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Cone Survival Despite Rod Degeneration in XOPS-mCFP Transgenic Zebrafish

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Abstract

PURPOSE—In animal models of retinitis pigmentosa, rod photoreceptor degeneration eventually leads to loss of cone photoreceptors. The purpose of this study was to characterize a transgenic model of rod degeneration in zebrafish.

METHODS—Zebrafish transgenic for XOPS-mCFP, a membrane-targeted form of cyan fluorescent protein driven by the *Xenopus* rhodopsin promoter, were generated by plasmid injection. Immunohistochemistry was used to detect cell type, proliferation, and TUNEL markers in larval and adult retinas. Rod- and cone-specific transcripts were detected by RT-PCR. Visual responses in transgenic adults were measured by electroretinogram.

RESULTS—The XOPS promoter directed specific expression of mCFP in rods by 55 hours post fertilization (hpf). Rods in XOPS-mCFP heterozygotes began dying at 3.5 days post fertilization (dpf) and were almost completely absent by 5 dpf. A few rods were observed at the retinal margin, and numerous immature rods were observed in the outer nuclear layer (ONL) of transgenic adults. Apoptosis was increased in the ONL of larval and adult transgenic animals, and an elevation of rod precursor proliferation in adults was observed. ERG analysis confirmed that rod responses were absent in this line. Cone morphology and electrophysiology appeared normal in transgenic animals up to 7 months of age.

CONCLUSIONS—The XOPS-mCFP transgene causes selective degeneration of rods without secondary loss of cones in animals up to 7 months of age. This raises important questions about the significance of rod-cone interactions in zebrafish and their potential as a model of human inherited retinal degenerations.

Vertebrate rod photoreceptors are highly specialized retinal neurons that mediate visual responses under scotopic conditions. Light capture by rhodopsin in the rod outer segment (ROS) initiates a phototransduction cascade that results in synaptic transmission in the outer plexiform layer to the second-order retinal neurons. Given its central role in rod phototransduction, it is not surprising that mutations in the rhodopsin gene are the most

frequently identified cause of retinitis pigmentosa (RP), a heritable retinal degenerative disease characterized by a progressive loss of vision. Patients with RP initially experience night blindness and tunnel vision, due to loss of rod photoreceptors, but eventually become completely blind after secondary degeneration of the cones. Although it has been 15 years since the first rhodopsin mutations that cause RP were identified,¹ the question of how mutations in a gene expressed specifically in rods lead ultimately to loss of cones remains unresolved.

Much has been learned about the molecular events leading to retinal degeneration through studies in animal models. In the mouse, several naturally occurring mutations, as well as targeted mutations and gene knockouts, display retinal degeneration phenotypes analogous to human RP. As in the human disease, rod photoreceptor degeneration is invariably followed by degeneration of the cones. The current research seeks to characterize what, if any, interactions between rods and cones are needed to promote cone photoreceptor survival. However, the applicability of this work to human retinal disease is limited by the fact that the mouse, a nocturnal animal, has a rod-dominated retina with only 3% cones, whereas humans have a retina that is more adapted to color vision and diurnal activity. Therefore, studies of the mechanism of photoreceptor degeneration would benefit from additional and complementary animal models of RP.

The zebrafish, a small, freshwater teleost, has good color vision and a cone-dense retina. Zebrafish retinal architecture is well conserved with that of other vertebrates, and the optical transparency of both the embryo and free-swimming larvae greatly facilitate studies of the visual system. Zebrafish possess one type of rod photoreceptor and four distinct classes of cones maximally sensitive to ultraviolet, short, medium, and long wavelengths of light.

Over the past 10 years, large- and small-scale genetic screens conducted by various laboratories have led to the isolation of numerous mutant lines of zebrafish displaying retinal degenerations.^{2–6} Although most mutations affect both rods and cones or are lethal (suggesting the mutated genes are not rod specific), two dominant mutations demonstrate an initial degeneration of rod photoreceptors,^{5,7} somewhat similar to RP and other human rod dystrophies. However, in homozygous embryos, these mutations display pleiotropic affects outside the retina and are lethal. A genetic approach that specifically targets the rod photoreceptors in zebrafish would therefore be beneficial.

With this goal in mind, we and others have generated lines of transgenic zebrafish that demonstrate rod-photoreceptor-specific expression of fluorescent reporter proteins such as green fluorescent protein (GFP).^{8–11} All show patterns of expression similar to the endogenous rhodopsin gene, and none cause any functional or structural abnormalities in the photoreceptors. Genetic manipulation of these lines should allow a more detailed study of the processes of rod development and degeneration. Furthermore, the rod-specific promoters may facilitate genetic ablation of the rods, which would further studies of the impact of rod loss on cone survival and may provide useful models for human RP.

In this report we describe a transgenic line of zebrafish expressing a membrane-targeted variant of cyan fluorescent protein (mCFP) driven by a portion of the *Xenopus* rhodopsin promoter that unexpectedly displays rod photoreceptor degeneration. Of interest, cones of transgenic animals appear to be morphologically and physiologically unaffected by the loss of the rods, at least up to 7 months of age.

