Long-Term Retinal Cone Survival and Delayed Alteration of the Cone Mosaic in a Transgenic Mouse Model of Stargardt-Like Dystrophy (STGD3)

Sharee Kuny,1 Marc-André Filion,1 Miyoung Suh,2 Frédéric Gaillard,1 and Yves Sauvé1

1Department of Ophthalmology and Visual Sciences, University of Alberta, Edmonton, Alberta, Canada
2Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, Manitoba, Canada

Purpose. To examine the pattern of cone degeneration in the retina of a transgenic mouse model of Stargardt-like dystrophy (STGD3).

Methods. Investigations were performed on ELOVL4/TG1-2 transgenic (TG) mice and wild-type (WT) littermates from 1 to 24 months of age. Phenotypes were assessed by fundus imaging, fatty acid analysis, and electroretinogram (ERG) recording. Cone degeneration pattern was determined on retina whole mounts using immunohistochemistry and Voronoi domain analyses.

Results. Consistent with low transgene expression, photoreceptors degenerate very slowly. At 1 month, anatomical structure and fatty acid composition of the TG retina is comparable with WT. Rod loss appears at 2 months, exhibiting a central to peripheral gradient, and fundus defects are observed at 3 months. In contrast, cone morphology, distribution and function are still normal at 12 months. Cone loss becomes apparent at 15 months when the outer nuclear layer is reduced to 3 to 4 photoreceptor rows. This process starts at the center of the retina and affects cone subtypes similarly. Very few cones remain at 24 months, after all rods have disappeared (18 months). Quantitative studies focusing on cones expressing M-opsin show a net increase in Voronoi domains and a significant decrease in regularity indexes only beyond 15 months.

Conclusions. Photoreceptor degeneration in this STGD3 mouse model follows the time course of a slow rod–cone dystrophy. The cone mosaic is preserved for almost 1 year after the onset of rod loss. This long delay provides an opportunity to examine rod–cone interactions during retinal degeneration and to test therapeutic effectiveness at protracting cone dysfunction.

Keywords: mouse, retina, cone photoreceptor, secondary degeneration, ELOVL4, fatty acids, Stargardt-like dystrophy, STGD3

Stargardt-like dystrophy (STGD3, Online Mendelian Inheritance in Man [OMIM] 600110) is a rare, inherited autosomal-dominant juvenile form of macular degeneration characterized by homogenous atrophy of the pigment epithelium in the macula (often associated with yellow flecks representing lipofuscin deposits) and temporal optic nerve pallor. In the early stages of the disease, patients experience progressive loss of visual acuity (20/200 or worse at the age of 30), but minimal color vision disturbance and minor changes in electroretinogram (ERG) responses. Marked central and peripheral visual impairment occur in the late stages.1,2

This retinal disorder is caused by mutations (797-801-delAACTT, 790ΔT+794ΔT, Y270ter) in exon 6 of the ELOVL4 gene3–5 located on chromosome 6q14. This widely conserved gene encodes a very long-chain fatty acid elongase.6–8 All of these mutations induce a truncation of the wild-type (WT) ELOVL4 protein at its C-terminus, and the subsequent deletion of a dileucine motif necessary for retention of transmembrane proteins in the endoplasmic reticulum (ER). Although recently challenged,9 cellular mislocation of the truncated ELOVL4 protein to perinuclear aggresomes10,11 and altered synthesis of very long-chain polyunsaturated fatty acids (VLC-PUFA) are currently considered to be at the origin of the disease.6,7,12–14 In mammals, including human,4,15–18 the ELOVL4 protein is abundant in the retina and is expressed at much lower levels in brain, skin, testis, and ocular lens. In the mouse, Elovl4 transcript is detected by embryonic day 7 (E7), and protein is expressed by E10.5 in the lens vesicle and presumptive retina. Expression of Elovl4 mRNA in the eye increases strongly up to 1 month of age (4-fold the level at birth) and then remains at a high level throughout life.17 As with monkey,4 rat,7 and pig,11 Elovl4 mRNA and protein in the adult mouse retina17,19 localize to the inner segments of both rods and cones.

One mouse model of STGD3 relies upon the addition into its genome of several copies of a mutated ELOVL4 gene (5 base pair deletion corresponding to the delAACTT human mutation) driven by the human interphotoreceptor retinoid-binding protein gene promoter present in all photoreceptors.13 The resultant transgenic (TG) mice were classified into three lineages according to the expression level of the ELOVL4 transgene. Based on this criterion, the TG line maintained in our laboratory appeared to be intermediate between original TG1 and TG2 lines,13 and was therefore referred to as ELOVL4/TG1-2.14
This TG1-2 line displays low ELOVL4 transgene expression relative to endogenous Elovl4 (ratio = 1.34 ± 0.21), resulting in a very slow photoreceptor degeneration over the lifespan of the mouse (2 years), and demonstrating reduced levels of the lipofuscin fluorophores, A2E and iso-A2E (due to the presence of the Leu450Met variant in the Rpe65 protein of C57BL/6 strain). Furthermore, full-field ERG recordings in these TG mice strongly suggest that, although introduced in all photoreceptors, the ELOVL4 mutation affects cones much later than rods.

Secondary cone photoreceptor loss is a common phenomenon observed in normal aging, rod-cone dystrophies, as well as, presumably, in age-related maculopathies. Because cones are accountable for daylight visual function, preservation of cone phenotype, function, and topography represents the key therapeutic target for preserving minimal visual perception in afflicted patients. The goal of the present work is to examine the time course of cone loss in the ELOVL4/TG1-2 mouse model of STGD3 to define an accurate time window for the application of putative therapies. Present results complement former descriptions of the degenerative process in ELOVL4/TG1-2 mice and show that alteration of the cone mosaic is delayed by almost 1 year from initial rod loss.

**MATERIALS AND METHODS**

**Animals**

Experiments were carried out on WT and heterozygous TG littermates (on a C57BL/6J background). Animals were raised on a 12:12 light/dark cycle and supplied with water and standard rodent Chow (Laboratory Rodent Diet 5001; PMI Nutrition International, Richmond, IN) without restriction. Proportions of arachidonic acid (AA; C20:4n-6), eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) in this chow were 0.06%, 0.165%, and 0.175% of the total fatty acids (our analysis), respectively. Experimental procedures were approved by the Institutional Animal Care and Use Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Fundus Photography**

Fundus images were taken at 1 month (WT, n = 2; TG, n = 4), 3 months (WT, n = 9; TG, n = 5), 6 months (WT, n = 7; TG, n = 4), 12 months (WT, n = 4; TG, n = 7), and 18 months (WT, n = 3; TG, n = 2) using a Micron III retina imaging system for rodents (Phoenix Research Laboratories, Inc., Pleasanton, CA) equipped with a Semrock FF01-554/211 filter (Semrock, Inc., Rochester, NY) for white light brightfield imaging (450–680 nm). StreamPix 5 software (NorPix, Montreal, Canada) was used for image capture and review.

