Tyrosine-Mutant AAV8 Delivery of Human *MERTK* Provides Long-Term Retinal Preservation in RCS Rats

Wen-Tao Deng,¹ Astra Dinculescu,¹ Qiubong Li,¹ Sanford L. Boye,¹ Jie Li,¹ Marina S. Gorbatyuk,² Jijing Pang,¹ Vince A. Chiodo,¹ Michael T. Matthes,⁹ Douglas Yasumura,⁹ Li Liu,³ Fowzan S. Alkuraya,^{4,5,6} Kang Zhang,⁷ Douglas Vollrath,⁸ Matthew M. LaVail,⁹ and William W. Hauswirth¹

PURPOSE. The absence of *Mertk* in RCS rats results in defective RPE phagocytosis, accumulation of outer segment (OS) debris in the subretinal space, and subsequent death of photoreceptors. Previous research utilizing *Mertk* gene replacement therapy in RCS rats provided proof of concept for treatment of this form of recessive retinitis pigmentosa (RP); however, the beneficial effects on retinal function were transient. In the present study, we evaluated whether delivery of a *MERTK* transgene using a tyrosine-mutant AAV8 capsid could lead to more robust and longer-term therapeutic outcomes than previously reported.

METHODS. An AAV8 Y733F vector expressing a human *MERTK* cDNA driven by a RPE-selective promoter was administered subretinally at postnatal day 2. Functional and morphological analyses were performed at 4 months and 8 months post-treatment. Retinal vasculature and Müller cell activation were analyzed by quantifying acellular capillaries and glial fibrillary acidic protein immunostaining, respectively.

RESULTS. Electrorretinographic responses from treated eyes were more than one-third of wild-type levels and OS were well

preserved in the injection area even at 8 months. Rescue of RPE phagocytosis, prevention of retinal vasculature degeneration, and inhibition of Müller cell activation were demonstrated in the treated eyes for at least 8 months.

CONCLUSIONS. This research describes a longer and much more robust functional and morphological rescue than previous studies. We also demonstrate for the first time that an AAV8 mutant capsid serotype vector has a substantial therapeutic potential for RPE-specific gene delivery. These results suggest that tyrosine-mutant AAV8 vectors hold promise for the treatment of individuals with *MERTK*-associated RP. (*Invest Ophthalmol Vis Sci.* 2012;53:1895-1904) DOI:10.1167/iovs.11-8831

Retinitis pigmentosa (RP) is a group of genetically heterogeneous, inherited ocular disorders typified by progressive photoreceptor cell death and eventual blindness. Mutations in more than 50 genes have been associated with RP (<http://www.sph.uth.tmc.edu/retnet/>). Many of these genes are either photoreceptor or RPE specific, and they encode proteins with diverse functions, including those involved in the phototransduction cascade, cellular adhesion, intracellular trafficking, structural proteins, transcription factors, and more.^{1,2}

A mutation in the *Mertk* gene was initially found in the Royal College of Surgeons (RCS) rat,³ a classical model of an autosomal recessive form of retinal degeneration in which the ability of RPE cells to phagocytize shed photoreceptor outer segment (OS) tips is almost completely abolished.^{4,5} *Mertk* is expressed strongly in two phagocytic cell types, RPE and macrophages, and encodes a transmembrane receptor that belongs to the TAM (Tyro3, Axl, and *Mertk*) family of receptor tyrosine kinases.⁶ In RCS rats, a deletion that includes part of *Mertk* exon 2 leads to loss of the protein, defective phagocytosis of OS tips by the RPE, and an accumulation of OS debris in the interphotoreceptor space. As a result, the close, dynamic interaction between photoreceptors and RPE cells is perturbed and retinal degeneration occurs. Photoreceptor cell loss starts at postnatal day (P) 18 and is virtually complete after 2 to 3 months, with electroretinographic (ERG) responses barely detectable by this age. Thinning and atrophy of the RPE begin after the onset of degeneration, and subretinal accumulation of OS debris becomes apparent at P12 to P13.^{4,7-9} The retinal vasculature develops normally but begins to show signs of degeneration as early as 3 weeks of age. By 3 to 4 months of age, retinal vessels proliferate abnormally toward the RPE.¹⁰⁻¹²

Most reported patients with *MERTK* RP have an early onset retinal dystrophy. Affected individuals frequently exhibit night blindness from childhood, with subsequent reduction in central vision.¹³⁻¹⁹ The rod ERG response is often affected at

