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Retina

Intravitreal Administration of AAV2-SIRT1 Reverses Diabetic Retinopathy in a Mouse Model of Type 2 Diabetes

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Received: October 3, 2022
Accepted: February 24, 2023
Published: April 18, 2023

Keywords: diabetic retinopathy; intravitreal drug delivery; electroretinography; hypoxia; SIRT1

Citation: Adu-Agyeiwaah Y, Vieira CP, Asare-Bediako B, Li Calzi S, DuPont M, Floyd J, Boye S, Chiodo V, Busik JV, Grant MB. Intravitreal administration of AAV2-SIRT1 reverses diabetic retinopathy in a mouse model of type 2 diabetes. Transl Vis Sci Technol. 2023;12(4):20, https://doi.org/10.1167/tvst.12.4.20

Purpose: The expression of silent information regulator (SIRT) 1 is reduced in diabetic retinopathy (DR). Previous studies showed that alterations in SIRT1 messenger RNA (mRNA) and protein expression are implicated in progressive inflammation and formation of retinal acellular capillaries. Treatment with the SIRT1 agonist, SRT1720, improved visual response by restoration of a- and b-wave responses on electroretinogram scotopic measurements in diabetic (db/db) mice. In this study, we investigated the effects of intravitreal SIRT1 delivery on diabetic retinal pathology.

Methods: Nine-month-old db/db mice received one intravitreal injection of either AAV2-SIRT1 or AAV2-GFP control virus, and after 3 months, electroretinography and optomotor responses were measured. Their eyes were then removed and analyzed by immunohistochemistry and flow cytometry.

Results: SIRT1 mRNA and protein levels were increased following AAV2-SIRT1 administration compared to control virus AAV2-GFP injected mice. IBA1$^+$ and caspase 3 expression were decreased in retinas of db/db mice injected with AAV2-SIRT1, and reductions in scotopic a- and b-waves and high spatial frequency in optokinetic response were prevented. Retinal hypoxia inducible factor 1α (HIF-1α) protein levels were reduced in the AAV2-SIRT1–injected mice compared to control-injected mice. Using flow cytometry to assess changes in intracellular HIF-1α levels, endothelial cells (CD31$^+$) from AAV-2 SIRT1 injected mice demonstrated reduced HIF-1α expression compared to db/db mice injected with the control virus.

Conclusions: Intravitreal AAV2-SIRT1 delivery increased retina SIRT1 and transduced neural and endothelial cells, thus reversing functional damage and improving overall visual function.

Translational Relevance: AAV2-SIRT1 gene therapy represents a beneficial approach for the treatment of chronic retinal conditions such as DR.

Introduction

Silent information regulator (SIRT) 1 is a member of the sirtuin (SIRT) family. The first sirtuin gene discovered was SIR2 from *Saccharomyces cerevisiae*, originally discovered as a spontaneous mutation that represses transcription at silent mating-type loci, telomeres, and ribosomal DNA.[1] Decades later, more proteins with similar functions were identified, creating the silent information regulator family of proteins whose functions are evolutionarily conserved from bacteria to humans.[2] Sirtuins are localized in a variety of subcellular compartments for specific roles. Their activity is directly linked to the metabolic state of cells based on their requirement or dependence on NAD for enzymatic reactions. The mammalian sirtuin family comprises seven members,

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SIRT1–7, among which SIRT1 is the most widely studied.[1]

SIRT1 is involved in numerous pathways and plays a role in oxidative stress, apoptosis, cholesterol regulation, cell metabolism, inflammation, glucose balance, and both as a tumor promoter and suppressor.[2] SIRT1 is found in mouse cornea, lens, iris, ciliary body, retinal inner and outer nuclear layers, and the ganglion cell layer.[3–5] SIRT1 deficiencies in mice result in lethal postnatal consequences, with mice dying soon after birth. Surviving mice are smaller than wild-type littermates. Ocular abnormalities include permanent or long-term eyelid closure and cornea, lens, and retina anomalies.[6] SIRT1 expression and activity decreased by over 40% in the retina of mice with 7 months of type 1 diabetes compared to their age-matched wild-type controls.[7] On the contrary, increased expression of SIRT1 is protective against various ocular diseases, especially those that are age related, including retinal degeneration, cataract, and optic neuritis.[8] Restoration of Sirt1 also retards development of diabetic retinopathy (DR).[8–11]

DR is the most common complication of diabetes mellitus and is largely recognized as a microvascular disease associated with vascular leakage, apoptosis, and neurodegeneration and in the late stages can contribute to blindness. Increasing Sirt1 by using Sirt1-overexpressing mice decreased matrix metalloproteinase 9 expression and formation of retinal acellular capillaries.[5] SRT1720, a Sirt1 activator, extends the life span of mice by decreasing inflammation and oxidative stress.[12,13] Previously, we showed that SRT1720 decreased the formation of acellular capillaries and protected retinal ganglion cell function in a model of type 2 diabetes (T2D). SRT1720 treatment restored function in the retina of diabetic mice, as assessed by improved b-wave amplitude in scotopic and photopic electroretinograms (ERGs) and recovery of the visual response by optokinetic nystagmus response.[10]

Adeno-associated virus (AAV) is a nonenveloped virus that was made to deliver DNA to target cells with low pathogenicity and ability to transduce several cell types. AAV gene therapy has been used for obesity and metabolic disease.[14–16] AAV-mediated gene therapy has already been described for its neuroprotection, modulation of ocular angiogenesis and inflammation, and protection of photoreceptors.[17] Therefore, we asked whether AAV2-SIRT1 could mitigate features of established DR in an animal model of T2D. Herein, we demonstrate improvement of visual function studies, as assessed by increased ERG and optokinetic nystagmus (OKN) responses, as well as a significant reduction of hypoxia, inflammation, and retinal apoptosis.

Methods

Animal Studies

All animal studies and experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham (IACUC #20917) and in accordance with guidelines set forth by the National Institutes of Health and followed the Association for Research in Vision and Ophthalmology Statement for the Use of Animals. Adult db/db mice, 8 weeks old, (strain B6.BKS (D)-*Lepr*^{db}/J Stock#000697; Jackson Laboratories, Bar Harbor, ME, USA) were housed in a standard laboratory environment and maintained on a 12-hour light/dark cycle. Mice were considered diabetic after recording two separate blood glucose levels greater than 250 mg/dL.