**Fatty Acid Analysis**

Transgenic animals were further characterized by comparing the fatty acid composition of their neural retina to that of WT littermates. Investigations were performed at 1 month (WT, n = 6; TG, n = 6), 3 months (WT, n = 3; TG, n = 8), 6 months (WT, n = 8; TG, n = 5), 9 months (WT, n = 8; TG, n = 8), 12 months (WT, n = 9; TG, n = 8), 18 months (WT, n = 8; TG, n = 9), and 24 months (WT, n = 6; TG, n = 7). Retina lipids were analyzed as previously published. In brief, lipids were extracted with chloroform:methanol (2:1, wt/wt) and total phospholipids (PL) were isolated on thin-layer chromatography plates (Analtech silica-gel G; MandleScientific Co., Guelph, Ontario, Canada) with petroleum ether/ethyl ether/acetonic acid (80:20:1, vol/ vol/vol). Fatty acid methyl esters were then prepared using BF3/methanol and separated by automated gas-liquid chromatography (Vista 6010 GLC and Vista 402 data system; Varian Instruments, Mississauga, Ontario, Canada) using a fused silica BP70 micro capillary column (15 m × 0.1 mm internal diameter [ID]). Hydrogen was used as the carrier gas at a flow rate of 0.5 mL/min using a split ratio of 20:1. The initial oven temperature was 130°C, then increased to 175°C at 20°C/min held for 1 minute, increased again to 200°C at 6°C/min with no hold and finally increased to 280°C at 30°C/min. Resulting peaks were identified by comparison of their retention times to those of standards (GLC-68A; Nu-Chek-Prep, Ely, MN). Data are expressed as micrograms per retina PL using C19:0 as an internal standard.

**ERG Recordings**

Dark- and light-adapted ERG recordings were performed as previously described. In brief, animals were dark-adapted overnight and prepared the next day for recordings under dim red light. Mice were anesthetized with a mixture of ketamine (75 mg/kg intraperitoneal [i.p.]) and xylazine (15 mg/kg i.p.) and pupils were dilated with 1% tropicamide. Simultaneous bilateral recording was achieved with active gold loop electrodes placed on each cornea, and subdermal platinum reference electrodes placed behind each eye. A subdermal ground platinum electrode was set on the mouse’s scruff. Light stimulation (white flashes delivered by a xenon bulb at 6500°K, 10 μs duration), signal amplification (0.3–300 Hz bandpass without notch filtering) and data acquisition were provided by a commercial system (Espion E2; Diagnosys LLC, Littleton, MA). Dark-adapted responses (flash strengths from −5.22 to 2.86 log cd s/m2) were collected first, then the light-adapted responses (10 minutes photopic adaptation, background illumination: 30 cd/m2, flash strengths from −1.6 to 2.9 log cd s/m2). For each animal, only responses from the best eye (yielding the largest dark-adapted b-wave) were considered for analysis.

**Primary Antibodies**

Immunogens, hosts and sources of primary antibodies are given in Table 1. Total cone population was labeled using a purified anti-gamma transducin antibody raised in rabbit against recombinant human Gyc protein. Cone subtypes were screened using anti-M-opsin and anti-S-opsin rabbit polyclonal antibodies. For double-labeling experiments, the former S-opsin antibody was replaced with an affinity-purified goat polyclonal antibody. These antibodies label specific types of cones, without cross-reactivity, in various mammalian species. Age-related levels of rhodopsin were assessed using common antirhodopsin monoclonal antibody.

**Immunohistochemistry**

Immunohistochemical investigations on whole-mounted retinas were performed as previously described. Following euthanasia (Euthanol; Bimeda-MTC Animal Health, Inc., Cambridge, ON, Canada), eyes were enucleated and the retinas carefully dissected from the eyecup and divided into four quadrants. After overnight fixation at 4°C in 4% paraformaldehyde, retinas were blocked for 3 hours at room temperature in a medium containing PBS+0.1% Triton X-100+5% nonfat milk, and incubated for 3 days at 4°C with the desired antibodies diluted appropriately in a 1:10 solution of the previous blocking medium. Next, the retinas were exposed for 2 days either to goat anti-rabbit Alexa488, goat anti-mouse Alexa594, donkey anti-rabbit Alexa594 or to donkey anti-goat Alexa688 secondary antibodies (Molecular Probes, Inc., Eugene, OR).
diluted 1:500 in a 1:10 solution of the blocking medium. Finally, retinas were mounted photoreceptor side-up on glass slides, covered with an aqueous antifade solution (Gel/Mount; Biomeda, Foster City, CA) and coverslipped. Control labeling without primary antibody remained negative. Only one retina per animal was used for analysis.

Additional eyes were fixed in 4% paraformaldehyde and prepared for cryosectioning. A few sections \( n = 2-3; 20-\mu m \) thick, passing through or close to optic nerve head (ONH) parallel to the nasotemporal axis, were mounted on glass slides and treated as above with the exception that they were reacted overnight with the primary antibodies and for 3 hours with the secondary antibodies. After washing, slides were covered with a 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI)-containing antifade aqueous medium (ProLong gold antifade reagent, P36939; Molecular Probes) and coverslipped.

**Imaging**

Representative retinal samples were imaged either with a laser confocal microscope (LSM 510 Axiovert 100M; Carl Zeiss Meditec, Inc., Dublin, CA) or a fluorescence microscope (DM6000 Leica; Deerfield, IL) equipped with a computer controlled motorized stage. Raw images were then processed for quantitative measurements using either ImageJ 1.44c (National Institutes of Health [NIH], Bethesda, MD; [in the public domain] http://rsb.info.nih.gov/ij/) or a dedicated subroutine of the microscope software (Leica Application Suite). Pictures and graphs were combined in Photoshop 8.0 (Adobe, San Jose, CA) for final illustrations. All data were collected from images corresponding to a single focal plane.

**Quantification of Photoreceptor Populations**

Photoreceptor loss in WT and TG mice was estimated by measuring the thickness and the number of rows of DAPI-stained nuclei of the outer nuclear layer (ONL) at 3 to 4 distinct locations, both in the center \( (=300 \mu m \) from the ONH) and the periphery \( (=300 \mu m \) from the ora serrata) of each cross-section.

Cones densities were determined from manual counts applied to raw images \( (224.3 \times 167.5-\mu m \) wide; area \( = 0.0376 \ mm^2) \) taken with a 40x oil immersion objective in the center \( (=500 \mu m \) from the ONH), in the mid periphery \( (=1200 \mu m \) from the ONH) and in the far periphery \( (=500 \mu m \) from the ONH) at each retinal quadrant of whole-mounted retinas (Fig. 4). Twenty-four to 48 images per retina \( (2-4 \) at each eccentricity) were considered for calculation. Every labeled cone outer segment in these frames was scored and tagged for additional Voronoi domain analysis by using the appropriate ImageJ plug-in (NIH). The area of the centermost domains (typically \( n = 100-150 \) in a frame was computed to generate an average Voronoi domain (VD) and regularity index (VDRI, also named “conformity ratio”); defined as the ratio of the mean VD area to the SD) in that frame. Voronoi domains related to elements located within the peripheral exclusion zone (15-\mu m wide) as well as those intersecting the outer limits of the sampling frame were always excluded from computation to minimize boundary effects. Moreover, computation did not consider frames containing less than 50 tagged elements (i.e., less than 20 domains in the central “safety” zone) for statistical reasons (Fig. 7). Measurements were performed by two different individuals. Variability between investigators (as assessed by comparing respective data in one WT and one TG mice at 12 months) was within 5% to 10%. Estimates of total cone population (Tables 2 and 3) are given for a 16.4-mm\(^2\) wide retina \( (+1.2 \ mm^2; \) average from 10 animals; WT, \( n = 4 \) and TG, \( n = 6; \) 3–24 months; see Jeon et al.\(^{37}\) for similar data).

