MicroRNA expression in human retinal pigment epithelial (ARPE-19) cells: Increased expression of microRNA-9 by N-(4-Hydroxyphenyl)retinamide

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Purpose: MicroRNAs (miRNAs) are important regulators of many cellular functions due to their ability to target mRNAs for degradation or translational inhibition. Previous studies have reported that the expression of microRNA-9 (miR-9) is regulated by retinoic acid and reactive oxygen species (ROS). We have previously shown that N-(4-hydroxyphenyl)retinamide (4HPR), a retinoic acid derivative, induces ROS generation and apoptosis in cultured human retinal pigment epithelial (RPE) cells, known as ARPE-19 cells. The aim of the present study was to investigate the expression of miR-9 in ARPE-19 cells in response to 4HPR treatment, and to identify other miRNAs normally expressed in these cells.

Methods: ARPE-19 cells in culture were treated with 4HPR, the total RNA fractions were isolated, and the expression of various miRNAs and mRNAs was analyzed using real-time PCR. The miRNA expression profile of ARPE-19 cells was analyzed using microarray hybridization.

Results: Treatment of ARPE-19 cells with 4HPR resulted in apoptosis characterized by the increased expression of HMOX1 and GADD153 genes. A twofold increase in the expression of miR-9 was also observed during this response. Potential binding sites for the transcription factors encoded by CEBPA and CEBPB genes were found to be present in the putative promoter regions of all three genes encoding miR-9. 4HPR-induced miR-9 expression was associated with parallel increases in the expression of these transcription factor genes. 5-Aza-2'-deoxycytidine, a methyl transferase inhibitor, also increased the expression of miR-9 in ARPE-19 cells. Microarray hybridization analysis identified let-7b, let-7a, miR-125b, miR-24, miR-320, miR-23b, let-7e, and let-7d as the most abundant miRNAs normally expressed in ARPE-19 cells. These miRNAs are known to regulate cell growth, differentiation or development. The 4HPR treatment increased the expression of miR-16, miR-26b, miR-23a, and miR-15b in ARPE-19 cells, although these increases were modest when compared to the increase in the expression of miR-9.

Conclusions: Our studies demonstrate that miR-9 is expressed in the RPE cell line ARPE-19, and its expression is increased by a retinoic acid derivative and by an inhibitor of promoter hypermethylation. Several miRNAs with inherent ability to regulate cell growth, differentiation and development are also normally expressed in ARPE-19 cells. Thus, miR-9 and other miRNAs could be important in maintaining RPE cell function.

MicroRNA (miRNA) is a class of single-stranded noncoding small (~22 nucleotides) RNA molecules known to regulate gene expression posttranscriptionally [1,2]. The miRNAs, encoded by genes localized to various chromosomes, are initially transcribed as primary transcripts (pri-miRNAs), then converted to pre-miRNAs and subsequently processed to mature miRNAs, which are essential components of the RNA-initiated silencing complex (RISC). An miRNA can function as a posttranscriptional silencer of gene expression either by destabilizing its target transcripts or by inhibiting their translation. A perfect complementarity between the miRNA and its target mRNA often results in the rapid degradation of the latter. A single miRNA can bind to the 3’-untranslated region (3’UTR) of many target gene transcripts and thereby inhibit their translation. The translational repression requires only a partial complementarity between the miRNA and its target miRNAs [2]. Recent studies have identified miR-9 as one such miRNA with an important role in cell growth, differentiation, neurogenesis, immunity and oncogenesis due to its ability to target translational inhibition of many genes, including ONECUT2 (one cut homeobox 2), REST (RE1-silencing transcription factor), TLX(NR2E1, nuclear receptor subfamily 2, group E, member 1), and NFKB1 (nuclear factor of kappa light polypeptide gene enhancer in B-cells) [3-9]. The expression of miR-9 is generally suppressed in cases of cancer due to hypermethylation of the promoter regions of genes encoding it [10-13]. The expression of miR-9 is altered in
brains affected by Alzheimer disease, and BACE1/beta-
secreatase is a target for translational inhibition by this
microRNA [14,15]. Decreased expression of miR-9 is
observed in presenilin-1 null mice [16]. Its expression is also
reported to be regulated by retinoic acid, oxidative stress,
alcohol and pro-inflammatory agents [7,9,17-19].

