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MicroRNAs of the mammalian eye display distinct and overlapping tissue specificity

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Purpose: In mammals, endogenous, noncoding RNAs, designated as microRNAs (miRNAs), inhibit the translation of a target messenger RNA, thereby silencing protein production. MiRNAs have been shown to regulate many aspects of development and differentiation in a wide range of tissues. Surprisingly, little consideration has been directed towards characterizing the expression of miRNAs in mammalian ocular tissues.
Methods: Low molecular weight (LMW) RNA isolated from the adult mouse corneal epithelium, lens/ciliary body, and a retina fractions of the eye was analyzed by miRNA arrays. The validity of the miRNA expression profiles were confirmed by northern blots and the tissue distribution of selected miRNAs was determined by in situ hybridization.
Results: MiRNAs exhibited distinct tissue and cell-type specificity in the ocular regions studied. MiRNA (mir)-184 had the highest hybridization signal in the corneal and lens arrays. In situ hybridization analysis revealed that mir-184 was expressed in the basal and immediately suprabasal cells of the corneal epithelium. In contrast, expression of mir-205 was detected throughout the anterior segmental epithelia as well as in the epidermis. Within the lens, expression of mir-184 was more strongly expressed in the epithelial cells of the germinative zone, whereas expression of mir-204 was uniformly expressed in all lens epithelial cells. Mir-181, -182, and -183 were detected in retinal and brain tissues, and their distribution patterns within the retina were both distinct and overlapping.
Conclusions: The tissue and cell specificity of ocular miRNAs suggests that these noncoding RNAs may be regulating aspects of development and differentiation.

MicroRNAs (miRNAs) are small (about 22 nucleotides in length), noncoding RNAs present in a wide variety of organisms ranging from plants to humans that have structural, enzymatic and regulatory functions (for reviews see [1-6]). MiRNAs represent a major class of regulatory molecules in animals that are phylogenetically conserved [2,7]. Mature miRNAs are produced from a larger precursor that is encoded in the genome. In vertebrates, miRNAs function to repress gene activity by binding with imperfect complimentarity to conserved sites in the 3' untranslated regions (UTRs) of target transcripts, thereby blocking the translation of mRNA transcripts into protein [3,8-10].

Many miRNAs appear to be expressed in a tissue-specific and/or developmentally regulated manner. One of the earliest insights into miRNA function was the identification of *lin-4* and *let-7* as miRNAs in *Caenorhabditis elegans* (*C. elegans*). These two miRNAs were demonstrated to be required for cell-fate switches at specific times during larval development [11,12]. Of the miRNAs that have thus far been identified in vertebrates, only a few have been assigned a specific function. For example, in the mouse, mir-181 is restricted to hematopoietic cells and the forced expression of this miRNA in progenitor cells favors the development of B over T cells [13]. Interestingly, this miRNA is also present in ocular tissues [14]. In cultures of human primary subcutaneous preadipocytes, increasing the mir-143 levels induced adipocyte formation, while inhibition of this miRNA blocked adipocyte differentiation [15]. A comprehensive study of the expression patterns of 119 miRNAs in adult organs from mice and humans revealed that at least 30 miRNAs were specifically expressed or enriched in a particular organ (e.g., brain, lung, liver, skeletal muscle), suggestive of tissue-specific functions [16]. Likewise, during zebrafish development, many miRNAs exhibit tissue-specificity indicating that these regulatory RNAs play significant roles in differentiation and/or the maintenance of tissue identity [17].

Although miRNAs are present in all mammals and have important regulatory roles in development and differentiation, little attention has been directed towards characterizing the expression of miRNAs in mammalian ocular tissues. In a single study of adult mice, miRNAs were identified by cloning low molecular weight RNA from a variety of tissues including the whole eye [14]. Six miRNAs were exclusively found in the eye; however, no further characterization of these eye-related miRNAs was carried out. In the present study, we analyzed the corneal epithelium, lens, ciliary, and retinal tissues from adult mice using miRNA arrays, northern blotting and in situ hybridization. Furthermore, we show that the expression of mir-184, the most abundant miRNA in both corneal and lens epithelia, is not altered by the proliferative status of the corneal epithelium. Our findings indicate that most ocular miRNAs are preferentially expressed in a tissue restricted manner. This is consistent with a regulatory function in ocular development and maintenance.

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METHODS

Animal care and use conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animal protocols were approved by the Institutional Animal Care and Use Committee of Northwestern University School of Medicine.

Isolation and preparation of ocular tissues: We dissected fresh whole eye globes, footpad, tongue, liver, hindbrain, kidney, heart, and spleens from 5-week-old mice. We isolated corneal epithelium from adult CD-1 mice eyes (n=100) by incubating whole globes in phosphate buffered saline containing 20 mM EDTA for 1 h at 37 °C, after which intact sheets of corneal epithelium were removed from the underlying stroma [18]. The entire lens was removed from the remaining ocular tissues. During removal of the lens, thin, thread-like material, most likely the ciliarus muscle (see Results) and possibly the ciliary body were occasionally attached to the surface of the lens. Accordingly, we have named this fraction "lens/ciliary" to reflect this fact. The material that remained following removal of the anterior segment and the lens/ciliary fraction was designated as the "retina-rich" fraction. We isolated relatively pure epithelium from mouse foot pads (n=6) by removing the underlying stroma, subcutaneous fat, and tendons with gentle scraping using a scalpel blade.

Isolation of low molecular weight (LMW) RNA: We extracted total RNA from the dissected tissues using Trizol (Invitrogen, Carlsbad, CA). LMW RNA was separated from the total RNA using mirVana miRNA purification columns (Ambion, Austin, TX). Samples (100 ng) of the starting total RNA and high molecular weight RNA depleted of miRNAs were analyzed by capillary electrophoresis (Agilent Bioanalyzer, Palo Alto, CA) to assess the degree of purification. Absorbance peaks corresponding to 5S, 18S, and 28S remained unchanged in the depleted flow-through fraction compared with the total RNA sample. However, a set of peaks corresponding to the small (micro) RNAs in the total RNA sample were absent in the fractionated flow through RNA.

