Identification and Characterization of MicroRNAs Expressed in the Mouse Eye

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Purpose. MicroRNAs (miRNAs) are a class of small, endogenous RNAs that negatively regulate gene expression post-transcriptionally by binding to target sites in the 3′ untranslated region (UTR) of messenger RNAs. Although they have been found to regulate developmental and physiological processes in several organs and tissues, their role in the eye transcriptome is completely unknown. This study was conducted to gain understanding of their eye-related function in mammals, by looking for miRNAs significantly expressed in the mouse eye by means of high-resolution expression analysis.

Methods. The spatiotemporal localization of miRNAs was analyzed in the murine embryonic and postnatal eye by RNA in situ hybridization (ISH) using LNA-modified oligonucleotide probes.

Results. Seven miRNAs were expressed in the eye with diverse and partially overlapping patterns, which may reflect their role in controlling cell differentiation of the retina as well as of other ocular structures. Most eye-expressed miRNAs overlap with or are in the near vicinity of transcripts derived predominantly from eye cDNA libraries. We found that these transcripts share very similar cellular distribution with their corresponding miRNAs, suggesting that miRNAs may share common expression regulatory elements with their host genes.

Conclusions. The data provide a detailed characterization of expression of eye-enriched miRNAs. Knowledge of the spatiotemporal distribution of miRNAs is an essential step toward the identification of their targets and eventually the elucidation of their biological role in eye development and function. (*Invest Ophthalmol Vis Sci.* 2007;48:509–515) DOI:10.1167/iovs.06-0866

One of the most striking results of genomic research over the past few years is the finding that noncoding RNAs (ncRNAs) represent a substantial component of all metazoan genomes and seem to control critical processes in animal biology. Among noncoding RNAs, miRNAs currently represent the class that is receiving the highest attention. miRNAs, a class of 21-25 nucleotide small RNAs processed from double-stranded hairpin precursors,[1] negatively regulate gene expression in animals by binding, with imperfect base pairing, to target sites in the 3′ untranslated region (UTR) of messenger RNAs leading to a reduction of translational efficiency.[1,2] Currently, over 300 miRNAs (miRNA registry,[3] http://microrna.sanger.ac.uk/sequences/ provided in the public domain by the Sanger Centre, Hinxton, UK) have been identified in the human and mouse genomes, but estimates suggest that their actual number may be close to 1000.[4] As each miRNA can regulate, on average, the expression of approximately 100 to 200 target genes,[5,6] the miRNA apparatus seems to participate in the control of gene expression for a significant percentage of the mammalian transcriptomes and proteomes. In all plant and animal species examined, defects in miRNA function have profound effects on development.[7–9]

Most miRNAs are expressed in a highly tissue-specific manner in vertebrates.[10,11] As a given cell type expresses a defined subset of miRNAs, which in turn control a defined repertoire of genes, an miRNA code has been proposed to provide the signature that allows the differentiation of specialized cells from common precursors (reviewed in Ref. 12). Hence, to define miRNA function in a specific organ, such as the eye, it is essential to determine a high-resolution profile of their spatial and temporal distribution by RNA in situ hybridization (ISH), which has been, until recently, hampered by their limited size. However, the observation that LNA (locked nucleic acid)-modified DNA oligonucleotide probes can be effectively used to perform RNA ISH represents a tremendous advancement in the recognition of the tissue-specific role of miRNAs.[11,13] Using this technique, Wienholds et al.[11] reported the expression profile of 115 miRNAs during zebrafish development and found that 13 of them showed significant expression in the zebrafish eye. Considering that mammalian development and physiology differs to an extent from that of other vertebrates, it is of uttermost importance to determine miRNA expression profiles in the mammalian eye to start shedding light on the possible role of miRNAs in eye development. To date, there are very few reports on the expression pattern of miRNAs in the eye and retina in mammals.[14] For this reason, we decided to analyze the expression of miRNAs during eye development in the mouse. The identification of the specific cell type and developmental stage in which each of the analyzed miRNAs is expressed will be very valuable to evaluate the biological significance of their predicted target genes and gain initial insight into their possible role in the control of eye development and function.

Methods

Bioinformatic Analysis

To analyze the genomic location of miRNAs and to identify the transcriptional units within which they are localized (host transcripts), we used the Mouse Genome Browser server (http://genome.ucsc.edu) and the Unigene database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene/ provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). Nucleotide sequences were assembled using the CAP assembly software (Infobiogen, Web site now closed). In silico expression data on the host transcripts analyzed were obtained from Unigene and from the GNF SymAtlas (http://symatlas.gnf.org/symatlas/ provided in the public do-

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main by the Genome Research Institute of the Novartis Foundation, San Diego, CA) databases.

