RESEARCH ARTICLE

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miRNA Mutations Are Not a Common Cause of Deafness

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Mutations in miRNA genes have been implicated in hearing loss in human families and mice. It is also possible that mutations in miRNA binding sites of inner ear targets alter gene expression levels and lead to hearing loss. To investigate these possibilities we screened predicted target genes of the miR-183 miRNA cluster known to be expressed in the inner ear sensory epithelium. In one Iranian family segregating autosomal recessive non-syndromic hearing loss (ARNSHL), we identified a homozygous variant in a predicted miR-96/182 binding site in the 3′UTR of the *RDX* (DFNB24) gene. However, in vitro functional studies showed that this site is not a functional target for miR-96/182. We extended our study to include the miR-183 genes themselves and 24 additional predicted target genes of the miRNA-183 cluster. Screening these miRNAs and target sequences in numerous families segregating either autosomal dominant non-syndromic deafness (ADNSHL) or ARNSHL did not identify any potential deafness-causing mutations. These results suggest that mutations disrupting gene regulation by the miR-183 cluster are not a common cause of human hearing loss. © 2010 Wiley-Liss, Inc.

Key words: radixin; ERM protein family; miRNA; ADNSHL; ARNSHL

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INTRODUCTION

Deafness is the most common human sensory deficit, and its social, economic, and quality-of-life consequences are severe [Morton, 1991]. It is estimated that globally 4 of every 10,000 children are born with profound sensorineural hearing loss (SNHL) [Smith et al., 2005]. Non-syndromic forms of SNHL (NSHL) account for ~70% of hereditary hearing loss.

Most cases of inherited deafness are monogenic. That global dysregulation of numerous genes can also result in human NSHL has only recently been discovered. Mencia et al. [2009] screened NSHL families with dominant, recessive, and unknown patterns of inheritance and identified separate mutations in two autosomal dominant non-syndromic hearing loss (ADNSHL) DFNA50 families in miR-96[Mencia et al., 2009, a member of the miR-183 miRNA cluster that is expressed in the inner ear sensory epithelium [Wienholds et al., 2005; Weston et al., 2006; Soukup et al., 2009]. Interestingly, expression of the miR-183 cluster is not limited to the ear, but is highly expressed in other sensory organs as well [Xu et al., 2007].

We therefore hypothesized that dysregulation of inner ear genes known to be regulated by the miR-183 cluster could lead to non-syndromic hearing loss. We focused on *RDX*, which encodes the radixin protein and causes DFNB24 ARNSHL [Khan et al., 2007]. Radixin and its related ezrin–radixin–moesin (ERM) protein family member ezrin are present in hair cell stereocilia of the mouse inner ear [Kitajiri et al., 2004; Pataky et al., 2004]. Although no

Abbreviations: ADNSHL, autosomal dominant non-syndromic hearing loss; ARNSHL, autosomal recessive non-syndromic hearing loss; SNHL, sensorineural hearing loss.

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deafness-causing mutations in ezrin have been identified to date, targeting of ezrin by miR-183 has been reported for some lung cancers [Wang et al., 2008], raising the possibility of miR-183 cluster involvement in the regulation of radixin.

We identified a variant in a predicted miR-96/182 binding site in the 3′UTR of *RDX* in an Iranian family segregating ARNSHL. While analysis with three algorithms predicted that this region is a miR-96/182 binding site, we performed in vitro functional studies that excluded this possibility. We then extended our study to include 24 additional predicted target genes. Screening the miR-183 cluster and their predicted targets in American ADNSHL and Iranian ARNSHL families, respectively, did not identify any potential deafness-causing variants. It appears that mutations disrupting gene regulation by the miR-183 family are not a common cause of human hearing loss.

