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Research Report

MicroRNA gene expression in the mouse inner ear

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ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs that function through the RNA interference (RNAi) pathway and post-transcriptionally regulate gene expression in eukaryotic organisms. While miRNAs are known to affect cellular proliferation, differentiation, and morphological development, neither their expression nor roles in mammalian inner ear development have been characterized. We have investigated the extent of miRNA expression at various time points throughout maturation of the postnatal mouse inner ear by microarray analysis. Approximately one third of known miRNAs are detected in the inner ear, and their expression persists to adulthood. Expression of such miRNAs is validated by quantitative PCR and northern blot analysis. Further analysis by in situ hybridization demonstrates that certain miRNAs exhibit cell-specific expression patterns in the mouse inner ear. Notably, we demonstrate that miRNAs previously associated with mechanosensory cells in zebrafish are also expressed in hair cells of the auditory and vestibular endorgans. Our results demonstrate that miRNA expression is abundant in the mammalian inner ear and that certain miRNAs are evolutionarily associated with mechanosensory cell development and/or function. The data suggest that miRNAs contribute substantially to genetic programs intrinsic to development and function of the mammalian inner ear and that specific miRNAs might influence formation of sensory epithelia from the primitive otic neuroepithelium.

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1. Introduction

The sensory endorgans of the vertebrate inner ear are mosaics of supporting cells and hair cells that transduce mechanical energy into electrical impulses destined for the central nervous system. Organization of terminally differentiated cell types in the sensory epithelia provides a model system for understanding the molecular processes that govern cell type specification and maturation from a simple epithelium to the complex organ of Corti. Much progress has been made in identifying the molecular constituents required for various aspects of inner ear morphogenesis, maturation, and homeostasis (Fritzsch and Beisel, 2003; Eatock and Hurley, 2003; Barald and Kelley, 2004). A number of transcription factors, morphogens, growth factors, receptors, ion channels, and cytoskeletal proteins have been identified that affect various aspects of inner ear development from primitive otic neuroepithelium to cell fate assignment and functional maturation of afferent neurons, hair cells, and supporting cells. Approximately 29,000 genes are expressed throughout ear development with about 4000 being differentially regulated (Chen Z.Y., personal communication). Yet to be considered in these processes is the probable influence of an extensive class of regulatory molecules termed microRNAs.

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MicroRNAs (miRNAs) are processed from the transcripts of endogenous genes and function through the RNA interference (RNAi) pathway to mediate post-transcriptional silencing of target genes (Ambros, 2004; He and Hannon, 2004). Among multi-cellular eukaryotic organisms, miRNA genes are evolutionarily conserved and abundant, supporting the view that miRNAs are part of an ancient and crucial genetic regulatory program (Zamore and Haley, 2005). Indeed, certain miRNAs are known to be critical determinants of developmental timing and cell fate specification (Grishok et al., 2001; Chen et al., 2004; Esau et al., 2004; Abbott et al., 2005), morphogenesis (Giraldez et al., 2005; Leaman et al., 2005), cell proliferation (Lee et al., 2005), or differentiated cell function (Poy et al., 2004). Notable examples are miRNAs of the *let-7* family that function as repressors of target genes involved in various aspects of biology (Banerjee and Slack, 2002; Abbott et al., 2005; Johnson et al., 2005). These include RAS-dependent cell signaling and proliferation, where *let-7* miRNA family members repress lin-60/RAS expression in *C. elegans* and human (Johnson et al., 2005), thus illustrating an expected conservation of miRNA function among eukaryotic organisms.

There are at least 270 mouse miRNA genes, many of which are orthologous to those of other vertebrate and mammalian species (Griffiths-Jones, 2004). MicroRNA genes are expressed as capped and polyadenylated RNA polymerase II transcripts (Cai et al., 2004; Lee et al., 2004). Among human and mouse miRNA genes, approximately 25% reside within introns and are presumably co-expressed with known genes, and approximately 40% reside in tandem with another miRNA gene(s) suggesting coordinated miRNA expression and function (John et al., 2004). From primary miRNA transcripts (pri-miRNA), the formation of precursor miRNAs (pre-miRNAs) precedes that of functionally mature miRNAs through successive processing events catalyzed by RNase III family members, Drosha and Dicer (Murchison and Hannon, 2004). Mature (~20 nucleotide) miRNAs associated with RNA-induced silencing complexes (RISC) primarily direct translational repression of partially complementary target mRNAs by a mechanism that is not yet well understood (Filipowicz, 2005). Additionally, miRNAs accelerate target mRNA deadenylation and degradation in a manner distinct from small inhibitory RNA (siRNA)-directed mRNA cleavage (Lim et al., 2005; Giraldez et al., 2006; Wu et al., 2006). Thus, miRNAs and their target genes have co-evolved

