

Prediction and Verification of miRNA Expression in Human and Rat Retinas

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PURPOSE. MicroRNAs (miRNAs) play a global role in regulating gene expression and have important tissue-specific functions. Little is known about their role in the retina. The purpose of this study was to establish the retinal expression of those miRNAs predicted to target genes involved in vision.

METHODS. miRNAs potentially targeting important “retinal” genes, as defined by expression pattern and implication in disease, were predicted using a published algorithm (TargetScan; Envisioneering Medical Technologies, St. Louis, MO). The presence of candidate miRNAs in human and rat retinal RNA was assessed by RT-PCR. cDNA levels for each miRNA were determined by quantitative PCR. The ability to discriminate between miRNAs varying by a single nucleotide was assessed. The activity of miR-124 and miR-29 against predicted target sites in *Rdb10* and *Impdb1* was tested by cotransfection of miRNA mimics and luciferase reporter plasmids.

RESULTS. Sixty-seven miRNAs were predicted to target one or more of the 320 retinal genes listed herein. All 11 candidate miRNAs tested were expressed in the retina, including miR-7, miR-124, miR135a, and miR135b. Relative levels of individual miRNAs were similar between rats and humans. The *Rdb10* 3'UTR, which contains a predicted miR-124 target site, mediated the inhibition of luciferase activity by miR-124 mimics in cell culture.

CONCLUSIONS. Many miRNAs likely to regulate genes important for retinal function are present in the retina. Conservation of miRNA retinal expression patterns from rats to humans supports evidence from other tissues that disruption of miRNAs is a likely cause of a range of visual abnormalities. (*Invest Ophthalmol Vis Sci.* 2007;48:3962-3967) DOI:10.1167/iovs.06-1221

MicroRNAs (miRNAs) are small (approximately 22 nt) non-coding RNAs that negatively regulate gene expression. Biogenesis of miRNAs¹ involves nuclear cleavage of pre-miRNAs by the Drosha RNase III endonuclease to release 60 to 70 nt stem loop pre-miRNAs² that are transported to the cytoplasm, where they are cleaved by another RNase III endonuclease, Dicer, to generate mature miRNAs.^{3,4} These miRNAs repress the translation of proteins by binding to the partially complementary 3' UTR of target mature mRNA.^{5,6} They can also influence the transcript levels of a large number of

mRNAs.^{7,8} The perfectly complementary, 2- to 8-bp “seed” region, located at the 5' end of the mature miRNA, is known to play a role in target selection.⁶ More than 2000 miRNA sequences have been described and assigned numerical identifiers. Orthologs have the same number, whereas paralogous miRNAs that differ at only one or two positions carry lettered suffixes.⁹

miRNAs are highly conserved and have been shown to have diverse temporal and tissue-specific expression patterns in a wide range of species.¹⁰⁻¹⁵ This suggests that they may regulate a large number of developmental and physiological processes. Indeed, loss of the miRNA-producing enzyme Dicer1 is lethal in mice and zebrafish.^{16,17} Since the discovery of their role in the development of *Caenorhabditis elegans*,^{18,19} miRNAs have been specifically implicated in the regulation of cell proliferation,²⁰ hematopoiesis,²¹ neural differentiation,²² and other functions.

In the eye, miRNAs have been implicated in *Drosophila* photoreceptor²³ and murine lens²⁴ differentiation. Little has been done to characterize the role of miRNAs in mammalian retina, though mir-9, -23b, -26a, -29, -30c, -124a, -125b, -181, -182, -183, -184, -204, and -205 have recently been demonstrated specifically in this tissue.^{25,26} Based on evidence from other tissues, it is probable that miRNAs are important for both development and maintenance of the retina. Mutations in miRNA genes or their target RNAs may potentially contribute to a range of retinal abnormalities. Indeed, SNPs that modify miRNA binding sites have been shown to alter phenotype²⁷ or to cause disease²⁸ in other systems.

Given that the 5' region of the miRNA is an important determinant of binding to target mRNA,⁶ the targets of miRNAs have been computationally predicted through analysis of sequence complementarity and the conservation of miRNA binding sites in the 3' UTR. Free energies of miRNA-mRNA binding duplexes have been used to verify these target sites.²⁹ As a first step in elucidating their role in the retina, we aimed to catalog the miRNAs predicted to target mRNAs important for retinal function and to confirm their presence experimentally.