MATERIALS AND METHODS

Clinical Evaluation of Families

One hundred fifty American ADNSHL and 576 Iranian ARNSHL families were studied. To document the degree of hearing loss audiologic testing was completed on consenting family members. A detailed family history was taken including any reported balance or visual problems. In some cases, caloric testing and funduscopy were completed. Ten milliliters of whole blood was obtained from family members by venipuncture and genomic DNA was extracted as described previously [Grimberg et al., 1989]. Human research institutional review boards at the Welfare Science and Rehabilitation University and Iran University of Medical Sciences, Tehran, Iran, the National Centre of Excellence in Molecular Biology, Lahore, Pakistan, the Quaid-I-Azam University, Islamabad, Pakistan, the Combined Neuroscience Institutional Review Board (IRB) at the National Institutes of Health, Bethesda, Maryland, USA, and the University of Iowa, Iowa City, Iowa, USA approved all procedures.

Target Gene Prediction

Target genes of the miRNA-183 miRNA cluster were chosen as all three miRNAs are expressed in the inner ear sensory epithelium [Wienholds et al., 2005; Weston et al., 2006; Friedman et al., 2009]. Three algorithms—miRanda (http://www.microrna.org/microrna/); PicTar (http://pictar.mdc-berlin.de/); and TargetScan (http://www.targetscan.org/)—that base predictions on thermodynamics, evolutionary conservation and target site-seed complementarity were used to select mRNA targets of miR-183/96/182 regulation. These algorithms identified hundreds of mRNA targets for each miRNA, although only some were common to all three algorithms. Inner ear expression and function were additional criteria used to select candidate target genes for screening (Table I).

PCR, DHPLC, and Sequencing

miRNA-183/96/182 genes and the 3′UTR of predicted target genes were amplified using gene-specific primers (Table II). Amplification reactions were cycled using a standard protocol on a GeneMate Genius thermocycler (ISC BioExpress, UT). For denaturing high performance liquid chromatography (DHPLC), all amplicons were pooled post-PCR and heteroduplexes were formed by denaturing at 95°C for 5 min in a thermal cycler and cooling at a rate of 1°C/min to room temperature as described previously [Prasad et al., 2004]. DHPLC analysis of each amplicon was performed at three different temperatures. The analysis was conducted using Navigator™ Software (Transgenomic™, Omaha, NE) to estimate optimal temperature, run time, and acetonitrile gradient. The best predicted temperature was bracketed by ±2°C to optimize sensitivity. Sequencing was completed with a BigDye™ v3.1 Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Sequencing products were read using an ABI 3730s sequencer (Perkin Elmer, Waltham, MA). All sequencing chromatograms were compared to published cDNA sequence; nucleotide changes were detected using Sequencher v4.5 (Gene Code Corporation, Ann Arbor, MI).

Luciferase Assays

We utilized RT-PCR to amplify and subclone a fragment (*Spe*I/*Hin*dIII) of the 3′UTR of *RDX* (nucleotides 2041–4247 of GeneBank™ accession number NM_002906, containing a potential binding site for *miR-96* and *miR-182*) into the luciferase reporter vector, pMIR-REPORT (Ambion, Austin, TX), 3′ to the firefly luciferase cassette. To introduce the c.*95C>A variation we used the QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer's guidelines. We plated HEK293 cells at a density of 5×10^5 cells/well in 24-well plates coated with poly-D-lysine (Sigma, St. Louis, MI) and transfected them with 150 ng of pMIR reporter construct (pMIR-REPORT-3′UTR/*Rdx* **95C* or pMIR-REPORT-3′UTR/*Rdx* **95A*), 15 ng of hpRL-SV40 (Promega, Madison, WI), and 5–50 pmol of the specified miRNA mimics or control oligonucleotide with a scrambled sequence (Dharmacom, Lafayette, CO) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Using the dual Luciferase Assay Kit (Promega), we measured firefly luciferase 48 hr post-transfection and normalized to *Renilla* activity. We performed three independent experiments for each assay.

