MicroRNA Profile of the Developing Mouse Retina

Laszlo Hackler, Jr,[1,2] *Jun Wan,*[1,2] *Anand Swaroop,*[3,4,5] *Jiang Qian,*[*,1] *and Donald J. Zack*[*,1,6,7,8]

Purpose. MicroRNAs (miRNAs) are short, noncoding transcripts that negatively regulate gene expression. They are implicated in diverse cellular processes. The purpose of this study was to obtain a global expression profile of miRNAs in the developing retina and identify differences in miRNA expression between adult rod and cone photoreceptors.

Methods. Locked nucleic acid (LNA) microarrays were used to investigate the miRNA transcriptome of the developing mouse retina and brain. Real-time PCR was used to validate the array findings. Laser capture microdissection was used to determine the miRNA spatial pattern of expression.

Results. One hundred thirty-eight miRNAs were expressed at at least one of the investigated time points. Several miRNAs showed significant changes in expression between embryonic day 15 and adult age in both retina and brain. Cluster analysis identified subgroups of miRNAs showing defined expression profiles. Globally, correlation of expression was higher, with increasing sequence similarity of the mature miRNAs. The miRNAs with identical seed sequences exhibited highly correlated expression profiles. The co-expression of selected host gene and intronic miRNA pairs was confirmed in adult retina. In some cases, expression profiles of miRNAs showed weak correlation with those of their host transcripts, suggesting posttranscriptional regulation of miRNAs during development. In addition, the miRNA transcriptome of rod- and cone-dominant retinas showed only minor differences, and no miRNAs specific for either cell-type were identified.

Conclusions. Global expression profiling revealed dozens of miRNAs with significant expression changes in the developing retina. Precise patterns of expression of miRNAs suggest their specific roles in development. (*Invest Ophthalmol Vis Sci.* 2010;51:1823–1831) DOI:10.1167/iovs.09-4657

From the Departments of [1]Ophthalmology, [6]Neuroscience, [7]Molecular Biology and Genetics, and [8]McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland; the [3]Neurobiology-Neurodegeneration and Repair Laboratory (N-NRL), National Eye Institute, National Institutes of Health, Bethesda, Maryland; and the Departments of [4]Ophthalmology and [5]Human Genetics, University of Michigan, Ann Arbor, Michigan.

[2]Contributed equally to the work and therefore should be considered equivalent authors.

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*Each of the following is a corresponding author: Donald J. Zack, Johns Hopkins University, Smith Building 400 N. Broadway, Baltimore, MD 21287; dzack@jhmi.edu.

Jiang Qian, Johns Hopkins University, Smith Building 400 N. Broadway, Baltimore, MD 21287; jqian2@jhmi.edu.

MicroRNAs (miRNAs) are small, highly conserved, noncoding molecules of 18 to 24 nucleotides that regulate gene expression in a wide variety of tissues and cell types.[1,2] The human and mouse genomes have been reported to have 885 and 689 miRNAs, respectively (miRBASE Release 13.0, March 2009; www.mirbase.org/ hosted in the public domain by the Faculty of Life Sciences, University of Manchester, Manchester, UK). miRNAs are encoded in either the introns of protein-coding genes or between genes as intergenic miRNAs. Bioinformatics studies have predicted that individual miRNAs can target hundreds of distinct mRNAs. It has been estimated that miRNAs may modulate the expression of 20% or more of the human genome.[3]

miRNAs are involved in a diverse set of cellular processes, ranging from proliferation, apoptosis, and malignant transformation[4] to neuronal development and fate specification.[5–7] Several groups have begun exploring the expression and function of miRNAs within the brain and retina. As one example, conditional inactivation of *Dicer* in Purkinje cells leads to a loss of miRNA expression and progressive cerebellar degeneration.[8] Loss of *Dicer* in the retina, identified by using the floxed *Dicer* allele and a *Chx10*-driven *Cre*, leads initially to the formation of photoreceptor rosettes followed by progressive functional and structural degeneration.[9] *Dicer* inactivation by morpholinos in *Xenopus* resulted in a more severe phenotype, with small retinas and associated lamination defects.[10] A more recent study has identified *miR-24a* as a regulator of apoptosis during *Xenopus* eye development.[10]

To gain a more complete understanding, several groups have begun to investigate global patterns of miRNA expression in the retina, particularly in the adult, and have compared these patterns to those in other tissues, identifying a number of miRNAs enriched in retina.[11,12] Other studies have focused on detailed analysis of the spatial and temporal expression patterns of a few selected miRNAs within the retina.[13]

Our goal in this report was to expand on this work by using microarray analysis to comprehensively characterize the retinal miRNA transcriptome during embryonic and postnatal development. In addition, we wanted to explore potential differences between the rod and cone miRNA transcriptomes. Therefore, we generated miRNA profiles of the developing retinas of wild-type (WT, C57BL/6) and $Nrl^{-/-}$ mice. *Nrl* is a basic motif-leucine zipper transcription factor that when knocked out leads to a retina in which essentially all photoreceptors are S-cones.[14–16] In addition, we have compared these retinal expression patterns to miRNA profiles derived from embryonic day 15 and adult brain.

Methods

RNA Isolation

Retinas from E15, E18, P1, P5, and P12 littermate embryos or pups were pooled and total RNA was purified (TRIzol; Invitrogen, Carlsbad, CA). The animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Two

independent biological replicates were generated for each time point. In the case of adult retina, three independent pools of retinas from the left and right eyes of individual 3-month-old mice were used to isolate RNA. The RNA quality was assessed with a Bioanalyzer (Agilent, Santa Clara, CA).

Laser Capture Microdissection

Enucleated eyes from three adult (3-month-old) C57BL/6 mice were flash frozen in embedding medium. Sections 7 μm thick were placed on slides (PEN-membrane; Leica, Wetzlar, Germany), dehydrated, and stained with Mayer's hematoxylin solution for visualization of cell nuclei. The sections were microdissected by using the laser capture method (LMD6000; Leica). The cells were collected from the ganglion cell layer (GCL), inner nuclear layer (INL), and the outer nuclear layer (ONL), and the RNA was purified (TRIzol; Invitrogen).

Microarray Production

The obtained miRNA probe (miRCURY LNA; Exiqon, Vedbaek, Denmark) was printed in quadruplicate on epoxy-coated slides (Corning, Lowell, MA) with an arrayer (MicroGrid II; Digilab Inc., Holliston, MA).

Labeling and Hybridization

One microgram total RNA was labeled (miRCURY LNA; Exiqon), using Hy5 fluorescent dye. Each sample was co-hybridized with a Hy3-labeled reference sample. For WT and *Nrl*$^{-/-}$ samples, hybridizations were performed in triplicate and duplicate, respectively. The arrays were washed and scanned (Scanarray ExpressHT; Perkin Elmer, Waltham, MA).

Quantitative Real-Time PCR

For cDNA synthesis, an miRNA first-strand cDNA synthesis kits (NCode; Invitrogen or miRCURY LNA; Exiqon) were used. Amplification and melting curve analysis were performed with standard procedures (IQ5; BioRad, Hercules, CA; see Supplementary Methods, http://www.iovs.org/cgi/content/full/51/4/1823/DC1). Primers sequences are listed in Supplementary Tables S1 and S2, http://www.iovs.org/cgi/content/full/51/4/1823/DC1.