Array analysis: We analyzed the LMW RNAs that we prepared from adult mouse corneal epithelium and compared it with epithelial-rich footpad using miRMAX™ (Bionomics Research and Technology Center, Rutgers University, Rutgers, NJ) and NCode™ Multi-Species miRNA (Invitrogen) arrays. In a separate series of experiments we used the NCode™ Mult-Species miRNA arrays to obtain miRNA expression profiles of the lens/ciliary and retina-rich fractions. These arrays contain all known miRNA genes from human, rat, mouse, Drosophila, and *C. elegans*. Briefly, the mature miRNAs in 200 ng of LMW RNA were labeled using either a Genisphere Array 900miRNA Direct kit for labeling or a NCode™ miRNA Labeling System, and then hybridized to the array chips. The corneal- and footpad-epithelial samples were labeled with either Cy5 or Cy3. Labeling and hybridizations were performed by the Bionomics Research and Technology Center, Rutgers University, Rutgers, NJ, and the Array Core Facility at Northwestern University, Chicago, IL.

Northern blots: Total RNA was fractionated on a 15% denaturing (8 M urea) polyacrylamide gel, transferred to nylon membranes (Nytran N; Amersham Biosciences, Buckinghamshire (Bucks), U.K.) and fixed by UV cross-linking. Membranes were probed with ^{32}P-labeled oligonucleotides complementary to the miRNAs. Hybridizations were carried out at 29 °C in 6X SSC, 5X Denhardt's, 0.2% SDS for 8 h. Membranes were washed three times in 6X SSC, 0.2% SDS at room temperature, and once at 42 °C in the same buffer.

In situ hybridization: Tissues for in situ hybridization were fixed in 4% paraformaldehyde, dehydrated in a graded series of ethanol baths, embedded in paraffin, and 5 μm sections were cut and picked up on Superfrost™ slides. Locked

Table 1.

MicroRNA	Cornea	Footpad
miR-184	6110	69
miR-205	3900	2070
miR-26a (b)	1940	2922
miR-200b (c,a)	1940	421
miR-31	1900	63
miR-24	1850	5187
miR-23a (b)	1840	8828
miR-204	1550	46
miR-30c (d,a,b)	1360	1525
miR-125b (a)	1280	2436
miR-450	1275	41
miR-217	1135	1435
miR-16	1110	1371
miR-341	1035	149
miR-203	910	17881
miR-27b (a)	875	4181
miR-29a	865	2597
miR-130a	745	445
miR-181a (b)	745	100
miR-182	720	68
let-7 (f)	1420	1325

The Highest Expressed miRNAs in corneal versus footpad epithelia. The numbers under the "Cornea" and "Footpad" column indicate the relative intensity of the hybridization signal. The letters in parentheses represent other family members with lower signal intensities and the "f" in parenthesis indicates entire family.

nucleic acid (LNA)-modified, digoxigenin (DIG)-labeled probes were generated by Exiqon (Vedbaek, Denmark). The LNA technology chemically modifies the sugar-phosphate backbone of the oligonucleotide to increase thermal stability, which improves hybridization properties [19]. Deparaffinized sections were treated for 5 min with proteinase K (10 μg/ml) followed by 30 s in 0.2% glycine. Sections were refixed in 4% paraformaldehyde for 10 min, washed twice in PBS, and were prehybridized for 2 h in hybridization buffer (50% formamide, 5X SSC, 0.4% Tween, 9.2 mM citric acid [pH 6], 50 μg/ml heparin, 500 μg/ml yeast RNA). Tissues were hybridized overnight in the presence of 20 nM digoxigenin labeled probe at 21 °C. Slides were washed twice in 2X SSC at 37 °C followed by a high stringency wash in 50% formamide, 2X SSC at hybridization temperature. Immunological detection was carried out with the anti-digoxigenin Fab conjugated to alkaline phosphatase (Roche, Mannheim, Germany) according to manufacturer's recommendation.

Corneal wounding and detection of rapidly cycling cells: Seven- to 10-week-old male and female CD-1 mice (n=6) were anesthetized with gamma-hydroxybutyric acid (ip injection of 100 μl of 10% solution in PBS). A 1.5 mm central corneal area was demarcated with a trephine and the corneal epithelium was physically removed with a rotating diamond burr. Twenty-four and 48 h following wounding, mice received BrdU (50 μg/g; ip), and were sacrificed 1 h after injection. The entire eyes were removed and processed for histology, in situ hybridization and immunohsitochemistry as described previously [20].

RESULTS

MicroRNAs and the anterior surface epithelia: microRNA expression profiling: To obtain a representation of the miRNAs present in the adult mouse corneal epithelium, we performed array analyses on LMW RNA isolated from corneal epithelium and compared it with LMW RNA isolated from the epithelial-rich footpad (Table 1). We chose the footpad epithelium as another example of a stratified epithelium for comparison with the corneal epithelium. Repeated analyses from two different facilities consistently revealed that miRNA (mir)-184 had the highest hybridization signal of all the corneal epithelial miRNAs; its corneal expression was about 90 fold greater than that detected in the footpad epithelium. Mir-31 and mir-204 also had high hybridization signals in corneal epithelium and relatively low signal intensities in the footpad fraction. Mir-205 had the second highest hybridization signal overall; however, this miRNA was also highly expressed in the footpad epithelium (Table 1). Within the footpad epithelium, mir-203 had a hybridization signal that was about 20 fold greater than that observed in corneal epithelium making it a preferentially expressed footpad epithelial miRNA (Table 1).

