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

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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)[3] 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 complementary inhibits translation of the targeted mRNA by an unknown mechanism (9), whereas binding with perfect complementary leads to cleavage of the corresponding mRNAs (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 mRNA 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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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1–14 and Fig. 1.

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[3] The abbreviations used are: miRNA, microRNA; RPE, retinal pigmented epithelium; pri-miRNA, primary miRNA; ZT, Zeitgeber time; qRT, quantitative reverse transcription; E, embryonic day; P, postnatal day; UTR, untranslated region; nt, nucleotide.

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 ectoderm, 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, *rd*1, were purchased from The Jackson Laboratories. 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 *rd*1 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-

ization artifacts on the array images: BA10458, BA10440, BA10339, BA10333, BA10283, BA10442, BA10376, BA10245, BA10479, and BA10351.

In the differential expression between day (ZT5) and night (ZT17) RNA samples, we calculated the fold of change for each miRNA as specific signal-ZT17/specific signal-ZT5. We evaluated the significance of differential expression using a two-tailed pairwise Student's *t* test between the specific signal intensity at ZT5 and the one at ZT17. The miRNA genes with $p < 0.1$ are identified as differentially expressed.

RT-PCR—We purchased *mir*Vana qRT-PCR primer sets of *mmu-miR-7, mmu-miR-9, mmu-miR-31, mmu-miR-96, mmu-miR-181c, mmu-miR-182, mmu-miR-185, mmu-miR-194, mmu-miR-210, mmu-miR-219, mmu-miR-320, mmu-miR-335, mmu-miR-361,* and mirVana qRT-PCR miRNA detection kit from Ambion and used 5 S rRNA as a normalizing control. For real time quantitative PCR (qPCR), we used 10 ng of total RNA and an Opticon 2 real time detector in a DNA engine 2 (Bio-Rad) with SYBR Green I for detection. For end product PCR, we used a DNA Engine Dyad Peltier thermal cycler and separated the product on 3.5% high resolution agarose electrophoresis gel. We used NCod SYBR Green miRNA first-strand synthesis and qRT-PCR system (Invitrogen) to amplify *mmu-miR-9-AS, mmu-miR-25, mmu-miR-92, mmu-miR-106b, mmu-miR-183, mmu-miR-184, mmu-miR-211, mmu-miR-140-AS,* and *rno-miR151-AS* with the forward primer (Sigma Genosys) sequences as the corresponding mature miRNA sequences.

For the circadian rhythm studies on *miR-182* and *miR-96*, we amplified 10 ng of total mouse retinal RNA prepared at ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21. Three RNA samples from each of three mice were amplified at each time point. The relative quantity of the *miR-182* and *miR-96* was normalized to the 5 S RNA quantitation. We used a nonparametric one-way analysis of variance test to determine the significance of the differences. For the circadian rhythm study on *Adcy6* expression, we employed a QuantiFast SYBR Green RT-PCR system (Qiagen) with Mm_Adcy6_1_SG QuantiTect primer set (Qiagen, QT00136850) and used 18 S rRNA amplified with primer set (Qiagen, QT01036875) to normalize. We followed the manufacturer's protocol for the qRT-PCRs.

In Situ Hybridization—We purchased 5′-digoxigenin-labeled miRCURY LNA detection probes for *mmu-miR-182, mmu-miR-183,* and *mmu-miR-96* from Exiqon and used ~6 pmol of probe on each section. We prepared fresh-frozen adult mouse retinal sections (10 μm) and followed the manufacturer's protocol with minor modifications. We prehybridized the sections in hybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 10 mM NaPO4, pH 8.0, 10% dextran sulfate, 1× Denhardt's, 0.5 mg/ml baker yeast RNA) for 1 h at room temperature. We diluted ~6 pmol of the probe in 300 μl of hybridization buffer for hybridization. The hybridizations were carried out at 52 °C overnight in a humidified chamber.

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.

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 2*A*); five were expressed in heart but not brain (supplemental Table 2*B*); 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. *miR-*

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. 3*D* 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.