MATERIALS AND METHODS

Wild-Type and Transgenic Animals

Rearing, breeding, and staging of zebrafish (*Danio rerio*) were performed as described.³ Wild-type zebrafish were inbred descendants of the Ekkwill strain, originally obtained from the Ekkwill fish farm (Gibsonton, FL). Zebrafish were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The XOPS-GFP line has been described previously.⁸ It expresses a transgene containing 5.5 kb of the *Xenopus* rhodopsin promoter¹² driving expression of enhanced GFP (BD-Clontech, Palo Alto, CA). The XOPS-mCFP plasmid was created by replacing the GFP coding sequence from XOPS-GFP with CFP. mCFP contains the N-terminal 20 amino acids of neuromodulin (GAP-43), which encode a posttranslational palmitoylation signal sequence that targets proteins to the plasma membrane.¹³ Supercoiled XOPS-mCFP plasmid was injected into one-cell-stage embryos, as described.¹⁴ Embryos expressing mCFP were raised to adulthood. Progeny of these fish were then screened for germline transmission of the transgene. A founder was identified, and its progeny were designated TG(XOPS::mCFP)Q01, hereafter referred to as XOPS-mCFP.

Histology

Transgenic and wild-type larvae and adults were killed by immersion in tricaine. Eyes were dissected from the adults. Larvae and adult eyes were fixed overnight at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7). The next day, larvae were dehydrated through a graded methanol series and stored in 100% methanol at -20°C for at least 1 day. After rehydration into 80% Hanks' buffered saline solution, samples were cryoprotected in 15% sucrose overnight at 4°C and then 30% sucrose overnight at 4°C. Samples were mounted in OCT medium (Miles Scientific, Elkhart, IN) and frozen on dry ice. Eight- to 10-µm sections were cut on a cryostat, mounted on gelatin-coated glass slides, and allowed to air dry at room temperature for 1 to 2 hours. Before immunolabeling, sections were postfixed in 1% para-formaldehyde for 10 minutes at room temperature. After two washes in PBS and two washes in PBST (0.05% Tween-20), slides were blocked in PBST containing 1% BSA for 30 minutes at room temperature. The slides were then incubated in primary antibody for 1 hour at room temperature in a humidified chamber. Slides were washed two times in PBST and then incubated in the appropriate fluorescent dye-conjugated secondary antibody for 1 hour at room temperature in the dark. Slides were washed two times in PBST, two times in PBS, counterstained with DAPI (4', 6-diamidino-2-phenylindole; Sigma-Aldrich, St. Louis, MO) or PI (propidium iodide; Molecular Probes, Eugene, OR) and mounted in 30% glycerol in PBS. When PI was used, slides were incubated with DNase-free RNase (100 µg/mL; Roche, Indianapolis, IN) for 30 minutes before counterstaining. Sections were imaged on either a fluorescence microscope (Axiovert) or a confocal microscope (model 510; both from Carl Zeiss Meditec, Inc., Dublin, CA).

The following primary antibodies and dilutions were used: 1D1 (1:100 dilution), a monoclonal antibody that recognizes an epitope on rhodopsin¹⁵; 4C12 (1:100 dilution), a monoclonal antibody that recognizes an unknown epitope on rods (Fadool J, Linser P, unpublished data, 1999); Zpr-1 (1:20 dilution), a monoclonal antibody that recognizes red and green cones (Oregon Monoclonal Bank); PCNA (1:100 dilution), a marker of mitotic cells (Santa Cruz Biotechnology, Santa Cruz, CA); a polyclonal antiserum against zebrafish blue cone opsin (1:50 dilution)¹⁶; and a polyclonal antiserum against zebrafish carbonic anhydrase (1:100 dilution) that labels retinal Müller cells.¹⁷ Alexa Fluor 488 goat anti-mouse, 546 goat-anti-rabbit (Molecular Probes), and Cy2 goat anti-mouse (Jackson ImmunoResearch, West Grove, PA) secondary antibodies were all used at a 1:100 dilution.

Terminal deoxynucleotide transferase (TdT)-mediated dUTP nick end labeling (TUNEL) was performed on retinal cryosections (ApopTag Red In Situ Apoptosis Detection kit; Chemicon, Temecula, CA), according to the manufacturer's instructions.

Microinjection of the Plasmid and Morpholinos

Microinjection of one- to two-cell-stage embryos was performed as previously described.⁸ Rhodopsin localization was analyzed by microinjection with a plasmid expressing a bovine rhodopsin-GFP (rho-GFP) fusion protein¹⁸ (a kind gift from David Papermaster, University of Connecticut, Farmington, CT). Injected embryos were kept in embryo medium containing 0.003% 1-phenyl-2-thiourea (PTU), to inhibit pigmentation. ¹⁹ Injected larvae were anesthetized at 3 days post fertilization (dpf) and imaged by confocal microscopy.

Morpholinos were synthesized by Gene Tools, LLC (Philomath, OR). The mCFP morpholino was complementary to the 25 bases surrounding the mCFP initiation codon. The standard control morpholino was complementary to human thalassemic β -globin pre-mRNA and has no biological activity in zebrafish. Morpholino oligos were resuspended in sterile water at a concentration of 1 mM. The sequences of the mCFP and control morpholinos were as follows: mCFP: 5'-ATACAGCACAGCATGGTGGCGACCG-3'; standard control: 5'-CCTCTTACCTCAGTTACAATTTATA-3'.

Morpholinos were microinjected into the yolk of one- to two-cell-stage embryos from a cross of XOPS-GFP homozygous and XOPS-mCFP heterozygous fish. The injected embryos were screened by fluorescence microscopy at 4 dpf for the presence of GFP-positive rods in the central retina. The significance of the results was assessed on the raw data by χ^2 test (with an expected 1:1 ratio of GFP-positive to -negative retinas).

