Transcriptional Profile Analysis of *RPGR*ORF15 Frameshift Mutation Identifies Novel Genes Associated with Retinal Degeneration

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PURPOSE. To identify genes and molecular mechanisms associated with photoreceptor degeneration in a canine model of XLRP caused by an *RPGR* exon ORF15 microdeletion.

METHODS. Expression profiles of mutant and normal retinas were compared by using canine retinal custom cDNA microarrays. qRT-PCR, Western blot analysis, and immunohistochemistry (IHC) were applied to selected genes, to confirm and expand the microarray results.

RESULTS. At 7 and 16 weeks, respectively, 56 and 18 transcripts were downregulated in the mutant retinas, but none were differentially expressed (DE) at both ages, suggesting the involvement of temporally distinct pathways. Downregulated genes included the known retina-relevant genes *PAX6*, *CHML*, and *RDH11* at 7 weeks and *CRX* and *SAG* at 16 weeks. Genes directly or indirectly active in apoptotic processes were altered at 7 weeks (*CAMK2G*, *NTRK2*, *PRKCB*, *RALA*, *RBBP6*, *RNF41*, *SMYD3*, *SPP1*, and *TUBB2C*) and 16 weeks (*SLC25A5* and *NKAP*). Furthermore, the DE genes at 7 weeks (*ELOVL6*, *GLOD4*, *NDUFS4*, and *REEP1*) and 16 weeks (*SLC25A5* and *TARS2*) are related to mitochondrial functions. qRT-PCR of 18 genes confirmed the microarray results and showed DE of additional genes not on the array. Only *GFAP* was DE at 3 weeks of age. Western blot and IHC analyses also confirmed the high reliability of the transcriptomic data.

CONCLUSIONS. Several DE genes were identified in mutant retinas. At 7 weeks, a combination of nonclassic anti- and pro-apoptosis genes appear to be involved in photoreceptor degeneration, whereas at both 7 and 16 weeks, the expression of mitochondria-related genes indicates that they may play a relevant role in the disease process. (*Invest Ophthalmol Vis Sci*. 2010;51:6038–6050) DOI:10.1167/iovs.10-5443

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The term retinitis pigmentosa (RP) refers to a group of many different inherited retinal diseases characterized by progressive rod or rod-cone photoreceptor degeneration that causes subsequent visual impairment and blindness. Some of the causative genes have clear, well-identified roles (e.g., involvement in phototransduction, in maintaining photoreceptor structure, or in RPE retinoid metabolism; RetNet: <http://www.sph.uth.tmc.edu/RetNet/> provided in the public domain by the University of Texas Houston Health Science Center, Houston, TX). However, there remain a large number of diseases caused by genes with poorly understood functions and for which the mechanism linking the genes and/or mutations with photoreceptor disease and degeneration is unknown.

Among these is the RP3 form of X-linked RP (XLRP), a uniformly severe, early-onset retinal disease in humans that is caused by mutations in the RP GTPase regulator (*RPGR*) gene.¹ Although estimates vary depending on the sample population and methods of testing, it is generally accepted that mutations in *RPGR* account for >70% of XLRP cases.^{2–4} Furthermore, the carboxyl-terminal exon open reading frame 15 (ORF15) of *RPGR*, a mutational hot spot, has been shown to be mutated in 22% to 60% of XLRP patients.^{2,5,6}

RPGR is essential for the maintenance of photoreceptor viability.⁷ The protein, which has a series of six RCC1-like domains (RLDs) characteristic of the highly conserved guanine nucleotide exchange factors, is found in the rod and cone photoreceptor connecting cilia.⁸ *RPGR* has complex interactions with other proteins that have microtubular-based transport functions in the retina and that are presumed to function in the photoreceptor centrosome, inner and outer segments, and ciliary axoneme region.^{9,10} Among these, the genes coding for nephrocystin-4,^{11–13} and *PDE6D*¹³; *RPGR* interacting protein (*RPGRIP1*)¹¹; and *RPGRIP1L*¹⁴ cause retinal disease when mutated, thus emphasizing the critical importance of this protein complex in maintaining photoreceptor structure, function, and viability.

One approach to developing insights into the cell- or tissue-specific functions of genes or to examining the molecular mechanisms of disease is microarray-based global profiling of gene expression in combination with bioinformatic analysis. In several studies, the transcriptome of the mouse and human retinas has been analyzed by characterizing changes in expression profiles during development and aging.^{15–17} More recently, transcriptomic data of distinct retinal cells^{18–20} and a web-based platform containing numerous retinal gene expression studies have been made available (<http://alnitak.u-strasbg.fr/RetinoBase/> provided in the public domain by University Louis Pasteur, Strasbourg, France). In addition, studies based on differential gene expression in mouse retinal disease models provide useful information to aid in discerning the role of disease-causing genes with respect to other genes and in evaluating their involvement in gene pathways and cascades.^{21–23}

These approaches have specific limitations in terms of human retinal diseases, not the least being the lack of adequate sample sizes at the appropriate disease stages. However, the constraints can be overcome by using animal models of homologous diseases. These models provide a powerful tool for translational studies, provided that the human disease modeled and the corresponding animal disease are comparable.

Natural mutations in *RPGRORF15* occur in humans and dogs,^{2,24} and X-linked progressive retinal atrophy (XLPRA) is the dog homolog of human XLRP. In dogs, two different *ORF15* microdeletions have been identified: XLPRA1 is a post-developmental, slowly progressive photoreceptor degeneration resulting from a 5-bp deletion in *ORF15* that truncates the translated protein, whereas XLPRA2 is an early-onset, progressive rod and cone photoreceptor disease caused by a 2-bp deletion that creates a frameshift and premature stop in the translated protein. The deduced peptide sequence is changed by the inclusion of 34 additional basic residues that increase the isoelectric point of the truncated protein.²⁵ Beltran et al.^{26,27} described in detail the course of retinal disease in canine XLPRA2, the phenotype of which replicates the salient features of *RPGR*-XLRP.^{28,29}

The purpose of the present study was to identify the genes and molecular mechanisms associated with disease onset and progression in normal and XLPRA2 mutant canine retinas. We examined the global retinal gene expression profiles at 7 and 16 weeks, the most relevant disease-related ages. Kinetics of photoreceptor cell death show a burst of dying cells between 6 and 7 weeks, whereas at 16 weeks, when the retina has lost approximately 40% of its photoreceptors, there is a constant but decreased rate of cell death.²⁶ For this, we used a validated custom retinal cDNA microarray^{30,31} and performed real-time quantitative reverse transcription-PCR (qRT-PCR), Western blot analysis, and immunohistochemistry, to confirm and expand the microarray results. We detected several genes that were differentially expressed (DE) at critical time points in the degenerating XLPRA2 retina and that are specific for the disease stages examined. The downregulation of rod-specific genes also suggests the differential and preferential damage of rods in the early stages of the disease.

MATERIAL AND METHODS

Tissue Samples

Retinas were obtained from age-matched normal and mutant dogs with a common genetic background that were maintained at the Retinal Disease Studies Facility (RDSF; Kennett Square, PA) and housed in 12-hour cyclic light conditions. To avoid potential fluctuations in retinal gene expression with time of day,³² we collected the eyes at a single time period (noon). Both eyes were enucleated after intravenous anesthesia with pentobarbital sodium, and the dogs were euthanized after enucleation with a barbiturate overdose. The retinas were collected within 1 to 2 minutes after enucleation, flash frozen in liquid nitrogen, and stored at -80°C until use. The research was conducted in full compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Analyses were performed at three critical time points in the disease: 3, 7, and 16 weeks of age.²⁶ The 3-week time point comes before the beginning of apoptosis, when the retina is comparable in structure to normal; we refer to this stage as the *induction phase*. The peak of cell death occurs at 7 weeks (*execution phase*) and decreases the number of photoreceptors by $\sim 10\%$ to 15% . At 16 weeks, in the *persistent execution-chronic cell death phase*, the mutant retina shows a sustained, albeit reduced, cell death rate and loss of 40% of the photoreceptor layer.

