Neovascularization, Enhanced Inflammatory Response, and Age-Related Cone Dystrophy in the Nrl−/−Grk1−/− Mouse Retina

Rosanne M. Yetemian,1 Bruce M. Brown,1 and Cheryl M. Craft1,2

PURPOSE. The effects of aging and light exposure on cone photoreceptor survival were compared between mouse retinas of neural retina leucine zipper knockout (Nrl−/−) mice and double-knockout mice lacking G-protein–coupled receptor kinase 1 (Grk1−/−/Grk1−/−).

METHODS. Mice were reared in total darkness, ambient cyclic light, or constant light, and their retinas were evaluated from 1 to 9 months of age using immunohistochemistry, electron microscopy, and fluorescein angiography. Retinal gene expression and statistically significant probe sets were categorized using analysis software. Select gene expression changes were confirmed with quantitative RT-PCR.

RESULTS. In contrast to retinas from Nrl−/−, those from Nrl−/−Grk1−/− exhibit a progressive loss of the outer nuclear layer, retinal physiology deficits, and a higher rate of degeneration with increasing age that is independent of environmental light exposure. Changes in retinal neovascularization occur in the Nrl−/−Grk1−/− at 1 month, before the onset of significant cone functional deficits. Microarray analyses demonstrate statistically significant changes in transcript levels of more than 400 genes, of which the onecostatin M signaling pathway and the inflammatory disease response network were identified.

CONCLUSIONS. These data demonstrate that the loss of functional Grk1 on the enhanced S-cone Nrl−/− background exacerbates age-related cone dystrophy in a light-independent manner, mediated partly through the inflammatory response pathway and neovascularization. According to these findings, Grk1 helps to maintain a healthy cone environment, and the Nrl−/−Grk1−/− mouse allows examination of the alternative roles of Grk1 in cone photoreceptor homeostasis. (Invest Ophthalmol Vis Sci. 2010;51:6196–6206) DOI:10.1167/iovs.10-5452

Significant advances in bioinformatics have identified essential genetic links and characterized basic molecular mechanisms driving the components of the visual G-protein–coupled receptor (GPCR) signal transduction cascade leading to rod photoreceptor cell death. However, with a population of 3% to 5% cone photoreceptors in the mouse retina, the manifestations of GPCR cascade disruption on cones have only recently been studied with the help of the neural retina leucine zipper knockout (Nrl−/−) mouse model.1 In humans, a loss-of-function mutation in the NRL gene leads to an autosomal recessive disorder, enhanced S-cone syndrome, which causes an excess number of S cones. Symptoms include night blindness, variable loss of visual acuity, and visual field abnormalities.2

Because of a developmental cell switch from rod to short-wavelength (SWL) pigment photoreceptors, the Nrl transcription factor provides a unique opportunity with which to study cone phototransduction mechanisms given that rod phototransduction components, including rhodopsin, are absent. Photopic electrophysiological analyses of Nrl−/− retinas reveal that the amplitude of light-adapted electroretinographic (ERG) responses elicited by maximum stimulus are stable up to 31 weeks of age, suggesting cones survive without rod function.1 Extensive ultrastructural, biochemical, and electrophysiological characterization of the Nrl−/− mouse photoreceptors demonstrate that the photoreceptors have many of the phenotypic hallmarks of cones.1,3,4 Results from gene expression profiling of the Nrl−/− mouse retina with microarray technology (GeneChip; Affymetrix, Santa Clara, CA) further support the concept that the rod precursors in the Nrl−/− retina differentiate as cones instead of rods and therefore exhibit an enhanced S-cone population, with the caveat that they are mutant cones and different from those found in a wild-type (WT) retina.2

Another essential regulatory component in the phototransduction cascade is the serine/threonine G-protein–coupled receptor kinase 1 (Grk1; rhodopsin kinase), which is expressed in both rods and cones in rod-dominant human, monkey, and mouse5–8 and in cone-dominant chicken.9 Grk1 phosphorylation of light-activated rhodopsin is required for normal phototransduction inactivation in vivo.10,11 Although the conespecific Grk7 is expressed in various species,6,11–12 only Grk1 is expressed in the mouse photoreceptor. With the creation of a double-knockout mouse lacking both Grk1 and Nrl, the electrophysiological analyses of mouse cone responses clearly demonstrated that Grk1 plays a critical role in the phosphorylation of S and M opsins and in the inactivation and normal recovery of cone pigments.3,5,8

The Nrl−/−Grk1−/− mouse provides an invaluable model not only for the study of cone GPCR signaling pathways but also for the molecular balance between cone photoreceptor survival and death in inherited retinal diseases. Unlike the rod photoreceptors

