Pathological Effects of Mutant C1QTNF5 (S163R) Expression in Murine Retinal Pigment Epithelium


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PURPOSE. The mutation S163R in complement C1q tumor necrosis factor–related protein-5 (C1QTNF5) causes an autosomal dominant disorder known as late-onset retinal degeneration (L-ORD). In this study, our goal is to evaluate the consequences of mutant S163R C1QTNF5 expression in mouse RPE following its delivery using an adeno-associated viral (AAV) vector.

METHODS. We generated AAV vectors containing either human wild-type C1QTNF5 or mutant S163R C1QTNF5 driven by an RPE-specific BEST1 promoter, and delivered them subretinally into one eye of adult C57BL/6 mice. Transgene expression was detected by immunohistochemistry. Pathological changes were further examined by digital fundus imaging and spectral-domain optical coherence tomography (SD-OCT).

RESULTS. We show that the AAV-expressed mutant S163R leads to pathological effects similar to some of those found in patients with advanced L-ORD, including RPE thinning, RPE cell loss, and retinal degeneration. In addition, we provide in vivo evidence that mutant S163R C1QTNF5 can form large, transparent, spherical intracellular aggregates throughout the RPE, which are detectable by light microscopy. In contrast to AAV-expressed wild-type C1QTNF5, which is secreted apically from the RPE toward the photoreceptor cells and the outer limiting membrane, the S163R mutant is primarily routed toward the basal side of RPE, where it forms thick, extracellular deposits over time.

CONCLUSIONS. Adeno-associated viral–targeted expression of mutant S163R in the RPE represents a useful approach for quickly generating animal models that mimic pathological features of L-ORD and offers the potential to understand disease mechanisms and develop therapeutic strategies.

Keywords: retina, adeno-associated virus, age-related macular degeneration, retinal pigment epithelium, late-onset retinal degeneration

The retinal pigment epithelium is a polarized monolayer of postmitotic epithelial cells located between the neural retina and the vascular choroid. On its apical side, it contains numerous microvilli extending into the interphotoreceptor matrix, and surrounding the outer segments of rods and cones. On the basal side is Bruch’s membrane, a thin, multilayered, extracellular matrix in which pathological changes are integral to AMD. The retinal pigment epithelium performs functions essential for both the structural integrity and survival of photoreceptor cells, including transport of nutrients, retinoids and waste products, phagocytosis of outer segments, synthesis of the visual chromophore retinal, and production of growth factors.

The mutation S163R in the globular gC1q domain of complement C1q tumor necrosis factor–related protein-5 (C1QTNF5/CTR5) causes a rare autosomal dominant disorder known as late-onset retinal degeneration (L-ORD). The earliest symptoms appear in midlife, manifested as difficulties in dark adaptation and loss of night vision. Over time, the disorder progresses to severe loss of both central and peripheral vision, with some patients also developing choroidal neovascularization in late stages of the disease that mimic neovascular AMD. In addition, patients also present with abnormally long anterior zonular insertions on the lens capsule which serve as an early disease phenotype. The hallmark of L-ORD is the presence of abnormal extracellular deposits between the RPE and Bruch’s membrane. These thick deposits, which are histopathologically similar to those found in AMD patients, have a widespread distribution, extending outside the macular region into the peripheral retina. Although there is evidence that supplementation with vitamin A may be beneficial early in the disease course when patients exhibit dark-adaptation abnormalities, there is no current treatment that prevents the progressive retinal degeneration leading to vision loss.

C1QTNF5 belongs to the C1q and tumor necrosis factor-related superfamily (C1q/TNF), characterized by an N-terminal signal peptide, a short variable region, a collagen repeat domain, and a C-terminal globular domain (gC1q) that is homologous to the immune complement component 1q. All members of the C1q/TNF family are secreted multimeric proteins, with specific tissue expression profiles, and many are found circulating in plasma, with levels influenced by genetic background, sex, and metabolic status. Members of the C1q/TNF family are of broad clinical interest since they play numerous roles in immunity and inflammation, glucose and lipid metabolism, and vascular maintenance. C1QTNF5 is a...
membrane-associated and secreted protein of unknown function, whose expression is the highest in the RPE and ciliary epithelium.\textsuperscript{14,17,18} It colocalizes with ZO1, an RPE tight-junction protein, at the hexagonal RPE membrane, and is also secreted into the interphotoreceptor matrix.\textsuperscript{17,19} C1QTNF5 can also be found in other ocular tissues, including the lens.\textsuperscript{17} It is secreted by the adipose tissue, and circulates abundantly in human serum, potentially playing an important role in energy homeostasis and obesity-related inflammation.\textsuperscript{16,20} The crystal structure of the trimeric globular domain of C1QTNF5 was determined at 1.34 Å resolution by Tu and Palczewski,\textsuperscript{21} who also showed that wild-type C1QTNF5 can multimerize into a bouquet-like octadecameric assembly containing six trimers.\textsuperscript{22} Previous in vitro studies have demonstrated that the S163R mutant is not secreted, has the tendency to aggregate, and is retained primarily within the endoplasmic reticulum.\textsuperscript{17,23}