Western Blots

We plated HeLa cells at a density of 8×10^4 cells/well in 12-well plates and transfected them with 20–200 pmol of the specified miRNA mimics or control oligonucleotide with a scrambled sequence (Dharmacom) using Lipofectamine 2000 (Invitrogen). We lysed cells 72 hr post-transfection in RIPA buffer, resolved extracts by SDS–PAGE, and transferred them to Hybond-P membranes (GE Healthcare, Piscataway, NJ). We used commercially available primary antibodies raised against Radixin (ab52495; 1:10,000; Abcam, Cambridge, MA) and acetylated alpha-tubulin (sc-23950; 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies conjugated to HRP (Santa Cruz Biotechnology) and ECL reagents (GE Healthcare) were used for detection as specified by the manufacturer. Three independent experiments were completed for each assay.

TABLE I. Candidate miR-183/96/182 Target Genes

Gene symbol	Gene name	OMIM number[a]	Predicted miRNA target sites[b]	Inner ear expression[c]	Known function	Number of families screened (ARNSHL)
AQP5	Aquaporin 5	600442	miR-96	Yes	Transmembrane water transport	192
ATP2B4	Plasma membrane calcium pump	108732	miR-183	Yes	Calcium ion transport	192
BTG1	B-cell translocation gene 1	109580	miR-183	Yes	Negative regulator of cell proliferation	192
CaBP1	Calcium-binding protein 1	605563	miR-96	Yes	Competitively inhibits calmodulin binding to calcium	192
Cav1.2	Voltage-dependent calcium channel 1.2.	114205	miR-96 miR-182	Yes	Calcium ion transport	192
Cav1	Caveolin 1	601047	miR-96	?	Integral membrane protein that interacts with G-proteins in cell signaling	192
CELSR1	Cadherin EGF receptor 1	604523	miR-96	?	Receptor involved in contact-mediated communication	192
CELSR2	Cadherin EGF receptor 2	604265	miR-96	Yes	Receptor involved in contact-mediated communication	192
EZRIN	Cytovillin	123900	miR-183	Yes	Component of microvilli and substrate for protein–tyrosine kinases	576
GNG5	Guanine nucleotide-binding protein 5	600874	miR-183	Yes	G-protein cell signaling	192
GRID1	Glutamate receptor delta 1	610659	miR-96	?	Synaptic transmission	192
KCC2	Solute carrier 12A5	606726	miR-96	Yes	Potassium/chloride transport	192
KCNJ14	Potassium inwardly rectifying channel (Kir2.4)	603953	miR-183	Yes	Potassium transport	192
MITF	Microphthalmia-associated transcription factor	156845	miR-96 miR-182	Yes	Basic helix–loop–helix transcription factor involved in regulation of development	192
MYRIP	Myosin VIIa and Rab-interacting protein	611790	miR-96 miR-182 (2)	Yes	Actin transport and cytoskeleton	192
NCS1	Neuronal calcium sensor 1	603315	miR-183	Yes	Calcium-ion sensor that modulates synaptic activity	192
NEFL	Neurofilament protein	162280	miR-183	Yes	Neurofilament assembly and axonal transport	192
PEX19	Peroxisome biogenesis factor 19	600279	miR-183	Yes	Cellular protein sorting	192
PPP2CA	Protein phosphatase, catalytic subunit, alpha	176915	miR-183	Yes	Regulation of protein phosphorylation	192
RDX	Radixin	179410	miR-96 miR-182	Yes	Cytoskeletal protein that may link actin to plasma membrane	576
RYK	Receptor-like tyrosine kinase	600524	miR-96	?	Growth factor receptor tyrosine kinase	192
SLC6A6	Solute carrier family 6	186854	miR-183	Yes	Taurine (neurotransmitter) transporter	192
TFCP2L3	Transcription factor CP2-like 3	608576	miR-96 (3)	Yes	Transcriptional regulation	192
TREK-1	Potassium channel KCNK2	603219	miR-183	Yes	Central nervous system (CNS) potassium transport	192
ZCCHC3	Zinc finger CCHC containing 3	—	miR-183	Yes	Transcriptional regulation	192

— denotes not available.
[a]Online mammalian inheritance in man identification number.
[b]Predictions from miRanda, PicTar, and TargetScan algorithms. Number of sites if multiple sites (brackets).
[c]Reported in database or publication.

