Characterization of a Dominant Cone Degeneration in a Green Fluorescent Protein–Reporter Mouse with Disruption of Loci Associated with Human Dominant Retinal Dystrophy

Daniel M. Lipinski,¹ Mohammed Yusuf,² Alun R. Barnard,¹ Christopher Damant,¹ Peter Charbel Issa,¹ Mandeep S. Singh,¹ Edward Lee,³ Wayne L. Davies,^{1,3} Emanuela V. Volpi,² and Robert E. MacLaren^{1,3}

PURPOSE. To characterize anatomically and functionally the retinal degeneration observed in a transgenic mouse line (OPN1LW-EGFP) expressing enhanced green fluorescent protein (EGFP) in a subpopulation of cone photoreceptors, and to map the location of the transgenic insertion.

METHODS. An anatomic comparison of cone survival was carried out between wild type (WT) and transgenic mice at three postnatal time points (P80, P140, and P245). Retinal function was assessed at P245 by ERG and included an ultraviolet flicker stimulus to isolate S-cone function. Chromosomal mapping by FISH and high-resolution mapping on DNA fibers (Fiber-FISH) were performed to identify the location of the transgenic insertion.

RESULTS. GFP expression was largely absent in S-cones. Cone numbers were significantly reduced in OPN1LW-EGFP mice at all time points compared to WT, with cone loss independent of GFP expression. Anatomic loss correlated with a functional deficit in dark- and light-adapted ERG responses, including a reduction in UV-flicker response, confirming the degeneration of S-cones. The phenotype of heterozygote mice was slightly less severe than in homozygotes, consistent with a dominantly inherited cone dystrophy. The transgenic insertion mapped to a specific region on chromosome 10 orthologous with loci for progressive bifocal chorioretinal atrophy and North Carolina macular dystrophy on human chromosome 6.

CONCLUSIONS. Cone loss is global in OPN1LW-EGFP mice and is independent of GFP expression. The mechanism underlying the degeneration remains elusive; however, disruption of loci associated with dominantly inherited retinal degenerations in humans makes this mouse of great interest. (*Invest Ophthalmol Vis Sci.* 2011;52:6617–6623) DOI:10.1167/iovs.11-7932

Inherited retinal degenerations, such as RP, affect an estimated 1 in 3000 individuals worldwide.¹ The degeneration of photoreceptors in these conditions typically results in severe visual impairment that carries with it a high economic and social cost. In many cases, the molecular mechanisms that cause photoreceptor degeneration are unknown; however, the use of animal models, specifically transgenic reporter lines, are key to understanding the genetic mechanisms that mediate retinal disease.

Because of its short half-life, enhanced green fluorescent protein (EGFP) is observed only in living cells where protein production is maintained at a constant level, making it a commonly used reporter for the study of tissue development and cell survival.^{2–4} Transgenic lines expressing GFP in retinal cell types⁵ have proven invaluable for the study of photoreceptor survival in vivo, where the unique properties of the eye allow for repetitive autofluorescence imaging⁶ and for examinations of donor cell engraftment in models of photoreceptor transplantation.⁷ In addition to a rod pigment (encoded by the *Rho* gene), mice possess two cone subpopulations that express SWS (S-cone) and MWS (M-cone) photopigments, encoded by *Opn1sw* and *Opn1mw* genes, respectively. A mouse model expressing EGFP driven by the human OPN1LW promoter was described previously, where EGFP expression is present in cone photoreceptors expressing MWS opsin.⁸ Although MWS opsin protein can be detected to some extent in the majority of cone photoreceptors across the mouse retina,⁹ in the human OPN1LW-EGFP reporter mouse, greater numbers of EGFP-expressing cells are observed in the dorsal retina than the ventral retina.⁸ Over time, a gradual loss of EGFP-expressing cells has been observed in OPN1LW-EGFP mice in vivo.⁶ This cone degeneration has been attributed to EGFP-mediated toxicity, as observed in other tissues.¹⁰ However, EGFP expression has not previously been associated with toxicity in the rodent retina,^{11,12} raising the question of whether EGFP expression is the direct cause of the observed cone loss.

We set out to investigate the contribution of EGFP toxicity to cone degeneration through anatomic and functional characterization of the OPN1LW-EGFP transgenic mouse. An anatomic quantification of cone survival over 9 months was performed. Light- and dark-adapted ERG were used to examine

From the ¹Nuffield Laboratory of Ophthalmology and Oxford Eye Hospital Biomedical Research Centre, University of Oxford, John Radcliffe Hospital, Oxford, United Kingdom; ²Wellcome Trust Center for Human Genetics, University of Oxford, Oxford, United Kingdom; and ³Moorfields Eye Hospital and University College London Institute of Ophthalmology Biomedical Research Centre, London, United Kingdom.

