Discrepant Expression of MicroRNAs in Transparent and Cataractous Human Lenses

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PURPOSE. Age-related cataracts are considered to be a pathological condition that arise as senescence progresses. However, little is known about the function of microRNAs (miRNAs) in the formation of age-related cataracts. The purpose of this study was to identify possible differences in miRNA expression in the central epithelium of transparent and age-related cataractous human lenses.

METHODS. Microarrays were used to determine the miRNA expression profiles of both transparent and cataractous lenses. The results were analyzed by significance analyses performed by the microarray software, and the results were confirmed by stem-loop RT-PCR. Algorithms were used to predict the target genes of the differentially expressed miRNAs.

RESULTS. Two hundred and six miRNAs were identified in all human lenses. The top eight miRNAs according to expression levels were miR-184, let-7b, miR-923, miR-1826, miR-125b, miR-1308, miR-26a, and miR-638 in transparent lenses. In contrast, the top eight miRNAs in cataractous lenses were miR-184, miR-1826, let-7b/c, miR-24, miR-23b, miR-923, and miR-23a. The expression levels of 20 miRNAs were increased and the levels of 12 miRNAs were decreased by more than 2-fold in transparent lenses relative to the levels in cataractous lenses. These findings were confirmed by stem-loop RT-PCR. In addition, several genes that were predicted to be targets of the identified miRNAs have been reported to be involved in lens development or cataract formation.

CONCLUSIONS. The authors report, for the first time, the distinct expression profiles of miRNAs in the central epithelium of transparent and age-related cataractous human lenses. Significant differences in miRNA expression were identified, and the genes targeted by the relevant miRNAs were predicted. The differential expression of miRNAs suggests that these miRNAs have potential roles in lens development and/or cataract formation. (Invest Ophthalmol Vis Sci. 2012;53:3906–3912)

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DISCLAIMER.

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MicroRNAs (miRNAs), a type of noncoding, small RNA in animals, plants, and fungi, regulate the expression levels of target genes by binding imperfectly to three prime untranslated region (3′ UTR) sequences in mRNAs and attenuating protein translation.1 The miRNAs are highly conserved and have been shown to have diverse temporal and tissue-specific expression patterns in a wide range of species.2,3 This evidence supports the hypothesis that miRNAs may play important roles in the regulation of diverse physiological processes (such as development and senescence) and pathological processes. For example, lin-4, a highly conserved miRNA among various species, controls the timing of larval development in Caenorhabditis elegans and regulates tissue aging in adult worms.4

Human lenses are relatively isolated tissues that contribute to the refraction of light to the retina, and are surrounded by the aqueous humor and the vitreous body. Lenses are transparent in young people, but changes occur as the body ages. These changes include the development of a hard, compact nucleus, local opacity, and, finally, the development of a pathological cataract. These characteristics allow the lenses to be used as adaptive models to study the function of miRNAs in cataracts. To date, several miRNAs have been identified in animal eyes5,6; miR-184, miR-205, miR-26a, miR-51, and miR-24 were detected in the cornea; miR-184, miR-125b, miR-31, miR-20h, miR-20a, let-7b, and others were detected in the lens; and miR-181a, miR-125b, miR-124a, miR-30c, miR-182, and miR-183 have been reported to be expressed in the retina. Moreover, the miRNA expression profiles in human lenses remain to be established, and little research has been performed to determine the miRNA expression profiles in cataracts. Cataracts are a condition caused by senescence, but the etiology has not been explained to date.

Several methods have been used to detect miRNA expression, including microarray analysis, stem-loop RT-PCR, northern blot analysis, and in situ hybridization.7,8 In the current study, the authors used a microarray-based approach, which is a revolutionary approach that combines molecular biology and computer technology to allow the high-throughput, simultaneous analysis of global miRNA expression.9 Prediction algorithms have been developed to predict the miRNAs’ target genes through analysis of the conservation degree of the 5’ region of the miRNAs, and the binding sites in
the 3′ UTR of mRNAs. In this study, the authors determined the miRNA expression profiles in the central epithelium of transparent and cataractous human lenses by microarray, and, subsequently, analyzed the results to identify the miRNAs that had distinct expression levels. Finally, several commonly used and accurate miRNA prediction algorithms, TargetScan, miRanda, and PicTar, were used to predict the target genes of these miRNAs and to assess their function in human lenses.

