Biochimica et Biophysica Acta 1862 (2016) 2119–2126

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

miR-431 is involved in regulating cochlear function by targeting *Eya4*

Yue Fan [a], Ying Zhang [a], Rimao Wu [b], Xingming Chen [a], Yong Zhang [b,*,1], Xiaowei Chen [a,*,1], Dahai Zhu [b,*]

[a] Department of Otolaryngology, Peking Union Medical College Hospital, Shuaifuyuan No.1, Beijing 100730, China
[b] The State Key Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and School of Basic Medicine, Peking Union Medical College, 5 Dong Dan San Tiao, Beijing 100005, China

ARTICLE INFO

Article history:
Received 24 March 2016
Received in revised form 15 August 2016
Accepted 17 August 2016
Available online 19 August 2016

Keywords:
miR-431
Eya4
Transgenic mouse
Cochleae
Spiral ganglion neuron

ABSTRACT

To understand the relationship between microRNAs and hearing loss and help clarify the causes of hereditary deafness, we studied the functions of miR-431 in cochleae. We first investigated the spatial-temporal expression profiles of miR-431 in spiral ganglion neurons (SGNs) in cochleae using real-time PCR and miRNA *in situ* hybridization. These studies showed that expression of miR-431 was high in SGNs in the cochleae of newborn mice, and decreased as development progressed. To test the functional effects of miR-431, we established miR-431 overexpressing transgenic (Tg) mice. Surface preparations of the cochlear basilar membrane and cochlear sections revealed no major structural differences between Tg and wild-type (Wt) mice. However, a comparison of auditory brain stem responses (ABRs) in Tg and Wt mice showed that ABR thresholds were significantly higher in Tg mice than in Wt mice. Notably, the density of SGNs was significantly lower in Tg mice than in Wt mice. We also found that the proportion of mature SGNs in cultures of primary SGNs from Tg cochleae was lower and their axons were shorter. A bioinformatics analysis predicted that the mRNA target of miR-431 was *Eya4*, a finding confirmed by luciferase reporter assays and western blotting. Importantly, overexpression of miR-431 in cochleae of Tg mice inhibited the translation of *Eya4* mRNA, leading to a deficiency of EYA4. Thus, excessive amounts of miR-431 in cochleae of Tg mice may be the cause of sparse SGNs, which in turn could be responsible for hearing loss.
© 2016 Published by Elsevier B.V.

1. Introduction

Sensorineural hearing loss is the most common disability in humans, affecting over 27 million people in China alone. The main reasons for sensorineural hearing loss are developmental disorders and degeneration of cochlear neurosensory cells resulting from genetic mutations, overstimulation, ototoxic drugs, infections, autoimmune diseases or aging. Of these contributors, genetic factors are involved in >50% of congenital hearing loss cases. Mutations in genes are also responsible for progressive hearing loss, and play an important role in presbycusis, highlighting the importance of the fine coordination and strict regulation of the expression of genes and regulatory factors for normal functioning of the inner ear [1,2].

DFNA10 is the tenth genetic locus identified for autosomal-dominant non-syndromic hearing loss (NSHL). The most recently discovered locus, identified in 1996 by linkage analysis of an American family and mapped to chromosome 6q22.3–q23.2, contains the gene *EYA4* (eyes absent 4) [3], which was subsequently found to be the causative gene. To date, six mutations in human EYE4 have been reported to cause DFNA10 [3–8]. *Eya* genes encode transcriptional coactivators that translocate into the nucleus in association with members of the SIX (sine oculis homeobox) family of transcription factors. EYA proteins interact *via* their eya-homologous region (eyaHR) with SIX family proteins to form transcriptional complexes that regulate the expression of target genes required for the development and maturation of the Organ of Corti [5]. Eya4-deficient ($Eya4^{-/-}$) mice also exhibit profound hearing defects [9]. Given the importance of EYA4 in maintaining normal hearing function, genes that regulate the expression of *Eya4* would also presumably affect hearing.

