Communication

Antioxidant and Cytoprotective Potential of Erythropoietin in Mitigating Oxidative Stress-Induced Changes in the Retinal Pigment Epithelium

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Abstract:

Erythropoietin (EPO) protects cells by inhibiting apoptosis, oxidative stress and inflammation in several models of retinal degeneration. In this study, we demonstrate the effects of recombinant Adeno Associated Virus (AAV) vector-mediated delivery of a modified form of erythropoietin (EPO-R76E) in an established mouse model of dry-AMD in which retinal degeneration is induced by RPE oxidative stress. Experimental vector AAV-EPO-R76E and control vector AAV-GFP were packaged into serotype-1 (AAV1) to enable RPE selective expression. RPE oxidative stress-mediated retinal degeneration was induced by exon specific deletion of the protective enzyme MnSOD (encoded by *Sod2*) by cre/lox mechanism. Experimental mice received subretinal injection of AAV-EPO-R76E in the right eye and AAV-GFP in the left eye. Western blotting of RPE/Choroid protein samples from AAV-EPO-R76E injected eyes showed RPE specific exogenous protein expression. Retinal degeneration was monitored by electroretinography (ERG). EPO-R76E over-expression in RPE delayed the progressive retinal degeneration as measured by light microscopy in RPE specific *Sod2* knockout mice. Delivery of EPO-R76E vector can be used as a tool to prevent retinal degeneration induced by RPE oxidative stress as seen in this mouse model.

Keywords: Dry-AMD, oxidative stress, MnSOD, RPE, retinal degeneration, Erythropoietin, gene therapy, Animal model, AAV, ERG.

1. Introduction

Age related Macular Degeneration (AMD) is one of the leading causes of irreversible vision loss among older adults [1]. Healthy communication between the photo-sensitive neural retina and underlying Retinal Pigment Epithelium (RPE) is crucial for proper vision. In the dry form of AMD, macular RPE atrophy leads to photoreceptors loss, thus affecting vision. Dysfunction and loss of RPE in AMD are associated with several genetic and environmental factors. These factors can induce oxidative stress and inflammation that play pathological roles in RPE degeneration [2]. Many endogenous and exogenous factors can damage mitochondrial DNA

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(mtDNA) in the neural retina and RPE cells resulting in reactive oxygen species (ROS) overproduction [3]. High mitochondrial ROS production imbalances antioxidant and cytoprotective systems in the RPE and play a pivotal role in AMD pathogenesis. Antioxidants, growth factors, and neurotrophic factors are widely proposed to protect RPE cells from oxidative damage-associated changes. [4].

Erythropoietin (EPO), a secreted cytokine, is FDA approved for the treatment of anemia. EPO has been shown to act as a novel agent in vascular protection against acute lung injury by promoting angiogenesis [5]. EPO provides neuroprotective effects in several animal models, as it blocks apoptotic pathways and indirectly induces endogenous antioxidants in neurons [6,7]. Intravenous EPO delivery improved visual acuity and color vision in patients following indirect traumatic neuropathy [8]. Studies show that systemic or retinal delivery of EPO or EPO-R76E, a modified form of EPO with reduced erythropoietic activity, can improve the function of retinal ganglion cells and photoreceptors cells [9–19].

We reported *Sod2*$^{flox/flox}$-*VMD2*^{cre} mice as an animal model of dry AMD by conditional genetic deletion of manganese superoxide dismutase (MnSOD, encoded by *Sod2*), a mitochondrial antioxidant enzyme in the RPE [20]. Loss of MnSOD in RPE leads to the induction of oxidative stress, further promoting progressive retinal degeneration seen as early as 4-6 months [21]. We have used this animal model to test various drugs, antioxidant genes, and nutritional supplements to improve the function of RPE and neural retina [22–26].

Eerythropoietin and the erythropoietin receptor (EPOR) are widely expressed within retinal cells, and several groups have tested the ability of exogenous EPO to ameliorate retinal degeneration associated in animal models of diabetic retinopathy, retinitis pigmentosa and other forms of retinal degeneration[17,27]. However, the potential of EPO to limit retinal degeneration associated with age-related macular degeneration (AMD) has not explored. Chronic oxidative stress in the RPE plays an important role in RPE loss in dry-AMD [2,3,28,29]. In response to sustained oxidative stress, RPE cells die by necroptosis[30,31]. We hypothesize that sustained expression of EPO-R76E in the RPE using an AAV vector will improve the health and survival of RPE and retinal photoreceptors. Thus, we evaluated the efficacy of the modified form of EPO in protecting RPE from oxidative stress-induced changes in our mouse model of dry-AMD. We show that EPO-R76E improves retinal function and preserves retinal thickness affected due to chronic oxidative stress in the RPE.

2. Materials and Methods

2.1 Study Design

Ten- to twelve-week-old mice of both sexes without were used to test the vector *in vivo*. The experimental vectors were delivered to the mice eyes by subretinal injection. The mice were analyzed for transgene expression by Western blotting six weeks following injection. The retinal function was recorded by scotopic ERG at 6 and 9 months of age (3 & 6 months after injection). At the end of the experiments (nine months of age), the retinal tissues were analyzed by histology. A schematic diagram of the experimental design is shown in **Fig-1**.

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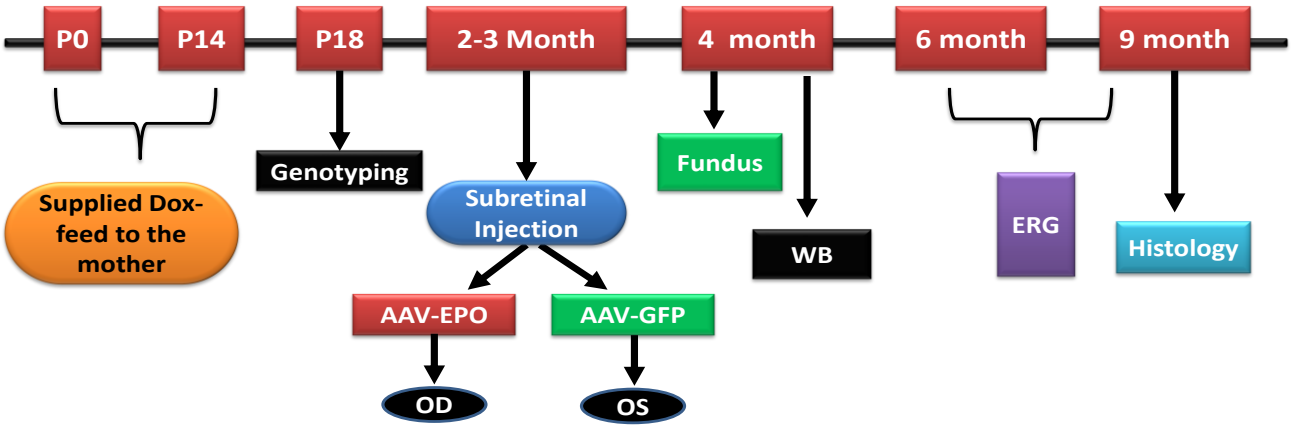

Figure 1: Experimental Design.