RNA Extraction

Total RNA from retinas was extracted by using standard procedures (TRIzol; Invitrogen-Life Technologies, Carlsbad, CA). RNA concentration was assessed with a spectrophotometer (model ND-1000; NanoDrop Technologies-Thermo Fisher Scientific, Wilmington, DE), and RNA quality was verified by microcapillary electrophoresis (model 2100 Bioanalyzer with RNA 6000 Nanochips; Agilent Technologies, Santa Clara, CA). Only high-quality RNA with an RIN greater than 7 and an A260/A280 ratio greater than 1.9 was used in both the microarray and the qRT-PCR analyses.

Microarray Procedures and Statistical Analysis

Expression profiles of age-matched 7- and 16-week-old normal and XLPRA2 mutant retinas (three biological replicates for each time point and group) were compared by using a canine retinal custom cDNA microarray containing ~ 4500 transcripts. Microarray construction and hybridization were performed as previously described.³⁰ Briefly, ~ 4500 transcripts from a normalized canine retinal EST database, including positive controls, were selected and used to construct the microarray.³¹ On the basis of initial validation studies,³⁰ pooled brain RNA, including equal amounts of total RNA from the occipital, temporal, and frontal regions collected from three 16-week-old beagles, was used as the reference sample. In each analysis, amplified and cleaned retinal RNA (RNeasy columns, Qiagen, Valencia, CA) was labeled with Cy5, and the amplified pooled brain reference RNA was labeled with Cy3. The two labeled samples were combined, and the mixture was hybridized to the slide microarray. Arrays were scanned (GenePix 4000B scanner; Molecular Devices Corp., Downingtown, PA), and the signal intensities were evaluated (GenePix Pro 6.0 software; Molecular Devices Corp.). Data normalization, using locally weighted linear regression (LOWESS) subgrid normalization,³³ which eliminates spatial- and intensity-dependent dye bias, and data filtering were performed (GeneSpring 7.3.1; Silicon Genetics-Agilent Technologies).

Significant changes in expression were identified with Significance Analysis of Microarrays (SAM 1.15, available at <http://www-stat.stanford.edu/~tibs/SAM/>, Stanford University, Palo Alto, CA). A two-class, unpaired *t*-statistic was applied to log 2-transformed expression data and ranked on the basis of 500 permutations, to identify significant gene expression differences between normal and mutant animals. For each gene, SAM calculated the *q*-value (in percent), which is the lowest false-discovery rate (FDR) at which an individual gene is called significant (calculated as the average of three biological replicates).³⁴ The false-negative rate (FNR) was also predicted by SAM for each comparison and resulted in 0.6% or less for all comparisons, indicating negligible type 1 error. DE genes were identified between XLPRA2 mutant versus control retinas at 7 and 16 weeks separately and between mutant and control retinas regardless of age (combined analysis). Only genes with *q*-values less than 10% and more than twofold change ratios were considered to be DE.

Microarray Data Submission

The complete microarray data set presented in this publication has been deposited in the Gene Expression Omnibus³⁵ (National Center for Biotechnology Information [NCBI], Bethesda, MD), according to the guidelines of the rationale of minimum information about a microarray experiment (MIAME),³⁶ and is accessible through GEO Series accession number GSE19124 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19124>).

Bioinformatic Analyses

The Database for Annotation, Visualization, and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov/>; National Institutes of Health, Bethesda, MD) was used to allocate DE genes with similar biological features in the different gene ontology (GO) categories (biological process, cellular component, and molecular function). A Fisher's exact test was applied to calculate the *P*-value ($P \leq 0.05$ was considered

statistically significant), which determined the probability that the association between the DE genes in the dataset and the category is explained by chance alone. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (<http://www.genome.jp/kegg/kegg2.html>) developed by the Bioinformatics Center, Kyoto University, and the Human Genome Center, University of Tokyo) and the pathway analysis databases (IPA; <http://www.ingenuity.com>; Ingenuity Systems Inc., Redwood City, CA) were interrogated to determine biological processes, pathways, and networks with possible involvement of DE genes. For the latter, gene identifiers were mapped to networks available in the Ingenuity Systems database and ranked by score, indicating the statistical significance of genes that were linked to the same network at better than chance. Using a 99% confidence level, we considered scores of >3 to be significant, and only networks containing more than two genes were further analyzed.

Quantitative Real-Time PCR

Eleven genes were selected for qRT-PCR to confirm expression patterns observed in the microarray data (*BNIP3*, *CRX*, *NDUFS4*, *PAX6*, *PFDN5*, *PLAGL2*, *SAG*, *SLC25A5*, *SPP1*, *TPD52*, and *ZBTB4*). To complement the microarray data and to evaluate crucial photoreceptor genes known to be involved in similar retinal diseases, we included seven additional genes not present in the microarray or that did not amplify in the brain pool reference tissue (*CNGA3*, *CNGB3*, *GFAP*, *OPN1LW*, *OPN1SW*, *RHO*, and *RPGR*). Either custom gene-specific assays (TaqMan; Applied Biosystems, Inc. [ABI], Foster City, CA) or gene-specific primers (Primer Express; ABI) were used (Supplementary Table S1; all Supplementary Material is available at <http://www.iovs.org/cgi/content/full/51/11/6038/DC1>). The primers for *CNGA3*, *CNGB3*, *OPN1LW*, *OPN1SW*, and *RHO* have been validated in ongoing studies of canine achromatopsia (András Komáromy, University of Pennsylvania, unpublished data, 2010). For *RPGR*, which is the gene mutated in XLPRA2 with a 2-bp microdeletion in exon ORF15, we used a probe in the junction between the 5' untranslated region (UTR) and exon 1 that is common to all known isoforms and has been validated in a parallel study of canine XLPRA (Shana Gilbert-Gregory, University of Pennsylvania, unpublished data, 2010).

To better understand the time course and early gene expression changes in the disease, we also performed qRT-PCR on 3-week-old mutant and normal retinas, in addition to the 7- and 16-week retinas. Three biological replicates were tested at each time point. In addition, at the 7- and 16-weeks time points, three technical replicates of one retina per group were tested, to control for technical errors.

RNA samples were treated with RNase-free DNase (Ambion, Austin, TX) and then reverse-transcribed with random hexamers (High Capacity cDNA Reverse Transcription Kit; ABI) according to standard procedures of the manufacturers. The real-time reaction (total of 20 μ L) included 20 ng of cDNA as a template, 1 $\times$ PCR reaction master mix (TaqMan Universal PCR Master Mix; ABI), 1 $\times$ custom gene-specific assay (TaqMan; ABI) or 900 nM of each forward and reverse primer, and 250 nM of probe (TaqMan; ABI). All the qRT-PCR reactions were performed in 96-well plates with a real-time PCR system (model 7500; ABI) and detection software (7500 ver 2.0.1; ABI). Four genes were initially selected as a reference: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; Supplementary Table S1), *18S* (TaqMan Gene Expression Assay Hs99999901_s1), *HPRT1* (TaqMan Gene Expression Assay Cf02626256_m1), and *ACTB* (TaqMan Gene Expression Assay Hs03023880_g1; all from ABI). Ultimately, *GAPDH* was selected because, for this specific disease, it performed the most accurately and with the least variation between samples (data not shown).

The CT values of the genes were normalized with those of *GAPDH*, and the ratio of mutant versus control was calculated by the $\Delta\Delta$ CT method.³⁷ An unpaired *t*-test was applied to each gene, to verify whether the differences between control and mutant samples at each time point were statistically significant, using thresholds previously described³⁸ ($P \leq 0.05$, statistically significant; $0.05 < P < 0.1$, trend toward statistical significance).

