ORIGINAL RESEARCH COMMUNICATION

Oral Monomethyl Fumarate Therapy Ameliorates Retinopathy in a Humanized Mouse Model of Sickle Cell Disease

Wanwisa Promsote,¹ Folami Lamoke Powell,¹ Satyam Veean,¹ Menaka Thounaojam,² Shanu Markand,³ Alan Saul,^{2,4} Diana Gutsaeva,² Manuela Bartoli,^{2,4} Sylvia B. Smith,²⁻⁴ Vadivel Ganapathy,⁵ and Pamela M. Martin^{1,2,4}

Abstract

Aims: Sickle retinopathy (SR) is a major cause of blindness in sickle cell disease (SCD). The genetic mutation responsible for SCD is known, however; oxidative stress and inflammation also figure prominently in the development and progression of pathology. Development of therapies for SR is hampered by the lack of (a) animal models that accurately recapitulate human SR and (b) strategies for noninvasive yet effective retinal drug delivery. This study addressed both issues by validating the Townes humanized SCD mouse as a model of SR and demonstrating the efficacy of oral administration of the antioxidant fumaric acid ester monomethyl fumarate (MMF) in the disease.

Results: *In vivo* ophthalmic imaging, electroretinography, and postmortem histological RNA and protein analyses were used to monitor retinal health and function in normal (HbAA) and sickle (HbSS) hemoglobin-producing mice over a one-year period and in additional HbAA and HbSS mice treated with MMF (15 mg/ml) for 5 months. Functional and morphological abnormalities and molecular hallmarks of oxidative stress/inflammation were evident early in HbSS retinas and increased in number and severity with age. Treatment with MMF, a known inducer of Nrf2, induced γ -globin expression and fetal hemoglobin production, improved hematological profiles, and ameliorated SR-related pathology.

Innovation and Conclusion: United States Food and Drug Administration-approved formulations in which MMF is the primary bioactive ingredient are currently available to treat multiple sclerosis; such drugs may be effective for treatment of ocular and systemic complications of SCD, and given the pleiotropic effects, other nonsickle-related diseases in which oxidative stress, inflammation, and retinal vascular pathology figure prominently. *Antioxid. Redox Signal.* 25, 921–935.

Keywords: retina, monomethyl fumarate, Nrf2, fetal hemoglobin induction, inflammation, oxidative stress

Introduction

SICKLE CELL DISEASE (SCD) is a common genetic mutation in the United States, principally among African Americans. Millions are affected worldwide (21, 45). There is no cure for the disease. Current treatment options are limited in that they are largely supportive in nature, targeting secondary manifestations of the disease that present after patients have already suffered much discomfort and irreversible damage to organs (21, 45). Pain medication, repeated blood transfusion,

and hospitalization during crises are the present standard for SCD management. This reduces the incidence of crises and hospitalization in some, but is ineffective in a high number of SCD patients (1, 5, 15, 21, 28, 45).

At present, hydroxyurea (HU) is the only United States Food and Drug Administration (FDA)-approved drug to treat SCD; the drug induces γ -globin and hence fetal hemoglobin (HbF) expression, consequently reducing erythrocyte sickling. However, HU has several deleterious side effects; furthermore, a significant proportion of SCD patients fail to

Departments of ¹Biochemistry and Molecular Biology, ²Ophthalmology, and ³Cellular Biology and Anatomy, The Medical College of Georgia at Augusta University, Augusta, Georgia.

⁴The Culver Vision Discovery Institute, The Medical College of Georgia at Augusta University, Augusta, Georgia.

⁵Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, Texas.

Innovation

The current work focuses on retinopathy in sickle cell disease (SCD) and the fetal hemoglobin-inducing properties of monomethyl fumarate (MMF). However, our findings demonstrate convincingly and are congruent with others that show MMF elicits a number of additional effects that would be desirable in other neurovascular diseases (12, 41, 44). Hence, the broad potential relevance of MMF to the prevention and treatment of non-SCD-related complications in which inflammation, oxidative stress and retinopathy are major features (*e.g.*, diabetes/diabetic retinopathy) should not be ignored.

respond to the drug (5, 15, 42). In addition, there is little evidence of benefit in the ocular environment following HU therapy, despite the commonness of retinopathy, vision loss, and blindness in SCD patients (4, 16, 45). Hence, the need for new therapies, whether as alternatives or adjuvants to HU, is overwhelming.

Here we demonstrate, using a humanized mouse model of SCD, the efficacy of monomethyl fumarate (MMF), a potent inducer of Nrf2 (12, 24, 31), as a novel drug for treatment of SCD based on changes in hematological parameters and SCD-associated retinal pathology. The clinical relevance of this study is heightened by the fact that FDA-approved drug formulations with MMF as the bioactive ingredient are currently available to treat multiple sclerosis (9, 32, 38); such formulations can be readily tested and repurposed for use in SCD patients.

Results

Retinal abnormalities in HbSS mice are consistent with retinopathy

Townes mice, a model genetically engineered to express human α -, β -, and γ -globin and therefore produce human hemoglobin (Hb), were used in this study (43). The absence of *Pde6b*^{rd1} (rd1) and *Crb1*^{rd8} (rd8), common mutations known to contribute to the development of abnormal retinal pathology in mice (11), was confirmed before study initiation. Retinal health was monitored in living normal Hb (HbAA)- and sickle Hb (HbSS)-producing Townes mice, using fluorescein angiography (FA), fundus imaging, and spectral domain optical coherence tomography (SD-OCT). FA imaging of the eyes of anesthetized mice revealed no overt signs of morphological or functional pathology in young HbAA mouse retinas (Fig. 1A, B). Indeed, HbAA retinas showed no sign of pathology early on and were generally healthy and asymptomatic even at older ages (Fig. 1I, J). However, pronounced pigmentary and microvascular anomalies were evident in young HbSS retinas (Fig. 1C–H) and increased in number and severity with advanced age (Fig. 1K–N). As with SCD in humans, there was some variability in the manifestation and severity of retinal symptoms among HbSS animals. In fact, some HbSS mice became extremely sick and were euthanized before desired experimental time points, died unexpectedly, or developed cataract or severe retinal hemorrhages precluding retinal imaging. As such, we present here the most consistently observed phenotypes, not the most unique or severe. SD-OCT was used to evaluate the neuroretinal characteristics of these HbAA and