For ocular injections, mice received anesthesia with inhalant isoflurane (1%–2%) vaporized in oxygen, and for electroretinography analysis, mice were anesthetized by ketamine (72 mg/kg)/xylazine (4 mg/kg) via intraperitoneal injection. Mice were euthanized by overexposure to isoflurane in a desiccation chamber in a fume hood followed by cervical dislocation. In this study, 9-month-old db/db mice received a single intravitreal injection of AAV2-SIRT1 (titer: 3.52E+12). AAV2-SIRT1 was generated and produced by the Powell Vector Core at the University of Florida. Briefly, human codon-optimized *SIRT1* complementary DNA (GenScript, Piscataway, NJ, USA) was amplified and cloned into an AAV expression plasmid. The cassette (TR-CBA-Sirt1-TR) is 4766 bp and within the 5.0-kb size limit for AAV2. The vector is wild-type AAV2, and the standard pDG helper plasmid was used for AAV2 production. The backbone that was used to create the clone is pTR-CBA-hGFP. The GFP was removed and human Sirt1 inserted. The AAV2-CBA-hGFP (UF11) is a standard control. This application has been previously used in Yin et al.[18] and Nieuwenhuis et al.[19] After 3 months, mice were euthanized, and eyes enucleated for analysis.

Electroretinography

ERGs were performed using a LED Ganzfeld stimulator (LKC Technologies, Gaithersburg, MD, USA). Mice were dark adapted for 12 hours overnight. The animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg total body mass) and xylazine (15 mg/kg total body mass). Pupils were then dilated with atropine/phenylephrine eye drops. A drop of Goniotaire solution (Altaire Pharmaceuticals, Northville, NY, USA) containing 2.5% Hypromellose

solution was applied to each eye to ensure a good electrical connection between the contact electrodes and the corneal surface of the eyes. The mice were kept on a 37°C heating pad throughout the procedure.

The mice were exposed to full-field white light flashes at intensities of −20, −10, and 0 diabetic (db) under scotopic conditions. Light adaptation was then done for 5 minutes, and the mice were exposed to full-field white light flashes at intensities of 3, 6, and 10 db under photopic conditions. Recordings were averaged and analyzed using the LKC EM software (LKC Technologies).

Optomotor Responses/Optokinetic Nystagmus Procedure

Optomotor responses/optokinetic responses were recorded using a computer-based visual acuity response test (OptoMotry; Cerebral Mechanics, Inc., Lethbridge, Canada) as previously described.[10] The visual acuity of the mice was assessed and recorded as the threshold spatial frequencies they could detect. Stimuli in the form of rotating sinusoidal gratings were presented to the animal via computer monitors. The mice were placed on a platform in the system, and head-tracking responses were recorded. The test was done under photopic conditions with the contrast between the gratings at 100% and speed of 12.0 d/s.

Immunohistochemistry

Frozen sections were prepared using previously published protocols.[20] Briefly, the eyes were immediately enucleated and fixed in 4% paraformaldehyde (PFA) overnight at 4°C. The eyeballs were then transferred to increasing concentrations of sucrose (15% overnight followed by 30% for 4 hours) in phosphate-buffered saline (PBS) to remove water and prevent crystals formation to preserve tissue morphology. Samples were then embedded in optimal cutting temperature (OCT) medium and kept at −80°C. Retinal sections (10 µm) were then prepared and stored at −20°C for immunohistochemistry.

SIRT1, IBA1, and Glial Fibrillary Acidic Protein Detection

Tissue sections were thawed at room temperature for 30 minutes and washed in PBS for 5 minutes. Nonspecific binding was then blocked by incubation in 5% goat serum (G9023; Sigma-Aldrich, St. Louis, MO, USA) in PBS for 90 minutes, and then goat serum solution was drained off the slides without washing. Primary antibody solutions were prepared with 1% goat serum in PBS (Sigma-Aldrich). SIRT1 1:500 (3931; Cell Signaling Technology, Danvers, MA, USA), IBA1 1:1000 (019-19741; Fujifilm Cellular Dynamics, Madison, WI, USA), and glial fibrillary acidic protein (GFAP) 1:500 (C9205; Sigma Aldrich). The slides were then kept at 4°C overnight.

For incubation with the secondary antibody, sections were washed three times in PBS for 5 minutes each. Sections were then incubated in fluorescent-labeled secondary antibodies for 2 hours at room temperature, followed by washing and a 10-minute incubation with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) 1:1000 (D1306; Invitrogen, Waltham, MA, USA) for nuclear staining, and finally coverslip mounted with Vectashield antifade mounting medium (H-1000; Vector, Burlingame, CA, USA). The quantification of GFAP intensity was performed using the Zen image acquisition software on the Zeiss Axio Imager.Z2 (Zeiss Oberkochen, Germany) epifluorescence microscope. The analysis was done using the module for region of interest (ROI) as described.[21] Briefly, the image was opened in the Zen software, and the ROI was selected by drawing. The fluorescence intensity was measured by the same software, and the data were then further analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The number of SIRT1-positive cells in the ganglion cell layer and IBA1-positive cells in the retina was counted by two trained independent observers masked to the conditions of the experiment.

Cleaved Caspase 3 Detection

Slides were thawed at room temperature for 30 minutes, and antigen retrieval was performed using sodium citrate buffer (pH 6) in a pressure cooker for 20 minutes. Sections were then permeabilized in 0.2% Triton X-100 in PBS for 20 minutes and blocked with 5% goat serum for 1 hour at room temperature. Tissue samples were then incubated overnight in primary antibody to cleaved caspase 3 (ASP175 #9661; Cell Signaling Technology) at 1:100 at 4°C.

Slides were then washed with PBS and then incubated with secondary antibodies for 2 hours at room temperature. Slides were washed again and incubated for 10 minutes with DAPI solution and finally mounted using Vectashield.

Enumeration of Acellular Capillaries

Mouse eyes were enucleated and fixed in 2% formalin and trypsin digestion of the neuro retina performed to expose the entire vascular network as previously described.[22] Briefly, the retina was isolated and incubated in elastase for 3 hours and then elastase activating solution overnight. The retina was cleaned of neural cells, and the vasculature was flat mounted on a glass slide. It was then stained with periodic

acid–Schiff for quantification of acellular capillaries by four independent observers masked to the samples' identity.