To validate our array findings, we assessed RNA isolated from preparations of corneal and footpad epithelia for the presence of some of the highest expressed miRNAs by northern analysis. We found high concordance between the array and northern data (Figure 1). For example, mir-184 was clearly abundant in the corneal epithelial RNA and absent in RNA from the footpad epithelium (Figure 1). Likewise, mir-204 and -31 were also expressed in corneal epithelium and were absent in footpad epithelium (Figure 1). Relatively equivalent signals for mir-205 and let-7, two highly expressed miRNAs, were observed in both preparations (Figure 1), consistent with the array data (Table 1). Converse to what was observed for mir-184, mir-203 was abundantly expressed in the RNA isolated from the footpad epithelium and was absent in corneal epithelium (Figure 1). Taken together, the northern analysis was uniformly consistent with the array findings and validated the miRNA expression profiles.

The high hybridization signal intensities seen for mir-184 and -205 compared to what was detected for let-7, a miRNA generally expressed in high levels in many tissues [21,22], prompted us to investigate whether other tissues expressed these miRNAs. Thus we determined the distribution of mir-184 and mir-205 in corneal epithelium, footpad, tongue, small intestine, epidermis, brain, heart, liver, kidney, and spleen (Figure 2A,B). Unexpectedly, mir-184 was absent from all tissues

Figure 1. MicroRNAs show varying distribution with corneal and footpad epithelia. Total RNA from adult mouse corneal (Co) and footpad (Fp) epithelia were analyzed by Northern hybridization with ^{32}P-labeled oligonucleotides to mir-184, -204, -31, 204, let-7, and -203. Expression of mir-184, -204, and -31 is restricted to the corneal epithelium whereas mir-203 is preferentially expressed in the footpad epithelium. Expression of mir-205 and let-7 are noted in both corneal and footpad RNA. The 5S ribosomal fraction serves as the loading control.

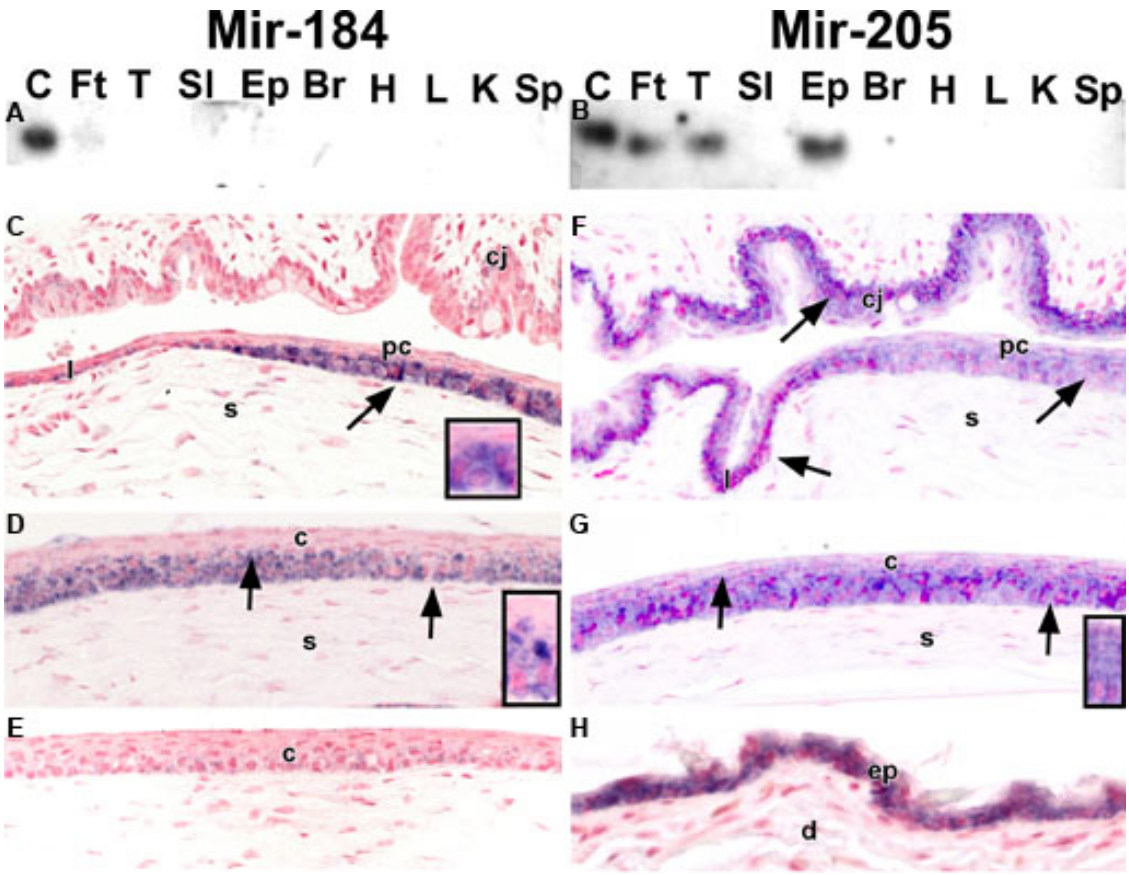

Figure 2. Epithelial miRNAs exhibit tissue and cell-type specificity. **A** and **B**: Total RNA (10 μg) from mouse corneal epithelium (C), footpad epithelium (Ft), tongue (T), small intestine (SI), epidermis (Ep), brain (Br), heart (H), liver (L), kidney (K), and spleen (Sp) were analyzed by northern hybridization with ^{32}P-labeled oligonucleotides to mir-184 (**A**) and -205 (**B**). Mir-184 was abundant solely in corneal epithelium (**A**), whereas mir-205 was present in the RNA from several epithelial tissues (**B**). **C**-**H**: Mouse anterior segmental epithelia (**C**-**G**) and epidermis (**H**) were processed for in situ hybridization with digoxygenin-labeled antisense probes for mir-184 (**C** and **D**) and mir-205 (**F**-**H**) and a scrambled oligonucleotide sense (**E**) control. Mir-184 is expressed in corneal (c) but not limbal (l) and conjunctival (cj) epithelia. Mir-184 RNA is observed (arrows) in basal and immediately suprabasal cells of the corneal epithelium. Little if any signal for mir-184 is seen in the wing and superficial cells of the corneal epithelium (**C** and **D**) Boxed areas in **C** and **D** show mir-184 expression restricted to basal and wing cells. Mir-205 RNA is present in all layers of the peripheral (pc) and central corneal (c) epithelia (**F** and **G**) as well as the limbal (l) and conjunctival (l) epithelia (**F**). Boxed area in **G** shows mir-205 expression in all layers of the corneal epithelium. A strong signal for mir-205 is also detected throughout the mouse epidermis (e; **H**). In the image, s denotes the stroma and d indicates dermis.