A normally functioning retinal pigment epithelium (RPE)
is indispensible for vision, and impaired function as a result
of oxidative stress is thought to be a major factor responsible
for the development of retinal degenerative diseases, such as
age-related macular degeneration [20]. ARPE-19, a cell line
derived from human RPE, has been widely used to investigate
the response of RPE to oxidative stress [21-25]. A previous
study from our laboratory has shown that the retinoic acid
derivative N-(4-hydroxyphenyl) retinamide (4HPR,
fenretinide) can generate oxidative stress, increase the
expression of HMOXI (heme oxygenase [decycling] 1) and
GADD153 (DDIT3, DNA-damage-inducible transcript 3)
genes and subsequently induce apoptosis in ARPE-19 cells
[26]. We reasoned that miR-9 expression could be altered
during this process since its expression is reported to be
regulated by both retinoic acid and oxidative stress [9,17,18].
Therefore, we investigated the expression of miR-9 in
ARPE-19 cells in response to 4HPR-induced HMOXI and
GADD153 expression and apoptosis. We then identified other
miRNAs normally expressed in ARPE-19 cells using
microarray analysis and determined if the expression levels of
some of these miRNAs were also affected by 4HPR.

METHODS

Cell culture: The ARPE-19 human RPE cell line at passage
18 was obtained from ATCC (Manassas, VA) and the
experiments were performed using cells from passages 20
to 24. The cells were grown in Dulbecco's modified
Eagle's medium containing nutrient mixture F12, 50/50 mix
(Cellgro, Herndon, VA) supplemented with 5% fetal bovine
serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM
non-essential amino acids and penicillin (100 U/ml) and
streptomycin (100 µg/ml), as described previously [26]. Cells
were seeded onto tissue culture plates at a density of 2×10⁴
cells/ml in complete medium and allowed to grow at 37 °C in
a humidified environment of 5% CO₂ in air to reach about 80%
confluence (1–2 days). The culture medium was then replaced
with fresh serum-free medium containing penicillin (100 U/
ml) and streptomycin (100 µg/ml) before treating the cells
with various agents. N-(4-hydroxyphenyl)retinamide (4HPR)
was purchased from Biomol (Plymouth Meeting, PA) and
dissolved at a concentration of 10 mM in DMSO before being
added to the cell culture medium to a final
collection concentration of 1 µM. This treatment lasted 3 days,
the culture medium being replaced every 24 h with fresh medium
containing the compound. Sodium meta-arsenite and
menadione were obtained from Sigma-Aldrich, their stock
solutions were prepared in water and the cells were treated
with these compounds for 24 h.

Apoptosis ELISA: Detection of apoptosis in ARPE-19 cells
was performed by quantitative sandwich-enzyme-
immunoassay using mouse monoclonal antibodies directed
against DNA and histones (Cell Death Detection Elisa kit;
Roche, Indianapolis, IN), as previously described [26].
ARPE-19 cells were grown on 24-well tissue culture plates,
treated with 4HPR for 24 h, and lysed by incubating with lysis
buffer (250 µl/well) for 30 min at room temperature. The cell
lysates were then centrifuged at 250× g for 10 min, and 10 µl
aliquots of supernatants were removed and analyzed using
ELISA. The absorbance at 405 nm was measured using a
VICTOR² Multilabel Counter (Perkin Elmer, Waltham, MA).

Microarray analysis: Microarray hybridization analysis was
performed using NCode miRNA Expression Profiling
Services (Invitrogen, Carlsbad, CA). Total RNA was isolated
from untreated ARPE-19 cells using TRIZol Reagent, and its
quality analyzed using agarose gel electrophoresis. Small
RNA was enriched from total RNA (20 µg) using the PureLink
miRNA Isolation Kit. The enriched miRNA fraction (2.4 µg)
was Poly(A) tailed, labeled with Alexa Fluor (NCode miRNA
Labeling System) and hybridized to NCode Multi-Species
miRNA Microarrays for 4 h at 62 °C. Two RNA samples were
analyzed using this procedure on different microarrays
containing duplicate spots of each miRNA probe. Hybridization
and washing of the arrays were performed
according to the standard protocol described in NCode
miRNA Labeling System Manual. The arrays were scanned
on a GenePix 400B Microarray Scanner (Molecular Devices,
 Sunnyvale, CA) and images were then analyzed using the
GenePix software.

