Timing of Antioxidant Gene Therapy: Implications for Treating Dry AMD

Manas R. Biswal,1 Pingyang Han,1 Ping Zhu,2 Zhaoyang Wang,3 Hong Li,1 Cristhian J. Ildefonso,2 and Alfred S. Lewin1

1Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, Gainesville, Florida, United States
2Department of Ophthalmology, University of Florida College of Medicine, Gainesville, Florida, United States
3Department of Ophthalmology, Shanghai Ninth People’s Hospital, Shanghai Jiaotong University School of Medicine, Huangpu District, Shanghai, China

Correspondence: Manas R. Biswal, Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, 1200 Newell Drive, Gainesville, FL 32610, USA; Biswal@ufl.edu.
Submitted: December 9, 2016
Accepted: January 23, 2017
Citation: Biswal MR, Han P, Zhu P, et al. Timing of antioxidant gene therapy: implications for treating dry AMD. Invest Ophthalmol Vis Sci. 2017;58:1237–1245. DOI:10.1167/iovs.arvojournals.org.

PURPOSE. To investigate whether antioxidant gene therapy protects the structure and function of retina in a murine model of RPE atrophy, and to determine whether antioxidant gene therapy can prevent degeneration once it has begun.

METHODS. We induced mitochondrial oxidative stress in RPE by conditional deletion of Sod2, the gene for manganese superoxide dismutase (MnSOD). These mice exhibited localized atrophy of the RPE and overlying photoreceptors. We restored Sod2 to the RPE of one eye using adenovirus- associated virus (AAV) by subretinal injection at an early (6 weeks) and a late stage (6 months), injecting the other eye with an AAV vector expressing green fluorescent protein (GFP). Retinal degeneration was monitored over a period of 9 months by electroretinography (ERG) and spectral-domain optical coherence tomography (SD-OCT). Immunohistochemical and histologic analyses were conducted to measure oxidative stress markers and to visualize retinal structure.

RESULTS. One month after delivery, the AAV-Sod2 injection resulted in production of MnSod in the RPE and negligible expression in the neural retina. Electroretinography and OCT suggested no adverse effects due to increased expression of MnSOD or subretinal injection. Decrease in the ERG response and thinning retinal thickness was significantly delayed in eyes with early treatment with the Sod2 vector, but treatment at 6 months of age did not affect the ERG decline seen in these mice.

CONCLUSIONS. We conclude that antioxidant gene therapy may be effective in preventing the detrimental effects of oxidative stress, but may not be beneficial once substantial tissue damage has occurred.

Keywords: gene therapy, age-related macular degeneration, superoxide dismutase 2, retinal pigment epithelium, geographic atrophy

Age-related macular degeneration (AMD) is one of the leading causes of irreversible visual dysfunction among the elderly.1 Visual dysfunction in AMD patients is characterized as either “dry” AMD or “wet” or neovascular AMD or both. The advanced form of dry AMD, also termed geographic atrophy (GA), is caused by death of macular retinal pigment epithelial (RPE) cells and degeneration of photoreceptors. In neovascular AMD, choroidal blood vessels invade the retina, causing vascular leakage and scarring within retina. In either of the forms, central vision is severely affected, leading to loss of independence. Even though treatments are available for wet AMD, there is no effective treatment for GA, though research on replacement of the RPE is in progress.2,3

Damage to the RPE and photoreceptors has been linked to progressive vision loss in the dry form of AMD. Animal models and the Age-Related Eye Disease Study (AREDS) trial strongly support a role for oxidative stress in the development of AMD.4,5 Because of the importance of oxidative stress as a contributing factor in AMD, treatments that reduce the accumulation of reactive oxygen species (ROS) may be valuable as therapeutics, though antioxidant therapy has shown more benefit in preventing progression to wet AMD than to GA.6–9