Protein Extraction and Western Blot Analysis

Because of limited sample availability, Western blot analysis was performed at two ages (7 and 16 weeks), with one normal and one mutant retina used at each time point. Protein extraction and Western blot analysis were performed as described elsewhere, with minor modifications.³⁹ Briefly, normal and mutant retinas were homogenized and sonicated at 4°C in a buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The homogenate was centrifuged at 13,000g for 15 minutes at 4°C, and the supernatant containing the proteins was collected. Total protein levels were determined by the Bradford method (ABC protein assay; Bio-Rad, Hercules, CA). For Western blot analysis, 20 μ g protein extract was boiled in SDS sample buffer (4% glycerol, 0.4% sodium dodecyl sulfate, 1% β -mercaptoethanol, and 0.005% bromophenol blue in 12.5 mM Tris-HCl buffer [pH 6.8]) and then separated, along with a biotinylated protein ladder (no. 7727, 1:1000; Cell Signaling Technology, Danvers, MA), by SDS-PAGE (4% stacking gel, 12% separating gel). The proteins were transferred to a polyvinylidene difluoride membrane (Trans-Blot Transfer Medium; Bio-Rad) in chilled transfer buffer (25 mM Tris base, 192 mM glycine, and 15% methanol). The membrane was blocked in 10% skim milk in Tris-buffered saline containing 0.5% Tween-20 overnight at 4°C and then was incubated for 1.5 hours with the primary antibodies. The antibodies included were NDUFS4 (ab55540, 1:1,000; Abcam, Cambridge, MA), SAG⁴⁰ (kindly provided by Igal Gery, 1:10,000), GFAP²⁷ (Z0334, 1:10,000; Dako Cytomation, Carpinteria, CA), and OPN1SW²⁷ (AB5407, 1:2,000; Chemicon, Temecula, CA). With the exception of NDUFS4 (specificity determined by the detection of the appropriate size product by Western blot analysis), the other antibodies had been validated and produced specific labeling in the canine retina using Western analysis and/or immunohistochemistry^{27,40} (Kathleen Boesze-Battaglia, School of Dental Medicine, University of Pennsylvania, Philadelphia, personal communication, 2010). ACTB (MAB1501, 1:10,000, Chemicon) was used as the loading control.

Signal was detected by incubating with the appropriate secondary antibody conjugated with horseradish peroxidase (1:2,000, Zymed, San Francisco, CA) and was visualized by the ECL method according to the manufacturer's recommendations (ECL Western Blot Detection Reagents Kit; Amersham, Piscataway, NJ). The blots were exposed on autoradiograph film (X-Omat; Eastman Kodak, Rochester, NY).

Immunohistochemistry

Seven-micrometer-thick cryosections of OCT-embedded retinas from normal and mutant animals at 7 and 16 weeks of age were used for immunohistochemistry (IHC); the sections were cut along the superior retinal meridian, as previously described.²⁶ We used the same antibodies as described in the Western blot section, but at different concentrations: NDUFS4 (1:500), SAG (1:3000), GFAP (1:1000), and OPN1SW (1:50).

Cryosections for NDUFS4 labeling were incubated with 5% H₂O₂/methanol for 30 minutes, blocked with 2% FBS/0.1 M Tris for 20 minutes, and incubated with the primary antibody overnight at 4°C. They were then incubated at room temperature for 30 minutes with secondary antibodies conjugated with biotin and for 30 minutes with avidin-biotin complex (Vector Laboratories, Burlingame, CA). Signals were detected with the HRP substrate 3, 3'-diaminobenzidine (DAB; Dako Cytomation).

Cryosections were washed with 1 $\times$ PBS and 0.25% Triton X-100, blocked for 20 minutes in 10% normal goat serum, 1 $\times$ PBS-0.25% Triton X-100, and 0.05% sodium azide and incubated overnight at 4°C with the primary antibodies. The antigen-antibody complexes were visualized with fluorochrome-labeled secondary antibodies (Alexa Fluor, 1:200; Molecular Probes, Eugene, OR).

Slides were examined (Axioplan microscope; Carl Zeiss Meditec, GmbH, Oberkochen, Germany) with transmitted light (NDUFS4) or epifluorescence (SAG, GFAP, and OPN1SW). Images were digitally captured (Spot 4.0 camera; Diagnostic Instruments, Sterling Heights)

and displayed with a graphics program (Adobe Photoshop, Mountain View, CA). Negative control slides (normal retinas at 7 weeks) without primary antibodies did not label and did not show any labeling with the secondary antibody.

RESULTS

Differentially Expressed Genes

We used a canine retinal cDNA slide microarray hybridization technique to generate a comprehensive gene expression profile of normal and *RPGR* mutant retinas at 7 and 16 weeks of age—the relevant time points for detection of degeneration-related genes. Using high stringency in the normalization and filtering of data, we identified greater than 3500 high-quality transcripts for the comparative expression analysis. The sequences of the DE transcripts were blasted against the canine genome, and the most likely represented gene was identified.

First, we characterized the gene expression changes during normal development by comparing the 7- and 16-week time points in normal retinas. At 7 weeks, the retina has just reached structural maturation, and at 16 weeks, it is considered developed.⁴¹ When compared with the 16-week normal retinas, the 7-weeks retina showed 245 transcripts upregulated, and 15 downregulated, with at least a twofold change and an FDR of less than 10% (Supplementary Table S2).

A total of 23 genes with an FDR of $q = 0$ were upregulated at 7 versus 16 weeks; of those, *PAX6* has a major role in eye development.⁴² With an increased q -value, some genes relevant to retinal transport (*KIF3A*, *KIFAP3*, and *IFT88*) and function (*CHML*) also showed increased expression at 7 versus 16 weeks (Supplementary Table S2). Eight DE genes at 7 versus 16 weeks are involved in retinal diseases in humans and, in some cases, in dogs and mice. Five were upregulated (*BBS9*, *EYS*, *PRSS11*, *RP1*, and *TUB*) and three were downregulated (*PRCD*, *NPHP4*, and *UNC119*). Among the 15 downregulated transcripts, 3 represented the same gene, *SAG* (S-arrestin, S-antigen, 48 kDa), indicating a high expression of this gene when the normal retina has matured.

On the other hand, in the mutant retinas, 28 transcripts were upregulated at 7 compared with 16 weeks, but none were downregulated (Supplementary Table S2). Four of these ($q = 0$) were unknown genes that were represented by the clones DR010015B20A11, DR010023B10B08, DR010024B20F07, and DR010020A10F02. With the exception of two genes, *VEZFI* and *TPR*, that were upregulated at 7 compared with 16 weeks in both the normal and mutant retinas, there was no commonality in the transcriptional profiles of the normal and mutant retinas at these two time points.

Next, we compared the gene expression changes that occurred between the mutant and normal retinas. At the 7- and 16-week time points, 56 and 18 transcripts, respectively, were downregulated twofold or more in the mutant compared with the normal retinas, but no statistically significant upregulated transcripts were found at an FDR <10% (Supplementary Table S3). Overall, 65 of the 74 transcripts that were downregulated at either time point were annotated in comparison with the ortholog genes in humans.

In general, although the total number of DE genes was lower at 16 weeks, significantly higher change ratios were observed when compared with those at 7 weeks. Of note, three transcripts with high change ratios (DR010015B20A11, DR010020A10F02, and DR010023B10B08), could not be associated with any known gene in the dog and other species. Furthermore, there was no overlap of DE genes at the two time points.

Finally, to determine disease-specific changes that were not influenced by age, we also compared the mutant retinas to the

normal ones, regardless of age (7 and 16 weeks: combined analysis). A total of 16 transcripts were DE, 7 upregulated and 9 downregulated, in the mutant retinas compared with the normal ones (Supplementary Table S3). Of these, *PDE6A* has a critical role in phototransduction and disease, and *RDH11* in vision, particularly during dark adaptation. Five genes—*NDUFS4*, *RDH11*, *RNF41*, *PCMT1*, and *SULT4A1*—were significantly downregulated at 7 weeks and in the combined analysis. However, none of the genes downregulated at 16 weeks was also DE in the combined analysis.