HbSS animals (Fig. 1O). These imaging studies revealed central (optic nerve head region) abnormalities in HbSS retinas. Differences in retinal thickness and abnormalities in the neuronal cell (outer nuclear layer [ONL] and inner nuclear layer [INL]) layers were also readily apparent throughout the retina. Associated OCT-guided morphometric analyses (Fig. 1P) supported these observations as total retinal thickness was significantly reduced in HbSS compared to HbAA retinas both in young and aged mice. The data further revealed that the degeneration (reduced thickness) of HbSS neural retina (NR) was attributed most notably to thinning of the ONL, the area of outer retina that contains the nuclei of the rod and cone photoreceptors (first-order neurons in the visual pathway), and the associated inner segments (IS) of these cells (located just below the ONL). In addition, the inner plexiform layer, the area of inner retina between the INL and ganglion cell layer (GCL) that comprises the synaptic connections between second- and third-order neurons in the visual pathway, was affected. To better appreciate the morphological features characteristic to the HbSS retina, hematoxylin and eosin (H&E)-stained JB-4 plastic retinal sections were prepared from the eyes of mice comparable in age to those used in OCT analyses (Fig. 1Q). The general laminar structure of the retina remained unaltered in HbAA animals. However, vascular and nonvascular anomalies were detected in HbSS retinal sections. Robust vessel tufts, indicative of neovascularization, were visible in the region of the nerve fiber layer, as were enlarged misshaped vessels within the INL (*black arrows* in bottom left panel of Fig. 1Q). Disruptions in outer retinal morphology were also quite prominent in many HbSS eyes; note specifically, the vacuolated appearance of the outer segments of the photoreceptors, the portion of the photoreceptor cell located just above the apical surface of the retinal pigment epithelium (also indicated by *black arrows*). The retinal pigment epithelium (RPE) underlying these disrupted areas of HbSS retina appeared to be largely intact; however, at higher magnification, abnormalities in the RPE-choroidal interface were evident. *Note:* The darkened vertical areas indicated by *white arrows* in HbAA and HbSS images are an artifact common to JB-4 histological processing.

Functional deficits are evident in HbSS mouse retina

Collectively, ophthalmic imaging and histological examination of HbAA and HbSS mouse eyes (above) demonstrate the development- and age-related progression of retinal pathology in HbSS mice. To determine whether these findings translate to altered visual function, electroretinogram (ERG) testing was performed (Fig. 1R). Because HbSS mice were found to be extremely sensitive to anesthesia-related death, particularly at older ages, ERG studies were performed using young animals. C-wave responses did not differ significantly between HbAA and HbSS animals. However, reduced a- and b-wave amplitudes, waves generated primarily by photoreceptor and bipolar cells, respectively, were readily detected in HbSS animals. Scotopic threshold responses, an index of retinal ganglion cell function in rodents, were also reduced in HbSS mice. The ERG data are congruent with OCT data and histological data (Fig. 1O–Q) demonstrating thinning and other morphological alterations in the cellular layers in which these retinal cell types are contained in HbSS mice, and importantly, with ERG studies conducted in human SCD patients (29, 30).

FIG. 1. Characterization of the retinas of live normal hemoglobin-producing (HbAA) and sickle hemoglobin-producing (HbSS) Townes humanized mice. Fluorescein angiography and fundoscopic imaging were conducted simultaneously on young (4–7-month old) and aged (8–12-month old) HbAA (A, B and I, J, respectively) and HbSS mouse eyes (C–H and K–N, respectively). $n=6-9$ mice for each genotype. Young HbAA eyes showed no overt signs of retinal pathology (A, B). The retinas of HbAA animals remained relatively healthy and asymptomatic with increased age (I, J). However, vascular anomalies were detected frequently in HbSS eyes even in animals of a young age. Tortuous vessels (asterisks), microaneurysms (red and white circles in E, K, G), arteriovenous crossings (upper left corner of E), and occluded vessels (red arrowhead in upper right of E) were observed commonly in HbSS retina. Digitally enlarged views of the regions highlighted by the white boxes in (E and F) are provided in (G and H), respectively. Pigmentary abnormalities were found commonly in HbSS eyes (white to yellowish-white patches present in F, H, L and N) that often coincided with areas in which, as indicated by fluorescein angiographic imaging of the same retinal area, microaneurysms were present (red circles in G and H; white circles in K and L). Significant fluorescein leakage and neovascularization at the optic disc (K) were also evident in some HbSS eyes. SD-OCT performed on young HbAA and HbSS animals (O) revealed frequent abnormalities at the optic nerve head (red arrowhead). Morphological disruptions (red arrows) were also common both centrally and peripherally in HbSS mouse retinas. SD-OCT aided quantification of retinal layer thicknesses (P). RNFL, retinal nerve fiber layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL + IS, outer nuclear layer plus inner segment; OS, outer segment; RPE, retinal pigment epithelium. Data in (P) are presented as mean distance in micrometers (μm) $\pm$ SE, $*p<0.01$ compared to HbAA control, $*p<0.001$ compared to HbAA control. Hematoxylin and eosin-stained retinal cross sections (Q). Black arrows, areas of morphological abnormality; white arrows, JB-4 processing artifacts. Electroretinographic (ERG) testing (R) revealed associated deficits in visual function in young HbSS mice compared to age-matched HbAA controls. Data in (R) are presented as mean $\pm$ SE, $n=6$ mice for each genotype. $*p<0.01$ compared to HbAA control. SE, standard error; SD-OCT, spectral domain optical coherence tomography. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Loss of inner and outer blood–retinal barrier integrity in HbSS mice

Like retinopathy in human SCD (10, 17), the above data show pigmentary and neuroretinal dysfunction to be prominent in HbSS mice. Retinal vascular and neuronal health is

critically dependent on the maintenance of inner and outer blood–retinal barrier integrity (20, 34, 36). Therefore, we evaluated in greater detail the cells responsible for preserving these barriers, endothelial and RPE cells, respectively (Fig. 2A–E). Trypsin digestion of HbAA and HbSS retinas to view the retinal vasculature independent of the nonvascular