Reverse Transcription–Polymerase Chain Reaction

Total RNA was prepared from 5-dpf zebrafish larvae and adult retinas (TRIzol reagent; Invitrogen-Gibco, Grand Island, NY) followed by treatment with RNase-free DNase (Roche). RT-PCR was performed as previously described.²⁰ The sequences of the PCR primers were as follows: rhodopsin forward: 5'-CCATGAACGGTACAGAGGGACC-3', rhodopsin reverse: 5'-AGAGTGTCTGGAAGGAGAGT-3'²¹; UV opsin forward: 5'-AGGCCTCCAACGGCACAACC-3', UV opsin reverse: 5'-TAAATGTGCTGCGGGAGGAT-3'²¹; and β -actin forward: 5'-TGGTATTGTGATGGACTCTGG-3', β -actin reverse: 5'-AGCACTGTGTTGGCATAACAGG-3'.

Electroretinography

Apparatus and procedures for collecting electroretinogram responses have been described in detail elsewhere.^{22,23} After light adaptation, single 200-ms flashes of 500-nm light of various irradiances were used to find the stimulus irradiance that produced a 50- μ V ERG b-wave response. A 500-nm stimulus was chosen because this wavelength represents the peak of the rod spectra. This irradiance was used for deriving dark-adaptation functions. Across all subjects, the range of stimulus irradiances used was from 12.40 to 12.44 log quanta $\cdot$ s⁻¹ $\cdot$ cm⁻². Once the appropriate stimulus irradiance was found, the broadband background light was turned off and the ERG response to a 500-nm, 200-ms flash was recorded every 2 minutes for up to 60 minutes.

Spectral sensitivity functions were derived, after 60 minutes of dark-adaptation, by presenting various wavelengths, ranging from 320 to 640 nm, at various irradiances. Sensitivity at each stimulus wave-length was defined as the stimulus irradiance that produced a b-wave amplitude

of 50 μ V.^{22,23} In several wild-type subjects after these procedures, the broadband background light was turned back on, and light-adapted spectral sensitivity functions were determined.

RESULTS

Absence of mCFP-Expressing Rods in 5 dpf Transgenic Larvae

Previously, our laboratory created a transgenic line of zebrafish expressing eGFP in rod photoreceptors, using a 5.5-kb portion of the *Xenopus* rhodopsin promoter to drive expression of eGFP.⁸ To create a transgenic line of zebrafish in which the fluorescent reporter was targeted specifically to the plasma membrane and ROS, the eGFP gene of XOPS-GFP was replaced with mCFP (a variant of cyan fluorescent protein), yielding the XOPS-mCFP transgene. mCFP contains the N-terminal 20 amino acids of neuromodulin (GAP-43), which encodes a post-translational palmitoylation signal sequence that targets proteins to the plasma membrane.

In XOPS-mCFP transgenic zebrafish, expression of the mCFP reporter initiated in a time-frame that was very similar to XOPS-GFP and to endogenous rhodopsin, with the first mCFP-positive cells appearing in the ventral patch at ~55 hpf. At 3 dpf, mCFP-expressing cells were located in the same regions of the photoreceptor cell layer as in the XOPS-GFP line (compare Figs. 1A and 1C) and colocalized with cells that immunolabeled for rhodopsin (data not shown). The subcellular localization of mCFP in some of the XOPS-mCFP rods indicated that the mCFP was being targeted to the plasma membrane and to the apical end of the cell in the developing ROS. However, at 3 dpf many of the mCFP-positive cells had an abnormal morphology. By 5 dpf, mCFP-positive cells were almost completely absent from the retina and were observed only at the retinal margin, a region of persistent neurogenesis in teleost fish (compare Figs. 1B and 1D). Furthermore, even at the margin, the mCFP-positive cells often had an abnormal morphology or were pyknotic, suggesting that these rods were dying as well.

Loss of Rod-Specific Transcripts

To confirm that the absence of mCFP-positive cells was due to rod loss rather than inhibition of mCFP expression, we performed RT-PCR on RNA isolated from 5-dpf transgenic animals (Fig. 2). RT-PCR of RNA isolated from siblings that did not inherit the XOPS-mCFP transgene showed that rhodopsin expression was detectable in these animals at 5 dpf, as expected (Fig. 2, lane 1). In the XOPS-mCFP transgenic larvae, however, expression of rhodopsin could not be detected by RT-PCR (Fig. 2, lane 2). This was not due to an overall decrease in RNA transcripts, as expression levels of UV opsin and β -actin in XOPS-mCFP animals were similar to the nontransgenic control subjects. Therefore, the absence of rhodopsin expression, which accounts for most of the protein content of rod outer segments, suggests that the rods indeed had degenerated in the XOPS-mCFP line by 5 dpf.

Mislocalization of a Rho-GFP Fusion Protein in XOPS-mCFP Rods

Degeneration of rod photoreceptors has been shown in some cases to result from aberrant vectorial sorting of rhodopsin.^{24–27} To investigate whether missorting of rhodopsin occurs in the XOPS-mCFP transgenic line, heterozygous embryos at the one- to two-cell stage were injected with a plasmid encoding a rho-GFP fusion protein (rho-GFP), which was previously shown to be vectorially sorted to the rod outer segments.¹⁸ In wild-type larvae that had been injected with rho-GFP, transient expression of rho-GFP was detected at the apical end of the elongating rod photoreceptors, in a smooth conical pattern indicative of sorting to the developing ROS, with a low level of GFP fluorescence observed at the plasma membrane (Fig. 3A).