Supported by Fight for Sight, the Wellcome Trust (Grants 075491/Z/04 and 086868/Z/08/Z), Health Foundation, Medical Research Council, Royal College of Surgeons of Edinburgh, Oxford Stem Cell Institute, and the National Institute for Health Research Ophthalmology and Oxford Biomedical Research Centres.

Submitted for publication May 24, 2011; revised June 11, 2011; accepted June 13, 2011.

Disclosure: D.M. Lipinski, None; M. Yusuf, None; A.R. Barnard, None; C. Damant, None; P.C. Issa, None; M.S. Singh, None; E. Lee, None; W.L. Davies, None; E.V. Volpi, None; R.E. MacLaren, None

Corresponding author: Robert E. MacLaren, Nuffield Laboratory of Ophthalmology, University of Oxford, The John Radcliffe Hospital, Headley Way, Oxford OX3 9DU; enquiries@eye.ox.ac.uk.

cone function in OPN1LW-EGFP animals compared to wild type (WT) controls, including a light-emitting diode (LED)-based approach using short wavelength flicker stimulus to preferentially activate cones in the ventral retina (S-cones).

An alternative cause for the observed cone degeneration was also explored. FISH was used to locate the chromosomal position of the transgenic insertion and to examine the potential disruption of a cone-specific locus as an underlying cause of the observed degeneration.

METHODS

Mice

All animal experiments were performed in compliance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. B6.Cg-Tg (OPN1LW-EGFP) mice have been described previously.⁸ Mice homozygous for the transgenic insertion are herein referred to as B6^{TgOPN1LW-EGFP+/+}, and mice heterozygous for the transgenic insertion referred to as B6^{TgOPN1LW-EGFP+/-}, where B6 is reference to the congenic strain. Founders of the colony were a kind gift from Rachel Pearson of the University College London Institute of Ophthalmology (originally obtained under MTA from Mutant Mouse Regional Resources Centre [MMRRC] code MMRRC: 000,043-MU Opm1.gfp). WT animals (C57Bl/6) were provided by the University of Oxford biomedical sciences division. F1 heterozygote mice were created by crossing B6^{TgOPN1LW-EGFP+/+} mice with C57Bl/6 WT mice. Mouse genotype was determined by PCR as previously described.⁸

Morphologic Studies

Histologic characterization of cone survival was performed on eyes harvested from OPN1LW-EGFP transgenic and WT mice at P80, P140, and P245 time points (see Supplementary Data, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7932/-DCSupplemental>). Immunostaining for SWS opsin (1:1500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and MWS opsin (1:1000 dilution; Millipore Corporation, Billerica, MA) was performed free floating on whole retinas which had been separated from the RPE. Automated quantification of cone number was carried out on multiple nonoverlapping fields ($\times 20$ objective) per retina.

ERG

ERG recording was carried out according to a standard protocol (see Supplementary Data, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7932/-DCSupplemental>) using an electroretinography system (Espion E2; Diagnosys LLC, Cambridge, UK). Animals with late-stage degeneration (P245) were used to establish the correlation between anatomic cone loss and retinal function. For dark-adapted responses, brief (4 ms) white flash stimuli were delivered over a six-log intensity series (-4 to 1 log cd.s/m²). For dim stimuli (-4 to -3 log cd.s/m²), 10 responses were averaged with an interstimulus interval (ISI) of 5 seconds; for all other stimuli (-2 to 1 log cd.s/m²), five responses were averaged with an ISI of 10 or 20 seconds. Dark-adapted flicker ERGs were carried out using 513- and 365-nm stimuli. Continuous 15 Hz and 30 Hz stimuli were delivered, and 20 response epochs (500 ms) averaged for each condition. The 513-nm (green) flicker stimulus intensity was 3 cd.s/m². Photometric units are inappropriate for quantification of the 365-nm (UV) flicker stimulus (because luminosity functions used do not extend below 400 nm). However, during preliminary testing, the forward voltage of the LED-array was altered so the UV flicker output evoked a response amplitude similar to the green flicker stimulus. For light-adapted responses, white flash stimuli of 3, 10, and 25 cd.s/m² were superimposed on a 30 cd/m² white background with 20 responses averaged using an ISI of 1 second. a- and b-wave amplitudes (flash stimuli), and trough-to-peak amplitudes (flicker stimuli) were quantified with software (Espion; Diagnosys LLC).