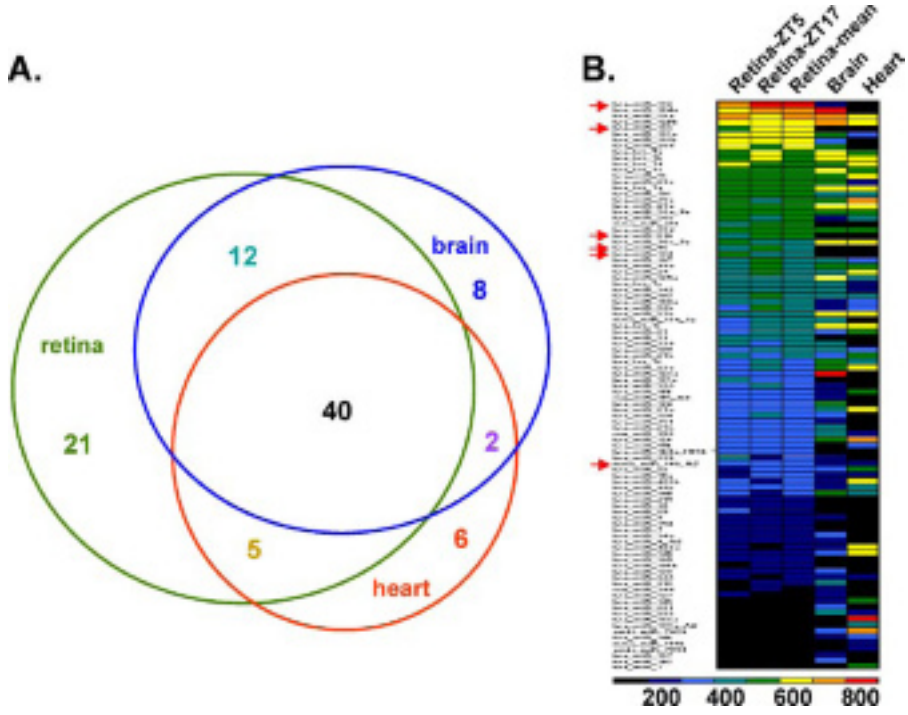

FIGURE 1. **miRNA profiling in adult mouse retina, brain, and heart.** *A,* Venn diagram summarizing overlapping expression of miRNAs; *B,* heat map of miRNA expression profile in all three tissues. *Arrows* indicate miRNAs expressed exclusively in retina as confirmed by RT-PCR. *Retina-ZT5*, retinal RNA isolated at ZT5 (local time 12:00 p.m.); *retina-ZT17*, retinal RNA isolated at ZT17 (local time 12:00 a.m.). The key for the color coding of the heat map is shown *below* with the numbers indicating relative signal intensity.

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. 4*A*). 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	9p21.3	325.6	303.5
8	hsa_miR_181c	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	16q22.1	273.2	208.1
12	hsa_miR_25	5qG1	7q22.1	263.3	116.0
13	hsa_miR_361	XqD	Xq21.2	260.0	255.8
14	hsa_miR_320	14qD1	8q21.3	249.4	305.5
15	hsa_miR_219	17qB2/2qA3	6p21.32/9q34.11	193.2	194.0
16	hsa_miR_92	14/XqA5	13q31.3/Xq26.2	192.7	188.3
17	hsa_miR_194	1qH4/19qA	1q41/11q13.1	189.1	155.6
18	hsa_miR_9_AS	9qF1/13qC3/7qD2	1q23.1	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