Interestingly, C1QTNF5 is expressed as a dicistronic transcript with MFRP encoding the membrane-type frizzled related protein.\textsuperscript{6,24} The complete open reading frame of C1QTNF5 is located within the 3' untranslated region of the final exon of MFRP. The two proteins were shown to directly interact; however, the functional significance of this interaction is not currently understood.\textsuperscript{18,22,23} Mutations in the two genes are associated with distinct disease phenotypes in humans, with the dominant S163R mutation in C1QTNF5 leading to L-ORD, whereas mutations in MFRP cause autosomal recessive retinitis pigmentosa (RP), nanophthalmos, foveoschisis, and optic disc drusen.\textsuperscript{25–27}

We have previously shown that subretinal delivery of a fast-acting AAV8-735F vector expressing wild-type MFRP led to robust transgene expression at its normal RPE location on the apical membrane and microvilli, prevented retinal degeneration, and rescued photoreceptor function in the rd6 mouse model of RP.\textsuperscript{28} In this study, we used the recombinant AAV vector technology to examine the pattern of mutant S163R transgene expression and its pathological effects following specific RPE targeting. We show that AAV-expressed wild-type C1QTNF5 is secreted apically toward photoreceptor cells and outer limiting membrane (OLM). In contrast, AAV-expressed S163R mutant forms large spherical intracellular aggregates within the RPE cells of normal mice, and gradually accumulates as thick, widespread extracellular basal deposits, leading to local regions of RPE atrophy and retinal degeneration. These findings have important mechanistic and therapeutic implications for L-ORD and perhaps for AMD as well.

**METHODS**

**AAV Vector Production and Subretinal Injections**

Self-complementary AAV2 quadruple capsid tyrosine mutant (Y272,444,500,730F) vectors (scAAV2quadYF) were used for packaging and expressing either human wild-type C1QTNF5 (hC1QTNF5wtHA) or the S163R mutant (hC1QTNF5(S163R)-HA), driven by an RPE-selective human BEST1 promoter.\textsuperscript{29} Both wild-type and mutant S163R C1QTNF5 were tagged with hemagglutinin (HA) at their C-terminal end for subsequent detection by immunostaining. The quadruplet capsid tyrosine mutant vector AAV2 containing point mutations of surface-exposed tyrosine residues was chosen for transgene delivery in all these experiments based on its higher transduction efficiency following a single injection, compared to wild-type AAV2.\textsuperscript{30} The viral vectors were packed, purified, and titered according to previously reported methods.\textsuperscript{31} The vectors were then subretinally injected in adult C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) as previously described.\textsuperscript{32} Each eye received 1 μL vector at a titer of 1 × 10\textsuperscript{12} genome copies/mL, leaving the left eye as an untreated control. Animals were maintained on a 12-hour light and 12-hour dark cycle in the University of Florida Health Science Center Animal Care Services Facility (Gainesville, FL, USA). All experiments were approved by University of Florida Institutional Animal Care and Use Committees and conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Immunostaining and Histologic Analysis**