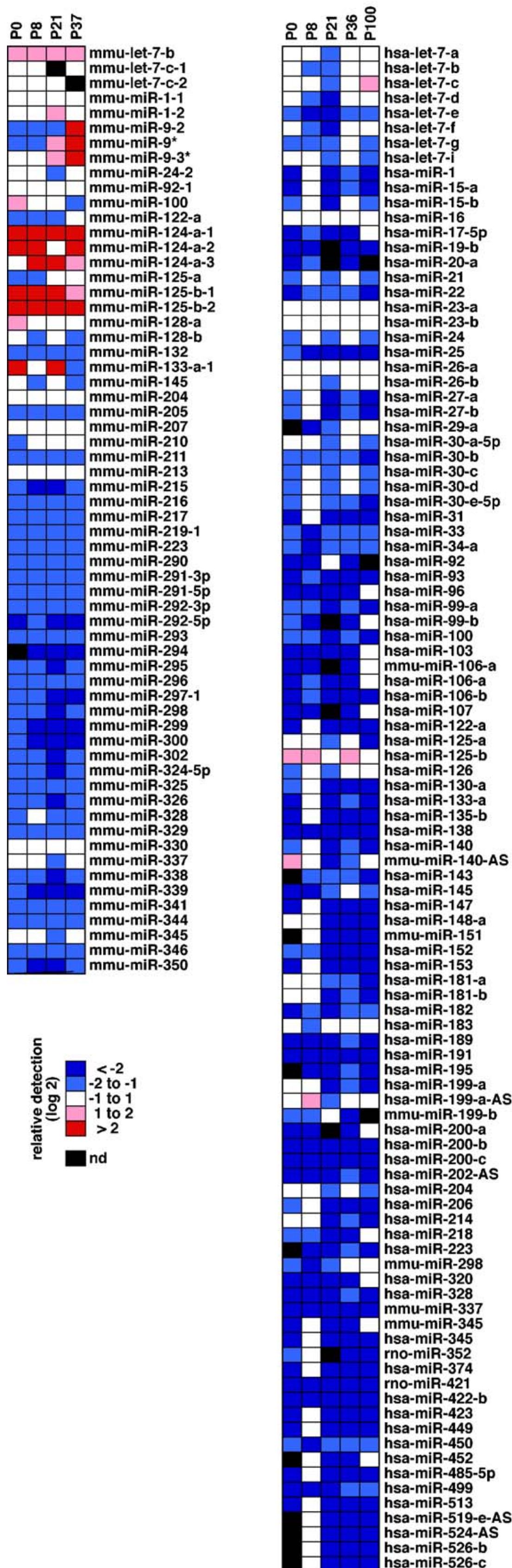

Fig. 1 – MicroRNA expression in the mouse inner ear determined by microarray analyses. Heat maps depict the relative expression level of precursor miRNAs (left) or mature miRNAs (right) indicated by microarray analyses of FVB mouse inner ear RNA isolated at various developmental time points. The color scale corresponds to miRNA detection level relative to endogenous tRNA^{met} for pre-miRNAs or an added control oligonucleotide for mature miRNAs. Depicted are only those miRNAs that were detected twofold greater than background for at least three of four time points between P0 and P37. Black boxes indicate those time points at which miRNAs were not detected twofold greater than background (nd). MicroRNA nomenclature provided by the microarray manufacturers corresponds to entries in The microRNA Registry (Griffiths-Jones, 2004). Mature miRNAs that are orthologous among human (hsa), mouse (mmu), and rat (rno) are listed as human miRNAs. An asterisk or 'AS' denotes the antisense strand or product of pre-miRNA processing.

such that their expression in specified tissues is mutually exclusive (Lewis et al., 2003; John et al., 2004). Analyses of miRNA expression and function in zebrafish have offered the first insights regarding the relevance of miRNAs to ear biology. The RNAi pathway can be disrupted by knockout of the *Dicer* gene, which encodes a ribonuclease required to produce functional microRNAs. Maternal zygotic *Dicer* knockout zebrafish have been shown to exhibit defects in organogenesis that include a lack of otoconia formation in the ear (Giraldez et al., 2005), demonstrating that an absence of functional miRNAs affects vertebrate ear development. Additionally, in situ hybridization analysis of miRNA expression in zebrafish shows that certain miRNAs are expressed in mechanosensory organs (Wienholds et al., 2005). In particular, a set of miRNAs (miR-96, miR-182, and miR-183) are expressed in the hair cells of zebrafish ears and neuromasts. While these studies suggest that miRNAs have cell-specific expression and may fulfill critical functions in vertebrate ear development, no analysis of miRNA expression in the mammalian ear has been performed.

We have examined the expression of miRNAs in inner ears from newborn to adult mice in order to identify relevant miRNAs. Microarray analyses that indicate either precursor or mature miRNA expression were performed to assess the extent of miRNA expression and processing in the inner ear. The expression of a subset of individual miRNAs is validated by Q-PCR and northern blot detection of mature miRNAs. Moreover, in situ hybridization demonstrates that the cell-specific distribution of miRNA expression can be assessed in whole-mounted sensory epithelia of the mouse inner ear.

2. Results

2.1. *Microarray analyses of miRNA expression*

To assess the breadth of miRNA expression in the mouse inner ear, two microarray analyses were performed. The first microarray analysis utilized probes designed to discriminate 213 mouse precursor miRNAs from total RNA samples, enabling identification of precursor miRNAs derived from different genetic loci that yield the same mature miRNA sequence. The second microarray analysis utilized probes designed to assess the expression of 344 mature miRNA sequences present in human, mouse, and/or rat from purified small (<40 nt) RNA samples. As a means of determining which miRNAs are expressed, a single-color analysis was performed where only those miRNAs detected twofold greater than background were considered expressed (Fig. 1). Analysis of miRNA expression throughout mouse inner ear maturation was achieved by examining four time points ranging from postnatal day zero (P0) to approximately 5 weeks, where detection level was normalized to that of an endogenous tRNA for precursor miRNA microarrays or that of an added oligonucleotide control for mature miRNA microarrays (Fig. 1).

Microarray analysis of precursors miRNAs indicates that 62 of 213 mouse miRNA genes are expressed in the inner ear at three or four of the time points examined (Fig. 1). A vast majority of precursor miRNAs were detectible at each of the four time points examined or undetectible at each of the four time points examined (Fig. 2A). Microarray analysis of mature miRNAs similarly demonstrates that 102 of 344 miRNAs are expressed at three of the four time points examined between P0 and P36 (Fig. 1) and that the subset of miRNAs expressed is relatively stable (Fig. 2A). The data illustrate that miRNA expression in inner ear tissues is abundant, likely owing to the wide variety of cell types, but that miRNAs expressed in the postnatal mouse inner ear remain almost constant.