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of the Grk1−/− mouse, which degenerate rapidly when exposed to light because of the inability to inactivate phototransduction,14 cones lacking Grk1 expression on the Nrl−/− background degenerate by apoptosis in the dark.15 The present study extends our initial observations and demonstrates that in addition to a delayed photoreceptor response, the absence of Grk1 expression leads to age-related and light-independent cone dystrophy with abnormal vascularization in the retinas of Nrl−/−/Grk1−/− mice compared with Nrl−/−. To facilitate the identification of potential genes involved in biological and disease pathways, we identified key molecular networks to characterize the Nrl−/−/Grk1−/− phenotype. Taken together, these data allow us to hypothesize that, aside from its known function in photoreceptor pigment recovery, Grk1 plays a functional role in the homeostasis of a healthy cone environment.

METHODS

Experimental Animals

All mice were treated according to the guidelines established by the Institute for Laboratory Animal Research and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The Institutional Animal Care and Use Committee of the University of Southern California approved all procedures involved in animal experiments. Control mice used for all experiments were C57/Bl6J (C57) mice that were born and maintained in 12-hour light/12-hour dark cycles. Knockout mice Nrl−/− (provided by Anand Swaroop) and Grk1−/− (provided by C.-K. Jason Chen) were created from a mixture of C57/Bl6J and 129/SvJ founders and are, therefore, on a similar mixed genetic background. The Nrl−/−/Grk1−/− mice were created by backcrossing Nrl−/− mice with Grk1−/− mice, as described previously.14 The Nrl−/− and Nrl−/−/Grk1−/− mice were born and maintained in total darkness, ambient light (5 lux white light measured at the cage level), 12-hour light/12-hour dark cycles, or constant bright light (approximately 8000 lux/white light measured at the cage level).

Electroretinography

Electroretinograms were recorded as previously described.15–17 The Nrl−/− and Nrl−/−/Grk1−/− mice maintained in total darkness, ambient (~5 lux) cyclic light, or bright (~8000 lux) constant light were recorded at 1, 2, 3, 5, 7, and 9 months of age. At least 10 mice were recorded for each genotype at each age. The Nrl−/− and Nrl−/−/Grk1−/− mice were dark adapted overnight, and their eyes were dilated with topical administration of phenylpropiolate (2.5%) and tropicamide (0.5%). Mice were anesthetized by intraperitoneal injection of ketamine hydrochloride (100 mg/kg body weight) and xylazine hydrochloride (10 mg/kg body weight), and their corneas were anesthetized with 0.5% topical tetracaine. Each mouse was placed in an aluminum foil-lined Faraday cage, and a DLT fiber electrode was placed on the right cornea. A platinum reference electrode was placed on the lower eyelid, and another ground electrode was placed on the ipsilateral ear. Photopic stimuli of 10 μs duration of a maximum light intensity (log 2.01 cd·s/m²) were delivered through a one arm of a coaxial cable using a xenon flash (PS22; Grass Instruments, West Warwick, RI). The cable delivered the flash 5 mm from the surface of the cornea, and a background light (200 cd/m²) with spectral peaks at 485, 530, and 543 nm and minimal transmission below 400 nm was used. Dark-adapted maximum responses were measured using the nonattenuated light stimulus. The b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave.

Eyecup Preparation

All mice were euthanized by overdose of carbon dioxide inhalation either at midday under fluorescent lights or in total darkness. Retinas were dissected either under room light using a dissecting scope or in total darkness with infrared goggles and an infrared-equipped dissecting microscope. Eyes from each genotype and age examined were enucleated, marked for orientation (if dissected in the light), and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for at least 2 hours. Each eyecup was washed twice in PBS for 5 minutes, then dehydrated in a solution of 50% (wt/vol) sucrose in PBS overnight at 4 °C. Each eyecup was then embedded in OCT media (Sakura Finetek, Torrance, CA)18 and frozen in liquid nitrogen. Retinal cryosections at 8-μm thickness were cut along the vertical meridian through the optic nerve and were placed on poly (L-lysine)-coated glass slides.

Retinal Histology

To examine the morphologic changes associated with a decrease in b-wave amplitude in Nrl−/−/Grk1−/− mice compared with Nrl−/− controls, six mice of both genotypes at 1, 5, and 9 months of age were used. Because the decrease in cone function by ERG analysis is light independent, retinal sections used in hematoxylin and eosin (H&E) studies were from mice born and maintained in ambient cyclic light.

Briefly, retinal sections were pre-rinsed in double-distilled water (ddH2O), then dipped in Harris hematoxylin for 45 seconds followed by a wash in ddH2O. Slides were subsequently dipped in acid alcohol (70% ethanol, 1% HCl) for 1 minute, washed in ddH2O, dipped five times in ammonium water (0.3% NH4OH), and washed. Slides were then dipped in eosin-philoxone for 1 minute, then dehydrated in a series of 95% ethanol and 100% ethanol followed by 6 minutes in xylene. Once the slides were dried, mounting medium was applied and slides were coverslipped.

