Gene Therapy Provides Long-term Visual Function in a Pre-clinical Model of Retinitis Pigmentosa

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Abstract

Approximately 36,000 cases of simplex and familial retinitis pigmentosa (RP) worldwide are caused by a loss in phosphodiesterase (PDE6) function. In the preclinical \( Pde6^{\alpha \, nmf363} \) mouse model of this disease, defects in the \( \alpha \)-subunit of PDE6 results in a progressive loss of photoreceptors and neuronal function. We hypothesized that increasing PDE6\( \alpha \) levels using an AAV2/8 gene therapy vector could improve photoreceptor survival and retinal function. We utilized a vector with the cell-type specific rhodopsin promoter: AAV2/8(Y733F)-Rho-Pde6\( \alpha \), to transduce \( Pde6^{\alpha \, nmf363} \) retinas and monitored its effects over a six-month period (a quarter of the mouse lifespan). We found that a single injection enhanced survival of photoreceptors and improved retinal function. At six months of age, the treated eyes retained photoreceptor cell bodies while there were no detectable photoreceptors remaining in the untreated eyes. More importantly, the treated eyes demonstrated functional visual responses even after the untreated eyes had lost all vision. Despite focal rescue of the retinal structure adjacent to the injection site, global functional rescue of the entire retina was observed. These results suggest that RP due to PDE6\( \alpha \) deficiency in humans, in addition to PDE6\( \beta \) deficiency, is also likely to be treatable by gene therapy.
Introduction

Loss of independence in activities of daily living resulting from some neurodegenerative diseases, such as retinitis pigmentosa (RP) or age-related macular degeneration (AMD), is due to photoreceptor cell death (1). In patients with RP, initially, death of the rod photoreceptors will cause night blindness. This is followed by a deterioration of the peripheral visual field and may progress until all visual response is extinguished. Approximately 36,000 cases of simplex and familial RP worldwide are caused by a mutation in the rod-specific cGMP phosphodiesterase (PDE6) complex (2). In spite of the occurrence of this disease, no effective treatments are currently available to ameliorate this loss of vision.

The PDE6 complex is composed of catalytic (PDE6α and PDE6β) and regulatory (PDE6γ) subunits (3-4). There are several mouse models with mutations in the gene encoding the β-subunit, such as Pde6β<sup>d1</sup>, Pde6β<sup>d10</sup>, and Pde6β<sup>H620Q</sup>. These Pde6β mouse models have been useful for testing various drug and gene therapies, but in spite of this, most viral-mediated gene therapy approaches have met with limited success (5-12). Recently, an adeno-associated viral (AAV)2/8 gene therapy vector with a Y733F capsid mutation was shown to promote long-term rescue compared with the earlier AAV2 and AAV5 vector serotypes (13). Pang et al. improved visual function in the Pde6β<sup>d10</sup> mouse for six months using an AAV2/8 gene therapy vector.

Mouse models have been described with mutations in the gene encoding the α-subunit of PDE6; Pde6α<sup>nmf282</sup> and Pde6α<sup>nmf363</sup>, which have similar mutations found in human RP patients (OMIM 180071, 14). These mice mimic the clinical phenotype of recessive RP found in humans, where there is a progressive loss of photoreceptors and visual function, at a faster rate of degeneration than occurs in the Pde6β<sup>d10</sup> mouse model (15). No therapeutic strategies have been tested on these Pde6α models, to either correct or slow the progression of the disease phenotype.
In this study, we hypothesized that an AAV2/8 gene therapy vector can increase both photoreceptor survival and neuronal function in a mouse model with a mutation in the PDE6α subunit. We utilized an AAV vector designed to specifically express wild-type PDE6α in the Pde6α mutant rod photoreceptor cells through the rhodopsin gene promoter: AAV2/8(Y733F)-Rho-Pde6α. We delivered the AAV2/8(Y733F)-Rho-Pde6α virus into the right eye of Pde6α<sup>nmf363</sup> mutant mice using a single subretinal injection. We then compared photoreceptor survival and visual function between the treated right eyes and the untreated left eyes of these mice.

**Results**

**AAV2/8(Y733F)-Rho-Pde6α vector map.** The AAV2/8(Y733F)-Rho-Pde6α gene therapy vector was created using a pZac2.1 plasmid. The Pde6α complementary DNA (cDNA) fragment was inserted into this plasmid, along with 1.1 kb of the murine rhodopsin promoter, a Simian virus 40 (SV40) site, and poly(A) tail flanked by inverted terminal repeats (ITRs). This pZac2.1 plasmid with these sites inserted was used for packaging into the viral construct before subretinal injections (Figure 1).

