Effective and Sustained Delivery of Hydrophobic Retinoids to Photoreceptors

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Purpose. Delivery of hydrophobic compounds to the retina/RPE has been challenging. The purpose of this study was to develop an effective method for the sustained delivery of retinoids to rod and cone photoreceptors of young mice lacking a normal supply of 11-*cis* retinal.

Methods. Solubilized basement membrane matrix (Matrigel; BD Biosciences, San Jose, CA) loaded with 9-*cis* retinal was administered subcutaneously into *Rpe65*$^{-/-}$ mouse pups for assessment of delivery to rods and cones and to *Rpe65*$^{-/-}$*Rho*$^{-/-}$ mouse pups for assessment of delivery to cones. Intraperitoneal injections of 9-*cis* retinal were used for comparison. Cone density and opsin localization were evaluated with immunohistochemistry. Cone opsin protein levels were assayed with immunoblots, and cone function was analyzed by electroretinography (ERG) recordings. Retinoid content was determined by high-performance liquid chromatography analysis of retinal extracts. Pigment levels were quantified in homogenized retinas by absorption spectroscopy before and after light exposure.

Results. Single administration of Matrigel loaded with 9-*cis* retinal to *Rpe65*$^{-/-}$ mice increased cone densities in all analyzed regions of the retina compared with mice treated using intraperitoneal delivery. Cone opsin levels increased to near wild-type levels. Similar treatment in *Rpe65*$^{-/-}$*Rho*$^{-/-}$ mice increased b-wave ERG amplitudes significantly, indicating the maintenance of cone function. Matrigel was shown to continuously release 9-*cis* retinal for periods up to 1 week.

Conclusions. As a method for sustained drug delivery, subcutaneous administration using Matrigel proved more efficacious than intraperitoneal injection for in vivo delivery of retinoids to cone photoreceptors. These experiments are the first to show a sustained delivery of retinoids in mice and suggest a strategy for potential clinical therapeutic development. (*Invest Ophthalmol Vis Sci.* 2010;51:5958–5964) DOI:10.1167/iovs.10-5766

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Sustained therapeutic dosing methods for long-term disease maintenance hold numerous advantages over bolus dosing, including a lower risk of toxicity and an increased duration of therapeutic efficacy. The retina poses unique challenges to sustained therapeutic dosing because of its anatomic isolation.[1] Injection of compounds directly into the vitreal chamber is a common method for treating the retina; however, long-term therapeutic maintenance necessitates multiple injections, which are associated with retinal detachment, hemorrhage, uveitis, endophthalmitis, and infections.[2] The dynamic nature of humoral flow and its subsequent change after vitreal liquefaction in the aged eye further complicate this method of therapeutic delivery.[3] Delivery of hydrophobic molecules such as retinoids presents an additional challenge.

Polymers provide an alternative route for administration that has the potential to be less invasive than intravitreal injection. This strategy has already been successfully demonstrated using in vitro periocular delivery models.[1,4] Thermosensitive hydrogels are a unique class of polymers that undergo phase change according to temperature. Studies have shown the potential clinical advantages of using gels for ocular drug delivery for glaucoma.[5]

We investigated the efficacy of Matrigel (BD Biosciences, San Jose, CA), a commercially available thermosensitive hydrogel, as a vehicle for sustained drug delivery to the retina. Matrigel is a solubilized basement membrane matrix originally developed to study endothelial cell differentiation in vitro and angiogenic processes in vivo.[6] The components are extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma and contain laminin, collagen, and heparin sulfate proteoglycan, as well as transforming growth factor-β (TGF-β), epidermal growth factor (EGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and other growth factors that occur naturally within the EHS tumor.[6] In our delivery protocol, Matrigel is loaded with therapeutic compounds in its liquid phase at 4°C and placed subcutaneously, where it solidifies into a plug and maintains the therapeutic compounds for sustained delivery.[6]

11-*cis* Retinal is the chromophore for rod and cone visual pigments and is a highly hydrophobic compound. The protein RPE65 is essential for the production of 11-*cis* retinal within the RPE cell.[7] Mutations in the *Rpe65* gene causes type 2 Leber congenital amaurosis (LCA2), an autosomal recessive form of retinal dystrophy that can cause severe blindness and visual impairment in humans within the first year of life because of decreased endogenous production of 11-*cis* retinal.[8] The *Rpe65*$^{-/-}$ mouse provides an excellent model for studying this disease and a model for testing the uptake of 11-*cis* retinal.[9,10]

The development of therapeutics such as gene therapy and pharmacologic agents aimed at treating LCA2 patients with RPE65 gene mutations has been greatly aided by the availability of animal models.[11,12] Mouse cones contain two visual pigments that, when bound to 11-*cis* retinal, are sensitive to either short-wavelength (S) or mid/long-wavelength (M/L) light. In mouse models for LCA2, both forms of cone pigments are

mistrafficked throughout the cone, and degeneration follows shortly afterward.[13,14] Previous studies have shown that initiating an early regimen of delivering exogenous 11-*cis* retinal or its naturally occurring functional analog 9-*cis* retinal can partially inhibit cone degenerative processes in mouse models for LCA2.[15,16] To maximize the efficacy of retinoid therapy, developing a sustained dosing protocol that is well tolerated at an early age is essential. We report here that Matrigel loaded with 9-*cis* retinal, applied subcutaneously (SC) to *Rpe65*$^{-/-}$ and *Rpe65*$^{-/-}$*Rho*$^{-/-}$ mice is efficacious for delivering retinoids to photoreceptors with a single administration.

