MicroRNA Expression in the Glaucomatous Retina

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Submitted: August 31, 2015
Accepted: November 5, 2015
Citation: Jayaram H, Cepurna WO, Johnson EC, Morrison JC. MicroRNA expression in the glaucomatous retina. Invest Ophthalmol Vis Sci. 2015;56:7971–7982. DOI:10.1167/iovs.15-18088

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 miRNAs 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 miRNAs 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,40–45 extracellular matrix (ECM) remodeling,46–48 and TGF-β signaling.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.51 and to enable meaningful comparison with our previously published data using the same experimental glaucoma model housed in identical light conditions.8,10,20,52 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 XI; Medtronic, Minneapolis, MN, USA). A weighted mean IOP elevation was calculated for each injected eye by dividing the cumulative IOP increase in conscious animals at least three times per week postinjection versus IOP measurement.

**Tissues**

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.8,55 This approach correlates strongly with total axon counting of optic nerve sections examined by transmission electron microscopy (TEM). 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.8,55 Therefore, this study represents the contrast between microRNA expression in retinae 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 retinae 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 retinae were disrupted by immediate sonication in ice-cold lysis/bind 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 Nanochip 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 multiscrue reverse transcriptase (50 U/μL), 1.5 μL 10× RT buffer, and 0.2 μL RNAse Inhibitor (20 U/μL) for 30 minutes at 16°C, 50 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. Pre-amplification was performed in 25-μL reactions containing 2.5 μL RT product, 12.5 μL 2× 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× 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× TaqMan MicroRNA Assay, 0.2 μL diluted pre-amplification product, 10 μL 2× TaqMan Universal Master Mix II, No Amperase UNG, and 8.8 μL nuclease-free water) by incubation in a thermal cycler (Chromo; Bio-Rad, Hercules,
calculated by relative quantification using the comparative CT expression between control and glaucomatous retinae was 0.9 to 1.0 (as close to 1.0 as possible). Differential microRNA values of

rno-miR-204-5p
GTGCTCGCTTCGGCAGCACATAT

Control Assays Sequence Assay ID
GUCCAGUUUUCCCAGGAAUCCCU
000591
rno-let-7a-5p
UGAGCAGCACGUAAAUAUUGGCG
000277

rno-miR-27a-3p
AUCACAUUGCCAGGGAUUUCC
000399
rno-miR-23a-3p
UAAUACUGCCUGGUAAAAUGAC
0001973

rno-miR-29b-5p
UAGCAGCACGUAAAUAUUGGCG
000277
rno-miR-29b-3p
UAGCUUAUCAGACUGAUGUUGA
000397

rno-miR-497-5p
UGUCAGUUUGUCAAAUACCCC
000482
rno-miR-223-3p
CAUUGCACUUGUCUCGGUCUGA
000377

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/zmi/mirwalk2/index.html], RNAhybrid [http://bibiserv.techfak.uni-bielefeld.de/rahybrid], miRanda [http://www.microRNA.org/microrna/], and Targetscan 6.2 [www.targetscan.org]) so as to identify potential gene targets.

The statistical basis of the mirWalk algorithm involves modeling the 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 targets based on an adjusted P less than 0.05, which incorporates extreme values of statistical normalization free energies of the microRNA-mRNA bond, a Poisson estimation of multiple binding sites, and a calculation of the effective number 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-fold change), and 50% or more probability of a log expression changes of at least −0.5 (−1.65-fold change).58 Targetscaon 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 Targetscaon 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,54 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 1. Taqman MicroRNA Probes Used for Quantitative RT-PCR**