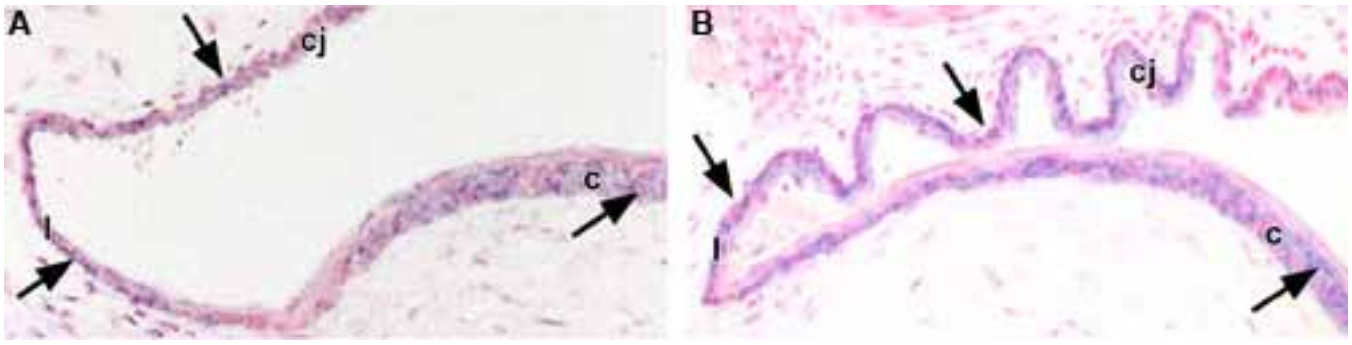

Figure 3. MiRNAs have varied expression patterns within the epithelium of the anterior segment. **A** and **B**: A portion of the adult mouse anterior segmental epithelium processed for in situ hybridization with a digoxygenin-labeled probes for mir-182 (**A**) and -217 (**B**). In the corneal (c) epithelium, mir-182 is expressed in the basal, wing and superficial cells (arrows), as well as throughout the limbal (l) and conjunctival (cj) epithelia. In contrast, within the corneal epithelium, mir-217 is expressed primarily in the basal and suprabasal cells (arrows) and not in the superficial cells. Mir-217 is also expressed in the limbal (l) and conjunctival (cj) epithelia.

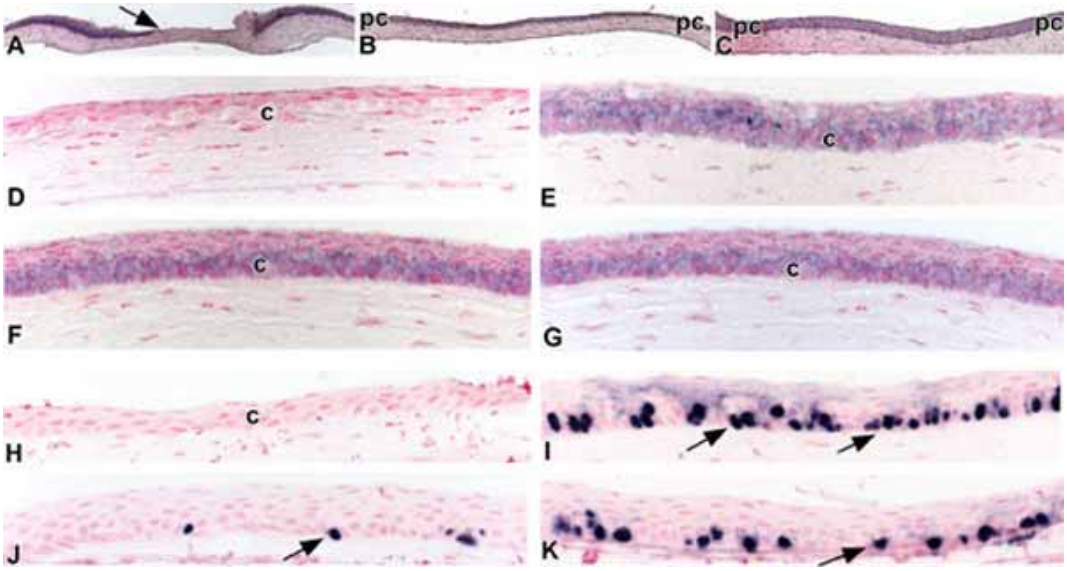

Figure 4. Mir-184 expression in mouse corneal epithelium is independent of proliferation. **A**-**C**: Application of a rotating diamond burr to the surface of the central cornea resulted in the removal of the corneal epithelium (**A**; arrow), whereas the peripheral corneal epithelium was intact (pc). Within 24 h (**B**) the corneal epithelial surface was reepithelialized, and by 48 h (**C**) the corneal epithelium returned towards a normal phenotype. **D**-**G**: Corneal tissues were processed for in situ hybridization with a digoxygenin-labeled antisense probe for mir-184. Twenty-four h post wounding, no signal for mir-184 was detected in the re-epithelialized central corneal epithelium (**D**); however, a strong signal was observed throughout the adjacent (pc) nonwounded corneal epithelium (**E**). Forty-eight h post wounding, strong expression of mir-184 is seen in both the reepithelialized (**F**) and adjacent (pc) nonwounded (**G**) corneal epithelia. **H**-**K**: Twenty-four (**H**,**I**) and 48 h (**J**,**K**) following the creation of scrape wounds, mice received a pulse of BrdU interperitoneally to label those cells in the S phase of division. Mice were sacrificed 1 h later. Twenty-four h postwounding, no BrdU labeled cells were detected in the reepithelialized central corneal epithelium (**H**); however, numerous BrdU labeled basal cells (arrows) were noted in the adjacent (pc) nonwounded corneal epithelium (**I**). Forty-eight h post wounding, some BrdU labeled basal cells (arrows) were noted in the reepithelialized corneal epithelium (**J**), while there was a reduction in BrdU-labeled basal cells (arrows) in the adjacent (pc) nonwounded (**K**) corneal epithelium.