Eyes were harvested, processed for paraffin embedding, and sectioned at a thickness of 4 μm, at the same retinal locations for treated and untreated eyes. For morphologic analysis, the sections were stained with hematoxylin and eosin, and visualized by light microscopy. For immunostaining, deparaffinized tissue sections were dewaxed in xylene and rehydrated in a graded series of ethanol, then incubated with 0.3% Triton X-100 for 15 minutes, followed by blocking with a solution of 2% albumin for 30 minutes. Hemagglutinin-tagged C1QTNF5 proteins in the AAV-treated eyes were detected by immunostaining with a high-affinity anti-HA–fluorescein antibody (AF3445, R&D Systems, Minneapolis, MN, USA), followed by the Alexa Fluor 594 secondary antibody (Molecular Probes/Invitrogen, Eugene, OR, USA). Negative controls to check for any potential autofluorescence in AAV-expressed S163R-treated retinas were also performed by omitting the anti-HA antibody, and purposefully overexposing those images to detect any background autofluorescence. The mouse monoclonal antibody B6-30 against rod opsin\textsuperscript{53} was used to label the outer segments. Fluorescence micrographs at higher magnification were also acquired using a ×100 oil immersion objective. All sections were examined by fluorescence microscopy using a microscope (Axiophot; Carl Zeiss Microscopy, LLC, Thornwood, NY, USA). Cryosections for Oil red O staining to detect neutral lipids in the AAV-expressed S163R-treated eyes were also used. Frozen liver sections were used as positive control for the lipid staining. The liver was from a mouse that received intraperitoneal injections with carbon tetrachloride (CCl\textsubscript{4}) to cause hepatocyte damage and liver fibrosis.\textsuperscript{54} Cryosections were air dried at room temperature for 30 minutes and rinsed with distilled water. Sections were then placed in polyethylene glycol (PEG), stained with Oil red O (Abcam, Cambridge, UK), rinsed with 85% PEG, then with distilled water, before adding an aqueous mounting medium (Dako, Carpinteria, CA, USA). Images were acquired using brightfield microscopy.

**Transmission Electron Microscopy**

For ultrastructural analysis, the injected eyes containing S163R aggregates and basal deposits that were identified at the light level were further examined by transmission electron microscopy (TEM). Paraffin blocks were submitted to the Electron Microscopy Core Laboratory, Interdisciplinary Center for Biotechnology Research, University of Florida. Areas of interest containing the RPE–choroid interface were excised from the paraffin blocks and placed in 100% xylene overnight. The dewaxed tissues were rehydrated through a decreasing ethanol series, water washed, buffer washed, and fixed with Trumps (Electron Microscopy Sciences, Hatfield, PA, USA). Fixed tissues were processed with the aid of a laboratory microwave (Pelco BioWave Pro; Ted Pella, Redding, CA, USA). The samples were washed in 0.1 M cacodylate buffer pH 7.24, postfixed with 2% OsO\textsubscript{4}, water washed and dehydrated in a graded ethanol series 25%, 50%, 75%, 95%, 100%. Dehydrated tissues...
were infiltrated with 1:1 ethanol/LR White hard acrylic resin (Electron Microscopy Sciences, Hatfield, PA, USA) then embedded in 100% LR White resin and polymerized at 60°C for 48 hours. The cured resin blocks were hand-trimmed and prepared for ultramicrotomy. Semi-thick (500 nm) and ultra-thin sections (100–120 nm) were cut using a diamond knife. Semi-thick sections were dried onto a glass slide and stained with toluidine blue. Ultra-thin sections were collected on carbon-coated Formvar copper slotted grids, poststained with 2% aqueous uranyl acetate, and Reynolds lead citrate. Sections were examined with a FEI Tecnai Spirit LaB6 at 120 kV (FEI, Hillsboro, OR, USA) and images acquired with a charge-coupled device camera (AMT XR41, 2k X 2k; Advanced Microscopy Techniques, Woburn, MA).

**Western Blot Analysis**

Whole eyecups from AAV-S163R-treated eyes (1 month post injection) were homogenized by sonication in RIPA buffer (Sigma-Aldrich Corp., St. Louis, MO, USA) containing a complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After centrifugation, the supernatant was mixed with 2X Laemmli’s sample buffer containing dithiothreitol (DTT) and subjected to SDS-PAGE. Protein extracts from AAV–wild-type C1QTNF5 or AAV-S163R-injected eyecups were also analyzed under non-denaturing conditions by sonicating the tissues in a sample buffer lacking the SDS and DTT, and loading the supernatants onto 4% to 15% gradient gels. Protein extracts from treated C57BL/6 mice were dark-adapted (>12 hours) and anesthetized by intraperitoneal injection. Full-field ERGs were recorded as previously described, using a UTAS Visual Diagnostic System (LKC Technologies, Gaithersburg, MD, USA). After protein transfer onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) and blocking by incubation for 1 hour in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA), the membranes were probed with a mouse monoclonal primary antibody against the HA tag (Covance, Gaithersburg, MD, USA). Signals were detected with the Odyssey infrared fluorescence imaging system (LI-COR Biosciences), using an anti-mouse antibody conjugated with CW800 dye (LI-COR Biosciences).