In the XOPS-mCFP line, the rods (identified by their expression of mCFP) displayed a range of abnormal morphologies (Fig. 3C). Some cells had an overall elongated appearance, but their

developing outer segments were shorter than in wild-type animals, displaying a knoblike appearance at the tip of the cell rather than having a smooth, conical shape. Other mCFP-positive cells were not elongated at all, but rather had a rounded-up appearance typical of dying cells. Thus, at 3 dpf, many mCFP-positive cells showed signs of degeneration. After injection of rho-GFP, XOPS-mCFP larvae displayed a range of rho-GFP localization patterns (Fig. 3B). In some rods, bright GFP fluorescence was spread over the entire cell body. In other rods, GFP fluorescence appeared to be localized to the abnormal, knoblike protrusion at the apical end of the cell. And in some rods, rho-GFP localized in the cytoplasm just below a misshapen outer segment, an arrangement suggestive of concentration within the Golgi network. The abnormal expression of rho-GFP was specific to the retinal rod photoreceptors, because pinealocytes transiently expressing the fusion protein displayed both a normal morphology and predominant apical distribution of the GFP signal (Fig. 3D). These data demonstrate that proper trafficking of rho-GFP is disrupted in XOPS-mCFP rods and raises the possibility that rod degeneration in this line is caused by aberrant protein targeting resulting from overexpression of palmitoylated mCFP. It should be noted that loss of mCFP-positive rods occurred even when transgenic embryos were reared in total darkness (data not shown), indicating that the cytotoxic effect of the transgene was light-independent.

Increase in Apoptosis in XOPS-mCFP Larval and Adult Retinas

To investigate the mechanism of rod cell death in XOPS-mCFP zebrafish, wild-type and transgenic retinas from 3 dpf larvae and from adults were subjected to TUNEL analysis. It has been shown that, in contrast to the developing mammalian retina, there is very little programmed cell death in the developing zebrafish retina, and the small amount of apoptosis observed in the outer nuclear layer (ONL) does not peak until 7 dpf.²⁸ Accordingly, we only rarely observed TUNEL-positive cells in wild-type retinas at 3 dpf (Fig. 4A). The number of TUNEL-positive cells observed in nontransgenic larval sections ranged from 0 to 9, with a range of 0 to 4 positive cells in the ONL (eight sections from six larvae examined). In contrast, in the XOPS-mCFP transgenic larvae we observed several TUNEL-positive nuclei in the photoreceptor cell layer (Fig. 4B, arrowheads). The number of TUNEL-positive cells ranged from 7 to 15 in retinal sections from XOPS-mCFP larvae, with a range of four to nine TUNEL-positive cells in the ONL (8 sections from 6 larvae examined). Double immunolabeling with an antibody that recognizes rhodopsin demonstrated that most of the TUNEL-positive cells in the ONL were rod photoreceptors.

We also observed an increase in TUNEL-positive nuclei in retinal sections from adult XOPS-mCFP animals (Figs. 4D, 4E). In retinal sections from adult wild-type animals, the number of TUNEL-positive nuclei ranged from zero to two (five sections from three eyes examined). However, in retinal sections from XOPS-mCFP adults, the number of TUNEL-positive nuclei ranged from 6 to 24, with an average of 14.1 (21 sections from four eyes examined). In wild-type adult retinas, the rod nuclei can be distinguished from the cone nuclei both by their position in the ONL (vitread to the cone nuclei in a layer roughly three to five cell bodies thick) and by their shape (rod nuclei are round whereas cone nuclei are elongated). In retinal sections from adult transgenic animals, the layer of rod nuclei was sparsely populated or contained only a single row of cells, whereas the cone nuclei retained their correct position and thickness. The TUNEL-positive nuclei observed in retinal sections from transgenic animals occupied the normal position of the rod cell bodies, suggesting that apoptosis occurred specifically in rods.

Double labeling of retinal sections with an antibody that recognizes rhodopsin showed very few positive cells in the retinal sections from transgenic adults, and those that labeled with this antibody were usually TUNEL positive as well (Fig. 4D). However, when XOPS-mCFP adult retinal sections were immunolabeled with a different antibody that recognizes an unknown protein in rods, we observed several labeled cells in the ONL in what would be the normal

position of the rod photoreceptors (Fig. 4E, arrows). Confocal microscopy revealed that some, but not all, of the labeled cells were also TUNEL positive (Fig. 4F). Furthermore, none of these labeled cells possessed a mature rod morphology or rod outer segments. It is possible that these cells were immature rods that had not yet initiated expression of rhodopsin and that the onset of rhodopsin (and mCFP) expression in XOPS-mCFP animals correlated with the induction of apoptosis.

Occasionally, we observed TUNEL-positive cells in the inner nuclear layer of retinal sections from larval and adult XOPS-mCFP animals (Figs. 4B, 4D, 4E, asterisks). These cells appeared to be connected to long, thin processes that stretched across the retina. Closer examination of these cells by confocal microscopy revealed a morphology typical of Müller cells (Fig. 4G). The processes of these cells overlapped with the TUNEL-positive rod nuclei in the ONL, suggesting that Müller glia scavenge the cell debris from the TUNEL-labeled rods, as has been reported.²⁹

Cell Proliferation in the ONL of XOPS-mCFP Adults

As discussed earlier, the presence of a substantial number of cells that immunolabeled with a rod-specific antibody but not with an antibody to rhodopsin raised the possibility that these were immature rods, perhaps generated through an increase in proliferation of the rod precursor pool,^{30,31} which has also been shown to possess the ability to regenerate most classes of retinal neurons in response to retinal injury.^{32,33} If this were the case, we would expect XOPS-mCFP retinas to demonstrate an increase in cells that label with markers of proliferation when compared to wild-type control subjects.

