MicroRNA (miRNA) Transcriptome of Mouse Retina and Identification of a Sensory Organ-specific miRNA Cluster

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Although microRNAs (miRNAs) provide a newly recognized level of regulation of gene expression, the miRNA transcriptome of the retina and the contributions of miRNAs to retinal development and function are largely unknown. To begin to understand the functions of miRNAs in retina, we compared miRNA expression profiles in adult mouse retina, brain, and heart by microarray analysis. Our results show that at least 78 miRNAs are expressed in adult mouse retina, 21 of which are potentially retina-specific. Among these, we identified a polycistronic, sensory organ-specific paralogous miRNA cluster that includes miR-96, miR-182, and miR-183 on mouse chromosome 6qA3 with conservation of synteny to human chromosome 7q32.2. In situ hybridization showed that members of this cluster are expressed in photoreceptors, retinal bipolar and amacrine cells. Consistent with their genomic organization, these miRNAs have a similar expression pattern during development with abundance increasing postnatally and peaking in adult retina. Target prediction and in vitro functional studies showed that MITF, a transcription factor required for the establishment and maintenance of retinal pigmented epithelium, is a direct target of miR-96 and miR-182. Additionally, to identify miRNAs potentially involved in circadian rhythm regulation of the retina, we performed miRNA expression profiling with retinal RNA harvested at noon (Zeitgeber time 5) and midnight (Zeitgeber time 17) and identified a subgroup of 12 miRNAs, including members of the miR-183/96/182 cluster with diurnal variation in expression pattern. Our results suggest that miR-96 and miR-182 are involved in circadian rhythm regulation, perhaps by modulating the expression of adenylyl cyclase VI (ADCY6).

MicroRNAs (miRNAs) are small, noncoding, regulatory RNAs of 18–24 nucleotides in length found in all metazoans. Since their discovery in 1993, at least 100 different miRNA genes have been documented in the genomes of Drosophila and Caenorhabditis elegans and more than 250 in vertebrate genomes (1) with recent estimates as high as 800 (2). By influencing translation and stability of mRNAs, miRNAs contribute a newly recognized level of regulation of gene expression affecting a variety of biological processes. miRNAs are transcribed by RNA polymerase II as transcripts (pri-miRNAs) that are capped, polyadenylated, and spliced (3). Pri-miRNAs fold into hairpin structures that are cleaved by an RNase III endonuclease, the Drosha-DGCR8 complex, to form 60–70-nt stem loop intermediates known as pre-miRNA (1, 4, 5) that are transported from the nucleus by an Exportin 5-dependent mechanism. In the cytoplasm they are cleaved by a second RNase III endonuclease, Dicer, to yield double-stranded miRNA: miRNA* duplexes that are loaded into the RNA-induced silencing complex where the miRNA* strand is degraded (6). Mature, single-stranded miRNAs in the context of RNA-induced silencing complex engage in base pairing with target sites, located typically in the 3′-UTR of their client miRNAs (1, 7). In plants, miRNAs base pair with target mRNAs by precise or nearly precise complementarity to specify cleavage of the target mRNAs by a mechanism that involves the RNA interference machinery (8). Most animal miRNAs have imperfect complementary to their mRNA target sites. miRNA binding with less than perfect complementarity inhibits translation of the targeted mRNA by an unknown mechanism (9), whereas binding with perfect complementarity leads to cleavage of the corresponding miRNAs (5, 10).

Expression of miRNAs appears to be highly regulated by developmental stage and tissue specificity (11). Some miRNAs are abundant with an estimated 10,000 molecules per cell, whereas others are barely detectable by hybridization to total cellular RNA (12). About 5,000 human genes (~20% of the total) are estimated to be subjected to miRNA regulation (13, 14) with each miRNA predicted to regulate the miRNA transcripts of ~200 genes (15, 16). These target relationships are the basis for major regulatory effects of miRNAs. For example, introduction of an abundant brain miRNA, miR-124, into HeLa...
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cells causes the expression profile of the recipient cells to shift toward that of brain, whereas introduction of a prominent muscle miRNA, miR-1, shifts the expression profile of the recipient cells toward that of muscle. In each case, about 100 mRNAs were down-regulated, suggesting that tissue-specific miRNAs help to define tissue-specific gene expression (10). Functional studies suggest that miRNAs play important roles in the control of development and function, including temporal pattern formation (17, 18), cell death and/or cell proliferation (19, 20), fat storage (21), sensory neuron specification (22, 23), hematopoietic lineage differentiation (24), stem cell division and maintenance (25, 26), and malignant transformation (27).

Microarrays have been used to profile normal miRNA expression in myoblast and preadipocyte differentiation (28, 29). Abnormal patterns of miRNA expression have been described in certain disease states, most notably in human cancers (30) where increasing evidence suggests that miRNA expression profiling can contribute to more precise tumor classification and predict therapeutic outcomes (27). Thus, genome-wide miRNA expression profiling is likely to become a powerful addition to the functional analysis of cellular differentiation, proliferation, and inherited and acquired disease states (27, 31–34).

Despite this progress in understanding miRNA functions and disease associations, little is known about the miRNA complement of mammalian retina. One recent report focused on miRNA expression of the anterior segment of the mouse eye but does list 10 miRNAs abundant in retina (35), another reports cloning of 9 miRNAs from the eye of the newt (36), and a third described the spatiotemporal expression of 7 miRNAs in embryonic and postnatal mouse eye (37). Retina is a derivative of the forebrain endoderm, and regulation of gene expression through intrinsic and extrinsic factors ensures a tightly controlled temporal and spatial developmental sequence (38). It is likely that, as in other tissues, miRNAs play important roles in the normal development and function of the retina. To better understand these functions, we used microarray analysis to determine the expression pattern of miRNAs in mouse retina as compared with brain and heart. We find at least 78 miRNAs are expressed in adult mouse retina, 21 of which are expressed preferentially or specifically in retina, including a cluster of three paralogous miRNAs that are also expressed in other neurosensory tissues. We also present evidence for circadian cycling in the expression of certain of these miRNAs.