**Electroretinographic Analysis and Fundoscopy**

Treated C57BL/6 mice were dark-adapted (>12 hours) and anesthetized by intraperitoneal injection. Full-field ERGs were recorded as previously described, using a UTAS Visual Diagnostic System (LKC Technologies, Gaithersburg, MD, USA). Dark-adapted ERGs were elicited with 0.02, 0.2 and 2 scot cd s m⁻² stimuli. Cone-isolated ERGs were elicited with a 25 phot cd s m⁻² (10 dB) stimulus on a rod-suppressing background light after a preadaptation period. Differences in b-wave maximum amplitudes between AAV hC1QTNF5(S163R)-HA–injected and uninjected contralateral left eyes were analyzed by Student’s t-test for paired samples (GraphPad Prism 6.0; GraphPad Software, San Diego, CA, USA), and considered statistically significant if P < 0.05. Electroretinographic data are presented as mean ± SEM. The Micron III fundus imaging system (Phoenix Research Laboratories, Pleasanton, CA, USA) was used to obtain digital images of the central retina.

**SD-OCT**

Spectral-domain optical coherence tomography (SD-OCT) was performed at 9 months post injection using a high-resolution SD-OCT instrument (Biotigen, Inc., Research Triangle Park, Durham, NC, USA) for image capture and to measure the thickness of the retina, as previously described. Neural retina thickness was determined by measuring the distance from the vitreal face of the ganglion cell layer to the retinal pigment epithelium apical side. Four measurements at the same distance from the optical nerve head were recorded from each eye and averaged. Statistical analysis was performed by paired t-test, with P < 0.05 considered statistically significant.

**RESULTS**

**Distribution of AAV-Expressed Wild-Type C1QTNF5 and S163R Mutant**

AAV2quadYF vectors containing either hC1QTNF5WT-HA or hC1QTNF5(S163R)-HA transgenes were delivered to wild-type mouse eyes in order to compare the distribution patterns of the wild-type and S163R mutant following specific RPE targeting, and to document potential pathological effects. In order to avoid detecting endogenous C1QTNF5, a C-terminal HA tag was added for the reliable detection of transgene expression by immunostaining in each case. We determined that wild-type C1QTNF5 was secreted apically from the RPE, reaching the outer limiting membrane and interdigitating with photoreceptor cells inner segments (Fig. 1A). Adeno-associated viral-expressed C1QTNF5 appears to diffuse further, beyond the outer limiting membrane, being detectable between photoreceptor nuclei (Fig. 1B).

In stark contrast to vector expressed wild-type protein, AAV-expressed S163R is predominantly retained within RPE cells, where it forms large spherical or oval-shaped intracellular aggregates (Figs. 1C–E). In some regions containing the aggregates, RPE microvilli were missing, as revealed by double immunostaining with an anti-MFRP antibody, which detects the endogenous MFRP found abundantly across the entire length of RPE apical processes (Fig. 1D). Aggregates of S163R are transparent when examined under brightfield microscopy (Fig. 1F). Interestingly, S163R mutant protein was also secreted toward the basolateral side of the RPE, where it formed a continuous layer (Fig. 2). Sections from AAV-S163R–treated eyes were examined at higher magnification (×100) using differential interference contrast (DIC) microscopy (Fig. 2A) and merged with the fluorescent image (Fig. 2B) to provide a clear view of the S163R extracellular basal deposit and globular aggregates. Transmission electron microscopy was also used to examine the ultrastructure of the RPE-Bruch’s membrane interface in S163R-injected eyes and confirmed that the S163R layer accumulates between the basement membrane of the RPE and its cell membrane, with large S163R aggregates being present inside the RPE cytoplasm (Figs. 2C, 2D).

Staining the AAV-S163R–treated sections with hematoxylin and eosin (H & E stain) showed that the S163R aggregates appear eosinophilic (pink; Fig. 3). Aggregates of S163R were found throughout the RPE after a single subretinal delivery of hC1QTNF5(S163R)-HA vector at 4 months post injection. The retinal pigment epithelium appears vacuolated in many regions, although some of these may be where the aggregates were lost during tissue processing (Fig. 3, center and right panels).

