

External ear microRNA expression profiles during mouse development

LEDA TORRES¹, ULISES JUÁREZ^{1,2}, LAURA GARCÍA^{3,4}, JUAN MIRANDA-RÍOS⁴ and SARA FRIAS^{*1,5}

¹Laboratorio de Citogenética, Depto. de Investigación en Genética Humana, Instituto Nacional de Pediatría, ²Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, ³Posgrado en Ciencias Biomédicas, Universidad Nacional Autónoma de México, ⁴Unidad de Genética de la Nutrición, Depto. Biología Molecular y Biotecnología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México e Instituto Nacional de Pediatría and ⁵Unidad de Genética de la Nutrición. Depto. Medicina Genómica y Toxicología Ambiental, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México e Instituto Nacional de Pediatría, México DF, México

ABSTRACT MicroRNAs (miRNAs) comprise a class of approximately 22 nucleotide regulatory non-coding RNAs that play several roles in diverse biological processes. Recent reports suggest that embryonic development in mammals is accompanied by dynamic changes in miRNA expression; however, there is no information regarding the role of miRNAs in the development of the external ear. The aim of this study was to determine the stage-specific expression of miRNAs during mouse external ear development in order to identify potentially implicated miRNAs along with their possible targets. miRNA expression profiles from fetal mice pinnae and back skin tissues at 13.5 dpc and 14.5 dpc were obtained using an Affymetrix GeneChip miRNA 3.0 array. Biological triplicates for both tissues, each collected from a litter averaging 16 fetuses, were analyzed. The results were analyzed with Affymetrix's Transcriptome Analysis Console software to identify differentially expressed miRNAs. We observed differential expression of 40 miRNAs including some predicted to target genes implicated in external ear development, such as *mmu-miR-10a*, a miRNA known to modulate *Hoxa1* mRNA levels, and *mmu-miR-200c* and *mmu-miR-205*. To our knowledge, this is the first miRNA expression profiling study of external ear development in mammals. These data could set the basis to understand the implications of miRNAs in normal external ear development

KEY WORDS: *miRNA*, *pinnae*, *auricle*, *microtia*, *GEO* (GSE64945)

In mammals, the outer ear is formed by the ear pinna (i.e., the auricle and the external ear), the external acoustic meatus (i.e., the ear canal), and the outer layer of the tympanic membrane (i.e., the eardrum). The outer ear develops as a result of complex tissue interactions between ectoderm and mesoderm during embryogenesis. Outer ear development is driven by the mesenchyme of the first and second pharyngeal arches and is controlled, at least in part, by the genes that determine the first and the second pharyngeal arch identity. The auricle is formed from six protuberances in the first and the second arches known as the auricular hillocks (Jones and Chuan, 1934; Mallo and Gridley, 1996). In mice, several genes involved in external ear development have been identified, including *Hoxa2*, *Hoxa1*, *Hoxb1*, *Prrx1*, *Dlx1*, *Dlx5*, *Six1*, *Six4*, *Sall1*, *Tbx1*, *Bmp5*, *Fgf3*, *Fgf8*, *Fgf10* and *Eya1*. The genetic and cellular mechanisms underlying normal morphogenesis of the external

ear are incompletely understood (Minoux *et al.*, 2013; Luquetti *et al.*, 2012). Recent reports suggest that embryonic development in mammals is accompanied by dynamic changes in the expression of an extensive class of regulatory molecules termed miRNAs (Lau *et al.*, 2001; Berezikov, 2011); therefore, these molecules could be influencing the external ear development process.

miRNAs are small (approximately 22 nucleotide long), highly conserved regulatory molecules that modulate gene expression, mainly by targeting mRNAs for translational repression or degradation (Lau *et al.*, 2001; Berezikov, 2011). Since their discovery, these non-coding RNA molecules have been shown to play important roles in several biological processes, including vertebrate develop-

Abbreviations used in this paper: dpc, days post coitum; *mmu*, *Mus musculus*.

*Address correspondence to: Sara Frias. Instituto Nacional de Pediatría México /Instituto de Investigaciones Biomédicas Universidad Nacional Autónoma de México. Insurgentes Sur 3700-C, CP 04530, Col. Insurgentes-Cuicuilco, Mexico D.F, México. Tel: +52-55-1084-5533. E-mail: sarafrias@biomedicas.unam.mx; sarafrias@yahoo.com

Accepted: 2 December 2015.

ment (Gavalas *et al.*, 1998; Petri *et al.*, 2014; Pasquinelli, 2012).

The study of miRNAs is a novel field, and little is known about their role in organogenesis (the development of organs or tissues) or the mechanisms that regulate their expression and function (Pasquinelli, 2012). However, there have been several mice studies that explored the role of miRNAs in the development of heart, retina, brain and inner ear. These studies have shown a particular spatiotemporal pattern of miRNAs expression, where specific sets of miRNAs are expressed in the developmental processes of each structure (Petri *et al.*, 2014; Sacheli *et al.*, 2009).

In the mouse, the study of miRNAs in the inner ear has shown temporal and spatial expression of the miR-96/-182/-183 cluster that correlates with the inner ear's functional maturation (Sacheli *et al.*, 2009). However, the miRNA expression profile during external ear development has not been assayed.

The purpose of this study was to determine the stage-specific expression of miRNAs during external ear development in mice under normal conditions and to identify the miRNAs along with their possible targets.

Results and Discussion

We describe the expression pattern of miRNAs involved in the development of the external ear in mice at two different stages of development; 13.5 days post coitum (dpc) and 14.5 dpc, using GeneChip miRNA 3.0 arrays from Affymetrix. We compared the expression profiles from pinnae and back skin tissues and observed, as expected, that there are distinct patterns of miRNA expression between the tissues and the stages of development. The differentially expressed miRNAs between pinnae and back skin tissues at 13.5 dpc and 14.5 dpc are shown as heat maps in Figs 1 and 2, respectively.

Spatial expression pattern showed by *in situ* hybridization of mmu-miR-10a, mmu-miR-205 and mmu-miR-200c at 13.5 and 14.5 dpc, confirmed the results observed by microarrays. We observed signal of mmu-miR-10a at both stages analyzed in the dorsal region and in the bones of anterior limbs, and almost undetectable signal in pinnae, the signal for mmu-miR-200c and mmu-miR-205 was present in lips and nose, as previously reported (Warner *et al.*, 2014) and in pinnae, with high levels of mmu-miR-205 and low levels in the case of mmu-miR-200c at both stages (Fig. 3).