Retinal sections from XOPS-GFP and XOPS-mCFP adults were immunolabeled with an antibody to PCNA, a marker of mitotic cells. As shown in Figure 5, we observed an approximate 3.5-fold increase in the number of PCNA-positive cells in retinal sections from XOPS-mCFP animals compared to XOPSGFP animals. The PCNA-positive cells were all located within the ONL, consistent with the location of rod precursor cells.

Effect of Injection of an mCFP Morpholino

If, as discussed earlier, rod cell death in XOPS-mCFP heterozygotes results from overexpression of mCFP, blocking expression of the reporter gene should slow the degeneration process. To test this hypothesis, we injected an antisense morpholino to mCFP into transgenic embryos to see whether rod degeneration could be delayed. XOPS-mCFP heterozygous females were crossed with XOPS-GFP homozygous males, and the resultant clutches of embryos were injected with various concentrations of mCFP or control morpholinos at the one- to two-cell stage. In these crosses, all the larvae should inherit the XOPS-GFP transgene and initially demonstrate rod-specific fluorescence, whereas one half also should inherit the mCFP transgene, leading to rod cell death and loss of GFP fluorescence. We examined uninjected, control-injected, and mCFP morpholino-injected larvae at 4 dpf and counted the number of animals in each group that contained GFP-positive rods in the central retina (we focused on the central retina because XOPS-mCFP animals continue to generate mCFP-positive cells at the margin throughout development, although these cells do not survive).

As expected, approximately one half of the animals in the uninjected group experienced rod degeneration with an accompanying loss of GFP fluorescence in the central retina by 4 dpf (Fig. 6). Similarly, only one half of the animals that were injected with a control morpholino still had GFP-positive rods in the central retina at 4 dpf. However, in the groups of zebrafish that were injected with the mCFP morpholino, a significant increase in the number of larvae that displayed GFP-expressing rods in the central retina at 4 dpf was observed. The number of

animals with GFP-positive rods increased with increasing concentrations of morpholino, and the difference from the uninjected control was determined to be significant (according to the χ^2 test) at all concentrations tested. We may therefore conclude that it is the expression of the XOPS-mCFP transgene itself that causes the rods to degenerate in transgenic zebrafish.

ERG Response after Dark Adaptation of XOPS-mCFP Zebrafish

In most vertebrates, the loss of rod photoreceptors ultimately results in a slow yet progressive loss of cone photoreceptors. To examine outer retinal function in XOPS-mCFP animals, ERGs were performed on 7-month-old wild-type and XOPS-mCFP heterozygotes. Figure 7 shows sample ERG waveforms from both wild-type and XOPS-mCFP fish at two different times during dark adaptation. During the initial stages of dark adaptation (Figs. 7A, 7C), the ERG waveforms of the wild-type and XOPS-mCFP subjects were very similar both in shape and amplitude. After 60 minutes of dark adaptation, the amplitude of the wild-type b-wave was much larger than at earlier time points (Fig. 7B). However, the b-wave amplitude of the XOPS-mCFP subjects after 60 minutes was only slightly larger than at the earlier time points (Fig. 7D).

The average dark-adaptation functions were determined for the wild-type and XOPS-mCFP animals, using the peak b-wave response amplitude elicited over the course of dark adaptation (Fig. 7E). During the early stages of dark adaptation, the two functions were very similar and gradually increased in response until approximately 10 to 12 minutes in the dark. After 12 minutes in the dark, the wild-type function continued to increase dramatically (indicating a rod–cone break and rod adaptation) with an eventual asymptote starting at approximately 40 minutes. However, the function based on the XOPS-mCFP response did not increase after 10 minutes in the dark, suggesting that no rod–cone break was present and that the peak b-wave amplitude was generated by only cone responses.

To examine further the XOPS-mCFP ERG response, the average spectral sensitivity function was determined, based on the b-wave response of dark-adapted wild-type, dark-adapted XOPS-mCFP, and light-adapted wild-type animals to various wavelengths of light (Fig. 7F). Dark-adapted spectral sensitivity functions from subjects with rod photoreceptors should indicate a peak sensitivity of approximately 500 nm (the peak sensitivity of the rod spectra) and should possess a higher absolute sensitivity than functions obtained under light-adapted conditions (when cone contributions dominate). This is demonstrated by the function derived from the wild-type, dark-adapted animals and by the model function derived from previous work investigating photoreceptor contributions to dark-adapted adult zebrafish.²³ This model shows a rod-dominated function from 400 to 640 nm. In contrast, the dark-adapted function of the XOPS-mCFP animals displayed a lower absolute sensitivity and did not peak at 500 nm. It also possessed several peaks near the peak sensitivity values of zebrafish cone subtypes. For comparison, the average spectral sensitivity function based on responses of light-adapted wild-type fish is shown. This function is similar to the XOPS-mCFP dark-adapted function, at least from wavelengths starting at 400 nm. The XOPS-mCFP function was slightly more sensitive because the broadband background reduced the overall absolute sensitivity of the normal light-adapted fish. Taken together, these data suggest that rod-mediated ERG responses are not present in XOPS-mCFP adult zebrafish, supporting the conclusion that the rods undergo degeneration in this line. Furthermore, the ERG responses from heterozygous adults indicate that cone function is not detectably impaired in these animals.