**Retinal transduction of AAV2/8.** To determine the transduction efficiency of AAV2/8 within the retina, a subretinal injection of AAV2/8-TurboRFP was administered to a litter of homozygous Pde6α<sup>nmf363</sup> mutant mice. This vector expresses red fluorescent protein (RFP), which allows for the visualization of the cells transduced by the virus. After a single subretinal injection at post-natal day (P) five, RFP expression remained visible within the retina at P55, indicating that the virus was taken up by the retinal cells and survived for at least two months.
(Figure 2A). RFP expression was detected within the portion of the retina that most likely correlated with the subretinal bleb, with only a minor scattering of fluorescence visible in other locations (Figure 2A; blue circle).

**PDE6α and RHO protein levels increased after treatment of Pde6α<sup>nmf<sub>363</sub></sup> mutant eyes.** Since AAV2/8-TurboRFP transduced retinal cells after a single subretinal injection, we tested whether AAV2/8(Y733F)-Rho-Pde6α could express PDE6α in Pde6α<sup>nmf<sub>363</sub></sup> mutant mice. In untreated mutant eyes at two months of age, PDE6α protein was undetectable in the retinal lysate (Figure 2B). In contrast, PDE6α was observed in the retinal lysate of the mutant eye treated with the gene therapy vector (Figure 2B). We also tested whether rhodopsin (RHO) is translated in the Pde6α<sup>nmf<sub>363</sub></sup> mutant mice, since RHO levels are indicative of the health of the photoreceptor outer segments. RHO protein levels were severely reduced in the untreated mutant eye, only detectable after over-exposure of the film, but RHO protein was detected at high levels in the treated mutant eye (Figure 2B). We then compared RHO levels in the treated mutant eyes at two and eleven months of age to that of a B6 control mouse (Figure 2C). RHO is lower in the two month old treated mutant eye, and RHO levels decline further by eleven months of age compared to a B6 control (Figure 2C). In summary, the untreated mutant eye expressed undetectable levels and low levels of PDE6α and RHO, respectively, while the AAV2/8(Y733F)-Rho-Pde6α treated mutant eye expressed higher levels of both PDE6α and RHO, although not as high as the levels of RHO in a B6 control mouse eye.

**Delay of retinal pigment epithelium (RPE) degeneration in treated Pde6α<sup>nmf<sub>363</sub></sup> mutant eyes.** Since PDE6α protein is detectable after viral transduction and RHO protein levels suggest
healthy photoreceptor neurons, we tested whether AAV2/8(Y733F)-Rho-Pde6α can rescue RPE degeneration visible upon fundus examination in RP patients. We examined infrared (IR) images of treated and fellow untreated Pde6α<sup>nmf363</sup> mutant eyes at five, seven, nine and eleven months of age (Figure 3). A B6 mouse IR image was shown as a control at eight months of age (Figure 3I). At five months of age, the untreated mutant eye shows the beginning signs of RPE atrophy compared to the treated mutant eye (Figure 3A, B; asterisks). Beginning at seven months of age and continuing through eleven months of age, extensive RPE atrophy is visible in the untreated mutant eye (Figure 3C, E, G; asterisks) while the treated mutant eye displays only a slight amount of RPE atrophy and appears to remain the same over time (Figure 3D, F, H).

Increased PDE6α protein improved photoreceptor survival in Pde6α<sup>nmf363</sup> mice. Since IR images displayed reduced RPE atrophy in treated Pde6α<sup>nmf363</sup> mutant eyes compared to the fellow untreated eye for at least eleven months, we tested whether AAV2/8(Y733F)-Rho-Pde6α is rescuing the photoreceptors in the Pde6α<sup>nmf363</sup> mice. We examined histological sections from both untreated and treated mutant eyes (Figure 4). When viewing the treated retina of a two month-old Pde6α<sup>nmf363</sup> mouse, the preservation of the photoreceptor cell bodies and outer segments (OS) is detectable in half of the retina (Figure 4A).

To determine the extent and longevity of this rescue, we examined the outer nuclear layer (ONL) thickness of the rescued portion of the treated retina compared with both the un-rescued opposing side of the treated retina (as an internal control) and fellow untreated Pde6α<sup>nmf363</sup> eyes in a longitudinal study (Figure 4B). There were significantly more photoreceptor nuclei in the rescued portion of the treated eye (3.79 ± 4.21) when compared to the untreated fellow eye (1.44 ± 5.46) for each of the six months examined, and the opposing side of the treated retina showed
similar ONL thickness to the untreated fellow eye as would be expected with little to no rescue (Figure 4B). The photoreceptor cell bodies declined between one and two months of age in the treated eye, then stabilized over the following months (Figure 4B).

