In Vivo Analysis of Cone Survival in Mice

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PURPOSE. To identify individual cone photoreceptors in a transgenic mouse line in vivo based on selective expression of green fluorescent protein (GFP) using cSLO (confocal scanning laser ophthalmoscopy) and to use this approach to monitor cone cell fate in mouse models of retinal degeneration.

METHODS. Transgenic mice expressing GFP under the control of a red-green opsin promoter (RG-GFP mice) were analyzed in vivo with respect to GFP expression in cone cells using cSLO and functional integrity using electroretinography (ERG). Histology was performed to correlate the pattern of GFP expression with light microscopic data. Longitudinal monitoring of cone survival was evaluated in crossbreds of RG-GFP mice with cpfl1 and Rpe65−/− mutant mice, respectively.

RESULTS. The authors found that RG-GFP transgenic mice had a stable GFP expression that did not interfere with retinal function up to at least 3 months of age. Thus, a longitudinal analysis of cone degeneration in individual RG cpfl1 and RG Rpe65−/− cross-bred mice in vivo was successfully performed and demonstrated distinct time frames of cone survival in the particular mouse model.

CONCLUSIONS. Monitoring GFP expression in cone photoreceptor cells, such as in the RG-GFP mouse, is a promising in vivo approach for the analysis of cone survival in mice. (Invest Ophtalmol Vis Sci. 2010;51:493–497) DOI:10.1167/iovs.09-4003

Green fluorescent protein (GFP) and its variant, enhanced GFP, are frequently used in basic research as a reporter protein. The expression of GFP under cell-specific promoters allows selective labeling of the cells of interest. Recently, a transgenic mouse line was generated that specifically expresses GFP under the control of a red-green (RG) cone opsin promoter. The in vivo analysis of cone-specific GFP expression presented here is a new tool for longitudinal in vivo evaluation of the pathophysiological processes in disorders affecting cones. Usually, cells labeled by GFP are evaluated by fluorescence microscopy in vitro, which requires sacrificing the animals. In this work we evaluated in vivo detection of GFP expression in cone photoreceptors by confocal scanning laser ophthalmoscopy (cSLO), a noninvasive imaging technique permitting repeated analyses at different time points in the same individual. This approach also helps to reduce the number of animals required for study, which has both ethical and economic implications.

In ophthalmic research, mice are frequently used for the investigation of hereditary retinal disorders that, in large part, involve photoreceptor degeneration and loss. As shown here, if such models are cross-bred with a GFP-expressing transgenic line, the fate of the photoreceptors may be studied over time. The cpfl1 mutant is a naturally arising mouse model initially identified by the Jackson Laboratory because of its practically complete lack of cone-mediated light responses; thus, it was given the name cone photoreceptor function loss 1 (cpfl1). Chang B, et al. IOVS 2001;42:ARVO Abstract 527). Recently, a 116-bp insertion in the gene encoding the α-subunit of the cGMP-phosphodiesterase of the cone photoreceptors (PDE6C) was identified as the molecular basis (Wissinger B, et al. IOVS 2007;48: ARVO E-Abstract 4521) for the deficiency, underlining the importance of the cpfl1 mouse as a genetic model for disorders with progressive loss of photoreceptors. Degenerative cone disorders are an important group of inherited retinal degenerations because they severely impair visual acuity and color vision, both key qualities of human vision. They are commonly classified as cone dystrophies or achromatopsia, depending on the degree of initial functional impairment. The functional impairment is usually accompanied by an early physical loss of cone photoreceptors, whereas rod function and morphology are not affected until late in the course of the disease.

Mutations in the gene encoding RPE65 cause LCA2, a major form of Leber’s congenital amaurosis. The protein RPE65 is expressed in the retinal pigment epithelium (RPE), where it plays a pivotal role in maintaining normal vision by regenerating the visual pigment rhodopsin. In Rpe65−/− mice, the blocked retinoid regenerating cycle causes an accumulation of retinyl esters in RPE cells. It was found that cone photoreceptors degenerate rapidly, whereas the remaining rods degenerate slowly.9

Cross-breeding with RG-GFP transgenic mice selectively expressing GFP in cone photoreceptor cells allowed long-term in vivo monitoring of cone survival in these two mouse models.