FISH

Chromosome slides for metaphase FISH analysis and high-resolution mapping on DNA fibers were obtained from short-term fibroblast cell cultures established from ear explants as previously described.¹³ To identify the chromosome holding the transgenic insertion(s), a clone for the OPN1LW gene and its upstream region from a human fosmid library was labeled and cohybridized to metaphase spreads with a panel of differently labeled mouse chromosome-specific probes. Once the chromosome holding the transgenic insertion was identified, the OPN1LW probe was cohybridized in dual color FISH experiments with a number of mouse fosmid and BAC clones mapping in the chromosomal region of interest. Clones were selected for their potential interest on the basis of both their cytogenetic band location and gene content.

Statistical Analysis

Two-way ANOVAs and *t*-tests were performed on full datasets to detect significant differences in the mean. Photoreceptor numbers were compared using age and genotype or opsin type as factors. ERG amplitudes were compared using genotype and light intensity or flicker rate as factors. Bonferroni post-hoc tests were applied in all instances. *P* = 0.05 was considered statistically significant.

RESULTS

Extent of Cone Loss is Identical in Dorsal and Ventral Cone Populations

We initially confirmed previous reports that GFP expression is observed in a greater number of cones in the dorsal retina compared to the ventral. Immunostaining on flat mounted retinas of young (< 2 month) B6^{TgOPN1LW-EGFP+/+} mice for the presence of MWS and SWS opsin allowed the quantification of total cone numbers across the retina regardless of cone class. The number of GFP-expressing cones was found to be significantly higher in the dorsal retina ($50 \pm 5\%$) than in the ventral retina ($9 \pm 3\%$; *P* = 0.005, two-tailed *t*-test; Figs. 1A, 1B).

The number of cone photoreceptors in the dorsal and ventral retina of WT and B6^{TgOPN1LW-EGFP+/+} mice was compared at three ages (P80, P140, and P245) by immunostaining for SWS and MWS cone opsins. WT mice showed no significant (ns) reduction in the number of cone photoreceptors in either the dorsal or ventral retina over the time period, indicating an absence of cone loss in WT mice up to 9 months of age (*P* > 0.05, two-way ANOVA; Figs. 1C, 1D). In contrast, a significant reduction in total cone number was observed in the ventral and dorsal retina of B6^{TgOPN1LW-EGFP+/+} mice over the same time period (*P* < 0.001 both groups, two-way ANOVA; Figs. 1C, 1D). There was no significant difference between the number of cone photoreceptors remaining in the dorsal and ventral retinas of B6^{TgOPN1LW-EGFP+/+} mice at P245 (*P* > 0.05, two-way ANOVA). In addition, the ratio of EGFP to non-EGFP expressing cone photoreceptors remained consistent throughout the time course in both the dorsal and ventral retina (Figs. 1C, 1D), indicating that cone degeneration occurs independently of EGFP expression.

The uniformity of loss across the retina strongly contraindicates the role of EGFP toxicity in cone degeneration, where significantly fewer cones express EGFP in the ventral than dorsal retina. Rod photoreceptors were not quantified in this study. However, the gross retinal morphology of B6^{TgOPN1LW-EGFP+/+} mice revealed no appreciable thinning of the outer nuclear layer compared to WT controls (data not shown), indicating that the observed cone degeneration is not secondary to the loss of rod photoreceptors.

FIGURE 1. Quantification of EGFP-expressing cone photoreceptors in the B6^{TgOPN1LW-EGFP/+} mouse retina (**A**) reveals significantly higher numbers of EGFP expressing cells in the dorsal retina compared to the ventral (**B**; ** $P < 0.01$, one-tailed t -test; error = SD; $n = 3$ mice). Immunohistochemical double labeling for MWS and SWS opsin allows quantification of cones across the whole retina. There is a significant reduction in total cone numbers in both the dorsal (**C**) and ventral (**D**) retina of B6^{TgOPN1LW-EGFP/+} mice in comparison to C57Bl/6 WT at all time points. The ratio of GFP-expressing cones (GFP+ve) to non-GFP expressing cones (OPN1LW) in OPN1LW-EGFP mice does not alter significantly over the time course (**C**, **D**). There is no significant decline of cone photoreceptors in the C57Bl/6 WT animals. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Two-way ANOVA with genotype and cone number as factors. Error = SD, $n = 3$ mice per group. Scale bar: (**B**) 50 μm; (**C**, **D**) 100 μm.