At five months of age, the ONL in mutant eyes was undetectable compared to a B6 control eye (Figure 4C, E). In contrast, the ONL was not only detectable in the treated mutant eye compared to the untreated mutant eye (Figure 4C, D), but it was approximately 50% of the ONL thickness of the control B6 eye (Figure 4D, E). Furthermore, OS were preserved in the treated regions of the mutant eye (Figure 4D) compared to the untreated mutant eye (Figure 4C), and of similar length as the B6 control (Figure 4E). Thus, photoreceptors are present in the treated mutant eyes at a time when there is a loss of photoreceptors in the untreated mutant eyes, and this significant improvement in photoreceptor survival persists through at least six months of age.

Despite focal rescue of retinal structure adjacent to the injection site, global functional rescue is observed in Pde6α<sup>nmf363</sup> mice. Since mutant photoreceptors survived after AAV2/8(Y733F)-Rho-Pde6α transduction, we tested whether these rescued photoreceptor cells were functional. We measured ERG responses in Pde6α<sup>nmf363</sup> mice after AAV2/8(Y733F)-Rho-Pde6α transduction beginning at one month of age (Figure 5). By two months of age when the untreated mutant eye lost almost all visual response, the treated mutant eye retained a strong visual response, although with a lower maximum b-wave amplitude and reduced a-wave amplitude than that of a control wild-type B6 mouse (Figure 5A). At five months of age, the treated mutant eye retained an approximately 100µV b-wave amplitude, similar to that observed at two months of age (Figure 5B). Each month, the overall visual response was significantly
enhanced in the treated eyes compared to untreated fellow eyes and this lasted through six months of age, the last age examined (Figure 5C).

Furthermore, since recessive RP caused by mutations in PDE6α is a rod-cone dystrophic disease, we tested whether the rod- and cone-specific visual responses were improved after a single subretinal injection with AAV2/8(Y733F)-Rho-Pde6α. The maximum ERG a-wave, which is a photoreceptor-dominated response, although substantially decreased in the ERG traces (Figure 5A, B), was increased in our treated mutant eyes compared to fellow untreated eyes over the six-month period (Figure 5D). Additionally, ERG responses from a dim light rod-only stimulation showed some waveforms where, at five months of age, the treated mutant eye retained a strong rod response, although with a lower maximum amplitude than that of a control wild-type B6 mouse (Figure 5E). However, not all treated mice displayed a strong dim light ERG trace, and between two and five months of age, the treated mutant eyes showed improved rod-specific b-wave responses over the fellow untreated mutant eyes, but these were not significant (Figure 5G).

Lastly, even if only a relatively small number of rod photoreceptor cells were functional after treatment, cone cells may still survive and function in these mice, so we tested for cone cell-specific visual function. We used light-adapted photopic ERG responses, which are cone-specific, and found that at five months of age, the traces from the treated mutant mice displayed a strong cone cell visual response compared to the untreated eye, which lost almost all visual response (Figure 5F). Additionally, the waveforms from the treated mutant eye were similar to that of a B6 control mouse, and only slightly reduced in amplitude (Figure 5F). The cone cell visual responses were enhanced in our treated mutant eyes compared to untreated fellow eyes, through at least six months of age, the last date tested (Figure 5H). Taken together, our data
suggests that treatment with AAV2/8(Y733F)-Rho-Pde6α, and its subsequent increase of PDE6α levels, rescues photoreceptor survival and inner retina visual function for at least six months in the Pde6αnmf363 mouse.

We also tested if injection of the viral vector alone is able to cause a functional restoration of the photoreceptor cells. We analyzed ERG responses from the AAV2/8-TurboRFP treated Pde6αnmf363 mice, to ensure that the injection of the AAV2/8 virus alone does not cause any functional rescue effect (Figure 6). At six weeks of age, the photoreceptor-specific and overall visual function in the AAV2/8-TurboRFP treated and untreated eyes were not significantly different (Figure 6). In contrast, the AAV2/8(Y733F)-Rho-Pde6α treated mutant eyes displayed significant improvement over the untreated eyes or those treated with the AAV2/8-TurboRFP virus alone (Figure 6). Thus, the AAV2/8(Y733F)-Rho-Pde6α vector-induced increase in PDE6α protein is likely responsible for the significant rescue in visual function through six months of age (Figures 3-5).