Quantitative real-time RT–PCR analysis: For quantitative
real-time RT–PCR analysis of various transcripts, 2 µg of total
RNA extracted from ARPE-19 cells with RNasey Protect
Mini Kit (Qiagen, Valencia, CA) was reverse transcribed
using the High Capacity cDNA Archive Kit (Applied
Biosystems, Foster City, CA). After reverse transcription, 5 µl
cDNA was used as a template for quantitative real-time
PCR, which was performed on an Applied Biosystems 7500
Real-Time PCR System using TaqMan Universal PCR Master
Mix and other reagents from Applied Biosystems. The
manufacturer's default thermal cycling conditions were
followed. Each PCR reaction (20 µl) was set up by using
validated TaqMan probes (labeled with reporter dye FAM at
the 5’ end) and primers specific for each gene (GADD153,
HMOXI, CEBPA, CEBPB, and TGM2 with assay
identification numbers Hs00358796_g1, Hs00157965_m1,
HMOX1, CEBPA, CEBPB, and TGM2 with assay
identification numbers Hs00358796_g1, Hs00157965_m1,
Hs00269972_s1, Hs00270923_s1, and Hs00190278_m1, respectively). A human GAPDH (part number: 4352934E) gene was used as the endogenous control. Gene amplification data were analyzed using Applied Biosystems 7500 System Sequence Detection Software version 1.2.3. The results were expressed as n-fold induction in gene expression, calculated using the ΔΔCT method.

For real-time PCR analysis of miRNAs, total RNA fraction containing miRNAs was prepared from ARPE-19 cells using miRNeasy Mini Kit (Qiagen). RT–PCR analysis was performed on an Applied Biosystems 7500 Real-Time PCR System using individual TaqMan MicroRNA Assays (miR-107, miR-125b, miR-15a, miR-16, miR-193b, miR-210, let-7a, let-7c, let-7d, miR-98, miR-9, miR-34a, miR-26b, miR-24, miR-23a, miR-223, miR-15b, miR-128a, miR-204, or miR-224), TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems). RNU48 was used as the endogenous control and the ΔΔCT method was employed to estimate miRNA expression. The expression of selected miRNAs in ARPE-19 cells was verified by real-time PCR employing NCode SYBR Green miRNA qRT–PCR Kit and NCode miRNA First-Strand cDNA Synthesis Kit (Invitrogen). Total RNA samples (1 μg) were first treated with DNase I and then subjected to a polyadenylation reaction before first strand cDNA synthesis with SuperScript III and Universal RT Primer (Invitrogen). Each PCR reaction was performed using a forward primer specific for the tested miRNA and the Universal qPCR Primer. The miRNAs were analyzed and their forward primer sequences were determined as: let-7b, TGA GGT AGT AGG TTG TGT GGT T; miR-125b, TCC CTG AGA CCC TAA CTT GTG A; miR-24, TGG CTC AGT TCA GCA GGA ACA G; miR-210, TGC TGC GTG TGA CAG CGG CTG A; miR-193b, TCC CTC AGT TCA GCA GGA ACA G; miR-23b, ATC ACA TTG CCA GGG ATT ACC; let-7e, TGA GGT AGG AGG TTG TAT AGT; miR-210, CTG TGC GTG TGA CAG CGG CTG A; miR-193B, AAC TGG CCC TCA AAG TCC GCC TTT; miR-423–3P, AGC TCG GTC TGA GGC CCC TCA G. For the analysis of statistical significance, Student’s paired t-test was used. All values have been expressed as mean±SD, n=4; p<0.05 denotes statistically significant differences. The results shown are representative of three different experiments.

Search for potential transcription factor binding sites: DNA sequences (1 kb) upstream of the genes transcribing miR-9 or other miRNAs were examined for the presence of potential transcription factor binding sites. The following Web-based software packages were used, each with default parameters: TESS - Transcription Element Search System [27] and the MatInspector program from Genomatix.
We analyzed the expression of miR-9 in ARPE-19 cells in response to 4HPR treatment. The cells were treated with varying concentrations of 4HPR for 24 h, and the miR-9 expression was measured using real-time RT–PCR. An increase in miR-9 expression was observed with increasing concentration of 4HPR (Figure 1). A ~2 fold increase in miR-9 expression was detected when the concentration of 4HPR reached 10 µM. Dimethyl sulfoxide, the vehicle used in this study to dissolve 4HPR, had no effect on the miR-9 expression by itself. ARPE-19 cells from passages 20 to 24 did not show detectable variation in miR-9 expression or its response to 4HPR treatment (data not shown). The 4HPR treatment induced apoptosis, as expected, as indicated by the generation of mono- and oligonucleosomes in the treated cells. We also analyzed the expression of two genes, HMOX1 (a marker for oxidative stress) and GADD153, since we have previously shown that their expression is highly increased during 4HPR-induced apoptosis of ARPE-19 cells [26]. The 4HPR-dependent increase in miR-9 expression paralleled increases in the expression of HMOX1 and GADD153. Thus, treatment of ARPE-19 cells with 4HPR induces expression of miR-9 along with established markers of oxidative stress and apoptosis.