EXPERIMENTAL PROCEDURES

Mouse RNA Samples—Wild type SVJ129 mice and retinal degeneration mice, rd1, were purchased from The Jackson Laboratory. Mice were kept in 12-h light/12-h dark cycles with lights on at 7:00 a.m. (ZT0) and the lights off at 7:00 p.m. (ZT12). Total retinal RNA was prepared from adult SVJ129 mice (~3 months old) at 12:00 p.m. (ZT5) or 12:00 a.m. (ZT17) using mirVana miRNA isolation system (Ambion). Mice were anesthetized with pentobarbital (75 mg/kg intraperitoneally) and sacrificed by cervical dislocation. The eyes were enucleated, and the retina was quickly removed and submerged in 300 μl of lysis/binding buffer, homogenized with a plastic pestle homogenizer. Thereafter, the manufacturer’s protocol for isolation of small RNA-enriched total RNA was followed (Ambion). Adult mouse total RNA samples from brain, heart, thymus, lung, liver, spleen, testicle, ovary, kidney, and embryonic day 10 embryos of Swiss Webster mice were purchased from Ambion. For the circadian rhythm study, total retinal RNA was isolated at ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21. At each time point, three mice were sacrificed, and total retinal RNA from each mouse was prepared separately. Adult rd1 mouse (~4 months old) retinal RNA was prepared as described above. Mouse olfactory epithelium total RNA was prepared as described previously (39) except that we used the mirVana miRNA isolation system. For mouse tongue epithelium total RNA preparation, we removed adult SVJ129 mouse tongues and soaked them in enzyme mixture (1 mg/ml collagenase A (Sigma), dispase (Invitrogen), and 1 mg/ml trypsin inhibitor (Roche Applied Science)) (40) for 10 min. We also injected the mixture under the epithelium of the tongue. We peeled the epithelium off and soaked it in the lysis buffer of mirVana miRNA isolation system (Ambion) and prepared total RNA as described above.

miRNA Labeling and Microarray Hybridization—We purified small RNA (~<40 nt) from the total RNA samples using a FlashPAGE fractionator (Ambion) according to the manufacturer’s protocol. We used the mirVana miRNA labeling kit (Ambion) and followed the Ambion protocol to label the miRNA with Cy3. We performed three independent microarray hybridizations with independent probes labeled from independent mouse retinal RNA preparations at ZT5 or ZT17. For the brain and heart profiling, we performed three independent microarray hybridizations with independently prepared probes from the total RNA purchased from Ambion. We used the mirVana miRNA bioarray (Ambion) for the miRNA profiling. This array contains 385 independent miRNAs each as a duplicate feature, representing a comprehensive panel of all human, mouse, and rat microRNAs in the miRNA Registry. We followed the manufacturers’ protocol for hybridization.

Microarray Scanning and Data Analysis—We used a Packard Biochip scanner at the Research Resource Center of the University of Illinois, Chicago, and ScanArray software to scan the arrays at 90–95% power and a photomultiplier of 75–80 and 5 μm resolution. The spot intensity was determined in QuantArray. The average intensity of the “empty” spots (negative controls) was calculated for each array as the background. We calculated the background-corrected signal intensity (BgCor Signal) according to the formula: BgCor Signal = spot intensity – (average of the empty spots). We reported values less than zero as zero. We normalized the BgCor Signal as a function of the average of the BgCor Signal of all noncontrol spots: normalized signal = BgCor Signal × 100/average of the BgCor Signal of all noncontrol spots. We calculated the average of the normalized intensity of the six spots for each miRNA gene (duplicate spots/array × three independent arrays) as the specific signal for each miRNA. We calculated significance of the specific signal as a two-tailed p value of Z-test (X = 0, σ = standard deviation of all noncontrol feature signals on array) for normalized signals. We identify a signal with p < 0.01 as a “positive” or as “expressed miRNA.” We removed the following features from analysis due to observation of apparent hybrid-
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**Results**

The miRNA Transcriptomes of Mouse Retina, Brain, and Heart—Using a microarray (Ambion), we profiled the expression of miRNAs in small RNA-enriched total RNA from mouse retina, brain, and heart. We identified at least 78 miRNAs expressed in retina (Fig. 1) (supplemental Table 1). Of these, 40 (~51%) are expressed in all three tissues (supplemental Table 8). These widely expressed miRNAs may represent a set of “housekeeping” miRNAs important for regulation of the basic cellular functions in all tissues. Of the remaining 38 retinal miRNAs, 12 were also expressed in brain but not in heart (supplemental Table 2A); five were expressed in heart but not brain (supplemental Table 2B); and 21 were detected only in retina making them candidate retina-specific miRNAs (Fig. 1 and Table 1). We also identified two miRNAs (mir-143 and Ambi_mir_7029) that were expressed in brain and heart but not retina (supplemental Table 7). Overall, the miRNA expression pattern of retina more closely resembled that of brain as compared with heart, in both the number of shared miRNAs and in the levels of their expression (Fig. 1, supplemental Tables 2 and 8–10).

**Confirmation of the Tissue Specificity of the Retinal miRNAs**—We performed quantitative RT-PCR assays for all apparently retina-specific miRNAs in RNA isolated from retina, brain, and eight other tissues (heart, thymus, lung, liver, spleen, kidney, testis, and ovary) and embryonic day (E) 10 whole mouse embryos. We confirmed that all except mir-361 are expressed in retina (Fig. 2 and supplemental Fig. 1). Interestingly, we found that mir-96, mir-182, mir-183, mir-184, mir-210, and mir-140-AS are all highly expressed in adult retina but are not detectable in RNA from brain or the other tissues. This high level of expression with apparent specificity suggests that these miRNAs may play important roles in the normal function of retina. One of the miRNAs, mir-210, is also expressed in E10 embryos (Fig. 2 and supplemental Fig. 1), but not in the other adult tissues and may have a role in early retinal development.

We also found nine miRNAs (mir-7, mir-9, mir-9-AS, mir-31, mir-181c, mir-211, mir-219, mir-320, and mir-335) that are preferentially expressed in retina or retina/brain with low levels of expression in various other tissues (Fig. 2 and supplemental Fig. 1). mir-25, mir-92, mir-194, and mir-151-AS are expressed in several other tissues in addition to retina.