2.2. Cell culture and vector production

AAV plasmid having a modified form of *EPO* (EPO-R76E) cDNA were provided by Dr. Tonia S. Rex (Vanderbilt University). This AAV plasmid consists of DNA sequences of inverted tandem repeats (ITR) Cytomeglao Virus (CMV) promoter, short intron sequence, human EPO-R76E cDNA, Woodchuck Hepatitis Virus (WHV) Posttranscriptional Regulatory Element (WPRE), bovine growth hormone polyadenylation (bgh-PolyA) signal and Ampicillin resistnace (Amp^r) genes.Each of the element has specific function in driving the gene expression by the plasmids. This plasmid was used for testing EPO expression *in vitro* and for generating the AAV used *in vivo*. Control AAV-GFP and experimental AAV-EPO-R76E plasmids were transfected to Stable3 cells to obtain enough plasmid DNA for testing in cell culture and animal experiments. HEK293T cells grown on 6 well plates were used in triplicates to transfect 4 micrograms of each plasmids using Polyethylenimine (PEI) cellular transfection reagent (Polysciences, Warrington, PA, catalog no: 23966-100) with a ratio of 1:2, DNA to PEI. Transfection medium with DNA & PEI was replaced with complete growth media after 24 hours and further incubated for another 24 hours to allow transgene expression. Next day, cells were checked for GFP fluorescence. After that, cells were dislodged using cold phosphate buffered saline (PBS). The cells were pelleted at 14,000 × *g* for 3 min at 4^0C and kept at -80^0C for western blotting. Around 1mg each of AAV-GFP and AAV-EPO-R76E plasmids were given to the Vector Core of the Center for Vision Research at the University of Florida to package into AAV1 serotype capsids. The packaged virus was purified using Iodixanol gradients and anion exchange chromatography [32] and achieved a stock concentration of 1 X 10^{12} viral genome copies per milliliter (VG/ml).

2.3 Animals and Injection procedures

All the procedures involving animals in this study followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the protocols were approved by Institutional Animal Care and Use Committee (IACUC) at the University of South Florida and University of Florida. Breeding pairs of RPE specific *Sod2* deleted mice (*Sod2*$^{flox/flox}$*-VMD2-cre*) on C57BL/6 background were set up to generate the mice for this project. These transgenic mice have the *VMD2* promoter driving inducible cre transgene [33] and loxP sites surrounding exon 3 of *Sod2* [34]. The cre transgene was induced by providing Dox food to the mother of newborn pups from P0-P14 that deleted *Sod2* as described in detail by Mao et.al [20]. The genotype of the transgenic pups showing mutations of *rd1*, *rd8* and *rd10* were removed from the study. Intraperitoneal injection of mixture of ketamine (95 mg/kg) and xylazine (8 mg/kg) was used to anesthetize the mice for *in vivo* procedures such as fundus imaging,

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electroretinography (ERG), and retinal injections. The procedures from one of our previous publications were followed for eye dilation and local anesthesia [23]. Mice received subretinal injection of 1 µL of 10^{12} VG/mL of AAV1-EPO-R76E (i.e., 1×10^9 total viral particles) in one eye and an equal dose of AAV1-GFP in the contralateral eye [23,35]. Any eyes with retinal detachment or any structural defect due to injection were excluded from further analysis.

2.4. Western blot analysis

Proteins from cells and retinal tissues were analyzed by western blotting to determine the expression of modified EPO. For cell culture analysis, the cell pellet was dissolved in 100ul of RIPA lysis buffer with protease inhibitors (Sigma, Cat no: P8340). The cells in lysis buffer were vortexed for three to four times with 10-minute intervals on ice to release the protein and then centrifuged at 14,000 × *g* for 30 min at 4^0C. The supernatant was collected to quantify the protein concentration. For *in vivo* expression studies, one month following subretinal delivery of vectors, the eyes were collected after euthanization. The retina and RPE/choroid were dissected out under a surgical microscope and collected separately in 100ul of RIPA lysis buffer with protease inhibitors. The tissues were sonicated for 30 -45 secs in lysis buffer while on ice and cell debris was pelleted at 14,000 × *g* for 30 min at 4^0C. Pierce™ 660nm Protein Assay Reagent (Thermo Fisher Scientific, Cat no: 22660) was used to quantify protein concentration using the supernatant collected from cell pellets and retinal tissues. Twenty micrograms of protein were separated on SDS-PAGE gels, and proteins were transferred to PVDF membrane. The membranes were blocked with Odyssey Blocking Buffer (a phosphate-buffered saline (PBS) based formulation, Li-COR) for an hour and incubated overnight with rabbit polyclonal *Epo* primary antibody (Santa Cruz Biotechnology, Cat no: sc-7956) and mouse monoclonal alpha *tubulin* (Abcam, Cat no: ab7291) primary antibody used as loading control. After washing in PBS-Tween 20 (0.05%) buffer, the membranes were incubated with species specific secondary antibody (LiCor; Cat no : 92532213 and Cat no: 92668170) diluted in PBS for one hour and washed three times in wash buffer (PBS-Tween 20 (0.05%)) before imaging. Labeled proteins were detected using the LiCor Clx Odyssey instrument that showed two different colors for two different protein bands depending upon size.

2.5. In vivo Fundus imaging.

GFP fluorescence fundus imaging was performed to check the spread and expression of control vector **(Fig. 2C)** using Phoenix Micron 3 fundus camera. For this, pupils of the mice were dilated once with 1% atropine and twice with 2.5 phenylephrine, then mice were anesthetized, the cornea was lubricated by one drop of artificial tears (GenTeal, Alcon). The eyes of the mice were positioned to face the fundus camera and images were receorded keeping the optic nerve at the center using GFP filter and normal bright field filter.

2.6. Scotpoic Electroretinography (ERG)

To monitor retinal function, the scotopic (dark adapted) ERG response was measured using the Espion full-field ERG system Espion ColorDome Ganzfeld ERG system (Diagnosys, Inc., Lowell, MA) according to an established protocol [23,24] . For this, mice were dark-adapted overnight, and pupils were dilated with one drop each of 1% atropine, 2.5% phenylephrine, and a local anesthetic (proparacaine) was applied. After that, mice were anesthesiszed with ketamine/xyalzine as described for ocular injections. ERG a-wave, b-wave and c-wave amplitudes from both the eyes recorded at a flash intensity of 20dB . The results were compared between control and experimental vector injected eyes at 3 months and 6 months following subretinal injction.

2.7 *Light microscopy*

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We used intraperitoneal injection of EUTHASOL® euthanasia solution (pentobarbital sodium and phenytoin sodium) to euthanize the experiemental mice. We followed the procedures for perfusions and tissue processing, embedding and sectioning as previously described[20]. Semithin cross-sections of 0.5 µm from resin embedded retinal tissue were cut through the optic nerves and mounted on glass slides. These sections were stained with 1% toluidine blue and 2% borate in distilled water. Stained sections were examined at 4X, 20X and 100X by light microscopy using Keyence All-in-One Fluorescence Microscope BZ-X800 (Itasca, IL 60143, U.S.A)

2.8.Statistical analysis.

GraphPad Prism 5.0 was used to illustrate the graph for ERG and light microscopy measurement data. Two-tailed student t tests were used to test the statistical significance of differences in results. All the data were represented as mean ± SEM unless otherwise indicated. A p-value of <0.05 was considered significant.