Functional Grouping and Assignment of the DE Genes to Biological Pathways

Using different approaches, we grouped the DE genes according to their functions and involvement in distinct pathways. We performed a literature screening with pathway analysis (IPA; Ingenuity Systems) of each DE gene and a formal functional clustering of the DE genes at each time point to the GO categories (biological processes, cellular compartments, and molecular functions) through the DAVID Bioinformatics resource at NIAID/NIH (National Institute of Allergy and Infectious Diseases, National Institutes of Health). Also, we investigated the relationships and common regulatory pathways of the DE genes with both the KEGG and IPA pathway databases.

Seven Weeks. The mutant retina showed expression changes in nine genes—*CAMK2G*, *NTRK2*, *PRKCB*, *RALA*, *RBBP6*, *RNF41*, *SMYD3*, *SPP1*, and *TUBB2C*—that directly or indirectly are involved in the apoptosis and cell death processes. These are defined as part of the signaling pathways that activate apoptosis, attempt to block apoptosis, or attempt to down- or upregulate protective cell functions (Table 1). Four genes with mitochondrial function—*ELOVL6*, *GLOD4*, *NDUFS4*, and *REEP1*—were downregulated in the mutant retinas at the same age, as well as a few genes (*CHML*, *PAX6*, *RDH11*, and *RBBP6*) that are relevant for visual system development and function (Table 1). *CHML* and *RDH11* are the paralogs of *CHM* and *RDH12*, two genes that, when mutated, cause X-linked choroideremia and RP, respectively.

The downregulated genes in the mutant retina that were DE were mainly associated with very general cellular functions such as secretion, cellular organization, homeostasis, protein modification, mRNA transcription, and different enzymatic functions (binding, deaminase, GTPase, glycosyltransferase, oxidoreductase, and nuclease). They were mainly localized in the cytoplasm, endoplasmic reticulum, and cellular membranes (Table 2).

To further characterize the DE genes, we interrogated the human KEGG pathways database. We found in the mutant retinas that several signaling (neurotrophin, ErbB, chemokine, and MAPK), focal adhesion, glioma, long-term potentiation, cancer, and metabolic pathways were significantly altered (Table 3). Relevant genes involved in several of these pathways included two kinases: *CAMK2G* and *PRKCB*. Together with *RAP1B*, these are part of the long-term potentiation pathway that is critical for neuronal synapses and interacts with the MAPK pathway. *RAP1B* and *RALA* also are part of, or are closely related to, the Ras signaling pathway, which affects many cellular functions, such as cell proliferation, apoptosis, migration, cell fate specification, and differentiation. Use of the IPA program showed networks that differed slightly from those in the KEGG analysis. The four networks of DE genes that were found with IPA at 7 weeks were (1) nervous system development and function, organ development, and cell morphology (12 DE genes); (2) amino acid metabolism, posttranslational modification, and small molecule biochemistry (11 DE genes); (3) infection mechanism, antimicrobial response, and gene expression (10 DE genes); and (4) cellular growth and prolif-

TABLE 1. Selected DE Genes Related to Apoptotic/Cell Death Processes or to Mitochondria Functions or Having Relevant Vision Functions in Mutant Retinas Compared with Age-Matched Normal Retinas at 7 and 16 Weeks and in a Combined (7 and 16 Weeks) Analysis

Gene	Name	Related Function	GO	<i>q</i> (%)	Change Mutant vs. Normal
Seven Weeks					
<i>NDUFS4</i>	NADH dehydrogenase (ubiquinone) Fe-S protein 4	Mitochondria	Mitochondrial electron transport; oxygen and reactive oxygen species metabolic process	0.0	−47.2
<i>PAX6</i>	Paired box 6	Vision	Eye development; protein and DNA binding; transcription regulator activity	0.0	−8.6
<i>ELOVL6</i>	ELOVL family member 6, elongation of long chain fatty acids	Mitochondria	Transferase activity; fatty acid elongation	0.0	−3.4
<i>GLOD4</i>	Glyoxalase domain containing 4	Mitochondria	Lyase activity	0.0	−3.2
<i>PRKCB</i>	Protein kinase C, beta	Proapoptosis	Protein kinase activity; nucleotide binding	7.0	−16.0
<i>TUBB2C</i>	Tubulin, beta 2C	Cell death	Nucleotide, GTP and MHC class I protein binding; gtpase activity	7.0	−2.8
<i>CHML</i>	Choroideremia-like (Rab escort protein 2)	Vision	Visual perception; regulation of gtpase activity	9.6	−12.6
<i>SPPI</i>	Secreted phosphoprotein 1, osteopontin	Antiapoptosis	Protein binding; cytokine activity; inflammatory response	9.6	−9.0
<i>CAMK2G</i>	Calcium/calmodulin-dependent protein kinase II gamma	Cell death	Protein kinase activity; nucleotide binding	9.6	−5.1
<i>RBBP6</i>	Retinoblastoma binding protein 6	Proapoptosis; vision	Nucleic acid and protein binding; ubiquitin-protein ligase activity	9.6	−4.4
<i>REEP1</i>	Receptor accessory protein 1	Mitochondria; cell death	Protein insertion into membrane	9.6	−4.3
<i>SMYD3</i>	SET and MYND domain containing 3	Antiapoptosis	Protein binding; transferase activity	9.6	−3.4
<i>RNF41</i>	Ring finger protein 41	Proapoptosis	Protein binding; ligase activity	9.6	−2.8
<i>RALA</i>	V-ral simian leukemia viral oncogene homolog A (ras related)	Cell death	Gtpase activity; protein and nucleotide binding	9.6	−2.6
<i>NTRK2</i>	Neurotrophic tyrosine kinase, receptor, type 2	Prosurvival	Protein kinase activity; nucleotide binding	9.6	−2.6
<i>RDH11</i>	Retinol dehydrogenase 11	Vision	Retinol dehydrogenase activity; catalytic activity	9.6	−2.0
Sixteen Weeks					
<i>NKAP</i>	NFKB activating protein	Prosurvival	Transcriptional repressor	7.5	−68.6
<i>TARS2</i>	Threonyl-tRNA synthetase 2, mitochondrial	Mitochondria	Nucleotide and ATP binding; ligase activity	7.5	−58.3
<i>SAG</i>	S-antigen; retina and pineal gland (arrestin)	Vision	Protein binding; signal transduction; visual perception	7.5	−5.7
<i>CRX</i>	Cone-rod homeobox	Vision	DNA and protein binding; transcription factor activity; visual perception	7.5	−5.0
<i>SLC25A5</i>	Solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 5	Mitochondria; pro-survival	Protein binding; transporter activity	7.5	−3.9
Combined Analysis					
<i>NDUFS4</i>	NADH dehydrogenase (ubiquinone) Fe-S protein 4	Mitochondria	Mitochondrial electron transport; oxygen and reactive oxygen species metabolic process	0.0	−37.0
<i>ACSL1</i>	Acyl-CoA synthetase long-chain family member 1	Mitochondria	Nucleotide and ATP binding	0.0	2.3
<i>PDE6A</i>	Phosphodiesterase 6A, cGMP-specific, rod, alpha	Vision	Catalytic activity; visual perception	8.0	−3.0
<i>PPP3CA</i>	Protein phosphatase 3, catalytic subunit, alpha isoform	Mitochondria; proapoptosis	Protein serine/threonine phosphatase activity	8.0	−2.3
<i>RNF41</i>	Ring finger protein 41	Proapoptosis	Protein binding; ligase activity	8.0	−2.3
<i>ADC</i>	Arginine decarboxylase	Mitochondria	Protein binding; lyase activity	8.8	2.5
<i>HSP90AA1</i>	Heat shock protein 90kDa alpha, class A member 1	Antiapoptosis; mitochondrial transport	Nucleotide binding; protein folding; response to stress	8.8	2.4
<i>TFAM</i>	Transcription factor A, mitochondrial	Mitochondria; antiapoptosis; vision	DNA binding; regulation of transcription	8.8	2.3
<i>ZBTB4</i>	Zinc finger and BTB domain containing 4	Proapoptosis	DNA and protein binding; regulation of transcription	8.8	2.0

Genes are listed from the lowest to the highest *q*-value per time point and are reported with the change ratios and the relevant GO functions. The entire list of DE genes is available in Supplementary Table S3.