**Detection of S163R Deposits in Older Mice**

By 9 months post injection, AAV-S163R–treated eyes display regions of RPE thinning and atrophy, as well as photoreceptor cell loss, particularly in those areas where thick, dome-shaped extracellular basal RPE deposits are present (Fig. 4B). In contrast, uninjected control eyes show normal RPE and retinal morphology (Fig. 4A). The basal RPE deposits consistently formed a continuous layer in every AAV-S163R–treated eye (Figs. 4C, 4D), and were determined to consist of S163R mutant protein by immunofluorescence following staining.
with the anti-HA antibody (Fig. 5A, Supplementary Fig. S1). We also tested for the presence of lipids following staining of cryosections with oil red O, and did not detect lipid accumulation in the AAV-S163R–injected eyes (Supplementary Fig. S1).

The thick S163R deposits located between the Bruch’s membrane and the deteriorating RPE layer appear transparent under brightfield microscopy (Fig. 5B) and pink following H & E staining (Fig. 5C). Interestingly, the spherical S163R C1QTNF5 aggregates are mostly absent at this stage, suggesting...
that the RPE may have developed a mechanism for their transport and deposition on their basal surface. The remaining photoreceptors retained their outer segments, as demonstrated by immunostaining with a rod opsin antibody (Fig. 5D). Low-magnification images show that the S163R deposit above the RPE is continuous and widespread (Figs. 6A–C). The endogenous MFRP protein is expressed throughout the RPE apical membrane (Fig. 6C).

Detection of AAV-Expressed S163R C1QTNF5 by Western Blot Analysis

We also evaluated the properties of AAV-expressed S163R mutant protein by Western blot analysis of whole eyecup protein extracts (Fig. 7). The adeno-associated viral-delivered mutant protein was detected primarily as a single immunoreactive band corresponding to the expected size of C1QTNF5.
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(slightly larger than 25 kDa) under denaturing and reducing conditions (Fig. 7A). We found S163R protein was present as high molecular weight aggregates when eyecups were sonicated in a buffer without SDS, a property consistent with results from previous in-vitro studies6,23 (Fig. 7B, first lane). In contrast, the AAV-expressed wild-type C1QTNF5 was detected as primarily a monomer, dimer, and trimer, as well as less abundant higher order oligomers in samples processed without SDS (Fig. 7B, second lane). In the presence of SDS, AAV-expressed wild-type C1QTNF5 was detected primarily as a strong monomeric immunoreactive band, and as less abundant signals corresponding to oligomeric and posttranslationally modified forms in a smeared appearance of immunoreactive bands (Fig. 7B, last lane).

Noninvasive Assessment of Pathological Effects

Noninvasive approaches were also used to analyze the structural and functional consequences of the mutant S163R expression in the RPE (Fig. 8). Full field ERG analysis was performed under scotopic dark-adapted, and photopic light adapted cone-mediated conditions to evaluate the effects of S163R protein on retinal function (Fig. 8A). There was a small but significant decrease in b-wave amplitudes in treated versus untreated mouse eyes by 2 months post injection under scotopic conditions at all light-intensities (Fig. 8A, left panel). For example, AAV-injected eyes had a rod-isolated b-wave amplitude (at 0.02 scot cd s m−2) of 238.5 ± 19.46 μV, which was 20% lower than the untreated controls, with a maximum b-wave amplitude average of 296.5 ± 26.12 μV (Average ± SEM, n = 13, P = 0.001). At 5 months

FIGURE 5. Examination of S163R expression at 9 months following AAV-hC1QTNF5(S163R)–HA delivery. (A) Detection of the AAV-expressed S163R by immunofluorescence. Note the thick, continuous S163R basal deposit, which appears transparent in the brightfield above the pigmented RPE (B). (C) Staining with H & E showing the thick basal deposit above a highly vacuolated, deteriorated RPE. (D) Double-labeling with an antibody against rod opsin showing the outer segments (red), and anti-HA (green) detecting the thick S163R deposit basal to the RPE. Scale bars: 20 μm.