Immunohistochemistry

Immunohistochemistry (IHC) is a well-established technique for the identification and localization of proteins expressed within the mouse retina. For all IHC experiments, our established protocol was followed with minor modifications.18 Frozen retinal sections of Nrl−/− and Nrl−/−/Grk1−/− mice were blocked in IHC blocking buffer (10% normal goat serum, 1% bovine serum albumin, 0.2% Triton X-100 in PBS) for 30 minutes, then incubated with the mouse anti-Arestin4 (Ar4) rabbit polyclonal antibody (pAb mCAR-LUMIJ) or the mouse anti-S or anti-M opsins rabbit polyclonal antibodies, as described previously.8 All three antibodies were diluted to 1:1000 in PBS and were incubated for 1 hour at room temperature. Animals were similarly stained using the mouse anti-VEGF antibody (Abcam, Cambridge, UK) at a dilution of 1:200 in PBS incubated overnight at 4 °C. After three 5-minute washes in PBS, the slides probed with pAb mCAR-LUMIJ and pAb-S or M-opsin were incubated in fluorescein anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. Slides were either mounted with mounting medium (Vectorshied; Vector Laboratories) for fluorescence with DAPI (Vector Laboratories) or incubated with monoclonal cyanine nucleic acid stain (To-Pro3 iodide; To-Pro3; Molecular Probes) for fluorescence with DAPI nuclear stain (Vector Laboratories). Slides were either mounted with mounting medium (Vectorshied; Vector Laboratories) or coveredslipped.

Slides prepared for IHC or H&E were photographed with a digital camera (SPOT model SP401-115, software version 3.5.0; Diagnostic Instruments, Inc., Sterling Heights, MI) mounted to a fluorescence microscope (DMR; Leica Microsystems, Wetzlar GmbH, Wetzlar, Germany).17

TUNEL Analysis of Apoptosis

Functional cone photoreceptor deficits in Nrl−/− and Nrl−/−/Grk1−/− mice were evident by 3 months; therefore, to verify cone apoptosis before outer nuclear layer (ONL) thinning, five 1-month-old mice from control C57/Bl6J, Nrl−/− and Nrl−/−/Grk1−/− genotypes each and three postnatal day (P) 21 mice from C57/Bl6J, Nrl−/− and Nrl−/−/Grk1−/− genotypes each were born and maintained in ambient cyclic light and euthanized in the light. Both eyes were fixed and embedded as described for retinal histology. Three adjacent sections cut through the optic nerve along the vertical meridian were used from each eye. Apoptotic cells were visualized by means of the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using a fluorescent TUNEL system (DeadEnd; Promega, Madison, WI) according to the manufacturer’s instructions.19 After labeling, the slides were mounted with mounting medium (Vectashield; Vector Laboratories) for fluorescence with DAPI nuclear stain (Vector Laboratories).
Apoptotic cells were quantified by counting TUNEL-positive cells from the entire section, and the average number of apoptotic cells per section was calculated from three sections of all eyes from each genotype. Statistical analysis was performed using the one-way ANOVA with Bonferroni’s correction for repeated measures.

**Microarray and Ingenuity Pathway Analysis**

Transcriptional variability when apoptosis was documented and before severe morphologic changes was examined in retinas from 1-month-old light-adapted *Nrl*−/− and *Nrl*−/−*Grk1−/− mice. Retinas were homogenized and total RNA isolated using a monophasic phenol and guanidine isothiocyanate solution extraction (TRIZol; Invitrogen) according to the manufacturer’s instructions. Retinas at this age also have ONLs of similar thickness, which allowed for comparable mRNA levels between the two strains. RNA purity and concentration were determined using spectrophotometry A260/A280 ratios. Microarray technology (Mouse Genome 430 2.0 GeneChips; Affymetrix, Inc.) was used for hybridization. RNA isolation and microarray hybridization were performed in triplicate for statistical background correction20 and quantile normalization algorithms.21 The log2-transformed data were then subjected to a two-way mixed-model analysis of variance (ANOVA) with a significance of canonical pathways was conducted using Fisher’s exact test by IPA.