Cone Morphology in XOPS-mCFP Transgenic Adults

To further investigate the impact of rod loss on cone survival in XOPS-mCFP zebrafish, we examined cone photoreceptor morphology and arrangement in adult animals by immunohistochemistry (Fig. 8). Retinal cryosections from wild-type and XOPS-mCFP 7-month-old adults

were labeled with antibodies to an epitope expressed by the red–green double cones, to carbonic anhydrase (a marker of Müller cells), and to blue cone opsin. Other than a noticeable decrease in thickness of the ONL in XOPS-mCFP retinas (due to the loss of rod photoreceptor nuclei) the morphology of the cones, tiering of cone photoreceptor nuclei, and the labeling pattern of each antibody were similar between wild-type and XOPS-mCFP animals. We examined tangential retinal sections as well and did not observe any alterations to the cone photoreceptor mosaic in adult transgenic animals (data not shown.) We did not perform immunohistology with UV cone-specific antibodies; however, the morphology of the UV-sensitive short single cones and their position within the photoreceptor mosaic were not different from wild-type control animals. Therefore, our data suggest that, at least at 7 months of age, rod degeneration in XOPS-mCFP transgenic zebrafish has no secondary effects on the cone photoreceptors.

DISCUSSION

In this study, we report the generation of a transgenic line of zebrafish demonstrating selective loss of rods without alterations in cone morphology or function. Results from immuno-labeling, RT-PCR, and ERGs confirmed that the rods had degenerated in XOPS-mCFP heterozygotes. The degeneration of rod photoreceptors in this line appears to be caused solely by transgene expression rather than by a position-of-integration effect, because injection of an mCFP antisense morpholino delayed rod cell death in heterozygous larvae. Strikingly, cone function appeared to be unaltered in transgenic animals.

The cytotoxicity of mCFP observed in this line is in marked contrast to numerous other transgenic lines in zebrafish, *Xenopus*, and mice, in which fluorescent reporter genes have been used successfully to study rod photoreceptor biology.^{8–11,18,34,35} In fact, a study specifically addressing the effects of uniform eGFP expression in the retina of mice found that it caused no functional or structural abnormalities.³⁶ In zebrafish, transgenic lines have been created using the XOPS promoter linked to GFP,⁸ the mCFP reporter driven by a Pax6 promoter,¹⁴ a cone-specific promoter linked to mCFP (data not shown), and a mGFP reporter driven by the Brn-3c promoter;³⁷ none of these constructs causes any abnormalities in transgenic animals. Therefore, the cytotoxicity observed with XOPS-mCFP seems to be specific to this particular transgene. Chan et al.³⁸ reported that mice homozygous for a knock-in of a human rho-GFP fusion protein displayed retinal degeneration a few months after birth. Human rhodopsin alone causes degeneration only when it is overexpressed.³⁹ Therefore, Chan et al.³⁸ hypothesized that some aspect of the structure of human rho-GFP was responsible for the retinal degeneration observed in this line. To our knowledge, our study is the first report of a fluorescent reporter gene that causes rod degeneration on its own in heterozygous animals.

The membrane targeting sequence of mCFP, derived from neuromodulin (GAP43), leads to palmitoylation of the N terminus of mCFP and subsequent plasma membrane association. Rhodopsin also contains a palmitoylation sequence at its C terminus, and this modification anchors the C terminus of rhodopsin in the plasma membrane.⁴⁰ Therefore, given our data showing mislocalization of injected rho-GFP in XOPS-mCFP heterozygotes, one might conclude that the transgene interferes with the proper palmitoylation and subsequent targeting of endogenous rhodopsin and that this is the cause of the rod degeneration. However, we do not believe this is the case, namely because mice transgenic for a mutant form of rhodopsin that cannot be palmitoylated demonstrate correct rhodopsin targeting to the rod outer segments and minimal changes in retinal morphology.⁴¹ Furthermore, of the dozens of different mutations in rhodopsin resulting in human RP, there are currently none that are located within the palmitoylation sequence of rhodopsin, suggesting that this modification is not essential for rod photoreceptor survival. Therefore, we suggest that the high levels of expression induced by the XOPS promoter, in combination with the membrane-targeting signal on the CFP reporter, result in a more global interference with intracellular protein trafficking, and the

mislocalization of the rho-GFP fusion protein in transgenic larvae is a marker of a more general phenomenon. Curiously, the rho-GFP fusion protein correctly localized to the apical ends of pineal photoreceptors, indicating that the defects caused by XOPS-mCFP are specific to the retina.

The increase of TUNEL-positive cells and of nuclei that immunolabel for the proliferation marker PCNA suggests a cycle of continued rod genesis and degeneration in the transgenic line. In larvae and adults, TUNEL labeling demonstrated that overexpression of mCFP led to apoptosis of the rods (Fig. 4), a frequent outcome for neurons or photoreceptors after metabolic damage. In transgenic adults, an increase in PCNA-positive cells was observed in the central retina, at a location vitread to the cone nuclei. This pattern is consistent with the location of ongoing rod genesis in teleost fishes, which normally functions to maintain a constant rod density as the retina is stretched within the expanding eye.⁴² Although we cannot exclude the possibility that the increase in PCNA immunoreactivity in transgenic retinas is reflective of attempted DNA repair in the degenerating rods (as has been seen in the *rd1* mouse⁴³), the fact that very few cells in adult transgenic retinas were rhodopsin positive (in contrast to the numerous cells that were PCNA positive) suggests that the PCNA-positive cells are rod progenitors rather than differentiated rods.