**Luciferase Reporter Assays of miRNA Targeting**—We utilized RT-PCR to amplify and subclone fragments (SpeI/HindIII) of the 3′-UTR of ADCy6 (nt 4206–6520 of GenBank™ accession number NM_015270, containing four potential target sites for mir-96 and mir-182) or MITF (nt 3219–4654 of GenBank™ accession number NM_198159, containing four potential target sites for mir-96 and for mir-182) into the luciferase reporter vector, pMIR-REPORT (Ambion), 3′ to the firefly luciferase cassette. We plated HEK293 cells at a density of 5 × 10^5 cells/well in 24-well plates coated with poly-d-lysine (Sigma) and transfected them with 150 ng of pMIR reporter construct (pMIR-REPORT-3′-UTR/Adcy6 or pMIR-REPORT-3′-UTR/Mitf), 15 ng of hpRL-SV40 (Promega), and 5–10 pmol of the specified miRNA mimics or control oligonucleotide with a scrambled sequence (Dharmacon) using Lipofectamine 2000 (Invitrogen). Using the dual luciferase assay kit (Promega), we measured firefly luciferase activity 48 h later and normalized to Renilla luciferase activity. We performed at least three independent experiments for each assay.

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106b and miR-185 are expressed at low level in most of the tissues.

Developmental Patterns of Expression—To study the developmental time course of the 21 miRNAs shown to be specifically or preferentially expressed in retina, we isolated mouse total retinal RNA from eye cups of embryos at E10, and the developing retinas of embryos at E14 and E18, as well as from retinas of postnatal days 1 (P1) and 10 (P10) mice and performed qRT-PCR (Fig. 3 and supplemental Table 13). In the E10 samples, all of the assayed miRNAs have little or no expression. Most (17/21) showed a pattern of increasing expression with development peaking at P10 or adult (Fig. 3, A–C). Of these, 14 showed at least a 10-fold increase of expression at the peak time (adult or P10) (supplemental Table 13), compared with E10. Eight (miR-183, miR-182, mir-96, mir-9_AS, miR-184, miR-211, miR-151_AS, and mir-140_AS) of the 12 miRNAs with peak expression in adult retina showed at least a 10-fold increase from P1 to adult, suggesting that these miRNAs correspond to the time when the late retinal progenitor cells are differentiating into the mature retinal cell types and functioning as mature retinal neurons and/or Muller glia. Four of the miRNAs (miR-335, miR-219, miR-194, and mir-185) showed peak expression at E14 and E18.5 (Fig. 3D and supplemental Table 13), whereas other miRNAs (miR-151_AS, miR-211, miR-184, miR-25, and miR-140_AS) showed a moderate increase at E14 or E18 (Fig. 3, B and C, and supplemental Table 13), suggesting that these miRNAs may have stage-specific functions.

Identification of a Sensory Tissue-specific, Paralogous miRNA Cluster—The genes for three of the highly expressed retinal miRNAs, miR-183, miR-96, and miR-182, are clustered within 4 kb on mouse chr6qA3, transcribed in the same direction (telomere → centromere), and located in an ~60-kb gap between the genes encoding nuclear respiratory factor-1 (Nrf1) and ubiquitin-conjugating enzyme E2H (Ube2H). The orthologous region in humans is 7q32.2, where hsa-miR-183, miR-96, and miR-182 have similar arrangement (Fig. 4A). The sequences of these three miRNAs are similar to one another as follow:

### TABLE 1
miRNAs expressed in retina but not in brain or heart

| No. | Name             | ms chr location | hs chr location | Average retina (ZT5) | Average retina (ZT17) |
|-----|------------------|-----------------|-----------------|----------------------|-----------------------|
| 1   | hsa_miR_182      | 6qA3            | 7q32.2          | 693.9                | 869.6                 |
| 2   | hsa_miR_183      | 6qA3            | 7q32.2          | 491.7                | 583.0                 |
| 3   | hsa_miR_184      | 9qE3.3          | 15q25.1         | 401.5                | 380.2                 |
| 4   | mmu_miR_211      | 7qC             | 15q13.3         | 391.4                | 445.6                 |
| 5   | hsa_miR_210      | 7F5             | 11q15.5         | 368.4                | 443.0                 |
| 6   | hsa_miR_96       | 6qA3            | 7q32.2          | 368.3                | 416.9                 |
| 7   | hsa_miR_31       | 4qC4            | 9q21.3          | 325.6                | 303.5                 |
| 8   | hsa_miR_184c     | 8C3             | 19p13.12        | 325.0                | 233.6                 |
| 9   | hsa_miR_335      | 6qA3            | 7q32.2          | 301.9                | 181.0                 |
| 10  | rno_miR_151_AS   | 15qE1/10?       | 8q24.3          | 281.3                | 254.6                 |
| 11  | mmu_miR_140_AS   | 8qD1            | 15q22.1         | 273.2                | 208.1                 |
| 12  | hsa_miR_25       | 5qG1            | 7q22.1          | 263.3                | 116.0                 |
| 13  | hsa_miR_361      | 5qD             | 12q21.2         | 240.0                | 253.8                 |
| 14  | hsa_miR_320      | 14qD1           | 8q21.3          | 249.4                | 305.5                 |
| 15  | hsa_miR_219      | 17qB2/2qA3      | 6q21.32/9q34.11 | 193.2                | 194.0                 |
| 16  | hsa_miR_92       | 14qXqA5         | 13q31.3/14qXq26.2 | 192.7               | 188.3                 |
| 17  | hsa_miR_194      | 1qhA/19qA       | 1qh1/19q13.1   | 189.1                | 153.6                 |
| 18  | hsa_miR_9_AS     | 9qF1/13qC3/7qD2 | 9q21.33        | 167.1                | 109.3                 |
| 19  | hsa_miR_7        | 13qB2/7qD2/17qD | 9q21.33        | 140.9                | 161.0                 |
| 20  | hsa_miR_185      | 16qB1           | 22q11.21        | 119.0                | 147.1                 |
| 21  | hsa_miR_106b     | 5qG1            | 7q22.1          | 57.4                 | 188.2                 |
in mouse, miR-96 has 52 and 56% identity with miR-182 and miR-183, respectively; miR-182 and miR-183 are 78% identical (Fig. 4B). All three of these miRNAs have a similar developmental expression pattern (Fig. 4D). The sequence similarity, clustered location, common orientation, and shared developmental pattern suggest that these three miRNAs comprise a paralogous cluster transcribed on a single pri-miRNA transcript. A similar arrangement has been observed for other miRNA clusters (41). To test this model we used RT-PCR of mouse retinal RNA to show that all three of these miRNAs are transcribed as a single polycistronic transcript (supplemental Fig. 2).