Probes

LNA-modified oligonucleotide probes for the miRNAs analyzed were purchased from Exiqon (Vedbaek, Denmark). One hundred picomoles of probe were digoxigenin (DIG)-labeled (DIG Oligonucleotide 3′-End labeling kit, second generation; Roche, Mannheim, Germany) according to the manufacturer's instruction, and the reaction was stopped with 5 μL 0.1 M EDTA (pH 8.0). The labeled probe was column purified (MicroSpin G-25; GE Healthcare, St. Giles, UK) and used in 1:100 (vol/vol) dilution in the hybridization mix. Host probes were designed on the clusters spanning the miRNA location. Templates for the host transcripts were generated using EST clones (Image ID 372321 for Unigene cluster Mm.221244, Image ID 3984161 for *Trpm3*). The template for cluster Mm.194050 was generated by PCR on mouse genomic DNA using primers 5′-ATCTCTGGAGTGAGCGAAGG-3′ and 5′-TACCCCCAAAATGGTACAGC-3′. PCR products were purified and used for RNA probe labeling. To detect the probe, a hapten tag (digoxigenin-labeled UTP) was incorporated into the RNA during the in vitro transcription reaction (DIG RNA labeling kit; Roche).

RNA In Situ Hybridization on Cryosections

RNA ISH experiments on adult eye sections using cRNA probes were performed essentially as previously described.[15,16] For RNA ISH using the LNA-modified probes we followed the protocol recommended by the manufacturer (Exiqon) with minor modifications. To this purpose, embryos, neonatal heads, and postnatal eyes (postnatal day [P]8, adult) were cryoprotected by treatment with 30% sucrose in phosphate-buffered saline (PBS) and embedded in OCT. Twenty-micrometer cryosections were collected on slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA) and postfixed with 4% paraformaldehyde (PFA) in PBS for 15 minutes. After being bleached with 6% H_2O_2 in PBS with 0.1% Tween-20 for 5 minutes, sections were treated with either 1 μg/mL (embryonic tissues) or 5 μg/mL (neonatal tissue) proteinase K for 15 minutes. After washes with 2 mg/mL glycine and postfixation with 4% PFA— 0.2% glutaraldehyde sections were prehybridized with 50% formamide, 5× sodium saline citrate buffer (SSC) and citric acid to pH 6, 1% sodium dodecyl sulfate (SDS), 500 μg/mL yeast RNA, and 50 μg/mL heparin. Hybridization with the digoxigenin-labeled probes was performed overnight at a temperature of approximately 21°C lower than the temperature of the probe (miR-9, 49°C; miR-29c, 50.3°C; miR-96, 53.7°C; miR-124a, 58.6°C; miR-181a, 56.5°C; miR-181b, 56.5°C; miR-182, 52°C; miR-183, 52°C; miR-184, 55°C; miR-204, 56.5°C; miR-213, 58°C; miR-216, 53°C; miR-217, 56.5°C). Hybridized sections were washed with 50% formamide, 2× SSC at the hybridization temperature. Sections were blocked for 1 hour with 1% blocking reagent (Roche) in 100 mM maleic acid, 150 mM NaCl, and 0.1% Tween 20 (pH 7.5; MABT) containing 10% sheep serum and incubated with alkaline phosphatase (AP)-labeled anti-digoxigenin antibody (1:2000; Roche) in 1% blocking reagent in MABT overnight at 4°C. After extensive washes with PBS containing 0.1% Tween 20 (PBT), sections were exposed to the substrate for AP, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indoyl phosphate (NBT-BCIP; Sigma-Aldrich, St. Louis, MO). Reaction was blocked by washes with PBS (pH 5.5), followed by postfixation in 4% PFA for 20 minutes. Slides were coverslipped with 70% glycerol in PBS or dehydrated and mounted (Eukitt Mounting Medium; EMS, Fort Washington, PA). The authors confirm adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Fluorescent RNA ISH and Immunofluorescence

Essentially the same protocol was followed as just described, with the difference that incubation with the anti-DIG antibody was performed at 37°C for 1 hour followed by fluorescence detection of the anti-DIG antibody (HNPP Fluorescent Detection Set; Roche). Subsequently, sections were blocked for 1 hour with 10% goat serum in PBS and incubated with the primary antibody (ID4, 1:400; National Cell Culture Centre, NIH, Bethesda, MD; PKCα, 1:1500, Sigma-Aldrich; GS6, 1:400, Chemicon International, Temecula, CA) in PBS containing 1% goat serum overnight at 4°C. After extensive washes with PBS, sections were incubated with the secondary antibody (anti-rabbit or anti-mouse Oregon Green; 1:1000; Invitrogen-Molecular Probes, Eugene, OR) in PBS for 1 hour. After washes with PBS, nuclei were 4′,6′-diamino-2-phenylindole (DAPI)–stained and mounted (Vectashield; Vector Laboratories, Burlingame, CA).

Wholemount RNA ISH

Wholemount ISH analysis was performed on embryos at 10.5 days of development, as suggested by Exiqon and previously described.[17]

Results

Selection and Expression Analysis of miRNAs with Significant Expression in the Retina

We selected 13 miRNAs (miR-9, -29c, -96, -124a, -181a, -181b, -182, -183, -184, -204, -213, -216, and -217) that had either been cloned from eye tissues[10] or had been found to be expressed at significantly high levels in the zebrafish eye by RNA ISH and/or microarray analysis compared with other tissues.[11] To define more specifically when and where miRNAs are expressed in the developing and adult eye, LNA-modified DNA oligonucleotide probes corresponding to the selected miRNAs were used for RNA ISH experiments on wholemount mouse embryos and on sections of murine eyes from different developmental stages. We determined that seven of the analyzed miRNAs were expressed in the mouse eye with different patterns (Figs. 1, 3). The expression data presented herein are partially summarized in Table 1. For miR-96, -183, -181b, -213, -216, and -217, we did not detect any signal in the eye at any of the developmental stages analyzed.