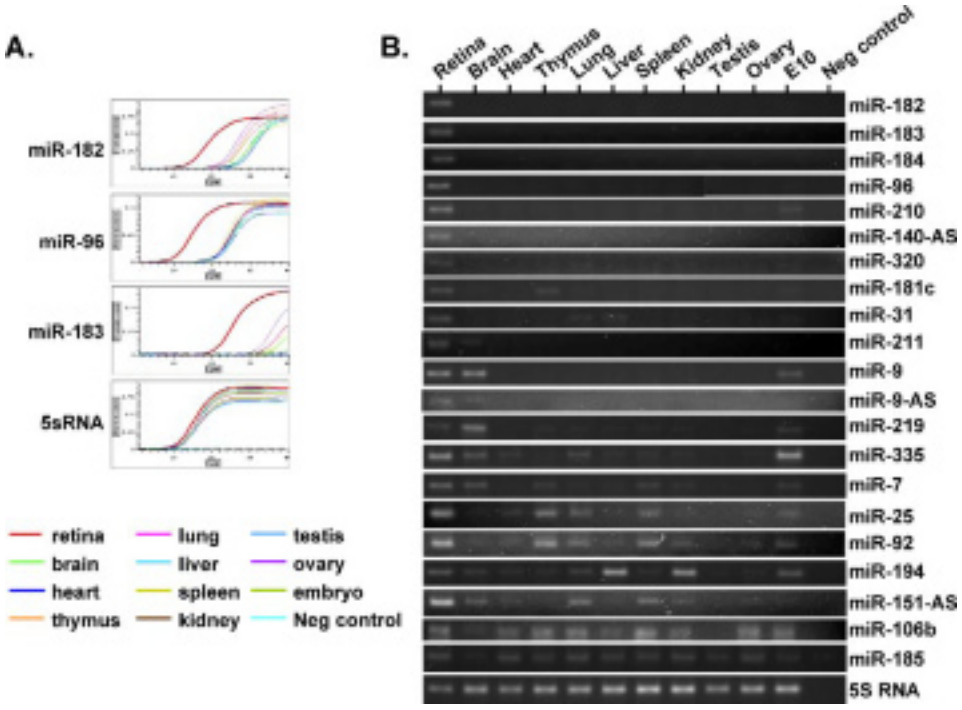

FIGURE 2. **Confirmation of tissue specificity by RT-PCR.** *A,* multitissue qRT-PCR of *miR-96*, *miR-182*, and *miR-183*; *B*, multitissue end product RT-PCR of candidate retina-specific miRNAs.

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. 4*B*). All three of these miRNAs have a similar developmental expression pattern (Fig. 4*D*). 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