Although very few cells in adult transgenic retinas were rhodopsin-positive, we observed several cells in the ONL of transgenic adults that labeled with an antibody recognizing an unknown epitope in rods and pineal photoreceptors. A subset of these cells were also TUNEL positive, but the rest did not appear to be undergoing apoptosis. Many of these cells had a single apical extension consistent with an inner segment; however, they did not possess outer segments. One explanation of these results is that the second antibody recognizes immature rods that have not yet initiated expression of rhodopsin (or mCFP). Given the increase in mitotic activity in the ONL, these results suggest that rod degeneration in this line causes an increase in rod precursor proliferation. The resultant immature rods must either arrest during their development or undergo apoptosis on initiation of mCFP expression, because we did not observe any healthy, mature rods in adult transgenic retinas.

It has long been known that injury to the retina of teleost fish stimulates neural cell regeneration.⁴⁴ The regenerative response has been elicited by exposure to pharmacologic agents,⁴⁵ surgical excision,⁴⁶ laser injury,³² and light damage.³³ However, these methods cause loss and regeneration of all retinal cell types or, in the case of laser or light damage, regeneration of both rods and cones. Our results demonstrate that selective loss of only rod photoreceptors is sufficient to provoke a regenerative response, which suggests that the trigger for rod precursor proliferation may come from the rods themselves, perhaps involving a density-dependent inhibitory mechanism.

The histologic absence of mature rods in XOPS-mCFP heterozygotes was strongly corroborated by the physiological data. ERGs recorded from XOPS-mCFP eyes confirmed that rod-mediated responses were absent from transgenic adults. Furthermore, the XOPS-mCFP dark-adapted spectral sensitivity function displayed a reduced sensitivity and showed no peak at 500 nm (the peak sensitivity of the rod spectra) although it possessed peaks near the peak sensitivity values of the zebrafish cone subtypes.

Two conclusions may be drawn from the ERG data. The first conclusion is that rod function is indeed lost from XOPS-mCFP animals; the second and perhaps more intriguing conclusion is that cone-mediated responses, at least up to 7 months of age, are not affected by the rod degeneration. Furthermore, the histologic data demonstrate that the position and morphology of the cones appears normal in adult XOPS-mCFP retinas.

The latter result is very interesting, given that in human RP and in mammalian and amphibian transgenic models of RP, defects in the rods lead eventually to secondary loss of the cones, although the timing of cone loss can vary, depending on the mutation.^{47–50} The mechanism of this secondary cone loss is not well understood; current theories include toxic extracellular substances released by dying rods, passage of toxic factors through rod–cone gap junctions, and loss of rod-derived cone viability factors.^{51–53} The existence of a diffusible photoreceptor survival factor has also been suggested by studies of some zebrafish retinal degeneration mutants.⁵⁴ Although it is possible that the cones of XOPS-mCFP animals will also eventually be affected by the rod degeneration, the fact that they are initially resistant to secondary degeneration suggests two possibilities. The first is that in zebrafish, rod–cone interactions may be different from other vertebrates. Although much of the architecture, physiology, and genetic regulation of rod development are conserved across species, the different developmental origin of the rods in adult teleosts may imply that the cones are less dependent on rod–cone interactions.

Alternatively, the cones may be protected by the increase in rod precursor proliferation. It has been suggested that during the course of human RP, cone function remains normal until approximately 75% of the rod outer segments are lost.⁵⁵ Perhaps in XOPS-mCFP zebrafish, the increase in rod precursor proliferation and immature rod production are sufficient to keep cone function normal. Even though the immature rods do not develop outer segments and there is no physiological evidence of light-evoked rod responses, the immature rods may still produce a cone survival factor of the type proposed by Mohan-Said et al.⁵⁶ Further studies of the importance of the rod precursor response in XOPS-mCFP animals should help resolve this question and may suggest new avenues of investigation into the causes of secondary cone loss during retinal degenerative diseases like RP.

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FIGURE 1.

Loss of rods in XOPS-mCFP transgenic larvae. Retinal cryo-sections of XOPS-GFP heterozygous larvae (**A**, **B**) at 3 and 5 dpf demonstrated an increase in GFP-positive rods in the ventral retina and scattered across the dorsal and central retina. In contrast, mCFP-positive rods initially appeared in the ventral retina of XOPS-mCFP heterozygotes at 3 dpf (**C**), but were present only near the marginal zone by 5 dpf (**D**). Some of the mCFP-positive rods have knoblike protrusions at the apical end instead of normally developing outer segments (**C**, **D**, *arrowheads*), whereas others appear pyknotic (**C**, **D**, *arrows*). Nuclei were visualized with propidium iodide. PCL, photoreceptor cell layer; INL, inner nuclear layer; GCL, ganglion cell layer; MZ, marginal zone.

FIGURE 2.

Absence of rhodopsin expression in XOPS-mCFP larvae at 5 dpf. RT-PCR was performed on RNA isolated from wild-type larvae (*lane 1*) or XOPS-mCFP larvae (*lane 2*) at 5 dpf.

Rhodopsin expression was not detectable in XOPS-mCFP larvae, but UV opsin and β -actin expression appeared similar to that in the wild-type control. *Lane 3*: PCR of wild-type adult retinal cDNA; *lane 4*: the no-reverse-transcriptase control.

FIGURE 3.