Discussion

Only recently, attempts at using AAV2/8 gene therapy to functionally rescue mouse models of retinitis pigmentosa (RP), in particular one Pde6β mouse model, has shown long-term rescue effects (13). Lentiviral, or other AAV serotypes, can delay photoreceptor degeneration in multiple Pde6β mouse models, but long-term efficacy has not been found in the majority of the reported studies (5-12). Although one AAV gene therapy case has now shown six-month rescue in a slower photoreceptor degeneration Pde6β mouse model, studies have not been conducted on animal models with mutations in the α-subunit of PDE6 to ameliorate the disease progression.
Here, we applied AAV-based gene therapy that can rescue photoreceptor degeneration and restore neuronal function in a $Pde6\alpha^{nmf363}$ mouse model of recessive RP. We demonstrate that after a single subretinal injection of AAV2/8(Y733F)-Rho-$Pde6\alpha$ into the $Pde6\alpha^{nmf363}$ mouse, the phenotype can be both structurally and functionally rescued. Sakamoto et al. showed that PDE6$\alpha$ protein was severely diminished in P12 $Pde6\alpha^{nmf363}$ mutant mice (14), and we found that PDE6$\alpha$ protein was undetectable in the two month-old $Pde6\alpha^{nmf363}$ mutant retinal lysates. This suggests that the PDE6$\alpha$ protein levels found in the treated mutant eye most likely came from the cells that were transduced by the AAV2/8(Y733F)-Rho-$Pde6\alpha$ vector. Additionally, the small amount of PDE6$\alpha$ protein provided by the viral injection protected the rhodopsin (RHO) protein expression in the treated $Pde6\alpha^{nmf363}$ mutant eye, since it was undetectable in the untreated mutant eye. Thus, AAV2/8(Y733F)-Rho-$Pde6\alpha$ partially rescues mutant photoreceptors in treated eyes as evidenced by the increase in both PDE6$\alpha$ levels, directly, and RHO levels, indirectly, both of which are necessary to correct the recessive RP disease phenotype in our $Pde6\alpha^{nmf363}$ mouse model.

Furthermore, our study replicates the results published by Sakamoto et al. on the retinal degenerative phenotype of the $Pde6\alpha^{nmf363}$ mouse model and extends the observation beyond one month of age (14). We found that RHO protein levels were diminished by two months of age, suggesting a loss of photoreceptor cells, which correlates with histological sections showing that the outer nuclear layer (ONL) photoreceptor cell nuclei decreased to approximately 1-2 cell thickness after two months of age. Additionally, a complete loss of ONL photoreceptor cell nuclei occurred in the mutant eye by five months of age. Electretinograms (ERGs) displayed similar findings, where the visual response was severely attenuated after two months of age, and cone cell-specific ERG responses declined dramatically by five months of age. Upon infrared
(IR) examination, retinal pigment epithelial (RPE) atrophy begins around five months of age, after the loss of the ONL and outer segments (OS) in the mutant eyes, and this RPE atrophy increases dramatically by seven months of age through eleven months of age, the latest time-point that was studied.

Effective photoreceptor rescue after AAV2/8(Y733F)-Rho-\textit{Pde6}\alpha transduction is confined to the location of the subretinal bleb, and the variability of the size of the subretinal bleb can account for gene transfer and therapeutic efficiency between different eyes. Photoreceptor survival occurred along one half of the treated retina (Figure 4), most likely on the side where the gene therapy injection site was located and the subretinal bleb was formed (Figure 2A). This correlates with the ERG results, where the treated \textit{Pde6}\alpha\textsuperscript{nmf363} mutant mice had enhanced visual responses long-term, at approximately 20% of that found in a B6 mouse (Figure 5).

Additionally, the amount of cells that are transduced by the virus is variable. The representative ERG waveforms (Figure 5A, B, E, F) display the ERG responses under each condition in a mouse with a strong visual response. The charts shown in Figure 5 (C, D, G, H) take into account all of the mice at each time examined, and this variation in the size of the subretinal bleb during the surgical procedure may account for the strength of significance and deviation in these charts.

For the dim light rod-specific ERG responses, statistical analysis of the treated and untreated eyes demonstrated no significant differences at any time-point in this pooled set of treated eyes (N ranges from 3-8 mice per time-point), although the treated mutant eyes showed some improvement over the untreated mutant eyes from two to five months of age. Additionally, we found three mice with evidence of rod-specific ERG responses much higher than the
untreated eyes (Figure 5E), and these three mice showed comparable rod-specific ERG responses at multiple time-points in the six-month study period. One interpretation of this result is a threshold level of transduction (number of rods transduced) is able to produce a more effective rescue of rod function and that the transduction levels of the majority of mice were below this level. This is likely due to variation during the surgical procedure. Alternatively, the variable ability of the vector to produce detectable rod-specific ERG rescue may be related to the capability of the vector to sustain PDE6α expression and/or survival of the transduced rods.