We observed that at embryonic day [E]10.5 miR-124a was expressed strongly throughout the nervous system, including the spinal cord, the rhombencephalon, mesencephalon, diencephalon, and weakly in the telencephalon (Fig. 1A). At this stage, only a faint staining was detectable in the optic cup. On sections of embryos at E14.5, expression of miR-124a was detected strongly in the eye, in the innermost cell layer where progenitors start differentiating into ganglion cells (see Fig. 4I, arrow). At this stage, expression of miR-124a was also strong in the brain and spinal cord. Of note, with the resolution obtained by ISH on sections, we observed that the subventricular zone of the brain, where pools of undifferentiated and proliferating cells reside, was devoid of any staining, suggesting that expression of miR-124a was limited to differentiated neurons (see Fig. 4I, arrowhead). Expression of miR-124a persisted in the neural retina during development up to adulthood (Figs. 1G, 1M). In the adult retina, miR-124a was strongly expressed in all cell layers with very intense staining in the outer (OS) and the inner (IS) segments of the photoreceptors, where most of the cytoplasm is found (Fig. 1M), whereas no expression was detected in the retinal pigment epithelium (RPE; data not shown). We observed a gap of staining in the middle part of the inner nuclear layer (INL) with a regular, "beads-on-a-string"–like distribution, suggesting that miR-124a is not commonly present in all retinal cell types. Based on the spatial arrangement of these blank spots, we hypothesized that they may correspond to Müller glia cells. To confirm further that miR-124a was not expressed in these cells, we performed fluorescent ISH for miR-124a followed by immunofluorescence using an antibody (GS6) that marks Müller cells. We did not detect any colocalization between miR-124a and GS6 (Figs. 2L–N) and this confirmed that miR-124a is expressed in the retina in differentiated neurons and not in glial cells.