FIGURE 6. Widespread distribution of basal S163R deposits at 9 months following AAV-hC1QTNF5(S163R)–HA delivery. (A–C) Low magnification images of retinal sections showing continuous, widespread S163R expression (green) above the RPE by immunofluorescence (A, C). (B) Brightfield image showing the transparent basal S163R deposit between the choroid and the pigmented RPE. (C) The RPE apical membrane is shown in red as labeled by the anti-MFRP antibody.
post injection (Fig. 8A, right panel), the maximum ERG b-wave amplitudes reflecting the rod-mediated function (at 0.02 scot cd·s·m⁻² flashes) further declined, being approximately 30% lower relative to control eyes (average of 134.3 ± 14.59 \( \mu \text{V} \) in AAV-treated versus 186 ± 9.02 \( \mu \text{V} \) in contralateral eyes, \( n = 5, P = 0.036 \)). Maximum cone b-wave amplitudes recorded under photopic conditions were similar in treated and untreated eyes. Similar to other cases of RPE-based retinal degeneration, such as the rd6 model caused by lack of functional MFRP, cone-driven ERG amplitudes are initially less affected compared with that of rods, perhaps because cones can also rely on an alternate visual cycle using Müller cells.36,37

Pathological changes in vector-treated eyes were also seen by fundoscopy and OCT (Figs. 8B, 8C). Eyes treated with AAV-S163R display an abnormal fundus with disperse pigmentary changes (Fig. 8B, right panel). By imaging with OCT, retinal thinning was seen in treated eyes by 9 months post injection, consistent with a loss of photoreceptor cells relative to untreated partner retinas (Fig. 8C). Comparison of retinal thicknesses showed that AAV-S163R treated eyes were 0.137 ± 0.021 mm thick, while untreated contralateral controls were 0.206 ± 0.014 mm (average ± SEM, \( n = 4, P = 0.01 \)).

**DISCUSSION**

Although L-ORD is a rare inherited disorder, some of its clinical features resemble AMD, a very common retinal degenerative disease that causes progressive loss of central vision that represents a major cause of blindness in the elderly.2,38,39 Specifically, both L-ORD and AMD exhibit age-related formation of drusen between the RPE and Bruch’s membrane, a key predictor of eventual AMD.6 In spite of the identification of numerous mouse models that exhibit some aspects of AMD, none mimic the disease accurately due to the lack of an anatomical macula in rodents and the complex nature of the disease, which results from an incompletely understood interplay among multiple genetic and environmental factors.39–42 One feature that makes L-ORD a potentially informative model for AMD is its well-established monogenic etiology that leads to retinal pathology due to sub-RPE deposits

**FIGURE 8.** Evaluation of pathological effects following AAV-hC1QTNF5(S163R)-HA delivery using noninvasive approaches. (A) Comparison of retinal function in AAV-S163R-C1QTNF5-treated and untreated eyes following ERG analysis. Bar graphs show the maximum ERG b-wave amplitudes in AAV-S163R injected eyes (black) compared with untreated control eyes (gray) at 2 months (left panel) and 5 months (right panel) post injection. Maximum b-wave amplitudes in AAV-S163R injected eyes were significantly lower than untreated controls at all light intensities under scotopic conditions (\( P < 0.05 \)), and were similar under light-adapted conditions. (B) Fundus photographs show pigmentary abnormalities in AAV-S163R-injected eyes (right) in contrast to the normal untreated age-matched control (left). (C) Evaluation of AAV-S163R-treated eyes by OCT imaging. The OCT scan intersecting the optic nerve head shows the normal retinal laminar architecture in the untreated eye (left). The ONL thickness was markedly reduced in the AAV-S163R-treated eye (right) at 9 months post injection, with numerous irregular deposits visible.
that extend into the peripheral retina, causing widespread photoreceptor cell loss and RPE atrophy. Consequently, mouse models of L-ORD represent a novel platform for developing treatment strategies that may apply to key aspects of AMD as well.