Materials and Methods

Animals

Rpe65$^{-/-}$ mice were a generous gift of T. Michael Redmond (National Eye Institute, National Institutes of Health, Bethesda, MD), and *Rpe65*$^{-/-}$*Rho*$^{-/-}$ mice were a generous gift of Mathias Seeliger (University of Tübingen, Tübingen, Germany). C57BL/6J (*Wt*) mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were reared under cyclic light (12 hours light/12 hours dark with the ambient light intensity at eye level of 85 ± 18 lux) conditions until the initiation of experiments, when they were transferred to a constant dark environment. Mice were killed by CO_2 exposure and cervical dislocation. Husbandry was performed under dim red light (GBX-2; Eastman Kodak, Rochester, NY). All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Medical University of South Carolina Animal Care and Use Committee.

Retinoid Delivery

All retinoids were handled under dim red light. 9-*cis* Retinal (Sigma-Aldrich, St. Louis, MO; 0.25 mg/animal) was dissolved in 20 μL of 100% ethanol and combined with 180 μL of either Matrigel or Matrigel with reduced growth factors at 4°C for a final volume of 200 μL. The mixture was injected SC into the dorsal torso region. 9-*cis* Retinal was omitted from the mixture for all control groups. For intraperitoneal (IP) delivery, 9-*cis* retinal (0.05–0.25 mg/animal) was dissolved in 200 μL vehicle (10% ethanol/10% bovine serum albumin in 0.9% NaCl) and injected into the peritoneal cavity.

Cone Density Measurements

After the cornea was removed, the retina-lens complex was separated from the RPE-choroid layer and fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) for 2 hours at room temperature (RT). After washing with PBS (3×, 20 minutes, 4°C), retinas were incubated with 0.02 mg/mL FITC-conjugated peanut agglutinin (PNA; lectin *Arachi hypogaea*; Sigma-Aldrich) overnight at 4°C. Retinas were washed with PBS (3×, 20 minutes, 4°C), flattened by relaxing cuts, mounted flat on a slide, and coverslipped after application of mounting medium (Fluoromount-G; Southern Biotech, Birmingham, AL). Samples were analyzed by fluorescence microscopy (Axioplan II; Zeiss, Jena, Germany) using a 100-W mercury light source and FITC filters. Cones were counted within six fields of view (Fig. 1A) at 20× magnification that spanned the entire length of the retina along the dorsal ventral axis. Cone densities are expressed as cones/mm^2.

Histologic Evaluation

To fixate the tissue, a flap was cut into the cornea, and the eye was immediately immersed in 4% PFA in 0.1 M PBS for 2 hours at RT. For morphologic analysis, eyes were dehydrated in ethanol (30%, 50%, and 70%, 15 minutes each) and left overnight in 70% ethanol at 4°C. Eyes were further washed in 90% and 100% ethanol (30 minutes each), and incubated in xylene (2 hours, RT). The eyes were then incubated in paraffin for 2 hours at 60°C under vacuum, embedded, and sectioned at 6-μm thickness. For dewaxing and rehydration, sections were dried under vacuum (20 minutes, 60°C) and washed in xylene followed by ethanol (100%, 90%, 80%, 70%, 50%) and finally deionized water (5 minutes each, RT). Sections were processed for staining with hematoxylin and eosin (H&E) and then dehydrated for coverslip mounting with medium (Permount; Sigma-Aldrich, St. Louis, MO). Slides were analyzed using a light microscope (Axioplan II; Zeiss, Jena, Germany).

For immunofluorescence analysis, eyes were cryoprotected, embedded, frozen, and sectioned at 10-μm thickness. Sections were incubated as previously described[16] with affinity-purified anti–M/L-opsin antibody (1:500 dilution;, Chemicon/Millipore, Temecula, CA) in a humidified chamber overnight at 4°C. After washing with PBS (1×, 10 minutes, RT), sections were incubated with solution containing Alexa594-conjugated secondary antibody (Molecular Probes, Eugene, OR) and 4′,6-diamidino-2-phenylindole (1:3000 dilution; Invitrogen, Carlsbad, CA) for 2 hours in RT, washed with PBS (2×, 10 minutes, RT), and coverslip-mounted with medium (Fluoromount-G; Southern Biotech) for analysis using a light microscope with appropriate filters.

Western Blot Analysis

Procedures were modified from methods previously described.[17] Two retinas were homogenized in 1% sodium dodecyl sulfate (Sigma-Aldrich) buffer using sonication (30 seconds). Total proteins (200 μg) from each lysate were loaded onto a 4% to 12% gel (NuPAGE Bis-Tris; Invitrogen) and run at 60 V for 30 minutes, followed by 100 V for 1.5 hours. The proteins were transferred to a nitrocellulose membrane and probed with rabbit anti–M/L-opsin primary antibody (1:2000 dilution; Chemicon/Millipore). The membrane was stripped and reprobed with mouse β-actin antibody (1:1000 dilution; Sigma-Aldrich). Secondary antibodies were goat anti-rabbit IgG (1:10,000 dilution; Vector, Burlingame, CA) and horse anti-mouse IgG (1:10,000 dilution; Vector). Bands were visualized with imaging equipment (VersaDoc; Bio-Rad, Hercules, CA). Densitometry analysis of the bands was performed with imaging and analysis software (Quantity One; Bio-Rad), and the data were expressed as a percentage of the *Wt* band.

ERG Recordings

Dark-adapted (12 hours) *Rpe65*$^{-/-}$*Rho*$^{-/-}$ mice (postnatal day [P] 30) were placed on a DC-powered 37°C warming pad and anesthetized using xylazine (20 mg/kg body weight) and ketamine (80 mg/kg body weight). Pupils were dilated with 1 drop of phenylephrine HCl (2.5%) and atropine sulfate (1%). A reference/ground needle electrode was placed SC at the forehead. ERG responses were measured on a universal testing and electrophysiological system (System 2000; LKC UTAS Visual Diagnostic System with BigShot Ganzfeld) using a contact lens electrode brought into electrical contact with the cornea with 1 drop of methylcellulose (Gonisol; Iolab Pharmaceutical, Claremont, CA).[18] Recordings were averaged in response to five 10-ms flashes using a white spectrum (450–750 λ) from 1.56 to 24.8 cd · s/mm^2 intensities with a 5-second-interval between each flash. B-wave amplitudes were measured from the trough to the highest peak and expressed in μV.