Proportions of single and dual opsin-expressing cones were assessed from double-stained retinal whole-mounts. Briefly, separate 8-bit grey pictures for S- and M-opsins were taken as above, the background was removed and the resultant images were combined using the ImageJ “Colocalization” plug-in (implemented by Pierre Bourdoncle, Institut Jacques Monod, Paris, and available on the ImageJ website; NIH). The plug-in returned a composite red-green-blue (RGB) image containing single- and double-labeled elements. Two points in this image were considered as colocalized if their respective intensities were higher than the threshold of their channels, and if their ratio of intensity was higher than the ratio setting value (which is 50% by default).

**Dorsoventral Gradient of M-opsin Expression**

This feature\(^{38}\) was examined in both WT and TG mice at 12 months (prior to cone degeneration) using a semi-quantitative approach based on fluorescence levels in frames \( (48.6 \times 335 \ \mu m; \times 20 \) objective) captured under identical illumination along the dorsoventral axis of selected whole-mounted retinas (3 frames per eccentricity; 18 frames per animal) reacted with the M-opsin antibody. After equalization of the background, fluorescence profiles (resolution: 0.32 \mu m/pixel) were collected along 11 equally spaced (30 \mu m) rows drawn parallel to the frame length and assembled graphically with a dedicated subroutine of the microscope software. The resulting histogram was imported into ImageJ (NIH) and the total area under the peaks was calculated. Finally, M-opsin expression for a given eccentricity was estimated by averaging the area values obtained in the 3 frames located at that eccentricity. Baseline for all measurements was set arbitrarily at \( +1 \) above the mean background level of the frame to account for local variability in the staining process.

---

**Table 1.** Primary Antibodies Used for Immunohistochemistry (IHC) and Western Blotting (WB)

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Antigen (Amino Acids)</th>
<th>Host</th>
<th>Source, Catalog Number</th>
<th>Dilution IHC</th>
<th>Dilution WB</th>
<th>Bands on WB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transducin</td>
<td>Full Gr/8 subunit (human)</td>
<td>Rabbit</td>
<td>CytoSignal*, PAB-00801G</td>
<td>1:1000</td>
<td>1:1000</td>
<td>10 kDa†</td>
</tr>
<tr>
<td>OPN1W/M-opsin</td>
<td>324-564 (human)</td>
<td>Rabbit</td>
<td>Millipore/Abcam®, AB 5405</td>
<td>1:500</td>
<td>1:1000</td>
<td>34, 42 kDa</td>
</tr>
<tr>
<td>OPN1SW/S-opsin</td>
<td>311-348 (human)</td>
<td>Rabbit</td>
<td>Millipore/Abcam, AB 5407</td>
<td>1:500</td>
<td>1:1000</td>
<td>39 kDa†</td>
</tr>
<tr>
<td>OPN1SW/S-opsin</td>
<td>8-27 (human)</td>
<td>Goat</td>
<td>Santa Cruz Biotech, sc-14363</td>
<td>1:200</td>
<td>1:1000</td>
<td>40 kDa†</td>
</tr>
<tr>
<td>Rhodopsin, clone 4D2</td>
<td>2-39 (bovine)</td>
<td>Mouse</td>
<td>Millipore/Abcam, MAB15</td>
<td>1:500</td>
<td>1:1000</td>
<td>35 kDa + multimers</td>
</tr>
<tr>
<td>α-Tubulin, TU-02</td>
<td>1-451 (porcine)</td>
<td>Mouse</td>
<td>Santa Cruz Biotech, sc-8035</td>
<td>1:500</td>
<td>1:500</td>
<td>54 kDa</td>
</tr>
</tbody>
</table>

* CytoSignal, Irvine, California.
† Manufacturer's data sheet.
‡ Millipore/Abcam, Cambridge, Massachusetts.
Table 2. Number of Cones in WT and TG Mice as a Function of Age and Retinal Location

<table>
<thead>
<tr>
<th>Age/Status</th>
<th>N</th>
<th>D3</th>
<th>D1</th>
<th>V1</th>
<th>V3</th>
<th>N3</th>
<th>N1</th>
<th>T1</th>
<th>T3</th>
<th>Mean</th>
<th>SD</th>
<th>Total</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Cones</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-Cones</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LOSS

-74% -76% -75% -77% -58% -72% -71% -80%

Counts from flat-mounted retinas stained either for cone transducin (all cone population) or OPN1MW opsin (M-opsin+ cones). Values in the mid periphery of the retina have been omitted for clarity. Bold values indicate significant loss of M-opsin+ cones in dedicated counting frames at 15, 18, and 24 months of age. N, number of animals examined; Mean, mean cone density per square millimeter; Total, total cone population in the retina periphery of the retina. D3, V3, retina periphery; D2, V2, mid periphery; D1, V1, retina center; N3, N1, T3, retina center.

Western Blotting

As previously described, 79 retinas from both TG and WT at 3, 12, and 18 months (WT, n = 4; TG, n = 5 at each age) were homogenized in PBS buffer. Protein extracts were boiled for 5 minutes and samples (1–10 μg total protein) were resolved by SDS-PAGE on 10% to 12% acrylamide gels. Proteins were transferred to polyvinylidene fluoride membranes, blocked for 1 hour with 5% nonfat milk diluted in TBS-T and incubated overnight with mouse anti-rhodopsin and rabbit anti-M-opsin antibodies diluted (1:1000) in the blocking solution. Alpha-Tubulin (1:500) was used as a loading control. The following day, membranes were washed and reacted for 1 hour with anti-mouse, anti-rabbit, or anti-goat immunoglobulin G (IgG), horseradish peroxidase-conjugated antibody (GE Healthcare, NA931 and NA934; GE Healthcare, Little Chalfont, UK; Santa Cruz sc-2354; Santa Cruz Biotech, Santa Cruz, CA, respectively; 1:5000 in the blocking solution). Finally, after extensive washing and addition of chemiluminescent reagent (Perkin Elmer, NEL 103; Perkin Elmer, Wellesley, MA), protein bands were visualized on a Kodak Image Station 440. Net intensity of bands was calculated using imaging software (Kodak, v.4.0.3; Eastman Kodak, Rochester, NY). Experiments were performed in duplicate.