From the Departments of ¹Ophthalmology and ³Pharmacology and Therapeutics, College of Medicine, University of Florida, Gainesville, Florida; ²Department of Cell Biology and Anatomy, University of North Texas, Fort Worth, Texas; ⁴Department of Genetics, King Faisal Specialist Hospital & Research Center, Riyadh, Saudi Arabia; ⁵Department of Pediatrics, King Khalid University Hospital and College of Medicine, King Saud University, Riyadh, Saudi Arabia; ⁶Department of Anatomy and Cell Biology, College of Medicine, Alfaisal University, Riyadh, Saudi Arabia; ⁷Shiley Eye Center, University of California San Diego, San Diego, California; ⁸Department of Genetics, Stanford University School of Medicine, Stanford, California; ⁹Departments of Anatomy and Ophthalmology, University of California, San Francisco, California.

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Corresponding author: Wen-Tao Deng, Department of Ophthalmology, College of Medicine, University of Florida, 1600 SW Archer Road, Gainesville, FL 32610; wdeng@ufl.edu.

the time of diagnosis, with a reduced cone ERG response seen later.^{13,14,16,19,20} The pattern ERG can be abnormally early in the disease.¹⁹ Patients also frequently demonstrate early macular atrophy.^{13,15,16,19} Spectral domain OCT and fundus examination may reveal photoreceptor degeneration, vascular attenuation, and hyperreflective layers adjacent to the RPE that resemble the debris layer seen in RCS rats.^{13,15,17,19} A wave-like appearance of the innermost retina can also be a distinctive feature in these patients.^{13,15}

RCS rats have been used as a model to evaluate the effects of *Mertk* gene replacement therapy. Previous studies have shown that recombinant adenovirus-, adeno-associated virus (AAV)2-, and lentivirus-mediated gene transfer of rodent *Mertk* to the RCS retina transiently improved visual function and retinal morphology as evidenced by ERG analysis and a slowing of photoreceptor loss.^{21–23} An early study with an adenoviral vector showed that the number of photoreceptors remaining near the area of injection was two- to threefold higher than in the uninjected eye at 1 month after treatment. At this time in treated eyes, RPE phagocytic function was restored and ERG responses were higher than in untreated control eyes.²³ AAV2-mediated gene therapy in this model resulted in up to 9 weeks of morphological improvement and functional rescue.²¹ The same group later employed lentiviral-mediated therapy in order to overcome a relatively slow onset of expression from AAV2 vectors. This led to more persistent photoreceptor preservation than the two previous reports.²² However, obvious differences in ERG responses were only observed up to 14 weeks, and there was a lack of organized rod OS beginning at 8 weeks post-injection, with survival of only a few photoreceptors at 7 months. Although these experiments provided proof of concept that gene replacement therapy for recessive *MERTK* RP was feasible, they also highlighted the need for a more robust and longer-lasting treatment modality.

Encouraging results from recent clinical trials involving one form of human LCA2 caused by defects in the *RPE65* gene suggest that replacement gene therapy mediated by AAV vectors represents a promising approach for the treatment of retinal degenerations, particularly those with mutant gene expression in the RPE.^{24–27} Studies in animal models and cell culture systems have shown that AAV vectors containing tyrosine-to-phenylalanine mutations in some of the seven surface-exposed capsid tyrosine residues in AAV2, AAV8, or AAV9 led to significantly increased transduction efficiencies relative to their wild-type counterparts.^{28,29} For example, an AAV8 Y733F mutant was found to provide rapid and efficient reporter gene expression when injected subretinally into adult mouse eyes.²⁸ In the present study, we evaluated whether AAV8 Y733F-mediated delivery to RCS rats at P2 of a human *MERTK* cDNA (*hMERTK*) targeted specifically to the RPE could prevent the loss of photoreceptors, stabilize the retinal vasculature by preventing retinal vascular capillary degeneration, and preserve retinal function. We demonstrate for the first time that an AAV8 mutant capsid serotype vector has a substantial therapeutic potential for RPE-specific gene delivery.

MATERIALS AND METHODS

Experimental Animals

Pink-eyed inbred RCS rats and its congenic wild-type strain *RCS-rdy*⁺ (www.ucsfeye.net/mlavaiiRDratmodels.shtml) were obtained from Dr Matthew LaVail. They were bred and maintained in the University of Florida Health Science Center Animal Care Services Facilities under a 12 hour/12 hour light/dark environment of less than 50 lux illuminance. All experiments were approved by the local Institutional Animal Care and Use Committee and conducted in accordance with

the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and NIH regulations.