**Endothelial Cell Staining**

To further characterize changes in retinal morphology, we used fluorescein-labeled GSL isoelectin B4 (Vector Laboratories) as a marker to selectively stain vascular cells. Retinas from P21 and 1-month-old and *Nrl*−/− and *Nrl*−/−*Grk1−/− mice raised in cycling light were dissected, fixed, and sectioned as described.8,22 Sections were dried, then blocked with 1% bovine serum albumin in PBS for 30 minutes. In a humidified chamber, slides were incubated with isoelectin B4 (1:1000) for 1 hour at room temperature, washed in PBS, and mounted with mounting medium (Vectashield; Vector Laboratories) for fluorescence with DAPI (Vector Laboratories) and coverslipped. For colocalization studies with VEGF, slides were incubated in a mixture of the mAb VEGF (1:200) and isoelectin B4 (1:100), followed by incubation with the AlexaFluor 568 goat anti–mouse secondary IgG (1:250; Invitrogen). Slides were photographed using a confocal laser scanning microscope (LSM 510; Zeiss, Oberkochen, Germany).

**Fluorescein Angiography**

Fluorescein angiography (FA) was used to visualize changes in retinal vasculature with increasing age in *Nrl*−/−, *Nrl*−/−*Grk1−/−, *C57/Bl6J*, and *Grk1−/−* mice. *Nrl*−/− and *Nrl*−/−*Grk1−/− mice were examined from 1, 3, 5, and 7 months of age, and *C57/Bl6J* and *Grk1−/−* mice were examined at 1 and 7 months of age. Before anesthesia, pupils were dilated with topical phenylephrine HCl (2.5%) and tropicamide (0.5%). After dilation, the mice were anesthetized with intraperitoneal injection of ketamine HCl (100 mg/kg body weight) and xylazine HCl (10 mg/kg body weight) and were kept on a heating pad for the duration of the experiment. The mice were placed in lateral recumbence with the visual axis of the eye upward. The focal axis of the hand-held camera (small animal camera; Kowa, Tokyo, Japan) was aligned with the visual axis of the eye. The rapid decrease of b-wave amplitude was elicited by high-intensity white fluorescent light. Note the rapid decrease of b-wave amplitude (approximately 40% from 1 to 3 months) with increasing age in the *Nrl*−/−*Grk1−/− mouse reared in the dark, in cyclic light, or in constant light.
Age-Related Light-Independent Cone Dystrophy

ERG analyses of retinal physiology and photopic function show that the b-wave amplitudes of dark-adapted ERG responses elicited by maximum light stimulus are largely stable until 7 months of age in Nrl−/− mice, regardless of the animals’ environmental lighting conditions (Fig. 1). In contrast, the photopic b-wave amplitude decreases rapidly in Nrl−/−/Grk1−/− mice in an age-dependent manner, with a 40% reduction in b-wave amplitude by 3 months of age (Fig. 1B). Importantly, the average b-wave amplitudes of 1-month-old Nrl−/−/Grk1−/− mice are similar to those of age-matched Nrl−/− controls in the three environmental light conditions tested (Fig. 1B). The ERG results illustrate a defect in cone function and suggest that the degeneration of cone photoreceptors in the Nrl−/−/Grk1−/− retina is related to increasing age under different light exposure, in contrast to the light-induced rod degeneration observed in the Grk1−/− mouse retina.14

Age-matched retinas of light-adapted Nrl−/− and Nrl−/−/Grk1−/− mice have a similar photoreceptor ONL thickness at 1 month (Figs. 2A, 2D); however, the Nrl−/−/Grk1−/− retinas had fewer rosettes and whorls at this age. At 5 months and older, the ONL appears thinner in the Nrl−/−/Grk1−/− mouse retina (Figs. 2E, 2F).15 Cone-specific Arr4 immunoreactivity and H&E staining confirm that the thinning of the retina in 5- and 9-month-old Nrl−/−/Grk1−/− mice is the result of cone photoreceptor loss (Figs. 2G–I).

Retinal cross-sections from Nrl−/− and Nrl−/−/Grk1−/− retinas were immunohistologically stained with pAB S- and M-opsin antibodies and confirmed our previous observations13 that both S and M cones degenerate in the Nrl−/−/Grk1−/− mouse retina in an age-dependent manner (Supplementary Fig. S1; Supplementary Figures and Tables available at http://www.iovs.org/content/51/12/6196/suppl/DC1). By 9 months of age, a significant loss of S- and M-opsin–expressing cones is observed in the Nrl−/−/Grk1−/− retinas compared with their age-matched Nrl−/− controls. Interestingly, more rosette structures are observed in the ONL of the Nrl−/− retina, which are reduced with age faster in Nrl−/−/Grk1−/− retinas (Supplementary Fig. S1).