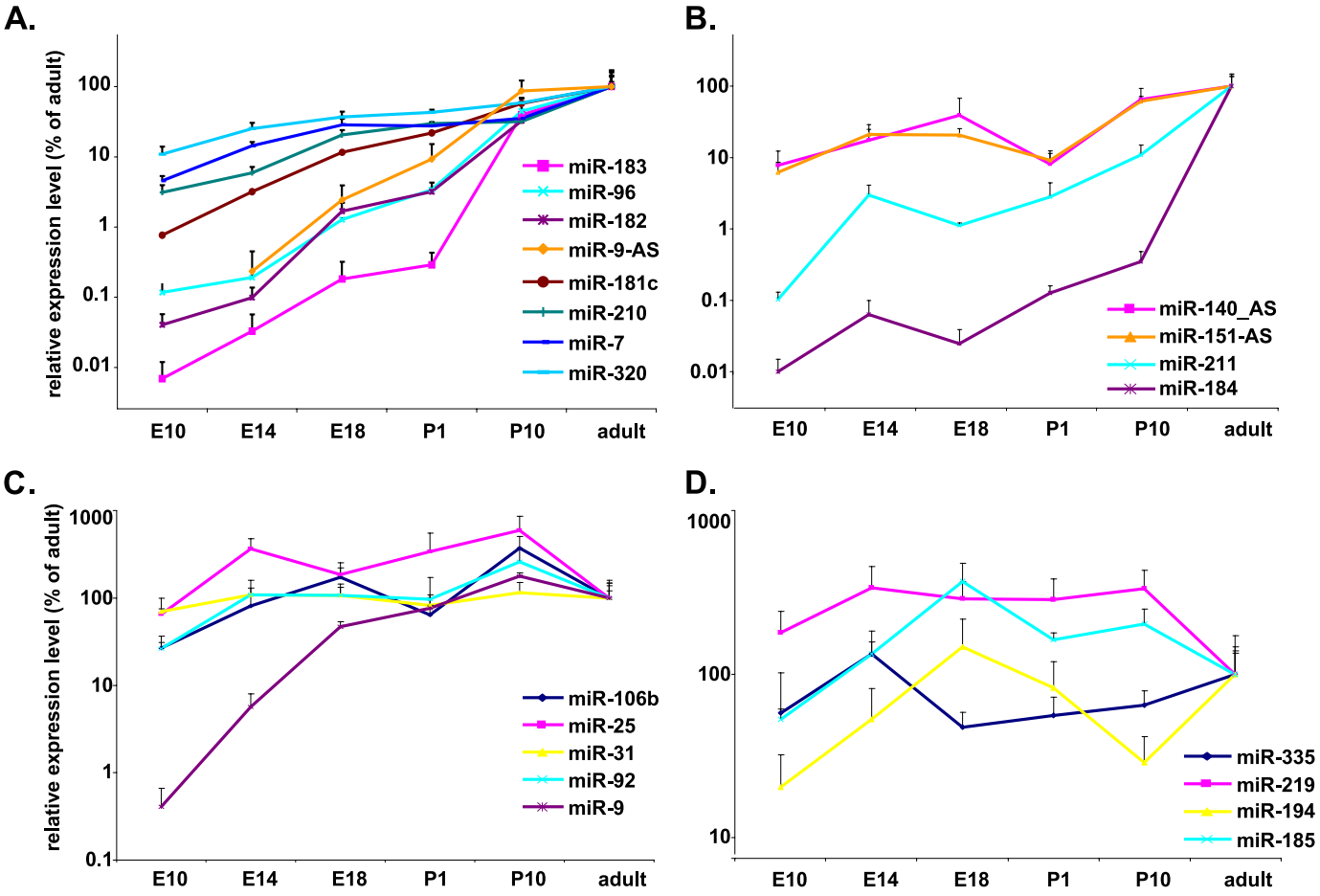

FIGURE 3. **Developmental expression pattern of miRNAs with preferential or specific retinal expression in mouse retina.** *A,* miRNAs with increasing expression and peak at adult retina; *B*, miRNAs with peak expression in adult retina and a surge at E14 or E18; *C*, miRNAs with peak expression at P10; *D*, miRNAs with peak expression at E14 or E18.

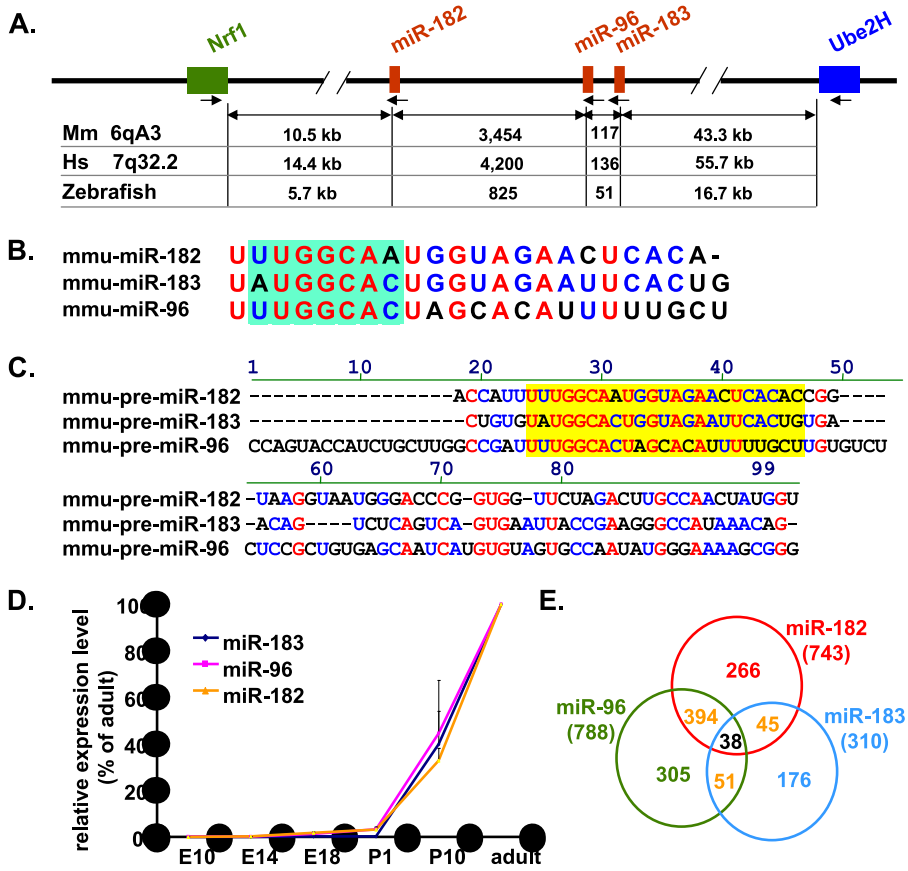

FIGURE 4. **Genomic organization, sequence, and predicted targets for the *miR-183/96/182* cluster.** *A,* genomic organization of the cluster is similar in mouse, human, and zebrafish. The *numbers* show the spacing in each of the three species in bp or kb. *B,* alignment of mature *miR-182*, *miR-96*, 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-miRNAs sequence 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.

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. 5*A*). 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 the remaining cellular constituents of neural retina (42). In support of our *in situ* results, we found easily detectable levels of all three miRNAs in the RNA from *rd1/rd1* animals (Fig. 5, *B* and *C*).