TABLE 2. Functional Assignment

GO Category	Term	Genes (<i>n</i>)	<i>P</i>
Seven Weeks			
BP	Secretion	5	0.01
	Cellular component organization and biogenesis	13	0.014
	Organelle organization and biogenesis	8	0.017
	Posttranslational protein modification	9	0.019
	Cellular monovalent inorganic cation homeostasis	2	0.036
	Establishment of localization	12	0.041
	Localization	13	0.042
CC	Protein modification process	9	0.047
	Organelle membrane	11	0.0007
	Cytoplasmic part	17	0.004
	Endoplasmic reticulum	7	0.01
	Endoplasmic reticulum membrane	5	0.016
	Nuclear envelope-endoplasmic reticulum network	5	0.018
	Endomembrane system	7	0.019
	Endoplasmic reticulum part	5	0.025
	Cytoplasm	21	0.034
	Intracellular organelle part	14	0.038
	Organelle part	14	0.039
MF	Membrane	23	0.047
	Protein binding	24	0.003
	GTP binding	4	0.047
	Guanyl ribonucleotide binding	4	0.049
Sixteen Weeks			
CC	Intracellular part	11	0.043
MF	Protein phosphatase regulator activity	2	0.031
	Phosphatase regulator activity	2	0.032
Combined Analysis			
BP	Mitochondrion organization and biogenesis	3	0.004
	Nitrogen compound metabolic process	4	0.011
	Cellular metabolic process	13	0.035
	Primary metabolic process	13	0.036
CC	Cytoplasmic part	10	0.0005
	Cytoplasm	11	0.006
	Endoplasmic reticulum	4	0.027
MF	Catalytic activity	10	0.044

DAVID was used to determine the GO categories of the DE genes in mutant retinas at 7 and 16 weeks, separately and regardless of age (combined analysis). For each category, the related term (BP, biologic process; MF, molecular function; and CC, cellular compartment), the number of DE genes, and the significance ($P \leq 0.05$) are shown.

eration, cellular development, and connective tissue development and function (9 DE genes).

Sixteen Weeks. The DE genes in the 16-week mutant retinas showed trends similar to those in the 7-week retinas. Some were also related to apoptosis (*NKAP* and *SLC25A5*), mitochondria (*SLC25A5* and *TARS2*), and visual perception (*CRX* and *SAG*) (Table 1). They also grouped in a cluster related to the protein-modification processes (e.g., the protein phosphatase regulator activities) and were located in the intracellular domain (Table 2). Although none of the KEGG pathways was represented by more than two genes, the IPA Cell Cycle, Genetic Disorder, Neurologic Disease network was identified with seven of the DE genes (Supplementary Fig. S1). Of note, qRT-PCR at 16 weeks confirmed downregulation of *OPN1SW* (opsin 1 cone pigments, short-wave-sensitive) and *RHO* (rhodopsin), two photoreceptor-specific genes that also belong to this network (see the Validation and Expansion section, to follow, and Fig. 1A).

Combined Analysis. As expected, the results of the combined analysis of DE genes which excluded age as a factor were in accord with the overall picture observed when the two ages were analyzed separately. Apoptosis-related genes (down-regulated: *RNF41* and *PPP3CA*; upregulated: *HSP90AA1*,

ZBTB4, and *TFAM*), mitochondria-related genes (downregulated: *PPP3CA* and *NDUFS4*; upregulated: *ACSL1*, *ADC*, *HSP90AA1*, and *TFAM*), and two genes shown to be relevant to visual processes (*PDE6A* and *TFAM*) were DE (Table 1). Furthermore, the DE genes play a relevant role in mitochondrial organization and biogenesis, in metabolic and catalytic processes, and in functions localized to the cytoplasm and the endoplasmic reticulum (Table 2). KEGG analysis identified the metabolic pathways as being represented with four DE genes: *ACSL1*, *ADC*, *NDUFS4*, and *RDH11* (Table 3). This pathway was already significant at 7 weeks with the same DE genes *NDUFS4* and *RDH11*, in addition to *ATP6V1H*. In contrast, IPA analysis identified Gene Expression, Cancer, Cellular Growth and Proliferation as the only significant network with 12 DE genes included.

In conclusion, these functional analyses showed a consistent correlation of the DE genes with processes and pathways known to be essential for the correct maintenance and regulation of retinal function. Furthermore, they indicate that the function of the identified DE genes alters and modifies several metabolic and cellular activities, as well as signaling pathways, in the retina that were not previously

TABLE 3. Significant Human KEGG Pathway

Seven Weeks

hsa04722 Neurotrophin signaling pathway
<i>CAMK2G</i> ; calcium/calmodulin-dependent protein kinase II gamma
<i>NRK2</i> ; neurotrophic tyrosine kinase, receptor, type 2
<i>RAP1B</i> ; RAP1B, member of RAS oncogene family
<i>SHC3</i> ; SHC (Src homology 2 domain containing) transforming protein 3
hsa04510 Focal adhesion
<i>PRKCB</i> ; protein kinase C, beta
<i>RAP1B</i> ; RAP1B, member of RAS oncogene family
<i>SHC3</i> ; SHC (Src homology 2 domain containing) transforming protein 3
<i>SPP1</i> ; secreted phosphoprotein 1
hsa04012 ErbB signaling pathway
hsa05214 Glioma
<i>CAMK2G</i> ; calcium/calmodulin-dependent protein kinase II gamma
<i>PRKCB</i> ; protein kinase C, beta
<i>SHC3</i> ; SHC (Src homology 2 domain containing) transforming protein 3
hsa04062 Chemokine signaling pathway
<i>PRKCB</i> ; protein kinase C, beta
<i>RAP1B</i> ; RAP1B, member of RAS oncogene family
<i>SHC3</i> ; SHC (Src homology 2 domain containing) transforming protein 3
hsa05200 Pathways in cancer
<i>PRKCB</i> ; protein kinase C, beta
<i>RALA</i> ; v-ras simian leukemia viral oncogene homolog A (ras related)
<i>TCEB1</i> ; transcription elongation factor B (SIID), polypeptide 1 (15kDa, elongin C)
hsa04010 MAPK signaling pathway
<i>NRK2</i> ; neurotrophic tyrosine kinase, receptor, type 2
<i>PRKCB</i> ; protein kinase C, beta
<i>RAP1B</i> ; RAP1B, member of RAS oncogene family
hsa04720 Long-term potentiation
<i>CAMK2G</i> ; calcium/calmodulin-dependent protein kinase II gamma
<i>PRKCB</i> ; protein kinase C, beta
<i>RAP1B</i> ; RAP1B, member of RAS oncogene family
hsa01100 Metabolic pathways
<i>ATP6V1H</i> ; ATPase, H ⁺ transporting, lysosomal 50/57 kDa, V1 subunit H
<i>NDUFS4</i> ; NADH dehydrogenase (ubiquinone) Fe-S protein 4
<i>RDH11</i> ; retinol dehydrogenase 11

Combined Analysis

hsa01100 Metabolic pathways
<i>ACSL1</i> ; acyl-CoA synthetase long-chain family member 1
<i>ADC</i> ; arginine decarboxylase
<i>NDUFS4</i> ; NADH dehydrogenase (ubiquinone) Fe-S protein 4
<i>RDH11</i> ; retinol dehydrogenase 11

The pathways were identified with the DE genes at seven weeks and regardless of age (7 and 16 weeks; combined analysis). Pathways having more than two genes are reported; no significant pathway was found at 16 weeks.

known to be involved with this particular or other retinal degenerative diseases.