Flow Cytometry Analysis

Cells isolated from the bone marrow and peripheral blood were incubated with ammonium chloride solution (07850; Stemcell Technologies, Vancouver, British Columbia, Canada) for 15 min on ice to lyse erythrocytes. Cells were washed with PBS supplemented with 2% fetal bovine serum and then incubated with a cocktail of primary antibodies (viability dye 510, Ly6G, CD45, CD11b, CCR2, CD31, CD133, Ly6C, and Flk-1) for 30 minutes at 4°C in the dark. The complete information of antibodies is provided in Supplementary Table S1. Cells were acquired on a FACS Celesta flow cytometer (BD Biosciences, Mississauga, Ontario, Canada) using the FACS Diva v.8.0.1.1 software (BD Biosciences), and flow cytometry data were analyzed using FlowJo Software, version 10.8.0.

Retina cells were isolated by incubating the entire retina with digestion buffer. Briefly, the retinas were isolated from the enucleated eye cup of the mouse. Papain (100 µL) per activator solution (60 µL) was added to each retina and heated at 37°C for 30 minutes. Ringer's solution (1 mL) was then added per retina and incubated at 37°C for 30 minutes. Retinas were dissociated by pipetting up and down and transferred into FACS tubes and spun at 200 × *g* for 5 minutes. The suspension of cells was filtrated in a 40-µm cell strainer. A membrane-permeant, fluorogenic probe (hypoxia inducible factor 1α [HIF-1α]; #NB100105PCP) that can detect cells adapting to a hypoxic environment and specific retinal cell surface markers was used. Cells were incubated with a cocktail of antibodies containing viability dye 510, CD31, CD144, F4/80, CD45, CD11b, HIF-1α, CD206, PKC-α, and rhodopsin. Details regarding antibodies are shown in Supplementary Table S1. Retinal cells were acquired on a BD FACSymphony A5 Cell Analyzer (BD Biosciences), and the flow cytometry analyses were performed using FlowJo software.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 7.0). The one-way analysis of variance testing was done for experiments with multiple groups. Statistical significance was defined as $P < 0.05$. All values are expressed as mean ± SEM.

Results

AAV2-SIRT1 Therapy Increases Retinal SIRT1 Expression

For these studies, db/db mice with a 7-month duration of diabetes were selected as at this time

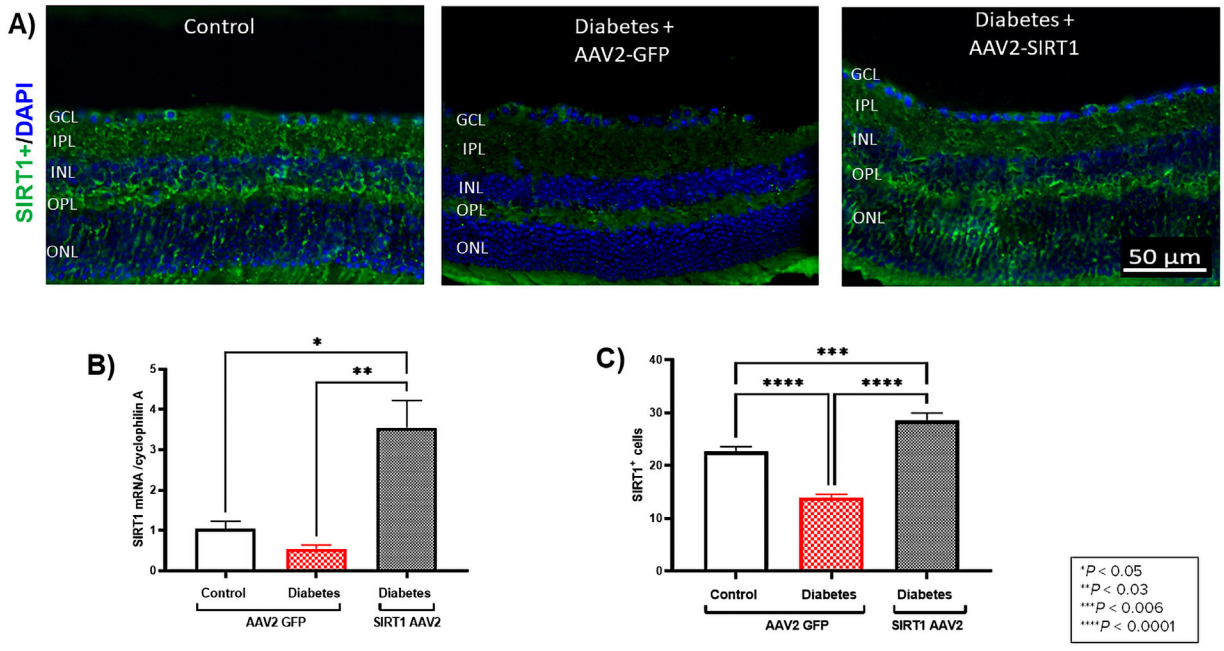

Figure 1. AAV2-SIRT1 intravitreal injection increases SIRT1 expression in retina cells. **(A)** The retina of mice injected with AAV2-SIRT1 shows increased SIRT1 immunohistochemical staining compared with diabetic retina. **(B)** SIRT1 mRNA expression is increased in the retina of AAV2-SIRT1–treated mice compared to diabetic mice with control virus treatment. **(C)** AAV2-SIRT1 increases the number of SIRT1 immunopositive cells.

