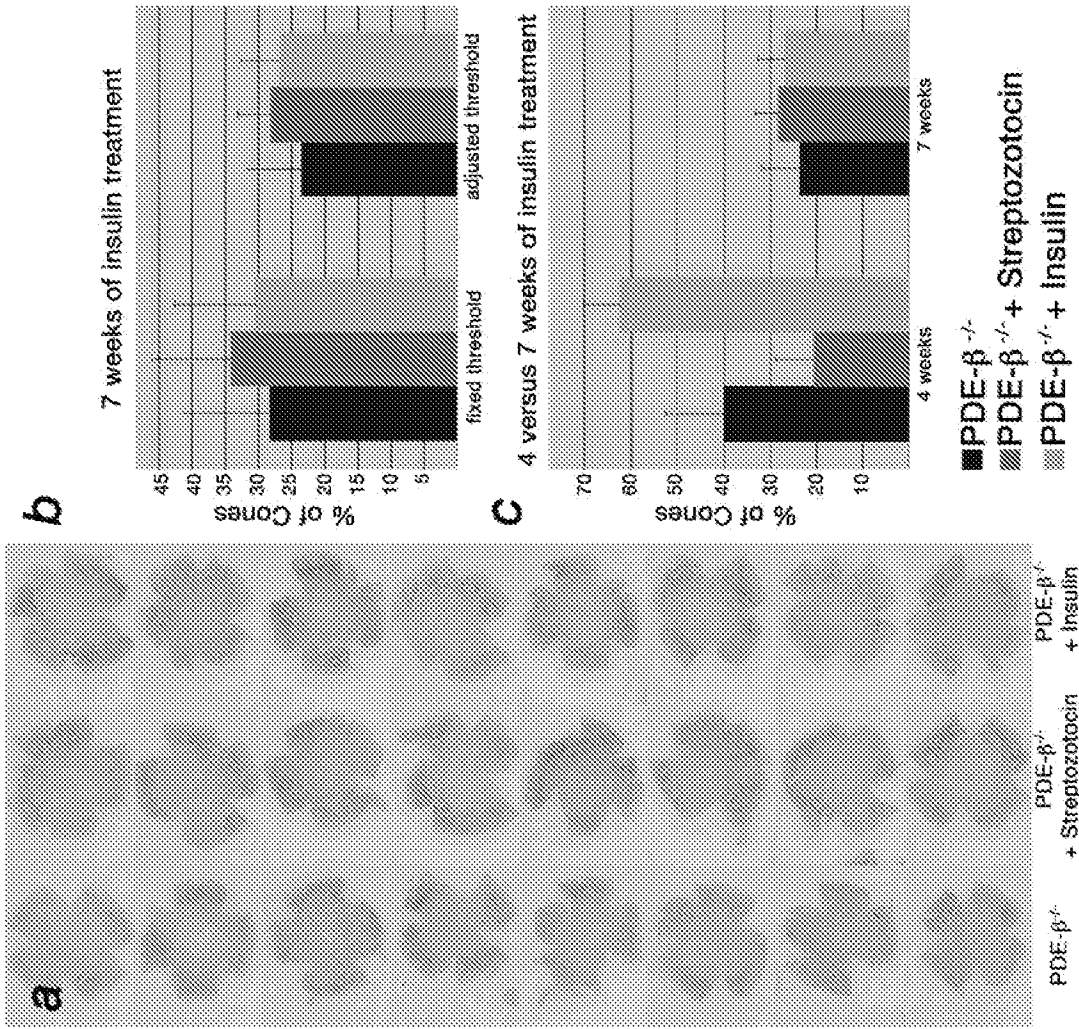


Fig. 18a-18c

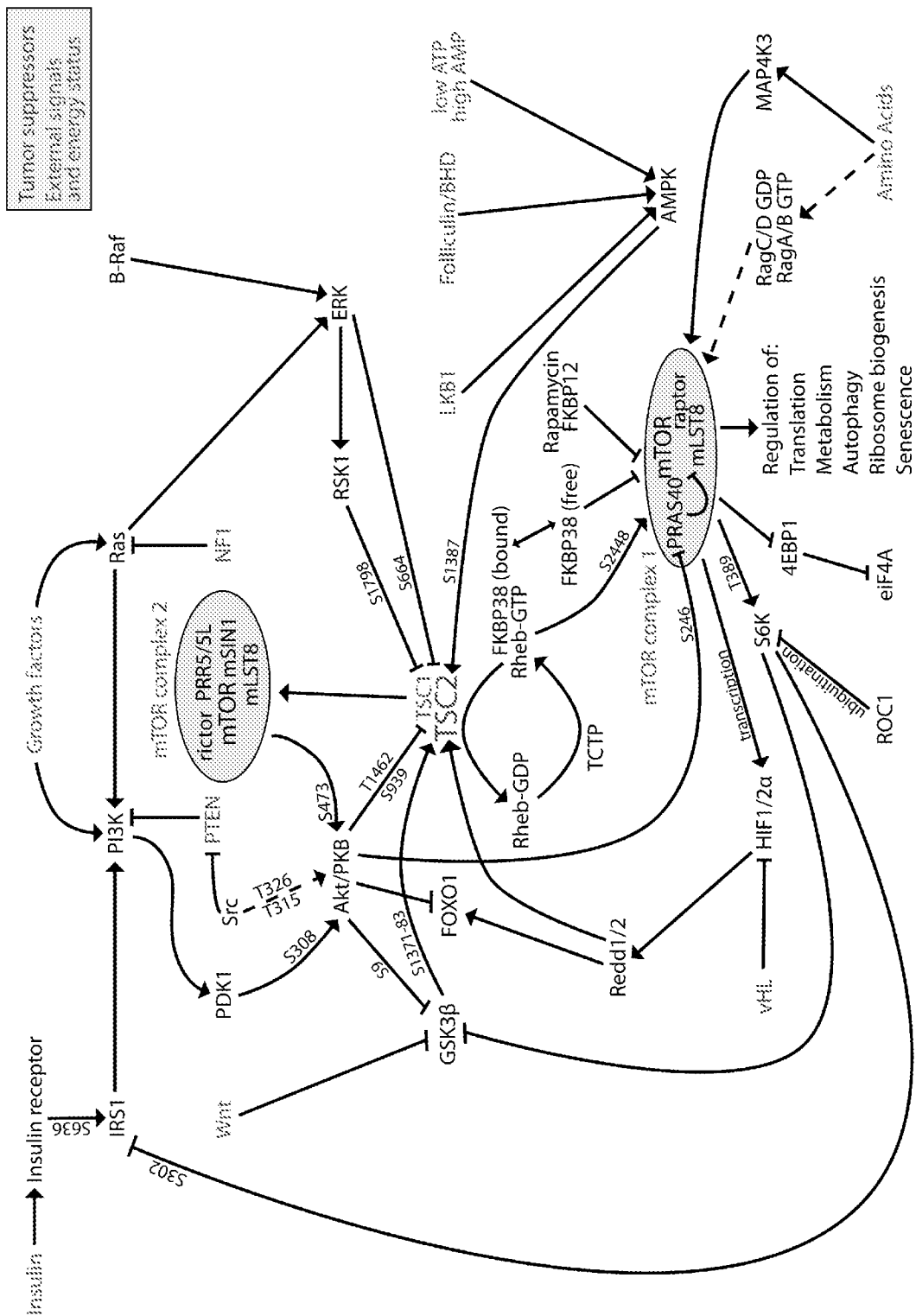


Fig. 19

20/24

Affymetrix ID	Gene Symbol	AVG_C0/R	AVG_C0/C1	AVG_C0/C2
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1432646_a_at	EG640370	4.46	3.22	3.83
1425833_a_at	Hpca	4.19	3.12	3.90
1425227_a_at	ATP6V0A1	3.93	2.49	3.41
1425405_a_at	ADAR	3.83	3.28	3.65
1426562_a_at	Olfm1	3.69	3.06	3.03
1415824_at	SCD2	3.66	2.23	2.99
1429385_at	Wdr68	3.54	3.67	3.88
1427754_a_at	DNM1	3.54	2.19	3.01
1420833_at	VAMP2	3.46	2.15	3.36
1454735_at	Odf2	3.45	2.47	2.98
1431107_at	Stk35	3.39	2.45	3.40
1454106_a_at	Cxxc1	3.39	2.72	3.58
1421255_a_at	Cabp1	3.39	2.00	1.98
1421780_a_at	Cabp5	3.36	2.21	2.28
1425338_at	PLCB4	3.33	2.58	2.49
1421349_x_at	Cend1	3.31	2.09	2.56
1425711_a_at	AKT1	3.27	2.23	3.28
1425457_a_at	GRB10	3.23	2.62	3.19
1451056_at	PSMD7	3.23	2.37	2.22
1448541_at	Kns2	3.23	2.27	3.04
1425659_at	Tom1l2	3.21	2.54	3.36
1448997_at	Pscd1	3.18	2.67	3.01
1442138_at	Gpr62	3.14	1.93	1.98
1426115_a_at	Kcnj9	3.13	2.87	2.69