Additionally, we found that the ONL and OS declined in the treated eyes until two months of age, and then remained at a similar thickness through six months of age. This suggests a halt in the progression of photoreceptor death after transduction with AAV2/8(Y733F)-Rho-Pde6α, where the treated eye has photoreceptor survival in the location of the subretinal bleb, but the remaining half of the retina continues to degenerate. This effect was also seen in the ERG results, where ERG traces decline until two months of age, then have a similar visual response to those at six months of age in the same mouse (Figure 4D, E, Figure 5A, B). At two months of age, the ERG tracing for the treated mutant eye has a similar maximum b-wave amplitude to that of a control B6 mouse, however a decreased a-wave amplitude as would be expected with the loss of photoreceptor cells in the unrescued half of the treated retina. The strength of the b-wave amplitude is likely due to the remaining photoreceptors and the inner retinal cells of the eye. By five months of age, the ERG tracing for the treated mutant eye retains the strong visual response from the remaining photoreceptors and inner retinal cells, but has a reduced maximum b-wave amplitude compared to the B6 control mouse response, which correlates with the thinner ONL seen in Figure 4D compared to Figure 4E, as the unrescued half of the treated retina is fully degenerated.
However, despite focal rescue of retinal structure adjacent to the injection site, we achieved remarkable global functional restoration as assessed by ERG that lasted over six months of age, a quarter of the mouse lifespan. RHO levels declined by eleven months of age in the treated mutant eyes, however IR imaging showed strong RPE atrophy in untreated mutant eyes, but healthy fundus images of the fellow treated mutant eye through eleven months of age; half of the mouse lifespan. Although only half of the retina retains photoreceptor cells after subretinal injection, it appears that these cells are enough to protect the treated eye from the secondary remodeling effects that occur after photoreceptor cell death in RP, such as RPE atrophy.

“Sham” injections have been found to produce rescue effects that last through a six-month testing period in the Royal College of Surgeons (RCS) rat model of RP (16-17). In RCS rats, a washing away of accumulated OS debris during the injection procedure, is the likely cause of the noticeable rescue effects. Injection of our AAV2/8 “blank” vector alone did not cause functional rescue in the Pde6a<sup>nmf363</sup> mouse model, therefore the rescue effects found for six months in this study are specific to AAV2/8(Y733F)-Rho-Pde6α transgene transduction.

Early rod photoreceptor dysfunction characterizes diseases that include RP and age-related macular degeneration (AMD). The reduction of PDE6 function is a common denominator in 36,000 cases of photoreceptor degeneration. Our study indicates that it may be possible to develop treatments based on correcting PDE6 levels using gene therapy vectors. Additional studies, however, are needed to examine the full duration of rescue, the timing of injections in respect to disease progression, the possibility of multiple injection sites, and re-injections if the virus begins to lose its ability to rescue the cells. While these studies need to be conducted, our results indicate a significant treatment potential for both photoreceptor cell survival and
functional rescue in mice carrying a mutant allele of Pde6α. Ultimately, the results from this study demonstrates potential for using gene therapy modifications to treat RP in the Cardigan Welsh Corgi, a dog model with a mutation in PDE6α, and in human patients with RP caused by PDE6α mutations.

Materials and Methods

Mouse lines and husbandry. C57BL/6J-Pde6αnmf363/nmf363, with a D170G mutation, herein referred to as Pde6αnmf363, mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained in the Columbia University Pathogen-free Eye Institute Annex Animal Care Services Facility under a 12/12-hour light/dark cycle. All experiments were approved by the local Institutional Animal Care and Use Committee (IACUC) under protocol #AAAB-4306. Mice were used in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research of the Association for Research in Vision and Ophthalmology, as well as the Policy for the Use of Animals in Neuroscience Research of the Society for Neuroscience.

Pde6αnmf363 mice used in this study were bred from a colony of mice that has been previously reported (14). Pde6αnmf363 are coisogenic in the C57BL/6J (B6) background, therefore, age-matched B6 mice were used as experimental controls (The Jackson Laboratory).