The expression profile of miRNAs in the mouse inner ear appears to be well established by P0, consistent with the fact that early inner ear development and cell fate specification mostly occur embryonically (Barald and Kelley, 2004). While the subset of miRNAs expressed in the postnatal mouse inner ear remain relatively constant, slight changes in expression level are indicated for some miRNAs (Fig. 1). This suggests that miRNA expression might be altered through maturational processes. An analysis of P100 mouse inner ear mature miRNAs shows that the subset of expressed miRNAs extends well beyond inner ear maturation through adulthood (Fig. 1), suggesting that the continued expression of miRNAs serves a role in homeostasis of inner ear cell types.

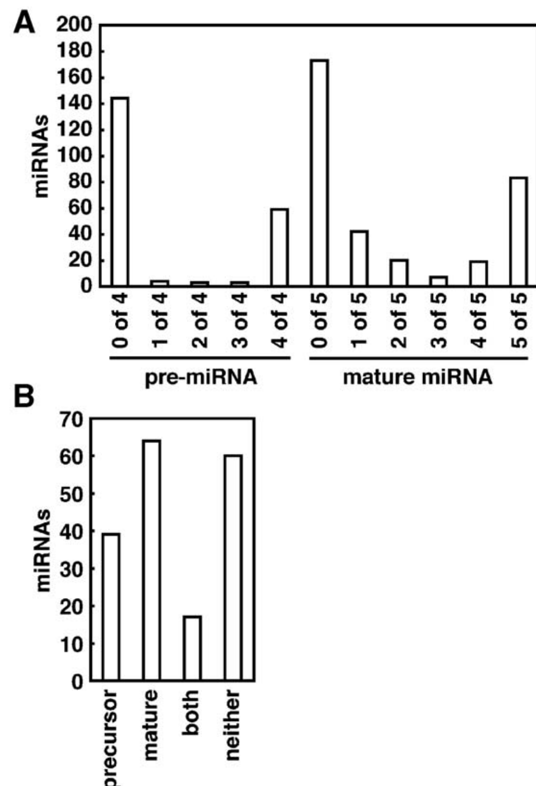

Fig. 2 – Comparison and contrast of miRNA detection between microarray experiments. (A) MicroRNAs in pre-miRNA or mature miRNA microarray experiments given as number detected twofold greater than background among 4 or 5 time points analyzed. (B) MicroRNAs in pre-miRNA or mature miRNA microarray experiments given as number detected only as precursor, only as mature, both as precursor and mature, or neither as precursor or mature. Only those miRNAs detected twofold greater than background for at least three of four time points between P0 and P37 were considered as detected in either precursor or mature form.

Microarray analyses of inner ear precursor and mature miRNAs exhibit a substantial degree of incongruence. Among those miRNAs interrogated by both microarray experiments, 103 miRNAs were detected as only precursor or only mature miRNA forms, while 77 miRNAs were either detected as both forms or neither form was detected (Fig. 2B). Detection of precursor miRNAs presumes that different miRNAs are similarly processed and persist as precursor and mature forms at similar ratios. The incongruence between microarray experiments either suggests that the relationship between precursor and mature miRNA forms is more complex or might be attributable to technical issues regarding detection among total RNA versus purified small RNAs. However, the detection of functionally mature miRNAs among purified small RNAs is likely to proffer the more biologically relevant data set.

2.2. Validation of mature miRNA expression

To validate the expression of mature miRNAs in the mouse inner ear, a subset of miRNAs indicated by microarray analyses was chosen for further study using quantitative PCR (Q-PCR) detection and/or northern blotting methodologies. Q-PCR detection utilized commercial primer sets for reverse transcription of specific mature miRNAs and amplification and detection of cDNA products by SYBR Green fluorescence. Among seven miRNAs chosen for Q-PCR