1450202_at	GRIN1	3.13	2.39	2.94
1428157_at	GNG2	3.12	2.95	3.22
1424359_at	OPLAH	3.08	1.42	1.75
1418174_at	Dbp	3.05	1.99	2.12
1426975_at	OS-9	2.97	2.63	3.11
1435276_a_at	Dger2	2.96	2.32	2.73
1449936_at	KIAA1467	2.94	1.87	2.30
1455410_at	Faim2	2.93	1.47	1.76
1453127_at	PPM1J	2.93	2.16	2.38
1435770_at	Txndc13	2.93	2.37	2.61
1457914_at	unknown	2.88	2.86	2.91
1458163_at	BC066028	2.86	2.10	1.89
1433714_at	SULT4A1	2.85	1.82	1.79
1436713_s_at	Gtl2	2.84	2.09	2.52

Fig. 20

1422756_at	SLC32A1	2.83	2.05	1.91
1452688_at	Prpf39	2.80	2.58	3.19
1421391_at	VIPR2	2.79	2.10	2.18
1446071_at	Steap2	2.78	1.86	2.02
1454891_at	CDS2	2.77	2.09	2.61
1421862_a_at	Vamp1	2.76	1.96	2.46
1440358_at	ARHGEF15	2.74	2.38	2.29
1431355_s_at	TRPM7	2.72	2.60	2.52
1422793_at	PAFAH1B2	2.72	2.25	2.78
1453199_at	Acbd6	2.71	2.09	2.23
1449615_s_at	Hdlbp	2.69	2.64	3.06
1432329_a_at	MATK	2.69	2.18	2.36
1459826_at	Kcnq2	2.68	2.37	2.59
1438205_at	C11ORF2	2.67	2.02	2.57
1426467_s_at	C1ORF123	2.63	2.35	2.74
1429761_at	Rtn1	2.62	2.39	2.27
1420148_at	SLC6A6	2.61	2.41	2.95