Construction and Packaging of AAV Vectors

The vector plasmid pTR-*BEST1*-*hMERTK* contains the human *BEST1* promoter upstream of the full-length human *MERTK* cDNA (*hMERTK*). The 585/+38 bp *BEST1* promoter has previously been shown to drive efficient transgene expression to the RPE.³⁰ The *hMERTK* cDNA was amplified by PCR from a human cDNA library isolated from peripheral lymphocytes of a normal-sighted individual. The resulting fragment was cloned into plasmid pCR2.1 TOPO (Invitrogen, Carlsbad, CA) via *Bam*HI and the DNA was sequenced. A BLAST search of the amino acid translation indicated the coding region was in agreement with previously published human *MERTK* protein sequences. The cDNA was subsequently removed from the subcloning vector by *Eag*I digest and ligated into an AAV vector plasmid already containing the *BEST1* promoter. Vector plasmid was packaged in AAV serotype 8 Y733F by transfection of H293 cells according to previously published methods.³¹

Subretinal Injections

Left eyes served as uninjected controls, and 1.5 μ L of AAV8 Y733F-*BEST1*-*hMERTK* vector (1.44×10^{12} vector genomes/mL) was injected subretinally into the right eyes of P2 RCS pups. Subretinal injections were performed as previously described with some modifications.^{32,33} Briefly, the eyelid was opened by separation with blunt forceps. The eye was slightly prolapsed by modest periocular pressure. The eyelids functioned to hold the globe prolapsed during the procedure. An aperture within the pupil area was made through the cornea with a 30-gauge disposable needle. A 33-gauge blunt needle mounted on a 5- μ L Hamilton syringe (Hamilton Co., Reno, NV) was then introduced through the corneal opening, avoiding the lens and reaching the subretinal space in the inferior retinal hemisphere. A small amount of neomycin, polymyxin B sulfates, dexamethasone ophthalmic ointment was applied to the eye following injection and then applied once daily for 3 days.

ERG Analysis

At 2 and 4 months post-injection, a UTAS-E 200 visual electrodiagnostic system (LKC Technologies, Gaithersburg, MD) was employed for ERG analysis. Detailed protocols have been previously described.³⁴ Briefly, rats were dark-adapted overnight and placed under dim red illumination (>650 nm). Dark-adapted animals were anesthetized with a mixture of 100 mg/kg ketamine, 20 mg/kg xylazine, and saline at a ratio of 1:1:3. ERGs from both eyes were recorded simultaneously. Gold contact lens electrodes were placed on the eyes with a drop of 2.5% hydroxypropyl methylcellulose (Eye Supply; Akorn, Inc., Decatur, IL). A reference electrode was placed subcutaneously between the two eyes and a ground electrode was placed in the tail. Rod- plus cone-mediated ERGs were recorded at stimulus intensity of 2.5 cd s/m² with 60-s intervals between stimulus flashes. Five ERG readings were averaged for each recording. Data were analyzed by paired *t*-test.

At 8 months post-injection, rod- and cone-mediated ERGs were recorded separately using a Toennies Multiliner Vision unit (Höchstberg, Germany) according to protocols previously described.³⁵ Scotopic rod recordings were performed with seven steps of increasing intensities of white light from 0.01 mcd s/m² to 5 cd s/m². Ten responses were recorded and averaged at each light intensity. Photopic cone recordings were taken after rats were adapted to a white background light of 30 cd/m² for 5 minutes. Recordings were performed with five steps of flash intensity from 100 mcd s/m² to 12 cd s/m² in the presence of 30 cd/m² background light. Fifty responses were recorded and averaged at each intensity. Scotopic and photopic b-wave amplitudes from untreated, treated RCS and wild-type *rdy*⁺ rats at each intensity were averaged and used to generate a standard deviation. The differences between

recordings from untreated, treated, and wild-type eyes were analyzed by repeated-measures ANOVA, with the Bonferroni post hoc test for ANOVA ($P < 0.05$) to compare means at individual flash intensities.