Apoptosis of Cone Photoreceptors

TUNEL analysis was performed on frozen retinal sections from P21 and P30 C57/Bl6j (C57), Nrl−/−, and Nrl−/−/Grk1−/− mice born and raised in cyclic ambient light to further characterize photoreceptor degeneration. A representative picture of a segment from P21 Nrl−/−/Grk1−/− and P30 Nrl−/− and Nrl−/−/Grk1−/− retinal sections demonstrate that apoptotic cells were observed only in the ONL of both Nrl−/− and Nrl−/−/Grk1−/− mice (Figs. 3A–C) and were statistically significantly different from Nrl−/− mice at P50 but not at P21 (Fig. 3D). On quantification of TUNEL-positive cells and repeated-measures ANOVA with Bonferroni correction, the Nrl−/−/Grk1−/− mouse retina was found to have significantly more apoptotic cells than C57 control retinas at both P21 and P30, which is consistent with the slower retinal degeneration phenotype (P < 0.05).1 However, the Nrl−/−/Grk1−/− retina shows a statistically significantly greater number of apoptotic cells compared with both Nrl−/− (P < 0.01) and C57 (P < 0.001) controls at P30 but was only significantly different from C57 mice at P21. These results suggest that the cone photoreceptors of these mice die through apoptosis, and the degeneration is exacerbated after ablation of Grk1 that is slightly higher at P21 but is only significantly higher by P30.

Microarray and Ingenuity Pathway Analysis

To identify potential cellular pathways involved in the cone dystrophy in the Nrl−/−/Grk1−/− mouse, we performed mi-
creased in the Nrt<sup>−/−</sup> Grk1<sup>−/−</sup> compared with the Nrt<sup>−/−</sup> group. On examination of the transcripts with the highest fold increases, Pttg1 appears twice with a 130.7- and 70.0-fold increase (Table 1). The Pttg1 gene encodes for a transcriptional regulatory protein that can shuttle between the cytoplasm and nucleus with alternative roles in sister chromatid segregation. Interestingly, Crumbs homolog 1 (Crb1) transcript levels were decreased in Nrt<sup>−/−</sup> Grk1<sup>−/−</sup> mice approximately 6.5-fold compared with Nrt<sup>−/−</sup> mice. This deregulation of Crb1 and Pttg1 has been observed in two separate photoreceptor dystrophy models, the Crb1- and Ras protein-specific guanine nucleotide releasing factor 1 (RasGRF1) null mutant mice, both of which demonstrate dystrophic retinas and are proposed models for retinal degeneration at an early age. Abnormal rod and cone findings on electroretinography and abnormal vascular endothelial growth factor (VEGF) expression studies were performed for TUNEL staining. Shown are images of the middle superior region of the retina. TUNEL-positive apoptotic cells (green) were counted under a fluorescence light microscope. The total number of TUNEL-positive cells from three sections of each mouse was recorded, and the average count (mean ± SEM) was calculated from three mice using one-way ANOVA of the same genotype (D). *P < 0.05; **P < 0.01; ***P < 0.001. Scale bar, 20 μm.

**Table 1.** Comparison of Upregulated Transcripts between Nrt<sup>−/−</sup> Grk1<sup>−/−</sup> and Nrt<sup>−/−</sup> Retinas

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<th>Symbol</th>
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naling, and nervous system development and function were ranked as the top categories according to \( P \) value and number of molecules categorized (Supplementary Table S2). The top canonical pathway identified was the oncostatin M signaling pathway \((P = 5.1 \times 10^{-4})\), which activates the Janus kinase (JAK)-signal transducer and activator of transcription 1 (STAT) signaling machinery in some retinal degenerative disease models.25,29 Other key canonical pathways associated with the \( Nrl^{-/-} Grk1^{-/-} \) retinal dystrophy model include Synaptic Long-Term Potentiation Signaling, the Pentose Phosphate Pathway, Rac signaling, and Ciliary Neurotrophic factor (Cntf) Signaling (Supplementary Fig. S2). The top networks identified with their respective number of focus molecules are presented in Supplementary Table S3. Of these identified networks, the Inflammatory Disease, Inflammatory Response network was the most relevant to our model system because of its known association with choroidal neovascularization. The upregulated transcripts from this network were organized into a schematic diagram and are presented in Figure 4. Interestingly, upregulation of Klk2 was identified in this pathway, as was the Oncostatin M signaling cascade with increased transcription of Stat1 along with other inflammatory response genes.

**Confirmation of the Gene Expression Changes by Quantitative PCR**

Quantitative PCR was performed for select genes of interest, Ptgs2 and Klk2, (Fig. 5) to validate the microarray results (Supplementary Table S1), and similar significant increases in both transcripts were observed compared with the \( \beta \)-actin reference standard. Notably, a similar trend in upregulation of both transcripts was observed in \( Grk1^{-/-} \) compared with WT retinas.