Construction of AAV vectors. AAV serotype 2/8 capsids containing a point mutation in surface-exposed tyrosine residues, AAV2/8(Y733F), exhibit higher transduction efficiency in photoreceptors and a faster onset of expression than other AAV serotypes when delivered into the subretinal space of the eye (13). Therefore, these vectors were used for packaging our Pde6α construct. Vector plasmids were constructed by inserting 1.1 kb of the murine rhodopsin
promoter region [Ensembl, rhodopsin, chromosome 6: (115, 930, 881-115, 931, 988); Ensembl, rhodopsin, ATG = 1: (-1125, -17)] and a full length murine Pde6α complementary DNA (cDNA) fragment into the pZac2.1 plasmid to generate pZac2.1.mRhodopsin.mPde6α.SV40 (18). AAV vectors were created, packaged and purified at the Penn Vector Corporation, to become AAV2/8(Y733F).mRhodopsin.mPde6α.SV40 (University of Pennsylvania, PA). The AAV2/8(Y733F).mRhodopsin.mPde6α.SV40 vector will be referred to as AAV2/8(Y733F)-Rho-Pde6α for the remainder of this publication. AAV2/8.CMV.TurboRFP.RBG was purchased from the Penn Vector Corporation as the AAV serotype control and will be referred to as AAV2/8-TurboRFP for the remainder of this publication.

**Transduction of AAV vectors.** To increase levels of wild-type PDE6α, we injected AAV2/8(Y733F)-Rho-Pde6α (0.7uL, 1.26e13 genome copy (GC)/mL) into the subretinal space of the right eye of Pde6αnmf363 mice at post-natal day (P) five. AAV2/8-TurboRFP (0.7uL, 7.28e12 GC/mL) was injected into the subretinal space of the right eye of Pde6αnmf363 mice at P5 for control experiments. Virus particles were injected at the 6 o’clock position of the eye, approximately 1.5 mm from the limbus, to produce a subretinal bleb in the mid-periphery of the retina. The left eyes of all mice were kept as a matched control for experimental analyses. Anesthesia and surgery were performed as previously described (6).

**Whole Mount Analysis.** Mice were euthanized according to established IACUC guidelines. Eyes were enucleated and placed in 2% paraformaldehyde for one hour at room temperature. The optic nerve, cornea, and lens were removed. The whole eyecup was then flattened by means of four radial cuts extending out from the optic nerve and mounted with mounting medium
Red fluorescent protein (RFP) visualization was achieved by fluorescence microscopy, and bright-field imaging was used to visualize the whole retina (Leica DM 5000B microscope). Pictures were taken at 2.5X magnification using the Leica Application Suite Software (Leica Microsystems Inc., Germany).

**Immunoblot Analysis.** Retinas were homogenized in 10% sodium dodecyl sulfate (SDS) by brief sonication and denatured at 100°C for five minutes. Following centrifugation, total protein content per sample was measured by the DC Protein Assay method (Bio-Rad Laboratories, Hercules, CA). Proteins were separated by SDS polyacrylamide gel electrophoresis. Samples were then transferred to nitrocellulose membranes, which were blocked in 3% bovine serum albumin (Santa Cruz Biotechnology, Santa Cruz, CA), 150 mmol/L NaCl, 100 mmol/L Tris (pH 7.4), and 0.5% Tween-20 (BSA-TTBS). Membranes were incubated with either anti-PDE6α (1:300, Abcam), rhodopsin (1D4) (1:500, Santa Cruz) or rabbit anti-cytoskeletal actin (1:300, Bethyl) antibodies in BSA-TTBS. After washing in TTBS, filters were incubated with goat anti-rabbit or goat anti-mouse conjugated horseradish peroxidase secondary antibodies (1:20,000, Santa Cruz Biotechnology). After washing, antibody complexes were visualized by chemiluminescence detection (Immobilon Western, Millipore Corporation, Billerica, MA) and Kodak BioMax film (Kodak, Rochester, NY).

**Infrared Imaging.** Infrared (IR) fundus imaging was obtained with the Spectralis Scanning Laser Confocal Ophthalmoscope (Heidelberg Engineering, Carlsbad, CA). IR imaging was obtained at 790 nm absorption and 830 nm emission using a 55 degree lens. Images were taken...
of the central retina, with the optic nerve located in the center of the image and the site of the subretinal bleb along the lower left-hand quadrant.