To determine which cells in the adult retina express the members of this miRNA cluster, we performed in situ hybridization with antisense probes corresponding to each and

**FIGURE 2.** Confirmation of tissue specificity by RT-PCR. A, multitissue qRT-PCR of miR-96, miR-182, and miR-183; B, multitissue end product RT-PCR of candidate retina-specific miRNAs.

**FIGURE 3.** Developmental expression pattern of miRNAs with preferential or specific retinal expression in mouse retina. A, miRNAs with increasing expression and peak at adult retina; B, miRNAs with peak expression in adult retina and a surge at E14 or E18; C, miRNAs with peak expression at P10; D, miRNAs with peak expression at E14 or E18.
found that the expression patterns of all three are similar and are limited to the photoreceptors and the interneurons in the inner nuclear layer with little or no expression in the ganglion cell layer (Fig. 5A). To confirm the expression in interneurons in the inner nuclear layer, we also assayed for their expression in RNA isolated from retinas of 4-month-old retinal degenerative mice, rd1/rd1. At this age, the retinas of these mutant animals have no photoreceptors but have a normal complement of inner nuclear layer and are also detectable in RNA isolated from tongue epithelium, presumably because of the inclusion of taste buds in this tissue (Fig. 6, B and C).

The hypothesis that expression of the miR-183/96/182 cluster is specific for sensory tissue is also supported by analysis of the sequence upstream of the most 5’ miRNA in the cluster (miR-183) (Fig. 6A). In both mouse and human there is a large upstream CpG island with multiple predicted transcription factor binding sites characteristic of genes expressed in neurosensory cells. These include at least one binding site for CHX10, a key transcription factor in retinal development (45–47), two binding sites for OLF1, an olfactory neuron-specific transcription factor (48), and multiple binding sites for OTX1 (49–53) and Pax2 (54–59), transcription factors important for the development of retina and inner ear. Other predicted binding sites for sensory organ related transcription factors in the 5’ region of the cluster include Pax5 (60–62), Pou3F2 (63), RXF1 (64–67), and LMO2 (68).

Target Prediction of the miR-183/96/182 Cluster—The members of this cluster have considerable sequence similarity, particularly residues 2–8 that comprise the “seed sequences” whose complementarity to target sites in miRNAs determines which transcripts will be regulated (1). At the 5’ end, 7 of the first 8 nucleotides are identical between miR-182/miR-96 and miR-182/miR-183 and miR-96/miR-183 (Fig. 4, A and C). This similarity predicts that these miRNAs will have overlapping sets of downstream targets. To examine this prediction more closely, we compared the results of four miRNA target prediction programs: PicTar, TargetScan, miRBase Targets, and Diana-MicroT, all available on line. The list of predicted targets was similar for all four programs. In the PicTar list (supplemental Table 11), miR-182 and miR-96 share 394 candidate target genes (56 and 67% of the total targets predicted for miR-182 and miR-96, respectively) (Fig. 4E). miR-182 and miR-183 share 45 predicted targets; miR-96 and miR-183 share 51 potential targets with 38 of the predicted targets shared by all three miRNAs. When we filter the list of predicted mRNA targets with the retinal transcriptome as determined by serial analysis of gene expression, the list of targets is reduced by about 50% with 20 retinally expressed transcripts that have predicted target sites for all three miRNAs in the cluster (supplemental

**Figure 4. Genomic organization, sequence, and predicted targets for the miR-183/96/182 cluster.** A, genomic organization of the cluster is similar in mouse, human, and zebrafish. The numbers show the spacing in each of the three species in bp or kb. B, alignment of mature miR-182, miR-96, and miR-183 sequences. Nucleotides in red are identical in all three; those in blue are identical in two of three. The aqua shaded rectangle denotes the seed sequence. C, alignment of the pre-miRNA sequences for miR-182, miR-96, and miR-183. The yellow rectangle denotes the mature miRNA sequence. D, expression of miR-182, miR-96, and miR-183 at different developmental stages. The relative expression level is normalized to the expression level in the adult retina. Results from three independent experiments were averaged and mean ± S.E. is presented as the error bar. E, Venn diagram of targets for miR-182, miR-96, and miR-183 predicted by PicTar.
Table 12). This set of highly enriched miR-96, miR-182, and miR-183 targets includes genes known to have important roles in various sensory organs (supplementary Table 14), supporting our hypothesis of the sensory organ specificity of this cluster.

**Circadian Variation Expression of Retinal miRNAs**—The expression of many genes in retina follows circadian rhythms, e.g. melatonin, the core circadian clock genes (Per1, Per2, Cry1, Cry2, and Bmal1), and activators of Bmal1 transcription (Rora, Rorb, and Rorc) (69–72). To determine whether the expression of any retinal miRNAs also exhibit diurnal variation, we harvested mouse retinal RNA ZT 5 and ZT17, respectively, and performed miRNA expression profiling. We identified 10 miRNAs whose levels were increased at ZT17, compared with those at ZT5 varying from 1.25-fold (miR-124a, miR-103, and miR-182) to 3.3-fold (miR-106b) (Table 2, part A). By contrast, two miRNAs (miR-422a and miR-422b) showed decreased levels at ZT17 as compared with those at ZT5 (51 and 25% decreased, respectively) (Table 2, part B).