Expression levels of miRNAs vary greatly.^{12,30,31} Less abundant miRNAs can be difficult to detect by cloning or by Northern or microarray hybridization. However, several methods have been described that make use of PCR amplification to enable accurate and sensitive detection of miRNAs or their precursors.³²⁻³⁴ In this study, we used a modified version of the real-time PCR approach described by Shi and Chiang³³ to detect miRNAs in the human and rat retina.

METHODS

Definition of Retinal Genes of Interest

To define the set of miRNAs involved specifically in retinal function, potential target genes were categorized based on known function, enriched expression in the retina, or association with inherited retinal disease. Lists of genes involved in phototransduction/vitamin A cycle and retinal diseases were downloaded from the RetinaCentral³⁵ and the Retinal Information Network (RetNet; Daiger SP, et al. *IOVS* 1998; 39:ARVO Abstract 1352) databases. EST databases indicated the level of expression of a gene in the tissue of origin. The online method, digital

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TABLE 1. miRNAs with Target Sequences in the 3' UTRs of Retinal Genes

let-7/miR-98*	miR-138	miR-18	miR-204/211	miR-26†
miR-1/206*	miR-139	miR-181†	miR-205	miR-27
miR-10	miR-140	miR-182†	miR-21	miR-27
miR-101	miR-141	miR-183†	miR-216	miR-29*†
miR-103/107*	miR-142-3p	miR-19	miR-217	miR-30†
miR-122	miR-143*	miR-190	miR-218	miR-33
miR-124*†	miR-144	miR-192/215	miR-219	miR-34/449
miR-125/351†	miR-145	miR-193	miR-22	miR-369
miR-126	miR-146	miR-194	miR-221/222	miR-375
miR-128	miR-148/152	miR-196	miR-223	miR-7*†
miR-130/301	miR-15/16/195	miR-199a*	miR-23*†	miR-9†
miR-133	miR-153	miR-200b*	miR-24	miR-93/302/372/373
miR-135*	miR-155	miR-203	miR-25/32/92/367	miR-96
miR-137	miR-17/20/106			

* Presence in the retina experimentally validated by RT-PCR.

† Previously reported in the retina.

differential display (DDD),³⁶ was used to access human dbEST libraries,³⁷ and the fractions of sequences corresponding to a particular Unigene cluster within pools of nonnormalized retinal and RPE libraries were compared with five other pools of tissue-specific libraries (Supplementary Table S1; supplementary tables are available online at <http://www.iovs.org/cgi/content/full/48/9/3962/DC1>). From these data, we defined categories of genes that were either specific to (not present in other libraries) or highly expressed in (top 10%) the retina or the RPE.

miRNA Target Prediction

The miRNAs that target the retinal genes in the above categories were predicted by the presence of conserved sites that match the miRNA seed region (TargetScan).^{38,39} Target sites for 67 different miRNAs were predicted (Table 1) from a nonredundant list of 320 genes (Supplementary Table S2).

RNA Extraction, Polyadenylation, and Reverse Transcription

Total RNA from human retina was purchased from BD Biosciences (Mountain View, CA) and total RNA was extracted from rat retina (two retinas pooled from two rats) using an extraction kit (mirVana; Ambion, Foster City, CA) according to the manufacturer's protocol (miRNA detection was not improved by use of a protocol selective for small RNAs). All animal procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. miRNAs are

short sequences (approximately 22 nt), and it is not possible to quantify them using conventional RT-PCR protocols. Therefore, we used a modified version of the method described by Shi and Chiang³³ in which mature miRNAs are polyadenylated and target sequences for a reverse primer are subsequently incorporated into cDNA by use of an oligo dT adapter.

One microgram of RNA was polyadenylated using poly(A) polymerase (PAP; Ambion, Foster City, CA) at 37°C for 1 hour in a 25- μ L reaction mixture. The RNAs were then reverse transcribed with 200 U reverse transcriptase (SuperScript III; Invitrogen, Paisley, UK) and 0.5 μ g poly (T) adapter (3' rapid amplification of complementary DNA ends [RACE] adapter in the FirstChoice RLM-RACE kit; Ambion).