TABLE II. Oligonucleotides Used for Amplification of Human miRNAs and Target Gene Binding Sites

Gene[a]	Forward primer (5′–3′)	Reverse primer (5′–3′)
miR-96	TACCGAAGGGCCATAAACAG	AGGCAGTGTAAGGCGATCTG
miR-182	AGCAGGAAGGGGGACTGT	GCAGGGAAACACAGAGTGTCA
miR-183	AAGGTCATCTTGGGCTGATG	GGCTCTCTGGGGACACACT
AQP5	TGGCTGCACAGTTAGAGAGG	CGGCATTCAATGAACCAGTC
ATP2B4	TCCTTGGTAGTCACTTGTCATTTT	TCACACTACTGGCGGATTTT
BTG1	TCTTGGGGATGGATTATGGA	TCTGGGAGAAACTGAAACCA
CaBP1	CAGGATGTACTGGCGGATG	GACATGCTGTGTGGGGTCT
Cav1.2 (96)	CAAGAAGGCATTTTGCTTCA	AAAGCTTGTCACACTCCAAATACA
Cav1.2 (182)	TTGACAGCATGTTGCAGTTTC	TTGGGCATACACAATGGTTG
Cav1	TGCATCAGCCGTGTCTATTC	TCAGACTGCCAAAAATAGATGAA
CELSR1	CTGCCCTTGAAGTGGAGTG	CCCTCTCAGTTCTGGCTTTG
CELSR2	CCTGCTCCTGTCTTGTGCTT	GGGAGTCAATTTCCAGCGTA
EZRIN	GAAACTTCATGCTGGCCTGT	CTGTGTGTGCGAGAGTGCTT
GNG5	TTCCCAAACCACTCCTTATGA	ATTGTATGCTGCTGCCAGTG
GRID1	TTCCCAATTTTCGAAGTCAG	GGTTCCTCGTCTTCCCTTCT
KCC2	GGTTGCAAACCAAATCAAGAG	ATTTTGTGCAGACGGGAGTC
KCNJ14	GTAGAGCACCCAGCCAAGAG	CCTTTTGGCATTACAGAACCA
MITF (96)	TTGGACTAGCACTGACTGAACTG	AGCATCACCAATGTTTCCAAG
MITF (182)	ATTTCTGCAGGTGGCAGGT	TTCCTTTGTGCTTTTAACTTCCTA
MYRIP (96)	TTGACAAAAATGTGTACTGTGTAAGC	GATCAAAATCACTTGATGACAAAA
MYRIP (182)	AAGTGCCTGCTCTGAAGGAG	GGAAATGCACATAGCAGCAA
MYRIP (182)	CAAGTGATTTTGATCTTTAGTGTCA	ATCTTGGCCCCTCCAGTTAC
NCS1	TTGCCATCTATCGACCTTCC	CAGGACAGGGGAGAGGAGAG
NEFL	CAGATGCAAGCTATGTGCAA	GTTAAAAGGGGCACTGACCA
PEX19	CAGCTATGGGGAACATCTGG	GGCAGAAACCACAATGGAGT
PPP2CA	CCTAATGGAAATGGGAAGAGC	TCCAATGATTGTTTGCTGCT
RDX	AGCTGAACCACCAACAGAGAA	TGGAAAAGAGGCAATGGAAC
RYK	TTGGACTAGGGGTACATTCTTACA	CAAGGCAGACCAGGTATCTTTT
SLC6A6	GATCAAGGGCCTTATGTGGA	TGTGAAAATTCTGCGGTCTG
TFCP2L3 (96)	GCCATGTGAGAGCTGTGAAC	GCATGTAGCAGGAGACCACA
TFCP2L3 (96)	GCCCTAAGGCAGAAGATGAA	TGTTTCGCTCAGGAAATTTTG
TFCP2L3 (96)	GCCCAGAAACTTAGGAAGCA	GATTCCCCCTCTCCCATTTA
TREK-1	GCTTGTTGAACGGTCCACTT	TGGTCCATATCTAGGCTCAGTT
ZCCHC3	GTCCCAAAGCAGTGCACAAT	TTTTAAAAGGGAGGGGCAAC

[a]Round brackets: miRNA binding site.