The miR-183/96/182 Cluster and Circadian Rhythm Regulation—Interestingly, miR-182 was one of the 10 miRNAs with increased expression at ZT17 (Table 2, part A). Adenylyl cyclase VI (Adcy6) is a target predicted by PicTar and TargetScan for miR-182, and for a second member of the cluster, miR-96, is with at least six target sites for each miRNA (Fig. 7A) (73). Adcy6 plays important roles in the differential regulation of serotonin N-acetyltransferase (acetyl-coenzyme A:arylalkylamine N-acetyltransferase) by bradykinin in rat pineal gland and has a circadian pattern of expression at the levels of mRNA and protein with the apex of expression around ZT5 and a nadir at ZT17 (74). This pattern is the inverse of that of miR-182 (Table 2) as expected if this miRNA contributes to circadian variation of Adcy6 expression (Table 2, part A). To
examine this more closely, we isolated mouse total retinal RNAs at ZT1, -5, -9, -13, -17, and -21 and performed quantitative RT-PCR on miR-182, miR-96, and Adcy6. Our results show that Adcy6 is expressed in retina with circadian variation (apex around ZT9, nadir at ZT17). miR-182 and miR-96 expression also displayed circadian variation with a pattern nearly inverse to that of Adcy6 (levels at ZT13 are ~1.28- and 2.69-fold, respectively, those at ZT5) (Fig. 7B). To demonstrate that Adcy6 is a direct target of miR-182 and miR-96, we subcloned a 3′-UTR fragment of Adcy6 (nt 4206–6520 of GenBank access number NM_015270 with four potential target sites for miR-96 and miR-182) into the luciferase reporter vector, pMIR-REPORT, to the luciferase cassette and co-transfected this construct (pMIR-REPORT-3′-UTR/Adcy6) with miR-96 and/or miR-182 mimics into HEK293 cells. The results showed that miR-96 or miR-182 down-regulate the expression of Adcy6 in the luciferase reporter assay.

### Table 2

| Name                     | ZT5 mean | ZT5 SD | ZT17 mean | ZT17 SD | -Fold Change (ZT17/ZT5) Change p Value |
|--------------------------|----------|--------|-----------|---------|-----------------------------------------|
| A, Candidate miRNAs upregulated at ZT17 comparing with at ZT5 |           |        |           |         |                                        |
| hsa_miR_106b             | 57.418   | 58.119 | 188.2     |         | 3.28 (0.019)                            |
| hsa_miR_30b              | 255.5    | 114.9  | 404.98    | 109.16  | 1.59 (0.022)                            |
| hsalet_7f                | 231.44   | 111.46 | 365.47    | 149.19  | 1.58 (0.054)                            |
| hsalet_7f                | 258.7    | 61.351 | 386.83    | 148.76  | 1.50 (0.040)                            |
| hsa miR_139              | 211.06   | 67.879 | 306.24    | 101.83  | 1.45 (0.043)                            |
| hsa miR_126              | 211.98   | 115.65 | 295.59    | 85.626  | 1.39 (0.093)                            |
| hsa miR_107              | 338.81   | 118.04 | 439.86    | 78.907  | 1.30 (0.056)                            |
| hsa miR_182              | 693.94   | 42.342 | 869.64    | 262.26  | 1.25 (0.068)                            |
| hsa miR_103              | 319.69   | 113.88 | 400.26    | 87.763  | 1.25 (0.100)                            |
| hsa miR_124a             | 551.98   | 165.86 | 690.09    | 175.65  | 1.25 (0.096)                            |
| B, Candidate miRNAs downregulated at ZT17 comparing to at ZT5 |           |        |           |         |                                        |
| hsa miR_422a             | 183.6    | 106.53 | 90.243    | 96.88   | 0.49 (0.072)                            |
| hsa miR_422b             | 249.19   | 80.212 | 187.08    | 58.359  | 0.75 (0.078)                            |

**FIGURE 7.** ADCY6 is a downstream target of miR-182 and miR-96. A, alignment of miR-182 and miR-96 with their potential target sites in ADCY6; the green box marks the seed sequences of miR-96 and miR-182 and the corresponding sequences in the potential target sites in ADCY6. B, relative expression level of miR-182, miR-96, and Adcy6 at ZT1, -5, -9, -13, -17, -21 (n = 3). The red bars indicate the mean value at each time point. *, p < 0.05 (Kruskal-Wallis test). C, miR-96 and miR-182 down-regulate the expression of Adcy6 in the luciferase reporter assay.
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A.

B.

Figure 8. Mitf is a target of miR-182 and miR-96. A, alignment of miR-182 and miR-96 with their potential target sites in Mitf; the green box marks the seed sequences of miR-96 and miR-182 and the corresponding sequences in the potential target sites in Mitf. B, miR-96 and miR-182 down-regulate the expression of mitf in the luciferase reporter assay.

DISCUSSION

Using miRNA microarray profiling, we identified at least 78 miRNAs expressed in adult mouse retina (supplemental Table 1), 21 of which are candidate retina-specific microRNAs (expressed in retina, but not in brain or heart) (Table 1). Using quantitative RT-PCR, we confirmed that six of these (miR-96, miR-182, miR-183, miR-184, miR-210, and miR-140-AS) are expressed in retina but not in other adult tissues (Fig. 2 and supplemental Fig. 1); four miRNAs are preferentially expressed in retina (miR-181c, miR-320, miR-31, and miR-211) and five in retina/brain (miR-7, miR-9, miR-9-AS, miR-219, and miR-335), whereas four miRNAs (miR-25, -92, -194, and miR-151-AS) are highly expressed in retina as well as in various other tissues. The 10 miRNAs identified in mouse retina by Ryan et al. (35) are included in the 78 retinal miRNAs we identified. This pattern of specific or preferential expression in retina suggests that this subset of retinal miRNAs may have especially important function in retina. We also detected expression of 10 of these miRNAs in E10 embryos (Fig. 2B), suggesting they may also function during retinal development. For example, miR-7 mediates epidermal growth factor receptor signaling and promotes photoreceptor differentiation in Drosophila (23).

