Rescue of M-cone Function in Aged Opn1mw<sup>−/−</sup> Mice, a Model for Late-Stage Blue Cone Monochromacy

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PURPOSE. Previously we showed that AAV5-mediated expression of either human M- or L-opsin promoted regrowth of cone outer segments and rescued M-cone function in the treated M-opsin knockout (Opn1mw<sup>−/−</sup>) dorsal retina. In this study, we determined cone viability and window of treatability in aged Opn1mw<sup>−/−</sup> mice.

METHODS. Cone viability was assessed with antibody against cone arrestin and peanut agglutinin (PNA) staining. The rate of cone degeneration in Opn1mw<sup>−/−</sup> mice was quantified by PNA staining. AAV5 vector expressing human L-opsin was injected subretinally into one eye of Opn1mw<sup>−/−</sup> mice at 1, 7, and 15 months old, while the contralateral eyes served as controls. M-cone-mediated retinal function was analyzed 2 and 13 months postinjection by full-field ERG. L-opsin transgene expression and cone outer segment structure were examined by immunohistochemistry.

RESULTS. We showed that dorsal M-opsin dominant cones exhibit outer segment degeneration at an early age in Opn1mw<sup>−/−</sup> mice, whereas ventral S-opsin dominant cones were normal. The remaining M-opsin dominant cones remained viable for at least 15 months, albeit having shortened or no outer segments. We also showed that AAV5-mediated expression of human L-opsin was still able to rescue function and outer segment structure in the remaining M-opsin dominant cones when treatment was initiated at 15 months of age.

CONCLUSIONS. Our results showing that the remaining M-opsin dominant cones in aged Opn1mw<sup>−/−</sup> mice can still be rescued by gene therapy is helpful for establishing the window of treatability in future blue cone monochromacy clinical trials.

Keywords: blue cone monochromacy, gene therapy, opsins, X-linked genetic disease, AAV
cones of Opn1mw−/− mice do not form cone outer segments, very much like the remaining cones in human BCM fovea with shortened outer segments; therefore, Opn1mw−/− mice can be used as a model for BCM. We also showed that AAV-mediated expression of either human OPN1LW or OPN1MW in Opn1mw−/− cones rescued M-cone function and promoted regeneration of cone outer segments in the dorsal retina.22 In the present study, we characterized the numbers of viable cones in dorsal and ventral retinas in young Opn1mw−/− mice and their rate of degeneration with age. We also assessed whether AAV-mediated delivery of human L-opsin can still rescue dorsal M-cones in aged Opn1mw−/− mice and the longevity of that rescue effect. These results provide important information on the age window for gene therapy in future clinical trials. We have used human L-opsin in this study because we believe this is a better choice of vector for future BCM clinical studies. We think it is best to treat BCM patients with only one opsin to minimize biologic variables and simplify the interpretation of study results, at least initially. L-opsin might be a better choice than M-opsin because when added to the endogenous S-cone frequencies, L-opsin response spectrum to light frequencies would give rise to a wider spectrum of perceivable frequencies than an M-opsin plus S-opsin combination.

METHODS

Animals

All mice used in this study were maintained in the University of Florida Health Science Center Animal Care Service Facilities on a 12-hour/12-hour light/dark cycle. All animals were maintained under standard laboratory conditions (18°C–23°C, 40%–65% humidity) with food and water available ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Florida and conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and National Institutes of Health regulations. The generation and characterization of Opn1mw−/− mice has been described previously.20

AAV Vector

The AAV vector expressing human OPN1LW driven by PR2.1 promoter (PR2.1-hOPN1LW) was described previously.21 This vector was packaged in serotype 5 AAV and was purified according to previously published method.22