METHODS
Inclusion Criteria, Classification, and Tissue Grouping
Fourty lens samples were collected from postmortem eyes (20 donors, donor age range was 58–65 years, free of ocular diseases) and age-related cataract patients (20 patients, patient age range was 60–68 years, free of other ocular diseases). The degree of lenticular opacification was determined by the Lens Opacities Classification System III (LOCSIII). Lenses from postmortem eyes were obtained from the Eye Bank of Zhongshan Ophthalmic Center in Guangzhou, China, 8 to 24 hours after death. The lenticular opacification of these lenses was graded 1 to approximately 2, and these samples were used as the transparent lens samples. The cataractous lens samples had lenticular opacification of grade 4 to approximately 6. These lenses were acquired during surgeries performed on age-related cataract patients after informed consent of each patient was obtained. In each cataractous lens, a nuclear cataract was obvious, and the nuclear cataract type was identified (see Supplementary Material and Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9178/-/DCSupplemental). There were no statistically significant differences between the transparent and cataractous human lenses with respect to the age of the donor (P > 0.05, Independent Sample t-test). This study was performed in accordance with the tenets of the Declaration of Helsinki for Research Involving Human Tissue. The authors also received consent for research use with the approval of the Sun Yat-sen University Institutional Human Experimentation Committee.

Tissue Preparation and RNA Extraction
All lens sample specimens were centered anterior capsules with a 5- to approximately 6-mm diameter obtained using anterior continuous curvilinear capsulorhexis. First, RNA samples sourced from the central epithelium of one lens do not contain sufficient RNA for a microarray assay, therefore, the authors randomly pooled the central epithelia of one lens do not contain sufficient RNA for a microarray assay; thus, creating three sets of pooled samples. The expression levels of the miRNAs, which were identified as invariant set normalization procedure was performed to normalize the hybridization data was analyzed using GeneChip Operating software (GCO S1.4; Affymetrix). The scanned images were first assessed by visual inspection and then analyzed to generate raw data files (saved as CEL files using the default setting of GCOS 1.4). An invariant set normalization procedure was performed to normalize the different arrays using a DNA-chip analyzer (dChip). Two hundred and six miRNAs were detected in both the transparent and cataractous samples.

To perform a comparison analysis, the authors applied a two-class unpaired method using Significant Analysis of Microarray (SAM) (CapitalBio Corporation, Beijing, China) to identify miRNAs that exhibited significant differences in expression between transparent and cataractous samples. The microarray assays were repeated three times, and the miRNA expression appeared to vary greatly among the assays. The authors first screened the results for miRNAs that had false discovery rate (FDR)-corrected P values less than 0.05 among the three samples. The expression levels of the miRNAs, which were identified using this method, varied only slightly among the assays. The authors next screened for miRNAs that exhibited a 2-fold change in expression levels. Thirty-two miRNAs fit the selection criteria, and these miRNAs were validated by an Independent Samples t-test to confirm the statistical significance.

Real-Time Quantification of MicroRNAs by Stem-Loop RT-PCR
The results of the microarray analysis were subsequently confirmed by stem-loop RT-PCR. Stem-loop RT-PCR is a highly sensitive RT-PCR method for the detection and quantification of miRNAs; this method was previously described by Erika Varkonyi-Gasic et al., and has also been performed extensively in the authors’ laboratory. The authors used three primers: a stem-loop specific RT primer, a miRNA specific forward primer, and a universal reverse primer (Table 1). This method is a two-step process. First, total RNA was extracted from the transparent and cataractous lens samples using TRIzol reagent according to the manufacturer’s instructions. Total RNA (1000 ng) hybridized with the stem-loop RT primer was reverse transcribed for 45 minutes at 37°C, and for 5 seconds at 85°C using the PrimeScript RT
reagent Kit (TaKaRa, Dalian, China) using the SYBR Green I assay (TaKaRa, Dalian, China). The results were obtained using the comparative threshold cycle method and were normalized to β-actin, which served as an internal control. The amplification conditions used were as follows: 5 minutes at 95°C; 39 cycles of 15 seconds at 95°C; 30 seconds at 60°C; and ramp up from 65°C to 95°C at 0.5°C intervals for 5 seconds each. All PCRs were performed in triplicate. The stem-loop RT-PCR was run five times for each sample (pooled central epithelium of five individual transparent and cataractous human lenses) and calculated the mean values for all samples.