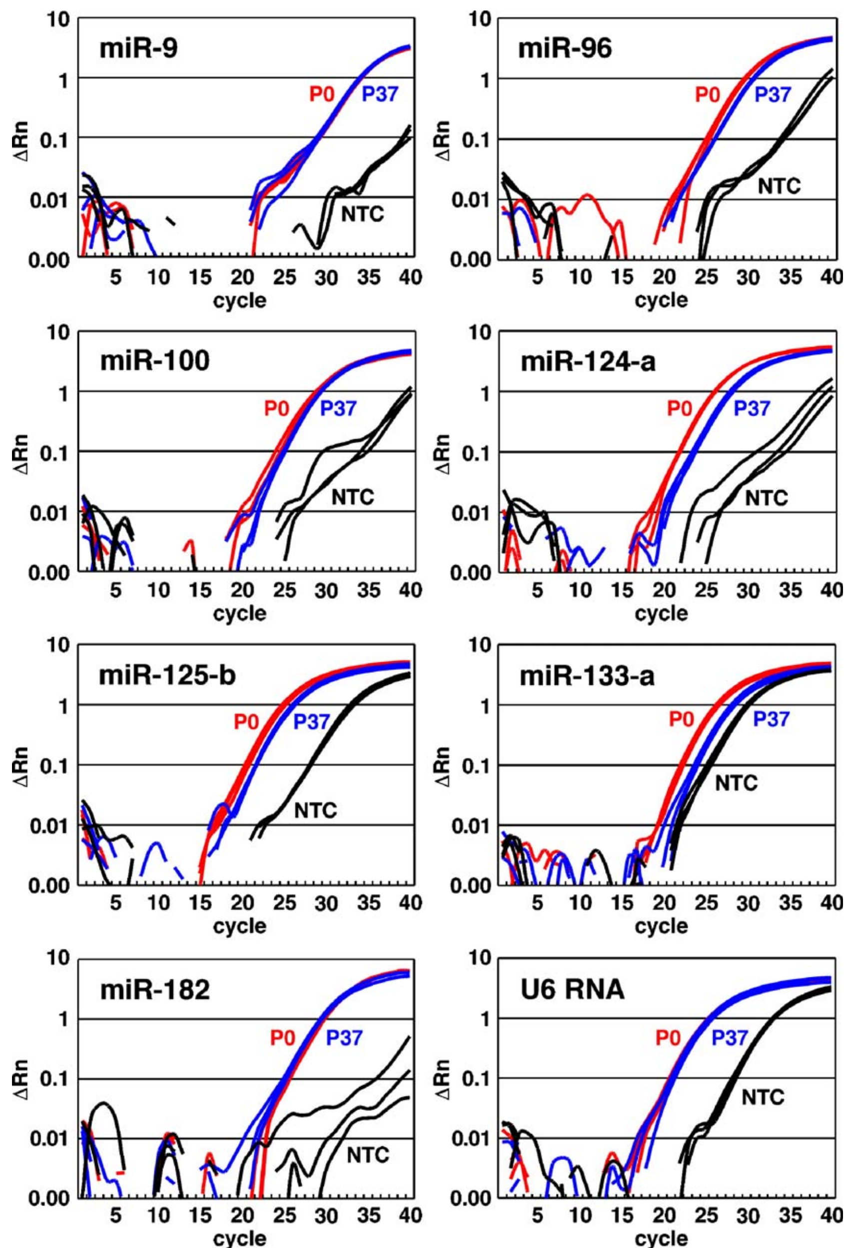

Fig. 3 – Q-PCR analysis of miRNA expression. Plots depict the relative change in fluorescence (ΔRn) indicative of product accumulation over 40 cycles of PCR amplification using primer sets specific for indicated miRNA sequences. PCR reactions were performed in triplicate using cDNA products derived from P0 or P37 mouse inner ear total RNA and miRNA-specific primers as indicated. NTC indicates no template control reactions. U6 RNA was similarly analyzed as an internal reference.

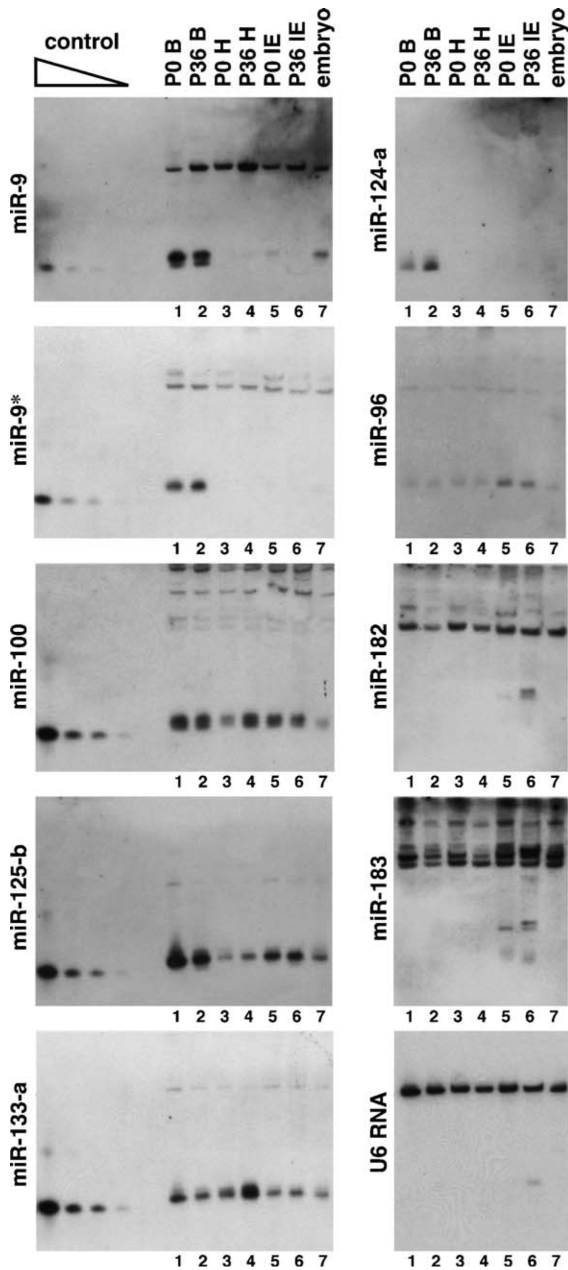

Fig. 4 – Northern blot analysis of miRNA expression. Indicated miRNAs were detected using DIG-labeled LNA probes and alkaline-phosphatase-conjugated anti-DIG antibody. Each lane (1–7) contains 10 μg total RNA prepared from P0 or P36 mouse brain (B), heart (H), or inner ear (IE), or 10 μg total RNA from mouse whole embryo as indicated. Controls represent DNA oligonucleotides homologous to miRNA sequences each in the range from 1 pmol to 1 fmol. The slight difference in electrophoretic mobility between DNA controls and miRNAs is attributable to their difference in chemical composition.

analysis, each exhibited threshold detection several cycles prior to control reactions lacking template (Fig. 3). This subset of miRNAs includes those detected by microarray analyses only as precursor form (miR-9 and miR-124-a), only as mature form (miR-96 and miR-182), and as both precursor and mature forms (miR-100, miR-125-b, and miR-133-a). Comparison of threshold detection between P0 and P37 mouse inner ear RNA suggests that the expression levels of each miRNA do not vary by more than twofold when normalized to U6 RNA as an endogenous control (Fig. 2 and data not shown).

As more definitive evidence for the expression of mature miRNAs in the mouse inner ear, a subset of miRNAs was further examined by northern blot analyses. Detection of mature miRNAs utilized locked nucleic acid (LNA) probes, which possess higher binding affinity than DNA probes (Valoczi et al., 2004). LNA probes were digoxigenin (DIG)-labeled and detected using alkaline-phosphatase (AP)-conjugated anti-DIG antibody and chemiluminescent AP substrate. Among nine miRNAs detected by microarray analyses, those detected only as mature forms (miR-96, miR-182, and miR-183) or as both precursor and mature forms (miR-100, miR-125-b, and miR-133-a) exhibited expression of mature miRNA in the mouse inner ear by northern blot analysis (Fig. 4). Those miRNAs detected by microarray analyses only as precursors (miR-9, miR-9*, and miR-124-a) were only weakly detectible as mature miRNA in the mouse inner ear by northern blot analysis (Fig. 4).