**Histochemical Analyses.** Mice were sacrificed and eyes enucleated as previously described (19). Excalibur Pathology, Inc. prepared Hematoxylin & Eosin retinal sections. The morphology of photoreceptors and amount of photoreceptor cell nuclei of AAV2/8(Y733F)-\(\text{Rho-}P\text{de}6\alpha\) treated eyes were compared to untreated fellow eyes. Quantification of photoreceptor nuclei was conducted on several sections that contained the optic nerve, as follows: the distance between the optic nerve and ciliary body was divided into four, approximately equal, quadrants. Three columns of nuclei (how many cell nuclei thick) were counted within each single quadrant. These counts were then used to determine the average thickness of the ONL for each individual animal at each time. For the treated eyes, the rescued half of the treated retina between the optic nerve and the ciliary body was quantified in this manner, with the opposite side of the retina, between the optic nerve and the ciliary body, being quantified similarly and considered the unrescued half of the treated mutant eye (the internal control). Averages and standard deviations were calculated from animals for each time-point using ratio paired \(t\)-test statistical analyses with statistical significance set at \(p < 0.05\). Sectioning proceeded along the long axis of the segment so that each section contained upper and lower retina as well as the posterior pole.

**Electroretinograms (ERGs).** Mice were dark-adapted overnight, manipulations were conducted under dim red light illumination, and recordings were made using Espion ERG Diagnosys equipment (Diagnosys LLL, Littleton, MA). Adult B6 control mice were tested at the beginning of each session to ensure equal readouts from the electrodes for both eyes before testing the
experimental mice. Pupils were dilated using topical 2.5% phenylephrine hydrochloride and 1% tropicamide (Akorn Inc., Lakeforest, IL). Mice were anesthetized by intraperitoneal injection of 0.1mL/10g body weight of anesthesia [1mL ketamine 100mg/mL (Ketaset III, Fort Dodge, IA) and 0.1mL xylazine 20mg/mL (Lloyd Laboratories, Shenandoah, IA) in 8.9mL PBS]. Body temperature was maintained at 37°C using a heating pad during the procedure. Handmade electrodes were placed upon the corneas and gonioscopic prism solution (Alcon Labs, Inc., Fort Worth, TX) was applied to each eye. Both eyes were recorded simultaneously. A total of 40-60 responses were averaged for each trial. All further detail on the ERG method has been described previously (20-21). We measured scotopic dim light maximal b-wave ERG responses to assess rod-only responses, scotopic maximal b-wave ERG responses to assess inner retina function, scotopic maximal a-wave ERG responses to assess photoreceptor-specific function, and photopic maximal b-wave ERG responses to assess cone-specific function. Maximal responses were taken from the Espion readout in µV and quantified using ratio paired t-test statistical analyses with statistical significance set at \( p < 0.05 \).

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References


**Figure Legends**

**Figure 1. Schematic representation of AAV2/8(Y733F)-Rho-Pde6α.** pZac2.1 vector plasmid displaying the Pde6α complementary DNA (cDNA) fragment driven by 1.1 kb of the murine rhodopsin promoter. The Simian virus 40 (SV40) polyadenylation signal is located at the 3’ end of the cDNA. Arrows indicate the direction of transcription. 5’- and 3’-ITR, inverted terminal repeats of AAV; AmpR, ampicillin resistant gene; F1 ori, origin of replication. Scale bar: 1 kb.

**Figure 2. Viral spread and enhanced levels of PDE6α in treated mutant eyes.** A single subretinal injection of AAV2/8-TurboRFP into the right eye of post-natal day (P) five Pde6α<sup>nmf363</sup> mice and visualized at P55. Red fluorescence was overlayed onto a bright-field image of the whole mount for localization of fluorescence in respect to the entire retina. Scale bar: 4000µm (A). A single subretinal injection of AAV2/8(Y733F)-Rho-Pde6α was performed in the right eye of P5 Pde6α<sup>nmf363</sup> mice. Immunoblot analyses using PDE6α, RHO, and β-actin antibodies were performed on retinal lysates from two month-old mice. β-actin was used as a loading control. 40µg of protein was loaded for both the treated and untreated mutant eyes. PDE6α antibody was not specific, and cross-reacted with the highly homologous PDE6β subunit (B). Immunoblot analyses using RHO and β-actin antibodies were performed on retinal lysates from a two month-old treated mutant eye and a control B6 eye, along with an eleven month-old treated mutant eye and control B6 eye. β-actin was used as a loading control. 9µg of protein was loaded for the two month-old samples and 20µg of protein was loaded for the eleven month-old samples (C).
Figure 3. Delay of RPE atrophy after AAV2/8(Y733F)-Rho-Pde6α transduction.

Representative infrared (IR) images of an untreated Pde6α<sup>nmf363</sup> mutant eye at five (A), seven (C), nine (E), and eleven (G) months of age compared to the fellow treated Pde6α<sup>nmf363</sup> mutant eye at five (B), seven (D), nine (F), and eleven (H) months of age. Images for five and seven months of age are taken from the same mouse, images from nine and eleven months of age are taken from a second mouse for replication purposes. Representative IR image of a C57BL/6J control mouse at eight months of age (I). Increased IR reflectance represents RPE atrophy (*).