Of the two knock-in mouse models of L-ORD generated to date, only one was found to display an ocular phenotype reflecting many of the early-stage characteristics of the disease, with the other model showing no abnormalities throughout its lifespan. Recombinant AAV vectors have several advantages over transgenic approaches, including the potential for quickly generating animal models, often with a faster time course of disease progression, controlling mutant protein expression using the appropriate vector serotype, promoter and titer, and producing graded levels of pathology, from early to late-stage disease phenotypes. Adeno-associated viral technology, combined with the use of an RPE-specific VMD2 (bestrophin) promoter, enabled us to examine the in vivo consequences of S163R C1QTNF5 mutant expression in wild-type mice. Our results show that S163R accumulates as intracellular aggregates within RPE cells, and gradually forms thick and widespread deposits on the basal side of the RPE. Interestingly, the S163R basal deposits are strikingly similar to those described in Efemp1 knock-in mice harboring the R345W mutation in the EFEMP1 protein, also known as fibrulin-3. Efemp1 knock-in mice are a model for Malattia leventinese, also known as Doyne honeycomb retinal dystrophy, an autosomal dominant inherited macular degenerative disorder that shares many similarities with AMD. Less prominent basal laminar deposits were also observed by electron microscopic evaluation in a previously characterized knock-in Ctrp5/−/− mouse model of L-ORD carrying the S165R mutation. The consequences of S163R C1QTNF5 overexpression were also examined in a previously described experiment using a lentiviral system. In that study, both photoreceptors and RPE appeared morphologically normal at 5 weeks following subretinal delivery in C57Bl6 mice. In addition to using a different delivery vector and promoter for transgene expression, most significantly, the postinjection analysis time was much longer in our study, with the phenotypic effects becoming readily apparent by 4 months post injection. Similarly to the lentivirus study, we did not see apparent changes at earlier time points by morphologic examination of the eyes using light microscopy, supporting the idea that the phenotype is moderately slow to develop.

Expressed AAV wild-type C1QTNF5 displays a directional secretion from RPE, moving apically toward photoreceptor inner segments, and reaching the outer limiting membrane, a pattern similar to that of the endogenous protein. Its interaction with MFRP and its polarized secretion toward the photoreceptor layer suggests that wildtype C1QTNF5 plays a significant role on the apical side of the RPE. Consistent with this view, S163R aggregates clearly have a detrimental impact on the apical RPE membrane, as seen by RPE microvilli being absent or sparse in some regions of AAV-hC1QTNF5(S163R)-HA treated eyes. The accumulation of mutant protein intracellular globules with time and the abnormal distribution of S163R toward the basal side of the RPE, suggest its presence could interfere with not only the transport of nutrients—including oxygen and vitamin A from the choroid to the photoreceptor layer—but also secretion of waste materials from the RPE, thus causing degeneration of both RPE and photoreceptor cells. Previous studies demonstrated that sub-RPE deposits from L-ORD patients are rich in oil red O-positive lipids, protein, and calcium. However, we did not detect the presence of lipids following staining of cryosections from AAV-S163R-injected mouse eyes, suggesting that the pathogenesis of L-ORD is incompletely recapitulated in this AAV-based mouse model.

Many factors that alter the stratified extracellular matrix at the RPE/choroid interface could lead to drusen formation. Critically, C1QTNF5 interacts with complement factor H, a secreted glycoprotein that functions as a negative regulator of complement activation. Single-nucleotide polymorphisms in genes encoding CFH and related complement proteins have been associated with an increased risk of developing AMD. In addition, other systemic factors may also contribute to the disease pathology in patients, as C1QTNF5 was found to circulate in the human sera with broad interindividual variation. C1QTNF5 has a collagen domain (residues 30–98) containing 23 uninterrupted Gly-X-Y repeats, and this is perhaps the reason why both S163R aggregates and basal deposits appear pink when stained with hematoxylin and eosin, similar to other collagen-containing structures. It is interesting to note that in L-ORD patients collagen deposits are detected within the inner part of the thick drusen accumulating between the basolateral side of the RPE and the Bruch’s membrane, suggesting that S163R deposits could be present at those sites in patients and act as a stimulus for drusen formation. By extension therefore, AAV-expressed S163R mutant, being misdirected toward the basal side of the RPE, may alter its normal binding to CFH, triggering complement activation over time, and thus accelerating the formation of the drusen seen in patients.

Protein aggregation is considered a pathological trigger in many neurodegenerative disorders, including Alzheimer, Parkinson, and Huntington diseases. Future experiments aimed at enhancing the autophagic pathway will determine if the disease progression can be slowed by increasing the ability of the RPE to degrade mutant S163R efficiently. Another approach is the use of small interfering RNAs to inhibit mutant protein expression.

In conclusion, we show that RPE-targeted expression of the S163R mutant of C1QTNF5 can generate a mouse model that displays pathological features similar to some of those found in patients with advanced L-ORD, including RPE thinning, RPE cell loss, and retinal degeneration. S163R mutant protein forms large, transparent, spherical aggregates throughout the RPE, and thick basal deposits, in contrast to the wild-type protein which is secreted in the opposite direction, toward the apical microvilli and photoreceptor cells. The mechanism leading to the formation of S163R basal deposits remains to be elucidated, but promises to yield valuable clues as to how to treat L-ORD and perhaps some aspects of AMD.

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