Table 3. Raw Number and Proportions of M-, S-, and M+S-Opsin Expressing Cones in WT and TG Mice Aged 12 and 18 Months

<table>
<thead>
<tr>
<th>Age/Status</th>
<th>Opsin in Cones</th>
<th>Number of Cones in Frames Along the Dorsoventral Direction</th>
<th>Total in All 12 Frames</th>
<th>Number of Cones in Retina</th>
<th>% Dorsal</th>
<th>% Ventral</th>
<th>% Across the Retina</th>
</tr>
</thead>
<tbody>
<tr>
<td>12mo-WT</td>
<td>M</td>
<td>291 312 255 0 5 6 1 1548 97.6% 1.0% 37.9%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>2 4 17 88 76 61 476 1.0% 22.8% 11.7%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M+S</td>
<td>0 9 106 296 278 180 2059 1.5% 76.2% 50.4%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12mo-TG</td>
<td>M</td>
<td>296 345 40 0 1 1499 86.4% 0.1% 31.1%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>9 13 137 122 125 156 142 859 3.0% 34.4% 17.8%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M+S</td>
<td>15 64 275 315 302 266 2469 6.5% 56.5% 15.1%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12mo-WT</td>
<td>M</td>
<td>263 287 37 3 0 0 1214 95.0% 0.0% 28.6%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>10 8 41 33 105 112 500 3.1% 31.4% 11.8%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M+S</td>
<td>0 11 400 341 306 168 2530 1.9% 68.6% 59.6%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12mo-TG</td>
<td>M</td>
<td>316 357 90 0 1 1531 90.7% 0.1% 31.3%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>9 13 87 102 146 122 774 3.0% 30.9% 15.8%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M+S</td>
<td>5 57 325 355 312 286 2587 4.8% 69.0% 52.0%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18mo-WT</td>
<td>M</td>
<td>192 291 266 2 0 1 1291 94.5% 0.2% 37.6%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>6 11 6 46 63 77 409 3.3% 24.6% 11.9%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M+S</td>
<td>1 10 6 254 275 153 1734 2.2% 75.6% 50.5%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18mo-TG</td>
<td>M</td>
<td>280 291 257 0 1 1320 96.9% 0.1% 35.4%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>3 8 3 61 131 107 625 1.9% 34.2% 16.7%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M+S</td>
<td>3 4 0 264 253 203 1789 1.2% 65.6% 47.9%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Counts were performed in 12 frames as drawn (black rectangles) in Figure 4 for M366 and M269. Values obtained along the nasotemporal direction have been omitted for clarity. D3, V3, retina periphery; D2, V2, mid periphery; D1, V1, retina center.
Cone Degeneration in STGD3 Mouse

Statistics

Statistics were performed using Statview software (SAS Institute, Inc., Cary, NC). Unless otherwise stated, differences were tested using one-tailed paired and unpaired t-tests. Significance was set at \( P \) less than or equal to 0.05. All values are given as the mean ± SD. Histologic measurements were not corrected for possible tissue shrinkage.

Results

Fundus Observations

Fundus images are presented in Figure 1. Transgenic retinas appear normal at 1 month, and then increasing numbers of granular-like vitreoretinal deposits are observed with aging (Fig. 1, top panel). There was no evidence of geographic atrophy. Fundus abnormalities in our TG1-2 mice therefore resemble those observed in the original TG1 line,\(^{13}\) which is characterized by a very low transgene expression level. We did however, see evidence of RPE mottling in one TG animal at 18 months (Fig. 1, bottom panel).

Retinal Profile of Fatty Acids in TG Mice

Concentration of the major long and very long chain polyunsaturated fatty acids (LC- and VLC-PUFA) in retina total phospholipids are illustrated in Figure 2 for TG and WT aged 1 to 24 months. Data are limited to AA, DHA, and fatty acid species involved in the ELOVL4-mediated elongation pathway.\(^{8}\) At 1 month of age, the concentration of LC- and VLC-PUFA in WT and TG do not differ significantly. In WT, AA and DHA are continuously increased until 18 months and then drastically decrease at 24 months. In TG, both LC-PUFA reach high concentration at 9 months and then start to decrease. While the loss of AA was seen at 12 months, DHA slowly decreased in a steady fashion from the age of 5 months until the end of the study by having only 55.8%, 80.8%, 77.0%, 51.9%, 36.4%, and 53.6% of WT level at 3, 6, 9, 12, 18, and 24 months, respectively. This led to much lower DHA/AA ratio in TG as early as 2 months. Moreover, ERG recordings done at 12 months indicated that the initial photoreceptor loss in TG was affecting only rods. Here we provide additional findings (Fig. 3) that complement and further strengthen this assertion.

Primary Defect in TG Mice Affects Rod Photoreceptors

Previous investigations from this laboratory\(^{20,39}\) demonstrated dramatic structural changes in the ONL of TG mice over a 2-year span. While normal at 1 month, this layer is reduced by one photoreceptor row in the central retina as early as 2 months, and is minimal at 24 months. Moreover, ERG recordings done at 12 months indicated that the initial photoreceptor loss in TG was affecting only rods. Here we provide additional findings (Fig. 3) that complement and further strengthen this assertion.

As generally reported,\(^{40,41}\) the thickness of the ONL in WT mice at 10 to 12 months (\( n = 2 \)) is 59.1 ± 5.1 \( \mu \)m in the center of the retina (10-12 photoreceptor rows; Fig. 3A) and 40.9 ± 5.2 \( \mu \)m in the periphery (6-8 photoreceptor rows). No significant change is observed in WT at 24 months (\( n = 2 \)). In contrast, the ONL in TG mice at 12 months (\( n = 2 \)) is evenly reduced on average to 24.1 ± 2.9 \( \mu \)m (≈4 photoreceptor rows). Compared with WT, this value denotes an approximate 60% decrease in the central retina and an approximate 41% decrease in the periphery (Figs. 3B, 3C). Few nuclei remain in TG at 24 months (\( n = 1 \)), aligned as a single row at discrete retinal locations. Total rhodopsin levels measured in TG at 3 months are markedly lower than normal (Fig. 3D). The decrease in total rhodopsin levels measured in TG with increasing age closely parallels the reduction in the number of photoreceptor rows (Figs. 3C, 3D). Counts performed in retinal whole mounts stained for cone transducin (Table 3) show that, at 12 months, WT (\( n = 4 \)) and TG (\( n = 3 \)) yielded similar number of cones per retina, approximately 178,100 ± 7947 and approximately 175,100 ± 20,240, respectively (\( P = 0.9 \); Mann-Whitney \( U \) test). Regardless of the retinal quadrant, periphery-to-center density gradients were comparable, ranging between 1.25 and 1.50. These values agree with those reported for C57BL/6 mouse strain using different approaches.\(^{8,34,42}\) With the exception of rare anomalous profiles (all located in the temporal periphery; Fig. 3E), cones in these TG mice at 12 months appeared normal: outer segment length did not differ from WT as illustrated by cone-specific opsin staining (Figs. 3F–H). Finally, M-opsin expression in both WT and TG at 3 months was similar; an approximately 20% reduction was observed at 18 months (Fig. 3I).