As we were preparing this manuscript, Weston *et al.* (43) reported expression of *miR-96*, *miR-182,* and *miR-183* in mouse inner ear, and Kloosterman *et al.* (44) showed that this same set of three miRNAs was expressed in the dorsal root ganglia. Based on these observations and our results, we considered the hypothesis that this miRNA cluster is a sensory tissue-specific miRNA cluster. To test this idea, we performed RT-PCR on RNA isolated from two additional sources of sensory neurons: mouse olfactory epithelium and lingual epithelium, which includes taste buds. Our results confirmed that *miR-96*, *miR-182,* and *miR-183* are highly expressed in olfactory epithelia 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. 6*A*). 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), RFX1 (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 mRNAs 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-183*/*miR-96* and 6 of the first 8 nucleotides are identical between *miR-182* and *miR-183* (Fig. 4, *B* 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. 4*E*); *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

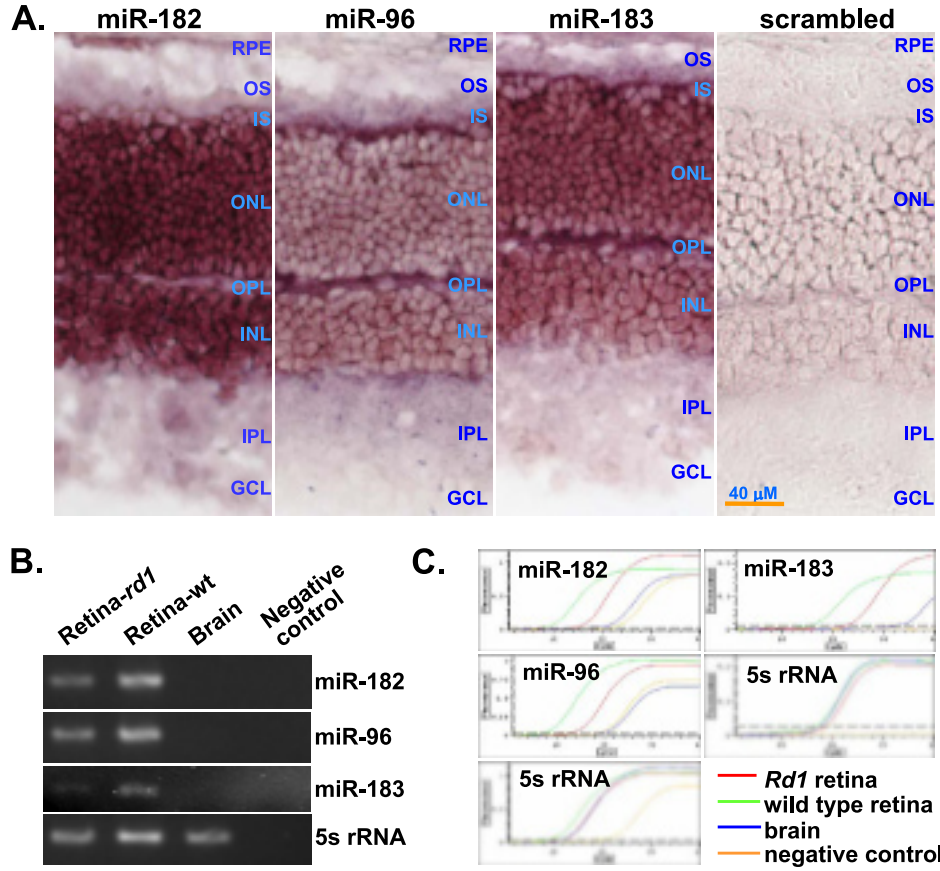

FIGURE 5. **Distribution of *miR-182, miR-96* and *miR-183* expression in retina.** *A, in situ* hybridization of *miR-182*, *miR-96,* and *miR-183* and a negative control probe with scrambled sequence to adult mouse retina. *RPE,* retinal pigment epithelium; *OS,* outer segment; *IS,* inner segment; *ONL,* outer nuclear layer; *OPL,* outer plexiform layer; *INL,* inner nuclear layer; *IPL,* inner plexiform layer; *GCL,* ganglion cell layer. *B*, RT-PCR for the indicated *miRNA* with RNA isolated from retina of *rd* mice (*Retina-rd1*), wild type retina (*Retina-wt*), and brain. *C*, qRT-PCR of *miR-182*, *miR-96,* and *miR-183* with RNA isolated from adult *rd*1 mouse retina, wild type mouse retina and a negative control.