Subretinal Injection

Eyes of Opn1mw−/− mice were diluted with Tropi-Phen (phenylephrine HCl 2.5%, Tropicamide 1% Ophthalmic Solution; Pine Pharmaceuticals, Tonawanda, NY, USA) 15 minutes before injection. Transcorneal subretinal injections were performed with a 33-gauge blunt-end needle attached to a 5-ml Hamilton syringe. First, an entering puncture was introduced at the edge of the cornea with a 29-gauge disposable needle, then 1 μl of viral vector mixed with fluorescein dye (0.01% final concentration) was injected through the corneal opening and delivered into the subretinal space as described before.23 An injection was considered successful if it detached at least 80% of the retina visualized by the fluorescence bleb that was monitored by video camera attached to the injection scope allowing real-time assessment of surgical procedures. Atropine eye drops (Akorn, Inc., Lake Forest, IL, USA) and neomycin/polymyxin B/dexamethasone ophthalmic ointment (Bausch & Lomb, Inc., Tampa, FL, USA) were applied after injection. One eye was injected, whereas the contralateral eye was un.injected and served as a control; 5 × 109 vector genomes (vg) in 1 μl was injected. Antisedan (Orion Corporation, Espoo, Finland) at 1 mg/kg was given intramuscularly following injection as an anesthetic reversal. We performed AAV5-GFP injections in Opn1mw−/− mice in our previous study20 and observed no adverse effect.

Electroretinography

M-cone ERG responses were analyzed using a UTAS Visual Diagnostic System with a Big Shot Ganzfeld dome (LKC Technologies, Gaithersburg, MD, USA). All mice were anesthetized with an intraperitoneal injection of ketamine (72 mg/kg)/xylazine (4 mg/kg). The pupils were dilated with Tropi-Phen. Mice were first exposed to white light for 5 minutes at 30 cd/m2 to suppress rod function. Then M-cone ERGs were recorded by stimulation of the green channel middle-wavelength light (510 nm) at intensities of −0.6, 0.4, and 1.4 log cd.s/m2. Twenty-five recordings were averaged for each of the three light intensities. ERG data were presented as average ± SEM. Seven mice were recorded for each treatment group. Statistical analysis was performed by 1-way ANOVA with the Bonferroni post hoc test to compare each treatment group with untreated control group. Significance was defined as a P value <0.01.

Our ERG machine has a 510-nm filter for detection of mouse M-cone. From our previous study,19 we found that the 510-nm filter can still detect human L-opsin delivered by AAV in Opn1mw−/− eyes because of the spectrum overlap between human L-opsin and mouse M-opsin. All animals were light-adapted before ERG to suppress any rod responses and we never detected any responses from contralateral untreated Opn1mw−/− eyes under photopic conditions when we performed M-cone ERG.

Preparation of Arrestin4 Monoclonal Antibody

Poly(A)+ RNA was isolated from C57/Bl6j mouse retinas (QuickPrep mRNA isolation kit; GE Life Sciences, Chicago, IL, USA) and reverse transcribed, priming with oligo(dT) (First-strand cDNA synthesis kit; GE Life Sciences). Arrestin4 cDNA was amplified from this cDNA using oligonucleotides specific to the 5’ and 3’ ends of the open reading frame murine arrestin4 cDNA, incorporating EcoRI restriction sites before the initiating methionine codon and after the stop codon. The cDNA was cloned into pET-28a (Novagen, Madison, WI, USA) to incorporate a His tag on the amino terminus. Arrestin4 protein was expressed in BL21(DE3) Escherichia coli in the presence of 50 μM IPTG. The protein was purified by nickel affinity chromatography (His GraviTrap; GE Life Sciences) in 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10 mM imidazole, and 6 M guanidine hydrochloride and then dialyzed in PBS. The arrestin4 protein was used to immunize BALB/c mice (Jackson Laboratory, Bar Harbor, ME, USA), as previously described.23 Hybridoma cells lines prepared from the splenocytes from the immunized mice were characterized by immunoblot and immunohistochemistry to identify monoclonal antibodies that were specific for arrestin4.