MicroRNAs (miRNAs) are important post-transcriptional regulators of protein expression that typically act through base pairing with the 3-UTR of their target mRNAs to inhibit protein expression through translational inhibition and/or mRNA degradation [10]. Bioinformatics analyses predict that ~30% of protein-coding mRNAs in humans are regulated by miRNAs [11,12]. Most research on miRNAs to date has been in the fields of cancer, cardiovascular diseases, and neural development. By comparison, the study of miRNAs in hereditary hearing loss has lagged. In the first studies of miRNA expression in the inner ear in 2005, Wienholds et al. reported the expression of miR-182, miR-96, and miR-183 in hair cell of the zebrafish [13]. Studies have also shown that miRNAs are essential for the development and function of the cochlea in invertebrates. Using microarray analyses, temporal changes in

* Corresponding authors.
E-mail addresses: dr_zhangyong@126.com (Y. Zhang), chenxw_pumch@163.com (X. Chen).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbadis.2016.08.015
0925-4439/© 2016 Published by Elsevier B.V.

miRNA expression during postnatal maturation of the mouse inner ear were described [14]. The most direct evidence of a relationship between miRNAs and hearing loss has come from a study of mutations of the miR-96 gene found in a Spanish deafness pedigree [15]. Subsequent studies have shown that mutations in the miR-96 gene result in autosomal-dominant, progressive hearing loss in both humans and mice [15,16].

An investigation of miRNA expression reported by Elkan-Miller et al. identified 157 miRNAs that are expressed in the inner ear sensory epithelia. A bioinformatics analysis also predicted that one of these miRNAs, miR-431, which was found to be expressed in both the cochlea and vestibule [17], targets *Eya4*. However, no previous studies have provided evidence of a role for miR-431 in cochlear function. In this study, we found that miR-431 is highly expressed in cochleae of mice, especially in spiral ganglion neurons (SGNs), the Organ of Corti, and the stria vascularis. Using miR-431 overexpressing transgenic (Tg) mice, we discovered that auditory brain stem response (ABR) thresholds were significantly higher in Tg mice than in Wt mice. Apart from a few missing hair cells in basal membranes of cochleae of Tg mice, the main structural effect of miR-431 overexpression was a significant decrease in the density of SGNs in Rosenthal canals. Using luciferase reporter assays and western blot analysis, we further confirmed that *Eya4* mRNA is the target of miR-431. We also found that cochlear EYA4 content was lower in Tg mice than in wild-type (Wt) mice. Collectively, our findings demonstrate that overexpression of miR-431 in cochleae of mice results in a deficiency of EYA4 and sparse SGNs, which may lead to hearing loss.

2. Materials and methods

2.1. Animals

All animal procedures were approved by the Animal Ethics Committee of Peking Union Medical College (ACUC-A01-2011-145). Mice were maintained in a low-noise, vented cabinet at 25 °C with a 12-hour light-dark cycle and were provided food water *ad libitum*. miR-431 transgenic (Tg) mice were generated by the Model Animal Research Center of Nanjing University. Overexpression of miR-431 in the Tg mice was driven by the β-actin promoter (pCAGGS) as previously described [18]. C57BL/6J mice were purchased from Vital River Laboratories Company in Beijing.

2.2. ABR measurement

For recordings of ABRs, animals were first sedated with a single intraperitoneal (IP) injection of 1.25% tribromoethanol (Sigma, USA) at a dose of 15 mL/10 g body weight. Mouse body temperature was maintained during the test using a heating pad. ABR recordings were performed in a sound proof environment using a Tucker-Davis Technologies (TDT) system and SigGen RP software (TDT, USA). Auditory stimuli (clicking sounds) were delivered to the ear by a speaker (TDT MF 1-1250), and threshold was determined by recording responses in 10-dB descending stimulus steps. The thresholds were determined by the occurrence of Wave II. Both mouse ears were tested, and the results were compared between genotypes.