Results

miRNA Profiling of C57BL/6 Mouse Retina and Brain

Previous studies identified miRNAs enriched in the adult retina and then determined the expression profiles of these specific miRNAs during development.[11,12] We took a different approach, performing global microarray analysis at a variety of developmental time points (E5, E18, P1, P5, P12, and adult), and as a result our miRNA profiling is not biased to a certain developmental stage. Of the 408 known mouse miRNAs (miRBASE Release 10.0) investigated in our study, 138 were expressed in the retina at some point in the studied timeframe, whereas 113 were detected in the brain (Supplementary Tables S3 and S4). (See Supplementary Methods for our criteria for determining expression.) In the retina, 50 miRNAs showed at least a fourfold change in expression, and 23 showed at least an eightfold change within the studied period (Supplementary Table S3). *miR-29b* showed the highest increase in expression during development in both retina and brain, 100- and 500-fold, respectively, whereas *miR-18a* exhibited the greatest decrease in expression, 100- and 40-fold in retina and brain, respectively.

miRNAs with differential expression patterns during development were selected for qPCR analysis. Expression profiles determined by the array analysis were successfully confirmed for 20 of the 23 miRNAs tested (Supplementary Table S1).

Hierarchical cluster analysis was performed on the microarray data to compare expression profiles of all miRNAs detected in the retina and/or brain (149 miRNAs; Fig. 1). The analysis catalogs miRNAs with related expression profiles and creates a dendrogram of all miRNAs. Two distinct groups of miRNAs were identifiable: brain-enriched and retina-enriched. The average expression values of brain- or retina-enriched genes were plotted on the side, showing a clear trend of preferential expression in brain or retina, respectively. Generally, miRNAs followed a similar decreasing or increasing expression profile both in retina and brain, with the exception of *miR-551b*. Its expression was higher at the adult stage in the brain, whereas in the retina it was enriched at E15 and E18.

Although we did identify miRNAs that were expressed preferentially in the developing retina compared with the adult retina, none of these embryonic stage-enriched miRNAs were retina-specific.

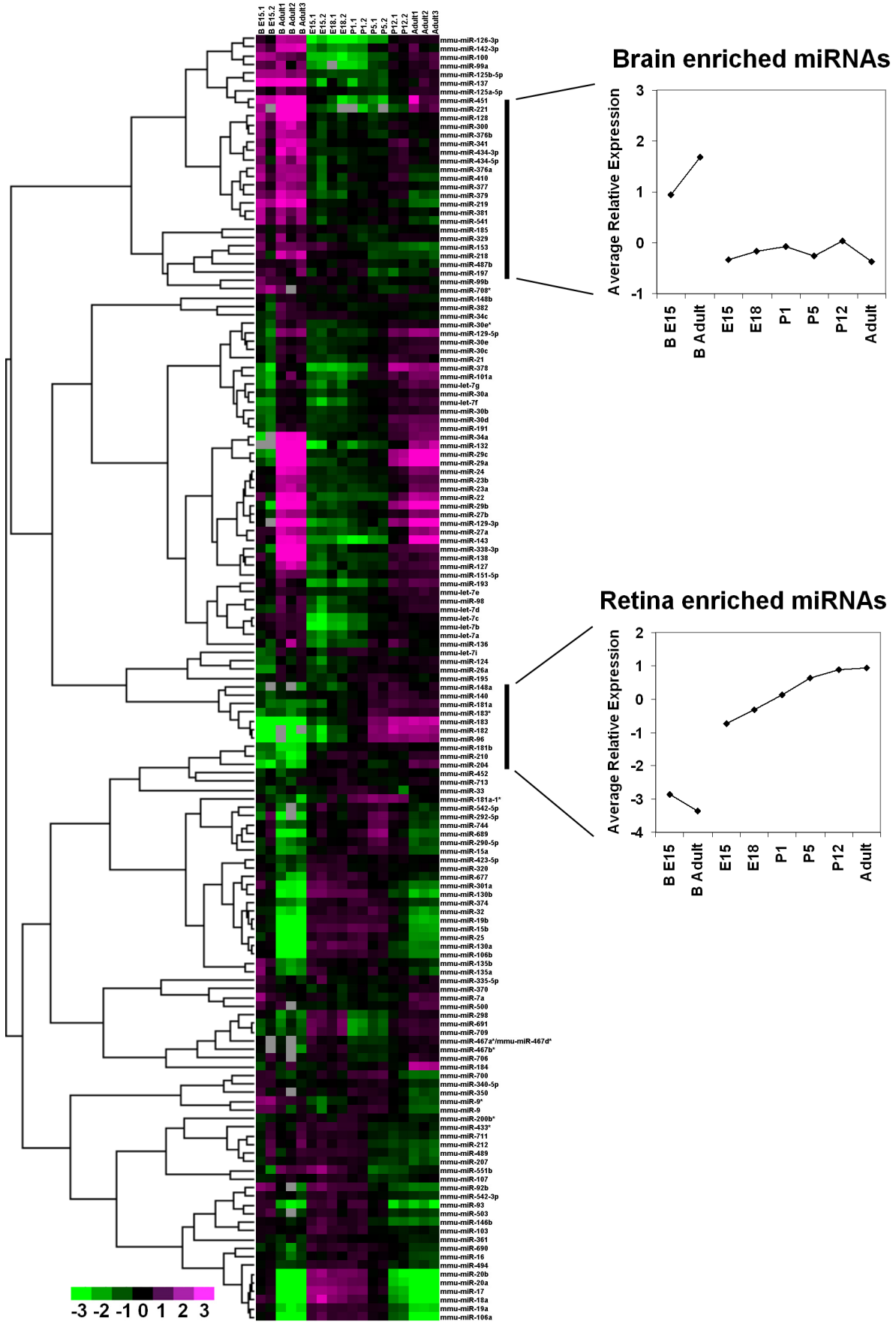
WT Retinas of C57BL/6 Mice

To further investigate the time course of retinal development, we used the Self Organizing Map (SOM) algorithm to analyze the microarray data. This analysis, after defining the number of clusters, assigns all miRNAs to a cluster according to their expression patterns. The created clusters represent the major expression profiles in the data set. The mean expression profile of the miRNAs is used as a descriptor of the cluster. The nine nonredundant, representative expression profiles revealed by the SOM analysis are shown in Figure 2 and Table 1.

The profiles described by clusters c1, c2, and c5 showed higher expression with time. Clusters c2 and c5 both showed increased retinal expression at P12; however, although c2 included miRNAs with a sharp and significant increase starting at P12, c5 includes miRNAs with a more moderate increase. Cluster c1 miRNAs demonstrated increasing expression from E18 until P12. miRNAs with high expression at embryonic time points were in cluster c6. They showed a steady decrease until P5 and a sharp drop at P12. Cluster c7 included miRNAs with a less pronounced decrease with time. Cluster c3 included miRNAs with no expression change from E15 to P5 and a steady decrease at P12 and adult age. Cluster c8 included miRNAs that displayed a significant decrease from E18 to P1 and a steady increase after that. Finally, clusters c0 and c7 showed only a moderate change with time, whereas c4 included miRNAs with no significant change in expression.