Our lab has developed a mouse model of GA in which the knockdown or deletion of Sod2, the gene for manganese superoxide dismutase (MnSOD), in the RPE leads to elevated oxidative stress, causing some of the features of GA including damage to the RPE and Bruch’s membrane and death of photoreceptors.10,11 Depletion of MnSOD in the RPE in mice shows accumulation of lipofuscin-like fluorescent aggregates containing A2E and Iso-A2E.12 While markers of oxidative stress are elevated by 2 months of age in this model, reductions in ERG a-wave and b-wave amplitudes and thinning of the outer nuclear layer (ONL) are not statistically significant until 6 months of age. Therefore, we reasoned that reducing oxidative stress in this interval (between 2 and 6 months) had the prospect of preventing retinal degeneration in these mice. We have also used this model for testing drug therapy for GA13–15 starting at weaning and have shown that treatment with antioxidants reduces free radical accumulation in the RPE and outer nuclear layer.16

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Because gene therapy can be targeted to the retina and RPE17–20 and potentially provide long-term protection against oxidative stress, this approach may be advantageous in treating a chronic condition like advanced dry AMD. Timing of therapy is a critical issue, however. Oxidative stress may contribute to the pathologic changes of RPE and retina, but will antioxidant therapy be beneficial in preventing GA after clinical signs have appeared? In this study we inquired whether adeno-associated virus (AAV)–mediated gene delivery of Sod2 could reverse the impact of RPE-specific deletion of the same gene at two time points, early and late after deletion of the gene. We found that early replacement of Sod2 in RPE delayed retinal degeneration, whereas replacement of Sod2 after substantial RPE damage had already occurred did not prove effective, suggesting that antioxidant gene therapy may be useful as a preventive but not as a therapy for GA.

METHODS

Study Design

Two age groups of mice were used to design the experiment, one group of mice at 6 weeks of age (early stage), and another group of mice at 6 months of age (late stage) (Fig. 1). The AAV-Sod2 myc experimental vector was injected in one eye, and the contralateral eye was injected with control vector AAV-GFP. The mice were examined by ERG and SD-OCT at 3, 6, and 9 months for early gene delivery and at 9 months of age for late gene delivery. After 9 months, changes in retinal ultrastructure in response to Sod2 delivery were examined using electron microscopy. Sod2 expression was determined by Western blotting and its effect in reducing markers of oxidative stress was quantified by ELISA.

AAV Vector

Self-complementary AAV vector was used to clone mouse Sod2 cDNA, a single copy of myc epitope inserted replacing stop codon of Sod2 (Supplementary Fig. S1A). A self-complementary AAV vector carrying GFP cDNA was used as a control to compare the effects of AAV injection. Expression of both genes was driven by a truncated version of the chimeric chicken β-actin proximal promoter and the immediate early enhancer of cytomegalovirus (the CBA promoter).22 Adeno-associated virus 1 was purified by the Vector Core of the Center for Vision Research at the University of Florida using iodixanol gradients and anion exchange chromatography.23 The stock concentration of purified AAV-Sod2 and AAV-GFP was 1 × 10^{12} genome copies (gc) per milliliter.

Subretinal Injection

Adeno-associated virus (10⁹ viral particles in 1 μL) was injected subretinally via the pars plana as described by Timmers et al.24 We examined all animals by SD-OCT 2 weeks after subretinal injection, and all animals with unhealed retinal detachments were excluded from further analysis.