MATERIALS AND METHODS

Mice

RG-GFP mice were kindly provided by Yijian Fei and Thomas Hughes (Department of Ophthalmology and Visual Science, Yale University...
School of Medicine, New Haven, CT). The mouse line was crossed with wild-type C57BL/6 mice; GFP-positive mice (heterozygous state) and GFP-negative control littermates from the F6 generation were used for this study. The cprf1 mice were obtained from Bo Chang (Jackson Laboratory, Bar Harbor, ME). Rpe65<sup>−/−</sup> mice were kindly provided by T. Michael Redmond (National Eye Institute, National Institutes of Health, Bethesda, MD). All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Electroretinography**

Electroretinograms were recorded according to previously described procedures.<sup>7</sup> The ERG equipment consisted of a Ganzfeld bowl, a direct current amplifier, and a personal computer–based control and recording unit (Multiliner Vision; VIASYS Healthcare GmbH, Hoechberg, Germany). Mice were dark-adapted overnight and anesthetized with ketamine (66.7 mg/kg) and xylazine (11.7 mg/kg). The pupils were dilated, and single-flash ERG recordings were obtained under dark-adapted (scotopic) and light-adapted (photopic) conditions. Light adaptation was accomplished with a background illumination of 30 cd/m<sup>2</sup> starting 10 minutes before recording. Single white-flash stimulation ranged from −4 to 1.5 log cd · s/m<sup>2</sup>, divided into 10 steps of 0.5 and 1 log cd · s/m<sup>2</sup>. Ten responses were averaged with an intersinus interval of either 5 seconds or 17 seconds (for 0, 0.5, 1, and 1.5 log cd · s/m<sup>2</sup>).

**Confocal Scanning Laser Ophthalmoscopy**

cSLO is a noninvasive imaging technique that allows the examination of specific tissues and layers and of their vascular structures in the human and murine retina.<sup>10</sup> cSLOs were obtained according to previously reported procedures.<sup>10</sup> Briefly, cSLO imaging was performed with a commercial scanning-laser ophthalmoscope (HRA I; Heidelberg Engineering, Heidelberg, Germany). The confocal diaphragm of the cSLO allows the visualization of different planes of the posterior pole, ranging from the surface of the retina to the RPE and the choroid. The HRA features two argon wavelengths (488 nm and 514 nm) in the short-wavelength range and two infrared diode lasers (795 nm and 830 nm) in the long-wavelength range. The laser wavelength of 795 nm was used for indocyanine-green angiography, with a barrier filter at 800 nm. GFP expression was detected in the autofluorescence mode of the cSLO (excitation at 488 nm, barrier filter at 500 nm). The resultant cSLO image was similar to a retinal whole mount stained for cones. For an overview of the fundus, a setting of 20° was used, and for a detailed view, the 10° setting was used. The dorsal-ventral orientation is indicated by D and V in the images.

**Immunohistochemistry**

Immunohistochemical staining was performed on retinal whole mount preparations, as described previously,<sup>11</sup> using the same materials and protocols. Anti-GNAT2 (transducin) antibody (Santa Cruz Biotechnology Europe, Heidelberg, Germany) was used to specifically label cone photoreceptors. Secondary detection of the antibodies was performed with Cy2- or Cy3-labeled anti-rabbit IgG (Dianova, Hamburg, Germany). Stained retinal whole mounts were analyzed by fluorescence microscopy (Axioplan 2; Carl Zeiss, Oberkochen, Germany). For cryostat sections, eyes were enucleated and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 6 to 8 hours at 4°C, followed by cryoprotection by soaking in 30% sucrose at 4°C overnight. Eyes were frozen in an OCT compound (Tissue-Tek; Sakura Finetek, Torrance, CA) on dry ice, and 20-μm-thick cryostat sections were cut. GFP expression was analyzed by fluorescence microscopy (Axio phot; Carl Zeiss).

**RESULTS**

**Morphologic and Functional Analysis of the RG-GFP Mouse Mutant**

RG-GFP transgenic mice selectively express GFP under control of an RG promoter.<sup>1</sup> In this mouse line, we were able to identify individual cone photoreceptors in cSLO autofluorescence imaging (Figs. 1A [overview], B [magnification]), similar to a whole-mount preparation with a cone-specific staining (Fig. 1C). In retinal sections of 8-week-old mice, strong GFP expression could be selectively detected in cone photoreceptor cells (Figs. 1D [overview], E [magnification]).

Longitudinal evaluation of GFP expression using cSLO imaging revealed that the expression remained nearly constant between 3 and 8 weeks of age (Fig. 2, see Fig. 4A). The dorsal-ventral gradient of GFP expression shown in SLO images reflected the distribution of the cones within the retina.<sup>1</sup> Furthermore, repeated analyses up to 3 months of age did not reveal changes of GFP expression (data not shown). However, after 10 months of age, the GFP expression was significantly decreased.
decreased in cSLO autofluorescence (Fig. 2), with no dorsal-ventral gradient of GFP expression detectable (Fig. 2).