Tissue Preparation

Eyes of vector injected RCS or RCS-*rdy*⁺ rats were enucleated at 2, 4, and 8 months after treatment. Retinal sections for immunohistochemistry were prepared according to previously described methods.³⁶ Briefly, immediately following sacrifice, the limbus of an eye was marked at "12 o'clock" with a hot needle to facilitate orientation later. Eyes were then enucleated and fixed in 4% paraformaldehyde. Cornea, lens, and vitreous were removed from each eye without disturbing the retina. The remaining eyecup was rinsed with PBS and then cryoprotected by placing in 30% sucrose in PBS for 4 hours at 4°C. Eyecups were then embedded in cryostat compound (Tissue TEK OCT, Sakura Finetek USA, Inc., Torrance, CA) and frozen at -80°C. Retinal sections for hematoxylin and eosin stain were fixed in a mixture of 4.0% paraformaldehyde and 0.5% glutaraldehyde, after whole retinas were paraffin-embedded and sectioned at 4 μ m through the optic nerve.

Immunohistochemistry

Retinal cryosections were rinsed in PBS and blocked in 2% normal goat serum, 0.3% Triton X-100 in 1% BSA in PBS for 1 hour at room temperature. Anti-GFP antibody (Invitrogen, Carlsbad, CA), monoclonal anti-MERTK antibody specific for human MERTK (Epitomics, Inc., Burlingame, CA), or glial fibrillary acidic protein (GFAP; Abcam, Cambridge, MA) was diluted in 0.1% Triton X-100 and 1% BSA in PBS and incubated with sections overnight at 4°C. The sections were then washed three times with PBS, incubated with IgG secondary antibody tagged with Alexa-488 or Alexa-594 (Molecular Probes, Eugene, OR) diluted 1:500 in PBS at room temperature for 1 hour, and washed with PBS. Sections were mounted with Vectashield Mounting Medium for Fluorescence (H-1000, Vector Lab, Inc., Burlingame, CA) and coverslipped. Sections were analyzed with an Olympus DSU Spinning Disk Confocal Scanner mounted on an Olympus IX81 inverted fluorescent microscope.

Western Blot Analysis

AAV8(Y733F)-*BEST1*-hMERTK-treated and untreated RCS eyes (two eyes each) were carefully dissected, and the eyecups were pooled and homogenized by sonication in a buffer containing 0.23 M sucrose, 2 mM EDTA, 5 mM Tris-HCl (pH 7.5), and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After centrifugation, aliquots of the extracts containing equal amounts of protein (50 μ g) were electrophoresed on SDS-PAGE, transferred, and probed with the same antibody used in immunohistochemistry. Visualization of specific bands was performed using the Odyssey Infrared Fluorescence Imaging System (Odyssey; Li-Cor, Lincoln, NE).

Light Microscopy and Transmission Electron Microscopy (TEM)

Eyes were immediately removed and immersed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate trihydrate buffer (pH 7.4). Corneas were removed and the eyes left in fixative for 24 hours. The lens was then removed followed by dehydration with a graded series of increasing ethanol concentrations. Eyecups were embedded in Epon epoxy resin. For each sample, 0.5- μ m sections were stained with toluidine blue for light microscopy followed by ultra-thin section preparation for TEM examination on a Hitachi 7600 (Hitachi, Tokyo, Japan).

Trypsin Digest Preparation of Retinal Vasculature

Retinal vessel preparation has been described previously.³⁷ In brief, eyes were fixed in 4% paraformaldehyde overnight, then the retinas

FIGURE 1. Detection of MERTK expression in RCS rat retinas following delivery of AAV8 Y733F-BEST1-hMERTK. (A) RT-PCR results from untreated and treated RCS retinas. Lane 1: uninjected RCS retinas, no RT control; lane 2: uninjected RCS retinas, RT reaction control; lane 3: injected RCS retinas, no RT control; lane 4: injected RCS retinas, RT reaction; lane 5: BEST1-hMERTK plasmid as positive control. (B) Western blot analysis of treated and untreated RCS retinas. (C) hMERTK expression (labeled as red) by immunofluorescence. Arrows indicate specific expression in the RPE from a treated eye (right panel). No labeling was detected in an untreated eye (left panel). Note the much thicker ONL in the treated eye compared to that in the untreated control. Scale bar, 20 μ m. RCS rats were injected at P2 and sacrificed 2 months post-injection in all three experiments.

