MicroRNA Expression in the Glaucomatous Retina

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PURPOSE. MicroRNAs are small, endogenous noncoding RNAs that modulate posttranscriptional gene expression. Although the contribution of microRNAs to the pathogenesis of glaucomatous damage is unknown, supporting evidence from central nervous system (CNS) research suggests they may play a role. It was therefore hypothesized that microRNAs known to be altered in CNS injury are also altered in experimental glaucoma.

METHODS. Intraocular pressure (IOP) was elevated in rats by unilateral injection of hypertonic saline and IOP monitored for 5 weeks. After rats were killed, retrobulbar optic nerve sections were graded for damage. MicroRNA was extracted from whole retinæ of eyes with advanced nerve damage ($n = 8$) and from normal, noninjected control eyes ($n = 8$). Quantitative PCRs were performed using a panel of 17 microRNAs, reported from CNS research to be implicated in mechanisms also linked to glaucomatous damage. Computationally and experimentally derived gene targets were identified for the differentially expressed microRNAs. These were then integrated with existing gene array data. Functional interpretation was performed using the Molecular Signatures Database and DAVID Functional Annotation Clustering.

RESULTS. Eight microRNAs were significantly downregulated in glaucomatous retinæ compared with controls (miR-181c, miR-497, miR-204, let-7a, miR-29b, miR-16, miR106b, and miR-25); miR-27a was significantly upregulated. Enrichment of targets associated with extracellular matrix/cell proliferation, immune system, and regulation of apoptosis were observed. Cholesterol homeostasis and mTORC-1 pathways showed reduced expression.

CONCLUSIONS. MicroRNAs are differentially expressed in retinæ of eyes with advanced glaucomatous damage compared with normal controls. Integrating microRNA with gene expression data may improve understanding of the complex biological responses produced by chronically elevated IOP.

Keywords: microRNA, glaucoma model, retina

Glaucoma is a leading cause of irreversible worldwide blindness, characterized by progressive loss of retinal ganglion cells (RGCs) and optic nerve degeneration that is usually secondary to elevated IOP.^{1–3} Current treatments for glaucoma are predominantly restricted to the modulation of IOP through pharmacological and surgical approaches. However, the effectiveness of this strategy can be limited in many patients by poor medication efficacy, intolerance, or surgical failure.

The primary insult leading to glaucomatous injury is thought to occur to RGC axons at the optic nerve head (ONH).^{4–7} In addition, patients with established glaucomatous damage may still suffer progressive loss of vision despite maximal IOP lowering. This progressive susceptibility of glaucomatous eyes to further damage may occur as a consequence of the molecular and structural mechanisms in the ONH and/or retina that occur during the disease process. An improved understanding of the biological processes in both locations may help direct future research toward the development of novel interventions to attenuate further loss of visual function in patients with glaucoma.

Experimental paradigms to model glaucomatous damage have been developed in a variety of species. In models ranging from rodents to primates, chronic elevation of IOP leads to loss of RGCs in a similar manner to that observed in human

glaucoma,⁸ with secondary cell death thought to ultimately occur due to apoptosis.^{9–12} Studies of gene expression within the whole retina in experimental glaucoma highlight the complex and dynamic nature of the changes that occur following the induction of glaucomatous damage, which serve to modulate both positive and negative regulatory pathways.^{13–19} These studies have reported common themes, including upregulation of genes associated with cell death, cell proliferation, glial activation, TNF- α signaling, immune and inflammatory responses, and downregulation of genes associated with lipid biosynthesis and cytoskeleton and light sensation.^{13–19}

However, with the initial IOP-related insult affecting RGC axons at the ONH, the impact of the primary injury would be expected to predominantly affect RGCs within the inner ganglion cell layer of the retina. Laser capture microdissection (LCM) has been used to isolate gene expression changes within the RGC layer following induction of experimental glaucoma,^{16,20} allowing further refinement of both the altered functional gene classes and the magnitude of any change, by reducing the potential dilution effect from gene responses in other retinal layers. Understanding the contribution of these various responses to the development of glaucomatous damage requires a detailed understanding of the variety of signals that may further impinge on their function.

MicroRNAs are small (~18–22 nt), endogenous noncoding oligoribonucleotides that are highly conserved across species and modulate the posttranscriptional silencing of gene expression.²¹ They function through recognition of specific sequences in their target mRNAs and predominantly act to reduce expression of their mRNA targets.²² Understanding how these molecules modulate the mechanisms associated with glaucomatous damage may facilitate future targeting of microRNAs to attenuate glaucomatous injury.

Although the influence of microRNAs on the biological processes that occur within the glaucomatous ONH and retina is not currently known, there is an emerging body of evidence in central nervous system (CNS) research that suggests they may play an important role. Experimental studies of brain and spinal cord injury have demonstrated that microRNAs influence several pathways that are also known to be modulated within the glaucomatous retina. These include apoptotic cell death,^{23–30} ischemia,^{31–39} inflammatory and immune responses,^{30,40–45} extracellular matrix (ECM) remodeling^{27,46–48} and TGF- β signaling.^{43,49,50} The interactions of these biological responses are highly complex. In isolation, interpretation and simplification should be performed with great caution. However, examining the pattern of microRNA expression in conjunction with gene expression changes may help in our interpretation and understanding of the mechanisms that develop following glaucomatous injury.

Eyes with established severe injury are likely to demonstrate maximal responses to the ongoing primary IOP-related stimulus, in addition to the secondary mechanisms that will develop throughout the whole retina and not just restricted to the RGCs. In this study, we therefore tested the hypothesis that microRNAs altered in models of CNS injury are also altered within the glaucomatous retina with advanced damage.

METHODS

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research with approval from the Oregon Health and Science University (OHSU) for use in all the experiments.