Next, we verified that GFP expression did not alter retinal function. For that purpose, flash ERGs were recorded from 8-week-old RG-GFP- and GFP-negative control mice under scotopic and photopic conditions (Fig. 3A). Under both conditions, no signs of impaired retinal function were found in the RG-GFP mice. To elucidate whether the late decrease in GFP expression was caused by downregulation or by loss of cone photoreceptor cells, analysis of the ERG responses of 10-month-old mice was also performed. It clearly revealed significantly reduced cone responses of RG-GFP mice compared with those of the controls (Fig. 3B). Thus, the decrease of GFP expression in 10-month-old mice was most probably caused by the loss of cone photoreceptor cells. Given that selective GFP expression in cone photoreceptor cells remained constant over at least 3 months, we went on to study the in vivo time course of cone survival in mouse models in which a loss of cone photoreceptor cells occurred.

**Longitudinal Cone Survival Analyses in cpfl1-GFP Mice**

*cpfl1* mice undergo a progressive, selective degeneration of their dysfunctional cone photoreceptor cells over time. To analyze these morphologic changes in the retina, in vivo cSLO analyses were performed between postnatal weeks 4 and 8. Because only approximately 3% of the murine photoreceptors are cones and these are relatively widely spaced, it was unclear whether selective loss may lead to detectable changes in vivo. No differences between WT and *cpfl1* mice could be detected, either in native fundus imaging (green laser at 514 nm, infrared laser at 830 nm, and autofluorescence mode) or in indocyanine-green angiography (data not shown). In addition, no changes could be detected in *cpfl1* mice between 4 and 8 weeks of age (data not shown). In particular, no enhanced autofluorescence was observed that would have indicated a substantial accumulation of lipid-rich photoreceptor debris, presumably lipofuscin, remained after the degradation of photoreceptor cells. To visualize cones specifically and to monitor the process of their degeneration in vivo, we cross-bred *cpfl1* mutants with RG-GFP transgenic mice. In this way, single cone cells were visible in the autofluorescence mode of the cSLO, and the changes in their number and distribution as part of the degenerative process in the *cpfl1* mutants were analyzed across ages in individual mice. The doubly mutant RG-GFP *cpfl1* mice initially displayed the same amount of GFP expression as their single RG-GFP counterparts at 3 weeks (Fig. 4). However, there was a marked decrease in GFP expression over time, leading to an almost complete loss of GFP-expressing cells in the ventral region and a strong reduction in the dorsal region (Fig. 4B) but no such change in RG-GFP animals (Fig. 4A). Thus, RG-GFP expression in *cpfl1* mice allowed in vivo follow-up of the number and distribution of cone photoreceptors during the course of progressive, selective degeneration in the *cpfl1* mouse model.

**Time-Course Analysis of Cone Survival in Rpe65−/−/−GFP Mice**

Constant activation of the phototransduction cascade by unliganded opsin is proposed to cause rod cell death in RPE65-deficient mice. However, considerable degeneration and loss of rod cells occurs relatively late, at approximately 6 months of age.
age and later. Cones, on the other hand, seem to be more sensitive to visual pigment deficiency because most of them degenerate much earlier.13 For in vivo analysis of cone survival in a RPE65-deficient background, Rpe65−/− mice were crossbred with RG-GFP transgenic mice. Initially, at 3 weeks of age, the Rpe65−/− GFP mice displayed nearly the same amount of GFP expression as observed in RG-GFP and cpfl1 GFP mice. In addition, a dorsal-ventral gradient of GFP expression1,14 was clearly visible. However, GFP signal detection by cSLO revealed that in Rpe65−/− mice, cone degeneration occurred more slowly than in cpfl1 mutant mice (Fig. 4). A significant decrease in GFP expression, which is representative of cone loss, was detectable only at 9 weeks of age. Even after 12 weeks, several cones were still present in the dorsal periphery of the retina (Fig. 4). Although almost no cones could be detected in the ventral retina at 12 weeks, a substantial number of cones did survive in the dorsal area. Apparently, the ventral retina is more prone to cone degeneration than the dorsal retina in RPE65-deficient mice.