Validation and Expansion of Selected DE Genes at the RNA Level

We performed qRT-PCR at 7 and 16 weeks on a subset of 11 genes, to validate the microarray results, and on seven additional genes, to better characterize the pathogenesis of XLPRA2. As well, we used qRT-PCR to compare the expression levels of all 18 genes between the normal and mutant retinas at 3 weeks, to identify possible early differences in gene expression that would provide additional insights into the disease (Fig. 1A). For this analysis, we examined three categories of genes:

1. Genes not DE in the microarray analysis, but with a potential role in the disease based on the functional analyses just detailed. These included *BNIP3* (a mitochondrial gene, hypoxia-responsive, and strong proapoptotic protein), *PLAGL2* (a zinc-finger protein inducer of cell death and promoter of *BNIP3* expression),⁴³ *PFDN5* (a subunit of prefoldin, a chaperone complex that binds and stabilizes newly synthesized polypeptides), and *TPD52* (a tumor protein with homodimerization activity present in the cytoplasm and the endoplasmic reticulum).
2. Genes downregulated (7 weeks: *PAX6*, *NDUFS4*, and *SPP1*; 16 weeks: *CRX*, *SAG*, and *SLC25A5*; combined analysis: *NDUFS4*) or upregulated (combined analysis: *ZBTB4*) in the microarray analysis in the mutant retinas.
3. Genes not contained in the microarray or not expressed in the brain reference tissue, but with a known role in photoreceptor function or retinal degenerative diseases (*CNGA3*, *CNGB3*, *OPN1LW*, *OPN1SW*, *RHO*, and *RPGR*) or that reflect an inner retinal glial response to outer retinal disease (*GFAP*).

High concordance between microarray and qRT-PCR results was found for all DE genes in the mutant retinas compared with the normal retinas at the corresponding ages (Fig. 1A). *SPP1* could not be confirmed by qRT-PCR, although the general pattern of expression was similar for both analyses at the 7- and 16-week time points (Fig. 1A). A trend toward a decreased expression in the mutant retinas at 16 weeks of *NDUFS4* ($P < 0.1$), identified by qRT-PCR but not by microarray, represented the only other difference between the two techniques in the mutant and normal retinas (Fig. 1A). We also confirmed the downregulation of *NDUFS4* and upregulation of *ZBTB4* ($P < 0.1$, trend toward statistical significance) identified in the combined microarray analysis (Fig. 1B). Furthermore, the qRT-PCR data confirmed the upregulation of *TPD52* ($P < 0.1$, trend toward statistical significance) and *PAX6*, and the downregulation of *SAG* in normal retinas between 7 and 16 weeks (Fig. 1C). *SAG* and *CRX* expression, determined by qRT-PCR, showed a trend toward upregulation in the mutant retinas at 7 compared with 16 weeks ($P < 0.1$; Fig. 1C), but these changes were not observed with the microarray analysis.

Regarding the analysis of genes not present on the array or not expressed in the brain reference tissue, we found that the expression of *GFAP* was increased in the mutant retinas at all three ages (Fig. 1A). Among the 18 genes tested, it was the only one altered at 3 weeks of age, and its expression was highly upregulated in the mutant retinas at 16 weeks compared with 7 weeks (Fig. 1C). The early increase in *GFAP* expression suggests that there is a response in the inner retina to the photoreceptor disease that occurs before the onset of overt degeneration.

Analysis of *RPGR* with a probe common to all retinally expressed isoforms (located in the junction between the 5' UTR and exon 1) showed increased expression in the mutant retinas compared with the normal retinas at 16 weeks, but no expression differences at 3 and 7 weeks (Fig. 1A). Similar results with a trend toward statistical significance ($P < 0.1$, results not shown) were found with an additional gene-specific assay (from ABI; Supplementary Table S1), which also identifies all known retinal isoforms but is located on the exon 3–4 junction.

In contrast to the finding at 3 weeks, which showed no differences in photoreceptor-specific gene expression between normal and mutants, at 7 weeks the rod-specific gene *RHO* was downregulated, whereas the S-cone specific *OPN1SW* ($P < 0.1$, trend toward statistical significance) and the cone-specific *CNGB3* genes were upregulated in the mu-

FIGURE 1. qRT-PCR results. The histograms represent the ratios of the changes in expression for the different genes analyzed. (▨) Genes not DE in the microarray analysis; (■) DE genes in the microarray analysis; (□) genes not on the microarray or that did not amplify in the brain reference tissue; (◻) genes that were only examined by qRT-PCR at 3 weeks, but not by microarray analysis. Values that significantly differ are indicated with asterisks (* $P \leq 0.05$; ** $0.05 < P < 0.1$, indicating a trend toward statistical significance). Error bars: ranges of the maximum and minimum change differences calculated based on the standard deviation of biological triplicates, as previously shown.³⁷ See Supplementary Tables S2 and S3 for the complete microarray results of the DE genes *CRX*, *PAX6*, *NDUF54*, *ZBTB4*, *SPP1*, *SAG*, *SLC25A5*, and *TPD52*. (A) Comparison of expression for 18 selected genes between mutant and normal retinas at 3, 7, and 16 weeks of age. (B) Comparison of *NDUF54* and *ZBTB4* expression between mutant and normal retinas in the combined analysis (7 and 16 weeks of age). (C) Expression comparison of eight genes between mutant and normal retinas at 7 and 16 weeks of age.

tant retinas (Fig. 1A). At 16 weeks, *RHO*, *OPN1SW*, and *SAG* were downregulated in the mutant retinas (Fig. 1A) and also were downregulated in comparison to their levels in the mutant retinas at 7 weeks (Fig. 1C). On the other hand, the cone-specific genes *CNGA3* and *OPN1LW* were equally expressed between the mutant and normal retinas at both 7 and 16 weeks.

Of interest, all the hybridization-based microarray analyses indicated much greater ratios of change in expression compared with the amplification-based technology (qRT-PCR). This result is in line with those reported in other retina studies.^{21,44}

Validation of Selected DE Genes at the Protein Level

To compare the differential RNA expression results at the protein level, we analyzed four DE genes (*NDUF54*, *SAG*,

GFAP, and *OPN1SW*) by Western blot analysis and IHC. For Western analysis, a single retina per status/time point was used. The results of Western analysis of *NDUF54* showed that a band of ~21 kDa was found for all four retinas. When compared using ACTB as a loading control, expression levels were lower in the mutant retinas at both ages, particularly at 16 weeks (Fig. 2A). This result is in accordance with the findings at the RNA level (e.g., at 7 weeks) and in combined analysis (microarray and qRT-PCR) and 16 weeks (qRT-PCR), as well as with the IHC results (Fig. 3A). *NDUF54* showed positive staining in cell layers where mitochondria are abundant (e.g., in the RPE and the photoreceptor inner segments; Fig. 3A). Compared with the other retinas, the normal at 16 weeks showed intense staining in the inner nuclear layer and in the photoreceptor inner segments. The loss of photoreceptors and the subsequent misalignment, disorganization, and shortened in-

FIGURE 2. Western blot analysis of single samples from normal (16 and 7 weeks) and XLPRA2 mutant (16 and 7 weeks) retinas with four antibodies (NDUF54, SAG, FAP, and OPN1SW) in addition to actin (ACTB), used as a loading control. *Dashed line* indicates that the gel was cut and ACTB placed below the protein of interest. The relevant molecular size markers are indicated. (A) NDUF54 levels were lower in the mutant retina at both ages. (B) Lower protein levels of SAG were found in the mutant retina at 16 weeks compared with either the normal age-matched control or the 7-week mutant. (C) GFAP was upregulated in both the 7- and 16-week mutant retinas. (D) The mutant retina at 16 weeks showed a downregulation of OPN1SW. The specific ~39 kDa band is absent, even though the quantity of the ACTB loading control was high.

ner and outer segments were observed in the mutant retinas at 16 weeks.

Western blot analysis of the SAG protein showed a single band at the expected molecular weight of ~48 kDa (Fig. 2B). Much lower SAG levels were found in the mutant retina at 16 weeks than in either the normal age-matched control or the younger mutant retina (Fig. 2B). On the other hand, Western analysis did not indicate an increase in this protein as a result of upregulation of SAG expression in the normal retinas at 16 weeks compared with 7 weeks. In the normal retinas, IHC showed high expression in the photoreceptor outer segment and in the synaptic terminals of the outer plexiform layer (Fig. 3B). In mutants, SAG was mislocalized to the outer nuclear layer, and loss of photoreceptors and outer nuclear layer at 16 weeks was reflected as a decrease in the label's intensity (Fig. 3B).