Preparation of Retinal Whole Mounts and PNA Staining

Mice were humanely euthanized, the eyes were marked at 12 o’clock on the cornea with a burn marker and then enucleated and fixed with 4% paraformaldehyde in PBS for 1 hour. The cornea and lens were then removed. To make flat mounts, the entire retina was carefully dissected from the eye cup and radial cuts were made from the edges to the equator of the retina. Retinal flat mounts were blocked in 3% BSA for 2 hours, then labeled with Biotinylated Peanut Agglutinin (PNA) (Vector...
Laboratories, Burlingame, CA, USA) and S-opsin antibody (MilliporeSigma, Burlington, MA, USA) in PBS overnight. The flat mounts were then washed with PBS three times, followed by incubating with Fluorescein Avidin D (Vector Laboratories) and immunoglobulin (Ig)G secondary antibody tagged with Alexa-594 (Molecular Probes, Eugene OR, USA) diluted 1:500 in PBS at room temperature for 2 hours, then washed with PBS. The flat mounts were flattened on slides with a fine brush, one drop of Vectashield Mounting Medium for Fluorescence (H-1400; Vector Laboratories) was applied and then covered with a coverslip. The flat mounts were imaged with a Leica (Wetzlar, Germany) Fluorescence Microscope LAS X Widefield System. Four images were taken from each flat mount, two from dorsal area and two from the ventral area, marked by a burn mark. PNA-positive cells from each image were counted in an area that is equivalent to 0.01 mm² of retina using the counting tool in Adobe Photoshop (Adobe Systems, Inc., San Jose, CA, USA). Counts of 12 images from six different mice (three males, three females) were average for dorsal and ventral regions, and the SD was calculated. Statistical analysis was performed by one-way ANOVA with Bonferroni posttests to compare the difference among each age group for dorsal or ventral regions. Significance was defined as \( P < 0.05 \).

**Frozen Retinal Section Preparation and Immunohistochemistry**

Mice were light-adapted at 2000 lux under white fluorescent light for 1 hour to facilitate cone arrestin (CAR) translocation to cone outer segments. They were then killed immediately and their eyes marked at 12 o’clock on the cornea with a burn marker and enucleated. The eyes were fixed in 4% paraformaldehyde for 30 minutes then the cornea and lens were removed without disturbing the retina. The retinas were further fixed for additional 2 to 3 hours at room temperature. The eyecups were then rinsed with PBS and cryoprotected with 30% sucrose/PBS for 3 hours at room temperature, then embedded in cryostat compound (Tissue TEK OCT; Sakura Finetek USA, Inc., Torrance, CA, USA) and frozen at \(-80^\circ\)C. Retinas were cut perpendicularly from dorsal to ventral at 12-μm thickness. For immunohistochemistry, retinal sections were rinsed in PBS and blocked in 3% BSA, 0.3% Triton X-100 in PBS for 1 hour at room temperature. Sections were then incubated with primary antibodies at room temperature overnight. The following primary antibodies were used: anti-L/M-opsin, anti-S-opsin (both from MilliporeSigma), cone arrestin, PDE6c (kindly provided by Bernd Wissinger, PhD). After incubation with first antibody, the slides were washed with PBS three times, followed by incubating with IgG secondary antibody tagged with Alexa-594 or Alexa-488 (Molecular Probes) diluted 1:500 in PBS at room temperature for 2 hours, then washed with PBS. Sections were mounted with Vectashield Mounting Medium for Fluorescence (H-1400; Vector Laboratories) and cover slipped. Sections were imaged with a Leica Fluorescence Microscope LAS X Widefield System.

**RESULTS**

**Rate of Cone Degeneration in Opn1mw−/− Mice**

Previously we showed that the dorsal M-cones of 2-month-old Opn1mw−/− mice had significantly shortened outer segments;
However, their cone sheaths were detectible by PNA staining, indicating that these cones remain viable.\textsuperscript{19} We also showed that ventral retinas (dominated by S-cones) of Opn1mw\textsuperscript{−/−} mice had normal cone structure. Here we stained retinal sections of 15-month-old Opn1mw\textsuperscript{−/−} mice with an antibody against cone arrestin. Cone arrestin staining was diffuse throughout the cone photoreceptors from the synaptic terminals to the cone outer segments,\textsuperscript{26} and is another marker for assessing cone viability. We found that in aged dorsal retinas, cone arrestin staining was still present in synaptic terminals, axons, cell bodies, and inner segments, an indication that these cones are viable. Similar to what we observed before, these dorsal cones had shortened outer segments compared with their ventral counterparts (Fig. 1A).