FIG. 2. Histological evaluation of HbAA and HbSS retinal vasculature and RPE. Trypsin digestion (**A**) revealed the presence of acellular capillaries (red arrows) and collapsed and/or degenerated vessels (red asterisks) that were detected frequently in young and aged HbSS mouse retinas but were less common in digested retinas prepared from HbAA mice of similar age as confirmed on quantitation in bar histograms found in (**B**). Data are presented as mean $\pm$ SE, $*p < 0.05$ compared to age-matched HbAA. Zonula occludin-1 (ZO-1; green) immunofluorescence in RPE flatmounts prepared from young and aged HbAA and HbSS mouse eyes to evaluate RPE morphology (**C**). DAPI nuclear counterstain (blue fluorescence). Quantification of RPE flatmounts; total cell number per image (**D** and **E**), number of multinucleate cells per image. Data in (**D** and **E**) are presented as mean $\pm$ SE, $*p < 0.05$ compared to age-matched HbAA, $**p < 0.05$ compared to young HbAA mouse RPE; ($n = 4$). DAPI, 406-diamidino-2-phenylindole. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

(i.e., neuronal and RPE) components of the tissue (13) revealed a significant number of acellular capillaries (red arrows) and capillaries that were degenerated or collapsed (asterisks) both in young and old HbSS mouse retinas, but not in HbAA mouse retinas of comparable age (Fig. 2A,B). As noted with other pathological features observed in HbSS retinas, the incidence and severity of these anomalies increased with age. Outer retinal barrier integrity was evaluated in RPE flatmounts prepared from HbAA and HbSS eyes and immunolabeled with antibody against the junctional protein zonula occludens 1 (ZO-1) (Fig. 2C–E). Uniformly stained monolayers of cobblestone-shaped RPE were apparent in HbAA flatmounts. This characteristic RPE phenotype persisted in HbAA mice until advanced age, at which time a greater number of hypertrophied and multinucleate RPE cells could be detected, but the relative uniformity of the ZO-1 labeling and the classical morphological shape of the RPE

remained intact. In contrast, numerous hypertrophied, abnormally shaped, and multinucleate RPE cells, features consistent with altered RPE tight junction formation and associated barrier dysfunction (34, 36), were detectable in HbSS RPE flatmounts at relatively early ages and worsened with advanced age. Subsequent transepithelial permeability assays performed using primary RPE cells isolated from HbAA and HbSS mouse eyes demonstrated definitively the compromised ability of HbSS RPE to form and maintain functional junctions and, in turn, barrier integrity (Fig. 3A). On daily observation of HbAA and HbSS primary RPE in culture, it was noted that HbAA primary RPE cells appeared to proliferate faster and therefore reach confluence sooner than HbSS primary cells cultured identically. To follow up definitively on this observation, MTT assays were performed, and the presence of fewer viable cells in HbSS compared to HbAA cultures was confirmed (Fig. 3B).

FIG. 3. Disruption of barrier properties in HbSS primary RPE. (A) FITC-Dextran (40-kD) transepithelial permeability assay performed on confluent monolayers of primary RPE cells established from HbAA and HbSS mouse eyes. (B) MTT cell viability assay of primary RPE cell cultures established from HbAA and HbSS mouse eyes. Data are presented as mean $\pm$ SE, * $p < 0.01$ compared to HbAA control; ** $p < 0.001$ compared to HbAA control. FITC, fluorescein isothiocyanate.

MMF treatments lead to improved retinal morphology and visual function in HbSS mice

Collectively, the data presented above coupled with our previous report (31) demonstrate that HbSS Townes mice recapitulate accurately a number of characteristic features associated with the development and progression of retinopathy in human HbSS patients, including intraretinal hemorrhage, venous tortuosity, pigmentary anomalies, increased inflammation and oxidative stress, and deficits in visual function (10, 17, 20, 30). Hence, the Townes SCD model can be considered a suitable one in which to study underlying mechanisms and therapies for sickle retinopathy (SR). With this in mind, we next evaluated the long-term effectiveness of MMF, a compound demonstrated previously to be acutely effective at suppressing oxidative stress and inflammation, reactivating γ -globin transcription, and increasing HbF production in cultured human retinal and primary erythroid progenitor cells and in intact HbSS Townes mouse retina following intravitreal injection (3, 24, 31).

Immediately after weaning, HbAA and HbSS mice were placed on drinking water containing MMF (15 mg/ml; made fresh and replenished daily) or maintained on regular drinking water (controls). Fundoscopy, FA, and ERG testing were

used to monitor the development and progression of retinopathy-like characteristics in HbAA and HbSS mice treated with MMF (Fig. 4). Untreated HbSS animals (Fig. 4A–D) developed retinopathy-like characteristics similar to those described in Figure 1. However, retinal health was much improved in HbSS animals that received MMF (Fig. 4E–H). In fact, many MMF-treated HbSS eyes did not differ significantly in appearance from those of HbAA control or MMF-treated HbAA animals. ERG analyses of control and MMF-treated HbSS animals demonstrated that these morphological improvements correlate with improvements also in visual function (Fig. 4I).

Retinal barrier functions are preserved with MMF delivery

Improvements in the retinal phenotype of MMF-treated HbSS animals were apparent also at the histological and morphological levels. Trypsin-digested retinas revealed fewer collapsed or acellular capillaries in HbSS mice that were treated with MMF than in untreated HbSS controls (Fig. 5A). The regularity of ZO-1 immunolabeling and RPE morphology was also preserved in MMF-treated HbSS eyes (Fig. 5B) and the number of multinucleate cells was significantly decreased (Fig. 5C). Since prior OCT and histological analyses of HbSS retinas (Fig. 1) showed evidence of retinal thinning and cellular loss, terminal dUTP nick end labeling (TUNEL) analyses were performed on cryosections prepared from control and MMF-treated HbSS mice to evaluate the influence of MMF on retinal cell viability (Fig. 5D). Few apoptotic (TUNEL positive) nuclei could be detected in control or MMF-treated HbAA cryosections, whereas many were evident in untreated HbSS mouse retina. Although not completely absent, there was a considerable reduction in the number of apoptotic nuclei detectable in MMF-treated HbSS retinal cryosections. Collectively, these data suggest that the vascular, RPE and neuroretinal dysfunction endogenous to HbSS Townes mouse retina are abrogated by prolonged MMF therapy.