We also assessed the protein expressions of GFAP and OPN1SW, two genes that were not on the microarray, but were DE in the qRT-PCR analysis. Western blot analysis of GFAP confirmed a band at ~50 kDa and an upregulation of this protein in the mutant retinas compared with the age-matched normal retinas at both 7 and 16 weeks (Fig. 2C). Similar results were found by IHC: In the mutant retinas, GFAP labeled the radial processes of Müller cells, which form a heavily labeled network of fibers that extend from the internal limiting membrane to the outer nuclear layer (Fig. 3C).

Western blot results of OPN1SW showed lower levels in the mutant retinas at 7 weeks, and no protein was detectable at 16 weeks, even though the quantity of the ACTB loading control was high (Fig. 2D). It appears that at the time the cones are degenerating and increasing *OPN1SW* expression at the mRNA level, the message is not translated or the protein degraded. Immunolabeling with OPN1SW antibody confirmed the localization and labeling of S-cone outer segments in the normal and 7-week mutant retinas (Fig. 3D). However, there was a marked decrease in the number of labeled S-cone outer segments at 16 weeks (Fig. 3D).

DISCUSSION

This study is the first report of a large-scale transcriptomic analysis at different critical ages in XLPRA2 mutant retinas and identifies the genes and pathways that are associated with photoreceptor degeneration in this relevant canine model of human *RPGR/XLRP*. In particular, our findings showed alteration, specifically downregulation, in mutant retinas of genes and several important cellular pathways (Tables 2, 3); these include mitochondria-related modifications, which might not be expected, based solely on the *RPGR* function. The alterations in the mutant retinas were specific for the disease stages examined, and no commonalities were found between the two ages.

Furthermore, Table 1 provides a list of DE genes that are known to be involved in nonclassic anti- and proapoptotic pathways, but, with the exception of *PAX6*, *SAG*, and *CRX*, have not been associated with photoreceptor degenerative diseases. The list of novel genes associated with XLPRA2 disease can serve as a useful reference for other comparative studies and for inter- and intraspecies meta-analyses.

The powerful tool of cDNA microarrays allows simultaneous analysis of thousands of genes, to look for those modified by a specific process (e.g., normal aging, disease, and disease stage). In this study, we applied this technology to expand our knowledge of the pathways and mechanisms involved in photoreceptor degeneration by examining the transcriptional profile of normal and XLPRA2 mutant retinas at 7 and 16 weeks of age. The two ages sampled represent key time points previously established for the disease.²⁶ At 7 weeks of age (*execution phase*), there is photoreceptor disorganization and disruption. The outer nuclear layer is 85% to 90% of its normal thickness, but cell death, assayed with the TUNEL method, is at its maximum. As nearly all photoreceptor cells present at this time are not dying, any detected alterations in gene expression are likely to represent early degenerative processes that are associated with or induce apoptosis. At 16 weeks (*persistent execution/chronic cell death phase*), there is loss of rod inner and outer segments, and narrowing of the outer nuclear layer to ~60% of normal thickness; at this time, the number of TUNEL-positive cells is significantly reduced, but remains constant until close to 1 year of age.²⁶

We used a custom canine cDNA microarray, derived from a normal retina library, that is the only canine and retina-specific array available.^{30,31} This approach is similar to that taken in other studies that have used custom slide microarrays of eye/retina-expressed genes.^{45–47} However, there are some limitations with the custom canine cDNA microarray, in that the number of genes that it contains is ~4500, and many belonging to classic anti- and proapoptotic pathways are not represented. For example, not included in the microarray are some of the major classic proapoptosis genes such as *BAX*; the calpains; caspase-3, -4, -8, and -10; *FADD*; *FAS/CD95*; *FASL*; *TNFSF10/TRAIL*; *TNFSF12/APO3L*; *TNFSF8/CD30L*; *TNFA*; *TNFRSF1A*; and *TRADD*. These genes, and others, are now being analyzed

FIGURE 3. Immunolabeling of normal and XLPR2 mutant 16- and 7-week retinas with the same four antibodies (NDUFS4, SAG, GFAP, and OPN1SW) as were used in Western analysis. Except for NDUFS4 (A), images were merged with DIC-transmitted light. (A) NDUFS4 was expressed in the mitochondrion inner membrane. Staining was observed in the retinal pigment epithelium (RPE), photoreceptor inner segment (IS), inner plexiform (IPL), and ganglion cell (GCL) layers. Lower NDUFS4 labeling was seen in the mutant retina at 16 weeks, particularly in the IS, owing to the loss of photoreceptors. Intense staining was found in the inner nuclear layer (INL) in the normal retinas at 16 weeks. (B) SAG was highly expressed in the photoreceptor outer segments (OS) and in the outer plexiform layer (OPL) in normal retinas. In the mutant, it mislocalized to the outer nuclear layer (ONL). (C) GFAP staining was weak and limited to astrocytes and Müller cell end feet in normal retinas at 7 weeks and was minimal at 16 weeks. In the mutant retinas, GFAP immunoreactivity was evidenced by intense GFAP labeling in Müller cells at both 7 and 16 weeks, and labeled radial processes extended from the inner retina into the ONL. (D) OPN1SW is exclusively expressed in the OS of S-cones, in the 16-week mutant retina OPN1SW were minimal to absent. Scale bar: 2.5 μ m.

as part of an ongoing, focused study on the expression of cell death and survival genes.

Changes in Normal and XLPR2-Mutant Retinas during Development

With these caveats in mind, we identified genes that were DE. In the normal retinas, 5% of the total genes on the array were DE when the 7- and 16-week samples were compared. Even though at both time points the retinas are structurally and functionally comparable, the higher level of DE genes at 7 weeks, most of which were upregulated, suggests that molecular changes are taking place as the retina completes postnatal development (7 weeks) and reaches maturity (16 weeks).⁴¹ In contrast, all the genes that were DE in the mutant retina were upregulated at 7 weeks when compared with 16 weeks. Moreover, in the mutants, we did not observe a similar pattern of change in gene expression with development, probably because disease-related molecular processes and pathways at this stage of development are altered secondary to the ongoing degenerative process.

Mitochondrial and Nonclassic Pro- or Antiapoptosis Genes Altered during XLPR2 Degeneration

One of the most important findings of this study highlighted a connection between mitochondria function and XLPR2 degeneration, with a clear emphasis on either pro- or antiapoptosis genes that are related to the death of the photoreceptors, but that do not contribute to the classic cell death and survival

events (Table 1). Mitochondria and their membrane integrity are critical for retinal cell function and survival. Dysfunctions, which can be caused by mutations in both mitochondrial and nuclear DNA, have been associated with the pathogenesis of hereditary neurodegenerative diseases⁴⁸ and with several outer retinal diseases, including age-related macular degeneration,⁴⁹ cone-rod dystrophy,⁵⁰ and light-induced retinopathy.⁵¹ The nine DE genes related to mitochondria that were identified in this study are located on the nuclear DNA, indicating that the mitochondrial DNA itself is not affected and providing new avenues for future investigation.

Age and Disease Stage Specificity of Gene Expression Profiles

A further relevant finding of this study indicated downregulation of genes in the mutant retinas compared with expression in the normal retinas at both ages. This result appears to be due to an overexpression of genes in normal versus mutant retinas at 7 weeks, and, at 16 weeks, probably reflects a general downregulation of gene expression in the mutant retina associated with ongoing photoreceptor degeneration and active cell death. These findings were further validated at the protein level, where downregulation of NDUFS4 at both ages, as well as SAG and OPN1SW at 16 weeks, was shown.