The expression of miR-9 genes could be suppressed by the hypermethylation of their promoter regions [10]. Treatment of cells with 5-aza-2’-deoxycytidine, a methyl transferase inhibitor, can alleviate this suppression. The expression of miR-9 in ARPE-19 cells increased approximately threefold in response to the treatment (Figure 5). TGM2, a gene known to be regulated by hypermethylation [28], was also induced as expected.

We next used microarray hybridization analysis to obtain a broad view of the microRNA species normally expressed in ARPE-19 cells. Small RNA preparation, which was enriched from total RNA isolated from untreated ARPE-19 cells, was employed for hybridization analysis on a microarray containing duplicate spots of each miRNA probe. The microarray analysis, when repeated using a second RNA sample, yielded similar results. The microarray data are provided at GEO (GSE23107). The relative expression levels of 62 miRNA (out of 366 human miRNAs tested) expressed substantially in these cells are shown in Figure 6. The most abundant miRNAs expressed in ARPE-19 cells were let-7b, let-7a, miR-125b, miR-24, miR-320, miR-23b, let-7e, and let-7d. The microarray analysis failed to detect the miR-9 expression in ARPE-19 cells. This could be due to the limited sensitivity of microarray analysis in comparison to real-time RT–PCR, where the target is detected after multiple cycles of amplification.
Figure 4. 4HPR-induced expression of miR-9 in ARPE-19 cells is associated with increased expression of CEBPA and CEBPB. The cells were treated with 10 μM of 4HPR for 24 h, and the gene expression was analyzed using real time RT–PCR. A: 4HPR increased miR-9 expression. B: 4HPR increased CEBPA expression. C: 4HPR increased CEBPB expression. *p<0.05 compared to control, n=4.

The microarray results were validated by analyzing the expression of selected miRNAs using real-time PCR analysis. Let-7b, miR-125b, miR-24, miR-23b, and let-7e represented the most abundant ones, while miR-210, miR-193b and miR-423 represented the less abundant ones. The amplification and dissociation plots (Figure 7) clearly indicate that all the miRNAs tested are present in the RNA preparations obtained from the ARPE-19 cells. Several miRNAs expressed in ARPE-19 cells are also reported or predicted to be neural retina-specific (Figure 8).

Figure 5. Expression of miR-9 increases in ARPE-19 cells following treatment with 5-aza-2’-deoxycytidine. Cells were treated with 1 μM 5-aza-2’-deoxycytidine for 3 days, and miR-9 expression was estimated using real time RT–PCR. The expression of TGM2, a gene known to be upregulated by this treatment, was also analyzed. A: 5-aza-2’-deoxycytidine increased miR-9 expression. B: 5-aza-2’-deoxycytidine increased TGM2 expression. *p<0.05 compared to control, n=4.
Figure 6. Microarray hybridization analysis of miRNAs normally expressed in ARPE-19 cells. The data shown is representative of hybridization analyses performed using two RNA samples isolated from untreated ARPE-19 cells. The microarrays contained duplicate spots for each miRNA probe. The average fluorescence intensities are shown; the view is filtered by name, which has multiple members selected.
Figure 7. Real-time RT–PCR validation of selected miRNAs. Amplification plot (left panel) and dissociation plot (right panel) for the indicated miRNA are shown.
We analyzed the response of selected miRNAs in ARPE-19 cells to 4HPR treatment using real-time RT–PCR analysis (Figure 9). Most of the miRNAs tested were selected from microarray results shown in Figure 6 to represent both the most abundant as well as the less abundant miRNAs. The miR-9, miR-223, miR-15b, and miR-128a were included to represent the ones not detected by microarray analysis. We also tested miR-204 and miR-224 because they are reported to be expressed in mouse RPE [29,30]. Although all the tested miRNAs, including miR-204 and miR-224, were found to be expressed in ARPE-19 cells, the majority did not respond to 4HPR treatment. Increases in the expression of miR-16, miR-26b, miR-23a, and miR-15b were observed following 4HPR treatment; however, these increases were modest when compared to the approximately twofold increase observed for miR-9. The 5′-flanking regions (~1 kb) of genes generating miR-16, miR-26b, miR-23a, miR-15b, and miR-223, and let-7a were analyzed for the presence of consensus binding sites for CEBP-α and CEBP-β. The genes for miR-16, miR-15b, and miR-223 as well as one of the three genes encoding let-7a were found to contain potential binding sites for these transcription factors (data not shown).