Glaucoma Model

Eight-month-old Brown Norway rats were housed in constant low-level light according to the standard protocol in our laboratory. This approach was adopted to attenuate circadian fluctuations of IOP^{8,51} and to enable meaningful comparison with our previously published data using the same experimental glaucoma model housed in identical light conditions.^{8,16,20,52} Aqueous outflow obstruction was created by injecting hypertonic saline unilaterally into episcleral veins, resulting in sustained elevation of IOP.⁵² The IOP was measured in conscious animals at least three times per week using a handheld tonometer (Tono-Pen XL; Medtronic, Minneapolis, MN, USA). A weighted mean IOP elevation was calculated for each injected eye by dividing the cumulative IOP by the number of postinjection days. The cumulative IOP was determined as the area under the curve of the plot of days postinjection versus IOP measurement.

Tissues were harvested for further analysis 5 weeks after injection. Optic nerve injuries resulting from IOP elevation were evaluated using the grading method as previously described.^{8,53} This approach correlates strongly with total axon counting of optic nerve sections examined by transmission electron microscopy (TEM).⁸ Briefly, optic nerves were removed and postfixed in buffered 2.5% glutaraldehyde/2% paraformaldehyde and embedded in Spurr's plastic. Nerve

cross sections were then graded from 1 (no injury) to 5 (active degeneration involving the whole nerve area) by five masked observers. Tissue from eyes with injury grades greater than 4.9 were used for these studies ($n = 8$; glaucoma model) along with noninjected control eyes with no injury ($n = 8$). Previous comparisons of nerves with similar advanced injury to actual axonal counts of TEM images from the same nerves, showed that a grade 5 injury is equivalent to a total intact axon count less than or equal to 45% of that within a normal optic nerve.^{8,53} Therefore, this study represents the contrast between microRNA expression in retinæ with extensive IOP-induced degeneration of at least 50% of optic nerve axons and that in fellow eyes with no detectable axon degeneration.

Isolation and Assessment of MicroRNA

Whole retinæ were quickly dissected from enucleated eyes and frozen at -80°C for subsequent analysis. Following evaluation of the extent of optic nerve injury, the *mirVana* microRNA isolation kit (Ambion; Thermo Fisher Scientific, Grand Island, NY, USA) was used to isolate total RNA (including microRNA). Whole frozen retinæ were disrupted by immediate sonication in ice-cold lysis/binding buffer. The manufacturer's protocol was modified to include a second aqueous phase extraction during the phenol-chloroform purification step, so as to maximize microRNA yield.⁵⁴ The DNase treatment was performed following RNA elution (TURBO DNase, Ambion; Thermo Fisher Scientific). Quality of RNA was confirmed on the Agilent Bioanalyzer using the RNA 6000 Nano chip and the Eukaryotic Total RNA program (Agilent Technologies, Santa Clara, CA, USA).

Reverse Transcription, Pre-amplification, and Quantitative Real-Time PCR

TaqMan MicroRNA Assays (Thermo Fisher Scientific) were used to detect 17 mature microRNA sequences identified from recent CNS research to influence biological pathways known to be modulated within the glaucomatous retina, and a panel of three endogenous controls (Table 1). A custom RT primer pool was prepared for these 20 individual probes according to the manufacturer's instructions. Reverse transcription was performed in 15- μL reactions containing 222 ng of RNA/4 μL nuclease-free water, 6 μL RT primer pool, 0.3 μL 100 mM dNTPs with dTTP, 3 μL multiscribe reverse transcriptase (50 U/ μL), 1.5 μL 10 $\times$ RT buffer, and 0.2 μL RNase Inhibitor (20 U/ μL) for 30 minutes at 16°C , 30 minutes at 42°C , and 5 minutes at 85°C in a thermal cycler (GeneAmp PCR System 9700; Thermo Fisher Scientific).

A custom pre-amplification primer pool was prepared for 20 individual probes by combining each TaqMan MicroRNA Assay (containing a mix of forward and reverse primers) according to the manufacturer's instructions. Reverse amplification was performed in 25- μL reactions containing 2.5 μL RT product, 12.5 μL 2 $\times$ TaqMan PreAmp Master Mix, 3.75 μL pre-amplification primer pool, and 6.25 μL nuclease-free water. These were incubated for 10 minutes at 95°C , 2 minutes at 55°C , 2 minutes at 72°C , and 5 minutes at 85°C , followed by 12 cycles of 95°C for 15 seconds and 60°C for 4 minutes, with a final 10-minute incubation at 99.9°C in the thermal cycler.

A total of 175 μL 0.1 $\times$ Tris-EDTA buffer was added to each reaction to form the diluted pre-amplification product used for PCR reactions. This was subjected to quantitative PCR performed in 20- μL reactions and performed in triplicate for each sample (1 μL 20 $\times$ TaqMan MicroRNA Assay, 0.2 μL diluted pre-amplification product, 10 μL 2 $\times$ TaqMan Universal Master Mix II, No Amperase UNG, and 8.8 μL nuclease-free water) by incubation in a thermal cycler (Chromo4; Bio-Rad, Hercules,

TABLE 1. Taqman MicroRNA Probes Used for Quantitative RTPCR

miRBase ID	Mature MicroRNA Sequence	Assay ID
rno-miR-204-5p	UUCCCUUUGUCAUCCUAUGCCU	000508
rno-miR-106b-5p	UAAAGUGCUGACAGUGCAGAU	000442
rno-miR-25-3p	CAUUGCACUUGUCUCGGUCUGA	000403
rno-miR-223-3p	UGUCAGUUUGUCAAAUACCCC	000526
rno-miR-497-5p	CAGCAGCACACUGUGGUUUGUA	001346
rno-miR-181c-5p	AACAUUCAACCUGUCGGUGAGU	000482
rno-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA	000397
rno-miR-29b-3p	UAGCACCAUUUGAAUACAGUGUU	000413
rno-miR-29b-5p	CUGGUUUCACAUUGGUGGUUAG	002166
rno-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG	000391
rno-let-7a-5p	UGAGGUAGUAGGUUGUAUAGUU	000377
rno-miR-22-3p	AAGCUGCCAGUUGAAGAACUGU	000398
rno-miR-200b-3p	UAAUACUGCCUGGUAAUGAUGAC	001800
rno-miR-23a-3p	AUCACAUUGCCAGGGAAUUC	000399
rno-miR-27a-3p	UUCACAGUGGCUAAGUUCGCG	000408
rno-miR-424	CAGCAGCAAUUCAUGUUUGGA	001076
rno-miR-145-5p	GUCCAGUUUCCAGGAUCCCU	002278
Control Assays	Sequence	Assay ID
U6 snRNA	GTGCTCGCTTCGGCAGCACATAT ACTAAATTTGGAACGATACAG AGAAGATTAGCATGGCCCCCTG CGCAAGGATGACACGCAAATT CGTGAAGCGTTCCATATTTT	001973
snoRNA234	CTTTTGGAACTGAATCTAAGTGA TTTAACAAAAATTCGTCCTA CCACTGAGA	001234
snoRNA202	GCTGTACTGACTTGATGAAAGTA CTTTTGAACCCCTTTTCC ATCTGATG	001232