It could be argued that the overall downregulation of gene expression in the mutant retinas was simply due to the loss of photoreceptors associated with the disease (i.e., ~10% and 40%, respectively, at 7 and 16 weeks). However, in most cases, the magnitude of the decreased expression was much greater

than could be accounted for by photoreceptor loss alone. This result suggests that downregulation of gene expression accompanies the disease at the time points examined and is not unique to the canine disease, as similar findings have been reported in other microarray studies of retinal degenerative disease: In rd1 mice during peak rod degeneration, 1 gene is upregulated and 69 downregulated⁵²; in Bbs4-null mice, 48 genes are upregulated and 306 downregulated⁵³; in R6/2 mice 81 transcripts are upregulated and 230 downregulated⁵⁴; and in R7E mice, 215 transcripts are upregulated and 324 downregulated.⁵⁴

Similar to studies of RP1 knockout mice,⁵⁵ our results showed very specific age- and disease stage-dependent changes in gene expression profiles, which further suggests that mechanisms triggered during the *execution phase* of the disease are not only different, but also have a broader influence on the cells than those during the *persistent execution/chronic cell death phase*. This possibility is reflected by the higher number of DE genes at the earlier stage, but also by the increase in the change ratio expression in the later stage, which may reflect the ongoing degeneration.

Important Cellular Pathways and Signaling Functions Altered by the *RPGRORF15* Frameshift Mutant Retina

The functional and pathway analyses were used to further characterize the gene expression changes. These mainly indicated a general modification of signaling, binding (in particular protein and DNA binding; Table 2), and metabolic functions, as well as alteration of homeostasis, cellular organization, and biogenesis in the mutant retinal cells. This result was in agreement with those in previous studies of photoreceptor cell death in mice, in which similar functional categories of genes were found to be altered.⁵⁶ Of particular interest, the 7-week XLPRA2 mutant retina showed an alteration in the neurotrophin pathway with the downregulation of *CAMK2G*, *NTRK2*, *RAP1B*, and *SHC3*. This pathway, which is closely related to the MAPK signaling pathway, is initiated by neurotrophins that promote cell survival by preventing the initiation of programmed cell death. Studies demonstrate that specific neurotrophins (e.g., pigment epithelium-derived factor [PEDF] and glial cell line-derived neurotrophic factor [GDNF]), induce neuroprotection in animal models of RP.^{57,58} Downregulation of the genes in this pathway may suggest that during the peak of photoreceptor loss, cell death can occur by both a lack of survival signaling and the induction of apoptosis cascades. The downregulation of another gene at 7 weeks, *ELOVL6*, confirms that a complex pattern of regulation occurs in mutant retinas. This gene belongs to the same family as *ELOVL4*, in which mutations have been shown to cause Stargardt disease-3 (STGD3),⁵⁹ and *ELOVL2*, a cone-specific gene that was upregulated in *NR2E3*^{-/-} and rd7/rd7 mice.⁶⁰

In the analysis, we also compared expression between normal and mutant retinas, regardless of age, to help identify genes that show a consistent change. Three of them, *HSP90AA1*, *TFAM*, and *ZBTB4*, are of particular interest with regard to apoptotic events, as they seem to have opposite functions. *HSP90AA1* is an antiapoptosis molecule related to mitochondrial pathways,⁶¹ whereas *TFAM* is involved in the maintenance of mitochondrial DNA and has been shown to attenuate apoptosis when upregulated.⁶² Increased expression of both genes in mutant retinas may suggest an antiapoptotic role in the mitochondria.

On the other hand, increased *ZBTB4* expression may suggest a proapoptosis role in mutant retinas, as depletion, in response to p53 activation, suppresses apoptosis and promotes long-term cell survival.⁶³

qRT-PCR Confirmation and Expansion of the Microarray Results

qRT-PCR results indicate high reliability of the microarray data at both ages, as the expression of almost all the genes tested was confirmed. The only exception at both ages was *SPP1*, which was downregulated in the mutant retinas at 7 weeks by microarray, but with qRT-PCR no statistically significant differences were found, because of an unusually high variation between samples. At 16 weeks, there was a 5.5-fold upregulation of *SPP1* in the mutant retinas by qRT-PCR; however, although upregulation was also found with the microarray analysis, it was not statistically significant because of a high *q*-value (42.3%).

The qRT-PCR analysis also identified DE genes that play a crucial role in the phenotype of the disease and that might have a bearing on photoreceptor degeneration. At 3 weeks (*induction phase*), when the mutant photoreceptors are developing, albeit abnormally, only *GFAP*, a marker of glial activation, was DE in the mutant retinas. Increased GFAP expression early indicates that signaling events from the outer retina to the Müller cells take place and suggests that early stress events in the photoreceptors may be transmitted to the Müller cells. GFAP expression was highly upregulated during the entire time course of the disease, as clearly confirmed at the proteomic level by Western and IHC analyses. Similar observations at different ages were also previously reported in mutant dogs.²⁶

In an interesting result, we found an upregulation of the expression of the mutated gene *RPGR* in the mutant retinas at 16 weeks. This result is in line with those in other studies of retinal degeneration caused by mutation in the *NR2E3* gene^{60,64,65} that also report an upregulated expression in mutant animals of the mutated gene and suggest that, in XLPRA2 mutants, the *RPGR* gene product is necessary for its own feedback mechanisms only during the *persistent execution/chronic cell death phase*.

Alteration of Photoreceptor-Specific Gene Expression

As expected, the qRT-PCR findings show misregulation of cone and rod photoreceptor-specific gene expression. In mutants, we found at 7 weeks of upregulation of *OPN1SW* and *CNGB3*, downregulation of *RHO*, and no change in *CNGA3*. These results suggest that during the photoreceptor death peak, a differential decrease in the number of rods with respect to cones occurs and confirms the results obtained with morphologic evaluation.²⁶ At 16 weeks, there was decreased expression of *SAG*, *RHO*, and *OPN1SW* in the mutant retinas, but expression of *OPN1LW*, *CNGA3*, and *CNGB3* did not change. These findings suggest a differential and preferential damage of rods and S-cones during this later phase of the disease.

The decreased expression at 16 weeks of the rod-specific gene *SAG* that was identified at both the RNA and protein levels is in line with the results reported in two *NR2E3* knockout mouse lines.⁶⁰ *SAG* encodes for one of the major soluble rod outer segment proteins; it binds as a cofactor to photoactivated-phosphorylated rhodopsin and interacts with *CRX*, a photoreceptor-specific protein. In the XLPRA2 retina at 16 weeks, both *SAG* and *CRX* showed comparable reductions in expression, a finding also reported in rd1 mice.⁵⁶ In general, downregulation of *CRX*, *PAX*, *RHO*, *OPN1SW*, and *SAG* have also been reported in other comparable retinal diseases.^{52,53,54,60}

Unknown EST Sequences and Potential Splice Variations of the 3' UTR Regions of Unidentified Genes

The identification of several DE transcripts that could not be assigned to any known gene suggests that some genes and/or pathways involved in the long-term regulation of disease are not yet known.

These unknown ESTs did not show any commonalities, including conserved domains and/or sequence homologies to known genes and are located on different chromosomes. The library used to construct the arrays is slightly biased toward the 3' UTR of the genes and the EST clones are unlikely to represent genomic contamination.³¹ As the translational data did not give any evidence that these clones could be in coding regions, we speculate that the unknown ESTs may be splicing variants of 3'-UTRs of genes not yet identified. Characterization and elucidation of these clones would be of particular interest and may help to identify novel genes that play pivotal roles in retinal maintenance and development not only in the dog, but possibly in other species. For example, in a previous study we identified an unknown and uncharacterized gene that subsequently was found to be a novel gene that causes retinal degeneration in dogs and humans.⁶⁶

CONCLUSIONS AND PERSPECTIVES

In conclusion, DE of retina-expressed genes in XLPRA2 provides useful information to begin to determine at the molecular level the sequence of events that link a mutation in a retina-expressed gene with the ultimate degeneration and loss of the visual cells. These studies now can be expanded so that some of the identified pathways can be examined in greater detail to identify the key signaling molecules and pathways responsible for the death of the photoreceptors.

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