Comparison of miRNA expression among P0 and P36 RNA samples from mouse brain, heart, inner ear, and whole embryo demonstrates that mature miR-100, miR-125-b, and miR-133-a are expressed in each tissue, while mature miR-96, miR-182, and miR-183 are predominantly expressed in the mouse inner ear (Fig. 4). These results are consistent with the previous observation that miR-96, miR-182, and miR-183 are expressed in hair cells of the zebrafish ear and neuromasts

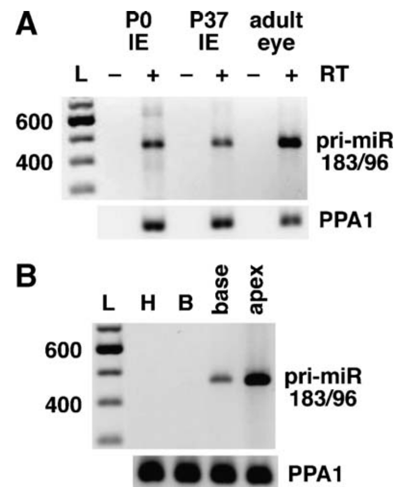

Fig. 5 – RT-PCR detection of primary miR-183/96 transcript. (A) Detection of primary miR-183/96 transcript in mouse ear and eye. Total RNA was reverse transcribed using random hexamer primers. PCR products derived from primary miR-183/96 or protein phosphatase A1 (PPA1) mRNA as an endogenous control are denoted. Control reactions lacking reverse transcriptase are shown and 100 bp ladder is indicated (L). (B) Detection of primary miR-183/96 transcript in P35 mouse cochlear apex and base, but not brain (B) or heart (H). Polyadenylated RNAs were reverse transcribed using an oligo(dT) primer. PCR reactions were performed as in panel A.

among other tissues (Wienholds et al., 2005). Detection of mature miR-9, miR-9*, and miR-124-a in the mouse brain (Fig. 4) is consistent with their characterization as neuronal-specific miRNAs (Lagos-Quintana et al. 2002; Sempere et al. 2004). Although expression of mature miR-9 and miR-124-a in the inner ear appears to be considerably less than that in the brain (Fig. 4), their detection by Q-PCR (Fig. 3) suggests that they are present at a level that is not detectible by microarray analysis (Fig. 1).

Northern blot analyses for certain miRNAs such as miR-9, miR-9*, miR-182, and miR-183 exhibit higher molecular weight species among the tissues examined that might represent either precursor miRNAs or non-specific binding to other transcripts (Fig. 4). It is tempting to speculate that the processing of these miRNAs might be differentially regulated in different tissues, as suggested for certain miRNAs (Yang et al., 2006). However, further analysis of the primary transcript containing miR-96 and miR-183, whose genes are clustered within a 500 bp segment of mouse chromosome 6, demonstrates detection by RT-PCR only in inner ear and eye (Fig. 5), consistent with in situ hybridization studies conducted in zebrafish (Wienholds et al., 2005). Therefore, hybridization of LNA probes to higher molecular weight species in northern blot analyses of RNA from other tissues (e.g. brain or heart) likely represents non-specific binding under the precise hybridization and wash conditions utilized.

2.3. *In situ hybridization of miRNAs*

To investigate the cell-specific distribution of miRNAs in mouse inner ear tissues, whole-mount in situ hybridizations were performed using DIG-labeled LNA probes detected with AP-conjugated anti-DIG antibody and colorimetric AP substrate. The expression of miRNAs examined by in situ hybridization demonstrates a variety of cellular distribution patterns and intensities (Fig. 6 and data not shown). For example, miR-124-a exhibits expression in neuronal cells demonstrated by staining of the spiral and vestibular ganglia (Fig. 6). Although miR-100 exhibited more ubiquitous expression throughout inner ear tissues (data not shown), it demonstrated preferential expression in neuronal cells with marked detection in the periphery of the vestibular ganglia (Fig. 6). In general, the LNA probes demonstrated good penetration of inner ear tissues likely attributable to their low molecular weight.

Of particular interest is the cellular expression pattern of miR-182. In situ hybridizations for miR-182 demonstrated its expression in all the inner and outer hair cells of the cochlea and in hair cells of the cristae, utricle, and saccule of the vestibular endorgans (Fig. 6A and data not shown). Additionally, miR-182 is detected less intensely in neurons of the spiral