<table>
<thead>
<tr>
<th>miRBase ID</th>
<th>Mature MicroRNA Sequence</th>
<th>Assay ID</th>
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<tbody>
<tr>
<td>rno-miR-204-5p</td>
<td>UUCUCCGUUGCACUAUAGCCU</td>
<td>000508</td>
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<tr>
<td>rno-miR-106b-5p</td>
<td>UAAGUGUGCCAGACUGGAU</td>
<td>000442</td>
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<tr>
<td>rno-miR-25-3p</td>
<td>CAUUGCCAGUGUUGCUGCCUGA</td>
<td>000403</td>
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<tr>
<td>rno-miR-225-3p</td>
<td>UGCAGUUGUGCAAAUACC</td>
<td>000526</td>
</tr>
<tr>
<td>rno-miR-497-5p</td>
<td>CAGCGCAACACUGUGUUGGUA</td>
<td>001346</td>
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<tr>
<td>rno-miR-181c-5p</td>
<td>AACAUUACCCUGCCUGGAGG</td>
<td>000482</td>
</tr>
<tr>
<td>rno-miR-21-5p</td>
<td>UCCCUACACGACUGUAUUGUA</td>
<td>000397</td>
</tr>
<tr>
<td>rno-miR-29b-5p</td>
<td>UAGCACAUIUGUAAACUGAGGU</td>
<td>000413</td>
</tr>
<tr>
<td>rno-miR-29b-5p</td>
<td>CUGUUIUCCAGUGGUGAUUGG</td>
<td>002166</td>
</tr>
<tr>
<td>rno-miR-16-5p</td>
<td>UAGCAGCACGUAUAUUGGCG</td>
<td>000391</td>
</tr>
<tr>
<td>rno-miR-12-5p</td>
<td>UAGCAGUGCAGUUGUAUGGUGG</td>
<td>000377</td>
</tr>
<tr>
<td>rno-miR-22-5p</td>
<td>AAGUGGUCACUGUAGAACGUCU</td>
<td>000398</td>
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<tr>
<td>rno-miR-200b-5p</td>
<td>UAUAUCUGCCGCUAGUAGAC</td>
<td>001800</td>
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<td>rno-miR-23a-3p</td>
<td>AUCACAUUGCCAGCGGUAUUC</td>
<td>000399</td>
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<tr>
<td>rno-miR-27a-3p</td>
<td>UCAACAGUGCCUAAUGUCGCCG</td>
<td>000408</td>
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<tr>
<td>rno-miR-424</td>
<td>CAGCAGCAAAUUGACUGUUGGA</td>
<td>001076</td>
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<tr>
<td>rno-miR-145-5p</td>
<td>GUCCGGUUGUCCCCAGAGAACCUC</td>
<td>002278</td>
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</table>

Control Assays Sequence Assay ID

U6 snRNA
GTGTCGCTTTCTGGCAGCAGCATAT
001973
ACTAAAATTTGGAAGATACAG
AGAAATTTAGCATGCTCGCCCTG
CGCAGAGTGACCAGCAGAATT
CGTGAAGCGTTCCATATTT

snoRNA244
CTTTTGGAACTGAATCTAAGTGA
001234
AGAGATTAGATCCGTTGCTG

snoRNA202
GCTGTAACGTATTTGATGTAAGTA
001232
CAGCTGACGCTGATTCCCTT
ACCTGATG

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 r² = 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 CT 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.
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 vs.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).

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

<table>
<thead>
<tr>
<th>miRNA ID</th>
<th>Accession Number</th>
<th>Fold-Change in Glaucomatous Retina Compared With Control</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>rno-miR-181c-5p</td>
<td>MIMAT0000857</td>
<td>−4.76</td>
<td>0.0038</td>
</tr>
<tr>
<td>rno-miR-979-5p</td>
<td>MIMAT0003383</td>
<td>−4.76</td>
<td>0.0065</td>
</tr>
<tr>
<td>rno-miR-204-5p</td>
<td>MIMAT0008577</td>
<td>−4.0</td>
<td>0.0080</td>
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<tr>
<td>rno-miR-29b-5p</td>
<td>MIMAT0004717</td>
<td>−3.57</td>
<td>0.0251</td>
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<tr>
<td>rno-miR-16-5p</td>
<td>MIMAT000785</td>
<td>−3.33</td>
<td>0.0052</td>
</tr>
<tr>
<td>rno-miR-106b-5p</td>
<td>MIMAT000825</td>
<td>−3.03</td>
<td>0.0445</td>
</tr>
<tr>
<td>rno-miR-25-3p</td>
<td>MIMAT000795</td>
<td>−2.63</td>
<td>0.0438</td>
</tr>
<tr>
<td>rno-miR-27a-3p</td>
<td>MIMAT000799</td>
<td>6.45</td>
<td>0.0103</td>
</tr>
</tbody>
</table>

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 (± SD) in control eyes was 28.2 ± 0.2/29.0 ± 0.3 mm Hg compared with 39.2 ± 3.5/51.7 ± 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, miR-106b, 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-25a, 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 ± 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 ± 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
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-κB 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-κB 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 retinae of rats with advanced glaucomatous
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 retinas 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
microRNA Expression in the Glaucomatous Retina