CA, USA) at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Fluorescence data were analyzed (Opticon Monitor 3 software; Bio-Rad) with a five-point dilution standard curve. A baseline was subtracted by using the average-over-cycle range method (2–20 cycles) and threshold-adjusted to give $r^2 = 0.9$ to 1.0 (as close to 1.0 as possible). Differential microRNA expression between control and glaucomatous retinae was calculated by relative quantification using the comparative C_T method with normalization performed using a panel of three endogenous controls (U6 snRNA, snoRNA234, and snoRNA202). Each experimental group consisted of eight biological replicates. Data were expressed as the change (x-fold) in glaucomatous retinae compared with retinae from control eyes by dividing the mean normalized expression value in experimental retinae by the mean value in control specimens. If this number (the relative quantity) was less than 1, the (negative) reciprocal was listed (e.g., 0.5, or a decrease of 50% compared with the control, is reported as –2-fold change). Statistical analysis was performed by comparing the normalized expression between glaucomatous and control retinae using an unpaired two-tailed *t*-test with significance considered for values of *P* less than 0.05.

Determination of Gene Targets of Differentially Expressed MicroRNAs

Experimentally validated gene targets for each differentially expressed microRNA in retinae from eyes with advanced glaucomatous damage were obtained by searching the freely available curated online database, miRWalk (v2.0) (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/index.html>).⁵⁵

Due to the highly conserved nature of microRNAs, we combined validated targets for both human and rat. In the context of this study, it is important to emphasize that these gene targets were not validated in retina, but within other cells or tissues derived from the either human or rat.

These were then augmented by including predicted gene targets for the rat using computational approaches that evaluate the interaction of the first seven nucleotides of the mature microRNA strand (seed sequence) with the 3'-untranslated region (UTR) of mRNA sequences, with prediction based on factors such as the degree of complementarity and thermodynamic stability of the putative bond. Four freely available algorithms were used (miRWalk 2.0 [<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/index.html>],⁵⁶ RNAhybrid [<http://bibiserv.techfak.uni-bielefeld.de/rna hybrid>],⁵⁷ miRanda [<http://www.microrna.org/microrna/>],⁵⁸ and Targetscan 6.2 [www.targetscan.org])⁵⁹ so as to identify potential gene targets.

The statistical basis of the miRWalk algorithm involves modeling the probability distribution of random matches between the 5' end of the microRNA sequence and the 3'-UTR of the mRNA sequence with a Poisson distribution. Longer predicted matches to the so-called seed sequence (a seven-nucleotide sequence comprising 5'-bases 2 to 8) of a microRNA would be associated with a more effective microRNA-mRNA interaction and lower probability. The algorithm output reports predicted genes with *P* less than 0.05.⁵⁶ The RNAhybrid reports targets based on an adjusted *P* less than 0.05, which incorporates extreme value statistics of normalized minimum free energies of the microRNA-mRNA bond, a Poisson estimation of multiple binding sites, and a calculation of the effective numbers of orthologous targets from comparative studies of multiple organisms.⁶⁰ The miRanda-predicted targets are ranked according to the mirSVR regression model, which generates a weighted sum based on features of the predicted microRNA-mRNA duplex.⁵⁸ The output was adjusted to report predicted targets with an mirSVR score of –1.0 or lower, which represent the top 7% of predicted targets, with 35% or more probability of having a log expression change of less than or equal to –1.0 (–2.7-fold change), and 50% or more probability of a log expression changes of at least –0.5 (–1.65-fold change).⁵⁸ Targetscan evaluates base-pairing complementarity within the seed sequence of a microRNA, the thermodynamic free energy of the putative microRNA-mRNA interaction, and identifies and ranks targets according to the predicted efficacy of targeting (context + scores) and the probability of conserved targeting across multiple genomes.^{61,62} Targetscan has an estimated sensitivity of 82% with a false-positive rate of approximately 21%.⁶³ To mitigate the risk of including excessive false positives and excluding false negatives, genes were accepted as potential targets only when predicted by two or more of the above algorithms.

Combination of Gene Targets With Existing Microarray Data and Functional Interpretation

Having identified potential gene targets for each differentially expressed microRNA in retinae from eyes with advanced glaucomatous damage, these were then compared against published gene array data of whole retinae from the same experimental model with an identical injury grade.¹⁶ Based on the assumption that the dominant action of microRNAs is to decrease target mRNA levels,²² we looked for significantly upregulated genes (≥ 1.3 -fold change) on the gene array where the microRNA was downregulated, and looked for downregulated genes (≥ 1.3 -fold change) on the gene array where the microRNA was upregulated. This comparison enabled the generation of lists of potential targets for each individual microRNA, which also showed significant change in the gene

TABLE 2. Differentially Expressed MicroRNAs in Whole Retinae of Eyes With Advanced Glaucomatous Damage

miRBase ID	Accession Number	Fold-Change in Glaucomatous Retina Compared With Control	<i>P</i>
rno-miR-181c-5p	MIMAT0000857	-4.76	0.0038
rno-miR-497-5p	MIMAT0003383	-4.76	0.0065
rno-miR-204-5p	MIMAT0000877	-4.0	0.0080
rno-let-7a-5p	MIMAT0000774	-3.57	0.0251
rno-miR-29b-5p	MIMAT0004717	-3.57	0.0295
rno-miR-16-5p	MIMAT0000785	-3.33	0.0052
rno-miR-106b-5p	MIMAT0000825	-3.03	0.0445
rno-miR-25-3p	MIMAT0000795	-2.63	0.0438
rno-miR-27a-3p	MIMAT0000799	6.45	0.0103

array analysis of whole retinae from eyes with advanced glaucomatous damage.