Table 2.

MicroRNA	Lens/Cil	MicroRNA	Retina
miR-184	5370	miR-181a	>6555
miR-125b (a)	2075	miR-125b (a)	>6555
miR-31	1965	miR-26a (b)	>6555
miR-204	1780	miR-124a	>6555
miR-26a (b)	1735	miR-204	4635
miR-181a (b)	1085	miR-30c	4370
miR-30c (d,a,b)	750	miR-182	4300
miR-23a (b)	655	miR-183	3970
miR-450	530	miR-23b	3550
let-7 (f)	1420	let-7 (f)	>6555

The highest expressed miRNAs in lens/ciliary and retina-rich preparations of adult mouse eyes. The numbers in the "Lens/Cil" and "Retina" columns indicate the relative intensity of the hybridization signal. The letters in parentheses represent other family members with lower signal intensities and the "f" in parenthesis indicates entire family.

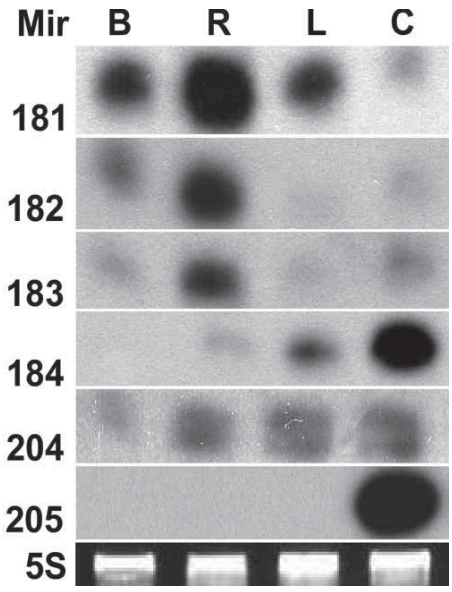

Figure 5. MicroRNAs show distinct tissue specificity. Total RNA from adult mouse brain (B), retina-rich (R), lens (L) and corneal epithelial (C) fractions were analyzed by northern hybridization with ^{32}P-labeled oligonucleotides to mir-181, -182, -183, -184, -204, and -205. Within the lens, strong expression is noted for mir-181, -184, and -204, while mir-181, -182, -183, and -204 are strongly expressed in the retina and brain. Note the absence of mir-205 expression in the lens. The 5S ribosomal fraction serves as the loading control.

surveyed with the exception of the corneal epithelium (Figure 2A). In contrast, mir-205 was detected in footpad, tongue, epidermis, and corneal epithelium; no signal was detected in RNA from small intestine, brain, heart, liver, kidney, and spleen (Figure 2B). This suggests that mir-205 might represent a stratified squamous epithelial miRNA.

MicroRNAs and the anterior surface epithelia: microRNA distribution patterns: We used in situ hybridization to determine the distribution patterns of some of the more highly expressed corneal epithelial miRNAs. The expression pattern for mir-184 confirmed and extended the array and northern data. A strong signal for mir-184 was detected primarily in the basal and immediately suprabasal cells of the corneal epithelium from a 21 day old mouse (Figure 2C,E). Very little signal was detected in the superficial cells (Figure 2C,E) In contrast, expression of mir-184 was absent in the limbal and conjunctival epithelia (Figure 2C), mucocutaneous junction of the eyelid, meibomian gland, and eyelid epidermis (data not shown). Thus, the overall expression pattern for mir-184 within the anterior segment appears to be highly restricted to the corneal epithelium.

One of the other miRNAs that we extensively analyzed (see retina, below) and that was expressed in corneal epithelium (Table 1) was mir-182. Thus we compared the expression patterns of this miRNA with mir-184. The expression patterns for mir-182 in the anterior segment (Figure 3A) were distinct from those of mir-184. Mir-182 was expressed in basal, wing, and superficial cells of the corneal epithelium (Figure 3), whereas mir-184 was restricted to corneal basal and immediately suprabasal cells (Figure 2C,E). Furthermore, in contrast to mir-184, mir-182 was also expressed in the limbal epithelium (Figure 3). Little if any signal for mir-182 was noted in the conjunctival epithelium. Signal for mir-182 was also detected among the stromal keratocytes (Figure 3A), whereas the other miRNAs that we examined were limited to the epithelial compartment. Thus, it clear that miRNAs are present within the corneal epithelium that are differentially expressed in limbal and corneal epithelia, as well as in different cell layers within the corneal epithelium.

The strong signal for mir-205 noted in both corneal and footpad epithelial northern blots (Figure 2), as well as the observation that this miRNA gave the second highest hybridization signal in the corneal epithelial arrays (Table 1), prompted us to investigate further its expression patterns. Mir-205 expression was detected throughout the entire corneal, limbal, and conjunctival epithelia (Figure 2D,F), as well as the mucocutaneous junctional epithelium of the eyelid (data not shown). In addition, mir-205 was also strongly expressed in the adult mouse epidermis (Figure 2H) as well as the bulge region of the hair follicle (data not shown). MiRNA-217 also had equal hybridization signal intensities in the corneal and footpad epithelial arrays. This miRNA was expressed in corneal, limbal, and conjunctival epithelia (Figure 3B). The corneal expression for mir-217 was most intense in the basal and suprabasal layers (Figure 3B), whereas mir-205 was expressed throughout all layers of the corneal epithelium (Figure 3D). The array, northern, and in situ data clearly demonstrate that several miRNAs are preferentially expressed across the epithelium of the anterior segment in a tissue and cell-type specific manner.