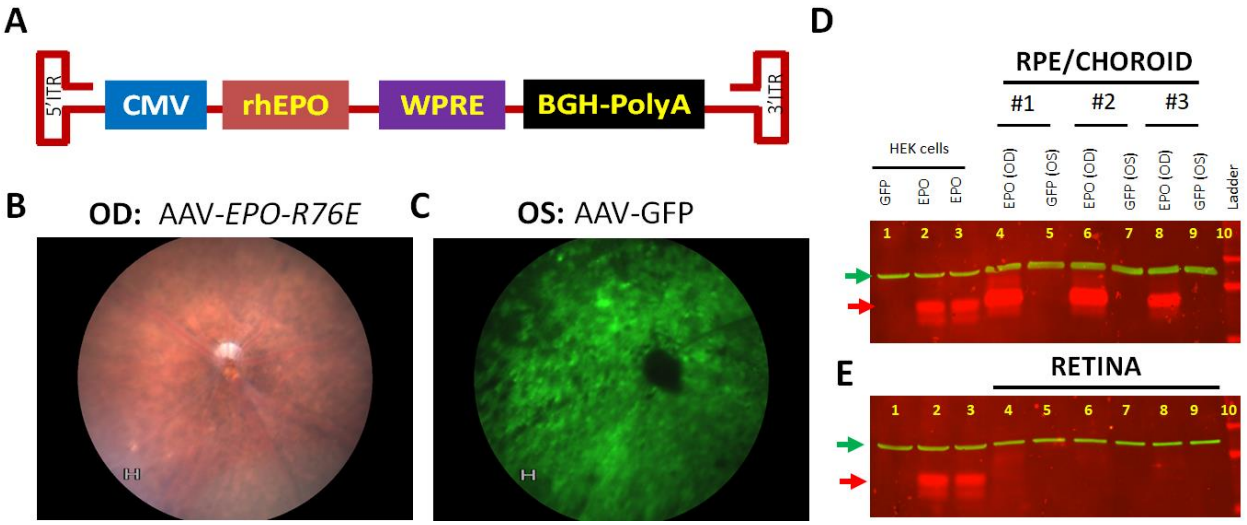

Figure-2: AAV-mediate protein expression in the retina. (A) The AAV plasmid contains rhesus EPO-R76E (rhEPO) cDNA driven by cytomegalovirus immediate early (CMV) promoter [24] and contains the Woodchuck Hepitis Postransciptional Regulatory Element (WPRE). The vector was packaged as serotype 1 (AAV1) promote RPE-specific gene expression. The AAV-EPO-R76E experimental vector was injected in one eye of 2-3-month-old mice (B), and the contralateral eye was injected with control vector, AAV-GFP. One month following subretinal gene delivery GFP fluorescence (C) was noticed around the optic nerve by fundus imaging. Exogenous EPO-R76E was significantly increased (D) in the RPE/choroid of *Sod2*$^{flox/flox}$/VMD2-cre mice (red arrow) injected with the AAV-EPO-R76E vector (lanes 4. 6 and 8) compared to eyes injected with the control AAV-GFP vector (lanes 5, 7, and 9), using an EPO specific antibody and beta actin used as a loading control (green arrow). (E) EPO levels were minimal (Lane 4, 6, 8) in the retinas of the same eyes. Proteins from GFP transfected HEK cells (lane 1) and EPO plasmid transfected HEK cells (Lane 2 and 3) were used in both the gels to have negative and positive control for retinal tissues.

3. Results

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3.1. EPO expression in RPE-specific Sod2 deleted mice.

Even though AAV1 transduces both Mueller glia and RPE following intravitreal injection, Mueller glia expression is much less than RPE [36]. In order to restrict transgene expression to RPE cells, we injected an AAV1 vector containing human modified EPO (EPO-R76E) into the subretinal space of 3-month-old RPE specific *Sod2* deleted mice [37]. The contralateral eyes from the same animals were injected with AAV1 expressing humanized GFP as a control to evaluate the impact of subretinal injection or virus induced effects. By using fluorescence fundus imaging, we observed GFP expression over 50-70% of the retina (Fig-2C) that suggested .the efficiency of subretinal viral delivery. To detect and quantify exogenous transgene expression, the level of AAV-delivered EPO protein expression was examined one month following subretinal injection using an EPO antibody. The control and experimental vector injected eyes were harvested from a cohort of mice 1 month following injection. The retina and RPE/choroid from each eye were collected separately for protein analysis. EPO antibody detected exogenous expression in RPE/choroid samples injected with AAV-EPO (Fig-2D) as we see a 37KD protein band. As expected, we found negligible expression of EPO in retina (Fig-2E) confirming the RPE specific tropism of AAV1 [38].

3.2. Improved Retinal and RPE function.

Under dark-adapted conditions, the ERG amplitudes of *Sod2* deleted mice are lower than control mice. Three months following *EPO* treatment, a-wave and b-wave ERG amplitudes were significantly different between eyes treated with the experimental and the control vectors (Fig-3). At 6 months of age (3 months following injection),

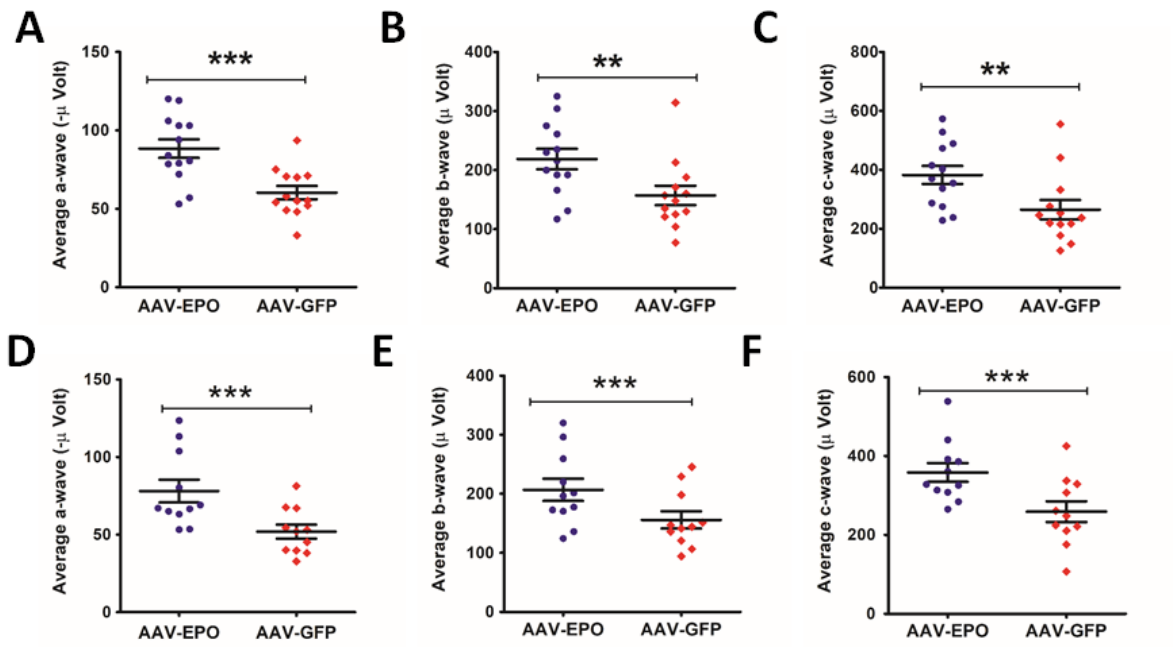

Figure 3: Improvement of Photoreceptor and RPE function. Dark-adapted full field electroretinogram (ERG) amplitudes measured at a light intensity of 20 cds/m2 at 6 months (6mo) and 9 months (9mo) of age after subretinal delivery of EPO-R76E. In the EPO treated group (n = 12), significant changes in (A) a-wave, (B) b-wave and (C) c-wave amplitudes were restored both at 6 months of age and 9 months of age (D, E, F) compared to untreated group injected with GFP vector.($P<0.01$)

the eyes treated with AAV-EPO vector showed 46% improvement in a-wave response and 39% increase in b-wave

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response compared to contralateral eyes injected with AAV-GFP vector **(Fig-3A and 3B)**. At 9 months of age (6 months following injection), the loss in a- and b-wave response were prevented **(Fig-3D and 3E)**. We found 50% improvement of a-wave response and 32% improvement of b-wave response. The c-wave ERG response reflects the health of RPE. We recorded a 44% **(Fig-3C)** and 38% **(Fig-3H)** improvement in c-wave ERG responses at 6 months of age (3 months following injection) and at 9 months of age (6 months following injection) respectively in eyes injected with AAV-EPO vector compared to untreated eyes injected with AAV-GFP vector. It should be noted that long-term expression of GFP in the RPE of rodents does not affect the ERG response [39,40].