Cone Distribution in 12-Month-Old TG Mice Is Normal

Cone photoreceptors in C57BL/6 mice are present throughout the retinal surface.\(^{37,45,44}\) They express M-opsin alone (\( \lambda_{\text{max}} = 508 \) nm; M-cones), S-opsin alone (\( \lambda_{\text{max}} = 560 \) nm; S-cones), or both opsin (M-S-cones). Some discrepancy exists, however, regarding the topology of opsin expression.\(^{28,38,45}\) Inspection of whole-mounted retinas from WT at 12 months (\( n = 2 \)) processed for cone opsin shows that M-opsin expression is evenly distributed across the retina, whereas S-opsin expression is fundamentally limited to the ventral retina. The S-opsin-rich area includes the ventral quadrant, most of the nasal quadrant and a small ventral fraction of the temporal quadrant. This area is sharply demarcated from the dorsal S-opsin-poor area (Fig. 4A). Of the total cone population, nearly 85% (average density: 8594 ± 1340/mm\(^2\)) express M-opsin (either alone or in combination with S-opsin) and 15% are genuine S-cones. The dorsal retina contains almost exclusively genuine M-cones (85%–99% of the local population). The ventral retina includes a majority of M-S-cones and a minority of genuine S-cones (65%–75% and 25%–35% of the local population, respectively). S-cones in the dorsal retina and M-cones in the ventral retina are always very sparse (1%–5% of the local population). These results are remarkably similar to those obtained by Havercamp et al.\(^{38}\) In addition to specific retinal patterning, M-opsin expression in WT mice is graded dorsoventrally. Extra investigations performed on whole-mounted retinas labeled for M-opsin (\( n = 3 \)) revealed that immunofluorescence levels in the dorsal periphery were on average 3.68-fold (±0.20) higher than those in the ventral periphery (Fig. 4C). This value agrees with that obtained by Fei et al.\(^{16}\) using a different approach.

Topography and proportions of the three cone subtypes observed in WT are maintained in age-matched TG retinas (\( n = 2 \)). In these animals, M+S cones, genuine M-cones, and genuine
FIGURE 1. Fundus images (right eye) in WT and TG mice. Note the first appearance of granular like vitreoretinal deposits in the paracentral to peripheral retina of TG mice at 3 months (arrows) and then the increasing density of these deposits with aging (top panel). Retinal pigment epithelial mottling in the central retina of a TG animal at 18 months of age (bottom panel).
S-cones account for 55.2%, 30.0%, and 14.8% of the total cone population, respectively. Genuine M-cones are strongly dominant dorsally (>95% of the local population), whereas 65% to 85% of the cones in the ventral retina contain M- and S-opsins (Fig. 4B). A normal cone pattern has also been observed in the rd1 mouse at a very early stage of degeneration. Finally, it is worthy to note that TG mice at 12 months exhibit a normal dorsoventral gradient in M-opsin expression (fluorescence ratio = 3.77 ± 0.43; n = 3; Fig. 4C).

Cone Degeneration in TG Mice Comes Beyond 15 Months of Age

The time course of cone loss was examined at 3 months (WT, n = 4; TG, n = 4), 12 months (WT, n = 5; TG, n = 5), 15 months (WT, n = 4; TG, n = 4), 18 months (WT, n = 5; TG, n = 4), and 24 months (WT, n = 5; TG; n = 8). Counts were performed on whole-mounted retinas reacted for M-opsin expression in both WT and TG mice. The number of retinas analyzed is given in text. Data are absolute amount of product (μg/retina PL). *Significant decrease from WT, P < 0.02.

Figure 2. Changes in long and very long chain PUFA in WT and degenerating TG retinas from 1 to 24 months of age. Number of retinas analyzed are given in text. Data are absolute amount of product (μg/retina PL). *Significant decrease from WT, P < 0.02.

Figure 3. Photoreceptor features in WT and TG mice. (A) At 12 months, WT displays approximately 11 photoreceptor rows. (B) In age-matched TG, the ONL is reduced to four rows. Double staining with calbindin (green) for horizontal cells and DAPI (blue) for other cell bodies. (C) Number of photoreceptor rows in the central retina as a function of age. Data computed from Kuny et al. and present work. (D) Expression of rhodopsin protein in mice of increasing age. The monoclonal antibody 4D2 detects both monomeric (35 kDa) and multimeric (70 and 105 kDa) forms of rhodopsin. Liver was used as a negative control. Percentages correspond to TG:WT ratios after normalization against tubulin (Tub). Decrease in total rhodopsin levels (sum of each band) correlates with the progressive rod loss in TG. (E) Degenerating M-cone in a TG at 12 months (mouse 348). This cone lacks outer segment (OS) and inner segment (IS). M-opsin is expressed at low levels in the cell body, axon, and synaptic pedicle. Cross-section reacted with Alex594-tagged secondary antibody. (F) Example of M-cones in a WT at 12 months (M237). (G) Example of M-cones in a TG at 12 months (M251). Whole-mounted retinas reacted with Alexa488-tagged secondary antibody. (H) Dimensions of cone outer segments in WT (black) and TG (grey) mice of increasing ages. Each column corresponds to the average length (±SD) of the 45 longest M-opsin+ cone profiles measured (arrows in [C, D], above) in three separate frames taken in the mid periphery of the dorsal quadrant in one representative animal. Selected WT are M39 (12 months), M407 (15 months), M556 (18 months), and M550 (24 months). Selected TGs are M347 (12 months), M434 (15 months; 6% cone loss), M370 (18 months; 30% cone loss), and M553 (24 months; 89% cone loss). The length of the cone profiles in WT remains constant (11.47 ± 1.30 μm) regardless of the age. Note the prevailing value for cone outer segments in C57BL/6 mouse is 13.4 ± 0.7 μm. Cone profiles in TG have similar length (11.44 ± 1.31 μm) until 15 months; they are significantly shorter (P < 0.0001; one-tailed unpaired t-test) in specimens 18 and 24 months old (9.12 ± 1.07 μm and 5.45 ± 0.75 μm, respectively). (I) Expression of M-opsin protein in mice of increasing age. Using our conditions, the polyclonal antibody AB5405 shows products at approximately 34 and 42 kDa (stars). Percentages correspond to TG:WT ratios after normalization against tubulin (Tub). PE, pigment epithelium; OPL, outer plexiform layer.
M- and M+ S-cones, a cone population thereafter referred to as the M-opsin+ cones. Prior to immunohistochemistry, some of the animals aged 12 months (WT, n = 1; TG, n = 5), 15 months (WT, n = 1; TG, n = 1), 18 months (WT, n = 1; TG, n = 2), and 24 months (WT, n = 2; TG, n = 5) were also tested for retina function.

**Figure 4.** Proportions of cone types as calculated from double-stained flat-mounted retinas. (A) Wild-type at 12 months (M366). (B) Transgenic at 12 months (M269). The grey zone in each retina corresponds to the extent of the S-opsin rich area. Numbers indicate the respective percentage of cones expressing M-, S- and M+S-opsins in the nearest counting frame (black rectangles). (C) Dorsoventral retinal gradient of M-opsin expression. Representative examples from WT (M39) and TG (M251) mice at 12 months. Frames were captured along the vertical diameter of the retina (central inset). Fluorescence profiles (11 per frame; color-coded plots) were assembled graphically, and the surface under the peaks was computed as described in text. Graphs evidence a similar dorsoventral gradient of M-opsin expression in the retina of both WT and TG. Ratios between dorsal (D3) and ventral (V3) fluorescence levels in the present mice are 3.87 (WT) and 3.62 (TG). D, dorsal; N, nasal; T, temporal; V, ventral.