We performed real-time quantitative RT-PCR analysis in the interest to know if mRNAs of *Hoxa1*, *Hoxa2*, *Tob1*, *Eya1* and *Six1* were present in pinnae or skin at the stages studied. The relative expression levels of mRNAs

Fig. 1. Heat map of miRNA expression at 13.5 dpc, showing differentially expressed miRNAs between pinnae and back skin tissues. Hierarchical clusters of 25 miRNA genes were found to be significantly differentially expressed (adjusted *p* value <0.05) in pinnae compared to back skin controls. Each row of the hierarchical clusters represents an individual miRNA and each column represents one biological replica of either pinnae tissue (1) or skin tissue (2). The scale represents the gene expression intensity (log₂ scale ranges between -1.5 and 1.5). Red color indicates an increase in miRNA gene expression (relative to the other expression measurements in the same row) and green indicates a decrease.

in relation to the *Actb* reference gene are presented in Fig. 4. We detected mRNA of the five genes studied in both tissues.

The microarrays analysis showed in fetal mice of 13.5 dpc, 25 different mmu-miRNAs differentially expressed in the pinnae with 11 of the mmu-miRNAs presenting elevated expression levels and 14 with lowered expression levels (Table 1). At 14.5 dpc, 31 different mmu-miRNAs showed differential expression in the pinnae with 14 mmu-miRNAs displaying elevated levels of expression while 17 exhibited decreased levels of expression (Table 2). We observed that mmu-miR-429, mmu-miR-375, mmu-miR-200a, mmu-miR-708, mmu-miR-27b-5p, mmu-miR-23b and mmu-miR-24 were highly expressed in both stages. These miRNAs have several possible targets according to the consulted databases; some of them (like *Eya1*, *Hoxa2* and *Six4*) are known to be related to external ear development. However, their interactions have not yet been corroborated.

Moreover, at both stages we detected in the microarrays some miRNAs with low expression levels, such as mmu-miR-7a-2-3p, mmu-miR-135a-2-3p, mmu-miR-10b, mmu-miR-615-3p, mmu-miR-9-3p, mmu-miR-219-3p and mmu-miR-10a. The low levels of expression of mmu-miR-10a in pinnae were also seen in *in situ* hybridization analysis at both stages analyzed, in detail the pinnae has significantly lower signal of mmu-miR-10a in the outline of the ear canal, as compared with back skin; note the cartilaginous region without signal. (Fig. 3C and 3E). The inquiry for possible targets of these miRNAs showed several genes. Of note is *Hoxa1*, a gene that participates in the development of the external ear that is a mmu-miR-10a target. In mice, the mmu-miR-10a gene-coding region is located at the 11qD locus within the *HoxB* gene cluster.