**Bioinformatics Analysis to Identify Possible MicroRNA Target Genes**

Three miRNA prediction algorithms were used to predict the miRNAs’ target genes. TargetScan predicts the biological targets of miRNAs by searching for the presence of conserved 8mer and 7mer sites that match the seed region of each miRNA. The miRanda algorithm incorporates current biological knowledge of target rules and uses an up-to-date compendium of mammalian miRNAs. The target sites predicted by miRanda are scored for the likelihood of miRNA-induced down-regulation using mirSVR, a regression model that is based on sequence and contextual features of the predicted miRNA to mRNA duplex. Finally, PicTar is an algorithm that identifies miRNA targets and provided details regarding (1) miRNA target predictions in vertebrates, (2) miRNA target predictions in seven Drosophila species, (3) miRNA targets in three nematode species, and (4) human miRNA targets that are not conserved but are co-expressed. These prediction algorithms have been previously cited in the literature.

**Statistical Analysis**

Differences between the two groups in microarray data were assessed using the FDR-corrected P values less than 0.05 provided by SAM. Differences between the two groups were also estimated with an Independent Samples t-test and a statistical computer program (SPSS 16.0; SPSS, Chicago, IL). A P value less than 0.05 was considered indicative of statistical significance.

**RESULTS**

**MicroRNA Expression Profiles in the Central Epithelium of Human Lenses**

The miRNA expression in transparent and cataractous lenses was initially determined by three separate microarray assays, which identified 206 miRNAs. All miRNAs were ranked according to average expression levels, and the authors found several differences between the expression profiles of transparent and cataractous lenses. The top eight differentially expressed miRNAs included miR-184, let-7b, miR-923, miR-1826, miR-125b, miR-130b, and miR-638 in transparent samples, and miR-184, miR-1826, let-7b/c, miR-24, miR-23b, miR-923, and miR-23a in cataractous lenses (Table 2). The expression levels of miR-184, let-7b, miR-923, and miR-1826 were high in both types of lenses, but the expression levels of these four miRNAs differed between transparent and cataractous samples. In particular, the expression levels of let-7b and miR-923 were significantly higher in transparent samples than in cataractous samples (P < 0.05) (Fig. 1). The level of miR-184, the most abundant miRNA in both transparent and cataractous samples, was not altered by the status of the lens, and exhibited an elevated level of expression relative to the levels of other miRNAs in the samples tested.

**Differential Expression of MicroRNAs between Transparent and Cataractous Samples**

The authors identified 206 miRNAs in the microarray analysis that exhibited distinct and varying expression levels between the two profiles (Fig. 2). They subsequently analyzed three separate microarrays and used an Independent Samples t-test. A 2-fold expression difference (FDR-corrected P values < 0.05) was used as a cutoff level. The results of these analyses demonstrated that 32 miRNAs (15.53%) were significantly differentially expressed between the transparent and cataractous samples. It was found that 20 (62.5%) of these 32 miRNAs were up regulated in transparent samples. The greatest change in expression was exhibited by miR-933, the level of which was approximately 19 times greater in transparent samples than in cataractous samples (Fig. 3). Twelve miRNAs that were down regulated in transparent samples were identified, miR-34a exhibited the greatest decrease in expression; its expression in transparent samples was approximately 4.65 times lower than in cataractous samples.

**Verification of Altered MicroRNA Expression Levels by Stem-Loop RT-PCR**

The miRNA expression profiles were first identified using microarray assays. As mentioned above, 32 miRNAs exhibited statistically significant differential expression between transparent and cataractous lenses. The authors then chose 10 of these 32 miRNAs, including those with the greatest differential expression (miR-953 and miR-145), and confirmed the results of the microarray using stem-loop RT-PCR. The primers were designed according to the methods described previously (Table 1). Five independent stem-loop RTPCR experiments for each

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**Table 1. Primer Sequences Used for Stem-Loop RT-PCR**