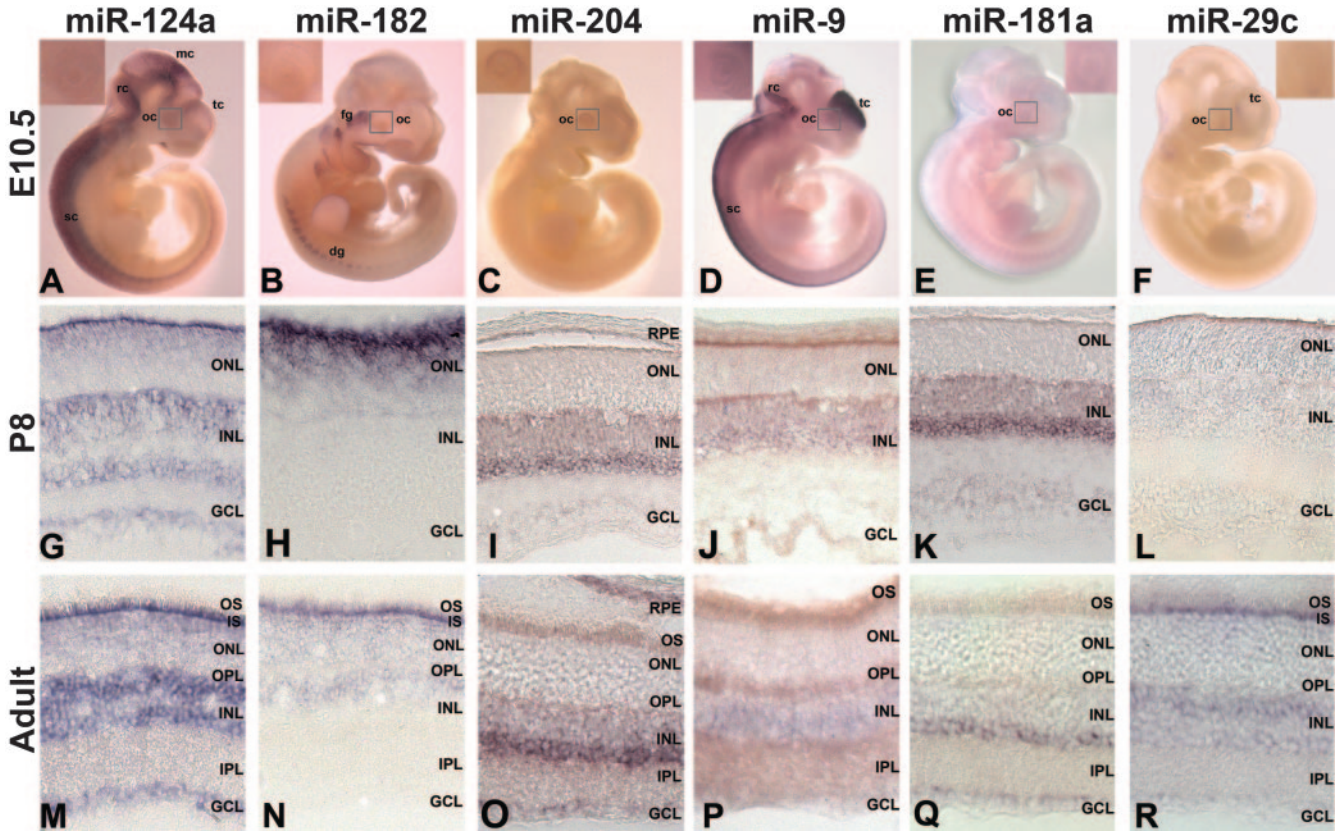

Figure 1. Expression analysis of six miRNAs during retina development in mouse. Expression analysis of six miRNAs by RNA ISH on whole E10.5 embryos (**A–F**) and on eye sections from P8 (**G–L**) and adult (**M–R**) on CD1 (albino) wild-type mice. *Insets*: close-up of the E10.5 optic. All miRNAs were expressed in the different retina cell layers with diverse and partially overlapping patterns, in both P8 and adult retinas. miR-124a was expressed throughout the nervous system (**A**) and in all differentiated neurons of the developing and adult retina (**G**, **M**). miR-182 at E10.5 was specifically expressed in dorsal root ganglia and cranial ganglia (**B**), whereas at P8 (**H**) and adult stage (**N**) expression of miR-182 was restricted to the photoreceptors and the limits of the INL. Wholemount ISH of E10.5 embryos for miR-204 resulted in an eye-specific staining (**C**). In situ analysis of eye sections in P8 (**I**) and adult (**O**) retinas confirmed the presence of miR-204 in the RPE and in the INL. In particular, in the adult neural retina, miR-204 was strongly expressed in INL and the GCL (**O**). miR-9 was expressed strongly in the telencephalon and in the spinal cord at E10.5 (**D**) and in the central region of the INL in the adult eye (**P**). miR-181a and -29c gave a weak staining at the ventronasal part of the optic cup and in the telencephalon for miR-29c (**E**, **F**). However, expression was detected in postnatal (**K**, **L**) and adult (**Q**, **R**) retinas. In adult, miR-181a was strongly expressed in the innermost part of the INL and the GCL (**Q**), whereas miR-29c was expressed throughout the retina and strongly in the IS of the photoreceptors and the outermost layer of the INL (**R**). oc, optic cup; tc, telencephalon; mc, mesencephalon; rc, rhombencephalon; sc, spinal cord; fg, facial ganglia; dg, dorsal root ganglia.

miR-182 was predominantly expressed in the cranial ganglia and dorsal root ganglia (Fig. 1B), with no detectable expression in the eye at E10.5 and E14.5 and at birth (P0; Table 1 and data not shown). However, at P8 as well as in the adult retina, miR-182 was strongly and specifically expressed in rod photoreceptors (Figs. 1H, 1N). In the adult, miR-182 also weakly stained the limits between the INL and the outer plexiform layer (OPL; Fig. 1N). To characterize the exact cell types that express miR-182, we performed fluorescent ISH followed by immunofluorescence with an antibody for rhodopsin. miR-182 colocalized with rhodopsin in the OS of rod photoreceptors (Figs. 2A–D). To define whether the weaker staining observed in the region of the OPL corresponds to bipolar cells of the INL or to synapses extending from the photoreceptors, we used an antibody for PKCα, a marker of bipolar cells. We did not observe any colocalization between miR-182 and PKCα in any of the sections analyzed both by standard (Figs. 2E–H) and confocal microscopy (Fig. 2I–K), suggesting that miR-182 may

Table 1. Summary of miRNA Expression during Eye Development

	E10.5	E14.5	P0	P8	Adult
miR-124a	OC	NR	NR	NR	NR
miR-182	ND	ND	ND	NR	NR
miR-204	OC	NR, RPE, LE	NR, RPE, LE	NR, RPE, CB, LE	NR, RPE, CB, LE
miR-9	OC	NR	NR	NR	NR
miR-181a	OC	NR	NR	NR	NR
miR-29c	OC	ND	NR	NR	NR
miR-184	OC	LE	LE, CO	LE, CO	LE, CO

ND, not detectable; OC, optic cup; NR, neural retina; RPE, retina pigment epithelium; LE, lens; CB, ciliary body.