MMF induces Nrf2 and downregulates ICAM-1 and vascular endothelial growth factor expression in HbSS mouse retina

Others and we have demonstrated MMF to be a potent inducer of Nrf2, a protein that regulates cellular antioxidant machinery in response to inflammation (3, 24, 31). As anticipated, extended oral administration of MMF was associated with induction of Nrf2 expression in HbSS mouse retina (Fig. 6A, B). To determine whether the increase in Nrf2 expression correlated with increased Nrf2 activity, we next evaluated the expression of several well-established downstream Nrf2 target genes, NAD(P)H quinone dehydrogenase 1 (Nqo1), heme-oxygenase 1 (HO-1), and thioredoxin reductase 1 (Txrdn), by real-time polymerase chain reaction (Fig. 6C–E). Indeed, the increase in Nrf2 expression in the retina of MMF-treated animals was associated with significant increases also in the expression of each of these genes. In addition, the expression of ICAM-1 (Fig. 6F–H) and vascular endothelial growth factor (VEGF) (Fig. 6I–K), molecules crucial to the potentiation of oxidant-induced inflammation and neovascularization and therefore highly relevant to the pathogenesis of SCD, was also evaluated. ICAM-1 mRNA was increased significantly in control HbSS eyes but reduced

FIG. 4. MMF preserves retinal health and function in HbSS Townes humanized mouse retina. Representative funduscopy and fluorescein angiographic images of the retinas of HbSS mice maintained for 5 months on regular drinking water (A–D; HbSS control) or drinking water containing MMF (15 mg/ml) starting from the age of 1 month (E–H, HbSS MMF). Pigmentary anomalies (red arrows), arteriovenous crossings and neovascular outgrowths (white arrows), and pronounced fluorescein leakages (C, red box) were found frequently in control HbSS eyes but not in MMF-treated HbSS eyes. (I) ERG data derived from testing of control and MMF-treated HbSS mice. $n = 6$ mice per group. Data are presented as mean $\pm$ SE, $*p < 0.01$. MMF, monomethyl fumarate. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

dramatically in association with MMF treatment (Fig. 6F), a finding verified by Western blot analyses of ICAM-1 protein (Fig. 6G, H). Analyses of VEGF protein expression using a modified Western blotting method in which retinal extracts are subjected to heparin binding affinity columns so that the protein/density of the band detected expressly represents the amount of active VEGF present within the sample rather than simply the total of amount of VEGF protein contained (6, 18), revealed trends in expression similar to those detected for ICAM-1 with respect to VEGF expression in HbSS controls compared to HbSS mice that received MMF (Fig. 6I, J). Immunolocalization studies of VEGF protein in retinal cryosections supported the VEGF blot data (Fig. 6K).

MMF-induces γ -globin expression and improves hematological profiles in HbSS mice

In keeping with our goal of evaluating MMF as a potential therapy for prevention and treatment of SCD-related complications, with emphasis at present on retinopathy, we next

evaluated whether MMF treatment was associated with changes in γ -globin and consequent HbF expression, key fundamental parameters of interest with respect to overall improvement in SCD (1, 35). Robust increases in γ -globin mRNA were detected in RPE tissues isolated from HbAA and HbSS mice treated with MMF (Fig. 7A). The fact that retinopathy is just one of several major complications that HbSS patients potentially face coupled with the fact that in this study, MMF was delivered to mice *via* an oral route, led us to investigate also whether the beneficial effects of the drug were evident also outside of the retina. High performance liquid chromatography (HPLC) analyses of blood samples collected from control and MMF-treated HbAA and HbSS mice demonstrated a two-fold increase in HbF and a significant reduction in the amount of sickle (abnormal) Hb present in HbSS whole blood (Fig. 7B–C). Also, hematological analyses revealed significant improvements in hematocrit (Hct), red blood cell (RBC) number, and Hb concentrations in MMF-treated HbSS mice (Fig. 7D). Reductions in white blood cell count, mean corpuscular volume, and mean corpuscular hemoglobin, additional indices of clinical improvement in human SCD, were also evident.

FIG. 5. Evaluation of endothelial RPE and neuroretinal cell morphology and viability in HbAA and HbSS mouse retina following MMF treatment. Trypsin digest (A) and RPE flatmount (B) preparations of control and MMF-treated HbSS eyes demonstrated improvements in retinal vascular and RPE cell integrity, respectively. *Black arrows* in panel (A) highlight degenerated or acellular capillaries. (C) Quantification of the number of multinucleate cells per RPE flatmount image. Data are presented as mean $\pm$ SE; * $p < 0.01$ compared to HbSS control; $n = 4$. (D) TUNEL analyses to evaluate apoptotic cell death in retinal cryosections prepared from the eyes of HbAA and HbSS mice treated with or without MMF. TUNEL-positive cells (*green fluorescence*). TUNEL, terminal dUTP nick end labeling. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Nrf2 expression is important for the MMF-induced upregulation of γ -globin and related suppression of Bcl11A in cultured RPE cells

Analyses of Nrf2 and related target genes (Fig. 6) support the MMF-induced limitation of inflammation and oxidative stress in SCD retina and we now know additionally that MMF effectively raises γ -globin and consequent HbF expression (Fig. 7); it remains to be determined whether there is any direct association between these two responses. Because some Nrf2-inducing agents have been found to reactivate γ -globin transcription in erythroid cells (26), we did investigate whether MMF effects on Nrf2 expression are similarly related to the ability of the compound to reactivate γ -globin expression and HbF production in RPE cells. To address this question, siRNA studies were conducted using cultured human RPE (ARPE-19) cells; cells demonstrated previously to, such as RBCs, produce Hb (31, 37). siRNA-mediated silencing of Nrf2 expression in ARPE-19 cells (Fig. 8A, B) blocked the MMF-induced effect on γ -globin expression (Fig. 8C), supporting the possible involvement of Nrf2 in the MMF-induced effects on γ -globin expression in this cell type. In addition, MMF treatment was associated with down-regulated expression of B-cell lymphoma/leukemia 11A (Bcl11A) (Fig. 8D); reduced Bcl11a is known to reverse γ -globin silencing and consequent switching from fetal to adult Hb (7, 8). The suppressive effects of MMF on Bcl11A expression diminished in association with siRNA-mediated silencing of Nrf2 in cultured RPE. As mentioned previously, a number of inducers of Nrf2 also induce γ -globin expression. The inverse relationship between γ -globin and Bcl11A expression is also established. However, these phenomena have

been demonstrated independently and the context of their interdependence, if any, has not been investigated; this is a subject worthy of additional detailed study.