**DISCUSSION**

Although microRNA expression and function in the mammalian eye has been investigated [29,31-37], information about miRNA expression and function in the RPE is limited. An exception is the reported detection of miR-204 and miR-224 in mouse RPE using in situ hybridization [29,30]. Here we demonstrate that miR-9 is expressed in ARPE-19 cells, and that its expression is increased when these cells are exposed to 4HPR. The increase in miR-9 expression by 4HPR was associated with apoptosis, as indicated by mono- and oligonucleosomes formation, and with large increases in HMOX1 and GADD153 expression. The 4HPR-induced apoptosis and increases in GADD153 and HMOX1 expression in ARPE-19 cells are preceded by an increase in reactive oxygen species generation in ARPE-19 cells [26]. Taken together, these facts are consistent with a model of miR-9 activation due to reactive oxygen species generated from 4HPR treatment. Indeed, increase of miR-9 expression following oxidative stress induced by metal sulfates has been reported [18]. However, our results eliminate oxidative stress as a direct regulator of the miR-9 expression in ARPE-19 cells. Menadione and arsenite, agents well known to cause oxidative stress, failed to increase the miR-9 expression in these cells. In contrast, the expression of HMOX1, a marker for oxidative stress, was highly increased in cells treated with these agents, as expected. 4HPR is a derivative of all-trans-retinoic acid, which has been shown to be a regulator of miR-9 expression [17]. All-trans-retinoic acid is also known to regulate gene expression through its effects upon the expression of CEBP transcription factors [38,39]. Our sequence analysis indicated that putative CEBP binding sites are present on potential promoter regions of all three genes encoding miR-9 precursors (miR9–1, miR9–2, and miR9–3). Our observation that the expression of genes encoding CEBP-α and CEBP-β increased in ARPE-19 cells in response to 4HPR treatment suggests that these transcription factors could be involved in the regulation of miR-9 expression. The 4HPR treatment also significantly increased the expression of miR-15b, miR-16, miR-23a, and miR-26b. However, putative binding sites for CEBP-α and CEBP-β were absent in the promoter regions of the genes encoding miR-23a and miR-26b. Thus, factors other than CEBP-α and CEBP-β are indicated in the regulation of expression of these miRNAs by 4HPR.

Expression of miR-9 is also known to be regulated by hypermethylation of the promoter regions of genes encoding this microRNA. This epigenetic repression of miR-9 expression has been reported to be a contributing factor to breast and colorectal cancer development [10-12]. Our results show that the miR-9 expression in ARPE-19 cells is regulated by this mechanism. Treatment of the ARPE-19 cells with 5-aza-2′-deoxycytidine to block DNA methylation resulted in increased expression of both miR-9 and TGM2, a gene known to be regulated by hypermethylation [28]. The potential role of epigenetic regulation of miR-9 or other microRNAs in RPE pathophysiology is not yet known, although it has been implicated in cancer and aging [40,41].

The ability of miR-9 to target many genes for translational repression makes it a potentially effective regulator of cell growth, differentiation, neurogenesis, immunity and cancer. There appears to be a large number of
potential targets for this microRNA; 936 conserved targets of miR-9 are reported in TargetScan release 5.2. However, only a few of them have been verified experimentally. MiR-9 is reported to control the secretory response of insulin-producing cells by diminishing the expression of the ONECUT2 transcription factor, a regulator of Granuphilin expression [3]. The transcription factor REST, a silencer of neuronal gene expression, is also a target of miR-9 whose expression is downregulated in Huntington’s disease [4]. MiR-9 is reported to accelerate neural stem cell differentiation by suppressing the expression of the nuclear receptor TLX [5]. Another target for miR-9 is BACE1/beta-secretase, whose activity is elevated in brains affected by Alzheimer disease [14]. The transcription factor NF-KappaB1 is reported to be targeted by this microRNA during growth of ovarian cancer cells and in monocytes and neutrophils exposed to pro-inflammatory agents [6,7]. Several components of the Fgf signaling pathway are targeted by miR-9 during late embryonic development in zebrafish [8]. Translational repression by miR-9 is also reported for E-cadherin in the SK-Hep-1 hepatoma cell line [42], Foxg1 in developing mouse brains [43] and the BK channel splice variant in rat striatal neurons during alcohol adaptation [19]. ONECUT2 appears to be an interesting target since it has been reported that this transcription factor regulates the expression of MITF, a gene important for RPE physiology [44]. \(\text{NF-KappaB1} \) could also be an important target during the response of RPE to pro-inflammatory agents or oxidative stress.