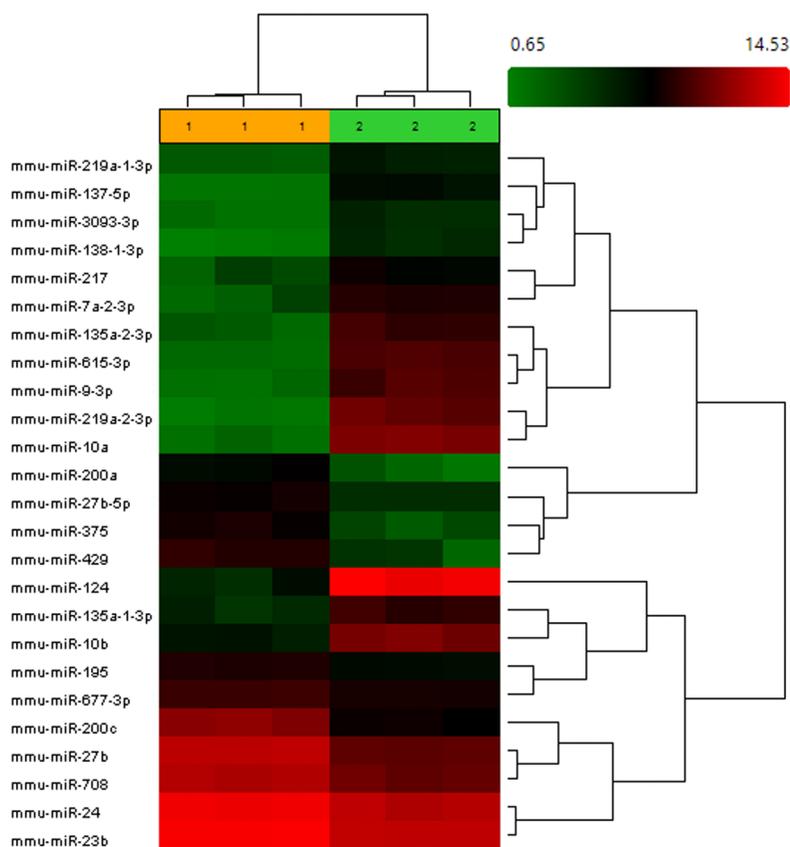


TABLE 1
THE miRNAs DIFFERENTIALLY EXPRESSED
IN PINNAE VERSUS SKIN BACK TISSUES AT 13.5 DPC

mmu-miRNA	Sequence (5' -3')	Length (nt)	Fold change
mmu-miR-200c	UAAUACUGCCGGUAAUGAUGGA	23	3.18
mmu-miR-429	UAAUACUGUCUGGUAUUGCCGU	22	2.80
mmu-miR-27b	UUCACAGUGGCUAAGUUCUGC	22	2.52
mmu-miR-375	UUUGUUCGUUCGGCUCGCGUGA	22	2.43
mmu-miR-200a	UAACACUGUCUGGUAACGAUGU	22	2.09
mmu-miR-708	AAGGAGCUUACAAUCUAGCUGGG	23	1.92
mmu-miR-27b-5p	AGAGCUUAGCUGAUUGGUGAAC	22	1.85
mmu-miR-23b	AUCACAUUGCCAGGGAUUACC	21	1.57
mmu-miR-24	UGGCUCAGUUCAGCAGGAACAG	22	1.56
mmu-miR-195	UAGCAGCACAGAAAUUUGGC	21	1.16
mmu-miR-677-3p	GAAGCCAGAUGCCGUUCUGAGAAGG	26	0.86
mmu-miR-3093-3p	UGUGGACACCGUGGGAGGUUGG	22	-1.06
mmu-miR-138-1-3p	CGGCUACUUCACAACACCGAGG	22	-1.17
mmu-miR-219a-1-3p	AGAAUUGCGUCUGGACGUCCCG	22	-1.29
mmu-miR-137-5p	ACGGGUAAUUCUGGGUGGAUAAU	23	-1.95
mmu-miR-217	UACUGCAUCAGGAACUGACUGGA	23	-2.07
mmu-miR-135a-1-3p	UAUAGGGAUUGGAGCCGUGGCG	22	-2.61
mmu-miR-7a-2-3p	CAACAAGUCCAGUCUGCCACA	22	-2.91
mmu-miR-135a-2-3p	UGUAGGGAUGGAAGCCAUGAA	21	-3.52
mmu-miR-10b	UACCCUGUAGAACCAGAAUUGUG	23	-3.75
mmu-miR-615-3p	UCGAGCCUGGGUCUCCUCUU	22	-4.17
mmu-miR-9-3p	AUAAAGCUAGAUAAACCGAAAGU	22	-4.23
mmu-miR-219a-2-3p	AGAAUUGUGGCUAGACAUCUGU	22	-4.96
mmu-miR-10a	UACCCUGUAGAACCAGAAUUGUG	23	-5.49
mmu-miR-124	UAAGGCACGCGGUGAAUGCC	20	-7.40

nt: nucleotides

It has been reported that in mouse embryo, miR-10a expression follows a HoxB-like expression pattern with a common regulatory element (Tanzer *et al.*, 2005). According to previous luciferase activity studies, it was suggested that miR-10a could be an inhibitor of the *HOXA1* expression in megakaryocytic differentiation (Garzon *et al.*, 2006). It seems that when mmu-miR-10a expression is low, as shown in our results, *Hoxa1* mRNA levels will not be downregulated, our results demonstrate that *Hoxa1* mRNA levels in pinnae was higher than that observed in back skin. Moreover, a report of a *Hoxa1* null mouse model revealed that the deficient mice present auricle hypoplasia (Gavalas *et al.*, 1998). These findings support that miR-10a has an important role during pinnae development in mammals, most likely by modulating the *Hoxa1* expression.

A spatiotemporal pattern of miRNA expression, similar to what has been described in other tissues, was found in the external ear. Some of the detected miRNAs changed their expression levels from one stage to the other and from one site to the other (Tables 1 and 2). For example, mmu-miR-200c was expressed only at 13.5 dpc, while mmu-miR-205 was expressed at 14.5 dpc.

We detected mmu-miR-200c and mmu-miR-205 expression in the pinnae tissues of healthy mice (Fig. 3). This correlates with the reported findings in human patients with microtia where hsa-miR-200c and hsa-miR-205 have reduced expression in the pinnae (Li *et al.*, 2013). Possible targets of mmu-miR-200c and mmu-miR-205 are the mRNAs of *Six1* and *Eya1*. In mouse, *Eya1* is expressed during outer and middle ear morphogenesis (13.5 dpc) the interaction between *Eya1* and *Six1* is crucial for the morphogenesis of the cochlea and the posterior ampulla during inner

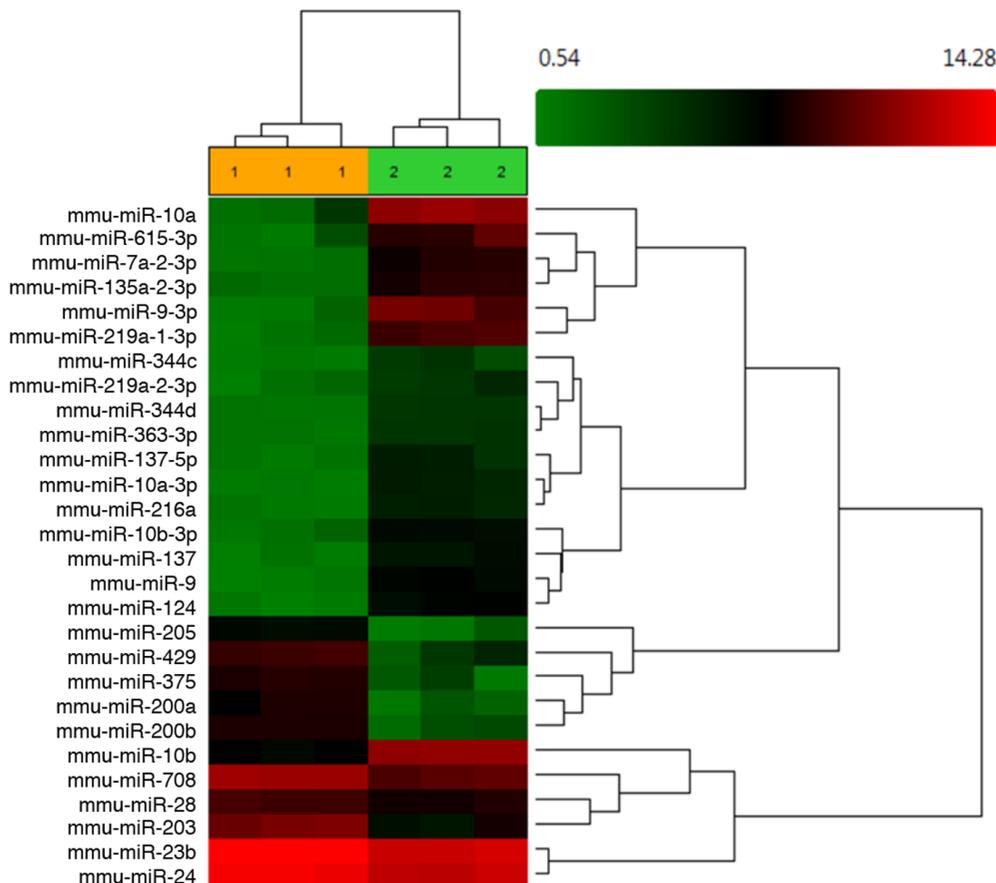


Fig. 2. Heat map of miRNA expression at 14.5 dpc, showing differentially expressed miRNAs between pinnae and back skin tissues. Hierarchical clusters of 31 miRNA genes were found to be significantly differentially expressed (adjusted *p* value <0.05) in pinnae compared to back skin controls. Each row of the hierarchical clusters represents an individual miRNA and each column represents one biological replica of either pinnae tissue (1) or skin tissue (2). The scale represents the gene expression intensity (log2 scale ranges between -1.5 and 1.5). Red color indicates an increase in miRNA gene expression (relative to the other expression measurements in the same row) and green indicates a decrease.