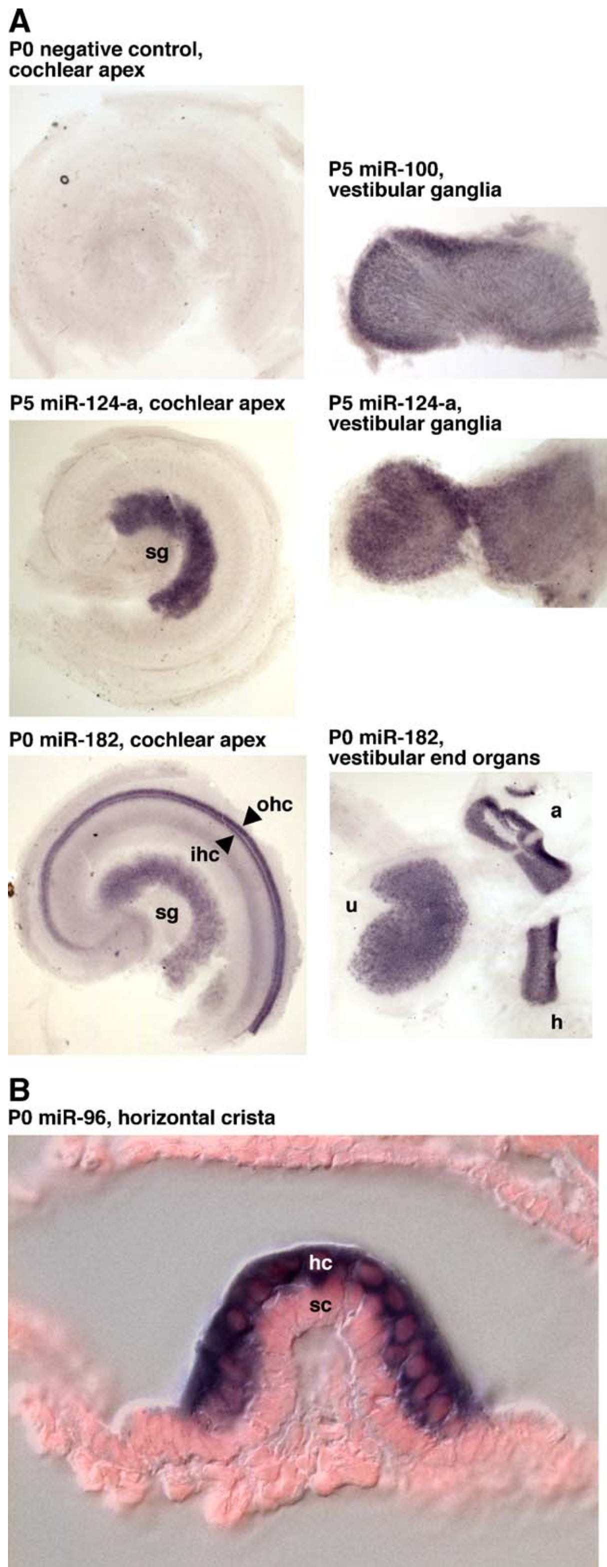

Fig. 6 – In situ hybridization of miRNAs in mouse inner ear. (A) Whole-mount in situ hybridizations. Depicted is the detection of miR-100, miR-124-a, or miR-182 in P0 or P5 mouse inner ear tissues as indicated using DIG-labeled LNA probes and alkaline-phosphatase-conjugated anti-DIG antibody. The negative control represents tissue similarly prepared without DIG-labeled probe. Denoted are spiral ganglia (sg), inner and outer hair cells (ihc and ohc, respectively), utricle (u), and anterior (a) and horizontal (h) cristae. The loss of hair cells in the anterior crista is a preparation artifact. (B) Cross-section of horizontal crista. Depicted is the detection of miR-96 with false-colored DAPI staining of nuclei (red) superimposed. Denoted are hair cell (hc) and supporting cell (sc) layers of the sensory epithelium.

and vestibular ganglia (Fig. 6A and data not shown). Identical expression patterns were observed for miR-96 and miR-183 (Fig. 6B and data not shown), consistent with miR-96, miR-182, and miR-183 being processed from a common primary transcript. The co-expression of these miRNAs in mammalian hair cells parallels expression in mechanosensory cells of zebrafish (Wienholds et al., 2005). These findings suggest that the miRNAs are evolutionarily associated with mechanosensory cell specification and function.

3. Discussion

MicroRNAs represent approximately 1% of the genes in mammalian organisms and a substantial component of abundant non-coding RNAs that contribute to the complexity of genetic regulation (Riddihough, 2005). The potential of miRNAs to regulate thousands of target mRNAs containing conserved complementary sites suggests that they contribute substantially to regulation of gene expression (John et al., 2004; Lewis et al., 2005). Our analysis of the mouse inner ear demonstrates that miRNA expression is extensive, where approximately one third of known miRNAs are detected in the inner ear. Throughout the postnatal stages of mouse inner ear development and maturation, the subset of miRNAs expressed appears to be relatively stable. The general profile of miRNA expression thus appears to be established during embryonic stages of development and cell differentiation. In other developing systems, certain miRNAs are known to contribute to cell fate specification. For example, expression of miR-124-a in the spiral and vestibular ganglia is consistent with its established role in neuronal differentiation (Krichevsky et al., 2006; Conaco et al., 2006). Therefore, the determination of cell-specific expression patterns of miRNAs in the maturing mouse inner ear is a useful avenue for inferring probable roles for miRNAs in development. Moreover, mouse inner ear miRNA expression remains relatively stable well into adulthood, suggesting that miRNA functions extend beyond developmental effects and contribute to differentiated cell functionality and stasis.

The detection of miRNAs by in situ hybridization using LNA probes is proving to be a useful methodology for revealing cell-specific miRNA expression patterns in vertebrate tissues (Wienholds et al., 2005; Kloosterman et al., 2006; Nelson et al., 2006). Our study demonstrates that LNA in situ hybridization can be readily extended to analysis of miRNA expression in the inner ear. Moreover, our analysis of miRNAs previously detected in zebrafish hair cells (Wienholds et al., 2005) demonstrates that miR-182, miR-183, and miR-96 are detected in hair cells throughout mammalian auditory and vestibular organs. This evolutionary conservation of miRNA expression in mechanosensory hair cells of zebrafish and mammals suggests an equal conservation of function in diverse organisms. The further detection of these miRNAs in zebrafish eye by in situ hybridization (Wienholds et al., 2005) and in mouse eye by RT-PCR analysis additionally indicates that the miRNAs are more generally associated with neurosensory cell fates and/or functions of possibly evolutionarily related cells (Fritzsch and Piatigorsky, 2005).