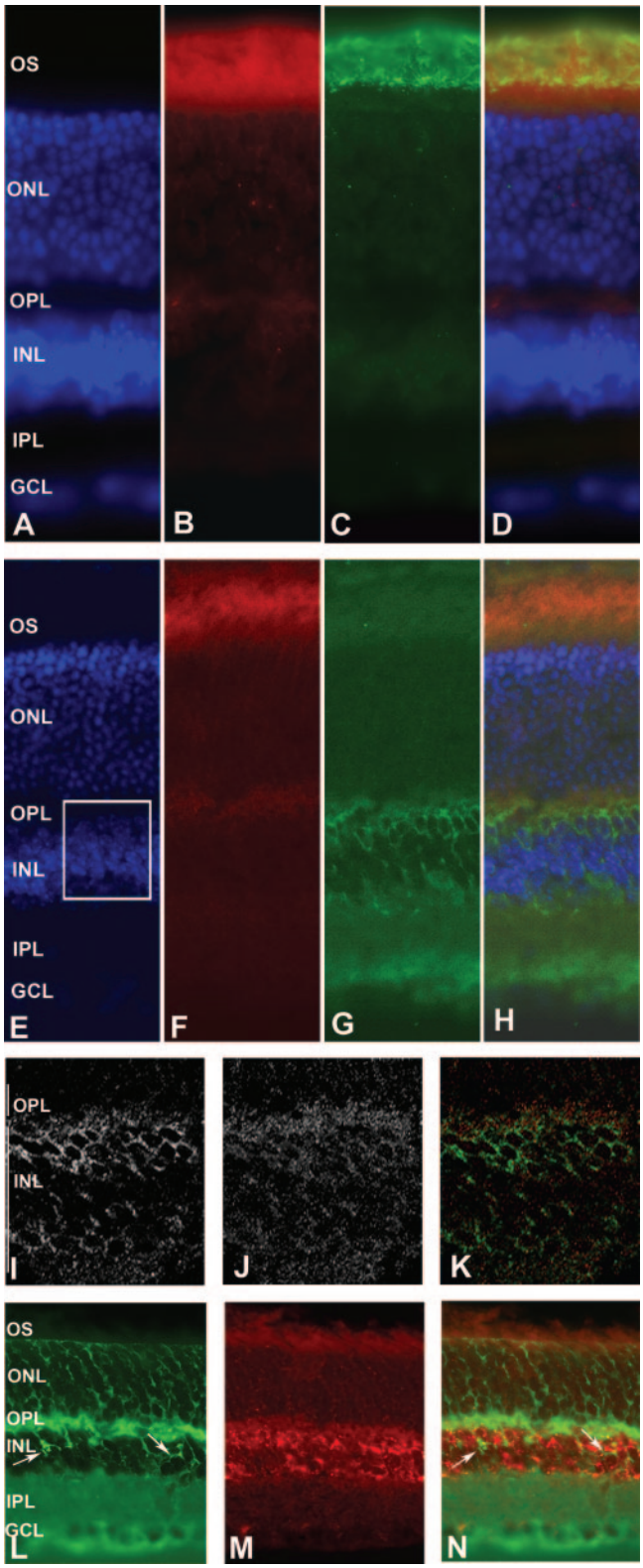

FIGURE 2. Characterization of retinal cell types expressing miR-182 and -124a. Fluorescence ISH for miR-182 and -124a followed by immunofluorescence with antibodies that recognize cell-type–specific markers performed on sections of adult murine retina. (**A–D**) In order, DAPI-stained nuclei (**A**; *blue*), miR-182 ISH (**B**; *red*), anti-RHO (ID4) immunofluorescence (**C**; *green*) and merged staining (**D**). miR-182 was strongly expressed in the photoreceptor OS, which were immunostained with anti-rhodopsin (ID4) antibody. miR-182 also gave some signal at the limits between the OPL and the INL (**B**, **D**). (**E–H**) In order, DAPI-stained nuclei (**E**; *blue*), miR-182 ISH (**F**; *red*), anti-bipolar (anti-PKCα) immunofluorescence (**G**; *green*), and merged staining (**K**). No colocalization was detected between the PKCα and miR-182. *White box*: the region analyzed by confocal microscopy in (**I–K**). (**I–K**) Confocal image of miR-182 (**I**), anti-bipolar PKCα (**J**) and merged staining (**K**). (**L–N**) anti-Müller GS6 antibody (**L**; *green*), miR-124a ISH (**M**; *red*) and merged staining (**N**). miR-124a was not detected in Müller cells (*white arrows*).

not be present in bipolar cells but, instead, in the synapses extending from the photoreceptors. miR-182 was also detected in the cochlea and the olfactory epithelium at E14.5 and in P0 (data not shown). Taken together, these observations suggest that miR-182 is specifically expressed in sensory neurons.

miR-183 and -96 were clustered with miR-182—that is, they were found in the mouse genome in very close proximity with each other (3.8 kb), they were transcribed in the same orientation, and they were probably coexpressed, as they have been shown to give very similar expression patterns in zebrafish wholemount experiments.[11] We detected a weak staining for these miRNAs on wholemount embryos at E10.5 that appeared almost identical with the one obtained by miR-182 (Fig. 1B). However, on sections of later stages we did not detect these miRNAs, probably because of the low level of expression or a reduced sensitivity of the corresponding probe.

miR-204 expression at E10.5 seemed to be restricted to the prospective RPE (Fig. 1C). However, from as early as E14.5 and at later developmental stages (P8 and adult) expression of miR-204 extended to the neural retina, and in particular to the ganglion cell layer (GCL) and to the innermost part of the INL where amacrine cells are localized (Fig. 1I, 1O). miR-204 is also very abundant in the ciliary body (see Fig. 4L). This observation combined with the strong expression of this miRNA both in the RPE and in cells of the neural retina suggest that miR-204 may contribute to the differentiation of pigment and neuronal cells from their common precursors. Finally, miR-204 is also expressed in the epithelial cells of the lens (see Fig. 4K, arrow). In sections of entire embryos at earlier developmental stages (E14.5), we could detect miR-204 expression in the choroid plexus of the lateral ventricles and in the sensory epithelia of the cochlea (data not shown).