Western Blot

Protein lysates from RPE/choroid were placed in sample buffer containing dithiothreitol and boiled for 6 minutes at 95°C. Equal amounts of protein were separated using SDS polyacrylamide gel electrophoresis and transferred into a polyvinylidene difluoride (PVDF) membrane using the iBlot system (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). This membrane was blocked with a blocking buffer from Li-Cor Biosciences (Lincoln, NE, USA) for 1 hour at room temperature and incubated overnight with the designated primary antibody at 4°C. To detect MnSOD we used a primary rabbit polyclonal antibody from Abcam (Cat. no. ab15533, 1:1000 dilution; Cambridge, MA, USA) and a donkey anti-rabbit secondary antibody from Li-Cor Biosciences (Cat. no. 926-32213, 1:5000 dilution); to detect the exogenously delivered Sod2-myc gene, we used a mouse anti-myc antibody from Invitrogen (Cat. no. 04-1117, 1:5000 dilution), and the secondary antibody was donkey anti-mouse from Li-Cor Biosciences (Cat. no. 926-68072, 1:5000 dilution). Rabbit anti-alpha-tubulin primary antibody from Abcam (Cat. no. ab7291, 1:10,000 dilution) and a donkey anti-rabbit secondary antibody from Li-Cor Biosciences (Cat. no. 926-32213, 1:5000 ratio) were used to detect tubulin.

Electroretinography

Scotopic ERG recordings were made as described in our previous paper.14 Briefly, we recorded scotopic a- and b-wave

FIGURE 1. Study design. Two age groups of mice were used to design the experiment, one group of mice at 6 weeks of age (early stage) and another group of mice at 6 months of age (late stage). The AAV1-Sod2-myc experimental vector was injected in one eye, and the contralateral eye was injected with control vector AAV1-GFP. The mice were examined by ERG and SD-OCT at 3, 6, and 9 months for early gene delivery and at 9 months of age for late gene delivery. After 9 months, changes in retinal ultrastructure in response to Sod2 delivery were examined using electron microscopy. Sod2 expression was determined by Western blotting and its effect in reducing markers of oxidative stress was quantified by ELISA.
ERG responses at three different light intensities (~20 dB [0.02 cds/m²], −10 dB [0.18 cds/m²], 0 dB [2.68 cds/m²]) from both eyes using an LKC UTAS Visual Electrodiagnostic System with a BigShot full-field dome (LKC, Gaithersburg, MD, USA). To examine RPE responses, a-wave amplitudes from each eye were measured using Espion full-field ERG system at a flash intensity of 20 dB (50 cds/m²), and the results were compared between treated and untreated eyes at one time point.

**Spectral-Domain Optical Coherence Tomography**

High-resolution SD-OCT images were obtained by employing Envisu SD-OCT ophthalmic imaging system (Bioptigen, Durham, NC, USA) as described in our previous paper.14 The outer nuclear layer (ONL) was measured at four different locations (temporal, nasal, superior, and inferior) at 0.35-mm distance from the optic nerve head (ONH). Upon averaging ONL thickness from each eye, the results were compared between treated and untreated eyes. We also used Bioptigen’s autosegmentation program to measure layers of the retina and RPE.

**Evaluation of Oxidative Stress Markers 8-OHdG and Nitrotyrosine**

Immunohistochemistry for 8-hydroxy-deoxyguanosine (8-OHdG) was conducted according to the previously published protocol.14 The nitrotyrosine (3-nitrotyrosine, 3NT) competitive enzyme-linked immunosorbent assay (ELISA) (Abcam, Cat. no. ab113848) was used to estimate the nitrotyrosine-modified protein levels from the retina/RPE/choroid sample, performed according to the manufacturer’s protocol. Briefly, 96-well microplates were coated with nitrotyrosine containing antigen overnight. The eye cup containing retina/RPE/choroid was dissected separately from the eyes treated with either AAV1-Sod2 or AAV1-GFP vector 45 days following subretinal injection. The samples were collected in 150 μL phosphate-buffered saline (PBS) and disrupted by sonication for 10 seconds on ice. The samples were further diluted for ELISA. Triplicates of each treatment and triplicates of each test standard were used in the assay. Being the competitive assay, the increased 3-NT in the sample results in a reduced colorimetric reaction at 600 nm.