Retinoid Analysis

All procedures were performed under dim red light on dark-adapted animals using methods modified from those previously described.[19] Retinas (4 retinas/sample) were dissected and homogenized in 200 μL PBS buffer using a microtissue grinder (Fisher Scientific, Pittsburgh, PA). Methanol (300 μL) and hydroxylamine (60 μL, 1 M in PBS buffer) were added, and samples were vortexed (30 seconds). After 5 minutes at 22°C, methylene chloride (300 μL) was added, and the samples were vortexed (30 seconds) and centrifuged (14,000*g*, 1 minute). The lower phase was separated, dried under argon, and dissolved in high-performance liquid chromatography (HPLC) mobile phase (11.2% ethyl acetate/2.0% dioxane/1.4% octanol in hexane, 90 mL). The *syn-* and *anti*-9-*cis* retinal oximes were separated using a 5-mm column (Lichrosphere SI-60; Alltech, Lexington, KY) and quantified by comparison of

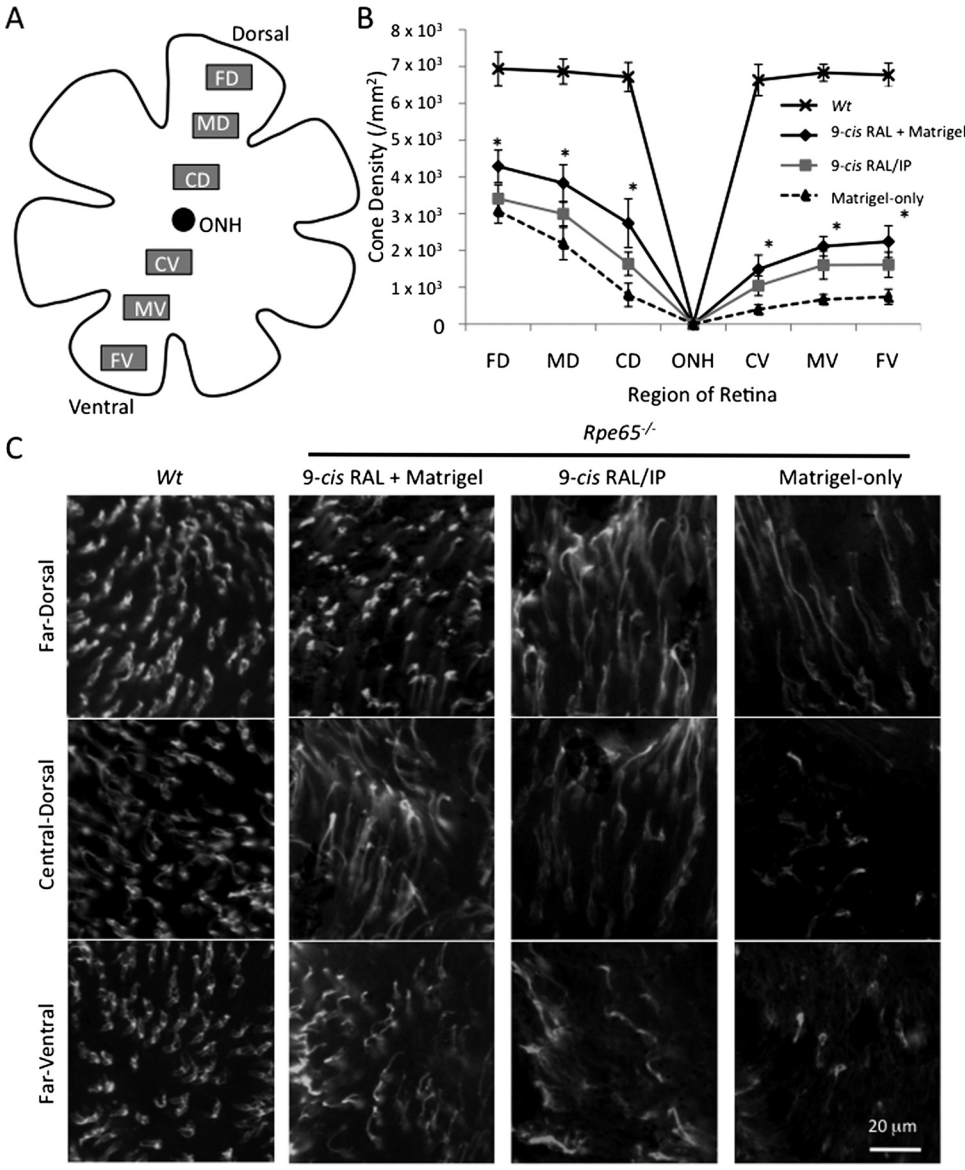

FIGURE 1. Total cone density increases in *Rpe65*$^{-/-}$ animals with delivery of 9-*cis* retinal by Matrigel. Data are obtained from P30 dark-reared mice that began treatment at P10. (**A**) Schematic representation of retinal flat mount, with six regions analyzed for cone density spanning the dorsal ventral axis. FD, far dorsal; MD, mid-dorsal; CD, central dorsal; ONH, optic nerve head; CV, central ventral; MV, mid ventral; FV, far ventral. (**B**) Quantification of PNA-positive cells by region from *Wt* ($n = 7$), *Rpe65*$^{-/-}$ mice treated with 9-*cis* retinal (0.25 mg/animal) using Matrigel ($n = 7$) or IP injection (0.05 mg/animal $\times 5$) ($n = 6$) and control *Rpe65*$^{-/-}$ mice ($n = 6$). Matrigel administration of 9-*cis* retinal significantly increased cone density at all analyzed regions of the retina compared with IP injection; $^{*}P < 0.01$. Two-way ANOVA was used to test for differences in cone counts by treatment group and retinal region with post hoc pairwise *t*-tests used to compare individual groups. Differences in maximum trough to peak, between left and right eyes, were averaged for each treatment group and analyzed using ANOVA with Tukey's post hoc tests to compare individual groups. A two-sided *P*-value of 0.05 was considered significant. Bars indicate ±SD. (**C**) Images of flat-mounted retina labeled with FITC-conjugated peanut agglutinin lectin.

retention times and absorption properties with pure retinoid isomeric standards.[20]

Pigment Measurements

Retinas were dissected under dim red light from dark-adapted animals and homogenized (2 retinas/sample) with a microtissue grinder in 1 mL of 10 mM Tris buffer (pH 7.5) containing 1 mM EDTA, 1 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride hydrochloride (AEBSF; Roche, Mannheim, Germany), and 10 μg DNase I (Sigma-Aldrich). Samples were shaken at 4°C for 2 hours and were centrifuged (88,000*g*, 15 minutes). The pellet was resuspended in 100 μL of 1% dodecylmaltoside in 0.1 M PBS, rotated for 2 hours at 4°C, and centrifuged (88,000*g*, 10 minutes). The supernatant was retrieved for analysis. Measurements were performed on a spectrophotometer (Cary 300; Varian, Walnut Creek, CA). Difference spectra were determined from measurements before and after bleaching with white light in the freshly prepared 20 mM hydroxylamine, pH 7.0. The concentration of isorhodopsin was calculated based on absorption at $\lambda_{max} = 487$ nm using the extinction coefficient ε (isorhodopsin) = 43,000 M/cm.[21]

Statistical Analysis