Functional Analysis of the Temporally Co-expressed miRNAs. To find common targets of the temporally co-expressed miRNAs identified by the SOM analysis, we made use of two target prediction databases: miRanda[17] and TargetScan.[18] To increase the specificity of the predicted target set, we defined as "targets" only the overlapping set of genes that were predicted by both miRanda and TargetScan. In addition, only targets expressed in the retina, as determined by the

Figure 1. Hierarchical cluster analysis of miRNAs expressed in brain and retina. *Lanes 1–5* (B E15.1–B Adult3): brain; *lanes 6–18* (E15.1–Adult3): retina. Scale on color bar represents $\log_2$ relative expression levels. Heat map depicts high (*magenta*), median (*black*), and low (*green*) values. *Gray*: missing values. Relative expression levels were median centered for each miRNA. Brain- and retina-enriched miRNAs are highlighted with bars beside annotations, and their average relative expression levels are plotted at each tested developmental stage.


B E15.1
B E15.2
B Adult1
B Adult2
B Adult3
E15.1
E15.2
E18.1
E18.2
P1.1
P1.2
P5.1
P5.2
P12.1
P12.2
Adult1
Adult2
Adult3
mmu-miR-126-3p
mmu-miR-142-3p
mmu-miR-100
mmu-miR-99a
mmu-miR-125b-5p
mmu-miR-137
mmu-miR-125a-5p
mmu-miR-451
mmu-miR-221
mmu-miR-128
mmu-miR-300
mmu-miR-376b
mmu-miR-341
mmu-miR-434-3p
mmu-miR-434-5p
mmu-miR-376a
mmu-miR-410
mmu-miR-377
mmu-miR-379
mmu-miR-219
mmu-miR-381
mmu-miR-541
mmu-miR-185
mmu-miR-329
mmu-miR-153
mmu-miR-218
mmu-miR-487b
mmu-miR-197
mmu-miR-99b
mmu-miR-708*
mmu-miR-148b
mmu-miR-382
mmu-miR-34c
mmu-miR-30e*
mmu-miR-129-5p
mmu-miR-30e
mmu-miR-30c
mmu-miR-21
mmu-miR-378
mmu-miR-101a
mmu-let-7g
mmu-miR-30a
mmu-let-7f
mmu-miR-30b
mmu-miR-30d
mmu-miR-191
mmu-miR-34a
mmu-miR-132
mmu-miR-29c
mmu-miR-29a
mmu-miR-24
mmu-miR-23b
mmu-miR-23a
mmu-miR-22
mmu-miR-29b
mmu-miR-27b
mmu-miR-129-3p
mmu-miR-27a
mmu-miR-143
mmu-miR-338-3p
mmu-miR-138
mmu-miR-127
mmu-miR-151-5p
mmu-miR-193
mmu-let-7e
mmu-miR-98
mmu-let-7d
mmu-let-7c
mmu-let-7b
mmu-let-7a
mmu-miR-136
mmu-let-7i
mmu-miR-124
mmu-miR-26a
mmu-miR-195
mmu-miR-148a
mmu-miR-140
mmu-miR-181a
mmu-miR-183*
mmu-miR-183
mmu-miR-182
mmu-miR-96
mmu-miR-181b
mmu-miR-210
mmu-miR-204
mmu-miR-452
mmu-miR-713
mmu-miR-33
mmu-miR-181a-1*
mmu-miR-542-5p
mmu-miR-292-5p
mmu-miR-744
mmu-miR-689
mmu-miR-290-5p
mmu-miR-15a
mmu-miR-423-5p
mmu-miR-320
mmu-miR-677
mmu-miR-301a
mmu-miR-130b
mmu-miR-374
mmu-miR-32
mmu-miR-19b
mmu-miR-15b
mmu-miR-25
mmu-miR-130a
mmu-miR-106b
mmu-miR-135b
mmu-miR-135a
mmu-miR-335-5p
mmu-miR-370
mmu-miR-7a
mmu-miR-500
mmu-miR-298
mmu-miR-691
mmu-miR-709
mmu-miR-467a*/mmu-miR-467d*
mmu-miR-467b*
mmu-miR-706
mmu-miR-184
mmu-miR-700
mmu-miR-340-5p
mmu-miR-350
mmu-miR-9*
mmu-miR-9
mmu-miR-200b*
mmu-miR-433*
mmu-miR-711
mmu-miR-212
mmu-miR-489
mmu-miR-207
mmu-miR-551b
mmu-miR-107
mmu-miR-92b
mmu-miR-542-3p
mmu-miR-93
mmu-miR-503
mmu-miR-146b
mmu-miR-103
mmu-miR-361
mmu-miR-690
mmu-miR-16
mmu-miR-494
mmu-miR-20b
mmu-miR-20a
mmu-miR-17
mmu-miR-18a
mmu-miR-19a
mmu-miR-106a
-3 -2 -1 0 1 2 3
Brain enriched miRNAs
Average Relative Expression
3
2
1
0
-1
B E15
B Adult
E15
E18
P1
P5
P12
Adult
Retina enriched miRNAs
Average Relative Expression
2
1
0
-1
-2
-3
-4
B E15
B Adult
E15
E18
P1
P5
P12
Adult

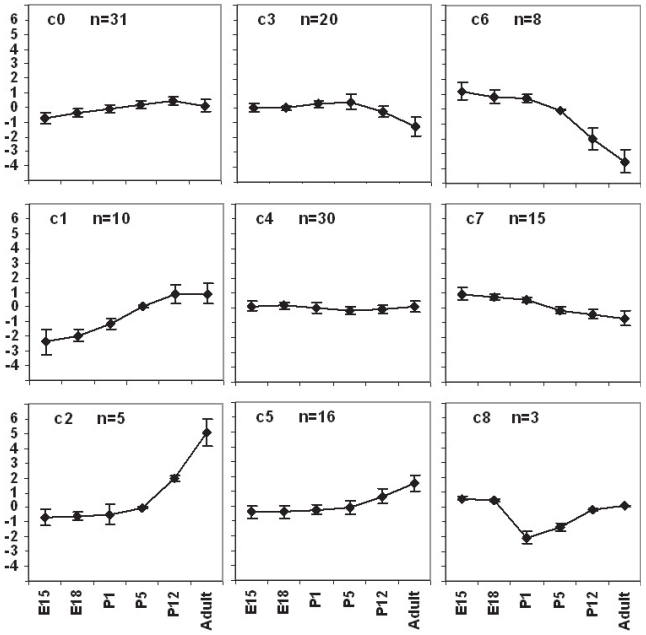


FIGURE 2. miRNA expression profiles identified by the SOM algorithm during retinal development. Average expression levels from the corresponding miRNAs are plotted in time. *y*-Axis indicates relative expression on $\log_2$ scale. *n*, number of miRNAs in the cluster. Error bars, SD from the plotted average expression.