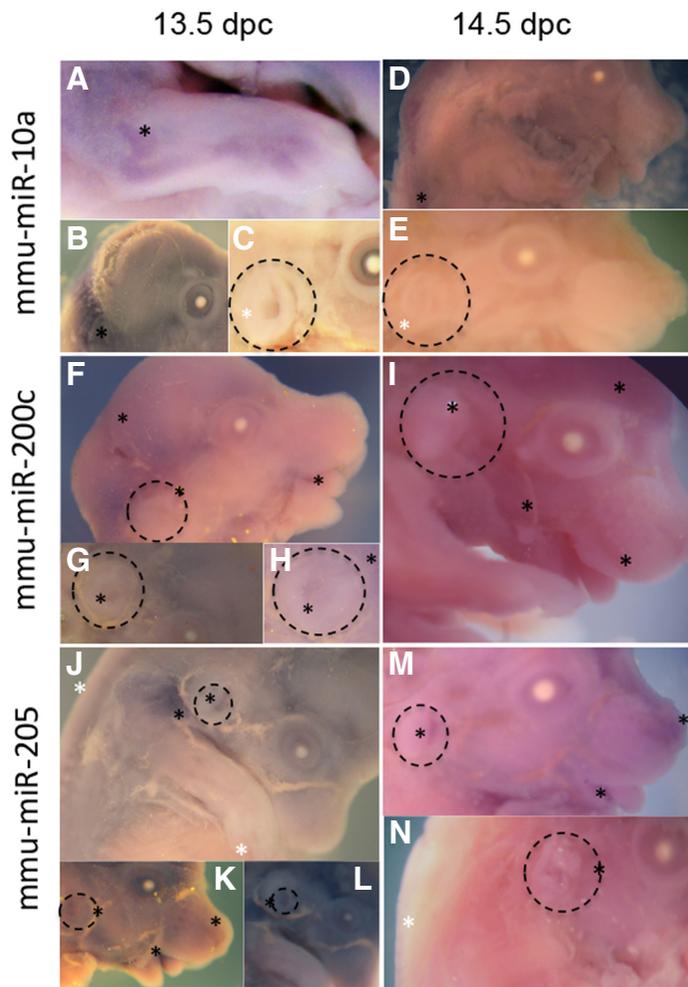


Fig. 3. miRNA *in situ* hybridization (ISH) at 13.5 and 14.5 dpc. Black (*) marks indicate positive signal; white (*) marks indicate absence of signal. Dashed circle surrounds pinna region. **(A-C)** ISH of *mmu-miR-10a* at 13.5 dpc; **(D,E)** ISH of *mmu-miR-10a* at 14.5 dpc; **(F-H)** ISH of *mmu-miR-200c* at 13.5 dpc; **(I-L)** ISH of *mmu-miR-200c* at 14.5 dpc; **(J-L)** ISH of *mmu-miR-205* at 13.5 dpc and **(M,N)** ISH of *mmu-miR-205* at 14.5 dpc. **(A)** Signal of *mmu-miR-10a* is present in the bone of anterior limbs. **(B)** Presence of *mmu-miR-10a* in the back skin, according with the microarrays results. **(C)** Pinna with significantly lower signal of *mmu-miR-10a* as compared with back skin. **(D)** Lateral view of fetus with strong *mmu-miR-10a* expression in the back skin. **(E)** Undetectable expression of *mmu-miR-10a* in pinna. **(F)** Presence of *mmu-miR-200c* in head and face 13.5 dpc, heavy mark in lips commissure, and light signal in skin surrounding pinnae. **(G)** and **(H)** Pinna closer view with detectable stain for *mmu-miR-200c*. **(I)** Head and face 14.5 dpc with *mmu-miR-200c* detection, positive signal was observed in all face and head, including the pinna. **(J)** Detectable signal of *mmu-miR-205* in pinna and surrounding skin, and undetectable signal in anterior limbs and back skin. **(K)** Nose, lips commissure and pinna with positive signal. **(L)** Closer view of pinna showing *mmu-miR-205* expression. **(M)** *mmu-miR-205* is present in pinna, lips and nose. **(N)** *mmu-miR-205* is almost absent in back skin and present in the pinna area.

ear development. (Kalatzis *et al.*, 1998; Zheng *et al.*, 2003). In addition, it has been observed that mutations in the human genes *EYA1*, *SIX1* and *SIX5*, cause the autosomal dominant syndrome Branchio-Oto-Renal, an early developmental defect characterized by varying combinations of branchial (fistulas, sinuses, and cysts),

outer, middle and inner ear, as well as renal anomalies. (Song *et al.*, 2013). The RT-PCR analysis confirms the presence of mRNA of *Six1* and *Eya1*, both possible targets of *mmu-miR-200c* and *mmu-miR-205*. All together, these findings suggest that miR-200c and miR-205 have an important role during normal external ear development in mammals.

Furthermore, we observed in the pinnae tissues that *mmu-miR-124* expression levels changed between the two analyzed stages. This miRNA remains low during 13.5 dpc and rises at 14.5 dpc, indicating that its activity is required in this stage. Recent reports have implicated this miRNA in neuronal development and aging processes (Neo *et al.*, 2014). A miR-124 knockout mouse model resulted in clear reduction of mature miR-124 levels that led to defective neuronal survival and axonal outgrowth, along with smaller brain size (Sanuki *et al.*, 2011). The participation of miR-124 in the development of the external ear has not been reported. However, *mmu-miR-124* is predicted to have several putative target genes implicated in external ear development, such as *Six4*, *Dlx5*, *Eya1* and *Prrx1*. Although none of these genes have been validated as direct targets of *mmu-miR-124*, these findings suggest that *mmu-miR-124* might contribute to external ear development.