RESULTS

Radixin—A Potential Target of miR-96/182 Regulation

We screened the predicted miR-96/182 binding site in the *RDX* 3′UTR in probands from 576 unmapped Iranian ARNSHL families (Table I). In one family, L-1007, we identified a homozygous c.*95C>A variation in the predicted binding site in affected individual II:1 (Fig. 1A,B). The c.*95C nucleotide is highly conserved between species (Fig. 1C,D). This variant was not present in 64 (128 chromosomes) Iranian controls or 191 (382 chromosomes) Centre d'Etude du Polymorphisme Humain (CEPH) controls.

Besides altering the predicted binding site for miR-96/182, the c.*95C>A variant also creates a novel binding site for miR-507/557. Thus, we hypothesized that the c.*95C>A mutation in RDX could result in hearing loss by (i) disrupting the binding of miR-96 and miR-182, and/or (ii) producing a new binding site for miR-507 and miR-557. The former has been demonstrated for a number of diseases including irritable bowel syndrome [Kapeller et al., 2008], while the latter has recently been described in Tourette syndrome, muscularity in sheep and in Parkinson disease [Clop et al., 2006; Chou et al., 2007; Wang et al., 2008].

Activity of the Predicted RDX miR-96/182 Binding Site In Vitro

To determine whether the predicted miR-86/182 binding site in the *RDX 3′UTR* is a biologically relevant target of regulation we performed luciferase assays (Fig. 2A). Neither miR-96 nor miR-182 affect luciferase activity of the chimeric luciferase reporter/*RDX* constructs as compared to scrambled control oligonucleotides. We also failed to observe any decrease in activity for the mutant construct when co-transfected with miR-507 or miR-557 mimics.