were dissected from eye cups, washed in water overnight, and digested in 3% trypsin (Invitrogen-Gibco, Grand Island, NY) for 3 hours at 37°C. After washing with water to isolate the network of vessels from adherent retinal tissue, the vessels were mounted on a slide, allowed to dry, and stained with periodic acid Schiff-hematoxylin and eosin (Gill No. 3; Sigma-Aldrich) according to the manufacturer's instructions. The tissue was then stained, washed in water, dehydrated, and mounted (Permount mounting medium; Fisher Scientific, Pittsburgh, PA). The prepared retinal vessels were photographed using an AxioCam MRC5, Axiovert 200 microscope (Carl Zeiss Meditec, Inc., Dublin, CA), using both 20 $\times$ and 40 $\times$ objective lenses. Six to eight representative, nonoverlapping fields from each quadrant of the retina were imaged and acellular capillaries were counted for each retina and expressed as the number of acellular vessels per square millimeter.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from injected and contralateral control eyes of RCS rats, using a Micro-to-Midi total RNA purification system (Invitrogen). First-strand cDNA was generated with SuperScript II reverse transcriptase and then amplified by PCR, using *Taq* polymerase (Invitrogen). DNase treatment before the reverse transcription was performed to prevent the amplification of the transgene present in the recombinant virus. The sequences of the primers were AGCTTGGGAGTCAGTGAGGA (forward) and GGGCAA-TATCCACCATGAAC (reverse), derived from human MERTK cDNA sequence to generate a 429-bp fragment. The cycling conditions for all reactions were 95°C for 5 minutes, followed by 35 cycles of 95°C for 1 minutes, 55°C for 1 minutes, and 72°C for 30 seconds. The PCR product was analyzed by agarose gel electrophoresis, subsequently subcloned into the pCR2.1-TOPO plasmid (Invitrogen) and verified by DNA sequencing.

FIGURE 2. Retinal function of RCS rats following AAV8 Y733F-BEST1-hMERTK delivery. (A) Representative dark-adapted ERG traces from an RCS rat at 2 months after treatment. (B) Average ERG a- and b-wave amplitudes from untreated and contralateral treated RCS rats at 2 months and 4 months after treatment. ERGs from a 2-month-old wild-type are shown as a control. (C, left and right panels) Average scotopic (C, left panel) and photopic (C, right panel) b-wave amplitudes as a function of flash intensity 8 months post-treatment.

RESULTS

AAV8 Y733F-mediated RPE-specific Expression of Human MERTK

An AAV8 Y733F vector containing hMERTK cDNA under control of an RPE-specific *BEST1* promoter was injected subretinally into one eye of RCS rats at P2, while the contralateral eyes were untreated and served as controls. Expression of hMERTK was analyzed by RT-PCR, immunostaining and Western blot analysis at 2 months post-injection. RT-PCR revealed the presence of hMERTK mRNA in injected eyes, but not in uninjected controls (Fig. 1A). Western blot analysis of untreated and treated RCS retinas with a human-specific MERTK antibody showed MERTK expression only in the treated retinas with a protein band at about 170 kDa (Fig. 1B). Immunostaining of untreated and contralateral treated RCS retinas with the same antibody showed hMERTK expression exclusively in the RPE cell layer and primarily localized to the apical side of the RPE (Fig. 1C, right panel). Preservation of the outer nuclear layer (ONL) in the treated retina is also obvious in this image, whereas the ONL in the untreated retina was already significantly degenerated at this age (Fig. 1C, left panel).

Retinal Function is Preserved in AAV8-treated RCS Eyes

To determine the effect of the treatment on retinal function over time, we performed ERG analysis at 2, 4, and 8 months post-treatment. The functional consequences of hMERTK treatment at 2 and 4 months post-injection were measured by ERG at a flash intensity of 2.5 cd s/m⁻², which records responses from both rods and cones. Figure 2A shows representative ERG traces measured simultaneously from untreated (left) and contralateral treated (right) eyes of an RCS rat at 2 months post-injection. There was considerable variation of the ERG amplitudes recorded from untreated eyes of 2-month-old RCS rats; some animals retained residual ERG responses while others had unrecordable ERG responses. In contrast, all eyes treated with AAV8 Y733F-BEST1-hMERTK exhibited significantly improved ERG responses. At 2 months, the average a-wave amplitude was 175 ± 36 μV in treated eyes, about 74% of the wild-type controls, vs. 16 ± 17 μV in untreated eyes ($n = 6$, $P < 0.005$). The average b-wave amplitude was 500 ± 150 μV in the treated eyes, about 83% of the wild-type controls, vs. 61 ± 48 μV in the untreated eyes ($n = 6$, $P < 0.005$) (Fig. 2B). At 4 months, we detected no ERG responses in any untreated eyes, whereas in treated eyes, ERG amplitudes were marginally decreased compared to those