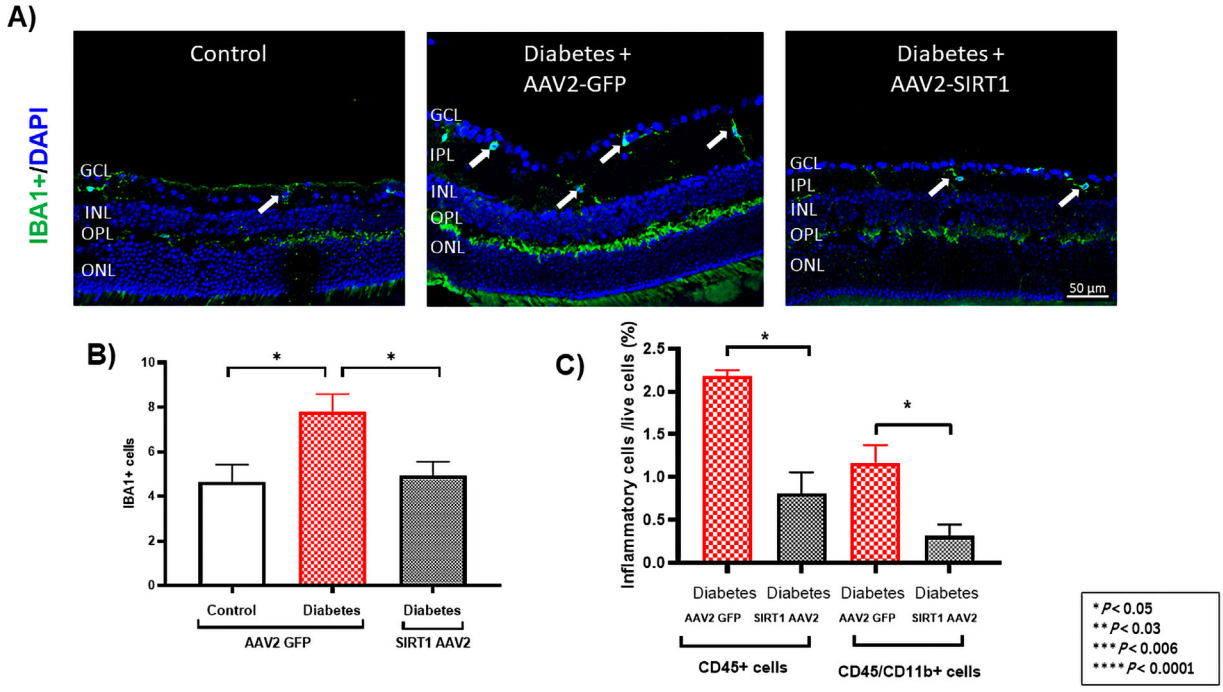

Figure 2. AAV2-SIRT1 treatment decreases retinal inflammation. **(A)** IBA1$^+$ cells in the retina of control mice, diabetic mice, and diabetic mice following AAV2-SIRT1 injection. **(B)** Quantification of IBA1$^+$ cells in each experimental cohort. AAV2-SIRT1 injection decreases the number of IBA1$^+$ cells in the retina. **(C)** Inflammatory cells are significantly reduced in the retina of AAV2-SIRT1–treated diabetic mice compared to control virus–treated mice.

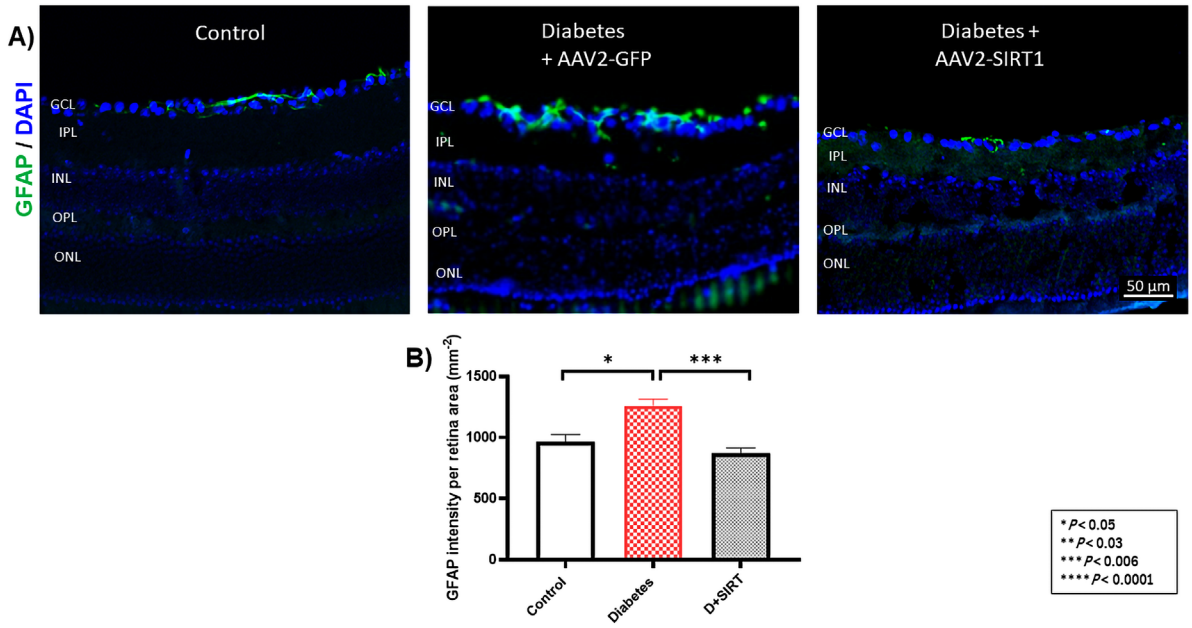

Figure 3. AAV2-SIRT1 treatment decreases retinal reactive gliosis. **(A)** GFAP staining in the retina of control mice, diabetic mice, and diabetic mice with the AAV2-SIRT1 treatment. **(B)** Quantification of GFAP intensity in each of the different experimental groups. AAV2-SIRT1 injection decreases the intensity of GFAP staining in the retina of treated diabetic mice.

DR is established in this model,[22,23] and the intention was to determine whether SIRT-1 overexpression could reverse aspects of DR pathology. Mice were euthanized at 10 months to allow sufficient time for the AAV2 virus to express SIRT1. Initially, to confirm adequate SIRT1 expression, the level of SIRT1 messenger RNA (mRNA) was assessed by quantitative reverse transcription polymerase chain reaction. AAV2-SIRT1–injected retinas of db/db mice demonstrated robust SIRT1 mRNA expression compared to control virus–injected db/db retinas (AAV-2-SIRT1: 3 $\pm$ 0.9 vs. AAV2-GFP: 0.5 $\pm$ 0.1, $P < 0.05$). The mice