<table>
<thead>
<tr>
<th>miRNA Name</th>
<th>miRNA Sequence (5′–3′)</th>
<th>Stem-loop RT Primer (5′–3′)</th>
<th>Forward Primer (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-105</td>
<td>UCAAAGUCUCAGACAGCUUGCU</td>
<td>ACCACA</td>
<td>TCAAAATGCTTGAGACT</td>
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<tr>
<td>miR-1207-5P</td>
<td>GGCACAGGAGGGACUGGAGG</td>
<td>CCCCCTC</td>
<td>TGCCAGGAGGGT</td>
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<td>miR-933</td>
<td>UGGGCGGCAAGGGACUCUCC</td>
<td>GGGAGA</td>
<td>TTGTCGCAGGGAGA</td>
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<tr>
<td>miR-145</td>
<td>CACGCGGUUUCAGGAGAAUCCCU</td>
<td>AGGAGT</td>
<td>GTCCAGGTTCCTCCAG</td>
</tr>
<tr>
<td>miR-22</td>
<td>AAGCCGCGCGGUAGAACGUG</td>
<td>AGAGTTC</td>
<td>JAGCCTTCAGGTTGA</td>
</tr>
<tr>
<td>miR-143</td>
<td>UGGCGGAGGAAGCAGUGCGC</td>
<td>GAGCTTA</td>
<td>TGGAATGAGGACAC</td>
</tr>
<tr>
<td>miR-339-5p</td>
<td>UCCUCGUGCUCAGGACUGUAGG</td>
<td>CCGCCG</td>
<td>CGCGCATCGCTCCAATTA</td>
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<tr>
<td>miR-84a</td>
<td>UUCCGCGUUCUACUGCGUGU</td>
<td>CAGCGT</td>
<td>TTTTGTCCCTCCCTTA</td>
</tr>
<tr>
<td>miR-1241</td>
<td>AUUCACUAGCUUUAUGUGGCCC</td>
<td>GGGCCGCC</td>
<td>ATCCACAGACATATTAA</td>
</tr>
<tr>
<td>miR-135a</td>
<td>UUCUGGGUCUCCUACCACGUC</td>
<td>CACCAGT</td>
<td>TTTTGTCCCTCCCTTA</td>
</tr>
</tbody>
</table>

* Additional sequence (GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATCAGAC) before the stem-loop RT primer.
† Additional sequence (GGGCG) before the forward primer.
Universal reverse primer GTCCAGGTTCCTCCAGG.
A pair of individual transparent and cataractous samples were repeated to obtain mean expression levels. The results were normalized using β-actin as an internal control. The expression levels of several miRNAs (e.g., miR-933, miR-1207-5p, and miR-133a) were significantly higher in transparent samples than in cataractous samples ($P < 0.05$). Other miRNAs, such as miR-34a and 339-5p, exhibited significantly lower expression levels in transparent samples than in cataractous samples ($P < 0.05$). The miR-933 miRNA also exhibited the greatest fold change in the stem-loop RT-PCR assay (the four miRNAs that exhibited the greatest change are shown in Fig. 4; for the other six miRNAs see Supplementary Material and Supplementary Fig. S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9178/-/DCSupplemental). PCR data for all detected miRNAs exhibited high concordance with the microarray data.

**Prediction of Target Genes**

Candidate target genes for several up regulated or down regulated miRNAs were identified using three commonly used prediction algorithms, TargetScan, miRanda, and PicTar, as described above in the methods section. Each prediction algorithm was established according to the principle that the 5′ region of the miRNA would pair with the 3′ UTR of target mRNAs of genes. The heteroduplexes formed between several miRNAs and their target mRNAs are shown in Figure 5 as examples. The authors found that one miRNA may regulate hundreds of genes based on the results of the prediction programs, and one gene may be regulated by several miRNAs. They then individually screened the entire set of putative target genes using the National Center for Biotechnology Information (NCBI) databases. The analyses showed that several screened genes appeared to correlate with lens development, aging, or disorders (e.g., cataracts), and may be involved in signal pathways for cell growth, apoptosis, cell proliferation, or the regulation of the cell cycle. The authors identified one possible target sequence for miR-933 in brain-derived neurotrophic factor (BDNF), and possible targets sequences for miR-145 in EYA3/4, MEIS1 and E2F3 genes, and for miR-204 in the MEIS1 gene (Fig. 5). The miRNAs and their putative target genes that correlated with lenses are presented in Table 3. The position of the heteroduplex and references detailing these genes’ functions are also shown.

**DISCUSSION**

The miRNAs have recently emerged as a prominent class of gene regulators. Although miRNAs have been identified in

![Figure 1](http://tvst.arvojournals.org/) Relative expression levels of miR-184, let-7b, miR-923, and miR-1826, which were highly expressed in both transparent and cataractous samples. The values (relative expression level) represent the mean ± SD of three independent microarray assays. The miRNAs examined are shown on the x-axis. In cataractous samples, the expression levels of let-7b and miR-923 were significantly higher than in cataractous samples ($P < 0.05$ versus cataractous samples). MiR-184 exhibited elevated expression levels in both samples relative to the levels of the other miRNAs.