**Neovascularization**

The identification of the Inflammatory Disease, Inflammatory Response Network by IPA as one of the top networks led us to examine \( Nrl^{-/-} Grk1^{-/-} \) retinas for neovascularization as a contributor to cone photoreceptor cell death because of its association with CNV. Endothelial cell staining using isocitulin B4 and neovascularization testing using FA were performed to analyze changes in retinal vasculature with increasing age. Isocitulin B4 staining (green) was observed in the inner nuclear layer (INL) and inner plexiform layer (IPL) in 1-month-old \( Nrl^{-/-} \) mice (Fig. 6). The morphology and retinal vasculature are similar to those of healthy retinas and are consistent throughout the entire section of \( Nrl^{-/-} \) controls. Age-matched \( Nrl^{-/-} Grk1^{-/-} \) mice reared under the same lighting conditions, however, exhibit choroidal anastomoses that infiltrate the retinal pigment epithelium (RPE). Blood vessels are present in the choroid, INL, outer plexiform layer (OPL), IPL, and inner limiting membrane (ILM). Compared with the controls, \( Nrl^{-/-} Grk1^{-/-} \) mice have atrophied RPE cells and breaks in the RPE monolayer coupled with anastomoses forming from both the choroid and the inner retina (Fig. 6). These studies demonstrate that by 1 month of age, \( Nrl^{-/-} Grk1^{-/-} \) retinas have blood vessel infiltration that penetrates all retinal layers (Fig. 6).

To correlate the observed photoreceptor TUNEL staining with choroidal anastomoses, we performed isocitulin B4 IHC on P21 \( Nrl^{-/-} Grk1^{-/-} \) and compared them with P30 \( Nrl^{-/-} Grk1^{-/-} \) sections. Supplementary Figure S3 demonstrates that overall, these mice have less blood vessel penetration from the choriocapillaris and have an unbroken RPE monolayer coupled with anastomoses forming from both the choroid and the inner retina (Fig. 6). These studies demonstrate that by 1 month of age, \( Nrl^{-/-} Grk1^{-/-} \) retinas have blood vessel infiltration that penetrates all retinal layers (Fig. 6).

**Table 2. Comparison of Downregulated Transcripts between \( Nrl^{-/-} Grk1^{-/-} \) and \( Nrl^{-/-} \) Retinas**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Entrez Gene Name</th>
<th>Fold</th>
<th>Change</th>
<th>ProbeSet ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grk1</td>
<td>G protein-coupled receptor kinase 1</td>
<td>9.36E-04</td>
<td>-72.593</td>
<td>1421361_at</td>
</tr>
<tr>
<td>Zn26</td>
<td>Zinc finger protein 26</td>
<td>3.73E-04</td>
<td>-30.101</td>
<td>1444902_at</td>
</tr>
<tr>
<td>Zfip874</td>
<td>Zinc finger protein 874</td>
<td>2.13E-04</td>
<td>-29.670</td>
<td>14354171_at</td>
</tr>
<tr>
<td>Skit212</td>
<td>Superkiller viridical activity 2 like 2 (Saccharomyces cerevisiae)</td>
<td>1.48E-04</td>
<td>-28.664</td>
<td>1475171_at</td>
</tr>
<tr>
<td>9430027B09RIK</td>
<td>RIKEN cDNA 9430027B09 gene</td>
<td>2.64E+03</td>
<td>-21.358</td>
<td>1454232_at</td>
</tr>
<tr>
<td>Mmt7</td>
<td>Myotubularin-related protein 7</td>
<td>1.67E-03</td>
<td>-19.189</td>
<td>1478313_at</td>
</tr>
<tr>
<td>Rbm45</td>
<td>RNA-binding motif protein 45</td>
<td>1.97E-03</td>
<td>-12.899</td>
<td>1453704_at</td>
</tr>
<tr>
<td>Aebp10</td>
<td>ATP-binding cassette, subfamily B (MDR/TAP), member 10</td>
<td>2.15E-03</td>
<td>-11.188</td>
<td>1416403_at</td>
</tr>
<tr>
<td>Ddoc2B</td>
<td>Doc1-related domain-containing 2B</td>
<td>2.18E-03</td>
<td>-9.736</td>
<td>1444714_at</td>
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<tr>
<td>Mat267</td>
<td>Mitogen-activated protein kinase kinase 7</td>
<td>1.36E-03</td>
<td>-9.381</td>
<td>1457182_at</td>
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<tr>
<td>C1orf47</td>
<td>Chromosome 4 open reading frame 47</td>
<td>2.31E-05</td>
<td>-8.472</td>
<td>1453168_at</td>
</tr>
<tr>
<td>Yipf4</td>
<td>Yip1 domain family, member 4</td>
<td>3.40E-04</td>
<td>-8.031</td>
<td>1426174_at</td>
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<tr>
<td>Elat11</td>
<td>ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R)</td>
<td>5.84E-04</td>
<td>-6.986</td>
<td>1404064_at</td>
</tr>
</tbody>
</table>