Not surprisingly, the miRNA transcriptomes of retina and brain were more similar to each other than either was to that of heart in terms of the number of shared miRNAs and their relative levels of expression (Fig. 1B and supplemental Tables 2, 7, and 8). For example, we found miR-124 to be the second most abundant in retina (supplemental Table 1) and, as others have reported (11, 76), the highest in brain. Among the miRNAs expressed in retina, we found that 12 were expressed in brain but not in heart (supplemental Table 2A) but only five that were expressed in heart but not in brain (supplemental Table 2B). Two miRNAs (miR-143 and ambi_miR7029) were expressed in brain and heart but not in retina (supplemental Table 7). We also identified eight candidate brain-specific (not expressed in the retina and the heart) miRNAs (supplemental Table 4) and six heart-specific (not in brain and retina) miRNAs (supplemental Table 5), including the well characterized heart-specific miR-1 (11, 77). Forty of the 78 miRNAs (~51%) we detected in retina are expressed in all three tissues (supplemental Table 8). These widely expressed miRNAs appear to represent a set of housekeeping miRNAs and may be important for regulation of the basic cellular functions in all or most tissues.

The relatively small numbers of the tissue-specific miRNAs is surprising but consistent with the diverse collection of targets predicted for each miRNA. The combinatorial complexity of the overlapping specificities of miRNAs may reduce dependence on tissue-specific miRNAs. Experimental and computational studies have shown that each miRNA can target hundreds of unique mRNAs (10, 14, 15), and the 3′-UTRs of many mRNAs have predicted target sites for multiple miRNAs (15, 78, 79) enabling combinatorial regulatory effects. Additionally, some genes have multiple poly(A) signal sites producing transcripts in which the 3′-UTR is composed of constitutive and alternative segments, allowing further diversity of the tissue-specific regulation by miRNAs on various mRNAs (80).

To characterize the developmental patterns of the miRNAs expressed exclusively or preferentially in retina, we performed qRT-PCR on RNA samples isolated from the eye cups of E10 embryos and from the developing retinas of E14 and E18 embryos, as well as from P1 and P10 pups and adult retina RNA (Fig. 3 and supplemental Table 13). These stages represent the
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major landmarks in mouse retinal development. Around E10, the retinal progenitor cells are actively proliferating and beginning to differentiate (38, 81–83). Differentiation of retinal ganglion cells, cone photoreceptors, horizontal cells, and amacrine cells peaks around E14–16. By E18, differentiation of rod photoreceptors, bipolar cells, and Müller glia is increasing to peaks around P1 (rod photoreceptors) and the first postnatal week (bipolar and Müller glia). By P10, all retinal progenitor cells have completed the last mitosis of their terminal differentiation (38, 81–84). Interestingly, we found that all of the 21 miRNAs tested have little or no expression at E10 with most (17/21) showing increased expression, in concert with retinal development, reaching peak levels at P10 or adult (Fig. 3, A–C). Eight of the 12 miRNAs (miR-183, miR-182, miR-9, miR-9 AS, miR-184, miR-211, miR-151 AS, and miR-140 AS) whose expression peaked in adult retina showed >10-fold increase from P1 to adult, suggesting that these miRNAs may play important roles in the differentiation, especially for the late-born retinal cells (rod photoreceptors, bipolar cells and Muller glia) and the maintenance of the phenotype and function of the mature retinal neurons and/or Muller glia. Consistent with this observation, we found few of these miRNAs are expressed in the retinal stem cell spheres (data not shown). Peak expression for four of the miRNAs (miR-335, miR-219 and miR-194, miR-185) (Fig. 3D and supplemental Table 13) occurred at E14 and E1, whereas the levels of five other miRNAs (miR-151 AS, miR-211, miR-184, miR-25, and miR-140 AS) showed a moderate increase at E14 or E18 (Fig. 3, B and C, supplemental Table 13), suggesting that these miRNAs may have stage-specific functions in retinal development.

Among the retina-specific miRNAs, we identified three (miR-96, miR-182, and miR-183) whose expression appears to be specific for neurosensory cells and whose structural genes map to a 4-kb genomic region on mouse chromosome 6qA3, with conservation of synteny and order to human chromosome 7q32.2 and a similar arrangement in zebrafish (Fig. 4A). These three miRNA genes are transcribed in the same direction and share high sequence homology, suggesting they are grouped in a paralogous cluster derived from duplications of a common ancestor. By in situ hybridization, we showed that all three miRNAs are highly expressed in photoreceptors and the interneurons of the inner nuclear layer (Fig. 5A). Moreover, using RT-PCR of mouse retinal RNA, we were able to connect all three of these miR transcripts in overlapping fragments of a single transcript (supplemental Fig. 2) indicating that they derive from a single polycistronic pri-miRNA. Consistent with this arrangement, qRT-PCR analysis of the expression over retinal development showed that all three members of this cluster have similar expression patterns with little or no expression in the early embryonic stages, and increasing expression postnatally to a peak in adult retina (Fig. 3A and 4D and supplemental Table 13). The postnatal change in expression of these miRNAs in retina is dramatic, increasing 30–350-fold from P1 to adult retina (Fig. 3A and 4D and supplemental Table 13). This time course suggests that members of this miRNA cluster may play important roles in the terminal differentiation of retinal progenitors and in the maintenance of mature retinal phenotype and function.

Consistent with the hypothesis that miR-96, miR-182, and miR-183 play important roles in sensory neural biology, the genomic sequence upstream of their polycistronic transcript in humans has a large CpG island (3.4 kb upstream from miR-183 in both mouse and human) associated with a cluster of predicted transcription factor binding sites, including sites for CHX10, a transcription factor important for retinal development and function (45); RORA1 and RORA2, transcription factors important regulators of circadian rhythm (71, 72); OLF1, a transcription factor involved in the regulation of several olfactory neuron-specific genes (48); and OTX1 (49–53), Pax2 (54–59), and other transcription factors involved in the development of both retina and inner ear (Pax5 (60–62), Pou3F2 (63), RFX1 (64–67), and LMO2 (68)) (Fig. 6A). Recently, Weston et al. (43) reported expression of these same miRNAs in the mouse inner ear, and Kloosterman et al. (44) showed that these same miRs are expressed in mouse dorsal root ganglia. Moreover, in zebrafish, miR-183 and miR-96 are expressed in the lateral line system, a mechanosensory organ (85). Stimulated by these observations, we performed RT-PCR on RNA isolated from mouse olfactory and tongue epithelium and found that all three members of the miR-183/96/182 cluster are also expressed in olfactory epithelium, which is largely composed of the olfactory receptor cells and in tongue epithelium which contains the taste buds (Fig. 6, B and C). The expression levels in olfactory epithelium are similar to or even higher than in the retina (Fig. 6, B and C). The much lower expression levels in RNA isolated from tongue epithelium likely reflects expression limited to the sensory neurons of the taste buds (<1% of tongue surface area), diluted by RNA from the nonsensory epithelial cells that make up the majority of this tissue (86, 87).