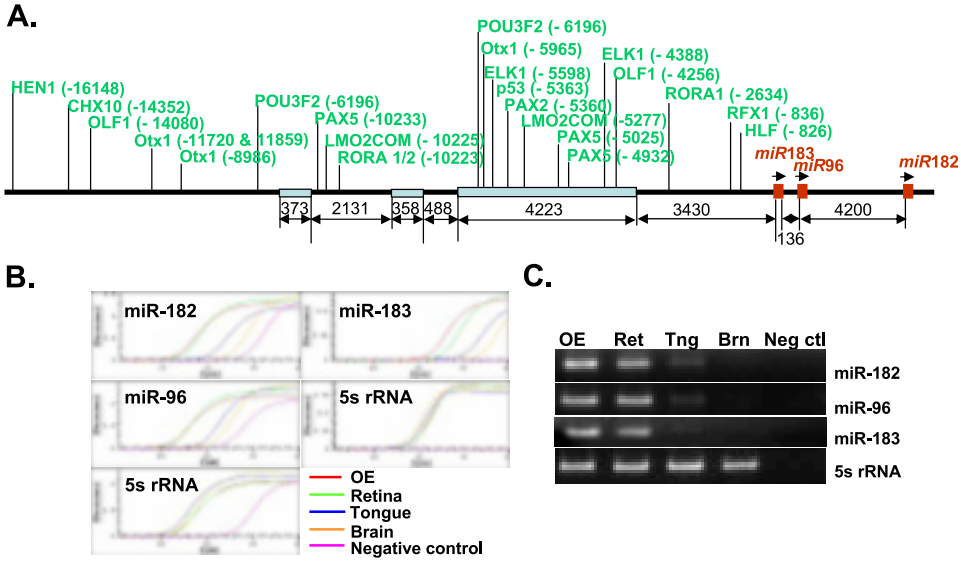

FIGURE 6. **The *miR-183/96/182* cluster may be sensory organ-specific.** *A,* potential 5′ upstream regulatory elements of the miRNA cluster on human Chr7q32.2 based on the March 2006, Human Genome Assembly of UCSC Genome Informatics. The *color rectangles* denotes CpG islands; the putative transcription factor binding sites are listed *above*; the *numbers* in the *parentheses* are the location of the putative binding sites in reference to the first nucleotide of *pri-miR-183*; the numbers labeled *below* are the sizes of the fragments in base pairs. *B*, detection of miRNA expression in the olfactory and lingual epithelia by qRT-PCR. *C,* detection of miRNA expression in the olfactory and lingual epithelia by end product RT-PCR.

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 (supplemental 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 BmalI 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. 7*A*) (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

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	121.57	3.28	0.019
hsa_miR_30b	255.5	114.9	404.98	109.16	1.59	0.022
hsa_let_7f	231.44	111.46	365.47	149.19	1.58	0.054
hsa_let_7i	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	183.4	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