VEGF is a hypoxia-induced cytokine that is strongly implicated in angiogenesis by virtue of its absolute requirement for retinal vasculization and its expression in spatial and temporal conjunction with developing retinal blood vessels.30 Therefore, IHC staining using an anti-VEGF antibody on P21 \( Nrl^{-/-} \) and \( Nrl^{-/-} Grk1^{-/-} \) retinas was performed. The level of VEGF

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expression in P21 Nrl<sup>−/−</sup> Grk1<sup>−/−</sup> mice is more apparent than the staining pattern observed in Nrl<sup>−/−</sup> mice of the same age (Fig. 7A). A control section without the primary antibody was performed to confirm that staining was not due to secondary background (data not shown). These results indicate an enhanced level of VEGF expression in Nrl<sup>−/−</sup> Grk1<sup>−/−</sup> mice that begins as early as P21. The section was focused on the nuclear plane; however, VEGF expression was localized throughout the retinal section but was highest in the ILM located below the IPL.

A higher magnification image of VEGF expression in the endothelial cells of a P21 Nrl<sup>−/−</sup> Grk1<sup>−/−</sup> retina is provided in Figure 7B. The staining pattern indicates that VEGF levels are enhanced and highly expressed near the blood vessels of these mice, particularly in the branching segments of the vessel. Age-matched Nrl<sup>−/−</sup> mice did not exhibit VEGF expression within their blood vessels (Fig. 7A).

Gross progressive changes that occur as a consequence of blood vessel penetration were examined using FA on Nrl<sup>−/−</sup> and Nrl<sup>−/−</sup> Grk1<sup>−/−</sup> mice at 1, 3, 5, and 7 months of age. Nrl<sup>−/−</sup> mice exhibit no detectable leakage and have healthy retinal vasculature at all ages examined (Fig. 8). In contrast, age-matched Nrl<sup>−/−</sup> Grk1<sup>−/−</sup> mice have a moderate level of leakage beginning at 1 month in the right upper quadrant (arrows), and by 3 months an excessive amount of leakage is observed that progresses with age (Fig. 8). FA staining on P21 Nrl<sup>−/−</sup> Grk1<sup>−/−</sup> mice could not be performed because of their small size. Leakage is first noticed in the right quadrant and spreads throughout the retina by 5 months (data not shown). Conversely, the retinas of C57 and Grk1<sup>−/−</sup> mice at both 1 month and 7 months of age demonstrate healthy vasculature in all animals tested (Supplementary Fig. S4).

**DISCUSSION**

The absence of Grk1 in retinas leads to a rapid light-dependent retinal degeneration of rod photoreceptors because of a defec-
and reduction in visual quality. We believe that the differences in both the rod-dominant and the "all-cone" mice lacking Grk1 expression degenerate with increasing age in a manner that is independent of their environmental light exposure (Fig. 1). Furthermore, we established that even when the Nrl-/- Grk1-/- mice are maintained in darkness, their retinas exhibit cone functional deficits and slower morphologic changes leading to the inevitable loss of the nuclei in the ONL (Fig. 2). The first indication of a defective photopic ERG function was apparent at 3 months in mice reared in all lighting conditions tested and led us to examine the molecular and morphologic changes at 1 month, when photopic ERG responses are similar to those of Nrl-/- controls (Fig. 1). Despite the well-characterized essential function in phototransduction recovery of Grk1, our data suggest alternative functions for Grk1, either directly or indirectly, in the homeostasis and survival of cone photoreceptors. Our results strongly suggest that even with a defective phototransduction shutoff pathway, the Nrl-/- mice are resistant to light-mediated damage, and the cone dystrophy is a manifestation of an alternative mechanism.

In most retinal degeneration (rd) models, cone cell death is often secondary to rod cell death because of the release of endotoxins from degenerating rods, environmental alterations, or deprivation of a rod-derived trophic factor. The loss of cone function, however, results in the more severe disability and reduction in visual quality. We believe that the differences found between the Grk1-/- and Nrl-/- Grk1-/- phenotypes are a demonstration of the dynamic role and protective mechanisms rods use in the prevention of cone cell death.