Given the conservation of miRNA genes, conservation of miRNA complementary sites within target mRNAs, and the conservation of tissue- or cell-type-specific miRNA expression patterns among organisms, it becomes possible to infer miRNA functions. Experimental approaches for individually validating predicted miRNA targets are generally labor-intensive (Bentwich, 2005), although microarray analysis of target gene expression has proven to provide a high-throughput methodology for examining the effects and elucidating the functions of miRNAs (Lim et al., 2005; Wang and Wang, 2006; Wu et al., 2006). Such analyses are generally limited to cell culture experiments and are not easily amenable to complex organs such as the inner ear, although similar analysis of single cells is possible (Tang et al., 2006). Applying predictive algorithms (John et al., 2004; Lewis et al., 2005) to miR-182, miR-183, and miR-96, which are likely co-expressed from a common primary transcript, reveals the potential for hundreds of target mRNAs, many of which encode transcriptions factors (data not shown). This suggests the miRNAs can contribute substantially to altering the expression profiles of hair cells relative to adjacent supporting cells or common precursor cells and that the miRNAs might thus facilitate hair cell differentiation and function.

This study demonstrates the extent and specificity of miRNA expression in the mammalian inner ear, where the regulatory functions of miRNAs are likely to contribute to developmental and functional aspects of ear biology. As gene therapeutic strategies for stimulating hair cell regeneration and hearing restoration advance (Maiorana and Staecker, 2005), they may benefit substantially from miRNA therapies (Hammond, 2006) designed to guide developmental or maturational processes that contribute to specified cell functions.

4. Experimental procedures

4.1. *Animals*

Animal care and handling complied with protocols approved by the Creighton University Institutional Animal Care and Use Committee (IACUC #0730) and employed measures to minimize pain or discomfort. FVB mice were purchased from Charles River Laboratories.

4.2. *RNA extraction*

Tissues were isolated from FVB mice in cold phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4). Inner ear included all tissues of the otic capsule (membranous and bony labyrinths) and cochlear and vestibular ganglia. Following rotor-stator homogenization of tissue, total RNA including microRNA was purified using the *mir*Vana miRNA Isolation Kit as suggested by the manufacturer (Ambion). The quality and quantity of each RNA preparation were determined using a Model 2100 Agilent BioAnalyzer and Nanodrop ND-1000 spectrophotometer.

4.3. *Microarray analyses*

For microarray analysis of inner ear precursor miRNA expression, 10 μg total inner ear RNA from P0, P8, P21, and P37 FVB mice was labeled and hybridized to a commercial microarray for analysis by the manufacturer (GenoSensor Corporation). The average of three mean fluorescence signal intensities for each precursor miRNA probe was normalized to that for $tRNA^{met}$ and log base 2 transformed. Precursor miRNAs detected twofold greater than background were considered to be expressed. For microarray analysis of inner ear mature miRNA expression, small RNAs (<40 nt) from 10 μg total inner ear RNA from P0, P8, P21, P36, and P100 FVB mice were isolated using a flashPAGE™ Fractionator (Ambion) as suggested by the manufacturer. Each RNA sample was labeled using the *mir*Vana miRNA Labeling Kit and hybridized to an oligonucleotide microarray containing the *mir*Vana miRNA Probe Set (Ambion) according to manufacturer's protocols. The probe set was printed on epoxysilane-coated glass slides E (Schott Nexterion), and sample hybridization and analyses were performed by the University of Nebraska Medical Center Microarray Core Facility. Oligonucleotide concentration for printing was 20 μM in spotting buffer "A" (MWG Biotech). Printing was performed at 50% humidity using an Omgrid 100 robotic printer (Genomic Solutions). Hybridization of labeled RNA samples was performed at 42 °C for 12 h in miRNA hybridization buffer (Ambion), and slides were washed according to the manufacturer's protocol. Microarrays were scanned using a GenePix 4000b Biochip Reader (Molecular Devices), and primary data were analyzed using the Digital Genome System Suite (Molecularware). The average of two mean fluorescence signal intensities for each mature miRNA probe was normalized to that for an added control oligonucleotide and log base 2 transformed. Mature miRNAs detected twofold greater than background were considered to be expressed.

4.4. *RT-PCR*

Quantitative PCR (Q-PCR) detection of miRNAs was performed using *mir*Vana qRT-PCR miRNA Primer Sets (Ambion). For each miRNA, 100 ng total RNA was treated with DNase I (Invitrogen) and reverse transcribed using a 10-fold dilution of *mir*Vana qRT-PCR miRNA-specific RT-primer and SuperScript III Reverse Transcriptase (Invitrogen) in a 10 μL reaction. Q-PCR was performed using a PRISM 7000 Sequence Detection System (Applied Biosystems) to analyze triplicate reactions (25 μL) containing 2× SYBR Green PCR Master Mix (Applied Biosystems), 0.5 μM *mir*Vana qRT-PCR miRNA Primer Set, and a fourth of the RT product. RT product was omitted from triplicate no template control (NTC) reactions. Following incubation at 50 °C for 2 min and 90 °C for 10 min, PCR products were analyzed throughout 40 cycles consisting of an incubation at 95 °C for 15 s and 60 °C for 30 s. Amplification plots for each miRNA and U6 RNA (Ambion) depict the change in SYBR Green fluorescence relative to the ROX internal standard and were generated using EXCEL (Microsoft). Results are representative of two independent assays.

Primary transcript containing miR-96 and miR-183 was detected by RT-PCR using primers 5′-TGCAGGCTGGA-GAGTGTGAC and 5′-CTCAGGCAGTGAAAGGTGATC yielding a 452 bp product. Transcript encoding protein phosphatase A1 was detected using primers 5′-CTGTTGCTGGCCTATAAGAT-CAG and 5′-AGCCCCAAAGGTAAAGGAGAC yielding a 372 bp product. 1 μg total RNA isolated from P0 or P37 inner ear, adult eye, or P35 basal cochlea, apical cochlea, brain, or heart was treated with DNase I and reverse transcribed using random hexamer or oligo(dT) primers and SuperScript III Reverse Transcriptase (Invitrogen). PCR reactions contained 100 ng cDNA, 0.2 μM primers, 10 mM Tris–HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, and 1 U FastStart Taq DNA polymerase (Roche). PCR products were analyzed by agarose gel electrophoresis following incubation at 95 °C for 4 min and 37 cycles consisting of incubation at 95 °C for 30 s and 55 °C for 30 s and 72 °C for 1 min.