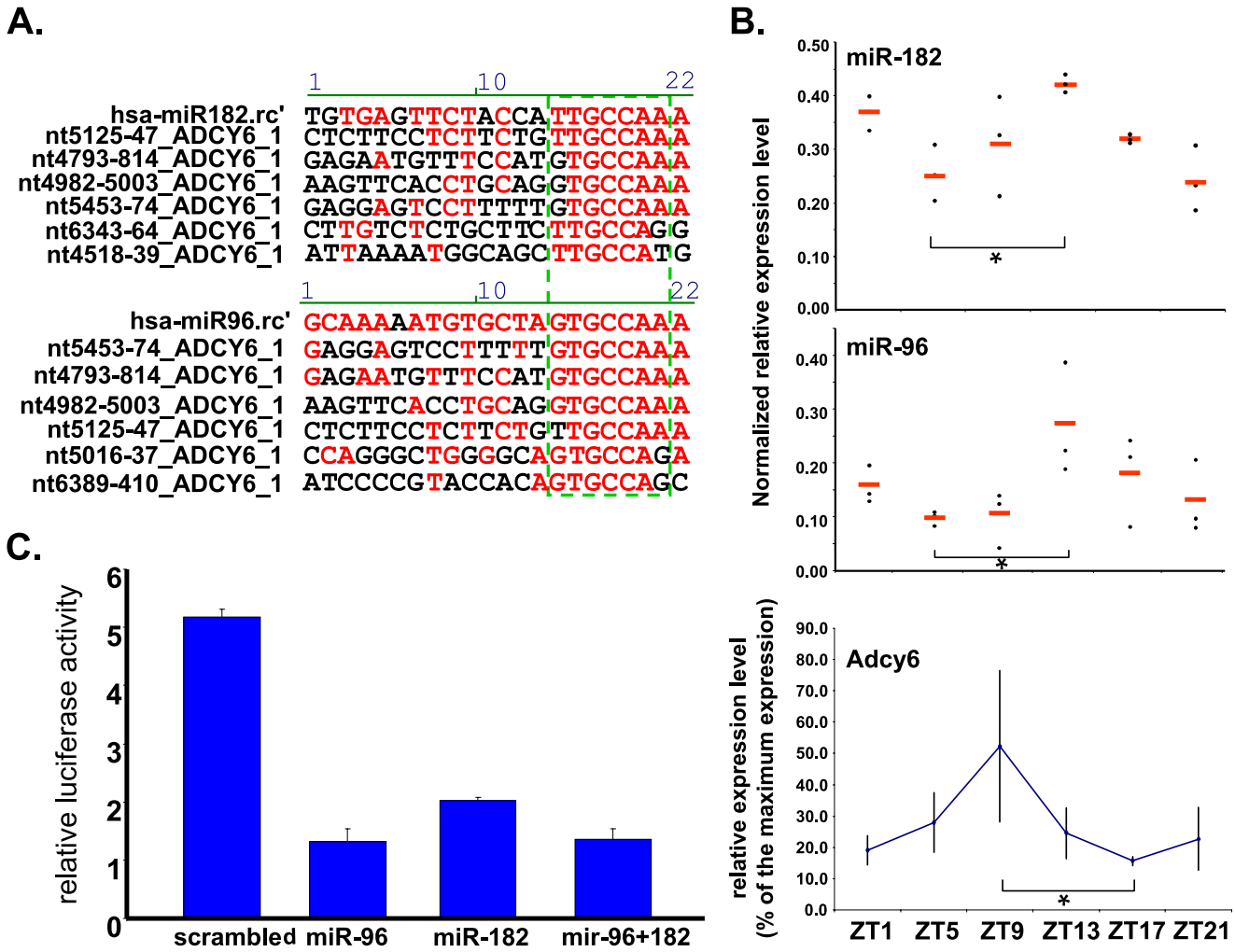

FIGURE 7. ***ADCY6* is a downstream target of *miR-182* and *miR-96*.** *A*, alignment of *miR-182* and *miR-96* with their potential target sites in *ADCY6*; the *green box* marks the seed sequences of *miR-96* and *miR-182* and the corresponding sequences in the potential target sites in *ADCY6*. *B*, 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.

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. 7*B*). To demonstrate that *Adcy6* is a direct target of *miR-183* and *miR-96*, we subcloned a 3′-UTR fragment of *Adcy6* (nt 4206–6520 of GenBank™ accession number NM_015270 with four potential target sites for *miR-96* and *miR-182*) into the luciferase reporter vector, pMIR-REPORT, 3′ 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-regulated luciferase activity by

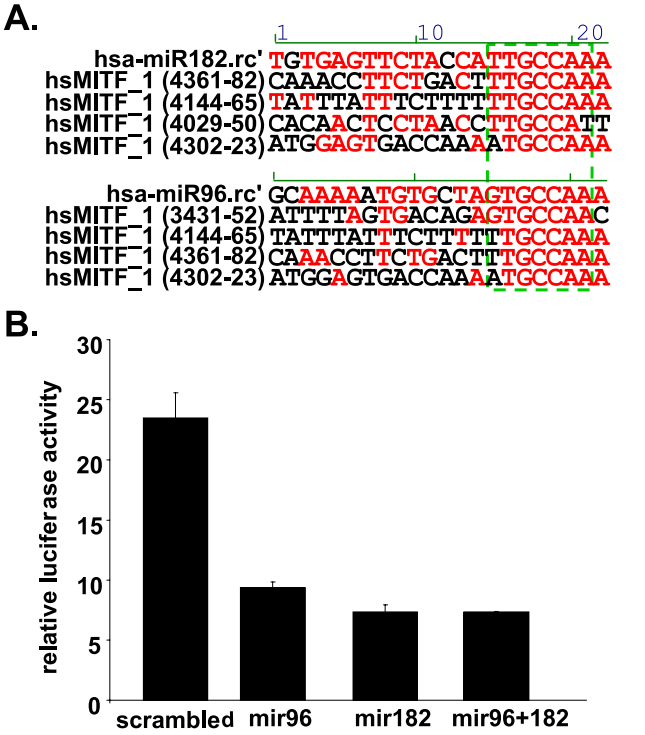

FIGURE 8. ***Mitf* is a targeted by *miR-182* and *miR-96*.** *A*, alignment of *miR-182* and *miR-96* with their potential target sites in *Mitf*; the *green box* marks the seed sequences of *miR-96* and *miR-182* and the corresponding sequences in the potential target sites in *Mitf*. *B*, *miR-96*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

~75% (±2%) and 61% (±7%), respectively (Fig. 7*C*). Co-transfection with *miR-96* plus *miR-182* had the same effect as *miR-96* alone (74 ± 4% inhibition). These results strongly support the prediction of *Adcy6* as a direct target of *miR-96* and *miR-182*.