FIGURE 3. (A–D) Retinal morphology based on light microscopy of the injected inferior hemispheres of RCS eyes following AAV8 Y733F-BEST1-hMERTK delivery. An untreated RCS retina at 4 months (A) shows a loss of almost all photoreceptors, with one surviving nucleus (arrow) and a persistent debris (D) layer in place of the inner segments (IS) and outer segments (OS) in the treated (B, C) RCS rat retinas and in the wild-type (D) retina. Treated RCS retinas are shown at 4 months (B) and 8 months (C) post-injection. The wild-type retina (D) is from an uninjected rat at 8 months of age. RPE, retinal pigment epithelium. Scale bar = 25 μm. (E) Measurement of thickness of ONL from one eye each of treated and untreated RCS and one wild-type control rat.

recorded 2 months prior, the a- and b-wave amplitudes being about 54% and 69%, respectively, of wild-type controls ($n = 6$) (Fig. 2B). At 8 months post-injection, rod- and cone-mediated ERG responses with a series of increasing light intensities were measured in RCS rats and in wild-type controls (Figs. 2C, 2D). The average dark adapted rod-driven b-wave amplitude at a flash intensity of 0.01 cd.s.m^{-2} in treated eyes was $223 \pm 56 \text{ μV}$ ($n = 4$), about 44% of the wild-type controls (Fig. 2c). The average light adapted cone-mediated b-wave amplitude at a flash intensity of 10 cd.s.m^{-2} was $30 \pm 11 \text{ μV}$ ($n = 4$), about 33% of the wild-type controls (Fig. 2D). Both rod- and cone-mediated b-waves in the untreated controls were undetectable at this age ($n = 4$; $P < 0.05$). These results demonstrate that AAV8 Y733F-mediated hMERTK treatment is able to preserve significant retinal function for at least 8 months.

Photoreceptors are Preserved and RPE Function is Restored in Treated RCS Eyes

To study the effect of treatment on photoreceptor cell preservation over time, we performed morphological analysis

using semi-thin retinal sections at 4 and 8 months post-injection. To ensure comparability among treated animals, all subretinal injections were targeted to the inferior retinal hemisphere. Vertical sections were cut through the cornea and retina parallel to the optic nerve and those containing the optic nerve head were analyzed. Figure 3A shows images taken from sections in the equatorial region of the inferior hemisphere where vector injections were targeted. At 4 months post-injection, the ONL in the untreated retinas was undetectable, indicating a near complete loss of photoreceptors (Fig. 3A). In these retinas, a thick debris layer replaced the OS and inner segment (IS) layers that are present in wild-type controls (Fig. 3D). In contrast, corresponding sections from treated retinas showed a well-preserved ONL in the inferior hemisphere containing about six to eight layers of photoreceptor nuclei at 4 months post-injection (Fig. 3B). The ONL decreased to about six to seven layers at 8 months post-treatment (Fig. 3C) compared to seven to eight layers in wild-type control (Fig. 3D). Well-organized OS and IS layers were observed in the treated retinas, and as noted in Figures 3B and 3C, in the best preserved regions, the outer retina was almost indistinguishable from normal (Fig. 3D).

FIGURE 4. Electron microscopic images of RPE and photoreceptor OS in treated and untreated RCS rats. Top panels: EM images ($\times 3500$); Bottom panels: EM images ($\times 8000$) of OS and RPE of untreated (A, D), AAV8 Y733F-BEST1-hMERTK treated (B, E) and wild-type control retinas (C, F). Well-organized OS and apical microvilli (MV) of RPE cells surround OS tips in the treated eye (B, E). Attenuated RPE and a thick debris (D) layer are evident in the untreated eye (A, D). Phagosomes (arrows) are present in the RPE cells of the treated eye (E) and normal control (F). In contrast, no phagosomes are seen in the untreated RCS control eye (D).

Photoreceptor cells were also preserved in the superior hemispheres but to a lesser extent than in the inferior hemispheres. This is consistent with the pattern of subretinal vector spreading as assessed by GFP expression after *BEST1*-GFP vector injection showing that 60% to 80% of the retinas were GFP positive when P2 rat pups were injected in the inferior hemisphere (see Supplementary Material and Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8831/-DCSupplemental>). In superior retinal areas of treated eyes, slightly disorganized OS were associated with reduced ONL thickness (data not shown). The ONL thickness measured in one treated eye of an RCS rat and an age-matched wild-type control at 8 months post-injection showed approximately 80% of normal in the inferior hemisphere and 40% in the superior hemisphere as shown in Figure 3E.