Dark- and Light-Adapted Flash ERG Reveals Significant Deficits in Cone Function in OPN1LW-EGFP Mice

We sought to correlate the observed anatomic loss of cone photoreceptors in OPN1LW-EGFP transgenic mice with functional response. WT, B6^{TgOPN1LW-EGFP/+}, and B6^{TgOPN1LW-EGFP/+} mice were examined at P245, the time point at which the anatomic degeneration was most pronounced and functional deficits had previously been reported.⁶

The dark-adapted flash series revealed no significant difference in a-wave amplitude across all intensities (−4 to 1 log cd.s/m²) between WT and transgenic animals ($P > 0.05$, two-

way ANOVA; Fig. 2A). Because the murine retina is rod-dominated (97% rods vs 3% cones), the a-wave is primarily a rod-driven response.¹⁴ Therefore, the absence of any differences in the a-wave amplitudes between WT and OPN1-EGFP transgenic mice strongly supports the morphologic evidence that there is no detectable degeneration of rod photoreceptors.

Dark-adapted b-waves showed a small but statistically significant reduction in amplitude in both B6^{TgOPN1LW-EGFP/+} and B6^{TgOPN1LW-EGFP/+} mice compared to WT mice at higher intensities ($P < 0.01$, two-way ANOVA; Fig. 2A). This might be expected because an increasing contribution from cones pathways at higher light intensities.¹⁵ Post-tests revealed no significant dif-

FIGURE 2. Specific cone pathway ERG deficits in OPN1LW-EGFP mice. The amplitude of the scotopic a-wave (A) is not reduced in B6^{TgOPN1LW-EGFP} or B6^{TgOPN1LW-EGFP/+} compared to C57Bl/6 WT mice at P245. The slight reduction in scotopic b-wave amplitude indicates a potential loss of cone photoreceptor function in both heterozygote and homozygote mice. Functional loss is confirmed by photopic b-wave where amplitude is significantly lower in both B6^{TgOPN1LW-EGFP} and B6^{TgOPN1LW-EGFP/+} animals compared to WT. Fifteen and 30 Hz cone isolating flicker series (C) with 513 and 365 nm stimuli were used to isolate cone populations based on opsin expression (B). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Two-way ANOVA with genotype and light intensity or flicker rate as factors. Error bars = SD, $n = 3$ to 6 mice per group.

ference in b-wave amplitude between B6^{TgOPN1LW-EGFP} and B6^{TgOPN1LW-EGFP/+} mice at any light intensity ($P > 0.05$, two-way ANOVA).

The light-adapted b-wave, which primarily reflects cone pathway activation, revealed a large reduction in amplitude in B6^{TgOPN1LW-EGFP} and B6^{TgOPN1LW-EGFP/+} mice compared to WT mice across all intensities ($P < 0.0001$, two-way ANOVA). Amplitudes in heterozygous and homozygous mice were reduced to a similar extent indicating that the degeneration follows a dominant inheritance pattern (Fig. 2A).

UV Flicker ERG Reveals a Functional Loss of SWS Opsin-Expressing Photoreceptors

Cone opsins are spectrally tuned to maximally absorb different wavelengths of light, with MWS and SWS photopigments having a spectral peak of absorbance ($\lambda_{\max}$) of approximately 508 nm¹⁶ and 359 nm¹⁷ respectively (Fig. 2B). Stimulating at the $\lambda_{\max}$ of the MWS photopigment is likely to evoke some degree of response from cones throughout the retina, where MWS opsin is expressed to a certain extent in cones in both dorsally and ventrally. By contrast, expression of SWS opsin is predominantly restricted to cones in the ventral retina, and stimulation at the $\lambda_{\max}$ of the SWS photopigment is likely to elicit a response from cones of the ventral retina only.^{18,19} Considering that there are far fewer EGFP-expressing cones in the ventral than the dorsal retina (Figs. 1A, 1B), a large reduction in a response driven by SWS opsin would strongly support the presence of a cone degeneration that is not directly related to EGFP toxicity. To test this hypothesis, a custom array of Transistor-Transistor Logic controlled UV LEDs with a narrow emission peak of 365 ± 3 nm was constructed. Flicker stimuli (15 and 30 Hz) were used to minimize contributions from rod pathways. Responses were quantified by measurement of trough-to-peak amplitude, because a- and b-waves of “traditional” appearance are not observed in response to flicker stimuli.