Moreover, bioinformatics analyses of putative mRNA targets for differentially expressed miRNAs, allowed us to propose nine new candidate genes (*Zeb1*, *Zeb2*, *Tob1*, *Snai1*, *Smad5*, *Runx1*,

TABLE 2

THE miRNAs DIFFERENTIALLY EXPRESSED IN PINNAE VERSUS SKIN BACK TISSUES AT 14.5 DPC

mmu-miRNA	Sequence (5' -3')	Length (nt)	Fold change
mmu-miR-205	UCCUUCAUUCCACGGAGUCUG	22	5.92
mmu-miR-203	GUGAAAUGUUUAGGACCAUAG	22	3.76
mmu-miR-429	UAAUACUGUCUGGUAUUGCCGU	22	3.37
mmu-miR-200a	UAACACUGUCUGGUAACGAUGU	22	2.74
mmu-miR-200b	UAAUACUGCCUGGUAUUGAUGA	22	2.73
mmu-miR-375	UUUGUUCGUUCGGUCGCGGUGA	22	2.63
mmu-miR-205-3p	GAUUUCAGUGGAGUGAAGCUCU	22	2.02
mmu-miR-708	AAGGAGCUUACAAUCUAGCUGGG	23	1.92
mmu-miR-27b-5p	AGAGCUUAGCUGAUUGGUGAAC	22	1.76
mmu-miR-24	UGGCUCAGUUCAGCAGGAACAG	22	1.46
mmu-miR-23b	AUCACAUUGCCAGGGAUUAAC	21	1.43
mmu-miR-497	CAGCAGCACACUGUGGUUUGUA	22	1.09
mmu-miR-28	AAGGAGCUCACAGUCUUAUUGAG	22	1.06
mmu-miR-324-3p	CCACUGCCCCAGGUGCUGCU	20	0.71
mmu-miR-363-3p	AAUUGCACGGUAUCCAUCUGUA	22	-0.76
mmu-miR-344c	UGAUCUAGUCAAGCCUGACAGU	23	-0.77
mmu-miR-344d	GAUUAUACCACUGCCAGACUGA	22	-0.85
mmu-miR-216a	UAAUCUCAGCUGGCAUCUGUGA	22	-1.39
mmu-miR-137	UUUUGCUUAAGAAUACGCGUAG	23	-1.44
mmu-miR-10a-3p	CAAAUUCGUUUCUAGGGGAAUA	22	-1.65
mmu-miR-10b-3p	CAGAUUCGAUUCUAGGGGAAUA	22	-1.92
mmu-miR-124-5p	CGUGUUCACAGCCAGCCUUGAU	22	-2.19
mmu-miR-9	UCUUUGGUUAUCUAGCUGUAUGA	23	-2.27
mmu-miR-7a-2-3p	CAACAAGUCCAGUCUGCCACA	22	-2.84
mmu-miR-135a-2-3p	UGUAGGGAUGGAAGCCAUGAA	21	-3.02
mmu-miR-615-3p	UCCGAGCCUGGGUCUCCUCUU	22	-3.22
mmu-miR-344	UGAUCUAGCCAAAGCCUGACUGU	23	-3.39
mmu-miR-10b	UACCCUGUAGAACCAGAAUUGUG	23	-3.81
mmu-miR-219-3p	AGAAUUGUGGUGGACAUUCUGU	22	-3.94
mmu-miR-9-3p	AUAAAGCUAGUAACCGAAAGU	22	-5.13
mmu-miR-10a	UACCCUGUAGAUCGAAUUGUG	23	-5.61

nt: nucleotides

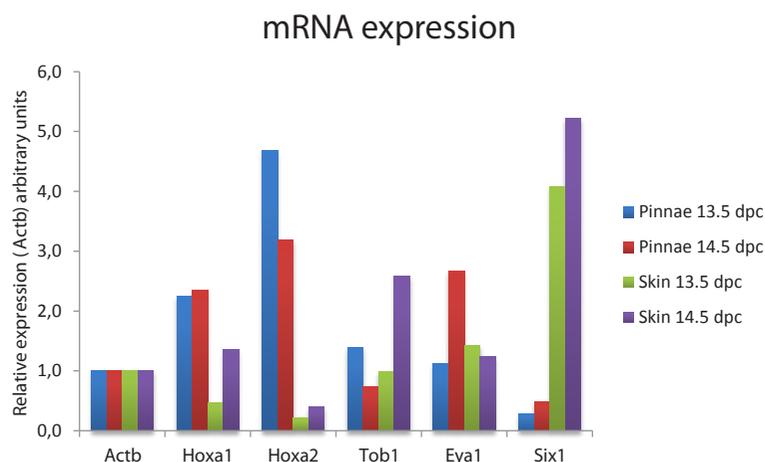


Fig. 4. mRNA relative expression levels at 13.5 and 14.5 dpc. Comparative mRNA expression levels in pinnae and back skin tissues of putative mRNA targets of the mmu-miR studied. Relative expression levels for each gene and each comparison between tissues were calculated using the formula of relative expression ($R=2^{-\Delta\Delta C_p}$) (Pfaffl, 2006).

Runx2, *Crim1* and *Dicer1*) that could be participating in external ear development in mice. *Zeb1* and *Zeb2* encode transcription factors that are involved in neural development and in the development of tissues derived from neural crest cells (Shin *et al.*, 2012). *Tob1* may negatively regulate the BMP/Smad signaling pathway in osteoblasts, a signaling pathway that has also been implicated in external ear development of early stage mice (Yoshida *et al.*, 2000; Minoux *et al.*, 2013) We found mRNA of *Tob1* in pinnae (Fig. 4), but the interaction with mmu-miR-200c, mmu-miR-205 and mmu-miR-429, their possible regulators, is still unknown. The remaining genes, *Sani1*, *Runx1* and *Runx2*, have been implicated in the process of chondrogenesis (Yoshida and Komori, 2005; Chen and Gridley, 2013).

In summary, we observed that there is a specific spatiotemporal pattern of miRNA expression during external ear development at 13.5 dpc and 14.5 dpc.