Discussion

As SCD is RBC related and hence a vascular disease, no organ system is spared. Vascular occlusion resulting from erythrocyte sickling impacts all organs. The retina, full of narrow blood vessels, is highly susceptible to damage in SCD. Our most recent work suggests that SCD-related dysfunction of RPE-generated Hb might also be involved (31). Vision loss and blindness are common, but largely understudied compared to other SCD-related complications (10, 17). Also, there is little evidence that strategies used routinely in the management of SCD confer any benefit in terms of preventing or treating retinopathy SR. Hence, therapies for SR are desperately needed. Progress in the field is hampered, however, by the lack of (a) animal models that accurately recapitulate human SR and (b) strategies for noninvasive but effective retinal drug delivery. We addressed both issues in this study. First, we characterized the retinal phenotype of the Townes humanized mouse model of SCD, a transgenic knockout mouse engineered to expression human α -, β -, and γ -globin and produce human Hb in place of mouse Hb (43). Although verified to mimic accurately many of the systemic characteristics of SCD and related pathology, the eyes have not been examined to determine whether SR-like pathological features occur in this animal model and whether the model is suitable to study morphological parameters consistent with human SR. Second, we used this animal model to evaluate the efficacy of oral administration of MMF, the principal

bioactive ingredient in FDA-approved formulations for treatment of multiple sclerosis, as a potential drug for SCD.

There are a number of available small animal models of SCD, and some have been demonstrated to develop a retinopathy-like phenotype consistent with features of human SR (25, 27). However, the Townes model affords the unique opportunity to evaluate human globin gene expression and human Hb production without the confounding presence of the mouse globin genes and mouse Hb (43) and therefore is highly attractive. To date, the retinal phenotype of this model has not been characterized. Comprehensive *in vivo* imaging, ERG and postmortem histological studies demonstrate the development and progression of a retinopathy-like phenotype in HbSS Townes mice. Like retinopathy in human SCD (10, 17), the retinal vasculature of Townes HbSS mice is prominently affected. Less well-investigated, neuroretinal dysfunction is also purported to occur also in human SR, often before clinically detectable vascular pathology is evident (29, 30). However, few have followed up on this phenomenon in recent years. Here we found pigmentary and neuroretinal dysfunction to be prominent in HbSS mice. Noteworthy is the fact that functional visual deficits, especially in outer retinal function, were noted in many HbSS mice even before vascular abnormalities could be detected *via* ophthalmic imaging. The findings obtained using *in vivo* imaging and ERG are highly consistent with findings reported commonly in human HbSS retina. Collectively, the present findings demonstrate for the first time that Townes mice recapitulate accurately a number of characteristic features associated with the development and progression of retinopathy in human HbSS patients (*i.e.*, intraretinal hemorrhage, venous tortuosity, pigmentary anomalies, increased inflammation and oxidative stress, and deficits in visual function) making it a suitable model to study underlying mechanisms and therapies for SR. In addition, our prior (31) and present studies suggest that the retinal pathology that develops in this SCD mouse model may be attributable to the combined detrimental consequences of erythroid- and RPE-generated HbS.

After validating the Townes SCD model with respect to its utility in the study of SR, we next evaluated the long-term effectiveness of MMF, a compound demonstrated previously to be acutely effective at suppressing oxidative stress and inflammation, re-activating γ -globin transcription, and increasing HbF production in cultured human retinal and primary erythroid progenitor cells, and in intact HbSS Townes mouse retina following intravitreal injection (3, 24, 31). Comprehensive *in vivo* imaging, ERG, and postmortem histological studies demonstrated significant improvements in

retinal morphology and function in HbSS mice in association with oral MMF therapy. In addition, hematological analyses of venous blood, performed and verified using two separate hematology analyzers, each equilibrated before use with the appropriate human complete blood cell count standards, revealed improvements in overall hematological profiles in these animals; improvements that associated HPLC analyses suggest might be attributable to reduced HbS concentrations and increased HbF levels. Although the increases in HbF produced following administration of MMF appeared to be modest (1–5% increase), the reductions in HbS were quite significant. This level of HbF induction is comparable to that reported for HU, the drug currently used for SCD management and one that elicits robust systemic improvements in SCD with HbF induction in the range of two- to four-fold (14). HU, unfortunately, does not appear to yield much benefit in the retina. On the contrary, our findings in retinal and erythroid cells suggest MMF to be capable of preventing or improving ocular as well as systemic complications of SCD.

Fumaric acid esters, especially MMF, have shown much promise in a variety of animal models of human disease and in human patients (9, 24, 31). Given MMF's demonstrated efficacy in inducing Nrf2 and elevating HbF in human retinal and erythroid cell types (31), here we tested and confirmed its efficacy in SCD in Townes HbSS mice. In some disease states in which oxidative stress and inflammation are prominent, Nrf2 levels are induced in what is thought to be a rescue attempt although such a response is still often not sufficient to prevent or combat cellular damage. Indeed, Vercellotti *et al.* (40) found levels of this redox-sensitive transcriptional factor and that of HO-1, a related downstream target, to be elevated in SS mice. Here we found, on the contrary, basal levels of Nrf2 to be significantly lower in HbSS compared to HbAA mice in the absence of treatment (Fig. 6), findings congruent with those reported by Keleku-Lukwete *et al.* (23) with respect to Nrf2 expression in HbSS mice. It is quite plausible that the expression of Nrf2 is highly temporal. Thus, while it may be elevated initially shortly after pro-oxidant insult, elevated Nrf2 expression might not persist over the long term in an environment of continued or exacerbated oxidative stress. Thus, differences in the age of SS animals used may partially explain the variability in basal levels of Nrf2 expression reported for SS mice in the different studies. SS animals in the Vercellotti study were examined between the ages of 8 and 17 weeks (2–5 months of age), whereas in the present study, we examined Nrf2 expression only at the conclusion of the MMF treatment period when mice were 6+ months of age. It is

