Expression analysis of microRNAs in murine cochlear explants

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Abstract

MicroRNAs (miRNAs) play functional roles in sound transduction in cochleae. This study focuses on the validity of cochlear culture as an *in vitro* experimental tool, in view of miRNA expression. E15 cochleae were dissected and maintained *in vitro* for 48 hr before extraction of micro RNAs. MiRNA expression was comprehensively screened in explanted cochleae using a miRNA array that covers 380 miRNAs. Strong correlation was observed between expression levels of miRNAs *in vitro* and those in *in vivo* cochleae. Levels of 43 miRNAs were altered *in vitro* and these changes were reproducible over three trials. These findings indicate that *in vitro* miRNA profiling is a viable method for analysis of gene expression and action of chemical compounds on cochleae.

Keywords: microRNA array; organ culture of cochlea; embryonic cochlea

Introduction

Cochlear culture is a useful tool to investigate the expression of genes [1] and the actions of chemical compounds in the cochlea. For example, it has been applied for investigations of cochlear-specific gene expressions [1] and action of dexamethasone on cochlear tissue [2]. Recent studies have found that the expression of particular microRNAs (miRNAs) plays critical roles in cochlea function [3-8]. We have already reported that mRNA expression in cochlear explants is well-preserved under the *in vitro* experimental conditions, as shown by DNA microarray assay [2]. However, the mechanism by which the expression of miRNAs is maintained *in vivo* is unknown. As a first step, it is important to test the validity of cochlear culture in view of miRNA expression as an *in vitro* experimental tool. In this study, the expression of miRNAs in cochlear explants was comprehensively evaluated using a miRNA array that covers 380 miRNAs, and the miRNAs were compared between *in vitro* and *in vivo* conditions.

Methods

Tissue dissection and culture of mouse cochleae

Timed pregnant female BALB/c mice were sacrificed using an excess of ketamine (150 mg/kg, intraperitoneal) on E15 (vaginal plug, E0). The fetal cochleae were immediately dissected under a binocular microscope and frozen in liquid nitrogen until total RNA

extraction, or placed into Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% KnockOut Serum Replacement (Invitrogen, Carlsbad, CA, USA) and penicillin G (0.06 mg/ml). The explanted cochlea included the cochlear duct and immature sensory epithelium, nonneuronal mesenchymal tissues, lateral wall of the cochlea and spiral neurons [2].

Explanted cochleae were placed in 250 µl of serum-free medium and maintained at 37ºC in 5% CO_2, with daily serum changes. Explanted cochleae were maintained *in vitro* for 48 hr (DIV2) before extraction of total RNA. In general, 12-14 cochleae from a single litter were processed in each sample. All experimental protocols complied with the guidelines of Okayama University's Committee on the Use and Care of Animals.

Comprehensive analysis of microRNA expression in cochlear tissue

To screen miRNA expression in the explanted cochleae, we performed a microarray analysis using *mir*Vana™ miRNA Bioarray V9.2 (Filgen, Inc., Aichi, Japan).This microarray carries the nucleotides for a total of 380 miRNAs with each miRNA spotted in quadruplicate. MiRNA purification and other experimental procedures were performed as follows.

MiRNA purification and labeling

Total RNA was extracted from the *in vitro* and *in vivo* cochlear tissue using an RNA-easy column (Qiagen, Hilden, Germany). The quality of the purified RNA was checked using

an Agilent 2100 Bioanalyzer (Agilent Technologies, Tokyo, Japan). Using the flash PAGE™ system (Ambion Inc., Austin, TX, USA), we purified the miRNAs from 22 μg of total RNA. The purified miRNA was labeled using a *mir*Vana™ miRNA Labeling Kit (Ambion, Inc.) and CyDye Mono-Reactive Dye Pack (GE Healthcare Bio-science Corp, Piscataway, NJ, USA) according to the manufacturer's protocol.