The hypothesis that members of the miR-183/96/182 cluster play important roles in sensory neural biology is further supported by analysis of their predicted targets, which includes many genes with important roles in the development and/or function of various sensory organs (e.g. PAX6 (88, 89), MITF (75), Hes1 (90) myosin 1C, (91) LMO3 (68), TFCP2L3 (92), and many others) (supplemental Table 13). One of these with predicted targets shared by miR-96 and miR-182 is Mitf (75) (Fig. 8A). Our target site assays showing that miR-96 and/or miR-182 inhibit the activity of a recombinant luciferase reporter construct with a 3’-UTR derived from human MITF support the prediction that this transcription factor is a direct target of miR-96 and miR-182 (Fig. 8B). MITF is essential for the acquisition and maintenance of RPE cell identity (46), and in mouse, Mitf mutations cause microphthalmia, due to failure of RPE terminal differentiation and transdifferentiation of RPE precursors to neuroretinal cells in the dorsal retina (75). Early in the development of the eye, Mitf is expressed across the entire optic vesicle, in precursors of neuroretinal and RPE cells, but is then down-regulated in the presumptive neuroretinal cells with continued expression in the RPE precursors (93). Reduction of Mitf expression in the neuroretinal cells is necessary to establish the identity of this tissue. Chx10, a transcription factor expressed in all neuroretinal progenitors in developing retina and in bipolar cells in adult retina, is required for neural retinal fate determination, retinal progenitor cell proliferation, and the differentiation of bipolar cells (45). Chx10-dependent fibroblast growth...
factor signaling has been shown to be important for the repression of Mitf expression in the neural retina (46, 93). In Chx10 mutant mice, Mitf expression persists in the precursors of the neural retina, and these cells fail to maintain their identity and transdifferentiate toward "RPE-like" cells (46, 94). The presence of at least one predicted binding site for in the putative promoter of the miR-183/96/182 cluster (Fig. 6A) suggests a regulatory role of Chx10 in the expression of the cluster. Thus, expression of miR-182 and miR-96 in neural retina may provide a mechanism for the down-regulation of Mitf by Chx10.

Although the absolute level of expression was low during the embryonic development, expression of miR-96, miR-182, and miR-183 increases by 1.6–4.7-fold from E10 to E14 and by 11–41-fold from E10 to E18 (supplemental Table 13), following the pattern of expression of Chx10 and development of the neural retina (47). The potential regulatory interaction and the coincidence of the developmental time courses suggest that miR-96 and miR-182 may play a key role connecting the functions of Chx10 and Mitf in the development and maintenance of the neural retina.

Retinal Circadian Rhythms and miRNA Expression—We found evidence for circadian variation in expression of 12 retinal miRNAs, including 10 miRNAs that were up-regulated at ZT17 from 1.25-fold (miR-124a, -103 and miR-182) to 3.28-fold (miR-106b), and two (miR-422a and -422b) that were down-regulated at ZT17 (51 and 25% respectively) (Table 2, part A). Although these changes in expression level are modest, they are in the same range as those of key circadian regulators such as Per2 (2.95-fold) and Bmal1 (1.26-fold) (70). Interestingly, miR-182 was among the group with higher level of expression at ZT17 and miR-96 and miR-183 also showed modest increases of expression at ZT17 as compared with ZT5 (1.13- and 1.19-fold, with p values of 0.30 and 0.14 respectively). To examine this more closely, we performed qRT-PCR profiling of miR-182 and miR-96 expression at 5-h intervals throughout the 24-h day and confirmed the diurnal variation in expression of both these miRNAs with peak and trough of expression for both at ZT13 and ZT5, respectively (Fig. 7B). The timing of the peak at ZT13 likely contributed to the fact that we only detected moderate differences in our expression array profile comparison between ZT5 and ZT17.

Stimulated by the circadian variation in expression of the miR-183/96/182 cluster, we examined the putative promoter of the polycistronic transcript and found several predicted binding sites for transcription factors known to be important in the regulation of circadian rhythm (Fig. 6A). These include multiple binding sites for RORA1 and RORA2, which act as activators of Bmal1 transcription in the suprachiasmatic nucleus (71, 72), and at least two potential binding sites for ELK1. Phosphorylation of ELK1 by ERK (extracellular signal-related kinase) is increased by photic stimulation and is involved in the regulation and photic resetting of free-running circadian rhythms (95). There is also a predicted binding site for hepatic leukemia factor, a member of the PAR bZip (proline and acidic amino acid-rich basic leucine zipper) transcription factor family, which is expressed rhythmically in liver and suprachiasmatic nucleus, and activates the expression of Per1 (96), a component of the core clock feedback loop. The presence of multiple predicted binding sites for transcription factors involved in the circadian clock mechanism is consistent with our observations of diurnal variation in miR-182 and miR-96 expression and supports our suggestion that these microRNAs may be involved in circadian variation in expression of genes in retina and other neurosensory tissues.