FIG. 6. Evaluation of Nrf2, Nqo1, Txrnd, HO-1, ICAM-1, and VEGF expression following MMF treatment of HbSS mice. (A) Representative blot from Western analysis of Nrf2 protein expression and (B) associated densitometric analysis. Real-time quantitative PCR (RT-qPCR) analysis of downstream Nrf2 target genes (C) NAD(P)H quinone dehydrogenase 1 (Nqo1); (D) thioredoxin reductase 1 (txrnd); (E) heme oxygenase (HO-1). The mRNA expression level of intercellular adhesion molecule 1 (ICAM-1) was also analyzed (F). Western blot and associated densitometric analyses were conducted to evaluate ICAM-1 protein (G and H, respectively). (I and J) Analysis of active VEGF protein present in retinal lysates prepared from control and MMF-treated HbAA and HbSS animals. (K) Immunofluorescence analyses of vascular endothelial growth factor (VEGF, *green fluorescence*) and isolectin-b4 (I-B4, *red fluorescence*) protein in HbAA and HbSS retinal cryosections. PCR and Western blot studies were performed using three independent tissue samples per group and were run in duplicate or triplicate. Data are presented as mean $\pm$ SE, * p < 0.05 compared to HbSS untreated control, ** p < 0.05 compared to HbAA untreated control. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

FIG. 7. MMF induces γ -globin mRNA expression and HbF protein production in HbSS retina and improves hematological profiles in HbSS mice. (A) RT-qPCR analysis of γ -globin mRNA expression in the RPE of HbAA and HbSS mice maintained either on regular drinking water (control) or drinking water containing MMF (15 mg/ml) for a period of 5 months. High performance liquid chromatography (HPLC) analysis of the percentage of HbF (B) and sickle hemoglobin (C) present in peripheral whole blood collected from HbSS mice maintained either on regular drinking water (control [CON]) or drinking water containing MMF (15 mg/ml: MMF) for a period of 5 months. (D) Complete blood cell and hematological profiles of control and MMF-treated HbAA and HbSS mice. Data are presented as mean $\pm$ SE, * p < 0.05 compared to control; ** p < 0.01 compared to control. WBC, white blood cell count; HCT, hematocrit; RBC, red blood cell count; HGB, total hemoglobin; MCV, mean cell volume; MCH, mean corpuscular hemoglobin; RDW, red cell distribution width; HbF, fetal hemoglobin.

additionally interesting that a number of the above-referenced related studies use mice heterozygous for the beta-globin gene mutation (HbAS) as study controls, whereas here, homozygous HbAA control mice were used.

MMF treatment was associated with improvements both in terms of the activation of Nrf2 and related downstream gene targets and the induction of γ -globin/associated HbF production. RPE cell culture experiments demonstrated an important role for Nrf2 in the regulation of γ -globin and Bcl11A; however, we still have not demonstrated definitely any interdependence or direct relationship between these phenomena in the living animal. Nrf2 knockout mice are readily available from commercial sources. Therefore, studies conducted using these animals would seem the ideal next step toward obtaining a reliable answer. However, available Nrf2 knockout animals express the mouse globin genes that are distinctly different from human globin gene expression and regulation, a factor that sparked the engineering of humanized mouse models of globin gene production for use in studies of human globin gene regulation such as the present. Even though our past (31) and present studies of MMF do not resolve the aforementioned issue, they do, without question,

demonstrate MMF to be beneficial for SCD in several different regards, most especially, HbF induction and reduced expression of pro-oxidant/proinflammatory molecules. The fact that the HbSS mice used in our current study received a relatively low dose of MMF *via* an oral route and a beneficial effect was achieved in the eye is also extremely significant, given the known difficulties associated with drug delivery to the posterior segment (22).

Sickle cell is a very serious and highly debilitating disease; hence, finding a therapy such as MMF that could possibly prevent and treat retinopathy in SCD would undeniably be good but would leave much to be desired if it only worked for retinopathy, given that the complications of SCD are numerous. At present, there is no single therapy that can treat effectively both ocular and systemic complications of SCD. Our prior work in primary human erythroid cells (31), coupled with our present findings in intact HbSS retina, suggests that MMF might represent one such potential compound. The pleiotropic nature of the benefits derived from MMF and the fact that FDA-approved formulations exist in which MMF is the main bioactive ingredient (32, 38) increase further the translational appeal of this work. The average life span of a

FIG. 8. The MMF-induced upregulation of γ -globin mRNA and suppression of Bcl11A mRNA in cultured RPE cells is dependent on Nrf2 expression. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) expression was knocked down in transformed human retinal pigment epithelial (ARPE-19) cells using siRNA technology as confirmed by (A) RT-qPCR analyses of Nrf2 mRNA expression and Western blotting and associated densitometric analyses (B) of Nrf2 protein. Treatment of control ARPE-19 (no siRNA) and nontargeted siRNA control ARPE-19 cells with MMF was associated with robust increases in Nrf2 mRNA and protein expression. However, the MMF-induced increases in Nrf2 mRNA and protein were abrogated significantly in ARPE-19 cells in which Nrf2 expression was knocked down using Nrf2-specific siRNA. Data in (A and B) are presented as mean $\pm$ SE, * p < 0.001 compared to corresponding untreated control, ** p < 0.01 compared to corresponding MMF-treated control or scramble siRNA samples. (C) γ -globin mRNA expression increased significantly in ARPE-19 cells in which Nrf2 expression remained intact but was reduced significantly in cells in which Nrf2 expression was knocked down using Nrf2-specific siRNA. (D) BCL11A mRNA expression was significantly reduced in ARPE-19 cells in which Nrf2 expression remained intact but was upregulated significantly in cells in which Nrf2 expression was knocked down using Nrf2-specific siRNA. Data in (C and D) are presented as mean $\pm$ SE, * p < 0.01 compared to control cells with no treatment, ** p < 0.01 compared to control + MMF. Bcl11A, B-cell lymphoma/leukemia 11A.

normal C57BL/6J mouse is approximately (24 months/2 years); Townes HbAA mice have a similar life span. SCD (HbSS) mice, in contrast, have a variable life expectancy, which like the human depends heavily on the severity of their disease (poor hematological profiles are associated with decreased life span); thus, as we mentioned previously, it was not surprising that some HbSS mice initially enrolled in our study did not survive until desired time points. However, the majority of our HbSS animals survived to at least 12 months of age. We administered MMF for a period of 5 months (1–6 months of age), a period of time roughly comparable to early childhood-adolescence in humans to early-middle adulthood; a time period during which SCD patients experience a lot of complications. The fact that there were no substantial detrimental effects when the compound was delivered to healthy HbAA mice (controls) within this same age range supports the notion that human SCD patients could possibly begin taking the compound at a relatively young age, even in the absence of hematological crises, given that low-grade in-

flammation and increased oxidative stress are present in adolescents with SCD in the absence of crises (2) and continue using the drug safely in the long term as a possible preventive. These notions, while highly speculative, are supported additionally by the fact that related fumaric acid ester formulations have been tested and proven to be well tolerated in humans at relatively high doses for extended periods of time, a stark contrast to HU (5, 15, 33). Conduction of clinical studies to confirm our preclinical findings is therefore important as the potential for the rapid extrapolation and use of MMF for treatment of SCD in humans appears to be high.