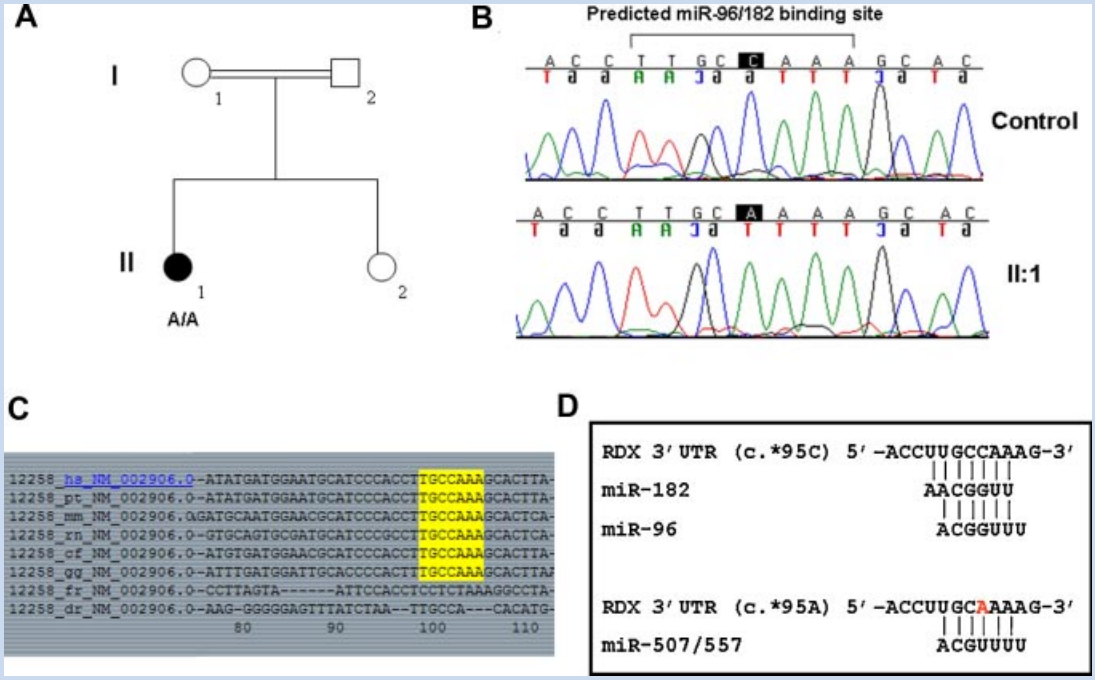

FIG. 1. Variation in the predicted miR-96/182 binding site of the *RDX* 3′UTR. A: Pedigree of Iranian family L-1007 with ARNSHL. Open symbols = unaffected; filled symbols = ARNSHL. The c.*95C>A genotype for affected proband II:1 is shown. B: Direct sequencing revealed a homozygous alteration (c.*95C>A) in the miR-96/182 seed sequence in proband II:1. C: Multisequence alignment generated using PicTar (http://pictar.mdc-berlin.de/) showing that the predicted miR-96/182 binding site in the 3′UTR of the *RDX* gene is highly conserved across species. hs, human; pt, primate; mm, mouse; rn, rat; cf, dog; gg, chicken; fr, pufferfish; dr, drosophila. D: Consequential pairing of the reference *RDX* target region (c.*95C) with miR-182/96 seeds (top), and the mutant *RDX* target region (c.*95A) with miR-507/557 seeds (bottom), predicted using TargetScan (http://www.targetscan.org/). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

We repeated transfections using a 10-fold increase in mimic but failed to observe any evidence for miRNA targeting (data not shown). In addition, we tested whether miRNA mimics could affect the endogenous expression of *RDX* in HeLa cells (Fig. 2B). As shown by Western blot, no significant reduction in RDX protein levels is detected in the presence of miRNA mimics compared to control. We also screened a family of Pakistani origin generously provided by Dr. Thomas Friedman and Dr. Zubair Ahmed (Laboratory of Molecular Genetics, National Institute on Deafness and Other Communication Disorders) that mapped to the DFNB24 locus. However, no 3′UTR variants were identified.

Screening of the miR-183 miRNA Cluster and Additional Predicted Targets

In addition to the *RDX* gene, we identified a large number of other potential targets of miR-96/182 regulation (Table I). Predicted miR-183 binding sites in a total of 24 candidate target genes were screened in 192 ARNSHL families; however, we identified no potential pathogenic variants in any of these genes. In the case of *EZRIN*, a related ERM family member, we screened 576 ARNSHL families without identifying any 3'UTR variants. Since mutations in the miR-96 seed sequence have been linked to ADNSHL in humans and mice [Lewis et al., 2009; Mencia et al., 2009], we also screened 150 American ADNSHL families for mutations in the miR-96, miR-182, and miR-183 genes (Table I). However, we found no potential pathogenic variants.

DISCUSSION

Radixin was selected as a potential target of miR-183 miRNA cluster regulation based on its association with DFNB24 ARNSHL, its interesting temporospatial expression pattern in the inner ear [Kitajiri et al., 2004; Khan et al., 2007], and the presence of a predicted miR-96/182 target site in its 3′UTR. Despite this, our in vitro assays results do not support a direct role for the miR-183 miRNA cluster in the regulation of radixin.

Mencia et al. [2009] did identify two mutations in adjacent nucleotides of the miR-96 seed sequence in two families segregating progressive ADNSHL. Supporting the disease-causing nature of these sequence variations was the simultaneous discovery by Lewis et al. [2009] of a single-base change in the miR-96 seed of the diminuendo (*Dmdo*) mouse model that also results in progressive hearing loss. However, investigation of families with hereditary deafness by Mencia et al. [2009], and now also in our laboratory has failed to find causative mutations in either miR-182 or miR-183. A possible explanation is that expression of other members of this