**Light and Electron Microscopy**

For light and electron microscopy, mice were injected with an overdose of sodium pentobarbital and perfused with a mixture of PBS containing 2% paraformaldehyde and 2.5% glutaraldehyde. The fixed eyes were collected and processed according to the procedure described in our previous paper.14

**Statistical Analysis**

The statistical software GraphPad Prism (version 5.0; GraphPad Software, Inc., San Diego, CA, USA) was used to analyze the data. All reported P values were calculated using the 2-tailed Mann-Whitney test as indicated in the legends, and a P value of <0.05 was considered significant. All data are represented as mean ± SEM unless otherwise indicated.

**RESULTS**

**RPE-Specific Sod2 Deletion and Overexpression**

Our mouse model (Sod2<sup>lox/lox</sup>/PVMD2<sup>Cre</sup>TA; tetO-P<sub>hCMV</sub>Cre) involves RPE-specific deletion of Sod2, using the cre-lox system, and results in a gradual retinal degeneration demonstrated by reduction in electroretinogram (ERG) amplitudes and in the thickness of the ONL that are statistically significant by 6 months of age.10 In this study, we examined whether Sod2 gene therapy could restore visual function and retinal degeneration in the Sod2<sup>lox/lox</sup>/PVMD2<sup>Cre</sup> mice. Serotype 1 AAV1 specifically transduces RPE cells following subretinal injection in mice.25 To enhance the expression of MnSOD in the RPE, we injected an AAV1 vector containing myc epitope-tagged mouse Sod2 cDNA into the subretinal space of one eye of 6-week-old or 6-month-old Sod2<sup>lox/lox/VMD2-cre</sup> mice (Supplementary Fig. S1A). To study the impact of injection or the delivery of AAV particles, we treated the contralateral eyes from the same animals with an AAV1 vector expressing humanized GFP. In control eyes, GFP expression was observed over 60% to 80% of the retina by fluorescence fundus imaging, suggesting efficient delivery of vector (Supplementary Fig. S1B). We found that MnSOD protein levels were reduced by 50% to 60% in eyes of doxycycline-induced Sod2<sup>lox/lox/VMD2-cre</sup> mice compared to Sod2<sup>lox/lox/VMD2-cre</sup> mice not induced with doxycycline (data not shown). To quantify exogenous Sod2 protein levels, the level of AAV-delivered MnSOD expression was examined 1 month following injection (Fig. 2A) using a MnSOD-specific antibody. To validate the RPE selectivity of the vector, retina and RPE/choroid tissue samples were collected separately from a cohort of mice at 1-month post injection. Using a myc antibody, myc-tagged MnSOD was detected in RPE/choroid samples treated with AAV1-Sod2 vector (Fig. 2B). As expected from the tropism of AAV1, negligible expression was observed in the neural retina.

**Reduction in Markers of Oxidative Stress**

By immunohistochemistry and ELISA, we have previously shown that Sod2 deletion in RPE increases markers of oxidative stress.13,14 Upon staining RPE flat mounts with antibody to 8-OHdG, we found a significant reduction in labeling of 8-OHdG fluorescence in treated eyes at the age of 3 months (Fig. 2C) compared to control eyes (Fig. 2D). Deletion of MnSOD increases the level of superoxide that reacts with nitric oxide to produce peroxynitrite (ONOO−), and adducts are formed by reaction of peroxynitrate with tyrosine residues. To determine if Sod2 vector treatment reduced the burden of modified proteins in these mice, we collected the eyes 1 month after injection of vector (injected at 6 weeks, tissue harvested at 2.5 months). The protein samples from the vectortreated eyes were assayed for 3NT by ELISA at the age of 3 months. We found a 54% reduction in levels of nitrotyrosine in treated eyes compared to control eyes (Fig. 2E). We expect that injection of AAV-Sod2 reduced oxidative stress in the mouse retina based on previous results.26-27 but we did not specifically measure the 8-OHdG and peroxynitrate in mice injected at 6 months of age.