**Figure 5.** Regional loss of M-opsin+ cones with age in TG mice. (A) Average density of M-opsin+ cones found in WT mice aged older than or equal to 12 months (control values). (B–D) Percentage of cone loss in TG mice at 15, 18, and 24 months.
Consistent with preceding double staining experiments, the average density of the M-opsin\(^+\) cones in WT at 12 months was found to be 8210 ± 972/mm\(^2\) (Table 2; Fig. 5A). Cone density does not change with age (\(P = 0.14\); one-way ANOVA), remaining stable at approximately 8155 ± 880/mm\(^2\). In contrast to WT, the number of M-opsin\(^+\) cones in TG mice decreases significantly (\(P = 0.0001\); one-way ANOVA) with age (Figs. 5B–D). There is no significant variation in cone density and distribution up to 12 months (\(P = 0.52\); ANOVA post hoc Fisher t-test; Table 2). As expected, photopic ERG and flicker responses in these mice remain within normal range (Figs. 6A, 6B). Threshold for light-adapted b-wave is just above \(-0.42\) cd s/m\(^2\) and flicker fusion frequency is close to 35 Hz (normal values in mouse: \(-0.36 ± 0.1\) cd s/m\(^2\) and 41 ± 5 Hz, respectively\(^{25}\)). In contrast, threshold values for mixed dark-adapted ERG a- and b-waves (Figs. 6C, 6D) differ strongly from normal, in agreement with early rod loss and critical alteration in the rod transduction system in these TG mice. A normal density of M-opsin\(^+\) cones is maintained in TG up to 15 months (\(P = 0.25\); ANOVA post hoc Fisher t-test). Compared with a massive 70% rod loss at this time point (Fig. 3C), TG mice exhibit a negligible loss (\(-2.5\%\) on average) of

![Figure 6](https://example.com/figure6.png)
M-opsin− cones. Decrease is however more important (~15%) close to the ONH, ventrally and nasally (Table 2; Fig. 5B). A significant (P = 0.004; ANOVA post hoc Fisher F-test) decrease in the density of M-opsin− cones occurs at 18 months: the average density of the M-opsin− cones in TG is approximately 70% compared to that of WT. Cone loss affects all retinal quadrants: it is maximal centrally and minimal in the nasal periphery (Table 2; Fig. 5C). By this age, cone function is still present, but ERG displays no detectable a-wave in light-adapted conditions (data not shown). Compared with WT, dark-adapted a-waves are barely detectable except at the highest flash intensity (Figs. 6C, 6E, 6F). Threshold values for light- and dark-adapted b-waves point to an unmixed cone-driven input. However, the flicker fusion frequency drops to 20 to 25 Hz (Fig. 6B), suggestive of abnormal cone function. By 24 months, the population of cones expressing M-opsin is further reduced (P = 0.001; ANOVA post hoc Fisher F-test) to 30% of the normal value on average (median = 22%). The density of these M-opsin− cones is, however, highly variable between animals: two with approximately 5000 cones/mm² (60% the normal value); two with approximately 2200 to 3200 cones/mm² (50%–40% the normal value); and four with 650 to 1370 cones/mm² (8%–15% the normal value). Cone loss in mice of this age affects the same retinal sectors, but to different degrees. In all cases, degeneration in the nasal periphery is less severe than around the ONH or in the periphery of the dorsal and temporal quadrants (Table 2; Fig. 5D). In the most affected mice, these retinal sectors contain extremely few degenerated cone profiles still expressing M-opsin (Fig. 7G). Only the less affected mice had measurable cone-specific ERG b-waves and flicker responses up to 10 to 15 Hz (Figs. 6A, 6B). Cone-driven ERG was extinguished in TG mice having less than 30% M-opsin− cones remaining in their retina.

Degeneration Affects Cone Subtypes Equally

By 18 months, rods are virtually absent from TG retinas or undergo strong remodeling (Fig. 7A). A similar advanced stage of degeneration is observed for cones at the very borders of the ora serrata and the ONH. These degenerated cones have lost their typical appearance and opsin is distributed throughout the cells. They display either a monopolar (Fig. 7B) or a multipolar shape (Fig. 7C) with new processes emerging from the cell body or pre-existing axon. In the rest of the retina, cone remodeling is less severe. Atrophy affects the outer segments, which appear either truncated and inflated (Fig. 3H), or filiform. By 24 months, degenerating cones are confined to one residual row of ONL where they organize into clusters (Fig. 7D). All have an abnormal morphology without exception (Figs. 7D–G).

Retinas from two additional TG mice at 18 months were stained for both M- and S-opsins to examine whether the mutated gene affects cone subtypes differentially as found in a knock-in model.48 In these two animals, the total cone population (n = 124,800 and n = 135,700, respectively) was almost 80% that found in TG aged 12 months (n = 154,260 and n = 177,800, respectively; Table 3). Cone subtypes were normally distributed: genuine M-cones were strongly dominant (96%) in the dorsal and temporal quadrants, whereas the rest of the retina was populated by genuine S-cones and by cones co-expressing M+S opsins (Fig. 4B). Moreover, the proportions of each cone type remaining were comparable with those found in TG at 12 months: 50.0% were M+S-cones, 35.5% were M-cones, and 14.5% were S-cones. Results at 24 months (n = 3) were less straightforward, in relation to the disparate degenerative conditions of the retina (Figs. 7E, 7G). Nearly normal proportions were found in retinas with 40% and 60% cone loss (mice M101 and M211, respectively; Fig. 7F), whereas only genuine S-cones remained in the dorso-temporal crescent of retinas with approximately 90% cone loss (M182; Fig. 7G). The last data suggests that S-cones are more resistant to degeneration than M-cones.

**Progressive Disorganization of the M-opsin− Cone Mosaic**

Because indexes of mosaic regularity have been assumed to be more sensitive than density estimates to detect early stages of photoreceptor degeneration (at least in human49), we examined potential changes in the Voronoi domains (VDs) of M-opsin− cones with age. Analysis was performed in parallel with density measurements.

Results are given in Table 4 and depicted in Figure 8. Average VDs for M-opsin+ cones in WT mice at 3 months ranged between 72 and 135 μm². They were significantly (P < 0.001) larger in the periphery than in the center. This centripetal decrease in VD area corresponds to a 1.32-fold increase in the M-opsin− cones density. In agreement with density measures (Table 2), a significant enlargement of VDs is observed at 12 months of age both in the periphery and the center (+13%; P = 0.015 and +24%; P < 0.001, respectively; one-way ANOVA; post hoc Fisher F-test) of the WT retina. No further changes occur at later ages. Regularity indexes in WT ranged between 2.35 and 3.8. Such low values denote an irregular distribution of the M-opsin− cones (P < 0.015; Kolmogorov-Smirnov test of normality). Regularity indexes, computed either from the periphery or the center, remain stable with age (P > 0.05; one-way ANOVA); values from the center are systematically higher than those from the periphery (on average 2.98 ± 0.29 and 2.86 ± 0.29, respectively; P = 0.002, one-tailed paired F-test). Of interest, average VDRIs computed for the total cone population in one WT mouse at 12 months stained for gamma-transducin (center: 2.96 ± 0.25; n = 4; periphery: 2.74 ± 0.22; n = 4) was comparable with those found for the M-opsin+ cone population in age-matched mice. This result indicates that the genuine S-cones are too sparse to disturb the general cone pattern to any extent.