Two-way ANOVA was used to test for differences in cone density evaluation by treatment group and retinal region with post hoc pairwise *t*-tests used to compare individual groups. Differences in maximum trough-to-peak b-wave amplitudes of ERG recordings between left and right eyes were averaged for each treatment group and analyzed using ANOVA with Tukey's post hoc tests to compare individual groups. Immunoblotting results were evaluated using two-sample *t*-tests. Retinoid concentrations and absorbance spectra were compared with their respective reference samples using two-sample *t*-tests. A two-sided *P*-value of 0.05 was considered significant. Statistical software (SAS, version 9.1; SAS Institute, Inc., Cary, NC) was used to conduct all analyses.

RESULTS

Increases in Cone Density after Early 9-*cis* Retinal Administration in Matrigel

Previous studies have shown that early administration of 9-*cis* retinal to young (P10) *Rpe65*$^{-/-}$ and *Lrat*$^{-/-}$ mice are effective for preserving rods and cones.[14,22,23] This study was undertaken to evaluate the efficiency of a single administration of Matrigel in delivering 9-*cis* retinal. Cone density was quantified by counts of PNA positively stained cells in six areas of the retina (Fig. 1A). Mice were dark-reared after the initiation of

treatment at P10 and analyzed at age P30. *Rpe65*$^{-/-}$ mice were given a single SC injection of Matrigel with and without 9-*cis* retinal. To determine the optimum dosage of 9-*cis* retinal for cone preservation in *Rpe65*$^{-/-}$ mice with the Matrigel delivery, a range of dosages (0.1–1.0 mg/mouse) were tested, and gains in cone density were observed to stabilize at 0.25 mg/mouse (data not shown). 9-*cis* Retinal (0.25 mg) with Matrigel in *Rpe65*$^{-/-}$ mice produced significant ($P < 0.01$) increases in cone densities at all six analyzed regions of the retina when compared with mice treated with Matrigel alone, although WT levels were not achieved (Fig. 1B). A multiple IP administration protocol was used for comparison, by which a dose-matched amount of 9-*cis* retinal was administered by five injections of 0.05 mg at ages P10, P14, P18, P22, and P26. The data show that the Matrigel delivery system produced significantly ($P < 0.01$) higher cone densities across all six analyzed regions of the retina compared with the IP delivery system (Fig. 1B).

To rule out the possibility that Matrigel exerts an independent effect on cone densities in the presence of 9-*cis* retinal, *Rpe65*$^{-/-}$ mice were treated by the IP protocol described, and volume-matched Matrigel was also injected SC into the dorsal torso region of the mouse at P10. There was no significant difference in the cone densities between the animals administered 9-*cis* retinal through IP alone and those coadministered 9-*cis* retinal through IP and SC Matrigel (data not shown). These data rule out the possibility of a synergistic effect between the two agents.

Prevention of Cone M/L-Opsin Loss in *Rpe65*$^{-/-}$ Mice

The immunoreactivity of cone M/L-opsin was evaluated in central dorsal retinal sections of P30 mice. In *Wt* mice, M/L-opsin was normally abundant within the cone outer segments (Fig. 2A, top). In P30 *Rpe65*$^{-/-}$ mice, there is advanced cone outer segment degeneration, highlighted by the decreased presence of M/L-opsin immunoreactivity (Fig. 2A, middle). However, 9-*cis* retinal administration with Matrigel at P10 resulted in increased M/L-opsin immunoreactivity in the outer segments of *Rpe65*$^{-/-}$ retina at P30, with opsin presence also in the pedicles (Fig. 2A, bottom).