**Early Delivery of AAV1-Sod2 Restores Retinal Function**

Three months following treatment with AAV1-Sod2, the experimental mice were examined by dark-adapted electroretinography (ERG). In eyes treated with AAV-Sod2, a-wave amplitudes decreased only 18% between 3 and 9 months following injection at 6 weeks of age. Electoretinogram signals from the control-treated eyes were lower at each time point relative to eyes to which Sod2 had been delivered. At the highest light intensity (2.68 cd/m²), treated eyes showed 56%, 59%, and 80% improved a-wave ERG responses compared to control eyes at 3, 6, and 9 months following treatment, respectively (Figs. 3A, 3C, 3E; Supplementary Figs. S2A, S2C,

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Similarly, the b-wave amplitudes at this light intensity in AAV1-Sod2–treated eyes were 38%, 32%, and 53% higher than the control-treated eyes at these intervals (Figs. 3B, 3D, 3F; Supplementary Figs. S2B, S2D, S2F, S2H).

**Improved RPE Functional Integrity in Early-Treated Eyes**

The c-wave component of the ERG has been correlated with RPE function. We found that the maximum amplitude of the c-wave component of the ERG was significantly increased in early-treated eyes compared to control-treated eyes (Fig. 3B). This indicates improved RPE functional integrity in early-treated eyes.

**Timing of Antioxidant Gene Therapy**

We investigated the timing of antioxidant gene therapy using Sod2 gene delivery. We found that early treatment with Sod2 gene delivery significantly preserved the a-wave amplitudes at 3 months (Fig. 3A, n = 18, ***P = 0.007), 6 months (Fig. 3C, n = 18, ***P = 0.007), and 9 months (Fig. 3E, n = 7, *P = 0.023) following flashes at a light intensity of 2.68 cd/s/m². At the same light intensities, we saw preservation of b-wave amplitudes at 3 months (Fig. 3B, n = 18, ***P = 0.003), 6 months (Fig. 3D, n = 18, ***P = 0.005), and 9 months (Fig. 3F, n = 7, *P = 0.016) following early treatment. We did not see any improvement in a-wave (Fig. 3G) and b-wave (Fig. 3H) responses from eyes at 9 months (3 months after treatment).
wave was reduced by 39% in doxycycline-induced Sod2^lox/lox^/VMD2-cre mice by 3 months (Supplementary Fig. S3A) compared to uninduced mice. Therefore, we recorded c-wave from the dark-adapted mice 3 months following early treatment. The average c-wave amplitudes were significantly increased (>2-fold) in AAV1-Sod2-treated eyes compared to control-treated eyes at this time point (Fig. 4A).

**Structural Rescue by Sod2 Vector (SD-OCT)**

At 5 and 9 months after early treatment, SD-OCT examinations were carried out to measure the thickness of the retinal layers (Figs. 5A, 5B). Visual inspection of the images revealed thinning of the entire retina and the outer nuclear thickness in control-treated eyes by 5 months post injection with the vectors. To compare structural differences, the ONL was
measured at four different locations (temporal, nasal, superior, and inferior) at 0.35-mm distance from the ONH. At 5 months following early treatment, the average ONL thickness was reduced by 13% in control-treated eyes relative to eyes treated with AAV1-Sod2 ($P < 0.01$) (Fig. 5C). At 9 months, the difference in ONL thickness between treated and control eyes was 18% ($P < 0.01$) (Supplementary Fig. S4A). Upon analyzing the OCT images by the Bioptigen autosegmentation program (Driver), we found preservation of total retinal thickness and RPE thickness in early-treated eyes at 5 months (Figs. 5D, 5E) and 9 months post treatment compared to eyes injected with control vector (Supplementary Figs. S4B, S4C). In control eyes, we observed accumulation of subretinal deposits (circle) and white hyperreflective spots (red arrows) (Figs. 6C, 6D), which were not seen in AAV-Sod2–treated eyes (Figs. 6A, 6B).