UniGene database of NCBI (http://www.ncbi.nlm.nih.gov/UniGene/ National Center for Biotechnology Information, Bethesda, MD) were considered for further analysis. We designated a gene as a common target if at least two thirds of the miRNAs in a given cluster were predicted regulators of that gene. We focused on the clusters showing the most significant change in expression, namely c1, c2, c6, and c8. Clusters c1 and c6, although having a similar number of member miRNAs (10 and 8), had a different number of common targets (3 and 175). Clusters c2 and c8 (number of members, 5 and 3) had 90 and 248 common targets, respectively. We also investigated the assigned gene ontology (GO) terms of the predicted common targets to gain functional insights. Significant enrichment of GO terms was found only for clusters c2, c6, and c8. In cluster c2 the GO term "potassium channel complex" showed the highest enrichment. In c6 the term "peptidyl-threonine modification" scored the highest, whereas in c8, the term "positive regulation of transcription from RNA polymerase II promoter" was the most enriched. Table 2 summarizes the top four enriched terms and the associated genes in clusters c2, c6, and c8.

miRNA Families. miRNAs can be assigned to families based on sequence similarity in their 5′ region. Among the retina-expressed miRNAs, there were several families that showed expression of at least two of the members (Supplementary Fig. S1, http://www.iovs.org/cgi/content/full/51/4/1823/DC1). Members of the same miRNA family appeared to share similar expression profiles, although there were examples of considerable variation. Also, expression of the detected family members followed a similar pattern during retina and brain development. It is worth mentioning that probes for very similar miRNAs may not be able to discriminate between highly similar sequences under the experimental conditions. This lack of specificity also occurs in vivo, since miRNAs sharing similar sequences tend to have an overlapping group of target mRNAs.

Genomic Clusters. Previous studies have described clusters of miRNAs (miRNA genes <10 kb apart in the genome) co-expressed under certain conditions or at specific developmental time points.[12,19] We have found several miRNA clusters showing well-defined expression patterns during retinal development. Supplementary Table S5 and Supplementary Figure S2 summarize genomic clusters with at least two miRNAs expressed. The previously described sensory organ–specific cluster on chromosome 6 (*miR-96*, *miR-182*, and *miR-183*)[12] was also identified in our study. With few exceptions, miRNAs belonging to the same genomic cluster generally showed similar expression profiles, suggesting that they originate from a common primary transcript.

Two copies of the *let-7a* gene are found on chromosomes 9 and 13 (Supplementary Fig. S2, clusters 13 and 12). Expression of *let-7a* is significantly different from that of *miR-100*, although it closely resembles the profile of *let-7d,* suggesting that expression is dominated by transcription from the gene on chromosome 13. A similar lack of correlation of the *let-7a-2* and *miR-100* pair is also observed in human tissues.[19]

Another example of differential expression among clustered miRNAs is *miR-132* and *miR-212* (cluster 20) in the first intron of *1700016P03Rik*. A possible explanation is that the two miRNAs are transcribed independently from the host gene and from different transcription start sites (TSSs), which is

TABLE 1. Member miRNAs in the Identified SOM Clusters

Cluster	Member miRNAs
c0 (*n* = 31)	*let-7a, let-7d, let-7e, miR-124, miR-136, miR-140, miR-148a, miR-151-5p, miR-181a-1*, miR-181a, miR-181b, miR-183*, miR-195, miR-21, miR-26a, miR-300, miR-30a, miR-30b, miR-30c, miR-30e*, miR-30e, miR-341, miR-376a, miR-377, miR-379, miR-410, miR-434-3p, miR-434-5p, miR-541, miR-9**
c1 (*n* = 10)	*let-7b, let-7c, let-7f, miR-100, miR-126-3p, miR-142-3p, miR-182, miR-183, miR-378, miR-96*
c2 (*n* = 5)	*miR-129-3p, miR-143, miR-29a, miR-29b, miR-29c*
c3 (*n* = 20)	*miR-135a, miR-15a, miR-15b, miR-16, miR-19b, miR-25, miR-290-5p, miR-292-5p, miR-32, miR-340-5p, miR-350, miR-374, miR-494, miR-542-5p, miR-689, miR-690, miR-700, miR-744, miR-9*
c4 (*n* = 30)	*let-7i, miR-107, miR-125a-5p, miR-135b, miR-148b, miR-185, miR-197, miR-200b*, miR-204, miR-210, miR-320, miR-329, miR-335-5p, miR-33, miR-34c, miR-361, miR-370, miR-376b, miR-381, miR-382, miR-423-5p, miR-452, miR-467a*, miR-467b*, miR-467d*, miR-487b, miR-706, miR-713, miR-7a, miR-99b*
c5 (*n* = 16)	*miR-125b-5p, miR-127, miR-138, miR-184, miR-191, miR-193, miR-22, miR-23a, miR-23b, miR-24, miR-27a, miR-27b, miR-30d, miR-338-3p, miR-451, miR-500*
c6 (*n* = 8)	*miR-106a, miR-130b, miR-17, miR-18a, miR-19a, miR-20a, miR-20b, miR-93*
c7 (*n* = 15)	*miR-103, miR-130a, miR-146b, miR-153, miR-207, miR-212, miR-218, miR-301a, miR-433*, miR-489, miR-542-3p, miR-551b, miR-677, miR-711, miR-92b*
c8 (*n* = 3)	*miR-298, miR-691, miR-709*

n, number of members in the cluster.

TABLE 2. Gene Ontology Term Enrichment of Common Predicted Target Genes

Cluster/GO Term	GO Roots	Enrichment Score	*P**	Genes (*n*)	Gene Symbols
Cluster c2					
Potassium channel complex	Cellular_component	11.274854	0.008249	4	*Kcnc3, Kctd21, Kcnc1, Kcnc2*
Voltage-gated potassium channel complex	Cellular_component	11.274854	0.009166	4	*Kcnc3, Kctd21, Kcnc1, Kcnc2*
Voltage-gated potassium channel activity	Molecular_function	6.694444	0.048917	4	*Kcnc3, Kctd21, Kcnc1, Kcnc2*
Transmembrane receptor protein: tyrosine kinase signaling pathway	Biological_process	6.22739	0.02355	5	*Ndst1, Ntrk2, Pik3r3, Plekha1, Ptpn11*
Cluster c6					
Peptidyl-threonine modification	Biological_process	10.328571	0.020003	3	*Akt3, Map3k12, Mtap2*
Peptidyl-threonine phosphorylation	Biological_process	10.328571	0.020429	3	*Akt3, Map3k12, Mtap2*
Erythrocyte differentiation	Biological_process	8.262857	0.034817	3	*Pknox1, Smad5, Sp1*
Receptor signaling protein Serine/threonine kinase activity	Molecular_function	6.25974	0.012762	5	*Bmpr2, Map3k12, Mapk14, Mapk4, Mapk9*
Cluster c8					
Positive-regulation of transcription from RNA polymerase II promoter	Biological_process	3.048703	0.008642	16	*E2f1, Klf12, Med12, Mll1, Ncoa2, Nfat5, Onecut2, Pbx1, Plagl2, Trp53, Usf2, Ablim1, Bmp2, Smad5, Sox11, Sp1*
Positive regulation of RNA metabolic process	Biological_process	2.939821	0.007289	18	*Aff1, E2f1, Klf12, Mecp2, Med12, Mll1, Ncoa2, Nfat5, Onecut2, Pbx1, Plagl2, Trp53, Usf2, Ablim1, Bmp2, Smad5, Sox11, Sp1*
Positive regulation of transcription, DNA-dependent	Biological_process	2.939821	0.014578	18	*Aff1, E2f1, Klf12, Mecp2, Med12, Mll1, Ncoa2, Nfat5, Onecut2, Pbx1, Plagl2, Trp53, Ust2, Ablim1, Bmp2, Smad5, Sox11, Sp1*
Positive regulation of transcription	Biological_process	2.650293	0.014793	18	*Aff1, E2f1, Klf12, Mecp2, Med12, Mll1, Ncoa2, Nfat5, Onecut2, Pbx1, Plagl2, Trp53, Usf2, Ablim1, Bmp2, Smad5, Sox11, Sp1*