3.3. Improvement in retinal structure after treatment with AAV-EPO-R76E is revealed by light microscopy.

Previously, we have reported the decrease in retinal thickness in RPE-specific *Sod2* knock out mice as the age progresses[20]. The effects of *Sod2* deleted changes in the retina were visible by light microscopy as progressive RPE and photoreceptor cell degeneration in all AAV-GFP injected eyes **(Fig-4A and B)**. We recorded around 32% preservation of retinal thickness in AAV-EPO injected eyes compred to control eyes **(Fig-4C)** . In control eyes injected with AAV-GFP vector, the RPE monolayer thinning along with irregular melanin pigment distribution was noticed **(Fig-5B)**. Thinning of the RPE monolayer are indicative of RPE loss and impaired RPE integrity. The changes in response to AAV-EPO-R76E was indicated by thicker RPE implying better structural Integrity **(Fig-5A)**. Melanin pigment distribution was quite uniform. Rounded RPE cells nuclei were visible in AAV-EPO treated eyes, whereas RPE cell nuclei were pyknotic in untreated eyes indicating the better health of RPE in treated eyes. The basal laminar layer in treated eyes exhibited well preserved structure compared to GFP injected eyes. Progressive disorganization of photoreceptor outer and inner segments and collapsed photoreceptor nuclei were indicated by the loss of outer and inner segments. More rows of photoceptor nuclei (ONL) were observed in AAV-EPO injected eyes compared to AAV-GFP injected eyes. Longer photoreceptor outer segments were seen in AAV-EPO injected eyes compared to AAV-GFP injected eyes. These results

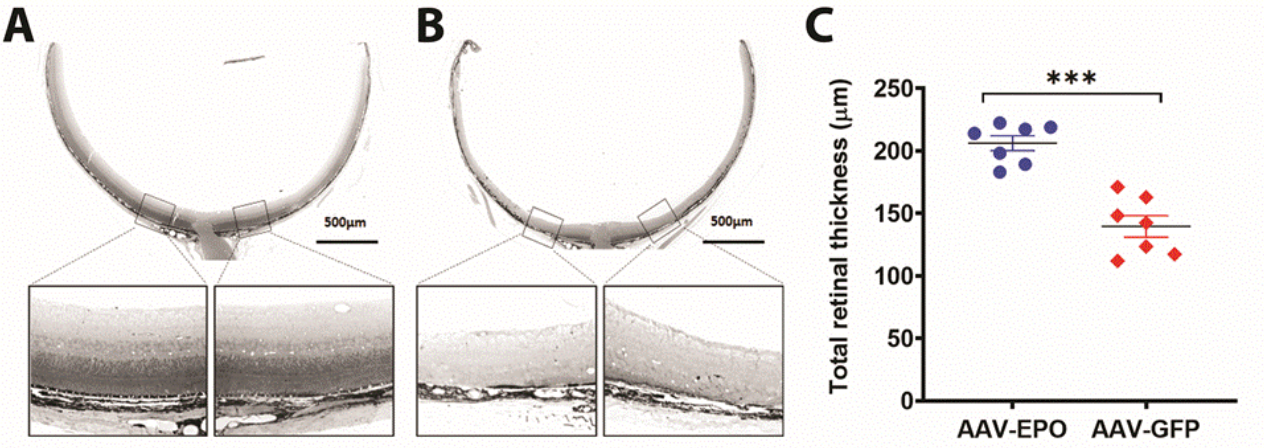

Figure-4:. **Preservation of retinal thickness:** Representative low magnification and merged images of retina sections (A, B) from contralateral eyes of one mouse through optic nerve and approximate areas (boxed and zoomed). (C) represents the measurement of retinal thickness of eyes treated with AAV-EPO compared to AAV-GFP injected eyes.Scale bar 500µm. *** P = ≤ 0.001.

suggested that prevention of retinal thinning predominantly occurs in the photoreceptor layer and retinal pigment epithelium (RPE).

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4. Discussion

Clinical and experimental evidence for both dry and wet form of age-related macular degeneration (AMD) demonstrates disruption of the structural and functional integrity of the RPE in addition to loss of photoreceptors [2,41–43]. We have shown that the deletion of mitochondrial form of superoxide dismutase (MnSOD or *Sod2*), an antioxidant gene especially in the RPE of an animal model impairs retinal structure and function. Oxidative stress is one of the key contributors to age-related retinal degeneration, particularly in dry-AMD [2,43]. Therefore, efforts are in progress to develop a therapeutic that can prevent further loss of structural and functional integrity of the RPE induced by oxidative damage. Growth factors offer the potential to prevent cell loss and degeneration of retinal cells from oxidative stress if they can be delivered to the specific cells. Therefore, using a cell-specific gene therapeutic approach, we have shown that subretinal delivery of serotype 1 AAV (AAV1) driving recombinant erythropoietin (EPO-R76E) can restrict EPO transgene expression in the RPE. This protects both the structural and functional integrity of RPE and retina impaired by RPE-specific oxidative stress.

Erythropoietin (EPO) is a hormone produced primarily by the kidneys, with small amounts made by the liver. EPO plays a key role in the production of red blood cells (RBCs), which carry oxygen from the lungs to the rest of the body. EPO is also expressed locally in the retina under the control of hypoxia inducible factor (HIF-1)[44]. EPO is present in considerably higher concentrations in eyes with diabetic macular edema than in eyes with exudative AMD or normal eyes [45]. The results from several studies indicate that systemic delivery of erythropoietic EPO is therapeutic for a broad range of neurodegenerative diseases [46–49]. The safe use of EPO is demonstrated in the clinic with other diseases as it can traverse the intact blood–brain and blood–retina barriers in therapeutic concentrations [44,50]. It was previously reported that the systemic delivery of EPO-R76E was able to provide successful preservation of retinal ganglion cells and visual function without significantly increasing hematocrit, unlike regular EPO [11]. Tao and colleagues recently demonstrated that pre-treament of mice by subretinal injection of AAV2-EPO, protected the retina from acute N-Methyl-N-Nitrosourea (MNU) toxicity [51]. There is a need, however, to demonstrate the best strategies for developing and delivering EPO or erythropoietic stimulating agents for the treatment of patients with atrophic or dry- AMD [52]. Adeno-associated virus (AAV) vectors can transduce a wide range of dividing and non-dividing cell types, which has made these vectors an important tool for ophthalmic gene therapy. A major advantage of AAV vectors is the long-term expression of therapeutic gene as episomes within cells that can be obtained after in vivo gene delivery [53]. AAV-EPO gene therapy vector offers the advantages of delivering and stably expressing EPO gene (or its protein product) to the physiologically relevant target tissues such as RPE using specific AAV serotypes (AAV1) or promoters (e.g. VMD2).

Our results indicate that stable expression of EPO-R76E in RPE cells protected the RPE and its nearby photoreceptors under the conditions of oxidative stress. EPO can protect the retina by acting directly on the RPE or by acting in a paracrine fashion on photoreceptors and Mueller glial. EPO has been shown to help maintain the barrier properties of the RPE, and this may contribute to protective role [27,54]. EPO protected RPE cells barrier integrity disrupted by oxidative stress by reducing intracellular ROS and restoring cellular antioxidant potential [55]. These authors also reported that there was a reduction in the secretion of inflammatory cytokines