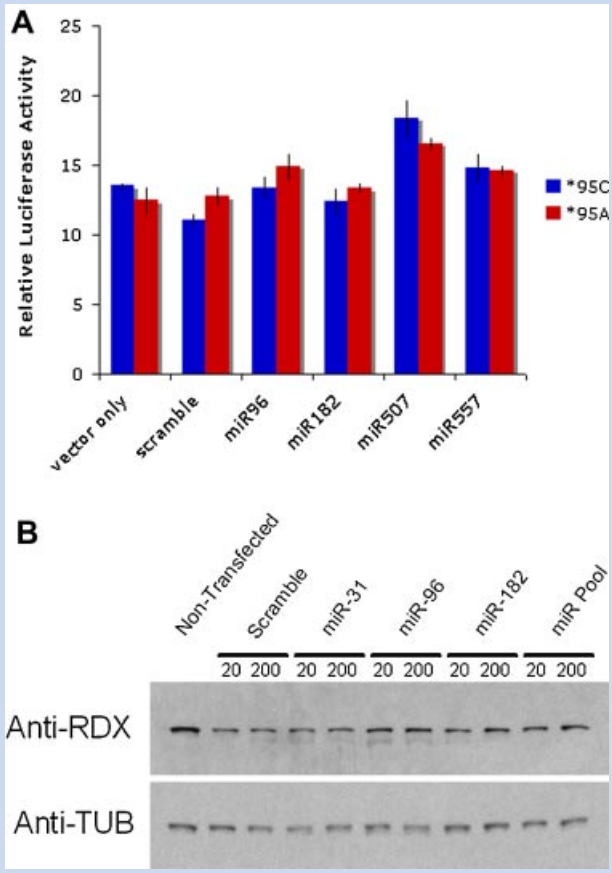

FIG. 2. Radixin is not targeted by miR-96 and miR-182 in vitro. A: Luciferase reporter activity of reference (*95C) and mutant (*95A) RDX 3′UTR constructs co-transfected with miRNA mimics in HEK293 cells. Blue bars = reference sequence; red bars = mutant sequence. Data are averages of six replicates. Error bars denote standard deviation (SD). B: Western blot analysis of endogenous radixin protein levels in HeLa cells treated with miRNA mimics (20, 200 pmol). miR-31 was included based on similarity to miR-96 and -182. miR Pool denotes cocktail of all three miR mimics. Cell lysates were collected 72 hr after transfection. Blots were stripped and reprobed with alpha-tubulin antibody as a loading control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cluster compensates for the loss of either miR-182 or miR-183 but not for the loss of miR-96. The identification of miR-96 mutations in only 2/568 genetically undiagnosed Spanish families with hereditary hearing loss in the Mencia et al. study and in 0/150 American ADNSHL families in our study suggests that mutations in this miRNA gene are a relatively rare cause of NSHL.

Detailed investigation of the downstream effects on gene regulation of the human and mouse miR-96 mutations revealed five genes containing predicted miR-96 binding sites that were upregulated in the presence of mutant miR-96 siRNA mimics [Lewis et al., 2009; Mencia et al., 2009]. The task of identifying true targets is difficult. Software algorithms such as miRanda, TargetScan, and Pictar can be used to identify candidate genes by calculating the statistical weighting of matches with the seed region of miRNAs [John et al., 2004], but these tools must be coupled with in vitro reporter assays. For example, Lewis et al. identified 132 potential target genes of miR-96 using miRanda and chose 13 for further characterization based on known inner ear expression and gene function. Of these 13 genes, only 5—*Aqp5, Celsr2, Odf2, Myrip*, and *Ryk*—were upregulated in the presence of siRNA mimicking the *Dmdo* miR-96 mutation, indicating loss of repression. Despite this data, analysis of human homologues of these genes in 192 hearing loss families in this study did not reveal any mutations.

The implications of miRNA involvement in the human auditory system are profound. Xu et al. [2007] discovered a cluster of miRNA genes whose expression is limited to sensory tissue including the inner ear. Mencia et al. and Lewis et al. showed that mutations in one member of this cluster, miR-96, lead to inherited deafness. These results identify a novel miRNA-mediated regulatory system essential to mammalian hearing. By studying animal models of miRNA-induced deafness, we hope to build on this foundation by understanding more about miRNA regulation and how its dysregulation leads to disease. The challenge remains to decipher whether over-expression of miR-96 target genes represents a dominant-negative effect or subtle, wide-spread dysregulation of gene expression in the pathogenesis of hearing loss.

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