* FDR multiple test correction.

supported by TSS predictions around and between the two miRNAs.[20]

Differentially Expressed miRNAs in Wild-Type and *Nrl*$^{-/-}$ Retinas

To explore possible differences in miRNA expression between rods and cones, we complemented our studies of WT retinas by profiling developing *Nrl*$^{-/-}$ retinas at postnatal days 1, 5, 12 and at adult age. miRNAs (n = 108) were expressed in *Nrl*$^{-/-}$ retinas between time points P1 and adult (Supplementary Table S6). Only a few miRNAs showed differential expression between WT and *Nrl*$^{-/-}$ adult retinas. *miR-200b**, *miR-298*, *miR-29a*, *miR-29b*, and *miR-29c* showed elevated expression in *Nrl*$^{-/-}$ retinas, whereas *miR-143*, *miR-184*, *miR-204*, *miR-211*, and *miR-335–5p* showed higher expression in WT retinas (Table 3). Although these differences were confirmed by quantitative (q)PCR analysis in all five tested cases on the same adult samples (Fig. 3A), differential expression of *miR-184* showed considerable variation in repeated measurements on independently collected samples (data not shown). Although *miR-182* has been reported to be specifically expressed in rods,[13] we found *miR-182* to be expressed similarly in both WT and *Nrl*$^{-/-}$ retinas in our microarray and qPCR experiments (Fig. 3B).

Spatial Distribution of miRNA Expression in the Adult Retina

To determine the spatial distribution of retina-enriched miRNAs, we used laser capture microdissection (LCM). Total RNA was collected from the GCL, INL, and ONL of the adult neuronal retina by LCM, and gene expression levels were determined by qPCR. Of the six miRNAs that were tested by this approach, we obtained reliable results with only three (*miR-204*, *miR-210* and *miR-211*), presumably due to low expression levels and limited amounts of available captured RNA (Fig. 4, all expression data were scaled to the corresponding INL sample).

Both *miR-204* and *miR-211* showed the highest expression in the INL (Figs. 4A, 4B). *miR-210* was detected in all three layers, but with a less pronounced enrichment in the INL (Fig. 4C). Biological replicates (Fig. 4, samples with numbers 1 through 4) showed considerable variation in expression levels. This variation may reflect different sub-

TABLE 3. Differentially Expressed miRNAs in *Nrl*$^{-/-}$ Retinas

Name	Mean Expression Change†		*P*‡
	Microarray	QPCR	
*miR-200b**	1.04	—	—
miR-29c	0.87	—	—
miR-298	0.63	—	—
miR-29b	0.59	—	—
miR-29a	0.53	2.32	0.054
miR-211	−0.71	−1.86	0.046
miR-204	−0.78	—	—
miR-335-5p	−1.63	−1.56	0.002
miR-184	−1.82	—	—
miR-143	−2.23	−2.31	0.007

† Log_2 scale.
‡ Student's *t*-test.

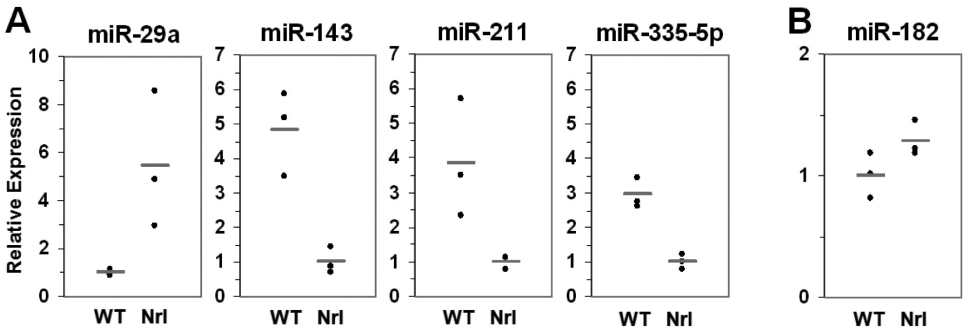


FIGURE 3. miRNAs differentially expressed in adult WT and $Nrl^{-/-}$ retinas. (**A**) Differentially expressed miRNAs. (**B**) *miR-182* is similarly expressed in WT and $Nrl^{-/-}$ retinas. *Horizontal bars*: the average expression; *solid circles*: biological replicates.

cellular localization of miRNAs; for example, synapses or inner segments may be collected to various degrees from sample to sample.

Correlation of Intronic miRNA and Host Gene Expression

Co-expression of host transcripts and corresponding intronic miRNAs was reported previously in retina.[13,19] We used LCM samples to explore further the relationship between host transcripts and intronic miRNAs. *miR-204* resides in an intron of *Trpm3* (transient receptor potential cation channel, subfamily M, member 3) on chromosome 19 in the mouse genome. We found an INL-enriched expression profile for *Trpm3* similar to that of *miR-204*, which is in good agreement with the reported co-expression in the GCL and the INL in adult retina.[13]

Similar to *miR-204*, *miR-211* is an intronic miRNA localized in an intron of the *Trpm1* (transient receptor potential cation channel, subfamily M, member 1) gene on chromosome 19. *Trpm1* showed expression similar to that of *miR-211* (Figs. 4A, 4B).

Several studies suggested that miRNAs are affected by post-transcriptional regulation.[21–23] In the case of intronic miRNAs, this effect can be detected by comparing expression profiles of the host genes to their respective miRNA in different conditions or at different developmental time points. To explore this relationship, we monitored relative expression of several gene/miRNA pairs during our retinal developmental series. Although *Trpm3* and *miR-204* were detected at all time points investigated, their profiles differed significantly (Fig. 5A). Expression of *miR-204* is fairly constant early, but then its expression increases sharply between P12 and adult, whereas expression of *Trpm3* shows little change. Both *miR-211* and *Trpm1* were detected at all time points, and both showed the highest expression at adult age (Fig. 5B). Expression of *Trpm1* showed a very sharp increase at P12, whereas expression of *miR-211* showed a more gradual increase from P5 to adult age.

We also investigated the brain-specific *miR-128*. The sequence of *miR-128* is found on mouse chromosomes 1 and 9 in introns of *Arpp21* (cyclic AMP-regulated phosphoprotein, 21) and *R3hdm1* (R3H domain 1). Identical mature miRNAs originate from the two locations expressing different precursor miRNAs. *miR-128* showed the highest expression level in adult brain, whereas it was not detected in retina by microarrays. qPCR showed an identical profile but detected *miR-128* in all retina samples (Fig. 5C). Expression of both host genes, *Arpp21* and *R3hdm1*, was detected in all brain and retina samples by qPCR. The expression level of *Arpp21* was highest in adult brain. In retina, at any time point *Arpp21* had lower expression than in E15 brain. *R3hdm1* had the highest expression in adult brain and had similar expression in E15 brain and retina at all times.