Polymerase Chain Reaction

Primers for specific miRNAs were based on miRNA sequences obtained from miRBase⁹ and were the same for humans and mice (Table 2). The reverse primer was the 3' adapter primer (3' RACE outer primer in the FirstChoice RLM-RACE kit; Ambion). PCR annealing temperatures were based on a predicted melting temperature (T_m) of 55°C. In the case of paralogous genes *miR-135a* and *miR-135b*, which vary by a single nucleotide primer, T_m was determined experimentally through melt-curve analyses with antisense oligonucleotides³³ (LightCycler 1.2; Roche, Basel, Switzerland). Each set of primer and antisense oligo (10 pmol) was mixed with 10 μ L master mix (SYBR Green Quantitect; Qiagen, Crawley, UK) in a total volume of 20 μ L. After 20-second denaturation at 95°C and 60-second annealing at 34°C, the temperature

TABLE 2. Primer Sequences

Name	5'–3' Nucleotide Sequence
let-7d	AGAGGTAGTAGGTTGCATAGT
miR-107	AGCAGCATTGTACAGGGCTATCA
miR-124	TAAGGCACGCGGTGAATGCC
miR-135a	TATGGCTTTTATTCCTATGTG
miR-135b	TATGGCTTTTCATTCCTATGTG
miR-143	TGAGATGAAGCACTGTAGCTCA
miR-200b	TAATACTGCCTGGTAATGATGAC
miR-206	TGGAATGTAAGGAAGTGTGTGG
miR-23a	ATCACATTGCCAGGGATTTCC
miR-29	TAGCACCATCTGAAATCGGTT
miR-7	TGGAAGACTAGTGATTTTGT
Poly(T) adapter	GCGAGCACAGAATTAATACGACTACTATAGGTTTTTTTTTTTTTTCG
Reverse RACE	GCGAGCACAGAATTAATACGAC
miR-135a antisense	CACATAGGAATAAAAAGCCATA
miR-135b antisense	CACATAGGAATGAAAAGCCATA
RDH10 forward	CATTAGTCTAGATGACATTTGTGATCCACCTGCC
RDH10 reverse	CAGGATTCTAGAAAACAGCCTTAAACATTGGCCACT
IMPDH1 forward	CATTAGTCTAGATTTCCAGGCAGAGAGGAGGG
IMPDH1 reverse	CAGGATTCTAGAGGAGGCGTTGAGACCTGCTT

was increased to 95°C at a rate of 0.05°C/s to produce a dissociation curve. The T_m for each 135a/b mismatched primer/antisense pair was also determined, and annealing temperatures for specific amplification were chosen accordingly. Conventional PCR was performed for 45 cycles using a PCR machine (ABI 2720; Applied Biosystems, Foster City, CA) with denaturing at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. Quantitative PCR was performed using a thermal cycler platform (LightCycler; Roche, Basel, Switzerland) with fluorescence detection (SYBR Green; Quantitect, Qiagen) at the same temperatures but with shorter steps (denaturation 15 seconds, annealing 15 seconds, elongation 10 seconds). PCR products were analyzed by polyacrylamide gel electrophoresis (20%; Invitrogen) to confirm the predicted size (approximately 60 bp, including mature miRNA and adapter sequences). Concentrations of validated products were determined with a fluorescent nucleic acid stain (PicoGreen; Invitrogen), and appropriate dilution series were prepared as standards for quantitative PCR.

Cloning

Sections of the 3'UTRs of *Impdb1* and *Rdb10* containing predicted miRNA binding sites for miR-29 and miR-124, respectively, were amplified by PCR using primers incorporating *Xba*I restriction enzyme sites (Table 2). After appropriate digestion and purification (QIAquick Gel Extraction and PCR Purification Kit; Qiagen), the products were cloned into the *Xba*I site of pGL3 (Promega, Southampton, UK) to generate pGL3-RDH and pGL3-IMPDH1. Plasmids were purified (Plasmid Purification Mini/Midi Kits; Qiagen), and their identities were confirmed by DNA sequencing.

Cell Culture, Transfection, and Luciferase Assays

HEK293 cells were cultured in Dulbecco modified Eagle medium (DMEM) low-glucose medium with 10% fetal bovine serum. Cells (6×10^5) were plated in each well of a 48-well plate (Nunc, Rochester, NY). After 24 hours, cells were transfected with 100 ng pGL3/pGL3-RDH/pGL3-IMPDH1, 40 ng pRL-TK, and 0.2 nM to 200 nM miRNA mimic (miR-124, miR-29 or miR-Control; Dharmacon, Lafayette, CO) using transfection reagent (Dharmafect Duo; Dharmacon). Luciferase activity was assayed after 48 hours (Dual-Luciferase Reporter assay system; Promega).