Two approaches were used to derive functional interpretations of the above lists of potential gene targets generated for each individual microRNA. First, significant overlaps between each microRNA-specific list of gene targets and so called “Hallmark Gene Sets” (developed at the Broad Institute, Cambridge, MA, USA) were investigated by using the Molecular Signatures Database v5.0 (<http://www.broadinstitute.org/gsea/msigdb/index.jsp>).⁶⁴ These “Hallmark Gene Sets” summarize and represent specific, well-defined biological processes and have been derived using a computational approach so as to reduce noise and redundancy for use in the formal gene set enrichment analysis of genomic data.⁶⁴ The investigation of gene sets uses a hypergeometric distribution of overlapping genes using the whole human genome as a background, so as to generate a significance value (*P* value) with an associated false discovery rate analogue to correct for multiple hypothesis testing (*q*-value < 0.00001).

Database for Annotation, Visualization, and Integrated Discovery (DAVID), an established online resource (<https://david.ncifcrf.gov/home.jsp>) that provides a comprehensive set of functional annotations for large gene lists, was also used.^{65,66} The DAVID functional annotation clustering module⁶⁷ was used to identify related annotation groups that were enriched for each list of microRNA-specific gene targets. This approach evaluates functional classification by examining relationships among annotation terms to cluster heterogeneous yet similar annotations into functional groups. This was performed for each microRNA-specific gene list by using annotations from the Gene Ontology (GO) consortium (<http://geneontology.org>), namely biological process, cell component, and molecular function, and those derived from the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database (<http://www.genome.jp/kegg/pathway.html>). A list of array probes included on the microarray platform (Supplementary Table S1) was uploaded as the background for the clustering analyses. The EASE method⁶⁵ was used to identify individual overrepresented GO categories or KEGG pathways by calculating a modified Fisher's Exact *P* value to demonstrate enrichment, where annotations showing *P* less than 0.05 were considered to be more enriched than by random chance. An enrichment score⁶⁵ for each cluster was calculated to rank the overall biological importance (enrichment) of the gene groups. Briefly, this represents the geometric mean of all enrichment EASE scores for each annotation term, with a negative log transformation applied to emphasize its nature as a relative score. Higher enrichment scores indicate that annotation term members play a more significant role. A score of 1.3 is equivalent to 0.05 on a nonlogarithmic scale and

it is suggested that more attention is given to groups with scores of 1.3 or higher, although annotation groups with lower scores may also be of potential interest.

RESULTS

History of IOP and Evaluation of Experimental Glaucoma

Whole retinae from eight unoperated control eyes and eight experimental eyes were analyzed in this study. The mean/maximum IOP ($\pm$ SD) in control eyes was $28.2 \pm 0.2/29.0 \pm 0.3$ mm Hg compared with $39.2 \pm 3.3/51.7 \pm 1.0$ mm Hg in experimental eyes (Fig. 1A). The cumulative IOP profile of all eyes by group across the duration of the experiment is shown in Figure 1B. Evaluation of optic nerve sections confirmed advanced glaucomatous damage in experimental eyes with a mean grade of 4.98, compared with a mean grade of 1.0 in control eyes (i.e., no evidence of injury).

Differential MicroRNA Expression Within Glaucomatous Retinae

Nine of the studied microRNAs showed significant differential expression in glaucomatous retinae when compared with noninjected controls (Table 2). Of these, miR-181c, miR-497, miR-204, let-7a, miR-29b, miR-16, miR106b, and miR-25 were significantly downregulated and miR-27a was the only microRNA found to be significantly upregulated. No significant change in expression of miR-21, miR-22, miR-23a, miR-145, miR-200b, miR-223, or miR-424 was observed.

Integration of Potential Gene Targets of Differentially Expressed MicroRNAs With Gene Array Data

A mean $\pm$ SD of 7602 ± 1320 experimentally validated targets were identified using the miRWalk database for each differentially expressed microRNA. Combining these genes with the additional predicted targets (identified by two or more of the computational algorithms) resulted in potential targets comprising 9502 ± 728 genes per individual microRNA (Supplementary Table S2).

Comparing these potential targets with published gene array data¹⁶ identified 112 ± 7 (mean $\pm$ SD) genes per microRNA that were upregulated on the array that were therefore either predicted or validated targets of the respective downregulated microRNAs (Supplementary Table S3). Interestingly, there was remarkable consistency among the genes targeted by the downregulated microRNAs identified, with 66 genes upregulated on the array being potential targets of all eight downregulated microRNAs. Forty-three of these shared gene targets were also significantly upregulated on a gene array of the RGC layer obtained by LCM from the same experimental model with an identical injury grade.¹⁶ Twenty-nine (78.4%) of 37 shared gene targets showed a significantly greater degree of change within the RGC layer when compared with the whole retina (unpaired two-tailed *t*-test, *P* < 0.008) (Table 3).

Eighty-six downregulated genes identified on the array were either predicted or validated targets of the single upregulated microRNA, miR-27a (Supplementary Table S3).