In opposition to this, VDs increase in TG mice as degeneration progresses (Table 4). While VD areas in TG mice were essentially normal up to 12 months of age (Fig. 8B), significant enlargement was first observed close to the ONH in mice at 15 months. Increase was small (<15%; P = 0.02), in agreement with the modest cone loss observed in this region. More significant changes occur by 18 months. On average, VD areas were found to be 20% to 35% larger (P < 0.01) than normal in nearly all retinal sectors, except at the periphery of the nasal quadrant. Enlargement was even greater (60%–80%) when the most affected TG mouse in this age group (M369; 60% M-opsin− cone loss; Fig. 7B) was considered. Finally, VDs derived from TG mice at 24 months were found to be 2-fold wider on average than those in aged-matched WT. The large variances observed in this sample are representative of strong interindividual variability. It must be noted, however, that the latter values are underestimates of the actual degenerating conditions in the old TG mice because they do not consider the four most affected specimens, which have less than 1330 labeled M-opsin− cones per square millimeter in their retinas (i.e., on average ≤ 50 tagged elements/counting frame; see Methods; Fig. 8C). Regularity indexes decrease as degeneration progresses, reaching significantly lower values than normal (P = 0.007; one-tailed F-test) only beyond 18 months (Table 4).
Figure 7. Characteristic shape of photoreceptors at advanced degeneration. (A) Rod phenotypes in a TG (M3) at 18 months. Whole-mounted retina stained for rhodopsin. Rods look like monopolar cells bearing a long, thin process decorated by tiny varicosities. Rhodopsin is still expressed in cell bodies (arrows), outer segment (arrowheads), and inner segments (stars). (B, C) Morphology of M-cones in a TG at 18 months, at the ora serrata (temporal quadrant) and the ONH (ventral quadrant) borders, respectively; whole-mounted retina M369 with 60% cone loss. Some cells show a branching pattern (open triangles) and still express M-opsin in cell bodies (arrows) and outer segment residues (filled triangles). Ora serrata in (B) is on right (dotted line). (D) Morphology of M-cones in a TG at 24 months; whole-mounted retina M553 with 90% cone loss. Degenerating cone profiles form large clusters. Outer and inner segments are very rare (stars). (E) Morphology of S-cones in a TG (M103) at 24 months. Cross-section stained for both M- and S-opsins; temporal periphery of the retina. This section contains only genuine S-opsin+ cones (green). All are misoriented; one has still an OS residue (arrow). (F) Cone distribution in the central retina (transition zone) of a TG at 24 months. Whole-mounted retina stained for both M- and S-opsins (M101; 40% cone loss). Although severely disfigured, cone profiles still expressing M-opsin (red), S-opsin (green), and M+S-opsins (yellow) distribute normally (7.5%, 15%, and 74.5% of the total cone population, respectively). (G) Cone distribution in the dorsal retina of a TG at 24 months. Whole-mounted preparation stained for both M- and S-opsins (M182; 92% cone loss). Only one S-cone (green) remains at this location (D5; Fig. 4C).
Despite intensive research, the primary cellular events triggering photoreceptor death in STGD3 have yet to be elucidated. Firstly, based on in vitro experiments showing an upregulation of various ER resident chaperones in hEK293 and COS cells transfected with mutant human gene, the defective ELOVL4 protein has been suspected to activate an unfolded protein response. Such ER-stress reaction, however, has not been observed in vivo, either in the heterozygous KI-1/2 knock-in mouse model (carrying the 5bp deletion plus two downstream point mutations) or in any transgenic mouse models. Secondly, consistent with the function of ELOVL4, studies using both KI-1/2 knock-ins and rod/cone-specific conditional knockouts reported that the 5bp deletion creates a selective reduction of retinal phosphatidylincholines with long acyl chains (>C28). Phosphatidylincholines are essential components of photoreceptor outer segments, but their exact function is still elusive.

However, investigations on mice generated by breeding KI-2 knock-ins with transgenic mice expressing the Elovl4 gene in the skin showed that the absence of PC had little effect on the structure of the neural retina but impaired visual function (Kedzierski W, et al. IOVS 2010;51:ARVO E-Abs 2939; and the recent study by Barabas et al. using conditional knockouts further demonstrated that the absence of retinal VLC-PUFA produces no structural, functional, or behavioral abnormalities.

We found no evidence in this study supporting a dysfunction of both LC- and VLC-PUFA synthesis in TG at 1 month. In these TG characterized by a normal ONL, the PUFAs remain normal. The decrease of both DHA and VLC-PUFA in TG was seen from 3 (parallel to starting cone degeneration) until 24 months. Interestingly, DHA still increased reaching maximum at 9 months and VLC-PUFA at 6 to 9 months. This indicates that the synthesis of these fatty acids are still functioning, although it may be weaker than in WT. Hence, it is very likely that the early rod loss seen in TG at 2 months involves mechanisms other than decreased levels of VLC-PUFA. This is in agreement with the findings of Barabas et al., in which close to complete depletion of very long chain fatty acids in conditional ELOVL4 knockouts did not result in photoreceptor cell loss. The drastic decline of the total n-3 C32 and C34 VLC-PUFA in TG was seen beyond 12 months. Concentration at 12, 18, and 24 months (59.5%, 41.2%, and 29.8% of WT, respectively) are parallel to starting cone loss and losing almost all rods. By 24 months, only 0.16 μg of these fatty acids are left in the retina PL in comparison with 0.52 μg in WT. At this age, there is only one residual row of ONL containing degenerating cones in the TG retina.

Our findings support the normal cone function observed in TG mice up to 12 months. Density distribution, opsin expression, light-adapted (cone-driven) ERG responses, subtype proportions, global topology, and local arrangement of retinal cones are all normal at that age. As such, it is a reasonable assumption that this STGD3 mouse model would experience nearly normal photopic and color vision over the first half of its life (based on a 2-year lifespan). Clinical investigations in STGD3 patients report a progressive loss of visual acuity but minimal color vision disturbance, suggesting reduced alteration of cone function in the early stages of the disease.

This 12-month time point might represent the latest period at which cone photoreceptors could be rescued from degeneration.