Altered rho-GFP localization in XOPS-mCFP rods. The rho-GFP fusion protein was expressed in wild-type or XOPS-mCFP transgenic embryos and examined at 3 dpf by confocal microscopy. Because injected embryos were mosaic for the fusion protein construct, only a subset of the rods expressed rho-GFP. In wild-type larvae (A), rho-GFP localized principally to the outer segments of developing rods. In contrast, rho-GFP was mislocalized in the XOPS-mCFP rods that expressed the fusion protein (B). In cell 1, most of the rho-GFP was observed in the cytoplasm, just below a knoblike protrusion at the apical end of the cell. In cell 2, rho-GFP was visible in a somewhat normal looking developing outer segment, but there was also a significant amount of rho-GFP expressed throughout the cell body. This cell also contained

an abnormal process that projected into the inner nuclear layer (*bracket*). Cell 3 displayed an abnormal, knoblike outer segment, with most of the rho-GFP concentrated in the knob. Cell 4 had a rounded morphology, with no outer segment and basal distribution of rho-GFP.

Arrows: apical regions of the cells. (C) mCFP expression of the cells in (B). In the pineal gland of XOPS-mCFP larvae (D), most of the rho-GFP was expressed at the apical ends of the pinealocytes. (E) mCFP expression of the cells in (D).

FIGURE 4.

Apoptosis increased in XOPS-mCFP larvae and adults. Retinal cryosections from wild-type (A, C) and XOPS-mCFP (B, D, E) 5-dpf larvae (A, B) and adults (C–E) were analyzed by TUNEL labeling (red) and by immunolabeling with the 1D1 antibody (A–D), which recognizes rhodopsin (green). Colocalization of the TUNEL label and the rhodopsin antibody gave an orange signal (arrowheads). Nuclei were visualized with DAPI. (E) A retinal cryosection from a transgenic adult, immunolabeled for TUNEL-positive cells and with the 4C12 monoclonal antibody (green, arrows). Several cells in the ONL labeled with the 4C12 antibody (arrows). (F) A confocal image of the section in (E). Some of the 4C12-labeled cells had apical processes indicative of inner segments. Some, but not all, of the 4C12-positive cells were also TUNEL positive (arrows). Some TUNEL-positive cells were observed in the inner nuclear layer of transgenic retinas (B, D, E,*). Confocal microscopy (G) demonstrates that these cells had a morphology typical of Müller cells. The cell colocalizes with a labeled rod in the ONL, suggesting that the Müller cells phagocytose the dying rods or rod debris. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; ROS, rod outer segments; RPE, retinal pigmented epithelium; HC, horizontal cells.

FIGURE 5.

Cell proliferation increased in XOPS-mCFP adults. Retinal cryosections from wild-type (**A**) and transgenic (**B**) adults were labeled with an antibody to PCNA and counterstained with DAPI to visualize the nuclei. *Arrows*; PCNA-positive cells in (**A**) and (**B**). (**C**) Average number of PCNA-positive cells per 100 μm is presented for wild-type (8 sections of three eyes) and XOPS-mCFP heterozygotes (10 sections of three eyes). *Significant increase in PCNA-positive cells in transgenic adults ($P < 0.005$; t -test).

FIGURE 6.

Injection of an antisense mCFP morpholino (MO) delayed rod degeneration in XOPS-mCFP larvae. Embryos from crosses between XOPS-GFP homozygous males and XOPS-mCFP heterozygous females were injected at the one- to two-cell stage with a control MO or the mCFP MO. Injected larvae were examined by fluorescence microscopy at 4 dpf for the presence of GFP-positive rods in the central retina. Injections were performed a minimum of three times. Significance was determined by the χ^2 test, based on an expected 1:1 ratio of GFP-expressing to -nonexpressing animals at 4 dpf. Injection of the control MO did not significantly increase the percentage of larvae with GFP-positive rods at 4 dpf. *a– c: $P \leq 0.005$.

FIGURE 7.

Rod-mediated visual responses are absent from XOPS-mCFP adults. Sample ERG waveforms at the initial stages of dark adaptation (**A**, **C**) and at later stages of dark adaptation (**B**, **D**) are shown for wild-type and XOPS-mCFP 7-mpf adults. The *horizontal bracket* on the abscissa depicts the 200-ms stimulus. (**E**) The average dark-adaptation functions of wild-type ($n = 5$) and XOPS-mCFP ($n = 3$) adults. Response amplitude is based on the peak amplitude of the ERG b-wave to a 500-nm, 200-ms stimulus. Error bars, SEM. (**F**) The average spectral sensitivity functions obtained from dark-adapted WT ($n = 8$), dark-adapted XOPS-mCFP ($n = 2$), and light-adapted WT ($n = 5$) subjects. Dark-adapted functions were obtained after 60 minutes of dark adaptation. Light-adapted functions were obtained with a broadband

background of $5 \mu\text{W}/\text{cm}^2$. *Dashed line*: the rod-dominated model based on the adult dark-adapted spectral sensitivity data of Saszik and Bilotta,²³ normalized at 500 nm with respect to the data. Sensitivity was defined as the stimulus irradiance that yielded an ERG b-wave peak amplitude response of $50 \mu\text{V}$. Error bars, SEM.

FIGURE 8.

Normal cone morphology in XOPS-mCFP adults. Retinal cryosections from wild-type (A, C) and XOPS-mCFP (B, D) 7-month-old adults were immunolabeled with antibodies to the red-green double cones (A, B; red), Müller cells (A, B; green), or blue cones (C, D) and imaged by fluorescence microscopy. Nuclei were visualized by staining with DAPI. Note the decrease in thickness in the ONL of transgenic adults due to loss of rod nuclei. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.