Cone M/L-opsin quantities in retina homogenates of P30 *Rpe65*$^{-/-}$ mice were analyzed by Western blot analysis (Figs. 2B, 2C). Multiple IP administration of 9-*cis* retinal to mice did not significantly increase overall M/L-opsin levels within the retina compared with mice receiving Matrigel-only injections, with both groups showing <60% of *Wt* levels (IP, 59% ± 14%; Matrigel-only, 52% ± 12%). Furthermore, no significant differences in M/L-opsin levels were detected between mice receiving Matrigel-only injections and those receiving saline injections after the dosing schedule of the 9-*cis* retinal/IP group (data not shown). Delivery of 9-*cis* retinal with Matrigel resulted in ($P < 0.05$) M/L-opsin levels near *Wt* levels (Matrigel, 91% ± 15%).

Maintenance of Cone Function in *Rpe65*$^{-/-}$*Rho*$^{-/-}$ Mice

Rpe65$^{-/-}$*Rho*$^{-/-}$ mice were used for ERG analyses to eliminate interference from rods.[24] Representative ERG traces are provided in Figure 3A, and the data are summarized in Figure 3B. *Rpe65*$^{-/-}$*Rho*$^{-/-}$ mice were treated at P10 as described with 9-*cis* retinal (0.25 mg/animal) using either a single Matrigel injection or five IP injections of 0.05 mg each and were maintained in darkness. Controls were animals injected with Matrigel alone. To gain maximum pigment formation and, therefore, maximum function, a single additional dose of 9-*cis* retinal (0.1 mg/mouse) was administered through the IP route to mice of all treatment groups, including the control group, 24 hours before cone function was evaluated by ERG. Delivery of 9-*cis* retinal using Matrigel produced significant ($P < 0.05$) increases in b-wave amplitudes beginning at a stimulus intensity of 1.56 cd · s/mm^2, whereas IP delivery of 9-*cis* retinal did not produce significant increases when compared to animals treated with either a Matrigel-alone injection or with multiple saline injections after the same dosing schedule (data not

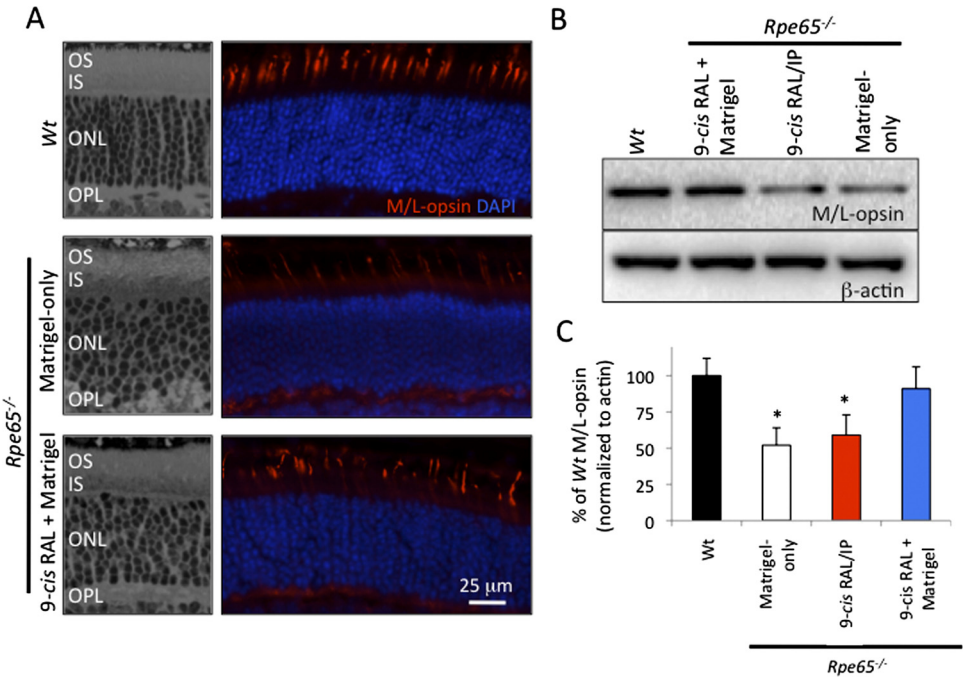

FIGURE 2. M/L-opsin protein increases with Matrigel delivery of 9-*cis* retinal in *Rpe65*$^{-/-}$ mice. Data obtained from *Rpe65*$^{-/-}$ P30 animals. (**A**) Cross-section images of the central dorsal retina are shown for *Wt* (*top*) and *Rpe65*$^{-/-}$ mice treated with 9-*cis* retinal using Matrigel (*bottom*). For control mice, 9-*cis* retinal was omitted from the Matrigel preparation (Matrigel-only; *middle*). Paraffin-embedded retinas were sectioned and stained with hematoxylin-eosin (*left column*); cryoprotected retinas were sectioned and stained with both M/L-opsin-specific primary antibody (*red*) and DAPI (*blue*) for nuclei (*right column*). OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer. (**B**) Western blot images for M/L-opsin (*top*) and β-actin (*bottom*) are shown for *Wt* and *Rpe65*$^{-/-}$ Matrigel, IP, and control mice. (**C**) Densitometry band analyses are displayed as percentages of *Wt*. Matrigel alone and 9-*cis* retinal (RAL) intraperitoneally injected *Rpe65*$^{-/-}$ mice displayed significantly decreased M/L-opsin protein levels compared with *Wt*; *$P < 0.02$. *Black bar*: *Wt*, $n = 3$. *White bar*: Matrigel alone, $n = 3$. *Red bar*: 9-*cis* retinal IP, $n = 3$. *Blue bar*: Matrigel plus 9-*cis* retinal, $n = 3$. Data are presented as mean ± SD and were analyzed by two-sample *t*-test, accepting a significance value of $P < 0.05$.