Next we characterized the number of viable cones in dorsal and ventral retinas in Opn1mw\textsuperscript{−/−} mice of different ages by labeling retinal flat mounts with PNA at 21 days, 3 months, 11 months, and 15 months (Fig. 2A). In the wild-type controls, we counted 100 to 140 cones per 0.01 mm\textsuperscript{2} of retina, similar to a previous reported cone density of 1.2 ± 0.2 × 10\textsuperscript{4} mm\textsuperscript{−2}.\textsuperscript{14,16,27} We found no statistical difference in cone densities between young and aged wild-type mice (Fig. 2C) (n = 6, P > 0.05 for both dorsal and ventral), also consistent with a previous report.\textsuperscript{28} We show here that at postnatal day 21 (P21), Opn1mw\textsuperscript{−/−} mice had similar numbers of viable cones in both dorsal and ventral retinas as in the 3-month-old wild-type controls (n = 6, P > 0.05). At 3 months of age, Opn1mw\textsuperscript{−/−} dorsal retinas had retained only ~70% viable M-cones compared with age-matched wild-type controls (n = 6, P < 0.01), whereas the density of ventral S-cones was not significantly different from wild-type controls (P > 0.05). Dorsal cones further degenerated with age, with 11- and 15-month-old Opn1mw\textsuperscript{−/−} dorsal retinas containing only ~50% of viable M-cones compared with the age-matched wild-type controls (n = 6, P < 0.0001),

![Figure 2](https://example.com/figure2.png)

**Figure 2.** The time course of cone degeneration in Opn1mw\textsuperscript{−/−} mice. (A) Representative images of retinal flat mounts taken from dorsal and ventral retinas of Opn1mw\textsuperscript{−/−} mice and wild-type controls at different ages. Retinal whole mounts were stained with S-opsin antibody (red) and PNA (green). S-opsin is primarily detected in the ventral retina. (B) A representative image of a flat-mounted 3-month-old Opn1mw\textsuperscript{−/−} retina with overlaid squares indicating regions analyzed for cone densities. (C) Quantification of PNA staining from dorsal and ventral retinas of Opn1mw\textsuperscript{−/−} mice at different ages and wild-type controls. Each bar represents PNA-positive signals counted in 0.01 mm\textsuperscript{2} of surface area of retina from 12 images taken from six different mice. Data show average ± SD. *P < 0.01. ns, not significant.
whereas the numbers of ventral S-cones were similar to their age-matched wild-type controls \( (P > 0.05) \).

**Expression of Human L-opsin Restores M-cone Function in Aged **\( \text{Opn1mw}^-\)** Mice

Consistent with above observation, AAV-mediated expression of human L-opsin rescued M-cone function in aged \( \text{Opn1mw}^-\) mice. Previously we showed that AAV5-mediated expression of either human M- or L-opsin promoted regrowth of cone outer segments and rescued M-cone function when delivered in 1-month-old M-opsin knockout (\( \text{Opn1mw}^-\)) mice.\(^{19}\) Here we show that subretinal injection of AAV vector expressing human OPN1LW driven by the cone-specific promoter PR2.1 rescued ERG responses to M-cone preferred middle-wavelength light stimuli in 15-month-old \( \text{Opn1mw}^-\) mice (Fig. 3). ERG responses in treated aged mice were lower than those in treated younger mice, consistent with the lesser number of viable dorsal cones in aged mice; however, significant ERG function was preserved in the aged mice, even in mice treated at 15 months. The averaged maximum M-cone b-wave amplitude in \( \text{Opn1mw}^-\) mice treated at 1 month of age and examined for ERG function at 2 months postinjection (\( \text{P1M} + 2\text{M} \)) was \( 96 \pm 4.3 \) \( \mu \text{V} \); b-wave amplitudes in

\[ \text{WT}, \text{P15M} + 2\text{M} \]
performed immunohistochemistry from mice injected at 15 months of age and killed at 12 months postinjection, only a few dorsal cones were found to contain outer segments, suggesting that treatment effect was gradually lost over time when mice were treated at this advanced age.