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

<table>
<thead>
<tr>
<th>Overlapping Hallmark Gene Set</th>
<th>No. Genes in Overlap</th>
<th>P</th>
<th>FDR q Value</th>
</tr>
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<tbody>
<tr>
<td>miR-181c (upregulated gene targets)</td>
<td>Complement</td>
<td>13</td>
<td>$1.71 \times 10^{-15}$</td>
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<tr>
<td></td>
<td>Epithelial-mesenchymal transition</td>
<td>12</td>
<td>$5.71 \times 10^{-14}$</td>
</tr>
<tr>
<td></td>
<td>TNF-α signaling via NF-κB</td>
<td>11</td>
<td>$1.73 \times 10^{-12}$</td>
</tr>
<tr>
<td></td>
<td>P53 pathway</td>
<td>10</td>
<td>$4.73 \times 10^{-11}$</td>
</tr>
<tr>
<td>miR-497 (upregulated gene targets)</td>
<td>Epithelial-mesenchymal transition</td>
<td>16</td>
<td>$5.11 \times 10^{-20}$</td>
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<td></td>
<td>TNF-α signaling via NF-κB</td>
<td>13</td>
<td>$2.80 \times 10^{-15}$</td>
</tr>
<tr>
<td></td>
<td>P53 pathway</td>
<td>12</td>
<td>$8.95 \times 10^{-14}$</td>
</tr>
<tr>
<td></td>
<td>Regulation of apoptosis</td>
<td>11</td>
<td>$2.42 \times 10^{-13}$</td>
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<td>miR-204 (upregulated gene targets)</td>
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<td>Complement</td>
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<td>G2/M checkpoint in cell cycle</td>
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...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 retinas, our experimental observations 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., Downregulated 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)

<table>
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<tr>
<th>Functional Annotation Cluster</th>
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<td>miR-106b (leading enriched functional annotation clusters)</td>
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<td>miR-204 (leading enriched functional annotation clusters)</td>
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<td>miR-181c (leading enriched functional annotation clusters)</td>
<td>3.16</td>
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<tr>
<td>miR-16 (leading enriched functional annotation clusters)</td>
<td>2.36</td>
</tr>
<tr>
<td>let-7a (leading enriched functional annotation clusters)</td>
<td>2.99</td>
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<td>miR-16, miR-29b, and let-7a</td>
<td>1.34</td>
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<td>miR-29b (leading enriched functional annotation clusters)</td>
<td>2.36</td>
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| 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 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 occurring early in the disease, by attempting to attenuate the extent of apoptotic cell death within the glaucomatous retina.
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. 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. Aside from its role on apoptotic pathways, reduced expression of miR-16 has also been shown to enhance susceptibility to damage from TNF-α-mediated pathways in human retinal vascular endothelial cell culture. This effect, in conjunction with proinflammatory effects of miR-181c, may lead to promote complement-mediated cell death in the context of retinae 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. 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. They may also be injurious, including mechanisms leading to axonal atrophy, RGC involution, or RGC death. Retinae 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, optic nerve itself in response to TNF-α signaling within the glaucomatous retina. We identified 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.

In this study, we observed that downregulation of several proapoptotic microRNAs (miR-16, miR-97, 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. 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.

Both in function as 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-κβ signaling but also with increased expression of proinflammatory mediators, including TLR-4. 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. 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. 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. Aside from its role on apoptotic pathways, reduced expression of miR-16 has also been shown to enhance susceptibility to damage from TNF-α-mediated pathways in human retinal vascular endothelial cell culture. This effect, in conjunction with proinflammatory effects of miR-181c, may lead to promote complement-mediated cell death in the context of retinae with advanced injury from chronically elevated IOP.
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 eyes.16 (Table 5). 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 conditions are not physiologically normal, due to circadian light 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; 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 retinas 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 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.

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.

Acknowledgments

Supported by the US-UK Fulbright Commission in conjunction with Fight for Sight, The Special Trustees of Moorfields Eye Hospital (HJ), National Institutes of Health Grants R01EY010145 (JCM) and P30EYO10572 (OHSU Core Grant), and an unrestricted grant from Research to Prevent Blindness, Inc (RPB). JCM is an RPB Senior Investigator.

Disclosure: H. Jayaram, Allergan (R); W.O. Cepurna, None; E.C. Johnson, None; J.C. Morrison, None

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16. Guo Y, Cepurna WO, Dyck JA, Doser TA, Johnson EC, Morrison JC. Retinal cell responses to elevated intraocular pressure: a gene array comparison between the whole retina...