1426888_at	EHMT2	2.61	2.55	2.68
1451989_a_at	Mapre2	2.59	1.80	1.81
1450512_at	Ntn4	2.58	2.25	2.12
1415795_at	Spin	2.57	2.49	2.59
1434467_at	Atcay	2.57	2.13	2.21
1426738_at	DGKZ	2.56	1.66	1.90
1419278_at	USP48	2.54	2.08	2.12
1417455_at	TGFB3	2.52	2.19	2.04
1427478_at	USP12	2.50	2.74	2.99
1437319_at	Unc13c	2.48	1.67	1.54
1427004_at	Fbxo2	2.47	1.68	1.55
1450177_at	NGFR	2.46	2.26	2.19
1415845_at	Syt4	2.46	1.78	1.82
1428717_at	Scrn1	2.45	2.11	2.11
1439160_at	4732496O08Rik	2.44	2.27	2.46
1450686_at	Pon2	2.43	1.93	1.68
1415803_at	CX3CL1	2.43	1.33	1.83
1421189_at	GABRB3	2.43	1.84	2.49
1416936_at	Aatk	2.42	1.67	1.84
1416190_a_at	Sec61a1	2.42	2.29	2.96
1452357_at	5-Sep	2.42	1.69	1.77
1450311_at	SLC8A3	2.41	2.10	2.51
1460384_a_at	Arid4b	2.37	1.91	2.19
1434563_at	Rps6kc1	2.37	2.26	2.27
1450080_at	Cxx1c	2.37	1.46	1.69
1444552_at	KIAA0240	2.36	2.28	2.28
1450055_at	Vsnl1	2.35	1.51	1.47
1448498_at	RPS6KA4	2.35	1.69	1.65
1424045_at	C6ORF35	2.33	2.04	2.52
1460321_at	Cntn4	2.32	1.75	1.91
1420387_at	Mpv17	2.32	2.06	2.12