This analysis is the first miRNA expression profiling study during external ear development in mammals. Thus, it could set up the basis for understanding the role of miRNAs in normal external ear development in mammals and for determining miRNA involvement in the etiology of external ear congenital malformations, such as microtia.

Materials and Methods

Mice

The CD1 mouse strain was used in this study. Scheduled mating was carried out in order to obtain fetuses at specific developmental stages. Pregnant females were sacrificed at 13.5 dpc or 14.5 dpc. The gestational age of the fetus was determined by the day of appearance of the vaginal plug (0.5 dpc) and confirmed by morphological criteria. All procedures were performed in accordance with the Mexican regulation NOM-062-ZOO-1999 norm and the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research of the National Academy of Sciences. The Institutional Animal Care and Use of Laboratory Animals and Research Committees from the Instituto Nacional de Pediatría approved this study.

Tissues samples and RNA extraction

Biological triplicates for each tissue and each developmental stage were analyzed. One litter represents one biological replica and each litter had an average of 16 fetuses. External ear tissue, the pinna (cartilage and skin), and back skin tissues were dissected under stereomicroscope in iced and cooled nuclease free PBS (Gibco, Waltham, MA USA) and were kept in RNAlater (Ambion, Waltham, MA USA) at -80°C until further processing. The region used as reference (back skin) is of an ectodermal origin, is close to the pinnae on the embryo and it differentiates at the same developmental stage. Using the reference region, we were able to identify the miRNAs that were specifically expressed in the external ear in these stages. Total RNA extraction including short RNAs (<200 bp) was performed with an RNA Mini elute kit (Qiagen, Venlo, Netherlands) and quantification was performed with a NanodropTM spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA quality was monitored by agarose gel electrophoresis.

miRNA array analysis

Affymetrix GeneChip miRNA Arrays 3.0 (Affymetrix, Santa Clara, CA, USA) were used to screen the miRNA profile expression during external ear development. A single array is comprised of 179,217 probes that represent 19,913 mature miRNA contained in miRBase V.17 (www.mirbase.org) with probes for 153 different organisms, including 1111 probes for *Mus musculus* miRNAs and 855 pre-miRNAs. The array analysis followed the manufacturer's instructions. Briefly, 500 ng of total RNA, including short RNAs, was labeled with the FlashTag[®] Biotin HSR kit (Genisphere/Affymetrix, Santa Clara, CA, USA) and the labeling was tested using an Enzyme Linked Oligosorbent Assay (ELOSA) QC Assay (Thermo Fisher Scientific Inc, Waltham, MA, USA). The denaturalized samples were then injected into array chips and hybridized at 48°C and 60 rpm for 17 hours in an Affymetrix Hybridization Oven 645. Arrays were then stained and washed in the Affymetrix GeneChip Fluidic Station 450 and scanned using an Affymetrix GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA, USA).

The array data were normalized by global normalization using the miRNA QC Tool of the Expression Console software (Affymetrix, Santa Clara, CA, USA). To identify differentially expressed miRNAs at each stage, linear regression analysis using the Bioconductor LIMMA (Lineal Models for Microarrays) algorithm was employed. Hierarchical clustering analysis was performed with the Bioconductor and Transcriptome Analysis Console software from Affymetrix. The microarrays data were deposited in the Gene Expression Omnibus database (accession number GSE64945).

Putative mRNA targets for differentially expressed miRNAs were identified using the bioinformatic predicting tools TargetScanMouse Release 6.2 (www.targetscan.org) miRNAbody Map (www.mirnabodymap.org), miRBase (www.mirbase.org) and DIANA Tools (Tarbase, microT v4 and microT-CDS), (<http://diana.imis.athena-innovation.gr/DianaTools>).

Real-time quantitative RT-PCR analysis

In order to know if target mRNAs are present in pinnae or skin at the stages studied, we realize real-time quantitative RT-PCR analysis; 500 ng of total RNA was treated with 0.1 U RNase-free DNase I (Invitrogen, Carlsbad, CA, USA) in 20 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1 mM MgCl_2 for 15 min at room temperature, the enzyme was then inactivated by adding EDTA to a final concentration 1 mM followed by incubation at $65^{\circ}\text{C}/10$ min. After this step total RNA was retro-transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, GmbH, Mannheim, Germany) using anchored-oligo (dT) 18 primer, 50-pmol/ μL and Random hexamer primer, 600 pmol/ μL , Protector RNase Inhibitor 20 U and 10 U of Transcriptor Reverse Transcriptase. The cDNA obtained was quantified using a Nanodrop ND 1000 spectrophotometer (Nanodrop

TABLE 3

**PRIMERS AND PROBES
USED TO DETECT mRNA EXPRESSION BY qRT-PCR**

Gene	Primer sequence 5'-3'	Probe sequence 5'-3'
<i>Hoxa1</i>	F: GCC CTG GCC ACG TAT AAT AA R: GAA GCA GGG GAA CGA CAG	CAG CCA CC
<i>Hoxa2</i>	F: ACC TGG AGG ACT CGG ACA A R: GGG CTT GCT CAA AGA GTG AC	GGA GGA AG
<i>Tob1</i>	F: ACT TTT GCT GCC ACC AAG TT R: GAG CTA CCT TGC TGC TAC GG	CTC CAC CA
<i>Six1</i>	F: GGT CTG CAA GCC CAT CAG R: AGT CCA CCA AAC TGG AGG TG	GCT CCA GG
<i>Eya1</i>	F: CGA GGT TCA GAT GGG AAG TC R: GGG AGG GGA GGG ATT ATT G	GGC AGA AG
<i>Actb</i>	F: GAT CAT TGC TCC TCC TGA GC R: CCG GAC TCA TCG TAC TCC TG	CTC CAC CA

Technologies, Wilmington, DE, USA).

Real-time quantitative-PCR (q-PCR) was performed on cDNA samples in duplicate for two biological repeats. The q-PCR was performed with the Universal Probes system (Roche Diagnostics, GmbH, Mannheim, Germany) and the Light Cycler Taq Man Master kit (Roche Diagnostics, GmbH, Mannheim, Germany). Besides target genes expression, *Actb* expression was quantified as reference gene. Primers for each gene were designed on line with the ProbeFinder Software (www.universalprobelibrary.com) and manufactured at Sequencing and Synthesis Unit (IBT, UNAM). The primers and the universal probe sequences for each gene are indicated in Table 3.