Photometric units (e.g., candela) are inappropriate for quantifying the intensity of a stimulus with a wavelength below 400 nm. The intensity of the 365-nm flicker stimulus delivered by our LED array was standardized by adjustment of the forward voltage until it elicited a similar amplitude trough-to-peak flicker ERG response as the 3 cd.s/m² 513 nm stimulus in WT mice ($P > 0.05$, one-tailed t -test).

The trough-to-peak amplitude in response to the 15 and 30 Hz 513-nm stimuli was significantly reduced in B6^{TgOPN1LW-EGFP} and B6^{TgOPN1LW-EGFP/+} mice compared to WT mice at P245 (Fig. 2C), indicating a loss of cones across the whole retina ($P < 0.001$ all groups, two-way ANOVA). Trough-to-peak amplitude in response to the 15 and 30 Hz 365-nm stimulus was similarly reduced in both heterozygote and homozygote transgenic mice compared to WT ($P < 0.001$ all groups, two-way ANOVA). The reduction in ERG response to a 365-nm stimulus strongly indicates that there is a significant loss of cones in the ventral retina, where few cones express EGFP.

Collectively, the flicker ERG results confirm the anatomic observations that cones of the ventral retina degenerate to a similar extent to those of the dorsal retina in OPN1LW-EGFP mice. This strongly contraindicates the role of EGFP-mediated toxicity as a causal mechanism of retinal degeneration in this mice model. Furthermore, the observation of a similar reduction in cone function in heterozygous and homozygous mice confirms that cone loss in transgenic mice is dominantly inherited.

FISH Localizes the Transgenic Insertion to Chromosome 10

The creation of transgenic animal strains necessitates the integration of an expression cassette, or transgene, within the host genome. The site of integration is often random and therefore the potential exists for insertional mutagenesis, where normal host genetic function is subverted by the introduction of the transgene into the genome. The possibility of insertional mutagenesis resulting in the disruption of a cone-specific locus was examined.

The primary strategy applied for localization of the OPN1LW-EGFP expression cassette was chromosomal mapping by FISH. Initially, we obtained a probe for the human OPN1LW promoter sequence that drives expression of EGFP in this transgenic strain. The OPN1LW sequence has no direct homolog in the mouse genome. The probe was validated on metaphase chromosome spreads from a human female and found to map to chromosome X, as expected (Fig. 3A). The probe hybridized specifically to metaphase chromosome spreads from several OPN1LW-EGFP transgenic mice (Fig. 3B). Cohybridization of OPN1LW probe with various differently labeled chromosome-specific probes or “chromosome paints”

FIGURE 3. Mapping of the OPN1-EGFP transgene by FISH. The OPN1 probe binds specifically to human (A) and transgenic (B) metaphase chromosomes and is localized to chromosome 10 in the OPN1LW-EGFP mouse (C). Various genes (red and yellow), such as nuclear receptor subfamily 2 group E member 1 (*Nr2e1*) (D), glutamate receptor ionotropic, kainate 2 (*Grik2*) (E, H), activating signal cointegrator 1 complex subunit 3 (*Ascc3*) (F, H, I), transformed mouse 3T3 cell double minute 1 (*Mdm1*) (G), and single-minded homolog 1 (*Sim1*) (I) were used as chromosomal landmarks to map the location of the OPN1-EGFP transgene (green) (B–I) through cohybridization. Fiber-FISH with multiple probes (labeled) refines the likely insertion point to a 40-kb region intergenic of *Ascc3* (white) and *Sim1* (yellow) (H–I). Analysis of the syntenic relationship between the chromosomal insertion point of the OPN1-EGFP transgene (green) on murine chromosome 10 and human chromosome 6 shows that the transgene has been inserted into a highly conserved region containing both *Sim1* and *Ascc3* genes (red) (J). Closer inspection reveals that this region in the mouse is orthologous to the progressive bifocal chorioretinal atrophy PBCRA (blue), flanked by prenyl (decaprenyl) diphosphate synthase subunit 2 (*PDSS2*) and POU class 3 homeobox 2 (*POU3F2*) genes, and the macular dystrophy retinal 1 (*MCDR1*; North Carolina macular dystrophy, NCMD) (yellow) loci in humans.

was used to unequivocally map the transgenic insertion to a specific subchromosomal region of mouse chromosome 10 (Fig. 3C).