Fig. 20

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1422694_at	Ttyh1	2.31	1.93	1.94
1433583_at	Zfp365	2.31	2.00	1.90
1431229_at	Ipw	2.30	1.87	1.76
1451975_at	MGC12966	2.30	2.26	1.71
1419399_at	MTTP	2.30	1.95	1.71
1433477_at	Abr	2.28	1.73	1.72
1426114_at	Hnrpab	2.27	2.16	2.03
1424500_at	Utp6	2.26	2.47	2.45
1417658_at	Tbrg4	2.26	1.96	1.74
1457744_at	Ddx46	2.25	2.01	2.25
L09192_3_at	Pcx	2.25	1.59	1.62
1421914_s_at	Mrpl19	2.24	1.79	1.90
1437091_at	Accn4	2.23	1.71	1.74
1426552_a_at	Bcl11a	2.23	1.68	1.81
1435045_s_at	MAPK8IP2	2.22	1.26	1.36
1439036_a_at	ATP1B1	2.22	1.60	1.62
1448581_at	Zfp346	2.21	2.01	2.25
1425846_a_at	Caln1	2.21	1.72	1.70
1416087_at	Ap1s1	2.20	1.37	1.74
1442622_at	STRBP	2.20	2.04	1.98
1437772_s_at	FUCA1	2.20	2.02	2.05
1419428_a_at	GAA	2.17	1.59	1.52
1415687_a_at	PSAP	2.17	1.59	1.71
1415908_at	Tspyl1	2.16	1.35	1.67
1424882_a_at	Nt5dc2	2.14	1.92	2.10
1449406_at	Cyhr1	2.13	2.27	2.76
1448605_at	RHOC	2.13	1.98	2.41
1425615_a_at	PCK2	2.12	1.58	1.54
1435532_at	FAM80B	2.12	1.69	2.01
1436343_at	Chd4	2.10	1.74	1.94
1435281_at	CPT1C	2.10	1.68	1.64
1451290_at	Map1lc3a	2.10	1.69	2.06
1417388_at	Bex2	2.10	1.66	1.48
1438671_at	PPP2R2C	2.10	1.40	1.50
1419812_s_at	Ccdc56	2.08	1.70	1.75
1425551_at	HIP1R	2.08	2.01	1.89
1422758_at	Chst2	2.07	1.33	1.63
1448656_at	Cacnb3	2.07	1.79	1.70
1417928_at	Pdlim4	2.06	1.70	1.72
1450368_a_at	PPP3R1	2.06	1.86	2.41
1420661_a_at	C17ORF39	2.06	2.32	2.54
1424161_at	Ddx27	2.06	2.19	2.19
1440871_at	MAGI1	2.05	1.75	1.68
1454677_at	TIMP2	2.05	1.57	1.36
1422102_a_at	STAT5B	2.05	1.52	1.73
1448185_at	HERPUD1	2.04	1.27	1.36
1418889_a_at	CSNK1D	2.03	1.95	2.57
1416364_at	HSP90AB1	2.02	1.61	1.50