The miR-183/96/182 Cluster Also Regulates Expression of Mitf—Microphthalmia-associated transcription factor (*Mitf*) (75), a key transcription factor required for the acquisition and maintenance of RPE cell identity (46), has four predicted target sites each for *miR-182* and *miR-96* (Fig. 8*A*), including two for *miR-182* (nt 4144–65 and nt 4361–82) and one for *miR-96* (nt 3431–52) that are perfectly complementary to the seed sequence of the respective microRNAs (Fig. 8*A*). To examine the possible regulatory function of *miR-182* and *miR-96* on Mitf, we subcloned a segment of the 3′-UTR of Mitf (nt 3219–4654 of GenBank™ accession number NM_198159, containing the potential target sites for both *miR-96* and *miR-182*) into the luciferase reporter construct. Co-transfection with *miR-96* and *miR-182* mimics inhibited luciferase activity by 60 and 69%, respectively, and by 69% in combination (Fig. 8*B*). These *in vitro* results strongly support the predictions of an inhibitory role of these miRNAs on *Mitf* function. The lack of additive inhibition suggests that, under the conditions of this assay, *miR-182* and *miR-96* compete for the same target sites.

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 *miR151-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 mRNAs 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 (*miR-7, -9, -25, -31, -92 -181c, -210, -219, -331,* and *-320*) in E10 embryos (Fig. 2*B*), 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. 1*B* 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 2*A*) but only five that were expressed in heart but not in brain (supplemental Table 2*B*). 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 retinal RNA (Fig. 3 and supplemental Table 13). These stages represent the

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-96*, *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. 3*D* 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. 4*A*). 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. 5*A*). 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. 3*A* and 4*D* 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. 3*A* and 4*D* 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. 6*A*). 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 epithelia 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. 8*A*). 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. 8*B*). 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. 6*A*) suggests a regulatory role of *Chx*10 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 *BmalI* (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 4-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. 7*B*). 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. 6*A*). These include multiple binding sites for RORA1 and RORA2, which act as an 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, adenylyl 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 A:arylalkylamine *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. 7*A*) 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. 7*C*). 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. 7*B*). 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. 7*B*). 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 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-Bmal1 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*

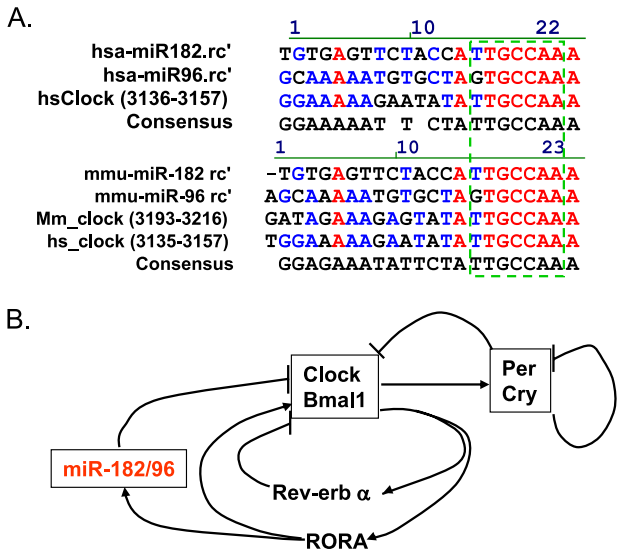

FIGURE 9. ***miR-182* and *miR-96* may be involved in the regulation of circadian core machinery.** *A,* alignment of *miR-182* and *miR-96* with their potential target sites in human (*top*) and mouse (*bottom*) *Clock* mRNAs; the *green box* marks the seed sequences of *miR-96* and *miR-182* and the corresponding sequences in the potential target sites in *Clock. B,* hypothetical involvement of *miR-182* and *miR-96* in the circadian clock feedback loop, modified from Sato *et al.* (72).

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. 9*A*), 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. 9*B*). *miR-182* may be further involved in circadian regulation through modulating basic helix-loop-helix domain containing class b,3 (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. 4*A*). 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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