Having determined the insertion site was on chromosome 10, probes were obtained for several genes in that subchromosomal region, including *Nr2e1*, *Grik2*, *Ascc3*, *Mdm-1* (Figs. 3D-G), *CDH23*, *BVES*, and *Gja1* (data not shown) and cohybridized with the transgene-specific probe on metaphase spreads. Cohybridization allows the position of the transgene insertion to be determined relative to known landmarks. The transgene is clearly proximal to the *Mdm-1* gene (Fig. 3G) and distal to *Nr2e1* (Fig. 3D). Overlapping signals in metaphase were observed with both *Grik2* and *Ascc3* probes (Figs. 3E, 3F).

Higher resolution mapping was carried by Fiber-FISH, which is a technique whereby individual genomic sequences can be visualized on DNA fibers. DNA strands are obtained by deproteinization of nuclear chromatin in a high salt environment, or through the use of detergents, resulting in the dissociation of the bound histones and unwinding of the genomic DNA. Localization of the transgene to the region containing *Ascc3* and *Sim-1* was carried out through cohybridization with pairs of genomic probes (Figs. 3H, 3I).

This region is located close to the macular dystrophy retinal 1 (MCDR1) locus, associated with North Carolina macular dystrophy (NCMD) in humans, and within the progressive bifocal chorioretinal atrophy (PBCRA) locus, associated with PBCRA^{20,21} (Fig. 3J).

DISCUSSION

The aim of this study was to anatomically and functionally characterize the cone degeneration previously observed in the B6.Cg-Tg(OPN1LW-EGFP) mouse model.⁶ A UV LED array was used to deliver a flicker stimulus, allowing isolation of the response from S-cones in the ventral retina, where significantly fewer cones express EGFP in comparison to the dorsal retina. Here, we show that cone degeneration affects cones of the dorsal and ventral retina alike. The degeneration follows a dominant pattern of inheritance and is independent of EGFP expression. With the likelihood that an underlying genetic cause for this degeneration is common to both cone subpopulations, multiple probe Fiber-FISH was used to map the insertion site of the OPN1LW-EGFP transgene. The transgene was mapped to chromosome 10 and the insertion site located to an intergenic region situated between *Ascc3* and *Sim1*.

Cone degeneration in the OPN1LW-EGFP transgenic mouse strain has previously been attributed to EGFP toxicity.⁶ While the expression of various fluorescent proteins results in light-induced cytotoxicity,²² there are few reports of EGFP-induced cytotoxicity either in vitro²³ or in vivo.¹⁰ Indeed, EGFP has previously been described as being nontoxic in the rodent retina,^{11,12,24} where its lack of cytotoxicity compared to other fluorescent proteins is most likely related to a limited production of damaging reactive oxygen species on photoactivation.^{25–27}

In the present study, cone survival was quantified by direct immunolabeling of the cone opsins on postmortem tissue, rather than by in vivo autofluorescence imaging, which allows only the quantification of cells expressing EGFP. Given that significantly fewer cones in the ventral retina express EGFP than in the dorsal retina, one would expect to observe a greater reduction in cone number in the dorsal retina if cone loss was caused by EGFP toxicity. We found that the rate and extent of cone loss was similar in the dorsal and ventral retina, and that the ratio of GFP to non-GFP expressing cones remained consistent over the time course, strongly indicating that cone loss

is independent of the presence of EGFP. Gross retinal morphology showed that the observed degeneration was not secondary to a primary cause of rod degeneration, as has been observed in animal models²⁸ and human patients with RP.²⁹ In addition, the absence of significant cone loss in WT mice of the same background (C57Bl/6) excludes the possibility that slow cone degeneration might be a feature of the background mouse strain.

The anatomic loss of photoreceptors corresponded to a functional deficit. ERG with a 365-nm flicker stimulus delivered using high-powered LEDs indicated the presence of a functional loss of SWS opsin-expressing cones. This deficit was equally as profound as the impairment in responses to a green (513-nm peak) flicker stimulus, likely to activate a far larger population of cones based on the more widespread expression of MWS opsin. The equivalence of the deficits strongly suggests that all cones are affected and supports the conclusion that the degeneration is independent of EGFP expression. A functional deficit in B6^{TgOPN1LW-EGFP+/-} mice has previously been observed,⁶ and our data supports that cone degeneration follows a dominant inheritance pattern. Similar to another recently described mouse model of dominant cone dystrophy,³⁰ a slightly more severe cone dysfunction is observed in B6^{TgOPN1LW-EGFP+/+} mice than heterozygote mice. That the presence of a WT allele confers little phenotypic compensation suggests that the degeneration is likely dominant negative (antimorphic).