![Figure 2](http://tvst.arvojournals.org/) Microarray assays were used to determine the genome-wide miRNA expression in the central epithelium of transparent and cataractous human lenses. The authors performed three separate microarray assays for each group. Colors indicate the expression relative to the mean for each miRNA. The blue color in the transparent samples indicates a lower expression level relative to the level in cataractous samples, and the yellow color indicates a higher expression level in the transparent samples than in the cataractous samples. Fold differences correspond to shading of the color. The expression levels appeared to vary greatly among the individual assays, and, therefore, the FDR-corrected $P$ values ($< 0.05$) for the three assays were used to avoid error.
miR-184 was also indicated that miR-184 was involved in the maintenance of transparency. The authors hypothesize that high expression of miR-184 in lens samples contributes to the maintenance of avascularity and transparency in human lenses. Recently, Hughes et al. reported that a mutation in the seed region of miR-184 is also responsible for severe familial keratoconus combined with early-onset anterior polar cataracts. The authors' research effectively confirmed that miR-184 is essential for the maintenance of transparent lenses.

The fact that miRNA expression profiles differ between the central epithelium of transparent and cataractous lens samples suggests that the miRNAs that are differentially expressed may play roles in lenticular development and cataractogenesis. The authors identified 32 miRNAs with a statistically significant fold change (> 2-fold changes) in expression. The miRNA expression levels of 20 (62.5%) of these 32 miRNAs were greater in transparent lenses than in cataractous lenses, and 12 miRNAs (37.5%) exhibited decreased expression in transparent samples (Fig. 3). These results suggest that the global expression of miRNAs in transparent lenses is increased relative to the expression levels in cataractous lenses. These findings were additionally confirmed by stem-loop RT-PCR. Conte et al. reported that miR-204 controlled lens cell differentiation by modulating the expression of lens placode differentiation genes in early embryogenesis, thereby revealing the function of miRNAs in lenticular development. The authors' data revealed that the expression level of miR-204 in transparent lenses was 2.31 times greater than those in cataractous lenses; this altered expression provides avenues for further research into the function of miRNAs in lenticular senescence.

The prediction of target genes is a key step toward understanding the function of specific miRNAs. Several prediction algorithms have been developed based on the principle that the 5' region of the miRNA pairs with the 3' UTR of targeted miRNAs to produce a marked effect on mRNA translation. Previous research into prediction algorithms revealed many potential miRNA/mRNA binding pairs, which were subsequently confirmed by additional experimental data. In the current study, three different algorithms were used to predict the target genes of miRNAs that exhibited a statistically significant change in expression between transparent and cataractous lenses. The results of each of the algorithms were then pooled together. The target genes that were involved in lens development, aging, or disorders (e.g.,

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Target Gene</th>
<th>Relationship</th>
</tr>
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<tbody>
<tr>
<td>miR-933</td>
<td>BDNF UTR</td>
<td>5' AUAGGAAAAACAGCAGAUU-UCGCGACA...</td>
</tr>
<tr>
<td>miR-933</td>
<td>EYA4 UTR</td>
<td>3' CACACAAUAGGUGUACACAGUAA...</td>
</tr>
<tr>
<td>miR-145</td>
<td>MEIS1 UTR</td>
<td>3' CACACAAUAGGUGUACACAGUAA...</td>
</tr>
<tr>
<td>miR-204</td>
<td>BDNF UTR</td>
<td>3' UCGGUAUCUCAGUACUGUUCCUU</td>
</tr>
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</table>
cataracts) were selected for additional study based on information obtained from the NCBI databases (Table 3). Previously reported data demonstrates that age-related nuclear cataracts may result from changes in a single major gene, and candidate genes have been identified to have altered expression in nuclear cataracts. The authors hypothesize that the genes found to be altered in their screen may become a single major gene because these genes are associated with changes in lens development, lens cell proliferation, differentiation, or apoptosis. For example, PAX6 is considered to be one of the most important genes in lens development and is a single major gene because these genes are associated with changes in lens development, lens cell proliferation, differentiation, or apoptosis. In conclusion, this study was conducted to investigate miRNA expression profiles in human lenses. Of the 206 miRNAs identified with microarray assays, 32 (15.53%) exhibited greater than 2-fold changes in relative expression between the two types of samples. The expression of most miRNAs was found to be similar between transparent and cataractous human lenses. This study is the first to date that different expression profiles of miRNAs in the central epithelium of transparent and age-related cataractous human lenses. The miRNAs that had significantly different expression levels between the two lens types were selected to focus the research on miRNAs which were specifically related to lens diseases. Additionally, special algorithms were adopted to analyze miRNAs and to predict their target genes. The miRNAs and their target genes that were predicted in this study have yet to be studied in vivo or in vitro to confirm the presence of any regulatory relationships.

### References