Additional evidence for this suggestion is provided by examination of the predicted targets of the miRNAs in the miR-183/96/182 cluster (supplemental Table 11). For example, adenyl cyclase VI (ADCY6), a predicted target for miR-182 and miR-96 by PicTar and TargetScan, has a circadian pattern of expression in the pineal body at both the mRNA and protein levels with an apex around ZT5 and nadir around ZT17 (74). These cyclical variations in ADCY6 expression contribute to the differential regulation of AANAT, which encodes serotonin N-acetyltransferase (acetyl-coenzyme Aarylalkylamine N-acetyltransferase), a key enzyme in the melatonin synthesis pathway in pineal gland (74). ADCY6 produces two transcripts, ADCY6_1 (GenBank™ accession number NM_015270) and ADCY6_2 (GenBank™ accession number NM_020983), differing by a 158-nt alternatively spliced exon that contributes to the open reading frame. There are six predicted target sites for each of miR-182 and miR-96 (Fig. 7A) in both ADCY6 transcripts. Among these are four predicted target sites in the 3’-UTR (nt 5125–5147, 5453–5474, 4793–4814, and 4982–5003) with complete complementarity to the seed sequences of miR-96 or miR-182 and overall complementarity of 55–59%. Our in vitro assays with a chimeric luciferase reporter/ADCY6 3’-UTR construct provide direct evidence that both miR-96 and miR-182 regulate ADCY6 (Fig. 7C). If ADCY6 is a physiological target of miR-182 and miR-96, we would expect that it would be expressed in retina and that it would show a circadian pattern that was roughly the inverse of that of miR-182 and miR-96 expression (Fig. 7B). To test this expectation, we performed quantitative RT-PCR of ADCY6 mRNA in the retinal RNA samples harvested every 4 h over 24 h. We found that ADCY6 is expressed in retina with a circadian pattern characterized by an ~3.3-fold variation with an apex at ZT9 and a nadir at ZT17 (Fig. 7B). As predicted, this expression pattern is the inverse of that of miR-96 and miR-182, which shows an apex at ZT13 and a nadir at ZT5 but about 4 h out of register. This delay may reflect the time required for miRNA-mediated process to reduce the level of a targeted mRNA in vivo. Importantly, these results support a model in which circadian variations in transcript abundance are achieved by variations in mRNA degradation rather than in or in addition to variations in mRNA synthesis.

There may be more general roles for miRNAs in the regulation of circadian rhythms. Many core circadian factors are themselves direct targets of transcriptional regulation by core oscillator components (72). In the current model, transcription activators, Clock and Bmal, heterodimerize and bind E box DNA elements in the promoters of Period (Per) and Cryptochrome (Cry) (72, 97, 98). The Period and Cryptochrome proteins associate in the Clock-Bmal complex to repress their own transcription, creating a 24-h-long feedback loop, the circadian rhythm (99–101). The orphan nuclear receptors RORA and Rev-erb balance Bmal1 expression through opposing activities: RORA activates (72) and Rev-erb suppresses Bmal1
expression (102). The involvement of the miR-183/96/182 cluster in circadian clock regulation may extend beyond its regulation on ADCY6. Interestingly, Clock is a predicted target for both miR-182 and miR-96 (Fig. 9A), and as discussed above, RORA may be a transcriptional activator for the miR-183/96/182 cluster. Clock is required for the normal expression of RORA (72). These observations suggest a model in which Clock, RORA, and members of miR-183/96/182 cluster constitute a feedback loop that adds another layer of regulation to the circadian clock machinery (Fig. 9B). miR-182 may be further involved in circadian regulation through modulating basic helix-loop-helix domain containing class b3 (BHLHB3, also called DEC2) (103, 104) and Casein kinase 1ε (CK1ε) (105), which are miR-182 targets predicted by PicTar (supplemental Table 11). BHLHB3 or DEC2, with DEC1, represses Clock/Bmal1-induced transactivation of Per1 expression (103), whereas CK1ε is involved in circadian clock regulation through phosphorylation of Per2 (106, 107). Finally, because each miRNA may regulate several hundred target mRNAs (14, 15), rhythmic changes in miRNA expression could produce rhythmic variation in expression for large numbers of genes amplifying the circadian pattern throughout the organism. These observations suggest that possibility of involvement of miRNA in the regulation of circadian variation merits additional investigation.

**Possible Disease Associations with miR-183/96/182 Cluster—**

The miR-183/96/182 cluster is localized to a 4-kb genomic region on mouse chromosome 6qA3, with conservation of synteny and order to human chromosome 7q32.2 and a similar but more compact arrangement in Zebrafish (Fig. 4A). Based on their expression level, specificity, and wide range of candidate targets important for sensory organ development and function, we anticipate that dysfunction of this miRNA cluster could contribute to a variety of disease phenotypes depending on the location and the nature of the mutations in this polycistronic miRNA gene. Mutations in the promoter region might interrupt the binding and action of certain transcription factors resulting in tissue-specific changes in the level of expression of the pri-miRNA, affecting all three mature miRNAs. Mutations in the mature miRNA sequence could change the strength and spectrum of the interactions with downstream targets. Changes in the regions flanking the mature miRNAs could influence the post-transcriptional splicing and maturation of the mature miRNAs in the cluster resulting in phenotypes specific for a single member of the cluster producing a phenotype that primarily involved a single sensory modality. For example, the locus responsible for a large multigenerational family with autosomal dominant nonsyndromic sensorineural hearing loss mapped to 7q32 (DFNA50) (108). The highest lod score was obtained from marker D7S530, only ~21 kb centromeric to miR-182. No disease-causing mutations were detected by sequencing the two flanking protein-coding genes, SMOH and UBE2H (108). Given our results and the mapping data, the miR-183/96/182 cluster is a strong candidate for DFNA50. Interestingly, Adcy6 has recently been shown to be expressed in the inner ear, interacting with a key component of the ankle-link complex in cochlear hair cells (117). Because our results showed that members of miR-183/96/182 cluster regulate the expression of Adcy6, mutations disrupting this regulatory mechanism may also result in abnormal functions of the inner ear.

Finally, several genome-wide studies on autism have provided evidence for an autism-susceptibility gene near 7q32.2 in a subgroup of patients (109–114), including a recent study in which the STS marker D7S530, located only 21 kb centromeric to miR-182, provided the most significant linkage score (110). Autism is characterized by impaired social interactions, communication defects, and repetitive behavior patterns with evidence of defects in sensory processing (115, 116). It is intriguing to speculate that a defect in the miR-183/96/182 cluster could account for the sensory processing defects and contribute to autism.

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Image 360x26 to 388x38
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