Hybridization to miRNA array and data analysis

The labeled miRNAs were dissolved in 1× miRNA Hybridization buffer, incubated for 3 min at 95°C and then cooled to room temperature before application onto the arrays. The arrays were then incubated for 16–20 hr at 42°C. After hybridization, the arrays were washed once with low stringency wash (nuclease-free water 376 ml, detergent concentrate 4 ml, salt concentrate 20 ml) for 30 sec and twice with high stringency wash (nuclease-free water 780 ml, salt concentrate 20 ml) for 30 sec. After the arrays were centrifuged at 600×g for 5 min for drying, they were scanned using a GenePix 4000B® scanner (Axon Instruments, Sunnyvale, CA, USA) and the signal data of each array was calculated using an Array-Pro Analyzer® Ver.4.5 (Media Cybernetics Inc., Bethesda, MD, USA). The array data was normalized and averaged using the Microarray Date Analysis Tool (Filgen, Nagoya, Japan). All the experiments, including tissue dissection, cochlear culture, and miRNA extraction on the array, were performed three times to test the reproducibility of the results.

Results

The overall correlation of the expression of the 380 miRNAs between the *in vitro* and *in vivo* cochleae was very high (r =0.902, p <0.001) (Figure 1). However, several miRNAs were expressed differently in cochlear culture. Among these, 27 miRNAs had increased by more than 2-fold, and 37 miRNAs had decreased to less than half, in the *in vitro* cochlear tissue compared to the *in vivo* cochleae in the first trial of the experiment. In the second trial, the expression levels of 30 miRNAs had increased and that of 43 miRNAs had decreased, while in the third trial, 29 miRNAs had increased and 40 miRNAs had decreased. In each of the 3 trials, the expression level of 19 particular miRNAs always increased by more than 2-fold and that of 23 particular miRNAs always decreased to less than half, with striking reproducibility (Table 1).

Figure 1,

Table1

Discussion

The present data demonstrates that the overall expression levels of miRNAs before and after explantation are strongly correlated. A small number of miRNAs demonstrated differential expression after explantation but these changes in the expression levels occurred with striking reproducibility throughout the three trials. Our data validates the

use of cochlear explants as a model to investigate gene expression and the actions of various chemical compounds on cochlear tissue.

In this study, the expression level of 19 miRNAs increased while that of 23 miRNAs decreased *in vitro* in all 3 trials with 100% reproducibility.

Among them, the expression level of miR-762 (Gene ID: 791073) always decreased. This miRNA is known to interact and inactivate the expression of Hsf1 (Gene ID: 15499, Heat-shock factor 1, Microcosm Targets, URL: http://www.ebi.ac.uk/enright-srv/microcosm). This protein activates heat-shock response genes under conditions of heat or other stresses such as hypoxia [9]. We previously examined mRNA expression in cochlear explants and reported that the expression level of *Gapdh* is increased in explanted cochleae, indicating that cochlear tissue in culture is in a state of relative hypoxia [1]. We believe that the expression level of miR-762 may have changed in response to the hypoxic conditions.

The expression levels of MiR-22 (Gene ID: 387141) and miR-683 (Gene ID: 751559) decreased in all three trials of the experiment. MiR-22 is known to interact and inactivate the expression of Dad1 (Gene ID: 13135, Defender against cell death 1, Microcosm Targets). MiR-683 is known to interact and inactivate the expression of Aven (Gene ID: 74268, Apoptosis, caspase activation inhibitor, Microcosm Targets). These proteins have been shown to play roles in preventing apoptotic cell death [10,11], suggesting that the

reduction in the expression levels of miR-22 and miR-683 may lead to prevention of apoptotic cell death. These miRNAs are thought to be regulated in cochlear explants as a protective response to the exposure of the tissue to *in vitro* conditions.