We demonstrated using microarray analysis that a large number of miRNAs are normally expressed in the RPE cell line, ARPE-19. RT–PCR analysis was employed to verify the expression of several miRNAs. The most abundant miRNAs that were detected in ARPE-19 cells were let-7b, let-7a, miR-125b, miR-24, miR-320, miR-23b, let-7e, and let-7d. The let-7 family of microRNAs are important regulators of differentiation and development, and their deregulation is often associated with cancer [45,46]. MiR-125b is a novel regulator of the tumor suppressor p53 [47] as well as many genes involved in neuronal differentiation [48]. MiR-24 has been shown to regulate several cell cycle genes, the activin type 1 receptor ALK4 and dihydrofolate reductase [49-51]. MiR-320 regulates cardiac ischemia/reperfusion injury and cell proliferation by targeting heat-shock protein 20 and transferrin receptor 1, respectively [52,53]. MiR-23b is reported to regulate cell differentiation by targeting Smad proteins [54]. The potential role of these microRNAs in regulating RPE function remains to be elucidated. It should be noted that microarray analysis did not detect miR-9 expression in ARPE-19 cells. The sensitivity of microarray analysis is highly limited when compared to real-time RT–PCR, the method we first employed to detect it.
Since ARPE-19 cells are a widely employed and useful experimental model, it is sensible to determine how well the population of miRNAs corresponds to what is observed in retinal tissue. Two groups of investigators have studied miRNA expression in mouse retinas, using both array hybridization techniques. Among the 69 miRNAs identified in this study, the 77 reported by Xu et al. [37] and 75 reported by Shen et al. [55], 30 are common to all three sets. The two studies based upon extraction of miRNA from retinal tissue have a total of 40 miRNAs in common. Interestingly, the ARPE-19 cells have a similar degree of commonality with the 2 data sets from retinas, having 43 in common with each.

The hybridization studies above have been complemented by an informatics approach. Working from a collection of retinal genes, Arora et al. [33] produced a list of miRNAs predicted to target the 3′-untranslated regions of their mRNA sequences. The authors then demonstrated the expression of a selection of the miRNAs using PCR. Several of the verified miRNAs are present in all three hybridization data sets (miR-29, miR-107, let-7d, miR-23a, and miR-143); three others (miR-124, miR-106, and miR-143) are present in at least one of the hybridization data sets. MiR-135 and miR-200, although validated, were not found in any of the hybridization data sets. However, this validation was performed using PCR, and like miR-9 in the present study, the expression of miR-135 and miR-200 may be undetectable by hybridization. Only 10 of the miRNAs found collectively in the three hybridization studies were not among those predicted. Interestingly, 5 of these (miR-184, miR-210, miR-31, miR-335, and miR-92) were deemed to be “retina specific” in expression, according to the tissue survey conducted by Xu et al. [37]. Thus, it appears that the predictive value of the informatics approach is fruitful.

In summary, we have shown that miR-9 is expressed in the RPE cell line known as ARPE-19 and that its expression increases during 4HPR-induced apoptosis. The 4HPR-induced miR-9 expression was associated with parallel increases in the expression of CEBPA and CEBPB transcription factor genes. Potential binding sites for these transcription factors were present in the putative promoter regions of all three genes encoding miR-9. The miR-9 expression in ARPE-9 cells was also increased in response to treatment with 5-aza-2′-deoxycytidine, a methyl transferase inhibitor, thus indicating that the gene(s) encoding this microRNA could be epigenetically regulated via hypermethylation of their promoter regions. Microarray analysis showed that a large number of miRNAs are normally expressed in ARPE-19 cells, the most abundant ones being let-7b, let-7a, miR-125b, miR-24, miR-23b, let-7e, and let-7d. The miR-9 and other microRNAs could play an important role in maintaining RPE cell function.

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