DISCUSSION

We have characterized the miRNA transcriptome of the developing mouse retina from embryonic day 15 through adult age using microarray technology. In addition, for comparative analysis, we have profiled E15 and adult brain and retinas of postnatal $Nrl^{-/-}$ mice. Hierarchical cluster analysis revealed dozens of miRNAs that are differentially expressed during the investigated timeframe of development. The majority of the temporally differentially expressed miRNAs in each system (WT retina, WT brain, $Nrl^{-/-}$ retina) showed overlapping profiles.

Numerous miRNAs grouped by cluster analysis were related to each other, either by sequence similarity (family members) or by their close positions on the genome (miRNA clusters), and sometimes by both. To further explore this relationship, we compared the sequences of all detected mature miRNAs in a pair-wise manner and plotted expression correlation as a function of sequence similarity. The correlation of expression was highest among miRNAs that are the most similar in sequence (Fig. 6A), as was also seen when individual families were investigated (see the Results section; Supplementary Fig. S1). A decreasing correlation is observed with increasing divergence in sequence, and a complete lack of correlation is apparent below ~60% sequence similarity.

The seed sequence of an miRNA (nucleotides 2–8 from the 5′ end of the mature miRNAs) is believed to be the most critical component in the formation of an RNA duplex between a miRNA and the 3′ UTRs of its target mRNAs.[24] Consistent with the importance of the seed sequence, the decrease in expression correlation with decreased sequence similarity between miRNAs was most striking when only the seed sequences were considered (Fig. 6B). The correlation declined to close to 0 if the seed sequences differed in only one base. The strong correlation in expression of miRNAs with the same seed sequence (family members) shows that their expression is precisely timed and suggests that some or all members may be under similar regulation. Their targets may be shared, providing a more effective and fine-tuned regulation of their translation. Findings in a recent study provide evidence for such a mechanism. The targeted deletion of the retina specific *miR-182* resulted in no detected deviations in the retinas of both heterozygous and homozygous mice.[25] *miR-182* is a member of the polycistronic *mir-183*, *mir-96*, and *mir-182* cluster on chromosome 6. In our studies, all three miRNAs showed close correlation of expression and their mature sequences are related (*miR-182* to *miR-96* and *miR-182* to *miR-183*: 0.99 and 0.978 expression correlation and 86% and 71% sequence similarity, respectively). In their seed sequences *miR-182* differs from *miR-96* and *miR-183* in one and two nucleotides, respectively. Furthermore, in vitro experiments suggest that *miR-182* and *miR-96* compete for the same targets.[12]

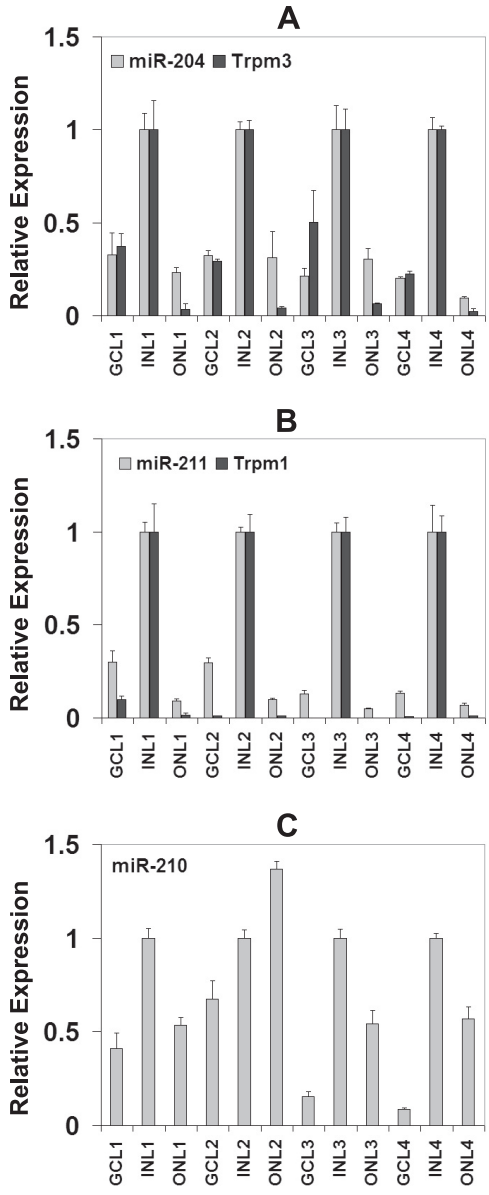


FIGURE 4. Spatial distribution determined by LCM and qPCR. (**A**) Expression of the *miR-204*, *Trpm3* miRNA host gene pair in dissected retinal layers. (**B**) Expression of the *miR-211*, *Trpm1* miRNA host gene pair. (**C**) Expression of the intergenic *miR-210*. Samples with the same number were dissected from the same retina. Numbers 1 through 4 represent biological replicates from four animals. All expression data are scaled to the corresponding INL sample. Error bars, SD from three measurements.

We also analyzed in a pair-wise manner the correlation of expression of miRNAs from the same chromosome as a function of the relative genomic distance between members of the pair. The expression correlation of miRNA pairs with identical orientation from the same chromosome was plotted versus their separation distance (Figs. 6C, 6D). Within a 5-kb distance, more than 70% of miRNA pairs exhibited a strong correlation (Pearson correlation coefficient, >0.7). The correlation dropped to the overall average correlation level after 5 kb (Fig. 6C), suggesting that miRNAs situated closer than 5 kb, in general, are likely to be derived from a common transcript, and this result may also reflect a general miRNA primary transcript size. This finding is consistent with a previous miRNA transcript analysis, where the average length of the primary transcript for intergenic miRNAs was estimated at 3 to 4 kb.[26] Of interest, an earlier study of miRNA expression in human organs suggested a 10-fold higher, 50-kb size as the upper limit of polycistronic primary transcripts.[19] Our plot of the average correlation versus genomic distance of miRNA pairs reveals a fluctuating pattern of correlation and anticorrelation (Fig. 6D). Among other explanations, this pattern may represent favored distances for coexpressed loci (duplication of miRNAs, chromosomal structure) or perhaps chance coexpression of those miRNAs.