miR-9 is expressed strongly in the forebrain and the spinal cord of E10.5 embryos (Fig. 1D). At this stage, only a faint staining was detected in the optic cup (Fig. 1D). However, at E14.5, all cells in the retina were clearly stained (data not shown, Table 1). In postnatal (Fig. 1J) and adult retina (Fig. 1P), miR-9 was detected in the middle part of the INL where Müller cells are localized. For miR-181a and -29c we did not detect any specific staining at E10.5 except for a spot in the ventronasal part of the optic cup and a weak staining of the telencephalon for miR-29c (Fig. 1E, 1F). In postnatal and adult retina, miR-181a stained weakly the GCL and strongly the innermost cells of the INL (Figs. 1K, 1Q). This staining probably corresponds to amacrine cells and we cannot exclude that the signal at the GCL may reflect expression of miR-181a in displaced amacrine cells. By contrast, miR-29c was detected throughout the retina and yielded a strong signal in the IS of the photoreceptors and the outermost cells of the INL which probably correspond to bipolar cells (Figs. 1L, 1R). Altogether, these data confirm that also in the mammalian retina miRNAs display highly specific cellular expression patterns.

miR-184 in the Lens and Cornea

miR-184 expression was not detectable in the neural retina at any stage analyzed. Instead, miR-184 was expressed in the lens, starting from early lens developmental stages (i.e., E10.5; Fig.

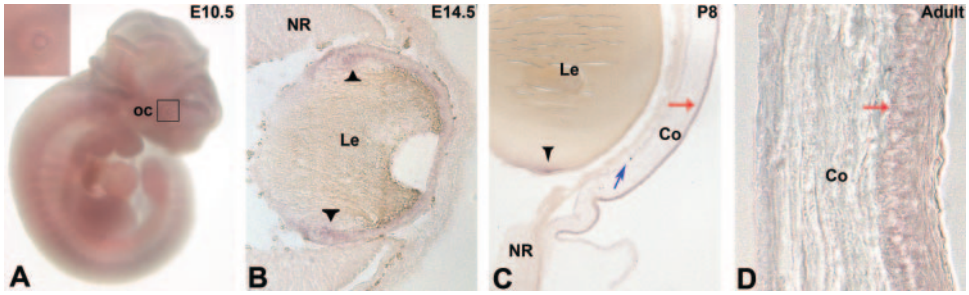

FIGURE 3. miR-184 expression in lens and cornea. Wholemount ISH of E10.5 embryos with miR-184 probe resulted in an eye-specific staining that appeared to correspond to the periphery of the lens (**A**; see magnified optic cup in the *inset*). In situ analysis of embryo sections at E14.5 (**B**) and eye sections at P8 (**C**) confirmed the expression of miR-184 in the epithelial cells of the lens (*arrowheads*). Expression of miR-184 was also detected in the cornea epithelia in P8 and adult (**C**, **D**; *red arrow*) and in endothelial cells (**C**; *blue arrow*). oc, optic cup; Le, lens; NR, neural retina; Co; cornea.

3A) up to adulthood in the epithelial cells of the equatorial zone (Figs. 3B, 3C; arrowheads). In P8 and adult eyes it was also strongly detected in the cornea epithelium (Figs. 3C, 3D; red arrows) and in the endothelial cells (Fig. 3C; blue arrow).

Expression Analysis of Host Transcripts

To understand the molecular mechanisms underlying the regulation of miRNA expression we analyzed the genomic regions that harbored these eye-expressed miRNA genes. In silico analysis (based on expressed sequence tag [EST] counts and publicly available microarray expression data) revealed that almost all miRNAs were localized within transcriptional units predicted to be highly expressed in eye tissues. We therefore verified whether the analyzed miRNAs show similar expression pattern with their host genes and, hence, share common functional elements controlling their expression.

The expression patterns of the host transcriptional units for miR-124a and -204 were analyzed by RNA ISH. miR-124a, is present in three copies in the mouse genome, two of which lay within clusters of spliced EST mostly derived from retina and brain cDNA libraries (Figs. 4A, 4B), whereas the third one is found in an intergenic region with no overlapping spliced transcriptional units. On chromosome 14, miR-124a overlaps with an exon of the transcriptional unit described by the Unigene cluster Mm.194050 and transcribed in the same orientation as the respective miRNA (Fig. 4A). In adult retina, this cluster gave a very similar expression pattern to miR-124a (compare Fig. 4D, 1M). Of note, expression was detected throughout the retina, with a gap of staining in the middle of the INL, as observed for miR-124a. Similar to miR-124a, a strong signal was obtained by Mm.194050 in the photoreceptor layers. However, Mm.194050 expression was not detected in the IS and the OS, where miR-124a is strongly detected, probably reflecting a difference in the distribution of miR-124a and of the Mm.194050 mRNA within photoreceptor cells. Mm.194050 mRNA was detected with an expression pattern very similar to that of miR-124a (Fig. 4I) in embryo sections at E14.5 (Fig. 4E, 4F) and, namely, in all differentiated neurons, whereas it was not detectable in the subventricular zone of the central nervous system where undifferentiated neuronal precursors reside (arrow). On chromosome 3, miR-124a is localized within an intron of the transcriptional unit represented by the Unigene cluster Mm.221244 (Fig. 4B) and is transcribed in the same orientation as the putative host transcript. The probe for the Mm.221244 mRNA yielded an identical expression pattern to miR-124a and Mm.194050 rendering though a signal of lower intensity (Figs. 4G, 4H).