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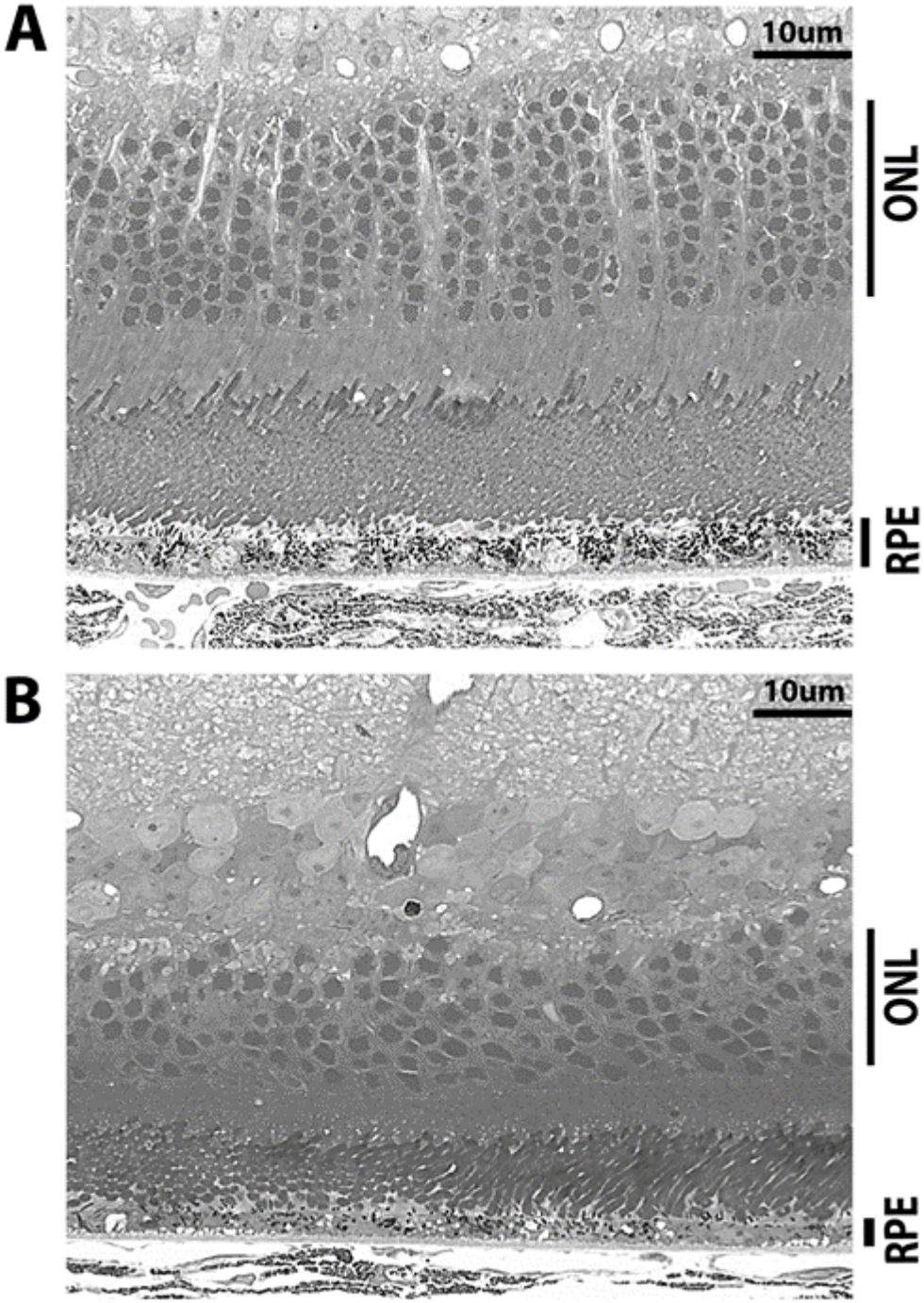

Figure 5. Photoreceptor and RPE preservation in AAV-EPO injected eyes. (A) represents the retinal sections from AAV-EPO injected eyes and (B) represent retinal section from AAV-GFP injected eyes. Scale bar 10µm.

(TNFα, and IL1-1β) and a decrease in caspase-3 activity under oxidative stress in response to EPO treatment. Since EPO is secreted, it may also protect retinal structure and function by acting directly on photoreceptors. Exogenous EPO could directly interact with the photoreceptors allowing them to maintain the metabolic activity despite increased oxidative stress-related effects. This may also activate a signal transduction cascade in the photoreceptors [56–59]. The eyes from *Sod2*$^{flox/flox}$-*VMD2*^{cre} mice evidenced an increase in oxidative stress as early

as two months of age. Protection from oxidative stress could be one of the reasons whereby EPO is permitting increased survival and prolonged function of photoreceptors. Another mechanism could be interactions with surrounding cells such as Mueller glia, which, in turn, can release proteins that support the survival of photoreceptors[60–62].

In AMD patients, the RPE and photoreceptors are comprimized in the macular region, causing loss of central vision. For treatment of macular degeneration, therefore, protection of cone photoreceptors is essential, because they are entiched in the maclula and are critical for visual acuity. Treatment with RPE-specific EPO-R76E using gene therapy may allow patients to have a useful vision for a longer period due to extended-expression of EPO in the RPE, thus further preventing loss of vision. We observed protection of scotopic a, b, and c wave full-field ERG response signifying the protection of photoreceptors and RPE. Histological analysis at nine months of age that clearly demonstrated visible preservation of the photoreceptor and RPE layers **(Fig-4A and 5A**) compared to control treated eyes **(Fig-4B and 5B)**. In the future, we aim to perform molecular analysis of RPE/ choroid and photoreceptors to evaluate changes in protective and inflammatory gene and protein expression as shown in other studies [63]. As *Sod2* deletion is related to mitochondrial dysfunction in RPE, it will be interesting to see whether supplementation of EPO-R76E can rescue mitochondrial dysfunction and improve bioenergetics, as noticed in RPE cells derived from AMD patients [64].

EPO signaling increases choroidal macrophages and cytokine expression and exacerbates choroidal neovascularization, conditions associated with the advanced wet-form of AMD [65]. EPO receptor signaling supports retinal function after vascular injury [66], but its pro-angiogenic properties may limit the usefulness of unregulated EPO expression as a therapy for dry AMD. We plan to determine if EPO-R76E stimulated choroidal neovasuclarization (CNV) using the laser-induced CNV model.

This pilot study showed the protective effect of EPO in preserving retinal structure and function while maintaining stable expression in the RPE. Even though we did not see any harmful effect of EPO-R76E in our animal model of dry-AMD, we must also study the impact of prolonged EPO expression in normal mice. In our study, we did not measure the level of EPO expression in retinal tissue by AAV-EPO. We aim to compare the expression levels using intravitreal or systemic injection of other EPO activating compounds or clinically approved EPO protein in further studies. Given that cone photoreceptor loss is prominent in dry-AMD, it will be necessary to perform focal ERG and/or optokinetic responses.to measure cone function and also include spectral domain optical coherence tomography (SD-OCT) to monitor progessive preservation of retinal layers in vivo [23]. Other than that, we also aim to check the contribution of different cell types (Mueller glia, photoreceptors, astrocytes, microglia, endothelial cells, ganglion cells, etc.) in protecting the retina by analyzing transcriptional landscape at a single-cell level. We predict that local stable EPO expression can impact the proteome changes in RPE under conditions of oxidative stress. Proteomics studies can supplement learning the proteome changes by EPO while protecting the cellular microenvironment.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

Author contribution: M.R.B., A.S.L; supervision, project administration, funding acquisition and writing. M.R.B., Z.W, R.J.P, Y,T, R.R.U., P.Z. , H.I., A.S.L.; Methodology, Data acquisition and analysis. M.R.B., A.S.L., R.J.P.; Review and editing.

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Institutional Review Board Statement: All the procedures involving animals in this study followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the protocols were approved by Institutional Animal Care and Use Committee (IACUC) at the University of South Florida (USF) and University of Florida (UF).