Materials and Methods

Animals

All experiments involving animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Georgia Regents University (Augusta, GA) Institutional Animal Care and Use

Committee. HbAA (normal hemoglobin)- and HbSS-expressing Townes humanized knockin SCD mice (Jackson Laboratories) were described previously (31, 43). For *in vivo* studies using MMF (Sigma-Aldrich), HbAA- and HbSS-expressing mice were maintained either on regular drinking water (controls) or MMF water (15 mg/ml) starting at the age of 1 month for 5 months. Fresh drinking water, with or without MMF, was prepared and given daily. At the end of the treatment period, the animals were euthanized by carbon dioxide asphyxiation followed immediately by cervical dislocation, and sera and eyes were collected for analyses. All groups contained six to nine animals each.

Reverse transcription–quantitative polymerase chain reaction

Total RNA was isolated from the NR and RPE/eyecup obtained from HbAA- and HbSS-expressing mouse eyes using TRIzol. RNA (1 μ g) was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative polymerase chain reaction (qPCR) was used to monitor steady-state mRNA levels of genes of interest. Primer sequences for all genes evaluated in this study have been published (3, 7, 31). Hypoxanthine phosphoribosyltransferase 1 or 18S was used as the internal control. Real-time qPCR amplifications, using detection chemistry (SYBR Green; Applied Biosystems), were run in triplicate on 96-well reaction plates. Gene expression was calculated by comparing Ct values ($\Delta\Delta$ Ct).

Western blot analysis

Protein was extracted from NR and RPE/eyecup isolated from control and MMF-treated HbAA- and HbSS-expressing mouse eyes. Protein concentration was determined using the bicinchoninic acid assay (Thermo Fisher Scientific). Protein samples were subjected to SDS–PAGE, transferred to nitrocellulose membranes, and then incubated with primary antibodies: HbF (1:1000; Bethyl Laboratories, Inc.), Nrf2 (1:1000; Abcam), ZO-1 (1:100; Abcam), and VEGF (1:100; Abcam) overnight at 4°C. Detection of HbF, Nrf2, and ZO-1 was done using horseradish peroxidase-conjugated secondary antibodies. After washing, the proteins were visualized using the enhanced chemiluminescence Western blot detection system (Thermo Fisher Scientific). β -actin served as the loading control. For the determination of VEGF protein levels, the retinal extracts were subjected to heparin binding affinity columns following previously described methods (6, 18).

JB-4 tissue processing

Mice were killed by CO₂ asphyxiation followed by cervical dislocation. Eyes were removed immediately, fixed in 4% paraformaldehyde/2% glutaraldehyde in 0.1 M cacodylate buffer, and then embedded in JB-4 embedding compound (EMS) as per the method of Ha *et al.* (19). Sections were stained with H&E and evaluated using a standard brightfield microscope.

Immunofluorescence assays

Retinal cryosections were prepared from HbAA- and HbSS-expressing mouse eyes as previously described (31). Sections were fixed with 4% paraformaldehyde, washed

with PBS–Triton X-100, incubated with Power Block (BioGenex), and then incubated with a primary antibody overnight at 4°C; the sections were washed three times with PBS–Triton X-100 followed by incubation with a secondary antibody for 1 h at 37°C. Sections were washed with PBS–Triton X-100 and coverslipped with Fluoroshield with 406-diamidino-2-phenylindole (DAPI; Sigma) to label nuclei. Sections were examined by a fluorescence microscope (Carl Zeiss Meditec).

TUNEL

Retinal cryosections were incubated in 50 μ l of reaction buffer containing terminal deoxynucleotidyl transferase (Promega) and fluorescein-12-dUTP (Roche) according to the manufacturer's instructions. Sections were incubated at 37°C for 1 h in a humidified chamber. After several washes in PBS and staining with DAPI, TUNEL-positive cells were observed by a fluorescence microscope.

RPE flatmount

RPE flatmounts were prepared by dissecting RPE/eyecups from unfixed eyes and fixing them in cold (–20°C) methanol overnight. Tissues were then placed in microtubes and processed for immunohistochemistry. Fixed RPE/eyecups were blocked with 0.1% bovine serum albumin, 2% goat serum, and 0.1% Triton-X in PBS for 1 h. Subsequently, the RPE/eyecups were incubated with a rabbit antibody against ZO-1 (1:100; Abcam) diluted in the blocking solution overnight at 4°C, and then with the goat anti-rabbit Alexa Fluor 488 secondary antibody (1:1500; Invitrogen) for 1 h at room temperature. Tissues were counterstained with DAPI and RPE/eyecups then cut partially at four places to allow the tissue to be flattened on the Superfrost microscope slides (Fisher Scientific).

Retinal trypsin digestion

Freshly enucleated eyes were fixed in 4% (wt/vol) paraformaldehyde in PBS overnight. After fixation, the retinas were dissected, washed in PBS, and incubated in trypsin (0.5% [Difco Trypsin 250] prepared in 20 mM Tris buffer pH 8) for about 45 min at 37°C. The vessel structures were isolated from the retinal cells by gentle shaking and rinsing in distilled water for overnight. After the vascular specimens were mounted on a slide and dried for 3–4 days at room temperature, periodic acid-Schiff staining was performed. Slides were imaged using traditional brightfield microscopy and the number of acellular capillaries per mm² of capillary area was determined by counting 10 randomly selected microscopic fields.