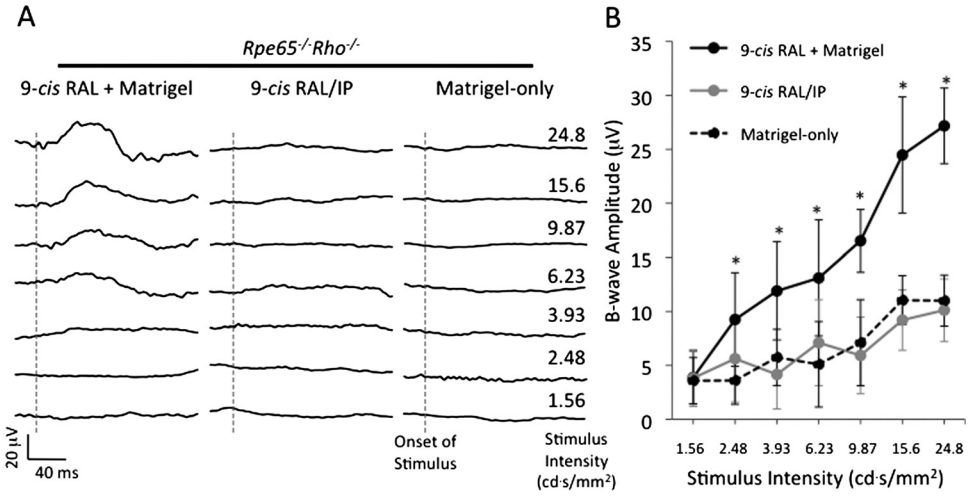

FIGURE 3. Maintenance of cone function in $Rpe65^{-/-}Rho^{-/-}$ mice with Matrigel delivery of 9-*cis* retinal. $Rpe65^{-/-}Rho^{-/-}$ mice were treated with 9-*cis* retinal using Matrigel (0.25 mg/mouse at P10) or by IP injection (0. 5 mg/animal, beginning at P10, ×5) and were analyzed at P30 with single white flash (intensity range, 1.56–24.8 cd · s/mm^2) ERG recordings under dark-adapted conditions. For control mice, 9-*cis* retinal was omitted from the Matrigel preparation (Matrigel-only). (**A**) Representative ERG traces from Matrigel/9-*cis* retinal (*left*), 9-*cis* retinal IP (*middle*), and control (*right*) animals. *Dashed line*: onset of flash. (**B**) Quantification of b-wave amplitudes from Matrigel/9-*cis* retinal (*black*, $n = 5$), 9-*cis* retinal IP (*gray*, $n = 5$) and control (*dashed*, $n = 5$) animals. Mice administered 9-*cis* retinal using Matrigel showed significantly increased cone responses from 2.48 to 24.8 cd · s/mm^2 flash intensities compared with those that did not receive 9-*cis* retinal therapy; $^*P < 0.05$. Data are presented as mean ± SD and were analyzed by two-sample *t*-test, accepting a significance value of $P < 0.05$.

shown). We did not detect a significant increase in b-wave amplitudes between animals treated with IP delivery of 9-*cis* retinal compared with those treated with Matrigel-only injections; however, this might have been because our equipment lacked the necessary sensitivity. Earlier studies have shown that treatment by multiple IP injections of 9-*cis* retinal produced some recovery in cone function; however, these animals were treated with greater doses than those of the present study and were evaluated at a younger age.[16]

Sustained Delivery of 9-*cis* Retinal by Matrigel

To evaluate the capability of Matrigel to maintain sustained release of 9-*cis* retinal to the retina, P45 $Rpe65^{-/-}$ mice treated with a single dose of 0.25 mg 9-*cis* retinal either through Matrigel or IP delivery were compared. Because most of the cones had degenerated by P45 in this model,[13,14] it is likely that the rod opsin was responsible for most of the 9-*cis* retinal uptake to form isorhodopsin, allowing for the quantification of retinoids delivered to the retina. The animals were reared in cyclic light for 2 days immediately after injection and then were transferred to dark-rearing conditions. After either 3 or 7 days of dark rearing, the harvested retinas were homogenized, and the retinoids were extracted for HPLC analysis (Fig. 4A). For controls, Matrigel was used without 9-*cis* retinal.

Untreated mice showed only trace levels of 9-*cis* retinal after 3 and 7 days of dark rearing (3 days, 2.86 ± 0.13 pmol/retina; 7 days, 2.23 ± 0.13 pmol/retina, $n = 6$). Administration of 9-*cis* retinal by IP injection resulted in a modest, but significant, increase of 9-*cis* retinal in mice after dark rearing for 3 or 7 days compared with untreated mice (3 days, 15.94 ± 3.02 pmol/retina; 7 days, 10.54 ± 1.78 pmol/retina; $n = 6$; $P < 0.05$). However, administration of 9-*cis* retinal in Matrigel showed a significant increase in 9-*cis* retinal levels at both time points compared with IP injection (3 days, 49.12 ± 5.74 pmol/retina; 7 days, 73.11 ± 14.36 pmol/retina; $n = 6$, $P < 0.05$) and with untreated mice ($P < 0.01$, Fig. 4B). Further, there was a significant increase in 9-*cis* retinal after 7 days of dark rearing compared with 3 days in the Matrigel-treated animals, showing that the retinoid was still being released. In the IP-injected animals, the levels were decreased at 7 days. This increase in 9-*cis* retinal levels observed in the Matrigel-treated mice was confirmed by quantification of isorhodopsin pigment levels through absorption difference spectra, where isorhodopsin levels doubled (3 days, 13.83 ± 2.20 pmol/retina; 7 days, 27.44 ± 3.14 pmol/retina; $n = 6$; $P < 0.01$; Fig. 4C).