Functional Interpretation of Altered Genes Targeted by Differentially Expressed MicroRNAs

The leading Hallmark Gene Sets showing significant overlap with genes that were either predicted or validated targets of

FIGURE 1. (A) Mean/maximum IOP of control eyes and those with experimental glaucoma over the duration of the experiment. Error bars represent $\pm$ SD. (B) Cumulative IOP profile of control and experimental eyes over the duration of the experiment.

the respective differentially expressed microRNAs, which also changed on the microarray, are shown in Table 4. Enriched gene sets potentially influenced by all eight downregulated microRNAs included those defining epithelial-mesenchymal transition (e.g., wound healing, fibrosis) and genes regulated by NF- κ B in response to TNF- α signaling. Six downregulated microRNAs were associated with enrichment of genes encoding components of the complement system and p53 pathways and networks, and others associated with genes regulating programmed cell death and genes upregulated in response to hypoxia. Analysis of the gene targets common to all eight downregulated microRNAs showed significant enrichment of gene sets associated with the complement system, epithelial-mesenchymal transition, genes regulated by NF- κ B in response to TNF- α signaling, and the regulation of apoptosis. The single upregulated microRNA, miR-27a, was associated with reduced expression of gene sets relating to mTORC-1 ("mechanistic target of rapamycin complex") signaling and cholesterol homeostasis.

Summaries of the major DAVID functional annotation clusters associated with predicted or validated targets of the respective differentially expressed microRNAs, which also changed on the microarray are shown in Table 5. Detailed information about specific annotation terms and the relevant

genes associated with each cluster are presented in Supplementary Table S4. Analysis of the gene targets that showed increased expression on the microarray for each individual downregulated microRNA showed robust enrichment of annotation clusters associated with ECM/cell proliferation and the immune system. Several downregulated microRNAs were associated with strong enrichment of annotation clusters associated with signal transduction/protein kinase cascades, with four microRNAs also associated with the regulation of apoptosis, although with lower enrichment scores. Analysis of the 66 gene targets common to all eight downregulated microRNAs demonstrated robust enrichment of clusters associated with ECM/cell proliferation, the immune system, and signal transduction/protein kinase cascades. The single upregulated microRNA, miR-27a, was associated with reduced expression of functional clusters relating cholesterol homeostasis, light responses, and axonal development.

DISCUSSION

The results of this study demonstrate for the first time that a range of microRNAs known to be altered in CNS injury are also altered within the retinæ of rats with advanced glaucomatous

TABLE 3. Of the 66 Altered Genes Within Whole Retina That Are Potentially Targeted By All Eight Downregulated MicroRNAs, 37 Were Also Significantly Upregulated in a Separate Gene Array Analysis of Retinal Ganglion Cell Layer (RGCL) Samples Obtained by Laser Capture Microdissection Using the Same Experimental Model With an Identical Injury Grade.¹⁶ All 37 Genes Were Experimentally Validated Targets (in Cells/Tissues Other Than Retina, Derived From Either Human or Rat) of All Eight Downregulated MicroRNAs, Although Computational Prediction of These Genes Varied Considerably as Shown in the Table. 29/37 Genes Identified Showed a Significantly Greater Degree of Change in the RGCL When Compared to Whole Retina ($P < 0.008$). The Extent of Change in the 8/37 Genes Showing a Greater Fold Change in Whole Retina Than the RGCL Did Not Differ Significantly

Gene	Fold Change in RGCL	Fold Change in Whole Retina	Gene Identified as a Predicted MicroRNA Target by 2 or More Algorithms?							
			miR-181c	miR-497	miR-204	let-7a	miR-29b	miR-16	miR-106b	miR-25
Atf3	28.38	3.24		•			•			
Gldc	12.41	1.39								
Col3a1	10.58	1.35			•					
Cdh2	6.80	1.38			•				•	
Kif22	5.20	1.66				•				
Fn1	5.17	4.27	•		•		•		•	•
Msn	4.90	2.43								
Cd63	4.83	2.78								
Clic1	4.31	2.23			•					
Jun	4.10	1.33								•
Ccnd2	4.01	2.54			•		•			
Zfp36l2	3.47	2.62			•		•		•	
Myh9	3.37	1.62	•							
Col5a2	2.84	1.43		•		•				
Zfp36l1	2.78	1.64			•					•
Adm	2.66	1.46					•			•
Smad3	2.56	1.42		•	•	•	•	•	•	
Anxa2	2.45	1.70								
Lcp1	2.38	1.70	•		•		•		•	
Flna	2.20	2.07	•							
Fosl2	2.20	2.09								
Igf1r	2.06	1.64								
Ets2	2.01	1.65	•	•	•		•			
H3f3b	1.87	1.30								•
Ern1	1.71	1.33								
Gna12	1.58	1.37		•				•	•	
Aprt	1.56	1.36								
Hsd17b4	1.55	1.37	•				•		•	
Lyn	1.52	1.34	•						•	•
Clu	3.18	4.08			•					
Ctsc	1.84	2.05	•							
Col4a1	1.81	1.84				•	•		•	
Lman1	1.64	1.84								
Itgb1	1.52	1.86	•	•				•		•
Asns	1.50	1.57	•							
Lamc1	1.48	1.62	•	•	•			•	•	
Cttna1	1.42	1.59								

29/37 genes with greater fold change in the RGCL compared to whole retina

8/37 genes with greater fold change in whole retina compared to the RGCL ($p = 0.48$)

damage. The integration and analysis of both differential microRNA and gene expression data also help provide a better understanding of the complex responses that are known to occur in retinæ with advanced injury. Despite recent

advances, the potential significance of microRNAs in modulating the pathogenesis of glaucomatous damage within the ONH and retina has yet to be investigated. However existing knowledge regarding the role of microRNAs in modulating

TABLE 4. Hallmark Gene Sets Showing Significant Overlap With Targets That Were Also Altered on the Microarray (i.e., Upregulated Genes) for Each Downregulated MicroRNA; List of Hallmark Gene Sets Showing Significant Overlap With Targets Common to All Eight Downregulated MicroRNAs (i.e., Upregulated Genes); Hallmark Gene Sets Showing Significant Overlap With Targets That Also Altered on the Microarray (i.e., Downregulated Genes) for the Single Upregulated MicroRNA