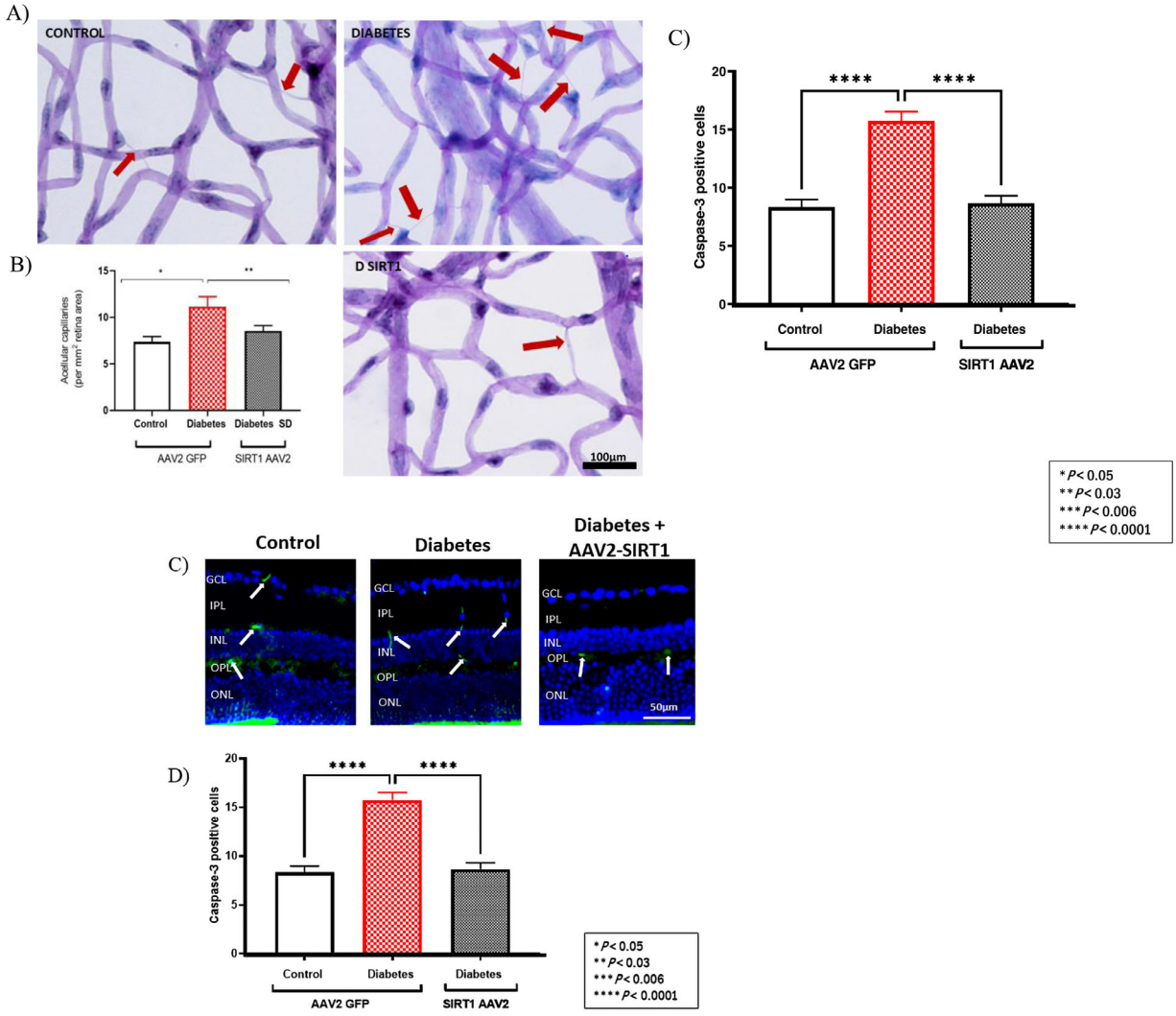

Figure 4. AAV2-SIRT1 treatment reduces the number of acellular capillaries and apoptosis. **(A)** Representative images of retinal acellular capillary staining in control, diabetic, and diabetic mice injected with AAV2-SIRT1. *Red arrows* point to acellular capillaries. **(B)** Quantification of acellular capillaries. Number of capillaries is significantly increased in the diabetic retina compared to the control retina and is decreased in the retina of the diabetic mice injected with AAV-2 SIRT. **(C)** Immunohistochemistry for caspase 3 in retinal sections. *White arrows* point to caspase 3^+ cells. **(D)** Quantification of caspase 3^+ cells in the retina. The number of apoptotic caspase 3^+ cells is reduced in the diabetic mice injected with AAV2-SIRT1 compared to control virus–injected mice.

were euthanized 3 months later to allow sufficient time for SIRT1 protein expression to affect the DR pathology. In the AAV2-SIRT1–injected retinas, as expected, the number of $SIRT1^+$ cells in the ganglion cell layer was significantly increased compared to control virus–injected mice (AAV-2-SIRT1: 28.4 ± 6 vs. AAV2-GFP: 13.9 ± 2, $P < 0.05$; Fig. 1).

Overexpression of SIRT1 Decreases the $IBA1^+$ Cells in Diabetic Retina

DR is characterized by increasing inflammation, and activated microglia/macrophages were assessed by enumeration of $IBA1^+$ cells (Figs. 2A, 2B). The db/db mice injected with AAV2-GFP showed higher numbers of $IBA1^+$ cells (7.79 ± 1.02 /mm^2) compared to AAV2-SIRT1–injected diabetic mice (4.92 ± 1.01/mm^2, $P < 0.05$; Fig. 2B). Flow cytometry was performed on the retinas from both cohorts of mice. $CD45^+$ cells and myeloid cells ($CD45^+/CD11b^+$) were increased in retinas of AAV2-GFP db/db mice, while mice injected with AAV-2 SIRT1 showed a reduction of both cell types (2.18 ± 0.15 vs. 0.81 ± 0.55, $P < 0.05$, and 1.17 ± 0.42 vs. 0.31 ± 0.30, $P < 0.05$, respectively) (Fig. 2C).