DNA Sequencing

miRNA PCR products were purified with a clean-up kit (ExoSAP-IT; GE Healthcare, Chalfont St. Giles, UK) and sequencing reactions, primed with the miRNA-specific primer and the reverse RACE primers (Table 2), performed using a terminator kit (ABI BigDye version 1.1; Applied Biosystems), and analyzed by capillary electrophoresis (ABI 3100 Genetic Analyser; Applied Biosystems).

RESULTS

Predicted Retinal miRNAs

A nonredundant list of retinal genes (Supplementary Table S2) was generated from the separate lists based on function and

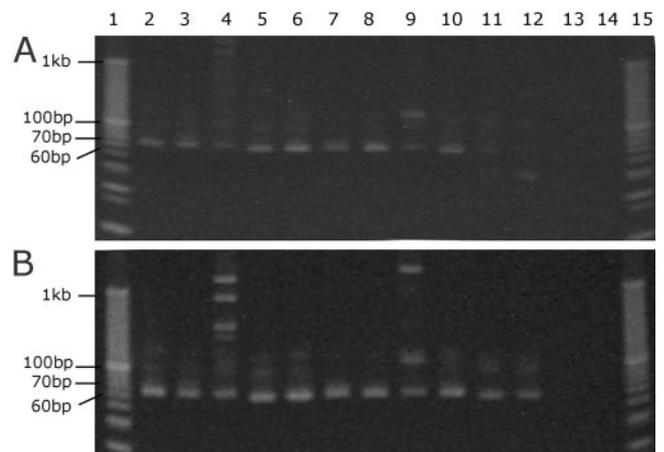


FIGURE 1. miRNA expression in the human (A) and rat (B) retina, detected by RT-PCR. Twenty percent polyacrylamide gel shows specific PCR products of the predicted sizes for miR-124, miR-7, miR-206, miR-143, miR-107, miR-23a, Let-7d, miR-200b, miR-29, miR-135a, and miR-135b (lanes 2-12, respectively). No product for any primer pair was detected in negative controls to which no reverse transcriptase was added (no RT controls). The no RT controls for miR-143 and miR-107 are shown in lanes 13 and 14, respectively. 10-bp marker (lanes 1 and 15).

expression, as described in Materials and Methods. Sixty-seven miRNAs were predicted to have target sites on one or more of the retinal genes (Table 1). Our aim was to demonstrate the expression of miRNAs in the retina. Therefore, we prioritized for experimental validation those miRNAs that potentially targeted highly expressed, retinal-enriched genes with known visual function and involvement in disease. For practical reasons, members of large miRNA families with similar sequences were avoided, and those with sequences conserved between rat and human were preferred. The miRNAs chosen for investigation were miR-23a, miR-29, miR-107, miR-124, miR-135a, miR-135b, miR-143, miR-200b, miR-206, and Let-7d. miR-7 was also investigated because of its known role in *Drosophila* photoreceptor development.²³ The predicted potential target genes of these miRNAs are listed in Supplementary Table S3.

All miRNAs tested were detected by conventional PCR (Fig. 1). All products were the predicted size, and their identities were confirmed by DNA sequencing. To provide an indication of the relative expression levels of different miRNAs, standards of known copy number were prepared from the PCR products. Relative copy numbers of the miRNAs in an aliquot of cDNA prepared from 1 μ g retinal RNA were determined by quantitative PCR (Fig. 2). Relative levels of expression followed a similar pattern in humans and rats, with let-7d the most highly expressed; miR-124 was considerably more highly expressed in rats.