miR-204 is localized within an intron of the *Trpm3* (transient receptor potential melastatin–like cation channels) gene and is transcribed in the same orientation (Fig. 4C). The *Trpm3* gene has a complex structure with several alternatively spliced products. The longest of these variants corresponds to an mRNA of 6.2 kb, composed of 25 exons and spanning a genomic region of 850 kb. We analyzed the expression of the *Trpm3* mRNA in sections of embryos at E14.5 and in adult retina using a probe that recognizes exons 2 to 7 of the longest transcript. At E14.5, *Trpm3* was strongly expressed in the choroid plexus of the lateral ventricles, as also reported by Deo et al.,[14] in the eye and in the cochlea, similar to our observations for miR-204 (data not shown). In the adult eye, *Trpm3* was expressed in the RPE, the INL, and GCL of the neural retina (Fig. 4J) as well as in the ciliary body and in the lens epithelial cells (Fig. 4K). This expression pattern was very similar to what we had previously observed for miR-204 (Figs. 1C, 1I, 1O, 4L). The only difference was that miR-204 rendered a diffuse staining that could underlie the cytoplasmic localization of the mature miRNA, whereas the *Trpm3* mRNA gave a more punctate pattern, which could reflect a putative nuclear distribution. Overall, this analysis establishes that miR-204 and *Trpm3*, its corresponding host gene, are coexpressed in the same tissues at the same developmental stages.

Taken together, these observations suggest that the host transcriptional units display an expression similar to that of the respective miRNAs.

DISCUSSION

We generated a high-resolution expression atlas of miRNAs significantly expressed in the mammalian eye. Our analysis demonstrated that RNA ISH can be effectively performed on mouse embryos and tissue sections using probes specifically recognizing the mature sequence of miRNAs, thanks to the use of LNA-modified DNA oligonucleotide templates. This chemistry renders the probes highly specific, and even single-base mismatches have been shown to abolish staining.[17] To our knowledge, this is the first demonstration of ISH using short, LNA-modified probes on sections of murine eye. To date, there has been only one published report[14] in which the investigators describe the expression of miR-124a and -204 on sections of murine retina, but a different probing method was used.

The analyzed miRNAs were found to display different and only partially overlapping patterns in the adult eye. miR-124a is expressed in all differentiated neurons and is not expressed in the glial Müller cells, as confirmed by immunofluorescence analysis. This observation is also consistent with other reports confirming that this miRNA is not expressed in brain glial cells.[13,14,18] By contrast, miR-9 was detected only in the middle part of the INL, probably reflecting its expression in Müller glia