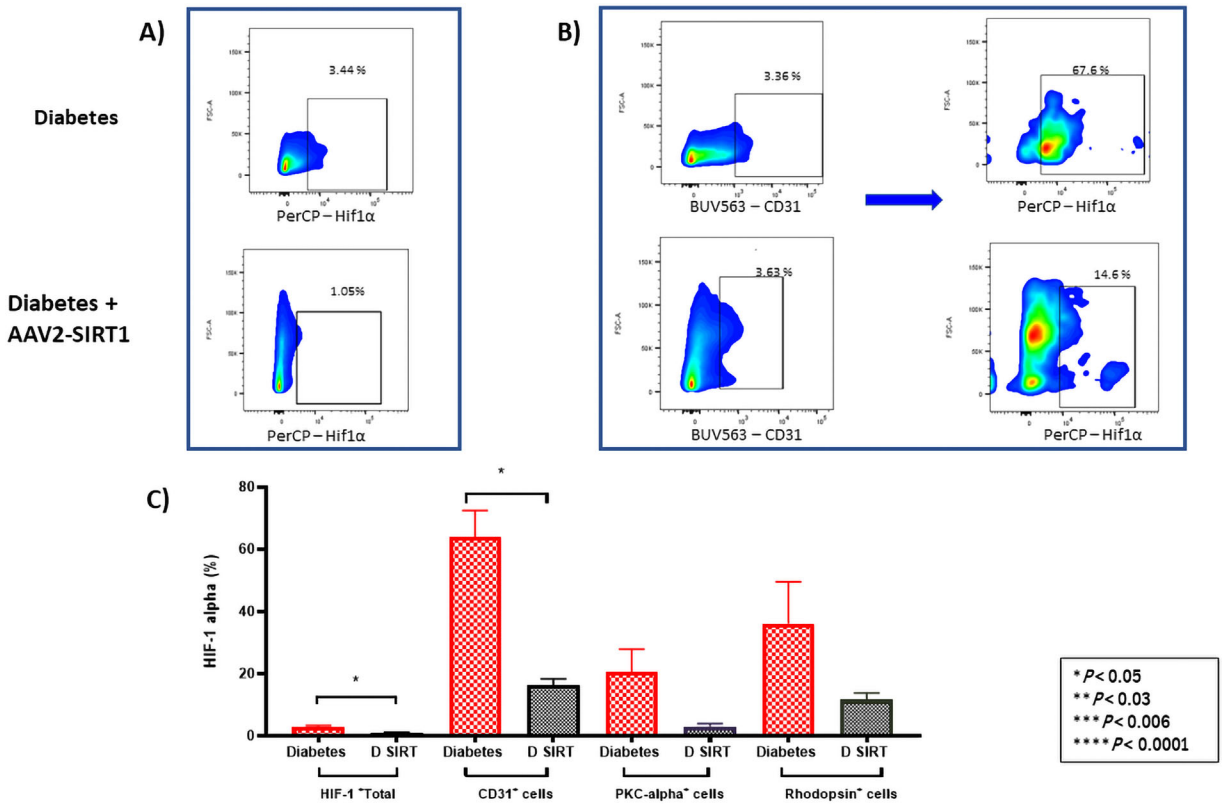

Figure 5. SIRT1 overexpression reduces retinal endothelial cell hypoxia. **(A)** Flow cytometry gating strategy for total HIF-1α in the retina. **(B)** Gating strategy for CD31$^+$ endothelial cells. The first column represents the gating for the percentage of CD31$^+$ cells in the retina. The second column shows the percentage of the CD31$^+$ cells that are HIF-1α^+. **(C)** Percentage of HIF-1α^+ cells in the total retina, CD31$^+$ cells, bipolar cells (PKC-α^+), and rod photoreceptors (rhodopsin$^+$ cells).

SIRT1 Treatment Reduces Retinal Reactive Gliosis

GFAP immunofluorescence was measured in the retina of the mice from the two cohorts to determine the effect of SIRT1 overexpression (Figs. 3A, 3B). GFAP staining was low and restricted to the retinal ganglion cell layer in the nondiabetic mice (964.1 ± 59.56/mm^2, $P < 0.05$). The diabetic mice, in contrast, had significantly higher expression of GFAP (1258 ± 54.68/mm^2, $P < 0.05$). Intravitreal injection of AAV2-SIRT1 in the diabetic mice resulted in a significant decrease in diabetes-induced retinal reactive gliosis compared to control-injected diabetic mice (869.9 ± 42.75 vs. 1258 ± 54.68/mm^2 $P < 0.05$).

SIRT1 Overexpression Reduces Apoptosis and the Number of Acellular Capillaries

The diabetic mice that received AAV2-SIRT1 showed a reduction in the number of acellular capillaries (8.54 ± 2.90, $P < 0.05$) when compared with the control-injected db/db mice (11.15 ± 4.65, $P < 0.05$), suggestive of a protective role of SIRT1 (Figs. 4A, 4B). Next, the number of apoptotic cells was examined using caspase 3 expression, which was reduced in AAV2-SIRT1–injected db/db mice compared to control virus–injected db/db mice (8.67 ± 1.0 vs. 15.74 ± 1.0, $P < 0.05$) (Figs. 4C, 4D).

SIRT1 Injection Attenuates Hypoxia of Retinal Cells

We identified that AAV2-SIRT1 administration resulted in a reduction of total Hif1α-positive retinal cells compared to AAV2-GFP administration (2.84 ± 0.51 vs. 0.87 ± 0.32, $P < 0.05$). This reduction was also observed in retinal endothelial cells (CD31$^+$) while bipolar cells and photoreceptors were not altered (Fig. 5). AAV2-SIRT1–treated mice had significantly reduced CD31$^+$ and Hif1-α positive cells as opposed to AAV2-GFP–treated mice (64 ± 8.55 vs. 16.30 ± 2.06, $P < 0.05$).