We examined the effects of corneal epithelial wound healing and the associated changes in epithelial proliferation [20,23,24] on the expression of mir-184. The central region of the corneal epithelium was physically removed with a rotating diamond burr (Figure 4A). Within 24 h post wounding, wound closure and reepithelialization of the corneal epithelium was evident (Figure 4B), and by 48 h post wounding, the corneal epithelium had returned towards normal (Figure 4D). No signal was detected for mir-184 in the reepithelializing corneal epithelium 24 h post wounding, whereas strong expression for mir-184 was observed throughout the corneal epithelium adjacent to the wound (Figure 4E), indicative that wounding down regulates mir-184 expression. Forty-eight h

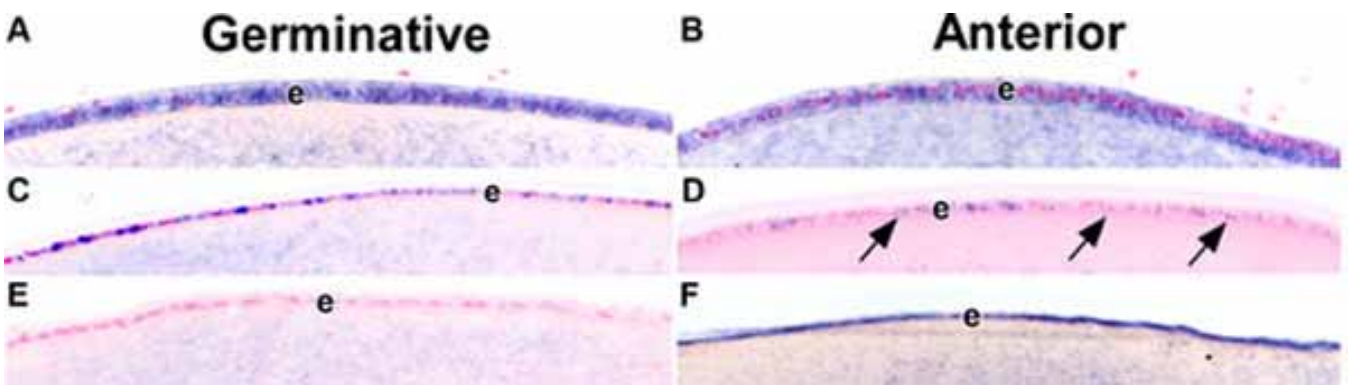

Figure 6. MicroRNAs reveal distinct expression patterns in the lens epithelium. Neonatal (**A** and **B**) and adult (**C**-**F**) mouse lens were processed for in situ hybridization with digoxygenin-labeled antisense probes for mir-184 (**A**-**D**) -204 (**F**) and a scrambled oligonucleotide sense (**E**) control. Uniform expression of mir-184 is seen in epithelial (e) cells from the germinative (**A**) and anterior (**B**) regions of the lens. In contrast, mir-184 expression is more uniform in the epithelial (e) cells of the germinative (**C**) compared with the anterior (**D**; arrows) regions. Expression of mir-204 (**F**) is uniformly detected in the epithelial cells of the anterior region of the lens. Staining detected in the region beneath the epithelial layer with the sense control (**E**), is believed to be non-specific.

after wounding, strong expression of mir-184 was observed throughout the reepithelialized corneal epithelium (Figure 4F) as well as the corneal epithelium adjacent to the wound (Figure 4G). Little if any proliferation was noted in the reepithelializing corneal epithelium 24 h after wounding (Figure 4H). However, numerous basal cells in the S phase of the cell cycle were observed in the adjacent corneal epithelium (Figure 4I) where a strong signal for mir-184 was also observed (Figure 4E). By 48 h post wounding, some proliferating basal cells were noted in the reepithelialized corneal epithelium (Figure 4J). At this time, proliferation in the adjacent corneal epithelium (Figure 4K) was still prominent. These data indicate that the expression of mir-184 is down-regulated in the reepithelializing corneal epithelium during the early stages of wound healing. Furthermore, the expression of mir-184 is not significantly altered in the epithelium adjacent to the wound site despite the marked increase in proliferation that occurred in this region during the healing process.

MicroRNAs and the lens: microRNA expression profiling: During our observations of mir-184 expression in the epithelia of the anterior segment, we consistently observed a prominent expression for this miRNA in the lens epithelium. When lens LMW RNA was analyzed by array hybridization, mir-184 was again the most abundant miRNA (Table 2), thus confirming our initial observation. Furthermore, many other highly expressed corneal epithelial miRNAs (e.g., mir-31, -181, -204) also gave strong signals in the miRNA lens array (Table 2). Northern analysis of lens RNA confirmed the array data (Figure 5); strong signals were detected for mir-181, -184, and -204. Interestingly, mir-205, which was strongly expressed in corneal and other epithelia (Figure 1, Figure 2B), was not detected in the lens (Figure 5), indicative that this miRNA is restricted to stratified squamous epithelia.

MicroRNAs and the lens: microRNA distribution patterns: In the adult (21 day) lens, mir-184 was differentially expressed, with a strong signal detected in the epithelial cells of the germinative zone (Figure 6C) contrasted by a more patchy expression in the epithelial cells of the anterior zone (Figure 6D). The expression pattern of mir-184 at an earlier developmental time point (PN day 7) was more equally distributed between the germinative (Figure 6A) and the anterior (Figure 6B) zones. Since we detected mir-204 expression throughout the epithelial cells of the anterior zone of the adult lens (Figure 6F), this eliminated the possibility that the patchy expression observed for mir-184 (Figure 6D) was due to tissue damage. Furthermore we observed an equally intense signal for mir-204 in both the anterior (Figure 6F) and germinative lens epithelial cells (data not shown), highlighting the cell-type restriction of mir-184 in the adult lens. We noted variable staining in the lens fiber cells beneath the epithelium (Figure 6A-C,F). However, since this staining pattern was also noted with a scrambled (control) oligonucleotide (Figure 6E), we believe such staining represents nonspecific background.