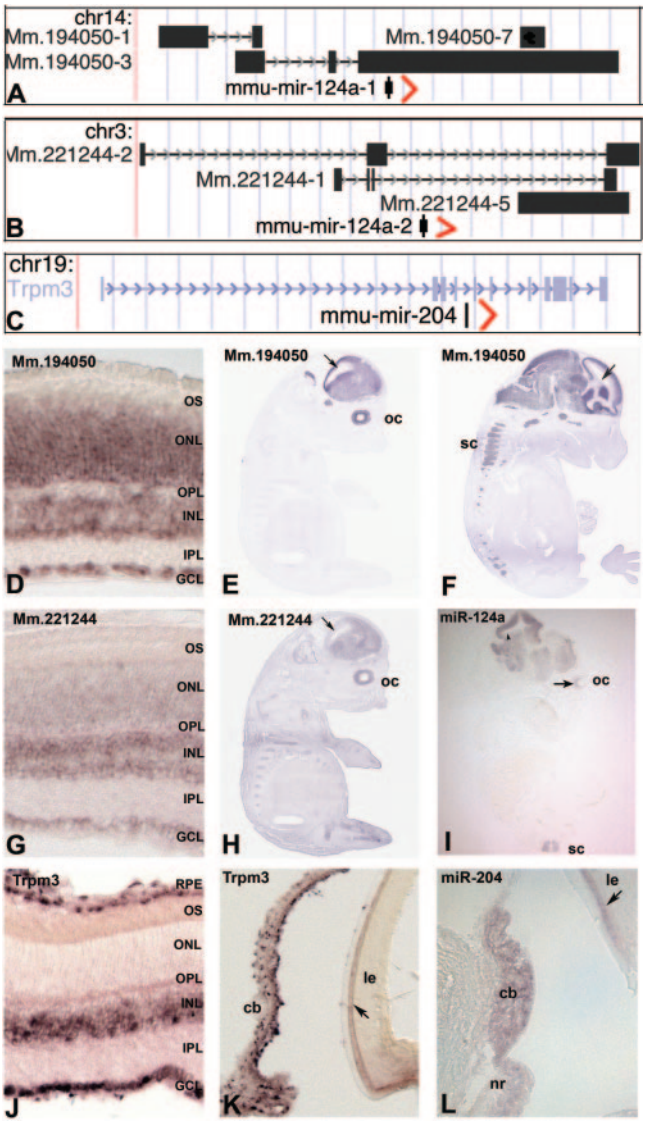

FIGURE 4. Expression pattern of miR-124a and -204 host genes. (**A–C**) Diagrams of the genomic location of miR-124a and -204. The sequence for miR-124a was found in three independent genomic locations in the mouse, and in two of those (**A**, **B**) were contained within clusters of spliced ESTs. On chromosome 14, miR-124a falls within Unigene cluster Mm.194050 (**A**), whereas on chromosome 3 it overlaps with an intron of the transcriptional unit described by Unigene cluster Mm.221244 (**B**). The *Trpm3* gene structure in (**C**) is simplified. *Red arrowheads*: indicate the orientation of miRNA transcription. (**D**, **G**, **J**) ISH on adult retinas with antisense probes for Mm.194050 (**D**), Mm.221244 (**G**), and *Trpm3* (**J**). (**E**, **F**, **H**, **I**) ISH on E14.5 embryos with antisense probes for Mm.194050 (**E**, **F**), Mm.221244 (**H**) and miR-124a (**I**). (**K**, **L**) ISH on adult eye with probes for *Trpm3* (**K**) and miR-204 (**L**). oc, optic cup; sc, spinal cord; le, lens; cb, ciliary body; nr, neural retina.

cells. This finding is in agreement with the reported preferential expression of miR-9 in glial cells.[13] Another interesting expression pattern was obtained for miR-182, which appears to be expressed exclusively in photoreceptors. miR-29c, -181a, and -204 were also expressed in the adult retina with variable patterns, and, of note, the latter was also strongly detected in the RPE. Finally, miR-184 was not detectable in the retina but was strongly expressed in nonneural tissues such as lens and cornea.

We also demonstrate that, in most cases, the analyzed miRNAs were overlapping with putative transcriptional units comprising eye-expressed cDNAs and ESTs. We find a direct correlation between the expression of miR-204 and the *Trpm3* mRNA in all tissues analyzed, consistent with the results reported by Deo et al.[14] *Trpm3* mRNA was detected in a punctate pattern, probably reflecting its nuclear localization. This finding was very intriguing, as most mRNAs, especially protein-coding ones, are cytoplasmic. However, there are examples of nuclear retained poly(A)$^+$ RNAs which on physiological stress are posttranscriptionally processed, rendering a transcript that is then exported to the cytoplasm.[19] Also, miR-124a was found to have an expression pattern very similar to that of the mRNAs corresponding to its two host transcripts. Remarkably, Mm.194050 and Mm.221244 mRNAs showed an expression pattern in embryonic sections very similar to that obtained for miR-124a. Also in adult retina these mRNAs were expressed in exactly the same cell types as miR-124a. It was already shown that miRNAs and their host transcripts share the same expression pattern at a tissue level.[20] Our data reveal that the coexpression of miRNAs and host genes is also found at the cellular level.

It is not yet completely clear whether all miRNAs have their own promoters or they highjack regulatory elements from the genes within which they reside. In our examples, the coexpression in the same cell layers prompts us to hypothesize that miRNAs share the same set of expression control elements (i.e., promoters and enhancers), with their host genes. We tend to favor this hypothesis over the alternative one that the coexpression could reflect the fact that eye-expressed miRNAs derive from loci where a combination of factors permit a preferential transcription in the eye. Further studies will be needed to determine whether host genes always have their own independent physiological role or, at least in some cases, their expression may serve only to provide a template for miRNA production. In any case, our data imply that for the identification of novel eye-expressed miRNAs attention should be oriented toward those miRNAs located within transcriptional units with predominant expression in the eye. Considering that Wienholds et al.[11] analyzed only 115 miRNAs and that the number of mouse miRNAs currently known (i.e., those deposited in the miRNA registry at the Sanger Centre[3]) is stably increasing (the latest release contains 358 mouse miRNA entries), this finding will facilitate the identification of additional miRNAs expressed in the mammalian eye.

After the systematic efforts for miRNA identification and cloning, mapping miRNA expression at high resolution in a given tissue is the next step toward designing appropriate approaches for elucidation of the precise function of these small regulatory elements in eye development and function. The variable spatial and temporal profiles obtained for these miRNAs suggest that they may modulate diverse aspects of ocular and retinal function. Knowledge of miRNA spatiotemporal expression in the eye in combination with target prediction data will provide a helpful tool in the identification of biologically significant targets. Of interest, a preliminary bioinformatic analysis revealed that *Mitf*, a transcription factor highly expressed in the RPE and whose repression is essential for the maintenance of a neuroretinal identity,[21] is predicted to be a target of miR-124a and -182, which are expressed in the neural retina, but not in the RPE. Depending on the cellular distribution of miRNAs, appropriate cell lines and experimental approaches can be used to elucidate miRNA function in the eye, both in physiological and in pathologic conditions. Overall, we believe that these data can be explored to gain a better understanding of the mechanisms controlling gene expression in the retina and eye and eventually may contribute to the identification of novel pathogenetic mechanisms.

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