Through the use of Fiber-FISH, a 40-kb intergenic region situated between *Ascc3* and *Sim1* was identified as the likely location of the transgenic insertion. Transgene integration is usually random; however, insertion has occurred within a region rich with photoreceptor genes, potentially through the recombination between sequences within the human promoter sequence and homologous sequences in the mouse genome.

The insertion site falls within the locus associated with PBCRA and close to the currently defined MCDR1 locus. NCMD is a congenital maculopathy resulting in the degeneration of cone photoreceptors,²¹ while PBCRA is characterized by progressive atrophy of the nasal retina and diffuse abnormalities of both rod and cone function.³¹ Both NCMD and PBCRA are dominantly inherited and affect cone function; however, while the OPN1LW-EGFP mouse has a retinal phenotype, one must be cautious when correlating phenotypic observations in mice, which have no macula, to human macular dystrophies.

In the OPN1-EGFP transgenic mouse line, disruption of the *Ascc3* gene is most likely related to the close proximity of the transgene, although neither the function nor the expression profile of this gene are well understood. Disruption of *Sim1* is highly unlikely, where homozygous knockout of this gene results in perinatal lethality.³² Alternatively, the OPN1LW-EGFP transgene insertion may not directly be the cause of the observed cone degeneration, but may segregate with the locus responsible—in this instance, PBCRA or MCDR1. However, this is unlikely considering the dominant nature of the degeneration and the absence of cone loss in WT mice of identical strain.

In conclusion, we have characterized a novel dominantly inherited cone degeneration in the B6.Cg-Tg(OPN1LW-EGFP) mouse model that is independent of EGFP expression. The OPN1LW-EGFP transgene is inserted in a region that is associated with a number of dominantly inherited retinal disorders. The mouse model we describe has a dominantly inherited cone degeneration that shares similarities with two human conditions, NCMD and PBCRA, which are associated with this locus. The genetic mechanism of the described cone degeneration remains elusive, but is nonetheless of great interest in the study

of dominant cone degenerations and may aid in investigating potential therapeutic strategies to prevent cone loss in human diseases.

Acknowledgments

The authors thank Stuart Peirson for providing the high-powered UV LEDs and Steven Hughes for his assistance in construction of the UV LED array. The fosmid and BAC probes used in FISH analysis were kindly provided by the Wellcome Trust Sanger Institute, Cambridge.