Overlapping Hallmark Gene Set	No. Genes in Overlap	<i>P</i>	FDR <i>q</i> Value
miR-181c (upregulated gene targets)			
Complement	13	1.71×10^{-15}	8.57×10^{-14}
Epithelial-mesenchymal transition	12	5.71×10^{-14}	1.43×10^{-12}
TNF- α signaling via NF- κ B	11	1.73×10^{-12}	2.88×10^{-11}
P53 pathway	10	4.73×10^{-11}	5.92×10^{-10}
miR-497 (upregulated gene targets)			
Epithelial-mesenchymal transition	16	5.11×10^{-20}	2.55×10^{-18}
TNF- α signaling via NF- κ B	13	2.80×10^{-15}	7.00×10^{-14}
P53 pathway	12	8.95×10^{-14}	1.49×10^{-12}
Regulation of apoptosis	11	2.42×10^{-13}	3.02×10^{-12}
miR-204 (upregulated gene targets)			
Epithelial-mesenchymal transition	17	6.20×10^{-21}	3.10×10^{-19}
P53 pathway	12	2.83×10^{-13}	7.07×10^{-12}
Complement	11	7.41×10^{-12}	9.26×10^{-11}
TNF- α signaling via NF- κ B	11	7.41×10^{-12}	9.26×10^{-11}
let-7a (upregulated gene targets)			
Epithelial-mesenchymal transition	17	3.55×10^{-22}	1.78×10^{-20}
Complement	11	1.26×10^{-12}	2.10×10^{-11}
TNF- α signaling via NF- κ B	11	1.26×10^{-12}	2.10×10^{-11}
Regulation of apoptosis	10	4.13×10^{-12}	5.16×10^{-11}
miR-29b (upregulated gene targets)			
Epithelial-mesenchymal transition	15	3.66×10^{-18}	1.83×10^{-16}
TNF- α signaling via NF- κ B	12	1.38×10^{-13}	2.30×10^{-12}
P53 pathway	12	1.38×10^{-13}	2.30×10^{-12}
Hypoxia	11	3.86×10^{-12}	4.82×10^{-11}
miR-16 (upregulated gene targets)			
Complement	14	2.70×10^{-17}	6.75×10^{-16}
Epithelial-mesenchymal transition	14	2.70×10^{-17}	6.75×10^{-16}
Regulation of apoptosis	10	3.74×10^{-12}	6.24×10^{-11}
TNF- α signaling via NF- κ B	10	3.23×10^{-11}	5.89×10^{-9}
miR-106b (upregulated gene targets)			
TNF- α signaling via NF- κ B	12	8.95×10^{-14}	4.48×10^{-12}
Complement	11	2.60×10^{-12}	3.25×10^{-11}
Epithelial-mesenchymal transition	11	2.60×10^{-12}	3.25×10^{-11}
P53 pathway	11	2.60×10^{-12}	3.25×10^{-11}
miR-25 (upregulated gene targets)			
Epithelial-mesenchymal transition	16	1.88×10^{-19}	9.39×10^{-18}
P53 pathway	12	2.31×10^{-13}	3.86×10^{-12}
TNF- α signaling via NF- κ B	12	2.31×10^{-13}	3.86×10^{-12}
Complement	11	6.71×10^{-12}	7.72×10^{-11}
All downregulated microRNAs (upregulated gene targets)			
Complement	10	3.32×10^{-13}	8.29×10^{-12}
Epithelial-mesenchymal transition	10	3.32×10^{-13}	8.29×10^{-12}
TNF- α signaling via NF- κ B	8	5.17×10^{-10}	8.62×10^{-9}
Regulation of apoptosis	7	3.72×10^{-9}	4.65×10^{-8}
miR-27a (downregulated gene targets)			
MTorc1 signaling	13	8.92×10^{-17}	4.46×10^{-15}
Cholesterol homeostasis	6	6.02×10^{-9}	1.50×10^{-7}
Androgen response	6	3.95×10^{-8}	6.59×10^{-7}
G2/M checkpoint in cell cycle	5	1.61×10^{-4}	4.86×10^{-4}

the sequelae of experimental CNS injury may provide a suitable paradigm for approaching these unanswered questions.

Although not providing evidence of a direct causal relationship in glaucomatous retinae, our experimental obser-

ventions provide correlations between microRNA and gene expression data that are consistent with experimentally validated responses in other tissues. It is also prudent to highlight that our findings reflect responses to chronically

TABLE 5. Summary of the Major Functional Annotation Clusters by Enrichment Score for Each Downregulated MicroRNA Obtained Using the DAVID Functional Annotation Clustering Module (Full Details in Supplementary Table S4), Using MicroRNA-Specific Gene Targets That Were Also Altered on the Microarray (i.e., Upregulated Genes); Summary of the Major Functional Annotation Clusters by Enrichment Score Using Upregulated Gene Targets Common to All Eight Downregulated MicroRNAs (Full Details in Supplementary Table S4); Summary of the Major Functional Annotation Clusters by Enrichment Score for the Single Upregulated MicroRNA (Full Details in Supplementary Table S4), Using MicroRNA-Specific Gene Targets That Were Also Altered on the Microarray (i.e., Downregulated Genes)

Functional Annotation Cluster	Enrichment Score
miR-181c (leading enriched functional annotation clusters)	
ECM/cell proliferation	3.16
Immune system	2.61
Regulation of apoptosis	1.22
miR-497 (leading enriched functional annotation clusters)	
ECM/cell proliferation	2.98
Signal transduction/protein kinase cascade	1.97
Immune system	1.55
Regulation of apoptosis	1.21
miR-204 (leading enriched functional annotation clusters)	
ECM/cell proliferation	3.11
Vasculature development	1.83
Immune system	1.53
Cell proliferation/phosphorylation	1.51
Signal transduction/protein kinase cascade	1.31
let-7a (leading enriched functional annotation clusters)	
ECM/cell proliferation	3.26
Immune system	2.26
Signal transduction/protein kinase cascade	1.91
Cell motility/migration	1.34
Regulation of apoptosis	1.01
Regulation of phosphorylation	1.01
miR-29b (leading enriched functional annotation clusters)	
ECM/cell proliferation	3.1
Immune system	1.58
B-cell, T-cell, MAPK signaling pathways	1.5
miR-16 (leading enriched functional annotation clusters)	
ECM/cell proliferation	2.8
Signal transduction/tyrosine kinase signaling	2.69
Signal transduction/protein kinase cascade	1.96
Vasculature development	1.75
Regulation of apoptosis	1.69
Immune system	1.67
miR-106b (leading enriched functional annotation clusters)	
ECM/cell proliferation	2.67
Signal transduction/protein kinase cascade	2.27
Immune system	1.69
Regulation of phosphorylation	1.47