Lack of Protection Following Late Treatment

Before late injection with AAV1-Sod2 we found significant reductions in ERG amplitudes (a-, b-, and c-waves) in our experimental mice (Sod2$^{flox/flox}$/VMD2-cre) compared to Sod2$^{flox/flox}$ without the inducible cre transgene (51% for a-wave; 47 % for b-wave, and 52% for c-wave) (Supplementary Figs. S3B, S3C, S3D). In contrast to treatment at 6 weeks of age, subretinal injection of AAV1-Sod2 at 6 months of age offered no evidence of retinal protection. Three months following late treatment, ERG a-wave and b-wave amplitudes were not significantly different between eyes treated with the experimental or the control vectors (Figs. 3G, 3H). Late treatment with Sod2 did not show any significant change in c-wave ERG responses (Fig. 4B) between AAV1-Sod2–treated and control-injected eyes. Similarly, we did not find a difference in ONL
thickness (Supplementary Fig. S5C), total retinal thickness (Supplementary Fig. S5D), or RPE thickness (Supplementary Fig. S5E) in late-treated eyes 3 months post treatment.

Sod2 Vector Preserved the Structure of the RPE and Photoreceptors

Since we found that early treatment preserved visual function and retinal structure, we processed eyes of mice treated at 6 weeks for microscopic analysis. At 9 months of age, in lower-magnification light micrographs (Figs. 7A, 7B), RPE vacuolization was apparent in both treated and control-treated retinas, but RPE atrophy was a feature only of the control eyes. In electron micrographs, areas of atrophy exhibited thinned RPE and highly vacuolized RPE with disorganized infoldings at the basal surface of RPE cells. Few, if any, mitochondria were detected. Outer segments of photoreceptors were very short, and only one or two layers of nuclei were present in the ONL (Fig. 7D). In the AAV1-Sod2–treated eyes (Fig. 7C), rod outer segments were full length but contained cystic spaces and some regions of apparent distension. The RPE layer was much thicker than in control eyes and contained mitochondria normally displaced at the basal surface of cells, and the basal infoldings were more compact than in the control eyes.

DISCUSSION

Mitochondrial oxidative stress has been implicated in tissue damage, and in the retina mitochondrial dysfunction is reported in AMD eyes. Oxidative damage to the RPE can impact photoreceptors. Induction of ROS in the absence of sufficient antioxidant system can stimulate the complement cascade and other inflammatory pathways. Retinal pigment epithelial cells derived from AMD patients have decreased antioxidative defenses. Normal aging or cigarette smoking can cause generation of ROS in the retina.

Modeling dry AMD in mice is difficult, since these nocturnal rodents do not rely on cones for central vision and have no anatomic structures analogous to the macula and fovea. Several gene knockout models have been developed that lead to RPE failure and atrophy due to oxidative stress. Mice deficient in Cu, Zn-superoxide dismutase (SOD1) showed oxidative damage and reduced barrier integrity of the RPE. Nuclear factor (erythroid-derived 2)-like 2 (NRF2)-deficient mice show age-related clinical phenotypes of RPE degeneration and increased presence of autophagic vacuoles in addition to spontaneous choroidal neovascularization. Mice carrying deletions in hephaestin and ceruloplasmin are also subject to RPE oxidative stress caused by iron overload, and these mice also show RPE atrophy leading to loss of photoreceptors. Other attempts to
model AMD using mice recapitulate other aspects of pathology. For example, mice knocked-in for the APOE4 gene and fed a diet high in fat and cholesterol exhibit drusen-like structures and retinal atrophy as well as signs of choroidal neovascularization. The retinal oxidative stress in our mouse model is caused by RPE-specific deletion of Sod2 using the cre-lox system. These mice exhibit an age-related decline in the scotopic a-wave and b-wave gradual thinning of the outer nuclear layer. Ultrastructural evidence shows localized atrophy of the retina associated with damage to the underlying RPE and Bruch’s membrane. We have used this model to test two drug therapies for retinal atrophy caused by oxidative stress.