Electron microscopic (EM) analysis was performed to examine at higher resolution the structure of OS, RPE, and RPE phagosomes in 4-month-old treated RCS rats. Rod OS disk shedding follows a circadian rhythm in rats with its peak occurring at about 1 to 1.5 hours after the onset of light in the

morning.³⁸ Hence, animals were sacrificed at the peak time of disk shedding to maximize the chance of observing any reversal of the RPE phagocytic defect seen in RCS rats. EM analysis at low magnification (Fig. 4, top panels) showed that an untreated eye had attenuated RPE, and a thick debris layer consisting of membranous waste material of shed OS accumulating as a result of the phagocytic defect (Fig. 4A). Retinal areas from the inferior hemisphere of a treated RCS retina display normal rod OS structure, with the apical microvilli of RPE surrounding OS tips (Fig. 4B). The OS length and RPE thickness were slightly reduced compared to those of the age-matched wild-type control (Fig. 4C). Further examination of RPE at higher magnification (Fig. 4, bottom panels) demonstrated that many round or oval phagosomes packed with OS disk membranes were evident in RPE apical processes and cell bodies of the treated eye (Fig. 4E), but not in the untreated eye (Fig. 4D). These phagosomes morphologically resembled those seen in the normal rats (Fig. 4F). In addition, the density of phagosomes in a given field was nearly equivalent to that in wild-type controls.

FIGURE 5. Retinal capillaries in treated and untreated RCS rats. Representative images of retinal vessels from trypsin-digested preparations of untreated RCS (A), treated RCS (B), and wild-type control retinas (C). RCS rats exhibited a significant increase in the formation of acellular capillaries (arrows in [A]). (D) Quantitative analysis of acellular capillaries. * $P < 0.001$.

Retinal Vascular Remodeling was Prevented in Treated RCS Eyes

The retinal vasculature initially develops normally in RCS rats. However, reduced numbers of endothelial cells and formation of acellular capillaries have been observed in RCS rats as early as 3 weeks. Subsequently, the retinal vasculature loses its normal density and architecture.^{39–41} To investigate the vasculature response to AAV8 Y733F-BEST1-hMERTK treatment in RCS eyes, we evaluated retinal vascular capillaries from untreated, treated, and wild-type eyes at 4 months post-injection (Fig. 5), using a trypsin-digested retinal vascular preparation. There was a marked increase in the numbers of acellular capillaries in the untreated RCS retinas (82 ± 8.2 , mean $\pm$ SD, Fig. 5A, 5B) compared to the wild-type control (1.4 ± 0.5 , Figs. 5C, 5D). The number of acellular capillaries was significantly reduced in the treated retina (34 ± 7 , $P < 0.001$, Figs. 5B, 5D). The retinal vasculature near the area of injection was very similar to that of the wild-type control, whereas areas farther away from the injection site showed less rescue (data not shown).

Müller Cell Activation was Inhibited in Treated RCS Rats

Activation of Müller cells in degenerating RCS retinas has been reported.^{42,43} We assessed the localization of GFAP in retinas by immunofluorescence. In a normal retina, GFAP (red staining) is found only in cell processes at the inner limiting membrane (astrocytes and Müller cell foot processes) (Fig. 6C). In untreated, degenerated 8-month-old RCS rat retinas (Fig. 6A), GFAP-immunostained Müller cell processes extended from

the inner limiting membrane throughout the retina into the subretinal space. In contrast, the GFAP staining pattern near the injection area of treated RCS retinas (Fig. 6B) was similar to that of normal (Fig. 6C).

DISCUSSION

In this study, we demonstrated that gene replacement therapy using an AAV8 Y733F-BEST1-hMERTK vector leads to preservation of photoreceptor numbers and function, rescue of RPE phagocytosis, and prevention of degenerative changes in the retinal vasculature. Importantly, these profound beneficial effects on retinal structure and function were still present and only modestly diminished at 8 months post-treatment, nearly half the animal's normal lifespan. To our knowledge, this is the longest and most dramatic rescue reported to date in this rapid photoreceptor degeneration model of *Mertk*-associated recessive RP.