2.3. Whole-mount Organ of Corti preparation

After killing mice by rapid decapitation, the brain was removed under a dissecting microscope, and the temporal bones were harvested and transferred to dishes containing sterile phosphate-buffered saline (PBS; pH 7.4). Cochleae were immediately dissected, and the membranous labyrinth was exposed by removal of the bony cochlear capsule. After the spiral ligament and stria vascularis had been removed, the Organ of Corti was carefully dissected from the modiolus using fine forceps and fixed by incubating at 4 °C in PBS containing 4% paraformaldehyde for 2 h.

Tissue was permeabilized by rinsing samples three times with PBS containing 1% Triton X-100 (PBST; Amresco, USA) at room temperature. Samples were then incubated in tetramethylrhodamine (TRITC)-labeled phalloidin (1:200 dilution; Invitrogen) for 30 min at room temperature. Nuclei were stained using the DNA-specific dye, 4′,6-diamidino-2-phenylindole (DAPI), diluted 1:1000. After rinsing three times with PBST, samples were slide-mounted with Citifluor and viewed under a confocal microscope.

2.4. Cochlear sections

Cochleae were perfused *via* the oval window with 4% paraformaldehyde in 0.1 M phosphate buffer, post-fixed at 4 °C overnight, and then decalcified by incubation at room temperature in 10% EDTA in 0.1 M phosphate buffer (pH 7.4) for 7 days. For mid-modiolar cross-sections, fixed cochleae were placed in 0.1 M phosphate buffer containing 15% sucrose for 2 days, and then in the same buffer containing 30% sucrose for 2 days. Sections were subsequently mounted in OCT (Miles, USA) or paraffin.

2.5. Immunohistochemistry and in situ hybridization

Cells and cryosections were fixed with 4% formaldehyde for 10 min and washed in PBST for 10 min at room temperature. Cells were then blocked with PBS containing 3% bovine serum albumin (BSA) for 10 min and incubated overnight at 4 °C with an anti-β-tubulin III primary antibody (Sigma, USA), diluted 1:100. After washing, cells were incubated at 37 °C for 20 min with fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibodies (Zhongshanjinqiao Corporation), diluted 1:50 in PBST containing 3% BSA. DAPI (diluted 1:1000) was then added for 1 min at room temperature. After several washes with PBS, cells were analyzed by fluorescence microscopy.

2.6. Primary cell culture

Newborn mice were killed by decapitation, after which their cochleae were removed and immersed in ice-cold Hank's balanced salt solution (HBSS; Invitrogen, Germany) for further dissection under a dissecting microscopic (Olympus, Japan). The bony cochlear capsule was carefully opened to remove the stria vascularis and the Organ of Corti from the modiolus.

Cells of the modiolus were seeded in a 6-well plate at 1×10^4 cells/cm^2 in Dulbecco's Modified Eagle Medium (DMEM)/F12 containing 10% fetal bovine serum (FBS). After 12 h, the medium was replaced with neurobasal media containing 1% glutamate and 2% B27. Cytarabine (final concentration, 5 mM) was added after primary cells had become well established to specify and support the differentiation of SGNs.

2.7. Western blot analysis

Cochleae were lysed in a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Nonidet P40, and protease and phosphatase inhibitors. Proteins in whole-cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride membrane. Immunoblotting was performed using primary antibodies against EYA4 (Abcam, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Millipore, USA). Membranes were washed with PBS for 30 min and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Zhongshanjinqiao Corporation) for 1 h at room temperature. After washing again with PBS for 30 min, immunoreactive proteins were detected using enhanced chemiluminescence (ECL; Thermo-Fisher, USA) by incubating in Detection Solution (1 min at room temperature) and subsequently exposing to X-ray film. The bands were quantified densitometrically using ImageJ software.

Download English Version:

https://daneshyari.com/en/article/1904453

Download Persian Version:

https://daneshyari.com/article/1904453

Daneshyari.com