A major focus of our study was to define the repertoire of miRNAs expressed at different time points of retinal development. In terms of broad classes with expression that changed during development, two major expression profiles were identified: miRNAs expressed predominantly early in retinal development and those expressed predominantly in the mature and

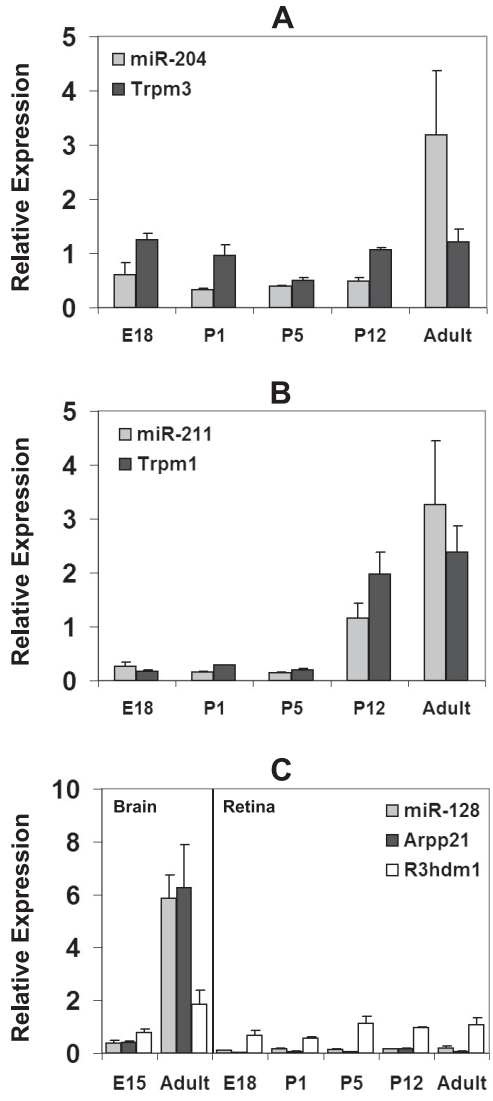


FIGURE 5. Correlation of expression of host genes and their intronic miRNAs during development. (**A**) Lack of correlation of expression of the *miR-204*, *Trpm3* miRNA host gene pair from E18 to adult age in retina. (**B**) Expression of the *miR-211*, *Trpm1* miRNA host gene pair from E18 to adult age shows good correlation in retina. (**C**) Contribution of two genomic loci to the overall expression. Expression of the brain specific *miR-128* and its host genes *Arpp21* and *R3hdm1* from E18 to adult age in retina and at E15 and adult age in brain. Expression data are scaled to the average expression from all time points. Embryonic and postnatal time points $n = 2$, adult $n = 3$. Error bars, SD.

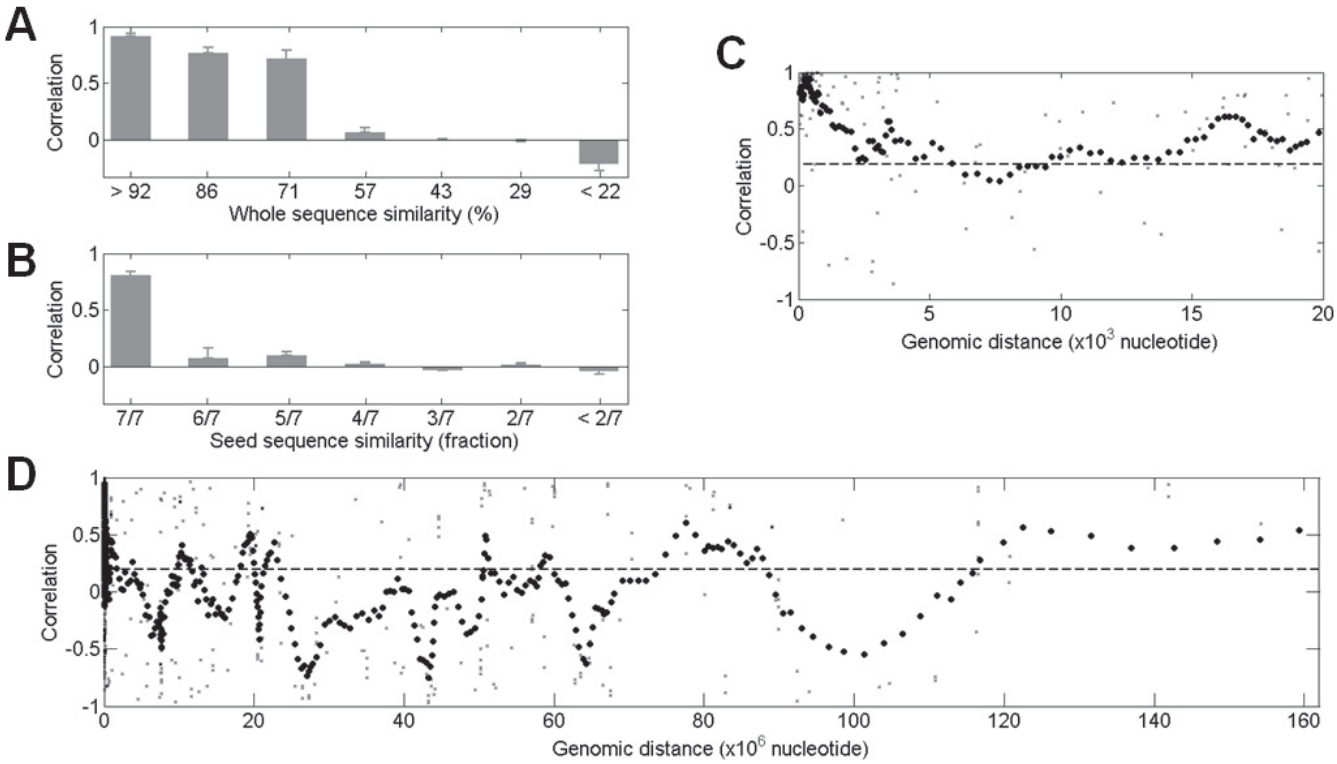


FIGURE 6. Correlation of expression in the function of sequence similarity and genomic distance. (**A**) Relationship between mature miRNA sequence similarity and correlation of expression. Pair-wise expression correlation is plotted in the function of the calculated miRNA sequence similarity. (**B**) Relationship between miRNA "seed" sequence similarity and correlation of expression. Sequence similarity is considered only from the nucleotides 2 to 8. Fraction indicates the number of identical nucleotides in the "seed" sequence. (**C**) Relationship between miRNA genomic distance and correlation of expression. Expression correlation of individual miRNA pairs from the same chromosome with identical orientation was plotted against their genomic distances (*small crosses*). *Dots*: 10-point moving average of correlation coefficients of 10 adjacent miRNA pairs against genomic distance. (**D**) Relationship between miRNA genomic distance and correlation of expression. The genomic distance investigated extended to 160×10^6 nucleotides.

developed retina. To evaluate the possible functions of miRNAs showing highly correlating expression profiles according to the SOM analysis, we searched for their shared target genes. We were able to identify common targets for several clusters, although the number of target genes varied greatly even in similar size clusters (cluster c1 and c6). In the case of clusters c2 and c6, the two profiles possessing the most significant change over time, our predictions show several biological processes with statistically significant enrichment suggesting specific roles for the co-expressed miRNAs. Unfortunately, no specific retinal events could be identified from these predictions for several possible reasons: the cellular complexity of the retina, "expression noise" from parallel events, the limitation of the GO analysis (incomplete GO annotations), or the ultimate lack of miRNA-retina specific biological event interaction.

One limitation of our analysis of target co-regulation of temporally co-expressed miRNAs is that it makes the simplistic assumption that there is spatial co-expression of the interacting partners—that is, it does not take into consideration the likely possibility that different miRNAs and corresponding target genes are expressed in different retinal cells. Thus, for better understanding of the functional role that miRNAs are playing in the retina, it is important to identify the expression patterns of miRNAs, not only in time but also in space. Unfortunately, however, the nature of miRNAs, especially their short sequence length and loop structure, makes it difficult to determine their spatial expression patterns by methods that are traditionally applied to mRNAs, such as in situ hybridization.