**Discussion**

In this study, we characterized the degeneration rate of mutant M-cones in Opn1mw−/− mice and explored the window of treatability. We found that mutant M-cones already degenerated at early life and they continued to degenerate with age. Aged Opn1mw−/− mice (15 months old) had ~50% of the viable dorsal M-cones compared with age-matched wild-type controls. These mutant M-cones, although having significantly shortened or no outer segments, remained viable and continued to express other essential cone outer segment proteins. We further demonstrated that delivery of AAV5 expressing human OPN1LW resulted in regenerated cone outer segments in the remaining M-cones in treated aged Opn1mw−/− mice. Moreover, other cone phototransduction proteins localized normally to the outer segments on regeneration, and treated M-cones responded appropriately to middle-wavelength light.

In previous studies on retinal structural changes in BCM human patients, it was found that there are a reduced number of cones early in life. Some patients showed evidence of progressive central retinal cone loss and macular atrophy at later stages. High-resolution retinal imaging in BCM patients confirmed the existence of inner segments as part of the remaining cone cells, albeit with significantly shortened but detectable outer segments. This evidence indicates that remaining mutant cones in human patients remain viable, suggesting potential value of gene therapy for BCM. We also detected degeneration of mutant dorsal cones early in young Opn1mw−/− mice, consistent with observations in BCM human patients. Our results confirm that gene therapy is still...
possible in older patients as long as they retain sufficient residual foveal cones.

There are two major mechanisms causing BCM: LCR deletion and C203R missense mutation. The *Opn1mw*+/− mouse model represents patients with LCR deletion. However, the C203R mutation might have a different disease mechanism because its corresponding mutation in rhodopsin (C187Y) causes early and severe autosomal dominant retinitis pigmentosa.\(^{29}\) Interestingly, a recent study in BCM patients comparing the phenotype caused by large deletion mutations with those having the C203R mutation showed that disease progression in patients with the C203R genotype appeared less aggressive than in their cohort of patients with deletion mutations.\(^{13}\) We are in the process of characterizing a mouse model representing the C203R mutation and performing proof-of-concept experiments.

In wild-type mice, the formation of the outer segments is complete at P21.\(^{30,31}\) We observed that at P21, *Opn1mw*+/− mice had a similar density of viable dorsal cones as in the 3-month old wild-type controls suggesting that differentiation and formation of M-cones was not affected initially in the absence of M-opsin. Mutant M-cones degenerated with age; however, the remaining cones, although having shortened or no outer segments, remained alive for a long time. This persistence is in sharp contrast to the fate of rod photoreceptors in rhodopsin knockout mice in which rods undergo rapid cell death.\(^{32,33}\) The hardness of cones maybe explained by the fact that most cones coexpress M-opsin and S-opsin.\(^{14,16–18}\) It was shown that in the S-opsin knockout mouse, cones in the distal ventral can survive for at least 1.5 years.\(^{16}\) In addition, knocking-out S-opsin led to a higher level expression of M-opsin.\(^{16}\) Consistent with this, we also noticed higher S-opsin expression in the *Opn1mw*+/− mice.\(^{20}\) Therefore, it is likely that some S-opsin expression in the dorsal cones of *Opn1mw*+/− mice contributes to their survival.

In conclusion, our results show that the remaining M-cones in aged *Opn1mw*+/− mice can be rescued by cone opsin gene therapy and this has important implications for defining the time window for possibly similar therapies in humans. Our results also provide important information for patient selection criteria for future BCM clinical studies.

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**References**