Degenerating cones are not detected until 15 months, almost 1 year after rod loss onset. Such long-term resistance to degeneration may be surprising since the mutant protein is expressed in both photoreceptor types. However, the findings of Harkewicz et al. indicate that the ELOVL4 transgene may...
differentially affect PC species in rods and cones. According to this study, cones would only be affected by a decrease in VLC-PUFA longer than C34. Interestingly, such a decrease occurs at 12 months in the present TG (Fig. 2). On another hand, it should be noted that cone loss in our model only begins when the ONL is reduced to almost three to four photoreceptor rows. Remarkably, (1) a very similar relationship has been reported with several, well-established rodent models of retinitis pigmentosa (RP),\textsuperscript{43.56–58} and (2) no cone loss was observed in aged (18 months) heterozygous P23H-3 rats with loss of under half of the rod complement.\textsuperscript{59} Hence, independently of the primary genetic defect, cones display the ability to survive until the rod population drops to a critical size corresponding approximately to 30% the normal value.

Secondary cone degeneration in rodent RP models has been attributed to various deleterious factors including toxic byproducts of dying rods, oxidative stress, reactive changes in glial and microglial cells, reduction in the amount of rod-derived cone viability factors,\textsuperscript{60,61} as well as defective cone-RPE interactions that could compromise the uptake of nutrients essential to cone survival.\textsuperscript{55,57} How these events lead to cone death in our model deserves further investigation. In two of these RP models (transgenic S334ter-line 9 and P23H-line 2 rat models of RP ([in the public domain] http://www.ucsfeye.net/mlavailRDratmodels.shtml),\textsuperscript{62} detection of early changes in photoreceptor mosaics of S334ter-line 9 and P23H-line 2 rat models of RP ([in the public domain] http://www.ucsfeye.net/mlavailRDratmodels.shtml).

In the present model, the ELOVL4 mutation affects cone subtypes equally, at least within the early phase of the degenerative process (15–18 months). This finding contrasts with the specific loss of S-opsin\textsuperscript{+} cones observed at the same time point in the heterozygous knock-in model only carrying the 5bp deletion.\textsuperscript{48} The latter result, however, is debatable, mainly because the analysis did not consider that most cones in C57BL/6 mice express both opsins.

To the best of our knowledge, quantification of the cone mosaic in mice has been performed in only one study\textsuperscript{66} using transgenic mice expressing green fluorescent protein (GFP) under the control of the 5’ regulatory sequence of the OPN1EW gene.\textsuperscript{64} Computation of nearest-neighbor distances between these GFP\textsuperscript{+} cones returned regularity indexes 30% to 50% higher than the ones presented here. Discrepancy with our study may result from a combination of experimental factors. Firstly, the mosaics examined may be different since the GFP\textsuperscript{+} cones (obviously genuine M-cones for their specific dorsal location\textsuperscript{64}) represent only a fraction of the total M-opsin\textsuperscript{+} cone population. Secondly, measurements based on the position of cell bodies might provide more regular indexes than those derived from the location of outer segments. Thirdly and finally, VD analysis relies on the position of all neighbors of a cell, whereas the nearest-neighbor approach considers only the location of the closest neighbor of that cell. This approach produces higher regularity indexes than VD analysis with small sample sizes.\textsuperscript{65,66} Taken together, these results support the necessity of investigating both WT and TG littermates with the same approach to accurately evaluate the effects of the mutated ELOVL4 transgene upon cone density and mosaic patterning.

Detection of early changes in photoreceptor mosaics of patients afflicted by various genetic defects (as performed with
adapted for precocious diagnosis of the disease and prompt delivery of accurate therapy. Significant disruption of the human cone mosaic has been observed when only approximately 5% of the cones are compromised.\(^\text{19}\) Using this method for the first time in a mouse model of retinal degeneration, we were able to show that the cone mosaic is not altered before 18 months, at which point almost 30% of the cones have disappeared. Such lack of sensitivity compared with human is likely related to the inherent irregularity of the normal mosaic pattern in the healthy mouse retina. Although regularity indexes in WT mice (Table 4) are larger than what would be expected for a totally random distribution of points (\(\approx1.9\)), these remain much lower than those found in human (7.3–8.6 for the 5-cones at 1.25° temporal from fovea\(^\text{19}\)) or chicken (6.4–7.8 regardless of the cone species\(^\text{20}\)). Low VDRI values reflect a large dispersion in the size of the Voronoi polygons drawn at a given retinal locus. As suggested from theoretical computation analyses,\(^\text{70}\) cone loss in mouse must be substantial to disturb significantly the low level of retinal organization.

Recently, Barabas et al.\(^\text{9}\) questioned whether the ELOVL4 transgenic mouse model of STGD3 is representative of the human condition because this model displays a typical rod-cone dystrophy not evidenced clinically in human.\(^\text{5}\) Indeed, with the exception of the macular atrophy that cannot be modeled in mice (because they do not have a macula), many of the human features are optimally mimicked by the phenotype of the heterozygous knock-in mouse model\(^\text{46}\) rather than by the TG phenotype. Although present in both rods and cones as in TG, the genetic mutation in knock-in mice induces primarily a cone–rod dystrophy with cone and rod defects starting at 2 and 10 months, respectively. The reasons why the knock-in mouse model differs both anatomically and functionally from the model presented here needs to be clarified. Both models can, however, be used in conjunction to examine the mechanisms of degeneration in rods and cones induced by the Elovl4 5-bp-deletion mutation.

Finally, of high relevance for clinical translation, is the presence of granular-like vitreoretinal deposits in the paracentral and peripheral TG retina as seen on fundus images at 3 months (Fig. 1, top panel). Tan et al.\(^\text{71}\) recently reported the occurrence of similar granular deposits confined to the periphery of the macula, in almost 50% of the AMD patients they studied. Moreover, the presence of these deposits correlated with the presence of drusen in this region. Since the occurrence of these deposits precedes cone remodeling and death in both human\(^\text{72–74}\) and the ELOVL4 transgenic mouse model, a more detailed study of their nature and time course could provide pertinent information into the link between rod and cone loss in rod–cone dystrophies as well as in AMD.

CONCLUSIONS

The present work clarifies the course of photoreceptor degeneration in the ELOVL4/TG1-2 transgenic mice, a model of STGD3 characterized by low transgene expression. The degenerative process is unquestionably a slow rod–cone dystrophy. Initial rod loss is visible at 2 months. It is likely not triggered by changes in retinal fatty acid composition. Yet, secondary cone degeneration does not begin until 15 months when the ONL is reduced by approximately 70% and is not completed until 9 months later. Voronoi analysis does not perform any better than density measures in terms of early detection: Significant alteration of the geometric order of the cone mosaic is not seen before 18 months, at which point most cones have strongly altered morphology. With regard to the slow time course of the degenerative process, this mouse model would be appropriate to investigate rod–cone interactions during retinal degeneration as well as to evaluate the efficiency of putative therapeutic treatments able to protract residual cone function.

Acknowledgments

The authors thank Morgan Murray for her extremely valuable work on fatty acid extraction and analysis.

Supported by grants from Canadian Institutes for Health Research (CIHR MOP 79278), also an Alberta Heritage Foundation for Medical Research Senior Scholar (YS).

Disclosure: S. Kuny, None; M.-A. Filion, None; M. Suh, None; F. Gaillard, None; Y. Sauvé, None.

References


52. Aveldano MI. Phospholipid species containing long and very long polyenoic fatty acids remain with rhodopsin after hexane extraction of photoreceptor membranes. Biochemistry. 1988;27:1229–1239.