TABLE 5. Continued

Functional Annotation Cluster	Enrichment Score
miR-25 (leading enriched functional annotation clusters)	
ECM/cell proliferation	3.08
Immune system	1.57
Phosphorylation	1.46
Signal transduction/protein kinase cascade	1.43
Regulation of phosphorylation	1.42
Vasculature development	1.39
All downregulated microRNAs (leading enriched functional annotation clusters)	
ECM/cell proliferation	3.41
Immune system	2.03
Signal transduction/protein kinase cascade	1.59
miR-27a (leading functional annotation clusters with reduced expression)	
Lipid biosynthesis	2.65
Nucleotide binding	1.8
Light response/neurological system	1.55
Neuronal and axonal development	1.48

elevated IOP that occur across the entire retina. Most cells within the retina survive this insult, with cell loss predominantly restricted to RGCs.¹ Improved understanding of the complex interplay between the responses to glaucomatous injury throughout the retina would be a significant advance, as future neuroprotective strategies for glaucoma may well involve the modulation of surviving retinal glia and neurons. Also, the findings of this study describe a snapshot of events within the advanced glaucomatous retina and therefore do not fully reflect the dynamic nature of mechanisms that occur during the early onset and progression of glaucomatous damage.

Upregulation of genes associated with apoptosis within the retina has been observed in a variety of experimental glaucoma models.^{9–12} Although apoptosis is known to occur in glaucoma, the observed alterations in the microRNA signature would suggest an attempt to shift toward an opposite, more protective environment (Fig. 2A). Upregulation of let-7a,²³ miR-16,²³ miR-29b,²⁹ and miR-497²⁴ have been observed following both CNS trauma and ischemia. Let-7a exerts its proapoptotic activity by inducing neurodegeneration through Toll-like receptor (TLR)-7 activity²⁷ and miR-16, miR-29b, and miR-497 promote cell death through negative regulation of the antiapoptotic protein Bcl-2.^{23,24,29} In addition, a reduction of miR-27a expression following traumatic brain injury leads to upregulation of proapoptotic molecules, whereas administration of exogenous miR-27a results in a reduction in lesion volume and neuronal loss following injury.²⁸ Our observed downregulation of these four proapoptotic microRNAs (miR-16, miR-29b, miR-497, and let-7a) and upregulation of the antiapoptotic miR-27a within the glaucomatous retina suggest that the microRNA expression may be working to adjust the microenvironment in a protective manner at this advanced stage of the disease, by attempting to attenuate the extent of apoptotic cell death throughout the retina.

Signaling of TNF- α , classified as a component of the immune response, has complex and contrasting roles in the response to elevated IOP, and may act to further modulate the equilibrium between RGC death and survival.⁶⁸ On one hand, this pathway plays a role in mediating RGC death within the glaucomatous human retina,⁶⁹ with recent clinical reports even suggesting an

FIGURE 2. Biological interpretations of differential microRNA expression in retinas with advanced glaucomatous damage. **(A)** Reduced expression of *proapoptotic* microRNAs (miR-16, miR-497, miR-29b, and let-7a) would aim to reduce apoptotic cell death. Increased expression of the *antiapoptotic* miR-27a would also enhance this effect. These changes in the microRNA signature are suggestive of a protective shift to limit apoptosis. **(B)** Reduced expression of several microRNAs (miR-106b, miR-25, and miR-204) is associated with enhanced TGF- β signaling and a reduction in let-7a expression is also known to enhance cell proliferation. These changes in the MicroRNA signature within glaucomatous retina are consistent with extensive ECM remodeling.

association between systemic anti-TNF therapy and reduced incidence of glaucoma.⁷⁰ However, through activation of NF- κ B pathways, although often considered as initiators of inflammation, TNF- α may also exert a protective effect by resolving inflammation and initiating tissue repair.⁷¹

Our findings appear to lend some clarity to the role of TNF- α signaling within the glaucomatous retina. We identified several downregulated microRNAs (miR-106b, miR-29b, miR-497, miR-181c, and let-7a) that were associated with increased TNF- α -mediated NF- κ B signaling. These included upregulation of NF- κ B1 and AP-1 complex components (cJun and cFos),⁷² which both function in an antiapoptotic manner. This also suggests an apparent protective shift in the cellular responses following advanced injury. In contrast, retinal glia may contribute a proinflammatory effect through these downregulated microRNAs and their correspondingly enriched genes. Rodent microglia activated by hypoxic culture conditions showed reduced miR-181c expression, which was associated not only with enhanced NF- κ B1 expression but also with increased expression of proinflammatory mediators, including TLR-4.⁴⁵ In addition, miR-181c knockdown led to an enhanced proinflammatory response in primary astrocyte cultures, with knockin studies resulting in increased production of the anti-inflammatory cytokine IL-10.⁴⁰ These observations suggest that the different constituent glia within the retina may have discrete roles in the modulation of the inflammatory responses to elevated IOP. Future investigation of the glaucomatous retinal milieu using *in situ* techniques may help to identify the specific microRNA and gene expression responses of the individual retinal cell types.

In this study, we observed that downregulation of several microRNAs, in particular miR-181c and miR-16, were associated with enriched expression of genes encoding components of the complement and immune system. Enhanced expression of the complement cascade and immune response has also been observed within the retina across a spectrum of experimental glaucoma models.^{13,14,16,18,73,74} As a part of the innate immune

response, complement pathways are also intrinsically linked with the process of glial activation in the pathogenesis of glaucomatous damage within the retina.^{13,75} Aside from its role on apoptotic pathways, reduced expression of miR-16 has also been shown to enhance susceptibility to damage from TNF- α -related pathways in human retinal vascular endothelial cell culture.⁷⁶ This effect, in conjunction with proinflammatory effects of miR-181c,⁴⁵ may lead to promote complement-mediated cell death in the context of retinæ with advanced injury from chronically elevated IOP.