Despite the fact that gene therapy is in clinical trial for inherited retinal degenerations (Clinicaltrials.gov reference numbers NCT00643747, NCT01482195, NCT02759952, NCT02416622), gene delivery has not been reported in animal models of GA. Our study demonstrates the development and testing of an antioxidant gene therapy vector in the RPE. This approach results in unambiguous structural and functional protection of rod photoreceptors in a mouse model of progressive retinal degeneration. Increased expression of Sod2 for a longer time did not have any adverse effect in retinal structure and function. Since RPE-specific expression of Sod2 rescued both the structure and the function of retina, antioxidant therapy in the RPE protects photoreceptors and bipolar cells, at least indirectly.

At a minimum, gene delivery of Sod2 to the RPE of mice in which this gene had been deleted revealed that reversion of the genetic lesion could prevent degeneration of the RPE and neural retina in this mouse model. Indeed, protection of photoreceptors and RPE was achieved within 3 months of gene transfer. But we also learned that timing is important: AAV1-Sod2 injection at 6 weeks, which is prior to the advent of clinical signs of injury in this model, led to substantial protection of the retina based on the ERG and SD-OCT measurements at subsequent times. Subretinal injection of the same vector at 6 months, which is after electrophysiological and structural decline can be documented, did not prevent continued retinal degeneration. We conclude that despite the fact that injury in this model is initiated by mitochondrial oxidative stress, once damage to the RPE is present, antioxidant therapy is not sufficient to protect the retina.

Dietary antioxidant treatment in patients with AMD has shown some benefit. Use of the AREDS formula of antioxidants plus zinc led to a 25% decrease in the number of patients progressing from midstage AMD to neovascular disease. Nevertheless, the same treatment did not prevent progression of early dry AMD to GA. By analogy to our results, we conclude that once tissue damage and inflammatory processes such as complement dysregulation are under way, antioxidant therapy is not sufficient to prevent death of RPE cells and damage to the choriocapillaris and photoreceptor cells. In contrast, dietary consumption of antioxidant nutrients may reduce the incidence of early AMD in people with high-risk alleles. We conclude that antioxidant gene therapy may prevent the progression of dry AMD if used early in the course of the disease, but such an intervention would require identifying patients with early AMD who are likely to progress to GA, and such prediction may be challenging. The contralateral eye of patients with unilateral choroidal neovascularization or GA would be a likely place to start.

**Acknowledgments**

The authors thank James Thomas for large-scale DNA preparation and Vince Chiado for production of AAV vectors. The authors also thank Tong Yao, Giovanni Q. Valdez, Brad Justis, and Chubul Ahmed for their help during ERG and SD-OCT data acquisition and Lindsey Buz for her help in genotyping of mice.

Preliminary data presented in abstract form at the annual meeting of the Association for Research in Vision and Ophthalmology, Orlando, FL, May 2014; Orlando, FL May 2015, and Seattle, WA, May 2016; the American Society for Gene and Cell Therapy meeting, Washington, DC, May 2014 and New Orleans, LA, May 2015; the Retinal Degeneration Symposium, Pacific Grove, CA, July 2014; the Association for Ocular Pharmacology and Therapeutics meeting, Charleston, SC, March 2015; and the International Society for Eye Research meeting, San Francisco, CA, July 2014 and Tokyo, Japan, September 2016. Supported by grants from the National Eye Institute (NEI) (R01 EY020825 [ASL]; R01EY026268 [ASL]; 1K99EY027013 [MRB]), an NEI core grant to the University of Florida (P50 EY02172 [ASL]), and the Shaler Richardson Professorship endowment (ASL).

Disclosure: M.R. Biswal, None; P. Han, None; P. Zhu, None; Z. Wang, None; H. Li, None; C.J. Ilefonso, None; A.S. Lewin, None

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