MicroRNAs and the retina: microRNA expression profiling: The most highly expressed retinal miRNAs were mir-181a, -125b, -26a, and -124a, with intensity signals that were

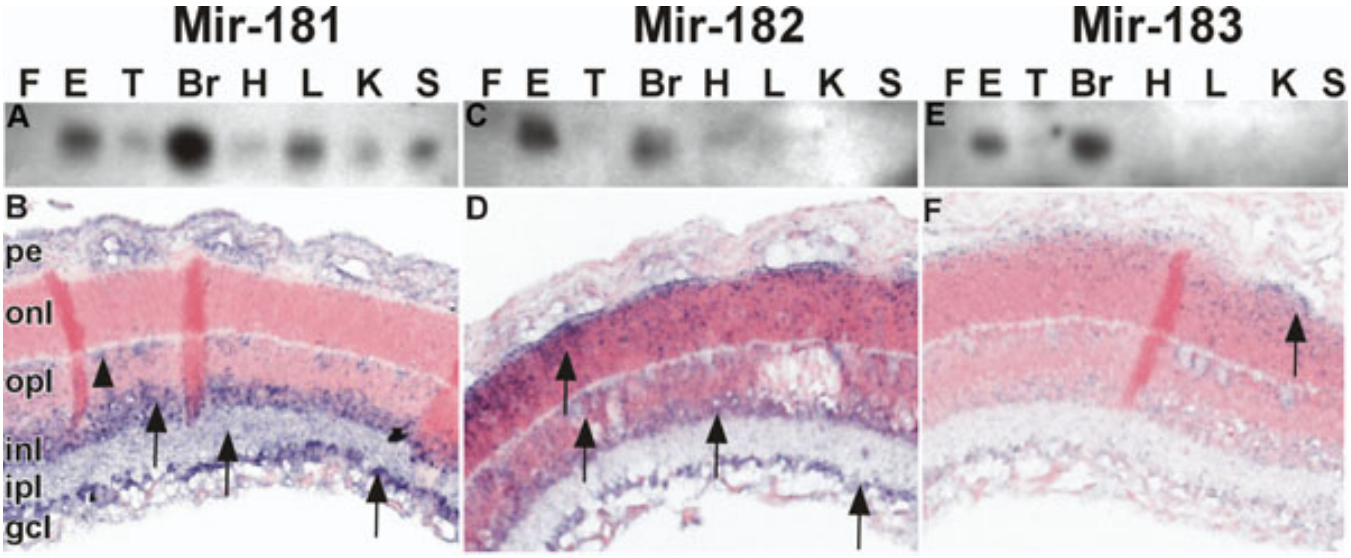

Figure 7. MicroRNAs reveal distinct distribution patterns within the adult mouse retina. **A**,**C**,**E**: Total RNA (10 μg) from mouse footpad epithelium (F), whole eye globe (E), tongue (T), brain (Br), heart (H), liver (L), kidney (K), and spleen (Sp) were analyzed by northern hybridization with ^{32}P-labeled oligonucleotides to mir-181 (**A**) -182 (**C**), and -183 (**E**). Mir-181 (**A**) was detected in most tissues with the most abundant signals in the brain, eye, and liver. In contrast mir-182 (**C**) and -183 (**E**) we restricted to the brain and eye. **B**,**D**,**F**: Mouse retinal tissues were processed for in situ hybridization with digoxygenin-labeled antisense probes for mir-181 (**B**) -182 (**D**), and -183 (**F**). Signal for mir-181 (**B**) is detected primarily in the ganglion cell (gc), inner plexiform (ip), and inner nuclear (in) layers (arrows). Patchy expression is noted in the outer nuclear (on) layer. Mir-182 (**D**) expression is seen in all retinal layers except the pigmented epithelial (pe) region. Mir-183 (**F**) has the most restricted expression pattern, localized primarily in the outer nuclear layer with a concentration at the outer limiting membrane (arrows).

above the saturation range (Table 2). In addition, mir-182, -183, and -204, which were strongly expressed in the corneal epithelial and lens fractions (Table 1, Table 2), also had high hybridization signals in the retina-rich fraction (Table 2). We analyzed the distribution of several of these miRNAs in RNA isolated from the hindbrain as well as the corneal epithelial, lens and retina-rich fractions of the eye (Figure 5). Mir-181, -182-, and -183 were detected in brain and retina (Figure 2), confirming a retinal (neuronal) relationship for these miRNAs. To learn more about the global distribution of mir-181, -182, and -183, we surveyed their expression in RNA from footpad epithelium, whole eye globes, tongue, brain, heart, liver, kidney, and spleen. Mir-182 (Figure 7C) and mir-183 (Figure 7E) had restricted distributions; both were abundant in the brain and whole eye globe and absent in the footpad epithelium, tongue, heart, liver, kidney, and spleen. In contrast, mir-181 was most abundant in brain and also exhibited strong signals in whole eyes and liver, and to a lesser degree in tongue, heart, kidney, and spleen (Figure 7A). The wide distribution of this miRNA is not surprising given its role in regulating skeletal myoblast terminal differentiation [25], as well as B-lymphocyte differentiation [13].

MicroRNAs and the retina: MicroRNA distribution patterns: Within the retina of a young mouse (PN day 7), mir-181 was strongly expressed in the ganglion cell, inner plexiform, and inner nuclear layers (Figure 7B). Little if any expression was detected in the outer nuclear layer. Expression of mir-182 was uniformly detected in all layers (Figure 7D). In contrast to the other miRNAs, mir-183 expression was restricted to the outer nuclear layer, with a concentration at the outer limiting membrane (Figure 7F). In the adult (day 21) mouse eye, the expression pattern seen in the outer nuclear layer with mir-182 and mir-183 localizes to the outer limiting membrane of the rods (data not shown). The distinct and overlapping retinal expression patterns of mir-181, -182, and -183 may reflect the special regulatory characteristics of these miRNAs.