4.5. *Northern blotting*

miRCURY LNA probes (Exiqon) were labeled with digoxigenin (DIG) using the DIG Oligonucleotide 3′-End Labeling Kit (Roche) and purified by size exclusion chromatography using CHROMA SPIN-10 columns (Clontech). U6 RNA was detected using a similarly DIG-labeled U6 DNA probe, 5′-TGGAACGCTT-CACGAATTTG. For northern blot analysis, 10 μg total RNA from P0 and P36 FVB mouse inner ear, heart, and brain, and 10 μg mouse whole embryo total RNA (Ambion) was resolved by 15% denaturing (7 M urea) polyacrylamide gel electrophoresis (PAGE) and transferred by electroblotting to GeneScreen Plus Charged Nylon Membranes (PerkinElmer). DNA oligonucleotides corresponding to miRNA sequences were included as controls to assess hybridization specificity and sensitivity. Blots were pre-hybridized by incubation with hybridization buffer (0.36 M Na_2HPO_4, 0.14 M NaH_2PO_4, 1 mM EDTA, and 7% w/v SDS, pH 7.2) for at least 1 h at 52 °C and hybridized by overnight incubation in hybridization buffer containing 0.2 nM DIG-labeled probe at 52 °C for each LNA probe or 25 °C for the U6 DNA probe. Blots were stringently washed twice in 0.5× SSC (7.5 mM sodium citrate, 75 mM NaCl, pH 7.0) containing 0.1% w/v SDS for 30 min at 52 °C for each LNA probe or 25 °C for the U6 DNA probe, rinsed in Wash Buffer, and incubated in Block Buffer for 30 min at 23 °C (DIG Wash and Block Buffer Set, Roche). Subsequently, blots were incubated with a 10,000-fold dilution of anti-DIG-AP Fab fragment (Roche) in Block Buffer for 1 h at 23 °C, washed three times for 15 min at 23 °C in Wash Buffer, and washed 2 times for 5 min at 23 °C with Detection Buffer (DIG Wash and Block Buffer Set, Roche). Anti-DIG-AP was detected using CDP-star chemiluminescent substrate for alkaline phosphatase (Roche). Blots were stripped by incubation for 10 min at 70 °C in 0.1× SSC containing 1% SDS and probed up to five times.

4.6. *Whole-mount in situ hybridization*

DIG-labeled miRCURY LNA probes (Exiqon) were prepared as described for northern blot analysis. Control DIG-labeled beta-actin mRNA riboprobe (633 nt) was prepared by in vitro transcription from DNA templates using T7 RNA polymerase and DIG-UTP (Roche) and purified using RNAeasy spin columns (Qiagen). DNA templates for in vitro transcription of the beta-actin riboprobe were generated by PCR using primers 5′-TAATACGACTCACTATAGGGACCGCTCGTTGCCAATAGTG

and 5′-TGGTGGGAATGGGTCAGAAG and random-hexamer-primed mouse cDNAs.

Whole-mount in situ hybridization of P0 or P5 FVB mouse inner ear tissue was performed essentially as previously described (Judice et al., 2002; Wienholds et al., 2005; Kloosterman et al., 2006). Anesthetized mice were transcardially perfused with PBS followed by PBS containing 4% PFA. Inner ears were dissected, fixed in PBS containing 4% PFA, decalcified in 120 mM EDTA, and stored in PBS at 4 °C. To improve tissue penetration, demineralized and cartilaginous labyrinthine tissue were removed and sensory epithelia were exposed by removing overlying acellular membranes. Tissues were defatted through a graded ethanol series, rehydrated in PBS containing 0.1% TWEEN 20, and digested in PBS containing 2.5 mg/ml proteinase K for 20 min at 37 °C. Digestions were terminated using PBS containing 2 mg/ml Glycine and 0.1% TWEEN 20, fixed in PBS containing 4% PFA, and re-equilibrated in PBS containing 0.1% TWEEN 20. Tissues were pre-hybridized by incubation for 1–3 h at 53 °C in 2 mL hybridization solution (50% formamide, 2× SSC, 0.1% TWEEN 20, and 150 μg/ml denatured herring sperm DNA). Hybridizations were incubated overnight at 53 °C following the addition of 100 pmol DIG-labeled LNA probe or 1 pmol DIG-labeled beta-actin mRNA riboprobe denatured for 10 min at 68 °C in hybridization buffer. Tissues were extensively washed at 50 °C to 0.5× SSC stringency, and RNA was digested by incubation for 1 h at 37 °C with RNase A (100 mM Tris–HCl pH 7.5, 500 mM NaCl, 0.1% TWEEN 20, and 10 μg/ml RNase A). Digestions were terminated by incubation for 10 min at 25 °C in 0.5× SSC containing 0.1% SDS. Tissues were incubated in Wash and Block Buffers (DIG Wash and Block Buffer Set, Roche) for 1 h at 4 °C and incubated overnight in a 1000-fold dilution of sheep anti-DIG-AP Fab fragment (Roche) in Block Buffer. Samples were extensively washed for 1–2 days at 4 °C with Wash Buffer. Alkaline phosphatase activity was detected using BM Purple AP Substrate (Roche) by incubation for 2–24 h at 25 °C. Reactions were terminated for at least 1 h in PBS (pH 5.5) containing 10 mM EDTA. Tissues were fixed by incubation overnight at 4 °C in PBS containing 4% PFA. Fixed tissues were mounted in glycerol under glass cover slips for image acquisition as described previously (Pauley et al., 2003). Prior to sectioning, tissues were cryoprotected in 20% sucrose, embedded in OCT medium (Sakura Finetek), and frozen in a dry ice ethanol bath. 20 μm frozen sections were mounted with VECTASHIELD containing DAPI (Vector Labs). Localization of BM Purple and DAPI staining was respectively visualized by differential interference contrast and epifluorescence microscopy.

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