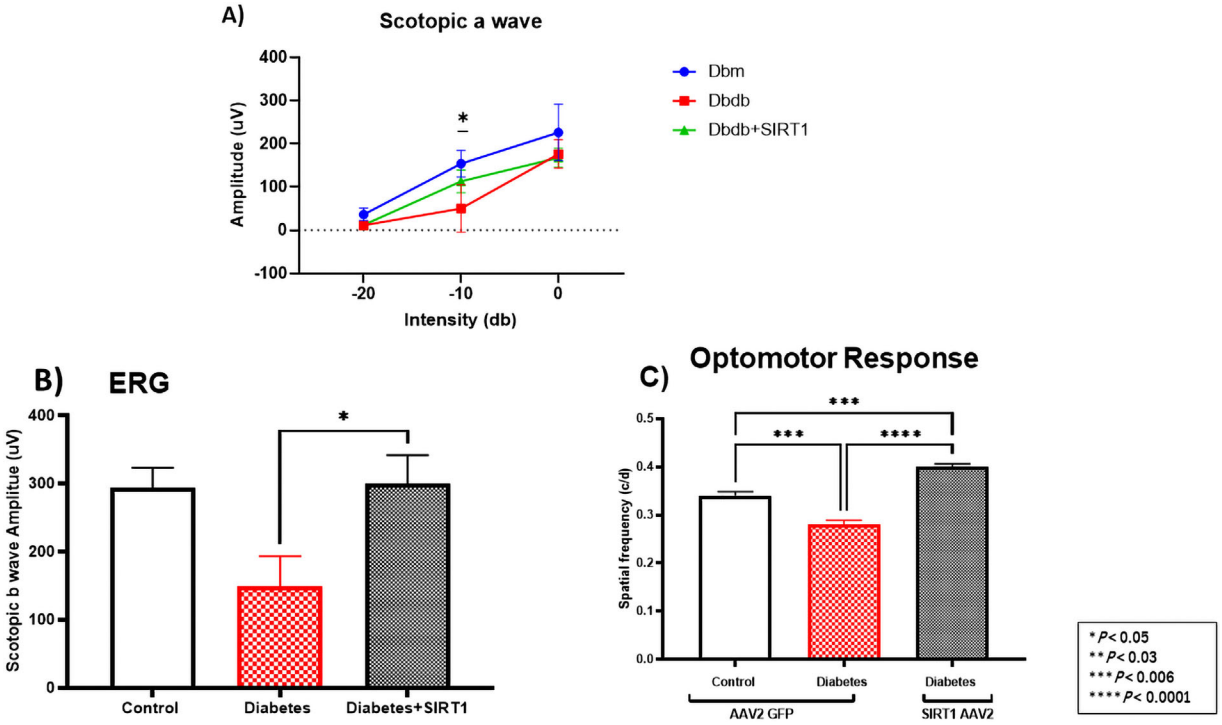

Figure 6. AAV2-SIRT1 improves visual recovery. **(A)** ERG scotopic a-wave amplitudes for mice at light intensities of −20, −10, and 0 db. Intravitreal AAV2-SIRT1 injection improves the amplitude of the scotopic a-wave at −10 db compared to diabetic mice; however, at 0 db, the amplitudes are similar for both diabetes and diabetes with AAV2-SIRT1 treatment. **(B)** Isolated ERG response amplitudes for the −10 db light intensity. **(C)** Optomotor response measurements. The spatial frequency observed by the diabetic mice is significantly reduced compared to the control mice and diabetic mice injected with intravitreal AAV2-SIRT1.

Visual Response Is Improved by AAV2-SIRT1 Administration

DR causes defective visual response due to neurodegeneration. The amplitude of the scotopic a-wave did not change with the AAV2-SIRT1 administration when compared to the AAV2-GFP control at –20 db and 0 db. However, there was a significant increase in the amplitude of the a-wave ERG at –10 db light intensity (Fig. 6A) and an improvement in the amplitude of the scotopic b-wave in the diabetic mice injected with AAV2-SIRT1 compared to the diabetic mice injected with control virus (Fig. 6B).

In addition, an increased optokinetic response was observed in AAV2-SIRT1–injected db/db mice compared with db/db injected with control virus. The spatial frequency was higher in AAV2-SIRT1 db/db mice (0.40 c/d) than the control db/m mice (0.35 c/d) and diabetic control virus–treated mice (0.28 c/d) (Fig. 6C).

AAV2-SIRT1 Does Not Affect Systemic Inflammation

AAV2 has the potential to activate a systemic immune response. Using flow cytometry, total monocytes, as well as the subgroups of classical and nonclassical monocytes, were quantified in peripheral blood and in bone marrow of mice injected with either AAV2-GFP or AAV2-SIRT1 and their uninjected counterparts. No statistical difference was noted between the cohorts (Fig. 7).

Discussion

The salient findings of this study include confirmation of successful delivery and expression of retinal SIRT1 following intravitreal injection of AAV2-SIRT1. This provides a rationale for using AAV2-SIRT1 to improve the visual response by decreasing retinal inflammation, reducing hypoxia, and minimizing apoptotic cells in retina of a diabetic mouse model.

The use of recombinant AAV vectors has emerged as a promising therapeutic strategy for retinal gene delivery targeted to treat monogenic retinal disease and restore visual function. The long-term expression and safety of AAV allow consideration of using gene therapy for chronic ocular conditions such as DR and AMD. The high transduction efficiency of AAV2

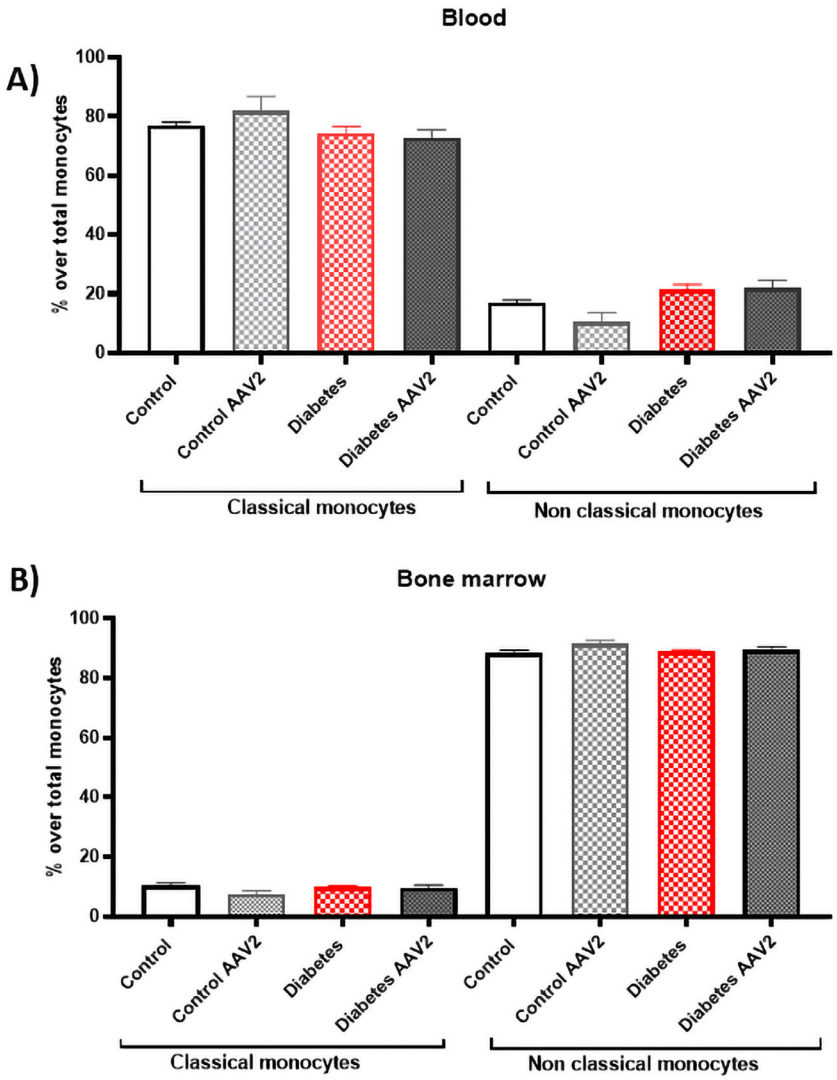

Figure 7. AAV2 injection does not cause systemic inflammation. **(A)** Percentage of classical and nonclassical monocytes in the blood. There were no significant differences in the percentage of classical and nonclassical monocytes in blood of diabetic mice compared to controls. **(B)** Percentage of classical and nonclassical monocytes over total number of monocytes. There were no significant changes in the percentage of classical and nonclassical monocytes in the bone marrow of control mice with and without treatment and diabetic mice with and without AAV2 treatment. The AAV2 injections did not alter the percentages of the classical and nonclassical monocytes in the bone marrow of the diabetic mice.