In the most successful previous study, a lentivirus-based gene replacement approach initiated at P10 showed morphological rescue in RCS rats for up to 7 months, while obvious differences in ERG responses were significantly diminished after just 14 weeks.²² Even at an early post-injection time (8 weeks), the average b-wave amplitude in lentivirus-treated animals relative to partner control eyes was significantly lower than the treated versus untreated amplitudes of AAV8 Y733F vector treated eyes at 8 months post-injection in the present study. Additionally, in the lentiviral study there was a marked decline in the number of photoreceptors reflecting the diminishing amplitudes of ERG responses during this post-treatment period. Moreover, a large amount of OS debris was visible in treated rats at 8 weeks post-treatment and beyond,

FIGURE 6. Changes in RCS rat Müller glial cell GFAP immunostaining following AAV8 Y733F-*BEST1*-hMERTK delivery. (A) In an 8-month-old untreated, degenerated RCS retina, the GFAP-immunostained Müller cell fibers (red) extend through the retina into the subretinal space. In the contralateral treated retina (B), GFAP staining was mostly restricted to the innermost retina at the inner limiting membrane, similar to that in an age-matched wild-type control (C). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar = 20 μ m.

suggesting incomplete correction of the RPE phagocytic defect. In contrast, in the RCS retinas treated with AAV8 Y733F-*BEST1*-hMERTK, no debris layer was observed in the region where photoreceptors were well preserved even 8 months post-treatment, indicating a long-term and complete rescue of the phagocytic defect in those areas.

The benefit of using an AAV serotype 8 appears to be in its increased transduction efficiency and ability to promote widespread transgene expression in the retina.⁴⁴ Previous studies employing this serotype and its capsid mutant for rescue of retinal morphology and function have focused on delivering therapeutic genes to photoreceptors,^{45–47} not to the RPE. For the first time, we show that an AAV8-Y733F vector can also efficiently and persistently rescue an RPE-based defect. This outcome can be attributed to several factors. First, the use of a mutant capsid serotype such as AAV8 Y733F significantly increases transduction efficiency and provides rapid onset of transgene expression compared to other AAV serotypes and wild-type AAV8.²⁸ Second, we injected at P2 in this study compared to P10 in previous AAV2- and lentivirus-based studies. We chose an early time of intervention to ensure that treatment began before the initiation of retinal degeneration. Moreover, because OS disk shedding normally starts at P12 in the rat,⁴⁸ we felt it was important to provide therapeutic transgene expression before that date. Therefore, the improved outcome achieved by our approach may derive from a combination of the choice of vector and the time of treatment. Clearly, it will be important in future studies to assess levels of therapy achievable at later stages of *Mertk* disease in the RCS rat.

In spite of its enhanced persistence, AAV8 Y733F-mediated gene therapy did not completely prevent the loss of photoreceptor cells in this study. There was a gradual decrease of photoreceptors in the treated area, and degeneration was more obvious in the area far away from injection site. Consequently, full-field ERG amplitudes also progressively decreased with age. There could be several reasons why photoreceptor degeneration proceeded despite early treatment. First, only 60% to 80% of the retina was transduced. Second, while use of an RPE-specific promoter should minimize potential toxicity due to ectopic expression in non-RPE cell types, transgene expression under the control of a *BEST1* promoter may still not be optimal for full MERTK function. Future studies will be needed to examine vector dosing and the effect of alternative RPE or constitutive promoters on the longer-term rescue of the RCS rat retina. RPE-specific localization of the RPE65 protein driven by CBA promoter has been shown in rd12 mouse model,³² and RPE65 LCA clinical trials have demonstrated the ability of the CBA promoter in restoration of visual function.^{25,26} It is possible that CBA promoter could drive more robust MERTK expression specifically in the RPE. However, we were not able to package

CBA-MERTK in any AAV vector, most likely because of the toxicity in host H293 cells caused by high levels of MERTK expression from a CBA promoter. Additionally, it may be useful to examine the retinal response to multiple, geographically dispersed vector injections to determine whether transducing RPE cells over a larger retinal area will prevent the observed gradual loss of photoreceptors.

In a previous study in RCS rats treated with Ad-*Mertk*,²³ a thin interphotoreceptor debris layer had already formed and OS no longer reached the apical surface of the RPE at P23 when the rats were injected. However, the debris layer resolved in areas of significant photoreceptor rescue at 1 month after treatment. Therefore, it appears possible for robust MERTK expression to be effective even after the underlying RPE defect has interrupted OS phagocytosis. Thus, although most individuals with mutations in *MERTK* have an early onset retinal dystrophy, it is possible that the therapeutic window may extend beyond childhood. As noted above, it will be important therefore to test AAV8 Y733F-*BEST1*-hMERTK treatment at more advanced stages of disease in RCS rats to fully evaluate the potential of this approach for human gene therapy.

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