Fig. 20

1460661_at	EDG3	2.01	1.90	2.50
1417103_at	DDT	2.01	1.53	1.59
1450632_at	LOC433749	2.00	2.11	2.35
1419746_at	Arhgap23	2.00	1.84	1.95
1426465_at	Dlgap4	2.00	1.67	1.67
1429187_at	Tmed7	2.00	2.15	2.23
1418297_at	Dpysl4	2.00	1.90	2.03
1439051_a_at	MARK4	1.99	1.78	2.01
1424928_at	C11ORF30	1.98	1.97	2.00
1423802_at	Camkv	1.97	1.27	1.13
1456869_at	Zfp787	1.97	1.84	2.07
1453008_at	2300002D11Rik	1.96	1.42	1.49
1416597_at	Hdgfrp2	1.96	1.69	1.65
1421971_a_at	Mrps34	1.95	1.42	1.45
1433921_s_at	Dph3	1.94	2.00	2.33
1429036_at	Otop3	1.94	2.41	3.05
1419753_at	Nfx1	1.90	1.57	1.85
1439375_x_at	ALDOA	1.89	1.79	1.65
1429643_a_at	PDE1C	1.89	1.48	1.39
1434554_at	Trim37	1.88	1.96	2.22
1435117_a_at	RBJ	1.88	1.59	1.29
1436775_a_at	Ankrd17	1.86	1.69	1.56
1427408_a_at	Thrap3	1.86	1.87	2.09
1449263_at	Ufml1	1.86	2.10	2.14
1424914_at	KIAA1737	1.84	1.82	1.59
1450878_at	Sri	1.83	1.53	1.61
1434027_at	Dscr1l2	1.83	1.52	1.67
1434027_at	RCAN3	1.83	1.52	1.67
1426343_at	STT3B	1.82	2.04	2.46
1452730_at	Rps4y2	1.81	1.52	2.03
1421014_a_at	CLYBL	1.79	1.54	1.66
1438016_at	Dkc1	1.79	1.38	1.22
1451364_at	Polr3gl	1.78	1.71	1.68
1460363_at	Tnrc6c	1.78	1.97	1.82
1450472_s_at	SMAD3	1.78	1.38	1.83
1417363_at	Zfp61	1.77	1.91	1.70
1448646_at	Wdr12	1.77	1.60	1.56
1417259_a_at	Capzb	1.77	1.74	1.98
1426306_a_at	Maged2	1.76	1.91	1.85
1460193_at	St13	1.73	2.34	2.73
1433539_at	Commd3	1.71	1.89	1.99
1454987_a_at	H2-Ke6	1.71	1.88	1.73
1415978_at	Tubb3	1.71	1.70	1.72
1417128_at	Plekho1	1.70	1.52	1.50
1424776_a_at	Slc25a28	1.70	1.60	1.49
1418632_at	UBE2H	1.69	1.27	2.07
1422456_at	NSF	1.68	1.46	1.40
1422679_s_at	Ctr9	1.67	2.06	2.07