vectors makes them ideal for diabetic mouse retina treatment.[24]

A large body of research shows that SIRT1 plays a protective role and enhances the survival of numerous cell types.[25] In a study using SH-SY5Y neuroblastoma cells, SIRT1 was found to function as a redox sensor modulating antioxidant defenses.[26] Oxidative stress promotes neural cell death via generation of reactive oxygen species and free radicals, which are damaging to cells, contributing to Parkinson disease progression.[26–28] SIRT1 transfection exerted a neuroprotective effect and enhanced cell survival.[26] Resveratrol, a SIRT1 activator, has been found in multiple studies as well to reduce hypoxia-induced mitochondrial damage and to reduce the expression of hypoxia inducible factor 1α and vascular endothelial growth factor proteins.[29–33]

In diabetes, multiple studies have suggested the protective effect of SIRT1. It was found to attenuate DNA damage-induced vascular calcification.[34] In another study, SIRT1 upregulation by exercise mitigated inflammation and metabolic dysregulation. There was a reduction in serum creatinine, urea, triglyceride, and hepatic aspartate aminotransferase and alanine transaminase activity, which is indicative of reduced progression of diabetic nephropathy

and hepatic steatosis.[35] Furthermore, previous studies from our lab have shown multiple benefits of SIRT1 in reducing serum hypercholesterolemia and reversing diabetic retinopathy. Fasting mimicking conditions increased SIRT1 activity and subsequently upregulated reverse cholesterol transport gene expression. In type 2 diabetic mice, SIRT1 activation reduced retinal vascular damage and inflammation and increased retinal electrophysiologic amplitudes by activation of the liver X receptor (LXR) pathway.[10]

In this study, we examined the impact of increasing SIRT1 on apoptosis of the retinal cells. Diabetic retinopathy is associated with apoptosis of a variety of retinal vascular and neural cells. Retinal vascular damage is characterized by the presence of acellular capillaries, which result from apoptosis and loss of pericytes and endothelial cells from retinal microvasculature.[36] We found that the control virus–treated diabetic mice had a significantly higher number of acellular capillaries representing severe retinal endothelial cell and pericyte loss compared to nondiabetic mice. The mice that received AAV2-SIRT1 virus injection demonstrated significantly reduced numbers of retinal acellular capillaries compared to the diabetic mice with control virus injection. This implies that sustaining SIRT1 expression was protective to retinal endothelial cells, which is consistent with findings from other studies.[5,37–39] SIRT1 was found to prevent apoptosis by regulating the mitochondria-related apoptotic signal.[25]

SIRT1 is a crucial player in providing resistance to stress-induced apoptosis occurring in systemic diseases.[5,7] Stressors such as hyperglycemia, oxidative stress, and reactive oxygen species production as well as caloric restriction trigger a compensatory increase in SIRT1 activity, causing deacetylation of factors involved in energy homeostasis such as the transcription coactivator PGC1α (peroxisome proliferator-activated receptor-γ [PPARγ] coactivator 1α). SIRT1 also deacetylates and activates the LXR pathway to increase cholesterol efflux by increasing reverse cholesterol transport activity.[10] SIRT1 activation using resveratrol in retinal cells reduced apoptosis, as observed by a significant decrease in the expression of caspase 3 expression compared to untreated cells.[31] This corroborates our findings in an in vivo model of diabetes that SIRT1 protects the retina from apoptosis.

Retinal neural dysfunction occurs early in diabetic eye disease, and this is characterized by a decrease in retinal response amplitudes. In this study, we observed that AAV2-SIRT1–treated db/db mice had increased retinal visual response amplitudes compared to control virus–treated db/db mice. This was significant in the b-wave amplitudes and implies that SIRT1 can improve retinal bipolar cell function in this type 2 diabetes model. Previous studies have shown that SIRT1 plays an important role in the function of bipolar cells[10] and, as shown in this study, improvement in b-wave amplitudes. This can aid retinal intercellular signal transduction. We also observed an overall improvement in the visual function of the mice treated with the AAV2-SIRT1.

Diabetes-induced inflammation has been implicated as a major contributor to the pathogenesis of DR.[9,36] Iba1 is a macrophage/microglia marker,[40] and microglial activation can be detected by changes in morphology and is associated with release of inflammatory cytokines contributing to the retinal neuronal and vascular damage in DR. In this study, the AAV2-SIRT1–treated mice showed decreased Iba1-positive cell immunohistochemistry staining. Abnormal Müller glial cells play an important role in the pathogenesis of DR. Müller cell dysfunction, which is characterized by high expression of GFAP, has been observed in the early stages of DR.[41,42] Intravitreal-delivered AAV2 has the potential to enter the systemic circulation and affect other organs.[43] We, therefore, analyzed the effect of AAV administration in the blood and the bone marrow. In both the blood and the bone marrow, we did not observe any variation in the amount of total, classical, or nonclassical monocytes.

Conclusions

Collectively, our results reveal that the administration of AAV2-SIRT1 can reduce retinal apoptosis, microglial activation, endothelial cell hypoxia, and the number of acellular capillaries. Additionally, it has the potential to promote retinal functional recovery. Retinal gene therapy with AAV2 vectors is efficacious in transducing different cells, including photoreceptors and endothelial cells in humans. This application has been tested in clinical trials and found effective in diseases such as Leber congenital amaurosis (LCA)[44] and choroideremia.[45] In the clinical trial to study choroideremia, the vector successfully transduced rods and ones resulting in improved function, and in LCA, there was increased visual sensitivity. In the aforementioned clinical trials as well as in our study, no vector-related ocular or systemic adverse effects were observed.

The intravitreal delivery of AAV2-SIRT1 therefore may provide a practical and useful therapeutic strategy for reversal of DR and has the potential to be tested in human studies.

Acknowledgments

Supported by National Institutes of Health grants R01EY012601, R01EY028858, R01EY028037, R01EY025383, and R01EY032753 to MBG and by an unrestricted grant from Research to Prevent Blindness Department of Ophthalmology and Visual Sciences VSRC Core grant P30 EY003039 from the National Eye Institute.

Disclosure: **Y. Adu-Agyeiwaah**, None; **C.P. Vieira**, None; **B. Asare-Bediako**, None; **S. Li Calzi**, None; **M. DuPont**, None; **J. Floyd**, None; **S. Boye**, None; **V. Chiodo**, None; **J.V. Busik**, None; **M.B. Grant**, None

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