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References

1. Friedman, D.S.; O'Colmain, B.J.; Muñoz, B.; Tomany, S.C.; McCarty, C.; de Jong, P.T.V.M.; Nemesure, B.; Mitchell, P.; Kempen, J.; Eye Diseases Prevalence Research Group Prevalence of Age-Related Macular Degeneration in the United States. *Arch. Ophthalmol.* **2004**, *122*, 564–572, doi:10.1001/archopht.122.4.564.
2. Datta, S.; Cano, M.; Ebrahimi, K.; Wang, L.; Handa, J.T. The Impact of Oxidative Stress and Inflammation on RPE Degeneration in Non-Neovascular AMD. *Prog Retin Eye Res* **2017**, *60*, 201–218, doi:10.1016/j.preteyeres.2017.03.002.
3. Kaarniranta, K.; Uusitalo, H.; Blasiak, J.; Felszeghy, S.; Kannan, R.; Kauppinen, A.; Salminen, A.; Sinha, D.; Ferrington, D. Mechanisms of Mitochondrial Dysfunction and Their Impact on Age-Related Macular Degeneration. *Prog Retin Eye Res* **2020**, 100858, doi:10.1016/j.preteyeres.2020.100858.
4. Abe, T.; Saigo, Y.; Hojo, M.; Kano, T.; Wakusawa, R.; Tokita, Y.; Tamai, M. Protection of Photoreceptor Cells from Phototoxicity by Transplanted Retinal Pigment Epithelial Cells Expressing Different Neurotrophic Factors. *Cell Transplant* **2005**, *14*, 799–808, doi:10.3727/000000005783982549.
5. Kakavas, S.; Demestiha, T.; Vasileiou, P.; Xanthos, T. Erythropoetin as a Novel Agent with Pleiotropic Effects against Acute Lung Injury. *Eur. J. Clin. Pharmacol.* **2011**, *67*, 1–9, doi:10.1007/s00228-010-0938-7.
6. Rex, T.S.; Wong, Y.; Kodali, K.; Merry, S. Neuroprotection of Photoreceptors by Direct Delivery of Erythropoietin to the Retina of the Retinal Degeneration Slow Mouse. *Exp. Eye Res.* **2009**, *89*, 735–740, doi:10.1016/j.exer.2009.06.017.
7. Bond, W.S.; Rex, T.S. Evidence That Erythropoietin Modulates Neuroinflammation through Differential Action on Neurons, Astrocytes, and Microglia. *Front Immunol* **2014**, *5*, 523, doi:10.3389/fimmu.2014.00523.
8. Entezari, M.; Esmaeili, M.; Yaseri, M. A Pilot Study of the Effect of Intravenous Erythropoetin on Improvement of Visual Function in Patients with Recent Indirect Traumatic Optic Neuropathy. *Graefes Arch. Clin. Exp. Ophthalmol.* **2014**, *252*, 1309–1313, doi:10.1007/s00417-014-2691-6.
9. Hines-Beard, J.; Bond, W.S.; Backstrom, J.R.; Rex, T.S. Virus-Mediated EpoR76E Gene Therapy Preserves Vision in a Glaucoma Model by Modulating Neuroinflammation and Decreasing Oxidative Stress. *J Neuroinflammation* **2016**, *13*, 39, doi:10.1186/s12974-016-0499-5.
10. Rex, T.S.; Allocca, M.; Domenici, L.; Surace, E.M.; Maguire, A.M.; Lyubarsky, A.; Cellerino, A.; Bennett, J.; Auricchio, A. Systemic but Not Intraocular Epo Gene Transfer Protects the Retina from Light-and Genetic-Induced Degeneration. *Mol. Ther.* **2004**, *10*, 855–861, doi:10.1016/j.ymthe.2004.07.027.
11. Sullivan, T.A.; Geisert, E.E.; Hines-Beard, J.; Rex, T.S. Systemic Adeno-Associated Virus-Mediated Gene Therapy Preserves Retinal Ganglion Cells and Visual Function in DBA/2J Glaucomatous Mice. *Hum. Gene Ther.* **2011**, *22*, 1191–1200, doi:10.1089/hum.2011.052.
12. Sullivan, T.A.; Geisert, E.E.; Templeton, J.P.; Rex, T.S. Dose-Dependent Treatment of Optic Nerve Crush by Exogenous Systemic Mutant Erythropoietin. *Exp. Eye Res.* **2012**, *96*, 36–41, doi:10.1016/j.exer.2012.01.006.
13. Sullivan, T.; Kodali, K.; Rex, T.S. Systemic Gene Delivery Protects the Photoreceptors in the Retinal Degeneration Slow Mouse. *Neurochem. Res.* **2011**, *36*, 613–618, doi:10.1007/s11064-010-0272-6.
14. Tsai, J.C.; Wu, L.; Worgul, B.; Forbes, M.; Cao, J. Intravitreal Administration of Erythropoietin and Preservation of Retinal Ganglion Cells in an Experimental Rat Model of Glaucoma. *Curr. Eye Res.* **2005**, *30*, 1025–1031, doi:10.1080/02713680500320729.
15. Weishaupt, J.H.; Rohde, G.; Pölking, E.; Siren, A.-L.; Ehrenreich, H.; Bähr, M. Effect of Erythropoietin Axotomy-Induced Apoptosis in Rat Retinal Ganglion Cells. *Invest. Ophthalmol. Vis. Sci.* **2004**, *45*, 1514–1522, doi:10.1167/iovs.03-1039.

16. Zhong, L.; Bradley, J.; Schubert, W.; Ahmed, E.; Adamis, A.P.; Shima, D.T.; Robinson, G.S.; Ng, Y.-S. Erythropoietin Promotes Survival of Retinal Ganglion Cells in DBA/2J Glaucoma Mice. *Invest. Ophthalmol. Vis. Sci.* **2007**, *48*, 1212–1218, doi:10.1167/iovs.06-0757.
17. Rex, T.S.; Kasmala, L.; Bond, W.S.; de Lucas Cerrillo, A.M.; Wynn, K.; Lewin, A.S. Erythropoietin Slows Photoreceptor Cell Death in a Mouse Model of Autosomal Dominant Retinitis Pigmentosa. *PLoS ONE* **2016**, *11*, e0157411, doi:10.1371/journal.pone.0157411.
18. Bond, W.S.; Hines-Beard, J.; GoldenMerry, Yp.L.; Davis, M.; Farooque, A.; Sappington, R.M.; Calkins, D.J.; Rex, T.S. Virus-Mediated EpoR76E Therapy Slows Optic Nerve Axonopathy in Experimental Glaucoma. *Mol. Ther.* **2016**, *24*, 230–239, doi:10.1038/mt.2015.198.
19. Hines-Beard, J.; Desai, S.; Haag, R.; Esumi, N.; D'Surney, L.; Parker, S.; Richardson, C.; Rex, T.S. Identification of a Therapeutic Dose of Continuously Delivered Erythropoietin in the Eye Using an Inducible Promoter System. *Curr Gene Ther* **2013**, *13*, 275–281, doi:10.2174/15665232113139990024.
20. Mao, H.; Seo, S.J.; Biswal, M.R.; Li, H.; Conners, M.; Nandyala, A.; Jones, K.; Le, Y.-Z.; Lewin, A.S. Mitochondrial Oxidative Stress in the Retinal Pigment Epithelium Leads to Localized Retinal Degeneration. *Invest. Ophthalmol. Vis. Sci.* **2014**, *55*, 4613–4627, doi:10.1167/iovs.14-14633.
21. Biswal, M.R.; Ildefonso, C.J.; Mao, H.; Seo, S.J.; Wang, Z.; Li, H.; Le, Y.Z.; Lewin, A.S. Conditional Induction of Oxidative Stress in RPE: A Mouse Model of Progressive Retinal Degeneration. *Adv. Exp. Med. Biol.* **2016**, *854*, 31–37, doi:10.1007/978-3-319-17121-0_5.
22. Biswal, M.R.; Ahmed, C.M.; Ildefonso, C.J.; Han, P.; Li, H.; Jivanji, H.; Mao, H.; Lewin, A.S. Systemic Treatment with a 5HT1a Agonist Induces Anti-Oxidant Protection and Preserves the Retina from Mitochondrial Oxidative Stress. *Exp. Eye Res.* **2015**, *140*, 94–105, doi:10.1016/j.exer.2015.07.022.
23. Biswal, M.R.; Han, P.; Zhu, P.; Wang, Z.; Li, H.; Ildefonso, C.J.; Lewin, A.S. Timing of Antioxidant Gene Therapy: Implications for Treating Dry AMD. *Invest. Ophthalmol. Vis. Sci.* **2017**, *58*, 1237–1245, doi:10.1167/iovs.16-21272.
24. Biswal, M.R.; Justis, B.D.; Han, P.; Li, H.; Gierhart, D.; Dorey, C.K.; Lewin, A.S. Daily Zeaxanthin Supplementation Prevents Atrophy of the Retinal Pigment Epithelium (RPE) in a Mouse Model of Mitochondrial Oxidative Stress. *PLoS ONE* **2018**, *13*, e0203816, doi:10.1371/journal.pone.0203816.
25. Ahmed, C.M.; Biswal, M.R.; Li, H.; Han, P.; Ildefonso, C.J.; Lewin, A.S. Repurposing an Orally Available Drug for the Treatment of Geographic Atrophy. *Mol. Vis.* **2016**, *22*, 294–310.
26. Young, B.M.; Jones, K.; Massengill, M.T.; Walsh, E.; Li, H.; Lewin, A.S.; Ildefonso, C.J. Expression of a CARD Slows the Retinal Degeneration of a Geographic Atrophy Mouse Model. *Mol Ther Methods Clin Dev* **2019**, *14*, 113–125, doi:10.1016/j.omtm.2019.06.001.
27. Zhang, C.; Xie, H.; Yang, Q.; Yang, Y.; Li, W.; Tian, H.; Lu, L.; Wang, F.; Xu, J.-Y.; Gao, F.; et al. Erythropoietin Protects Outer Blood-Retinal Barrier in Experimental Diabetic Retinopathy by up-Regulating ZO-1 and Occludin. *Clin. Experiment. Ophthalmol.* **2019**, *47*, 1182–1197, doi:10.1111/ceo.13619.
28. Zhang, M.; Jiang, N.; Chu, Y.; Postnikova, O.; Varghese, R.; Horvath, A.; Cheema, A.K.; Golestaneh, N. Dysregulated Metabolic Pathways in Age-Related Macular Degeneration. *Scientific Reports* **2020**, *10*, 2464, doi:10.1038/s41598-020-59244-4.
29. Jarrett, S.G.; Boulton, M.E. Consequences of Oxidative Stress in Age-Related Macular Degeneration. *Mol Aspects Med* **2012**, *33*, 399–417, doi:10.1016/j.mam.2012.03.009.
30. Tong, Y.; Wang, S. Not All Stressors Are Equal: Mechanism of Stressors on RPE Cell Degeneration. *Front Cell Dev Biol* **2020**, *8*, 591067, doi:10.3389/fcell.2020.591067.
31. Hanus, J.; Anderson, C.; Wang, S. RPE Necroptosis in Response to Oxidative Stress and in AMD. *Ageing Res Rev* **2015**, *24*, 286–298, doi:10.1016/j.arr.2015.09.002.