One particularly interesting miRNA observed in this study is mir-204 because it was abundant in all three ocular regions studied (Table 1, Table 2, Figure 5). Furthermore, it was highly abundant in corneal epithelium and absent in footpad epithelium (Table 1), suggesting an ocular preference. Finally, of the miRNAs that we characterized thus far, only mir-204 was expressed in the nonpigmented epithelium of the ciliary body (Figure 8). This observation is consistent with a recent study that focused on miRNAs expressed in the developing and adult nervous system [26]. In this study, mir-204 was observed in the epithelium of the choroid plexus and the ciliary epithelium, tissues with similar functions.

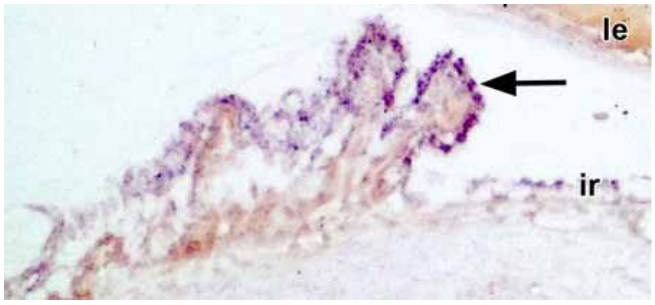

Figure 8. MiRNA expression in the ciliary epithelium. Of the miRNAs studied, only mir-204 was detected in the non-pigmented portion of the ciliary epithelium (arrow). In the image, le indicates the lens and ir indicates the iris.

DISCUSSION

In the present study we have characterized the miRNA environment that exists in the adult mouse eye. An earlier report identified through cloning several miRNAs in mouse eyes, in particular mir-181, -182, -183, -184, and -204 [14]. We have extended these findings by characterizing the location of this group of miRNAs within the corneal epithelium, lens, and retina. Furthermore, we have identified the major miRNAs present in these ocular tissues using array hybridization. Our findings clearly demonstrate that miRNAs exhibit distinct tissue and cellular specificity within the corneal epithelium, lens, and retina. This is consistent with the idea that miRNAs may be important in maintaining tissue identity [16,17].

Mir-184 was unique in that it was the most abundant miRNA in both the corneal and lens epithelia. The epithelial specificity for miRNAs is clearly illustrated by the varied distributions of mir-184 -203, and -205. Whereas mir-184 is enriched in the corneal epithelium, mir-203 is preferentially expressed in footpad epithelium. In contrast, mir-205 exhibits a broad range of expression within many stratified squamous epithelia, suggestive that it represents a more generalized epithelial miRNA. Consistent with this finding, when small RNAs (18-25 nt) were cloned and sequenced from embryonic (E17.5) mouse epidermis, mir-205 was highly expressed [27]. Therefore, with respect to the corneal epithelium, the restricted expression of mir-184 highlights the distinct nature of this ectodermal lineage.

Similar to mir-184, several of the highly expressed corneal epithelial miRNAs (e.g., mir-26a, -31, -125b, and -181) also had strong hybridization signals in the lens epithelium. This is not surprising given the role that the lens plays in organizing the anterior segment [28,29]. It is tempting to speculate that the transcriptional regulation of these miRNAs may be governed by “eye specific” transcription factors such as Pax6 [30] and references therein. Furthermore, the observed overlapping expression patterns of mir-182 and mir-183 in the retina together with the known clustering of these miRNAs on human chromosome 7 and mouse chromosome 6 [14] also suggests a common transcriptional control.

Understanding the function of the miRNAs is best achieved by defining their regulatory target mRNAs. The differential expression of certain ocular miRNAs may provide help in assessing potential function as well as targets for future study. For example, mir-184 was exclusively restricted to the corneal epithelium and was absent from the adjacent limbus. It is well accepted that the corneal epithelial basal cells are more differentiated than the limbal epithelial basal cells [31-36]. Our observation that mir-184 is restricted to the cor-

neal epithelium distinguishes further these two adjacent epithelia and suggests that this miRNA may be involved in corneal epithelial differentiation. Further evidence to support this proposal can be obtained from the wound healing experiments. The observation that mir-184 expression is down-regulated in the reepithelializing cells of the wounded corneal epithelium highlights the similarities in the regulation of this gene with more commonly encountered genes such as the corneal keratins [37,38]. Our observation that increased corneal epithelial proliferation during wound healing did not alter the expression of mir-184 indicates that the role of mir-184 is independent of the proliferative status of the corneal epithelium, further arguing for a role in demarcating the differentiated corneal epithelial phenotype. The tightly restricted and abundant expression of mir-184 to corneal and lens epithelia suggests that this miRNA might be involved in some aspect of physiology that is unique and common for both of these tissues; maintenance of transparency, avascularity, and extracellular matrix synthesis are just a few examples. Our studies highlight the potential importance of miRNAs in regulating ocular development and function. The RNase III enzyme (Dicer) is necessary for the processing of short miRNAs from double stranded precursors [11,39] and therefore mutating Dicer will effectively halt miRNA production [39-41]. Recently $Dicer^{flox}$ mice were crossed to a transgenic mouse expressing Cre recombinase driven by the keratin-14 promoter [27,42]. This resulted in the absence of miRNAs in the epidermis and severely affected hair follicle development. Therefore it will be important to investigate the global effects that miRNAs have on ocular tissues by disturbing the overall production of miRNAs. Use of PAX6-driven Cre expression to disrupt the Dicer-1 gene should effectively silence miRNA production in the corneal and lens epithelial cells [43], and therefore provide insight into the roles that miRNAs play in the development of these ocular structures.

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