References

1. Delyfer MN, Leveillard T, Mohand-Saïd S, Hicks D, Picaud S, Sahel JA. Inherited retinal degenerations: therapeutic prospects. *Biol Cell*. 2004;96:261–269.
2. Megason S, Amsterdam A, Hopkins N, Lin S. Uses of GFP in transgenic vertebrates. *Methods Biochem Anal*. 2006;47:285–303.
3. Corish P, Tyler-Smith C. Attenuation of green fluorescent protein half-life in mammalian cells. *Protein Eng*. 1999;12:1035–1040.
4. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. *Science*. 1994;263:802–805.
5. Akimoto M, Cheng H, Zhu D, et al. Targeting of GFP to newborn rods by Nrl promoter and temporal expression profiling of flow-sorted photoreceptors. *Proc Natl Acad Sci U S A*. 2006;103:3890–3895.
6. Beck SC, Schaeferhoff K, Michalakakis S, et al. In vivo analysis of cone survival in mice. *Invest Ophthalmol Vis Sci*. 2010;51:493–497.
7. MacLaren RE, Pearson SA, MacNeil A, et al. Retinal repair by transplantation of photoreceptor precursors. *Nature*. 2006;444:203–207.
8. Fei Y, Hughes TE. Transgenic expression of the jellyfish green fluorescent protein in the cone photoreceptors of the mouse. *Vis Neurosci*. 2001;18:615–623.
9. Applebury ML, Antoch MP, Baxter LC, et al. The murine cone photoreceptor: a single cone type expresses both S and M opsins with retinal spatial patterning. *Neuron*. 2000;27:513–523.
10. Huang WY, Aramburu J, Douglas PS, Izumo S. Transgenic expression of green fluorescence protein can cause dilated cardiomyopathy. *Nat Med*. 2000;6:482–483.
11. Dreyer EB, Vorwerk CK, Zurakowski D, Simon PD, Bennett J. Infection with adeno-associated virus may protect against excitotoxicity. *Neuroreport*. 1999;10:2887–2890.
12. Daniels DM, Shen WY, Constable IJ, Rakoczy PE. Quantitative model demonstrating that recombinant adeno-associated virus and green fluorescent protein are non-toxic to the rat retina. *Clin Exp Ophthalmol*. 2003;31:439–444.
13. Jefferson A, Volpi EV. Fluorescence in situ hybridization (FISH) for genomic investigations in rat. *Methods Mol Biol*. 2010;659:409–426.
14. Brown KT. The electroretinogram: its components and their origins. *Vision Res*. 1968;8:633–677.
15. Biel M, Seeliger M, Pfeifer A, et al. Selective loss of cone function in mice lacking the cyclic nucleotide-gated channel CNG3. *Proc Natl Acad Sci U S A*. 1999;96:7553–7557.
16. Sun H, Macke JP, Nathans J. Mechanisms of spectral tuning in the mouse green cone pigment. *Proc Natl Acad Sci U S A*. 1997;94:8860–8865.
17. Yokoyama S, Radlwimmer FB, Kawamura S. Regeneration of ultraviolet pigments of vertebrates. *FEBS Lett*. 1998;423:155–158.
18. Jacobs GH, Neitz J, Deegan 2nd JF. Retinal receptors in rodents maximally sensitive to ultraviolet light. *Nature*. 1991;353:655–656.
19. Lyubarsky AL, Falsini B, Pennesi ME, Valentini P, Pugh EN Jr. UV- and midwave-sensitive cone-driven retinal responses of the mouse: a possible phenotype for coexpression of cone photopigments. *J Neurosci*. 1999;19:442–455.
20. Gehrig A, Felbor U, Kelsell RE, Hunt DM, Maumenee IH, Weber BH. Assessment of the interphotoreceptor matrix proteoglycan-1 (IMPG1) gene localised to 6q13–q15 in autosomal dominant Stargardt-like disease (ADSTGD), progressive bifocal chorioretinal atrophy (PBCRA), and North Carolina macular dystrophy (MCDRI). *J Med Genet*. 1998;35:641–645.
21. Small KW. North Carolina macular dystrophy: clinical features, genealogy, and genetic linkage analysis. *Trans Am Ophthalmol Soc*. 1998;96:925–961.
22. Strack RL, Strongin DE, Bhattacharyya D, et al. A noncytotoxic DsRed variant for whole-cell labeling. *Nat Methods*. 2008;5:955–957.
23. Liu HS, Jan MS, Chou CK, Chen PH, Ke NJ. Is green fluorescent protein toxic to the living cells? *Biochem Biophys Res Commun*. 1999;260:712–717.
24. Nour M, Quiambao AB, Al-Ubaidi MR, Naash MI. Absence of functional and structural abnormalities associated with expression of EGFP in the retina. *Invest Ophthalmol Vis Sci*. 2004;45:15–22.
25. Tour O, Meijer RM, Zacharias DA, Adams SR, Tsien RY. Genetically targeted chromophore-assisted light inactivation. *Nat Biotechnol*. 2003;21:1505–1508.
26. Bulina ME, Lukyanov KA, Britanova OV, Onichtchouk D, Lukyanov S, Chudakov DM. Chromophore-assisted light inactivation (CALI) using the phototoxic fluorescent protein KillerRed. *Nat Protoc*. 2006;1:947–953.
27. Wiedenmann J, Oswald F, Nienhaus GU. Fluorescent proteins for live cell imaging: opportunities, limitations, and challenges. *IUBMB Life*. 2009;61:1029–1042.
28. Punzo C, Kornacker K, Cepko CL. Stimulation of the insulin/mTOR pathway delays cone death in a mouse model of retinitis pigmentosa. *Nat Neurosci*. 2009;12:44–52.
29. Hartong DT, Berson EL, Dryja TP. Retinitis pigmentosa. *Lancet*. 2006;368:1795–1809.
30. Buch PK, Mihelec M, Cottrill P, et al. Dominant cone-rod dystrophy: a mouse model generated by gene targeting of the GCAP1/Guca1a gene. *PLoS One*. 2011;6:e18089.
31. Godley BF, Tiffin PA, Evans K, Kelsell RE, Hunt DM, Bird AC. Clinical features of progressive bifocal chorioretinal atrophy: a retinal dystrophy linked to chromosome 6q. *Ophthalmology*. 1996;103:893–898.
32. Michaud JL, Boucher F, Melnyk A, et al. Sim1 haploinsufficiency causes hyperphagia, obesity and reduction of the paraventricular nucleus of the hypothalamus. *Hum Mol Genet*. 2001;10:1465–1473.