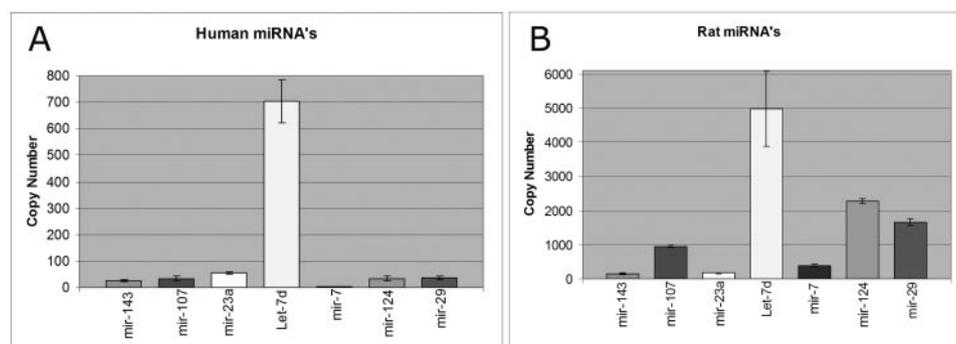
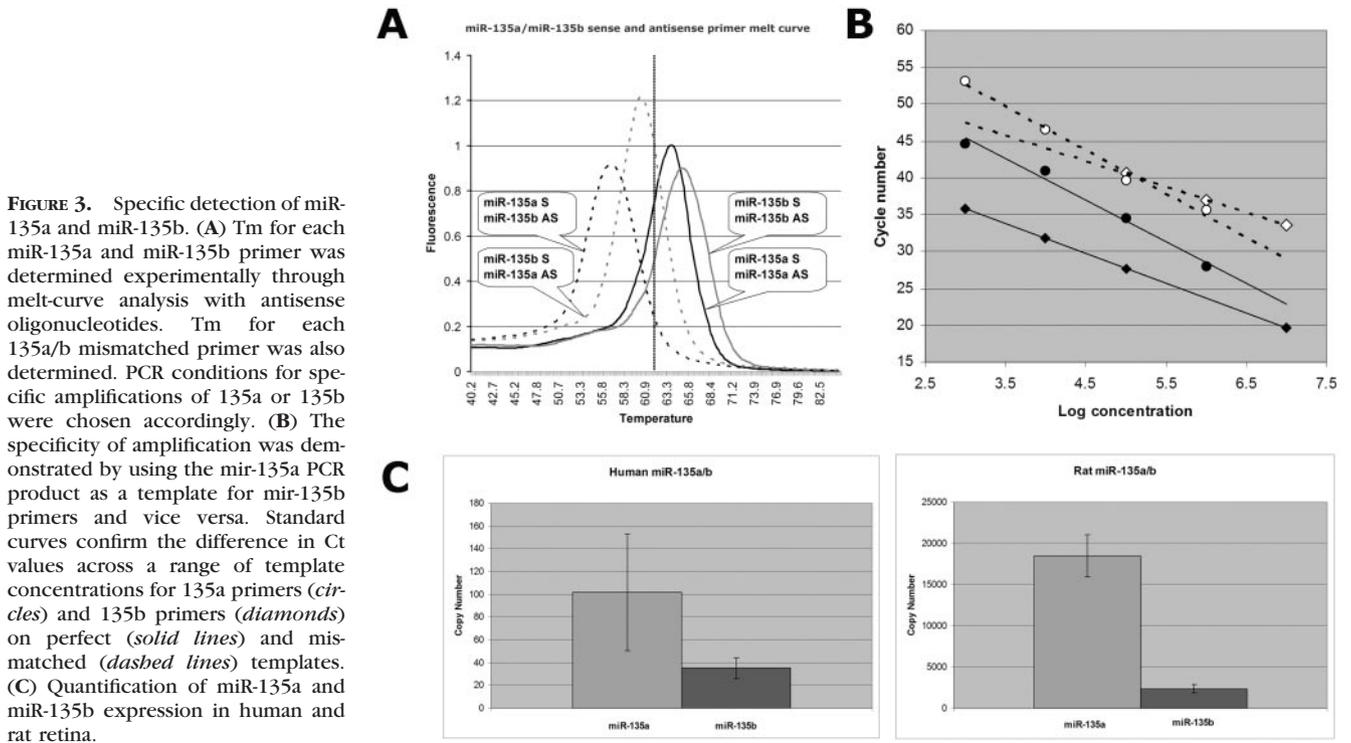


FIGURE 2. Relative quantification of miRNA expression. An indication of the expression of the selected miRNAs (x-axis) in human (A) and rat (B) retina was obtained by estimating cDNA copy number through the use of PCR standards. Let-7d was the most highly expressed miRNA in humans and rats.