The q-PCR was carried out in the Light Cycler 2.0 Carousel Roche equipment and the conditions were as follows: 1 cycle at 95°C for 10 minutes, denaturalization at 95°C for 10 seconds, annealing at 60°C for 30 seconds and extension and detection at 72°C for 5 seconds for a total of 45 cycles. Relative expression levels for each gene and for each tissue were calculated using the formula of relative expression $R=2^{-\Delta\Delta C_p}$ (Pfaffl, 2006).

Whole mount in situ hybridization

The protocol followed was the recommended by Exiqon (Exiqon, Vedbæk, Denmark) (Thisse *et al.*, 2004; Kloosterman *et al.*, 2006) with modifications. Briefly, embryos were fixed overnight at 4°C in 4% paraformaldehyde in PBS and subsequently transferred to 25%, 50%, 75% and 100% methanol in PBST (PBS containing 0.1% Tween-20), and stored at -20°C until further processing. At the first day of the *in situ* hybridization embryos were rehydrated by successive washes for 5 min at room temperature (RT) in 75%, 50%, 25% and 100% PBST (4 x 5 min). Embryos were treated with 10 µg/ml proteinase K (Roche Diagnostics, GmbH, Mannheim, Germany) in PBST for 45 min at 37°C, refixed for 20 min in 4% paraformaldehyde in PBS at RT and washed 5 x 5 min with PBST at RT. The embryos were then transferred to hybridization mix (HM)(50% Formamide, 5x SSC, pH 6.0, 0.1% Tween, 9.2 mM citric acid, 50 µg/ml heparin, 500 µg/ml yeast RNA) and prehybridized for 2-3 hour at the hybridization temperature (57°C for probes mmu-miR-205 and mmu-miR-200c, 53°C for probe mmu-miR-10a). Hybridization was performed in fresh pre-heated hybridization buffer containing 10 nM of DIG labeled LNA probe (Exiqon, Vedbæk, Denmark) overnight, at the hybridization temperature. Post-hybridization washes were done at the hybridization temperature by successive incubations for 15 min in HM without heparin and yeast RNA, 75% HM-/25% 2x SSC, 50% HM-/50% 2x SSC, 25% HM-/75% 2x SSC, 100% 2x SSC, twice in 0.2x SSC 30 min. Subsequently, embryos were transferred to PBST through successive incubations for 10 min at RT in 75% 0.2x SSC/25% PBST, 50% 0.2x SSC/50% PBST, 25% 0.2x SSC/75% PBST and 100% PBST. After washing the embryos were exposed for 1 hour in blocking buffer (1X blocking in PBST) (Roche Diagnostics, GmbH, Mannheim, Germany), then were incubated overnight at 4°C in blocking buffer containing anti-DIG-AP FAB fragments (1:2000) (Roche Diagnostics, GmbH, Mannheim, Germany). The next day, embryos were washed once in PBST 5 min RT, after 5 x 1 hour in PBST and then at 4°C in PBST overnight. After the post-antibody

washes, the embryos were washed 3 x 5 min in staining buffer (100 mM Tris HCl pH9.5, 50 mM MgCl₂, 100 mM NaCl, 0.1% tween 20). Staining was done in buffer supplied with 4.5 µl/ml NBT (50 mg/ml stock)(Roche Diagnostics, GmbH, Mannheim, Germany) and 3.5 µl/ml BCIP (50 mg/ml stock)(Roche Diagnostics, GmbH, Mannheim, Germany). The reaction was stopped with 1 mM EDTA in PBST and the embryos were refixed for 60 min in 4% paraformaldehyde in PBS at RT and washed 5 x 5 min with PBST at RT. For observation and imaging the embryos were mounted in 50% glycerol. The embryos were observed under a stereoscopic microscope over petri dishes coated with a layer of 1% agarose (\pm 2 mm thick) prepared in PBS; pictures were taken using a digital camera, first for the complete embryo and then for specific structures as pinnae and limbs.

Acknowledgments

Financial support: Federal Funding from the Instituto Nacional de Pediatría 053/2013. U. Juárez received a CONACyT scholarship 270077. We would like to thank the personnel of the animal care facility of the Instituto Nacional de Pediatría, for their professional work with the mice, to Dra. Diana Escalante for their valuable advice for in situ hybridization technique and to Dra. Ana Valeria Martínez Silva from Instituto de Fisiología Celular, UNAM for technical support, to Dr. Benilde García de Teresa for their valuable comments and revision of the manuscript, to Dr. Pablo González for bioinformatics analysis, and to the Sequencing and Synthesis Unit from the Instituto de Biología, UNAM, for primer synthesis.