Fig. 20

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1435009_at	Slc9a6	1.67	1.64	1.92
1435525_at	Kctd17	1.65	1.40	1.43
1431212_a_at	TRMT6	1.64	2.05	1.92
1427077_a_at	AP2B1	1.63	1.89	2.01
1416284_at	Mrpl28	1.61	1.72	1.66
1415974_at	MAP2K2	1.61	1.71	1.65
1415956_a_at	PCTK1	1.61	1.46	1.80
1427073_at	Lace1	1.61	1.30	1.23
1435698_at	Rictor	1.60	1.48	1.50
1425068_a_at	Tex264	1.60	1.89	1.80
1418432_at	Cab39	1.60	1.60	1.56
1432464_a_at	KIAA1543	1.60	1.43	1.54
1427258_at	Trim24	1.59	1.79	2.00
1427050_at	Txndc16	1.58	1.65	1.66
1422510_at	Ctdspl	1.58	1.28	1.33
1417223_at	Cd2bp2	1.57	1.39	1.34
1417148_at	PDGFRB	1.57	1.47	1.41
1417063_at	C1QB	1.56	2.26	2.57
1453209_at	Brf1	1.55	1.55	1.69
1417504_at	CALB1	1.55	1.26	1.36
1449097_at	TXNRD2	1.55	1.45	1.45
1427149_at	Plekha6	1.55	1.36	1.27
1417870_x_at	Ctsz	1.55	1.79	1.95
1448122_at	Tcp1	1.55	1.62	1.46
1428461_at	PPP2R5E	1.54	1.65	1.68
1456867_x_at	Ergic3	1.53	1.72	1.93
1422489_at	GCS1	1.52	1.73	1.83
1427987_at	Safb2	1.50	1.41	1.14
1416493_at	DDOST	1.50	1.56	1.71
1431137_at	Rusc1	1.47	1.30	1.47
1421148_a_at	Tial1	1.46	1.69	1.98
1452448_at	Aqr	1.46	1.50	1.91
1451048_at	Metap2	1.43	1.62	1.61
1460553_at	1700025K23Rik	1.42	1.75	1.94
1428894_at	Selenoprotein O	1.40	1.54	1.43
1452168_x_at	Gspt1	1.39	2.14	2.25
1432029_a_at	Smap1	1.38	1.60	1.50
1423212_at	Phc1	1.38	1.98	1.86
1451050_at	NT5C3	1.38	1.56	1.63
1429455_at	Gapvd1	1.37	1.78	1.65
1423961_at	Wdr26	1.36	1.98	1.97
1427934_at	Lym2	1.32	1.54	1.64
1417258_at	Cct5	1.31	1.54	1.50
1448934_at	NDUFA10	1.31	1.51	1.55
1434894_at	Zc3h13	1.22	1.34	1.26
1423081_a_at	Tomm20	1.17	1.45	1.68
1418528_a_at	Dad1	1.16	1.27	1.28

Fig. 20

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 09/66557

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/00 (2010.01)

USPC - 514/1

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61K 31/00 (2010.01)

USPC - 514/1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 514/\$

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

PubWEST(USPT,PGPB,EPAB,JPAB); Google Scholar

Search terms: mTOR, mammalian target of rapamycin, viability, survival, death, apoptosis, glucose, energy, metabolic, metabolism, retinal disorder, retinal disease, retinitis pigmentosa, rod, cone, photoreceptor, insulin, IGF-1, IGF-2, mitogen, phosphatidic acid, leucine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ECKMILLER, Defective cone photoreceptor cytoskeleton, alignment, feedback, and energetics can lead to energy depletion in macular degeneration. Progress in Retinal and Eye Research, September 2004, Vol 23, No 5, pp 495-522. p 498, right col, para 1; p 498, left col, para 4- right col para 1; p 503, left col, para 4 - right col, para 1-2; p 516, right col, para 1; p 516, left, col, para 4 - right col, para 1.	16-25, 29-30 -----
Y		1-11, 14-15, 26-28, 31-33
Y	WOODS et al., Regulation of Food Intake Through Hypothalamic Signaling Networks Involving mTOR. Annual Review of Nutrition, 2008 (Published online 22 April 2008), Vol 28, pp 295-331. p 300, left col, para 4; p 304, left col, para 1-2; p C-2, Fig 2.	1-11, 14-15, 26-28, 31-33

☐ 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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 January 2010 (18.01.2010)

Date of mailing of the international search report

25 FEB 2010

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 09/66557

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 12 and 13
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.