The responses of retinal glia to a glaucomatous insult are complex, and may also change as the disease progresses from early to advanced injury.⁷⁷ Responses during this time may be protective to both RGC axons and the glia themselves, such as regulation of ionic imbalances and limitation of glutamate-induced excitotoxicity by Müller glia or astrocytes.⁷⁸ They may also be injurious, including mechanisms leading to axonal atrophy, RGC involution, or RGC death. Retinæ from our eyes with advanced glaucomatous damage demonstrate a marked shift toward mechanisms associated with ECM remodeling and repair (Fig. 2B), as shown by the enrichment of gene sets associated with the ECM and cell proliferation. These genes are potentially influenced by all eight downregulated microRNAs, and would be consistent with a potential causal relationship. Extracellular matrix remodeling has been demonstrated within the ONH^{79,80} and optic nerve itself⁸¹ in response to glaucomatous injury. Similar changes have been described within the glaucomatous retina,^{16,82} although this area has been less intensively studied.

The let-7a also has been established as a master regulator of cell proliferation pathways⁴⁸ and plays a crucial role in neural progenitor cell differentiation.⁸³ These characteristics are consistent with our observed reduction in let-7a expression leading to enhanced expression of genes associated with remodeling and cellular proliferation within the glaucomatous retina. Signaling of TGF- β is integral to the CNS response to injury, with blockade of this pathway shown to reduce glial scarring.⁸⁴ Several of the microRNAs we observed to be downregulated in glaucomatous retinæ influence TGF- β signaling, with repression of miR-204,⁴⁹ miR-106b,^{43,50} and miR-25⁵⁰ known to stimulate downstream components of this pathway.

The only microRNA observed to show increased expression in glaucomatous retinæ was miR-27a. Functional analysis of its altered gene targets demonstrated repression of genes associated with mTORC-1 signaling, lipid biosynthesis, and axonal development. Autophagy is implicated in the pathogenesis of several neurodegenerative diseases,⁸⁵ and its complex role in the pathogenesis of glaucomatous damage is becoming a focus of emerging research.^{86,87} Signaling of mTORC-1 is a major regulator of autophagy, and observations that *in vivo* inhibition may attenuate damage in animal models of neurodegeneration⁸⁸ has led to considerable interest in modulating this signaling pathway as a potential therapeutic intervention. Further investigation of the interactions between miR-27a and autophagy in the context of experimental glaucoma models is an area that merits further investigation.

There is significant interplay between microRNAs and their gene targets, in that an individual microRNA may influence multiple targets and, conversely, an individual gene target may be modulated by multiple microRNAs. This makes the discovery of biologically significant microRNA targets a challenging process, as different microRNAs may regulate individual targets in a cooperative manner, but with differing hierarchy in terms of relative effect. This concept may explain why we observed no significant change in expression of several microRNAs anticipated to be altered in the glaucomatous retina on the basis of existing CNS research.

It is also worth noting the consistency of genes targeted by all the downregulated microRNAs identified, with 66 shared potential targets showing a significant increase in expression on the gene array of whole retina. It is also important to emphasize that almost two-thirds of these shared targets were also significantly upregulated within the RGC layer of glaucomatous eyes¹⁶ (Table 3). Interestingly, a closer comparison of the relative upregulation of these genes between the RGC layer and whole retina suggests that analysis of whole retina most likely represents a dilution of a larger change in gene/microRNA expression that is occurring within the RGC layer.¹⁶ It is therefore reasonable to propose that the downregulated microRNAs identified within our study are likely to modulate the specific gene expression changes that occur within the RGC layer of eyes with advanced glaucoma. An improved understanding of the hierarchical role of these and other microRNAs may be achieved by the assay of additional microRNAs within this tissue in the future. The behavior of individual microRNAs may also vary across tissue subtypes, leading to variations in biological function even within tissues of similar developmental origin, such as the retina and CNS.

It may be that animals housed in constant light are not physiologically normal, due to circadian cycle disruption.⁸⁹ In this study, both groups were housed in identical light conditions, therefore controlling for any differences attributable to the use of constant light. This approach may well minimize the fluctuation of microRNAs known to have circadian or diurnal variations^{90–92}; however, these specific targets were not investigated in this study. Nevertheless, the groups examined differ only in the extent of IOP-related damage as characterized by the extent of optic nerve injury, and are therefore able to successfully differentiate microRNA and gene expression between two groups of animals differing only in the extent of glaucomatous damage.

In the context of human glaucoma, the differential microRNA expression observed in this study may be comparable to what could be expected in a head-to-head comparison between the retinæ of patients with refractory advanced glaucoma and healthy controls. These observations provide further insight into the regulation of the retinal microenvironment in the context of advanced glaucomatous damage. The integration of both microRNA and gene expression data also offers further understanding of the complex biological responses that occur in response to chronically elevated IOP.

The therapeutic application of microRNA biology to other aspects of human disease is a rapidly emerging field, with early clinical trials currently under way.⁹³ Further research in experimental glaucoma models may potentially lead to future opportunities to explore novel neuroprotective approaches using microRNA-targeted techniques aimed toward modulating the retinal milieu during advanced disease. However, it is important to emphasize that glaucomatous changes within the retina are secondary to a primary insult at the ONH. Detailed study of the dynamic nature of alterations in microRNA and gene expression during the early responses to an elevated IOP insult, in the ONH as well as the retina, would enhance our understanding of the mechanisms responsible for the initiation and development of RGC damage. This may provide a real opportunity to correlate changes in both microRNA and gene expression in response to elevated IOP at early time points and provide a basis for future translational research toward attenuating the impact of the mechanisms associated with the development of glaucomatous damage.

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