References

- BEREZIKOV E (2011). Evolution of microRNA diversity and regulation in animals. *Nat Rev Genet* 12: 846-860.
- CHEN Y, GRIDLEY T (2013). Compensatory regulation of the *Snai1* and *Snai2* genes during chondrogenesis. *J Bone Miner Res* 28: 1412-1421.
- GARZON R, PICHIORRI F, PALUMBO T, IULIANO R, CIMMINO A, AQEILAN R, VOLINIA S, BHATT D, ALDER H, MARCUCCI G, CALIN GA, LIU CG, BLOOMFIELD CD, ANDREEFF M, CROCE CM (2006). MicroRNA fingerprints during human megakaryocytopoiesis. *Proc Natl Acad Sci USA* 103: 5078-5083.
- GAVALAS A, STUDER M, LUMSDEN A, RIJLI FM, KRUMLAUF R, CHAMBON P (1998). *Hoxa1* and *Hoxb1* synergize in patterning the hindbrain, cranial nerves and second pharyngeal arch. *Development* 125: 1123-1136.
- JONES FW, CHUAN WI (1934). The development of the external ear. *J. Anat* 68:525-533.
- KALATZIS V, SAHLY I, EL-AMRAOUI A, PETIT C (1998). *Eya1* expression in the developing ear and kidney: towards the understanding of the pathogenesis of Branchio-Oto-Renal (BOR) syndrome. *Dev Dyn* 213: 486-99
- KLOOSTERMAN WP, WIENHOLDS E, de BRUIJN E, KAUPPINEN S, PLASTERK RH (2006). *In situ* detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes. *Nat Methods* 3: 27-29
- LAU NC, LIM LP, WEINSTEIN EG, BARTELD DP (2001). An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science* 294: 858-862.
- LI C, HAO S, WANG H, JIN L, QING F, ZHENG F, ZHANG P, CHEN L, MA D, ZHANG T (2013). MicroRNA expression profiling and target genes study in congenital microtia. *Int J of Pediatr Otorhinolaryngol* 77: 483-487.
- LUQUETTI DV, HEIKE CL, HING AV, CUNNINGHAM ML, COX TC (2012). Microtia: Epidemiology and genetics. *Am J Med Genet* 158A: 24-139.
- MALLO M, GRIDLEY T (1996). Development of the mammalian ear: Coordinate regulation of formation of the tympanic ring and the external acoustic meatus. *Development* 122: 173-179.
- MINOUX M, KRATOCHWIL CF, DUCRET S, AMIN S, KITAZAWA T, KURIHARA H, BOBOLAN, VILAIN N, RIJLI FM (2013). Mouse *Hoxa2* mutations provide a model for microtia and auricle duplication. *Development* 140: 4386-4397.
- NEO WH, YAP K, LEE SH, LOOI LS, KHANDELIA P, NEO SX, MAKEYEV EV, SU IH (2014). MicroRNA miR-124 controls the choice between neuronal and astrocyte differentiation by fine-tuning *Ezh2* expression. *J Biol Chem* 289: 20788-20801.
- PASQUINELLI AE (2012). Non-Coding RNA MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. *Nat Rev Genet* 13: 271-282.
- PETRI R, MALMEVIK J, FASCHING L, AKERBLMOM M, JAKOBSSON J (2014).

- miRNAs in brain development. *Exp Cell Res* 321: 84-89.
- PFÄFFL MW (2006). Relative quantification. In *Real-time PCR*. (Ed. M. Tevfk Dorak), Taylor & Francis Group. New York, N Y. pp 63-80.
- SACHELI R, NGUYEN L, BORGS L, VANDENBOSCH R, BODSON M, LEFEBVRE P, MALGRANGE P (2009). Expression patterns of miR-96, miR-182 and miR-183 in the developing inner ear. *Gene Expr Patterns* 9:364-370.
- SANUKI R, ONISHI A, KOIKE C, MURAMATSU R, WATANABE S, MURANISHI Y, IRIE S, UNEO S, KOYASU T, MATSUI R (2011). miR-124a is required for hippocampal axogenesis and retinal cone survival through *Lhx2* suppression. *Nat Neurosci* 14: 1125–1134.
- SHIN JO, KIM EJ, CHO KW, NAKAGAWA E, KWON HJ, CHO SW, JUNG HS (2012). BMP4 signaling mediates Zeb family in developing mouse tooth. *Histochem Cell Biol* 137: 791-800.
- SONG MH, KWON TJ, KIM HR, JEON JH, BAEK JI, LEE WS, KIM UK, CHOI JY (2013). Mutational analysis of EYA1, SIX1 and SIX5 genes and strategies for management of hearing loss in patients with BOR/BO syndrome. *PLoS One* 8: e67236
- TANZER A, AMEMIYA CT, KIM CB, STADLER PF (2005). Evolution of microRNAs located within *Hox* gene clusters. *J Exp Zool B Mol Dev Evol* 304: 75-85.
- YOSHIDA CA, KOMORI T (2005). Role of Runx proteins in chondrogenesis. *Crit Rev Eukaryot Gene Expr* 15: 243-254.
- THISSE B, HEYER V, LUX A, ALUNNI V, DEGRAVE A, SEILIEZ I, KIRCHNER J, PARKHILL JP, THISSE C. (2004). Spatial and temporal expression of the zebrafish genome by large-scale *in situ* hybridization screening. *Methods Cell Biol* 77: 505-519.
- WARNER DR, MUKHOPADHYAY P, BROCK G, WEBB CL, PISANO MM, GREENE RM (2014). MicroRNA expression profiling of the developing murine upper lip. *Develop Growth Differ* 56: 434-447.
- YOSHIDA Y, TANAKAS, UMEMORI H, MINOWAO, USUIM, IKEMATSUN, HOSODA E, IMAMURA T, KUNO J, YAMASHITA T, MIYAZONO K, NODA M, NODA T, YAMAMOTO T (2000). Negative regulation of BMP/Smad signaling by Tob in osteoblasts. *Cell* 103: 1085-1097.
- ZHENG W, HUANG L, WEI ZB, SILVIUS D, TANG B, XU PX (2003). The role of Six1 in mammalian auditory system development. *Development* 130: 3989-4000.

Further Related Reading, published previously in the Int. J. Dev. Biol.

The molecular biology of ear development - "Twenty years are nothing"

Fernando Giraldez and Bernd Fritsch

Int. J. Dev. Biol. (2007) 51: 429-438

<http://www.intjdevbiol.com/web/paper/072390fg>

Pocket proteins and cell cycle regulation in inner ear development

Sonia M.S. Rocha-Sanchez and Kirk W. Beisel

Int. J. Dev. Biol. (2007) 51: 585-595

<http://www.intjdevbiol.com/web/paper/072387sr>

Patterning and morphogenesis of the vertebrate inner ear

Jinwoong Bok, Weise Chang and Doris K. Wu

Int. J. Dev. Biol. (2007) 51: 521-533

<http://www.intjdevbiol.com/web/paper/072381jb>

Molecular evolution of the vertebrate mechanosensory cell and ear

Bernd Fritsch, Kirk W. Beisel, Sarah Pauley and Garrett Soukup

Int. J. Dev. Biol. (2007) 51: 663-678

<http://www.intjdevbiol.com/web/paper/072367bf>

Mouse models to study inner ear development and hereditary hearing loss

Lilach M. Friedman, Amiel A. Dror and Karen B. Avraham

Int. J. Dev. Biol. (2007) 51: 609-631

<http://www.intjdevbiol.com/web/paper/072365lf>

5 yr ISI Impact Factor (2013) = 2.879