**DISCUSSION**

In summary, we have developed and tested a novel method for the in vivo longitudinal follow-up of cone survival in mouse models of retinal degeneration. We show that the technique of selective GFP expression in the RG-GFP mouse allows in vivo visualization of cones for an extended period. At the late stages observed here (approximately 10 months of age), GFP expression may have deleterious effects, as observed for other organs such as heart15 and muscle.16,17

Use of the RG opsin promoter limits the production of GFP protein to cones that also produce green (MWS) opsin. In mice, there are only two cone opsin types, the MWS opsin (corresponding to the green opsin in humans) and the SWS opsin (corresponding to the blue opsin in humans); there is no red opsin in mouse cones.18,19 Accordingly, we found that the staining of cones with GFP closely followed the distribution of MWS opsin, which has been described in previous studies.1,20–22 The fact that many cones have been shown to coexpress MWS and SWS opsin20 suggests that all such cones will also produce GFP in the transgenic and that only the few “true blue” cones21 would be completely missed. An interesting aspect is that the ratio of MWS and SWS opsin expression in the mixed opsin cones follows a dorsoventral gradient. It is unclear whether topographic activity differences of the RG-GFP promoter are responsible for the MWS opsin gradient22; that, in turn, would explain the GFP gradient found.

For the present study, it is important to note that we did not experience a time-dependent fluctuation of GFP expression in individual cells and that a loss of staining was correlated to degeneration of the respective cell. Given that we conduct repetitive follow-up studies in the same animals, the actual topographic distribution pattern is less important than the relative change of the given pattern over time because this reflects the degenerative process.

In the two examples of degenerative disorders presented, cone survival could be followed very well in vivo with this approach. In addition, cpfl1 mice, bearing a mutation in the Pde6c gene, are a naturally occurring mouse model specifically for human achromatopsia. The functional assessment in this model is uninformative regarding physical cone cell status over time because function is practically absent from birth. In this study, we used selective GFP expression to follow the fate of the cone photoreceptor cells in the cpfl1 mouse. cSLO autofluorescence imaging allowed direct correlation of GFP-marked cells with cone numbers. With this method, we found early, progressive cone degeneration that was marked after 4 weeks of age in this model (Fig. 4). It appears that the decrease in cone number was initially evenly distributed across the whole retina, whereas later more cone cells appeared to have degenerated within the ventral part of the retina. With further progression of cone loss, the dorsal-ventral gradient appeared
more prominent: this may suggest that in the dorsal part of the retina more cones were able to survive (Fig. 4).

Mutations in the gene encoding RPE65 account for 10% to 15% of Leber congenital amaurosis (LCA), the most serious form of autosomal recessive childhood-onset retinal dystrophies. In studies on early intervention in Rpe65−/− mice, rescue of cone function and preservation of cones against degeneration have been demonstrated. Therefore, suitable methods and methods of examination are important for the further development of therapeutic strategies with respect to cone functionality. In the Rpe65−/− GFP mouse, cone photoreceptor cells were also well detectable by GFP expression analysis, but the time frame of cone degeneration differed markedly. Cones in the Rpe65−/− mouse survived longer than those in the cpfl1 mouse. Although considerable loss of cones was detected after 9 weeks of age, even after 12 weeks a substantial number of cones were still present in the RPE65-negative background (Fig. 4). However, the same topographic pattern of cone survival observed in cpfl1 mice was observed in this mouse model, with more cones appearing to survive in the dorsal half of the retina. This pattern is in line with observations by Jiménez et al. and Znoiko et al.; the latter found that the susceptibility of cone photoreceptors to degeneration was dependent on SWS and MWS opsin mRNA levels. The molecular and biochemical processes that control this differential susceptibility across the retina, however, remain to be elucidated.

To conclude, a novel method of selective GFP expression in cones and their detection and follow-up with cSLO in vivo was established and tested. This technique appears promising to facilitate the characterization of disease dynamics and the evaluation of putative therapeutic effects after experimental interventions. Furthermore, noninvasive imaging helps to reduce the number of experimental animals because cross-sectional evaluations by Jime´nez et al. and Znoiko et al. found negative background (Fig. 4). However, the same topographic pattern of cone survival observed in cpfl1 mice was observed in this mouse model, with more cones appearing to survive in the dorsal half of the retina. This pattern is in line with observations by Jiménez et al. and Znoiko et al.; the latter found that the susceptibility of cone photoreceptors to degeneration was dependent on SWS and MWS opsin mRNA levels. The molecular and biochemical processes that control this differential susceptibility across the retina, however, remain to be elucidated.

To conclude, a novel method of selective GFP expression in cones and their detection and follow-up with cSLO in vivo was established and tested. This technique appears promising to facilitate the characterization of disease dynamics and the evaluation of putative therapeutic effects after experimental interventions. Furthermore, noninvasive imaging helps to reduce the number of experimental animals because cross-sectional studies may be at least partially replaced by longitudinal studies with repeated imaging of the same individuals.

References