Many miRNAs form families and may vary by as little as a single base pair.⁹ This provides a challenge for the detection of specific members because the short length of the miRNA necessitates that the primer span the whole sequence.^{33,34} It is, therefore, not possible to amplify both variants and to detect the differences within the amplicon. We used the example of miR-135a and miR-135b to demonstrate that such isoforms can be discriminated. The strategy we used was to determine the optimum annealing temperature that distinguishes between perfect and mismatched primers. To this end, we determined the real T_m for each 135a and 135b primer with perfect and mismatched antisense oligonucleotides by performing melt-curve analysis³³ (Fig. 3A). From these data, a T_m of 61°C was chosen (63°C did not improve discrimination between templates). The specificity of amplification of 135a and 135b primers was demonstrated by generating standard curves with either 135a or 135b PCR product as template (Fig 3B). The threshold cycle (Ct) for 135a amplification of perfect template (135a PCR product) was approximately seven cycles earlier than that for mismatched template (135b PCR product) across a range of starting concentrations. Similarly, for 135b primers, the Ct for perfectly matched templates was approximately 13 cycles earlier than that for the mismatched 135a template. Relative concentrations of miR-135a and 135b in retinal cDNA, assuming only correct priming occurs, are shown in Figure 3C. These maximum estimates are, in fact, close to the true values because the effect of mispriming is negligible given the large difference in Ct values between perfect and mismatched templates.

miRNA Activity on Selected Target Genes

Two candidates with known relevance to retinal disease, *Rdb10* and *Impdh1*, were chosen to validate the identity of predicted target genes. Their 3'UTR sequences, which contain predicted target sites for miR-124 and miR-29, respectively, were cloned into the 3'UTR of the luciferase gene in plasmid pGL3 (Promega). Transfection of miR-124 mimics (Dharmacon), together with pGL3-RDH, reduced luciferase activity, and

the effect increased as the concentration of miR-124 was raised (Fig. 4). In contrast, a negative control miRNA mimic with no predicted targets (Dharmacon) did not reduce pGL3-RDH luciferase activity. Maximal inhibition was achieved with 20 nM miR-124, and a similar effect, $0.68 (\pm 0.11 \text{ SD})$ of control levels, was replicated in an independent experiment. Transfection of miR-29 mimics (0.2–200 nM) did not cause a reduction in luciferase activity expressed by pGL3-IMPDH (data not shown).

DISCUSSION

The RT-PCR approach was specific for mature miRNAs except for miR-200b and miR-206, for which larger amplification prod-

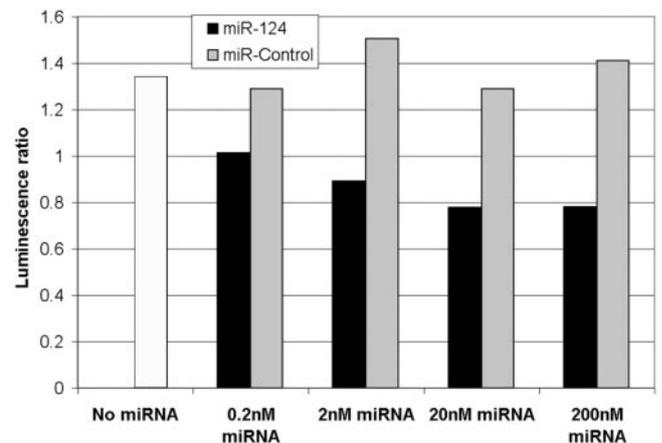


FIGURE 4. Assay of RDH10 reporter plasmid luciferase activity (representative of multiple independent experiments). Addition of miR-124 mimics, but not miR control, caused inhibition of luciferase activity (ratio of firefly/*Renilla* luciferase activities). The extent of inhibition increased as the concentration of miR-124 was elevated.

ucts were observed (Fig. 1). However, purification and sequencing of the bands from miR-206 showed that they were not derived from precursors. Contamination with nonspecific products cannot be alleviated by use of alternative primers. Therefore, it must be accepted that the concentrations of mature miRNAs may in these cases be underestimated because of competition during PCR amplification. Copy numbers calculated for the different miRNAs, though not accounting for variable RT efficiency, do provide an estimate of relative abundance. Direct comparison between humans and rats is not possible because of the different RNA extraction protocols, which likely account for the lower absolute human levels, but the relative trends are remarkably similar. This is consistent with the high evolutionary conservation of miRNA sequences and may reflect a conserved functional role within the mammalian retina.