Grk1 expression is essential for light-dependent phosphorylation of SWL and medium wavelength (MWL) pigments in the mouse retina and controls the rapid rate of cone recovery analogous to its rod function. Immunohistochemical studies using antibodies for S- or M-opsin plus Arr4 prove that both SWL- and MWL-expressing photoreceptors of these mice (Supplementary Fig. S4) and do not exhibit a fluorescein dye leakage like that of Nrl-/- Grk1-/- retinas, vascular cell staining and FA experiments were performed. Nrl-/- Grk1-/- mice harbor breaks in the RPE with fluorescein-labeled GSL isoclect B4 (green) shows penetration of blood vessels into the retina from the choroid (arrows) in Nrl-/- Grk1-/- mice. There are two known types of neovascularization in the retina: retinal neovascularization (RNV) and choroidal neovascularization (CNV). RNV is characterized by sprouting blood vessels that penetrate the ILM and grow into the vitreous, angiogenesis is specific to the loss of Grk1 in Nrl-/- mice. Retinas were harvested from 1-month-old C57 mice are resistant to ischemia, VEGF expression, hyperoxia-, and hypoxia-mediated gene regulation the key players in the characterization of the disease. In Nrl-/- Grk1-/- mice, we observed an
enhanced level of VEGF expression beginning at P21 that localized to both the blood vessels and the ILM (Fig. 7).

Alternatively to RNV, the molecular participants in the pathogenesis and stimulation of CNV are less well understood. One possible contributor to CNV is inflammation. Previous reports have indicated that deposits in and around Bruch’s membrane and the ILM (Fig. 6) and a subsequent increase in blood vessel penetration after an insult, in this case the loss of Grk1. We also observed a decrease in expression of Crb1 and Pttg1, with an up-regulation of more than 29-fold that was validated by qPCR (Table 1; Fig. 5B), is an autoantigen that was recently described as an inducer of Sjögren’s syndrome-like keratoconjunctivitis sicca in Lewis rats. Tissue kallikreins inhibit apoptosis and promote cell survival though the activation of the mitogen-activated protein kinases p44/p42 and p42/p44 (Erk1/2) signaling pathways. We believe that the upregulation of Klk2 and Pttg1 is noteworthy because of the high AFC values for each and for their prevalence in microarray studies from other mouse models of retinal degenerative disease; nevertheless, the precise role and causal mechanisms behind differential expression of these transcripts require further study.

ONcostatin M signaling was the top pathway identified (Supplementary Fig. S2) and is a well-characterized mechanism for both light-mediated and inherited forms of retinal degeneration. Previously, the retinal degeneration 1 (rd1) mouse, with a mutation in the β-subunit of phosphodiesterase leading to a rapid degeneration of photoreceptors, and the VPP mouse, a transgenic strain carrying three rhodopsin mutations (V20G, P23H, P27L) leading to a slow degeneration, were examined for JAK-STAT signaling as independent forms of inherited retinal degeneration. Phosphorylation of STAT3 and viral proto-oncogene 1 (Akt), along with an induction of leukemia inhib-
ity factor (LiF), Cntf, fibroblast growth factor-2, OsMr, Gp130, and Ch3H1, are some of the family members of the oncostatin pathway that are upregulated in both mouse models and can be identified in the immune response pathway found in the Nrl/−/−Grk1/−/− retina (Tables 1, 2; Fig. 6). It is unclear from the microarray and qPCR data which cell types exhibit upregulation or downregulation of the transcripts characterized; however, we postulate that these changes in gene expression are associated in the cone photoreceptor degeneration observed in the Nrl/−/−Grk1/−/− mouse. We understand the challenging caveats when identifying potential signal transduction pathways using either microarray studies or proteomic approaches because of the volume of information acquired using either technique. However, data presented here lay the groundwork for further studies.

Grk1 is only one of at least seven members of the Grk superfamily expressed in the mouse retina and pineal gland, including Grk2. The ubiquitous Grk2 may be a relevant modulator of inflammatory responses because of its ability to attenuate chemokine-induced migration in T cells and monocytes. Chronic downregulation in Grk2 protein expression in immune cells leads to an aberrant inflammatory response. Despite the limitations of studying a model that does not mimic the natural structure of a rod-dominant retina, the Nrl/−/−Grk1/−/− mouse is an invaluable tool that demonstrates the relevance of Grk1 in cones and supports the theory that Grk1 has alternative roles in the retina, analogous to Grk2, in the regulation of inflammatory response genes in maintaining healthy cone structure and function.

In conclusion, we have identified a retinal neovascularization phenotype that most closely resembles retinal angiomaticous proliferation in AMD. Although future studies are required, our working hypothesis is that Grk1 ablation in cones leads to a hypoxic or metabolically compromised environment that subsequently stimulates increased blood vessel penetration into the retina, leading to increased cone apoptosis. It is important to note that the OS discs of Nrl/−/− mice have been characterized to misalign and abnormally associate with the RPE monolayer, which may facilitate blood vessel penetration by way of a weakened Bruch’s membrane. Closer inspection of the membrane itself must be conducted to further examine this possibility. Forthcoming studies will extrapolate the causal relationship and relevance of Grk1 in the cone photoreceptor and will provide a model for potential pharmacologic interventions to either slow or rescue photoreceptors from cell death.

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References