32. Zolotukhin, S.; Potter, M.; Zolotukhin, I.; Sakai, Y.; Loiler, S.; Fraites, T.J.; Chiodo, V.A.; Phillipsberg, T.; Muzyczka, N.; Hauswirth, W.W.; et al. Production and Purification of Serotype 1, 2, and 5 Recombinant Adeno-Associated Viral Vectors. *Methods* **2002**, *28*, 158–167, doi:10.1016/s1046-2023(02)00220-7.
33. Le, Y.-Z.; Zheng, W.; Rao, P.-C.; Zheng, L.; Anderson, R.E.; Esumi, N.; Zack, D.J.; Zhu, M. Inducible Expression of Cre Recombinase in the Retinal Pigmented Epithelium. *Invest. Ophthalmol. Vis. Sci.* **2008**, *49*, 1248–1253, doi:10.1167/iovs.07-1105.
34. Strassburger, M.; Bloch, W.; Sulyok, S.; Schüller, J.; Keist, A.F.; Schmidt, A.; Wenk, J.; Peters, T.; Wlaschek, M.; Lenart, J.; et al. Heterozygous Deficiency of Manganese Superoxide Dismutase Results in Severe Lipid Peroxidation and Spontaneous Apoptosis in Murine Myocardium in Vivo. *Free Radic. Biol. Med.* **2005**, *38*, 1458–1470, doi:10.1016/j.freeradbiomed.2005.02.009.
35. Timmers, A.M.; Zhang, H.; Squitieri, A.; Gonzalez-Pola, C. Subretinal Injections in Rodent Eyes: Effects on Electrophysiology and Histology of Rat Retina. *Mol. Vis.* **2001**, *7*, 131–137.
36. Pang, J.; Lauramore, A.; Deng, W.; Li, Q.; Doyle, T.J.; Chiodo, V.; Li, J.; Hauswirth, W.W. Comparative Analysis of in Vivo and in Vitro AAV Vector Transduction in the Neonatal Mouse Retina: Effects of Serotype and Site of Administration. *Vision Res* **2008**, *48*, 377–385, doi:10.1016/j.visres.2007.08.009.
37. Lebherz, C.; Maguire, A.; Tang, W.; Bennett, J.; Wilson, J.M. Novel AAV Serotypes for Improved Ocular Gene Transfer. *J Gene Med* **2008**, *10*, 375–382, doi:10.1002/jgm.1126.
38. Auricchio, A.; Kobinger, G.; Anand, V.; Hildinger, M.; O'Connor, E.; Maguire, A.M.; Wilson, J.M.; Bennett, J. Exchange of Surface Proteins Impacts on Viral Vector Cellular Specificity and Transduction Characteristics: The Retina as a Model. *Hum Mol Genet* **2001**, *10*, 3075–3081, doi:10.1093/hmg/10.26.3075.
39. Seo, S.; Krebs, M.P.; Mao, H.; Jones, K.; Conners, M.; Lewin, A.S. Pathological Consequences of Long-Term Mitochondrial Oxidative Stress in the Mouse Retinal Pigment Epithelium. *Experimental Eye Research* **2012**, *101*, 60–71, doi:10.1016/j.exer.2012.05.013.
40. Rolling, F.; Shen, W.Y.; Barnett, N.L.; Tabarias, H.; Kanagasingam, Y.; Constable, I.; Rakoczy, P.E. Long-Term Real-Time Monitoring of Adeno-Associated Virus-Mediated Gene Expression in the Rat Retina. *Clin Exp Ophthalmol* **2000**, *28*, 382–386, doi:10.1046/j.1442-9071.2000.00341.x.
41. Young, R.W. Pathophysiology of Age-Related Macular Degeneration. *Surv Ophthalmol* **1987**, *31*, 291–306, doi:10.1016/0039-6257(87)90115-9.
42. Handa, J.T.; Bowes Rickman, C.; Dick, A.D.; Gorin, M.B.; Miller, J.W.; Toth, C.A.; Ueffing, M.; Zarbin, M.; Farrer, L.A. A Systems Biology Approach towards Understanding and Treating Non-Neovascular Age-Related Macular Degeneration. *Nat Commun* **2019**, *10*, 3347, doi:10.1038/s41467-019-11262-1.
43. Ferrington, D.A.; Sinha, D.; Kaarniranta, K. Defects in Retinal Pigment Epithelial Cell Proteolysis and the Pathology Associated with Age-Related Macular Degeneration. *Prog Retin Eye Res* **2016**, *51*, 69–89, doi:10.1016/j.preteyeres.2015.09.002.
44. Grimm, C.; Wenzel, A.; Groszer, M.; Mayser, H.; Seeliger, M.; Samardzija, M.; Bauer, C.; Gassmann, M.; Remé, C.E. HIF-1-Induced Erythropoietin in the Hypoxic Retina Protects against Light-Induced Retinal Degeneration. *Nat Med* **2002**, *8*, 718–724, doi:10.1038/nm723.
45. Jonas, J.B.; Neumaier, M. Erythropoietin Levels in Aqueous Humour in Eyes with Exudative Age-Related Macular Degeneration and Diabetic Retinopathy. *Clinical & Experimental Ophthalmology* **2007**, *35*, 186–187, doi:https://doi.org/10.1111/j.1442-9071.2006.01437.x.
46. Bayer, A.U.; Ferrari, F.; Erb, C. High Occurrence Rate of Glaucoma among Patients with Alzheimer's Disease. *Eur Neurol* **2002**, *47*, 165–168, doi:10.1159/000047976.