Role of Growth Factors within Matrigel for Sustained Delivery

Given that Matrigel is derived from the EHS mouse sarcoma, numerous growth factors—such as TGF-β, EGF, IGF, VEGF, and FGF—are present within the compound.[6] To investigate whether these growth factors were involved in the sustained delivery of compounds by Matrigel, P45 $Rpe65^{-/-}$ mice were treated with a single administration of 9-*cis* retinal (0.25 mg/animal) using growth factor-reduced (GFR) Matrigel, reared in cyclic light for 2 days immediately after injection, and then transferred to dark-rearing conditions. After either 3 or 7 days of dark rearing, the retinas were homogenized and the retinoids were analyzed by HPLC for comparison with animals treated using normal Matrigel. Results show significantly decreased 9-*cis* retinal levels per retina at both 3 and 7 days of dark rearing (3 days, 26.80 ± 5.30 pmol/retina; 7 days, 22.84 ± 4.63 pmol/retina) using GFR Matrigel when compared with mice administered 9-*cis* retinal using normal Matrigel ($P < 0.01$), and there was no significant difference in 9-*cis* retinal levels between 3 and 7 days of dark rearing ($P = 0.39$) in animals treated with GFR Matrigel.

Growth factors within Matrigel have previously been shown to trigger the infiltration of blood vessels into the solidified compound when injected into the mouse as a plug.[25] To investigate whether the infiltration of blood vessels contributed to the sustained release of 9-*cis* retinal by Matrigel in our delivery model, plugs from mice treated with normal or GFR Matrigel were retrieved and processed for immunohistochemical analysis. Vascular infiltration of the normal Matrigel plug was observed to be more abundant than was the GFR Matrigel plug (Supplementary Figs. S1A and S1B, http://www.iovs.org/cgi/content/full/51/11/5958/DC1). The observed structures within the normal Matrigel plug were confirmed to be blood vessels through the histologic identification of endothelial cells (Supplementary Fig. S1C), the presence of erythrocytes in the vessel lumen (Supplementary Fig. S1E), and positive staining of platelet endothelial cell adhesion molecule-1 (PECAM-1, Supplementary Fig. S1D). The presence of 9-*cis* retinal within the normal Matrigel plug did not produce an observable effect on the amount of vascular infiltration (data not shown).

DISCUSSION

Our studies have shown that using a collagen gel such as Matrigel increases the efficiency and duration of delivery of