As an alternative approach, we used qPCR to analyze miRNA expression levels in LCM samples of adult retina. In the case of three miRNAs, we were able to succeed with this approach. It seems that the known retina-enriched miRNAs that have been examined do not show specificity to an individual cell type; rather they show expression in multiple retinal cell types although at quantitatively distinct levels. Notably, a comparison of the miRNA transcriptomes of rod (WT) and cone dominant ($Nrl^{-/-}$) retinas revealed only a handful of miRNAs showing differential expression, and none of them showed specificity to one or the other subtype.

Intronic miRNAs are generally co-expressed with their host transcription units.[13,19,27] This finding proved to be the case with the tested miRNA host gene pairs in adult retinas. The combination of LCM with qPCR was an effective tool, providing quantitative information on miRNA expression in the retina.

Posttranscriptional regulation of miRNAs can happen either in the nucleus before transport to the cytoplasm[28] or in the cytoplasm by blocking pre-miRNA processing by *Dicer*.[21] Co-expression of host genes and intronic miRNAs suggest that *miR-204* and *miR-211* are not differentially regulated at the posttranscriptional level in adult retinas. Although *Trpm1* and *miR-211* showed largely similar expression patterns during development, with *Trpm3* and *miR-204*, there was a clear dissociation of expression profiles, suggesting that the maturation of the miRNA is blocked or inhibited during the period from P12 to adult (Fig. 5).

In addition to changes during development, there can be differences in miRNA processing between tissues. Lee et al.[29] reported that the precursor of the brain-specific miRNA *miR-128* is expressed in a several tissues, but processing into the mature

form occurs predominantly in the brain. In the retina, although it is part of the CNS, we found that the expression of mature *miR-128* was minimal. In fact, it was only detected in the retina by highly sensitive qPCR and not by microarray analysis.

Taken together, the lack of correlation of host and miRNA expression in the mentioned examples suggests that posttranscriptional regulation may affect the maturation of some miRNAs during retinal development. Further studies are needed to validate these findings and to identify the molecular mechanisms that regulate the expression of miRNAs within the retina.

Acknowledgments

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References

1. Ambros V. The functions of animal microRNAs. *Nature.* 2004;431:350–355.
2. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* 2004;116:281–297.
3. Xie X, Lu J, Kulbokas EJ, et al. Systematic discovery of regulatory motifs in human promoters and 3′ UTRs by comparison of several mammals. *Nature.* 2005;434:338–345.
4. Hwang HW, Mendell JT. MicroRNAs in cell proliferation, cell death, and tumorigenesis. *Br J Cancer.* 2006;94:776–780.
5. Fiore R, Siegel G, Schratt G. MicroRNA function in neuronal development, plasticity and disease. *Biochim Biophys Acta.* 2008;1779:471–478.
6. Leaman D, Chen PY, Fak J, et al. Antisense-mediated depletion reveals essential and specific functions of microRNAs in Drosophila development. *Cell.* 2005;121:1097–1108.
7. Li Y, Wang F, Lee JA, Gao FB. MicroRNA-9a ensures the precise specification of sensory organ precursors in Drosophila. *Genes Dev.* 2006;20:2793–2805.
8. Schaefer A, O'Carroll D, Tan CL, et al. Cerebellar neurodegeneration in the absence of microRNAs. *J Exp Med.* 2007;204:1553–1558.
9. Damiani D, Alexander JJ, O'Rourke JR, et al. Dicer inactivation leads to progressive functional and structural degeneration of the mouse retina. *J Neurosci.* 2008;28:4878–4887.
10. Decembrini S, Andreazzoli M, Barsacchi G, Cremisi F. Dicer inactivation causes heterochronic retinogenesis in Xenopus laevis. *Int J Dev Biol.* 2008;52:1099–1103.
11. Ryan DG, Oliveira-Fernandes M, Lavker RM. MicroRNAs of the mammalian eye display distinct and overlapping tissue specificity. *Mol Vis.* 2006;12:1175–1184.
12. Xu S, Witmer PD, Lumayag S, Kovacs B, Valle D. MicroRNA (miRNA) transcriptome of mouse retina and identification of a sensory organ-specific miRNA cluster. *J Biol Chem.* 2007;282:25053–25066.
13. Karali M, Peluso I, Marigo V, Banfi S. Identification and characterization of microRNAs expressed in the mouse eye. *Invest Ophthalmol Vis Sci.* 2007;48:509–515.
14. Mears AJ, Kondo M, Swain PK, et al. Nrl is required for rod photoreceptor development. *Nat Genet.* 2001;29:447–452.
15. Swaroop A, Xu JZ, Pawar H, Jackson A, Skolnick C, Agarwal N. A conserved retina-specific gene encodes a basic motif/leucine zipper domain. *Proc Natl Acad Sci U S A.* 1992;89:266–270.
16. Yoshida S, Mears AJ, Friedman JS, et al. Expression profiling of the developing and mature $Nrl^{-/-}$ mouse retina: identification of retinal disease candidates and transcriptional regulatory targets of Nrl. *Hum Mol Genet.* 2004;13:1487–1503.
17. Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. *Nucleic Acids Res.* 2008;36:D149–153.
18. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* 2009;19:92–105.
19. Baskerville S, Bartel DP. Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. *RNA.* 2005;11:241–247.
20. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ. miRBase: tools for microRNA genomics. *Nucleic Acids Res.* 2008;36:D154–158.
21. Obernosterer G, Leuschner PJ, Alenius M, Martinez J. Post-transcriptional regulation of microRNA expression. *RNA.* 2006;12:1161–1167.
22. Wulczyn FG, Smirnova L, Rybak A, et al. Post-transcriptional regulation of the let-7 microRNA during neural cell specification. *FASEB J.* 2007;21:415–426.
23. Ding XC, Weiler J, Grosshans H. Regulating the regulators: mechanisms controlling the maturation of microRNAs. *Trends Biotechnol.* 2009;27:27–36.
24. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. *Cell.* 2003;115:787–798.
25. Jin ZB, Hirokawa G, Gui L, et al. Targeted deletion of miR-182, an abundant retinal microRNA. *Mol Vis.* 2009;15:523–533.
26. Saini HK, Griffiths-Jones S, Enright AJ. Genomic analysis of human microRNA transcripts. *Proc Natl Acad Sci U S A.* 2007;104:17719–17724.
27. Bak M, Silahtaroglu A, Moller M, et al. MicroRNA expression in the adult mouse central nervous system. *RNA.* 2008;14:432–444.
28. Tang GQ, Maxwell ES. Xenopus microRNA genes are predominantly located within introns and are differentially expressed in adult frog tissues via post-transcriptional regulation. *Genome Res.* 2008;18:104–112.
29. Lee EJ, Baek M, Gusev Y, Brackett DJ, Nuovo GJ, Schmittgen TD. Systematic evaluation of microRNA processing patterns in tissues, cell lines, and tumors. *RNA.* 2008;14:35–42.