Detection of different members of miRNA families with similar sequences presents a challenge. Selective PCR amplification for detection of different alleles is usually achieved by designing a primer such that the primer will match/mismatch one of the alleles at the 3' end of the primer.^{40,41} This was not possible in our study because the primer had to span the entire mature miRNA sequence. However, we have demonstrated that by careful choice of annealing temperature, guided by experimental determination of melting temperatures of perfectly and mismatched primer-template duplexes, it is possible, at least in some cases, to discriminate between miRNAs differing by a single internal nucleotide. Target sequences of miR-135a/miR-135b are present in the rhodopsin 3' UTR, and we have demonstrated that both these miRNAs are present in the retina, with 135a at higher levels, particularly in the rat. The rhodopsin gene is extremely highly expressed and critical to retinal function; hence, any potential regulatory mechanism is of great interest.

Limited data from the retina^{25,26} and from other tissues suggested that a range of miRNAs would be expressed in the retina, and we have now demonstrated that this is the case. A reasonable estimate based, for example, on studies in the inner ear⁴² is that one third of all miRNAs might be expressed in the retina. We have focused on miRNAs predicted to be involved in retinal-specific functions, and it is likely that many more are involved in housekeeping functions. Although even miRNAs expressed at a very low level could potentially be detected by the PCR approach we used, the fact that all 11 of those assayed were present increases confidence that many of the predictions based on potential retinal target genes may be correct. Indeed, all nine of the most highly expressed miRNAs in retina reported by Ryan et al.²⁵ were among those predicted to target retinal genes. Hence, most of the miRNAs predicted to target retinal genes are expressed in the retina.

The presence of miR-29 and miR-124 in the retina is consistent with previous reports in the eye.^{26,31} Investigation of just one potential target gene for each of these miRNAs confirmed that the 3' UTR of *Rdb10* mediates miR-124 inhibitory activity in a cell culture system. This adds weight to the argument that miRNAs in the retina do regulate those target genes with which they are coexpressed. The lack of activity observed for miR-29 against *Impdb1* in cell culture may reflect limitations of this experimental system, such as the cell type or plasmid/miRNA copy number, or may reflect that not all target sites predicted by sequence are physiologically active.

The observed retinal miRNA expression and demonstration of miR-124 activity with *Rdb10* suggest that miRNAs are likely to regulate at least some of the predicted target genes in vivo. What are the possible functional roles for miRNAs in regulation of retinal gene expression? miRNAs have been shown to switch gene expression during development through reciprocal negative feedback loops.²³ Several predicted targets of miR-124 are

developmental genes and may act in a similar fashion during retinal development. More generally, miRNAs are thought to be involved in fine-tuning of gene expression. Specifically, a single miRNA may target multiple genes involved in a particular process; modulation of this miRNA could regulate this process. For example miR-29 is predicted to target several extracellular matrix proteins.

Disruption of a mature miRNA seed sequence or mutations affecting its expression would alter regulation of all its target genes. For example, the disruption of mir124, which is neural specific, would likely be detrimental for the brain and retina. Mutations within a target sequence could remove a gene from regulation by the cognate miRNA, leading to overexpression. Alterations in gene expression levels can have major effects on phenotype.^{43,44} For example, the miR-135 site in *Rbo* or the mir-124 or miR-107 sites in *Rdb10* may lead to retinal degeneration. Hence, greater attention should be given to miRNA genes and potential miRNA target sites within the 3' UTRs of retinal-expressed genes, which are located within disease loci.

We used high relative expression in the retina as one approach for assessing retinal-specific function. One might expect to detect miRNAs that target genes repressed in the retina.⁸ The possible functional implications of the observed expression of miRNAs predicted to target retinal genes have been discussed. Other explanations which could contribute to this observation are that these genes may not be true targets in vivo and that the cellular distribution of miRNA expression within the retina may vary from that of the cognate retinal genes. Alternatively, these miRNAs may be involved in the regulation of circadian patterns of gene expression.

Now that the presence of multiple miRNAs within the retina has been confirmed, it will be important to determine their cellular distribution and temporal expression. Not only will this hint at possible functions, it may provide candidates for involvement in disease. For example, miRNAs whose expression patterns are restricted to photoreceptors may be implicated in retinal degenerative disease.

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