The attenuation of the arterioles is also arrested in the treated eyes at age examined. IR imaging was obtained at 790 nm absorption and 830 nm emission using a 55 degree lens. Images were taken of the central retina, with the optic nerve located in the center of the image and the site of the subretinal bleb along the lower left-hand quadrant.

Figure 4. Improved photoreceptor survival after AAV2/8(Y733F)-Rho-Pde6α transduction.

H&E stained retinal section of a two month-old treated Pde6α<sup>nmf363</sup> mouse eye (A). Images were composited together to show entire retina. Arrows show ONL and OS regions. Scale bar: 1000µm. Quantification of the thickness of the ONL by counting columns of photoreceptor nuclei in the rescued half of the treated retina, the un-rescued half of the treated retina (labeled internal control), and fellow untreated eyes from one to six months of age (B). Error bars show SEM for each time-point and significance was calculated for the rescued half of the treated retina compared to the untreated fellow eyes using ratio paired t-test analysis. N ≥ 3 mice. H&E stained retinal section of a five month-old untreated Pde6α<sup>nmf363</sup> mouse retina (C), the fellow Pde6α<sup>nmf363</sup> mouse retina treated with AAV2/8(Y733F)-Rho-Pde6α (D), and a B6 control mouse.
Figure 5. Rescued visual function after AAV2/8(Y733F)-Rho-Pde6α transduction.

Representative scotopic maximum ERG traces for a B6 control mouse (black), the treated eye (light grey) and the untreated eye (dark grey) of a Pde6αnmf363 mouse at two months of age (A), and five months of age (B). Maximum scotopic b-wave amplitudes in a B6 mouse, the treated Pde6αnmf363 eyes and fellow untreated eyes monthly between one and six months of age (C). Maximum scotopic photoreceptor-mediated a-wave amplitudes (shown as positive values) in a B6 mouse, the treated Pde6αnmf363 eyes and fellow untreated eyes monthly between one to six months of age (D). Representative scotopic dim light rod-specific ERG traces for a B6 control mouse (black), the treated eye (light grey) and the untreated eye (dark grey) of a Pde6αnmf363 mouse at five months of age (E), and representative photopic single flash cone-mediated ERG traces for a B6 control mouse (black), the treated eye (light grey) and the untreated eye (dark grey) of a Pde6αnmf363 mouse at five months of age (F). Dim light rod-specific scotopic b-wave amplitudes in a B6 mouse, the treated Pde6αnmf363 eyes and fellow untreated eyes monthly between one and six months of age (G). Photopic cone-specific b-wave amplitudes in a B6 mouse, the treated Pde6αnmf363 eyes and fellow untreated eyes from three to six months of age (H). B6 mouse ERG results did not vary over the six month testing period so only the first result is shown in the charts. Error bars show SEM for each time-point and significance was calculated using ratio paired t-test analysis. N ≥ 3 mice.
Figure 6. No rescued visual function from a control vector injection. Maximum photoreceptor-specific a-wave amplitudes (shown as positive values) in six week-old treated $Pde6\alpha^{nmf363}$ eyes compared to their fellow untreated eyes and $Pde6\alpha^{nmf363}$ mice treated with AAV2/8-TurboRFP alone (A). Maximum b-wave amplitudes in six week-old treated $Pde6\alpha^{nmf363}$ eyes compared to their fellow untreated eyes and $Pde6\alpha^{nmf363}$ mice treated with AAV2/8-TurboRFP alone (B). Error bars show SEM for each time-point and significance was calculated using ratio paired and two-tailed $t$-test analysis. $N \geq 8$ mice.

Abbreviations

AMD, age-related macular degeneration; B6, C57BL/6J wild-type mouse strain; cGMP, cyclic guanosine 3',5'-monophosphate; ERG, electroretinogram; INL, inner nuclear layer; IR, infrared; IS, inner segment; ONL, outer nuclear layer; OS, outer segment; P, postnatal; PDE6, cGMP phosphodiesterase complex; PDE6A, human gene encoding PDE6\(\alpha\); PDE6\(\alpha\beta\), \(\alpha\) and \(\beta\) subunits of PDE6; Pde6\(\alpha^{nmf363}\), hypomorphic Pde6\(\alpha\) allele; Pde6b, mouse gene encoding PDE6\(\beta\); Rho, gene encoding rhodopsin; RP, retinitis pigmentosa; RPE, retinal pigment epithelium.