47. Keswani, S.C.; Buldanlioglu, U.; Fischer, A.; Reed, N.; Polley, M.; Liang, H.; Zhou, C.; Jack, C.; Leitz, G.J.; Hoke, A. A Novel Endogenous Erythropoietin Mediated Pathway Prevents Axonal Degeneration. *Ann Neurol* **2004**, *56*, 815–826, doi:10.1002/ana.20285.
48. Campana, W.M.; Li, X.; Shubayev, V.I.; Angert, M.; Cai, K.; Myers, R.R. Erythropoietin Reduces Schwann Cell TNF-Alpha, Wallerian Degeneration and Pain-Related Behaviors after Peripheral Nerve Injury. *Eur J Neurosci* **2006**, *23*, 617–626, doi:10.1111/j.1460-9568.2006.04606.x.
49. Yin, Z.-S.; Zhang, H.; Bo, W.; Gao, W. Erythropoietin Promotes Functional Recovery and Enhances Nerve Regeneration after Peripheral Nerve Injury in Rats. *AJNR Am J Neuroradiol* **2010**, *31*, 509–515, doi:10.3174/ajnr.A1820.
50. Brines, M.L.; Ghezzi, P.; Keenan, S.; Agnello, D.; de Lanerolle, N.C.; Cerami, C.; Itri, L.M.; Cerami, A. Erythropoietin Crosses the Blood-Brain Barrier to Protect against Experimental Brain Injury. *Proc Natl Acad Sci U S A* **2000**, *97*, 10526–10531, doi:10.1073/pnas.97.19.10526.
51. Tao, Y.; Zhu, Q.; Wang, L.; Zha, X.; Teng, D.; Xu, L. Adeno-Associated Virus (AAV)-Mediated Neuroprotective Effects on the Degenerative Retina: The Therapeutic Potential of Erythropoietin. *Fundam Clin Pharmacol* **2020**, *34*, 131–147, doi:10.1111/fcp.12494.
52. Wang, Z.-Y.; Zhao, K.-K.; Song, Z.-M.; Shen, L.-J.; Qu, J. Erythropoietin as a Novel Therapeutic Agent for Atrophic Age-Related Macular Degeneration. *Med Hypotheses* **2009**, *72*, 448–450, doi:10.1016/j.mehy.2008.09.055.
53. Deyle, D.R.; Russell, D.W. Adeno-Associated Virus Vector Integration. *Curr Opin Mol Ther* **2009**, *11*, 442–447.
54. Garcia-Ramírez, M.; Hernández, C.; Ruiz-Meana, M.; Villarroel, M.; Corraliza, L.; García-Dorado, D.; Simó, R. Erythropoietin Protects Retinal Pigment Epithelial Cells against the Increase of Permeability Induced by Diabetic Conditions: Essential Role of JAK2/ PI3K Signaling. *Cellular Signalling* **2011**, *23*, 1596–1602, doi:10.1016/j.cellsig.2011.05.011.
55. Wang, Z.; Shen, L.; Tu, L.; Hu, D.; Liu, G.-Y.; Zhou, Z.; Lin, Y.; Chen, L.-H.; Qu, J. Erythropoietin Protects Retinal Pigment Epithelial Cells from Oxidative Damage. *Free Radic. Biol. Med.* **2009**, *46*, 1032–1041, doi:10.1016/j.freeradbiomed.2008.11.027.
56. Junk, A.K.; Mammis, A.; Savitz, S.I.; Singh, M.; Roth, S.; Malhotra, S.; Rosenbaum, P.S.; Cerami, A.; Brines, M.; Rosenbaum, D.M. Erythropoietin Administration Protects Retinal Neurons from Acute Ischemia-Reperfusion Injury. *Proc Natl Acad Sci U S A* **2002**, *99*, 10659–10664, doi:10.1073/pnas.152321399.
57. Shah, S.S.; Tsang, S.H.; Mahajan, V.B. Erythropoetin Receptor Expression in the Human Diabetic Retina. *BMC Res Notes* **2009**, *2*, 234, doi:10.1186/1756-0500-2-234.
58. Scheerer, N.; Dünker, N.; Imagawa, S.; Yamamoto, M.; Suzuki, N.; Fandrey, J. The Anemia of the Newborn Induces Erythropoietin Expression in the Developing Mouse Retina. *Am J Physiol Regul Integr Comp Physiol* **2010**, *299*, R111-118, doi:10.1152/ajpregu.00108.2010.
59. Ruscher, K.; Freyer, D.; Karsch, M.; Isaev, N.; Megow, D.; Sawitzki, B.; Priller, J.; Dirnagl, U.; Meisel, A. Erythropoietin Is a Paracrine Mediator of Ischemic Tolerance in the Brain: Evidence from an in Vitro Model. *J Neurosci* **2002**, *22*, 10291–10301.
60. Wang, M.; Ma, W.; Zhao, L.; Fariss, R.N.; Wong, W.T. Adaptive Müller Cell Responses to Microglial Activation Mediate Neuroprotection and Coordinate Inflammation in the Retina. *J Neuroinflammation* **2011**, *8*, 173, doi:10.1186/1742-2094-8-173.
61. Hauck, S.M.; von Toerne, C.; Ueffing, M. The Neuroprotective Potential of Retinal Müller Glial Cells. *Adv Exp Med Biol* **2014**, *801*, 381–387, doi:10.1007/978-1-4614-3209-8_48.
62. Hooper, M.J.; Ash, J.D. Müller Cell Biological Processes Associated with Leukemia Inhibitory Factor Expression. *Adv Exp Med Biol* **2018**, *1074*, 479–484, doi:10.1007/978-3-319-75402-4_59.

63. Kauppinen, A.; Paterno, J.J.; Blasiak, J.; Salminen, A.; Kaarniranta, K. Inflammation and Its Role in Age-Related Macular Degeneration. *Cell Mol Life Sci* **2016**, *73*, 1765–1786, doi:10.1007/s00018-016-2147-8.
64. Ferrington, D.A.; Ebeling, M.C.; Kapphahn, R.J.; Terluk, M.R.; Fisher, C.R.; Polanco, J.R.; Roehrich, H.; Leary, M.M.; Geng, Z.; Dutton, J.R.; et al. Altered Bioenergetics and Enhanced Resistance to Oxidative Stress in Human Retinal Pigment Epithelial Cells from Donors with Age-Related Macular Degeneration. *Redox Biol* **2017**, *13*, 255–265, doi:10.1016/j.redox.2017.05.015.
65. Bretz, C.A.; Divoky, V.; Prchal, J.; Kunz, E.; Simmons, A.B.; Wang, H.; Hartnett, M.E. Erythropoietin Signaling Increases Choroidal Macrophages and Cytokine Expression, and Exacerbates Choroidal Neovascularization. *Sci Rep* **2018**, *8*, 2161, doi:10.1038/s41598-018-20520-z.
66. Bretz, C.A.; Simmons, A.B.; Kunz, E.; Ramshekar, A.; Kennedy, C.; Cardenas, I.; Hartnett, M.E. Erythropoietin Receptor Signaling Supports Retinal Function after Vascular Injury. *Am J Pathol* **2020**, *190*, 630–641, doi:10.1016/j.ajpath.2019.11.009.