Conclusion

The present data demonstrated strong correlation in the expression levels of miRNAs between *in vitro* and *in vivo* cochleae (r=0.902, $p<0.001$). The levels of 43 of the 380 miRNAs screened were altered *in vitro* but these changes were highly reproducible over the three experimental trials. These finding indicate the validity of using cochlear explants for investigations regarding gene expression and the actions of various chemical compounds on the cochlea.

Acknowledgements

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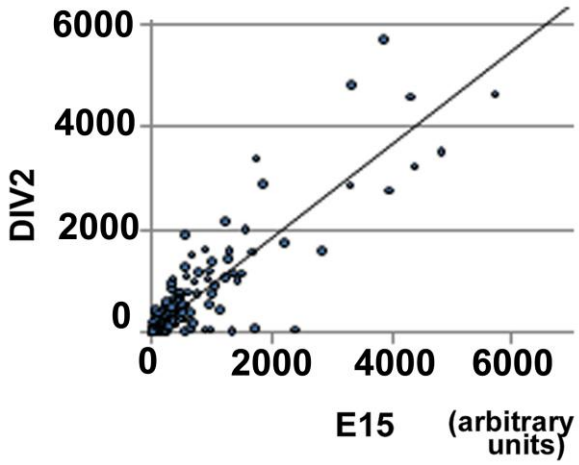
Legends

Figure 1. Overall levels of microRNA expression in the cochlear before and after explantation. The overall expression levels of the 380 miRNAs were highly correlated between the *in vitro* and *in vivo* cochleae (r =0.902, p <0.001).

Table 1. MicroRNAs increased by more than 2-fold, or decreased to less than half in the *in vitro* cochleae compared to the *in vivo* cochleae.

The expression of 19 miRNAs was upregulated by more than 2-fold, and those of 23 miRNAs were downregulated to less than half *in vitro*, compared to the levels *in vivo*. These data were completely reproducible over the three experimental trials.

Figure 1



microRNAs increased more than 2 fold in vitro (RefSeq ID)	ratio of normalized intensity. first trial	second trial	third trial	microRNAs decreased less than 0.5 fold in vitro (RefSeq ID)	ratio of normalized intensity. first trial	second trial	third trial
miR_20a	2.03	2.46	2.33	miR_224	0.24	0.43	0.38
miR_106b	2.29	2.93	2.56	miR_297	0.09	0.07	0.04
miR_92	2.76	2.64	2.83	miR_341	0.23	0.23	0.24
miR_181a	2.16	2.06	2.20	miR_34a	0.33	0.38	0.40
miR_106a	2.13	2.54	2.35	miR_22	0.36	0.34	0.40
miR_18	3.62	5.12	5.12	miR_346	0.17	0.12	0.14
miR_19b	2.49	2.75	2.91	miR_468	0.04	0.03	0.02
miR_19a	2.50	2.01	2.88	miR_370	0.49	0.48	0.41
miR_124a	5.82	7.71	6.76	miR_210	0.30	0.35	0.32
miR_126-3p	3.82	5.31	3.52	miR_204	0.19	0.15	0.40
miR-301	2.52	2.00	4.04	miR_122a	0.16	0.12	0.10
miR_20b	2.09	2.63	2.45	miR_26b	0.34	0.23	0.47
miR_503	2.72	2.91	3.21	let_7f	0.29	0.26	0.40
miR_302c	2.44	2.25	3.76	miR_669b	0.05	0.04	0.03
miR_720	2.22	2.20	2.51	miR_679	0.35	0.47	0.35
miR_690	2.63	3.01	2.31	miR_297b	0.11	0.07	0.05
miR_138	4.59	4.74	3.57	miR_683	0.27	0.23	0.35
miR_301b	2.38	2.79	4.13	miR_669c	0.03	0.02	0.02
miR_804	3.10	3.61	4.00	miR_669a	0.08	0.06	0.05
				miR-711	0.43	0.28	0.37
				miR-672	0.14	0.10	0.07
				miR-762	0.37	0.35	0.24
				miR-760	0.46	0.47	0.35

list of microRNAs reproducible in 3 isolated trials