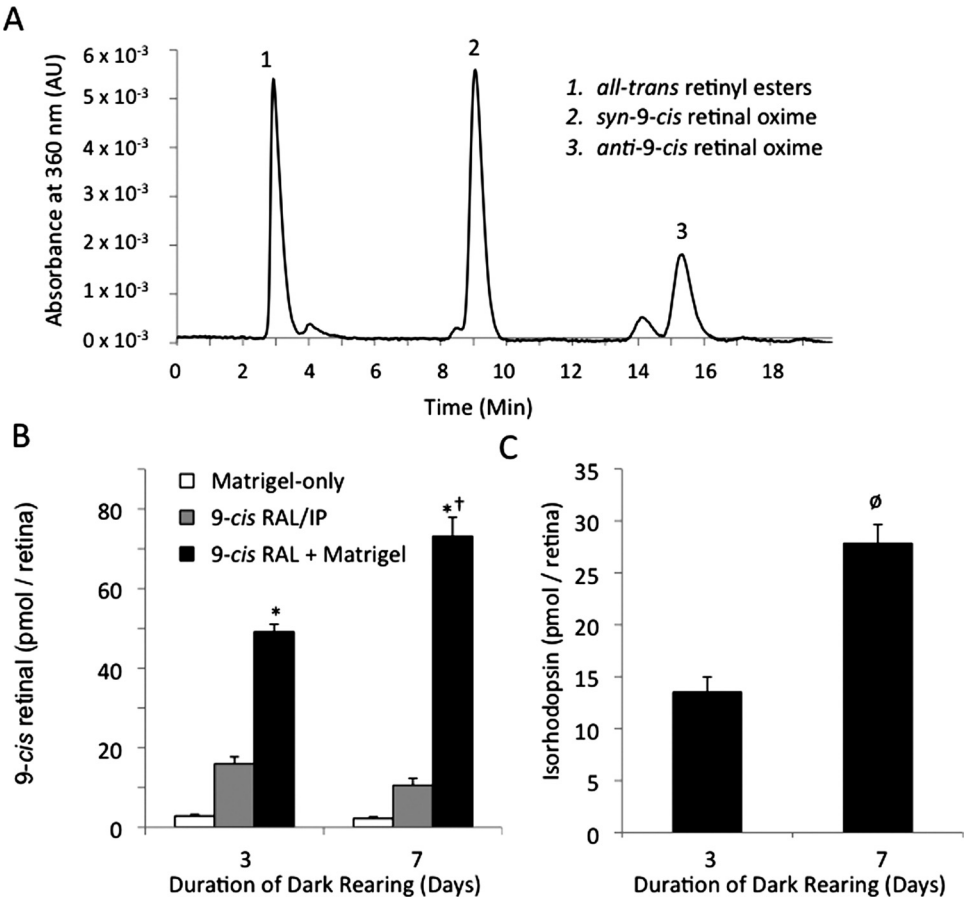

FIGURE 4. Matrigel maintains a sustained delivery of 9-*cis* retinal to photoreceptors. P45 $Rpe65^{-/-}$ animals were administered 9-*cis* retinal (0.25 mg/animal) with a single subcutaneous Matrigel or IP injection. For control animals, 9-*cis* retinal was omitted from the Matrigel preparation (Matrigel-only). Animals were reared under cyclic light until 2 days after treatment and then were dark reared for either 3 or 7 days, and the retinas were analyzed for extracted retinoids or isorhodopsin levels (4 eyes/sample). (**A**) Representative HPLC profile of retinoid extract from $Rpe65^{-/-}$ mice dark reared for 3 days and administered 9-*cis* retinal using Matrigel. Peaks were identified by comparison with known standards. Detection was at 360 nm. (**B**) *syn*-9-*cis* Retinal oxime levels after 3 and 7 days of dark rearing in $Rpe65^{-/-}$ mice administered 9-*cis* retinal with a single injection of Matrigel (*black*, $n = 6$), IP administration (*gray*, $n = 6$), or control (*white*, $n = 6$). Administration of 9-*cis* retinal with Matrigel significantly increased *syn*-9-*cis* retinal oxime levels compared with IP administration; $^{*}P < 0.002$. Mice administered 9-*cis* retinal with Matrigel showed significantly increased *syn*-9-*cis* retinal oxime levels between 3 and 7 days of dark rearing; $\dagger P < 0.05$. (**C**) Isorhodopsin levels from whole retinas of mice administered 9-*cis* retinal using Matrigel injection after 3 ($n = 3$) and 7 ($n = 3$) days of dark rearing. Analysis showed a significant increase in isorhodopsin levels between both time points, indicating sustained delivery of 9-*cis* retinal; $\text{ø}P < 0.01$. Data are presented as mean ± SD and analyzed by two-sample *t*-test, accepting a significance value of $P < 0.05$.

retinoids to photoreceptors. We have used the $Rpe65^{-/-}$ mouse as a model, both to track delivery of a hydrophobic compound and as a model for a clinical disorder. The drawback of this model is that early delivery is critical for cone photoreceptors because cones degenerate rapidly in the absence of the opsin chromophore.

The retinospatial patterning of cone loss within the $Rpe65^{-/-}$ retina follows the dorsal ventral axis over time. Previous studies have shown no detectable differences in cone densities within the retinas of 2-week-old $Rpe65^{-/-}$ and *Wt* mice.[13] By P30, $Rpe65^{-/-}$ mice exhibit significant loss of cone densities across all regions of the retina, highlighting the importance of innate 11-*cis* retinal production for early cone survival. There was no significant variation in the cone densities across the *Wt* retina, whereas $Rpe65^{-/-}$ retina exhibited significantly reduced cone densities in central and ventral regions. This pattern of cone loss may be related to the spatial distribution of S- and M/L-opsin expression within cones across the retina. Although mouse cones are capable of expressing both forms of cone opsins, the ratio of M/L- to S-opsin is much higher in cones of the dorsal retina.[26] Our results suggest that a high concentration of M/L-opsin may be favorable for cone survival in the absence, or with limited amounts (<0.1%), of chromophore.[27] This is supported by our finding that cones are present in the far and mid-dorsal retinal regions (rich in M/L opsin cones) of untreated $Rpe65^{-/-}$ mice that were dark reared for 5 months; however, complete absence of cones was observed in the central and ventral regions (data not shown). Clinical data from LCA2 patients with the RPE65 gene mutation also lend support, whereas loss of cones within the fovea containing high levels of S-opsin occurred earlier than those containing high levels of M/L-opsin.[8] However, variation in the supply of chromophore to the different areas of the retina remains a possibility.

Opsin mistrafficking may play a key role in the pathophysiology of cone loss in diseases involving a disrupted visual cycle. Previous studies in both $Rpe65^{-/-}$ and $Lrat^{-/-}$ mice indicate that trafficking of phototransduction proteins, including S- and M/L-opsins, to the cone outer segment is disrupted, with opsins distributed throughout the entire length of the cell at ages younger than P30.[14] This pathologic characteristic may underlie the subsequent onset of rapid cone loss, highlighted by the lack of M/L-opsin immunoreactivity we observed in central dorsal sections of the retina from P30 untreated $Rpe65^{-/-}$ mice. Cone opsins are speculated to contribute to cone outer segment stability in a manner analogous to rhodopsin, promoting the development and stability of the rod outer segment.[28] Inappropriate trafficking of opsins throughout the cone results in a decreased quantity of opsin reaching the cone outer segment region, causing destabilization and, presumably, triggering degenerative processes. IP administration to mice of either 9- or 11-*cis* retinal immediately after birth has been shown to partially correct for opsin mistrafficking, resulting in the arrest of cellular degeneration.[13,14,16]

Our data suggested that growth factors present within Matrigel have a role in its ability for the release of hydrophobic compounds. Matrigel is derived from the EHS mouse sarcoma, and it contains numerous growth factors (e.g., TGF-β, EGF, VEGF, and FGF) that have previously been shown to trigger the infiltration of blood vessels into Matrigel when it is injected

into the mouse as a plug.[25] Vascular infiltration of the Matrigel plug may contribute to the sustained release of loaded compounds into systemic circulation, as newly formed vessels are characterized by increased permeability and a fenestrated phenotype leading to vascular leakiness, a characteristic implicated in the pathophysiology of neovascularizing diseases such as diabetic retinopathy.[29] When leaky vessels initially infiltrate the periphery of the plug, 9-*cis* retinal entrapped within the region may be deposited into the vessel lumen through fenestrations, carried into systemic circulation, and ultimately delivered to the retina through the ophthalmic artery. As the growing vasculature continues to infiltrate adjacent regions of the plug, this may possibly result in the release of further amounts of 9-*cis* retinal into systemic circulation. The vascularized Matrigel plug is completely absorbed by the animal by 4 weeks after injection, with no health detriments observed during this time.

This demonstration that Matrigel delivers hydrophobic compounds to the retina in a sustained fashion provides an important tool for animal studies that require long-term drug maintenance and suggests approaches to drug delivery for various human ocular diseases.

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