Transepithelial permeability assay

Transepithelial permeability assays were performed following the method of Trudeau *et al.* (39). In brief, HbAA- and HbSS-expressing primary RPE cells were seeded on noncoated membranes with 0.4 μ m pores (Transwell; Corning Costar), in Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F-12). The permeability of the cells was determined after 28 days in culture by measuring the apical-to-basolateral movement of fluorescein isothiocyanate–dextran (40 kDa; Sigma-Aldrich) using a

microplate reader (ELx800; Bio-Tek) with absorbance set to 485 nm of excitation and 528 nm of emission. The diffusion rate was calculated as (amount of dextran lower chamber)/(amount of dextran upper chamber). A minimum of three wells were used for each time measurement and each experiment was repeated four times.

MTT assay

HbAA- and HbSS-expressing primary RPE cells were seeded in 96-well plates and cultured for 72 h with a fresh culture medium supplied every 24 h. Cells were washed with PBS twice followed by MTT reagent (ATCC). Treatment and lysis of the cells were done as per the manufacturer's instructions. Absorbance of the lysate was measured at 570 nm. The experiments were conducted using passage 2–4 of primary RPE cells.

Fundus and angiography analyses

To evaluate retinal integrity as well as vasculature and permeability *in vivo*, HbAA- and HbSS-expressing mice were anesthetized using a 20 μ L intramuscular injection of rodent anesthesia cocktail (ketamine 100 mg/mL, xylazine 30 mg/mL, acepromazine 10 mg/mL). Pupils were dilated using 1% tropicamide (Bausch and Lomb). The mouse was placed on the imaging platform of the Phoenix Micron III retinal imaging microscope (Phoenix Research Laboratories), and Goniovisc 2.5% (hypromellose; Sigma Pharmaceuticals, LLC) was applied liberally to keep the eye moist during imaging. For angiography analysis, mice were administered 10–20 μ L fluorescein sodium (10% Lite) (Apollo Ophthalmics) while also receiving Goniovisc 2.5%, and rapid acquisition of fluorescent images ensued for $\sim$ 5 min.

Electrophysiological studies

Mice, dark adapted overnight, were anesthetized by intraperitoneal injection of ketamine–xylazine solution (80:12 mg/kg). Proparacaine (0.5%) drops were applied to both eyes and pupils dilated with 1% tropicamide and 2.5% phenylephrine hydrochloride. Animals were placed on a heating pad controlled by a rectal thermometer and DTL electrodes were placed on the corneas and needle electrodes in the cheeks (references) and tail (ground). All experiments used a series of full-field light flashes presented in a Ganzfeld (LKC). Flashes were presented from dim to bright with the interstimulus interval increasing with brightness. After dark-adapted testing, animals were light adapted for 10 min with a background light in the Ganzfeld (30 cd/m²). To record cone-isolated responses, a series of full-field flashes with increasing intensity were presented in the presence of the background light. Stimuli were generated by a custom LED-based system. A 5500° white LED was used for bright stimuli and a 470-nm blue LED for dim stimuli. The light from the blue LED was passed through neutral density filters and defocused before collection by the optical fiber launcher to further diminish the light intensity. LED flashes of 5-ms duration were used.

Nrf2 siRNA in ARPE-19 cells

The transformed human RPE cell line ARPE-19 was cultured in DMEM/F12 supplemented with 10% fetal bovine

serum (FBS), 100 U/mL penicillin, and 100 μ g/mL streptomycin, and maintained at 37°C in a humidified chamber of 5% CO₂. Cells were plated in 60-mm plates and allowed to reach 70% confluency. Nrf2 small interfering RNA (siRNA) and control nontargeted (scramble) siRNA were obtained from Santa Cruz Biotechnology. Preparation of siRNA was achieved by combining 160 pmol siRNA with equal volumes of RNAiMax (Invitrogen) diluted in Opti-MEM media. The latter mixture was incubated at room temperature for 30 min before being added to ARPE-19 cells. Dishes were mixed gently and incubated for 24 h. MMF (100 μ M) diluted in culture media was then added to cells and allowed to incubate for another 24 h. Finally, cells were harvested for Western blot or qPCR analysis as described above.

Data analysis

All experiments were repeated three to five times with independent cell or tissue preparations and samples run in duplicate. Data are presented as mean $\pm$ standard error of the mean. Statistical significance was determined with the Student's *t*-test, and one-way ANOVA with Tukey–Kramer's *post-hoc* tests for comparisons between two groups or multiple groups, respectively. Differences were considered statistically significant at $p < 0.05$.

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Author Disclosure Statement

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Address correspondence to:

Dr. Pamela M. Martin

Department of Biochemistry and Molecular Biology
The Medical College of Georgia
at Augusta University
1120 15th Street
Augusta, GA 30912

E-mail: pmmartin@augusta.edu

Abbreviations Used

Bcl11A = B-cell lymphoma/leukemia 11A
DAPI = 406-diamidino-2-phenylindole
DMEM/F-12 = Dulbecco's modified Eagle's medium: nutrient mixture F-12
ERG = electroretinogram
FA = fluorescein angiography
FDA = United States Food and Drug Administration
FITC = fluorescein isothiocyanate
GCL = ganglion cell layer
Hb = hemoglobin
HbAA = normal (homozygous) human hemoglobin genotype
HbF = fetal hemoglobin
HbSS = homozygous sickle human hemoglobin genotype
HPLC = high performance liquid chromatography
HU = hydroxyurea
ICAM-1 = intercellular adhesion molecule 1
INL = inner nuclear layer
IPL = inner plexiform layer
MCH = mean corpuscular hemoglobin
MCV = mean corpuscular volume
MMF = monomethyl fumarate
NR = neural retina
Nrf2 = nuclear factor (erythroid-derived 2)-like 2
ONL = outer nuclear layer
qPCR = quantitative polymerase chain reaction
RBC = red blood cell
rd1 = *Pde6b*^{rd1}
rd8 = *Crb1*^{rd8}
RPE = retinal pigment epithelium
RT-qPCR = real-time quantitative PCR
SCD = sickle cell disease
SD-OCT = spectral domain optical coherence tomography
SE = standard error
SR = sickle retinopathy
TUNEL = terminal dUTP nick end labeling
VEGF = vascular endothelial growth factor
WBC = white blood cell

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