Analysis of microRNA expression in organ of Corti in neonatal and adult rats

GUO Wei-wei, Hou Zhao-hui, YANG Shi-ming,
Department of Otolaryngology, Head & Neck Surgery, Institute of Otolaryngology, Chinese PLA General Hospital, Beijing 100853, China

Abstract Objective To characterize microRNA (miRNA) expression profile in microdissected auditory epithelia from the Corti’s Organ in new born and adult rats. Methods The TaqMan® MicroRNA Arrays were used to identify expression of microRNA in the new born and adult groups. GO analysis was applied to analyze the main function of the differential expression genes according to the Gene Ontology which is the key functional classification of NCBI. Similarly, Pathway analysis was used to find out the significant pathway of the differential genes according to KEGG, Biocarta and Reatome. Results Increased expression was seen in 16 miRNAs in mature rat compared to new born rats, with increased folding ranging from 17 to 600 folds. Expression levels in 2 miRNAs were reduced in mature rats, namely rno–miR–29c and rno–miR–29a. The high-enrichment GOs targeted by over-expressed miRNAs were negative regulation of epithelial cell differentiation, common-partner SMAD protein phosphorylation, mesenchymal–epithelial cell signaling, regulation of transforming growth factor beta2 production, etc. Functional analysis of miRNAs by KEGG revealed that 19 signal transduction pathways were upregulated and 14 were downregulated. Conclusions The difference in miRNA expression patterns in the organ of Corti between neonatal and adult rats may be closely related to maturation of the organ of Corti and loss of proliferative capacity of inner ear hair cells, and TGFβ signaling may play an important role in hair cells regeneration.

Key words miRNA; microarray; Rat; cochlear; TGFβ

Introduction

In mammals, embryonic hair cell (HC) and supporting cell (SC) proliferation within the sensory epithelia culminates between embryonic day 13 (E13) and E15 and stops after birth. It has been shown that acutely dissociated cells from the newborn rat organ of Corti can develop into otospheres consisting of 98% nestin positive cells when plated on a non-adherent substratum in the presence of either epidermal growth factor or fibroblast growth factor, although replacement of lost hair cells does not occur spontaneously in disorders such as age-related hearing loss.

The microRNA (miRNA) are small noncoding RNA molecules (~22 nucleotides) that regulate posttranscriptional gene expression by relatively nonspecific binding to the 3’–untranslated region of mRNA. A single miRNA may regulate several genes because of the sequence similarity. It has been proposed that over one-third of all protein encoding genes are under translational control by miRNA (Friedman et al., 4. A recent study using conditionally knocked out Dicer only inner ear sensory epithelium (SE) hair and SCs after their normal differentiation from progenitor cells revealed the importance of miRNA in the development and function of inner ear hair cells in vertebrates. Using an in silico prediction model that integrates miRNA, mRNA and protein expression, Elkan-Miller et.al has discovered 157 miRNAs expression in the inner ear sensory epithelia, with 53 miRNAs differentially
expressed between the cochlea and vestibule. Six miRNA families appear to be functionally important in the inner ear. Currently, there is no study regarding the impact of miRNA differential expression during the auditory epithelia stem cell development in postnatal rat.

In this study, we characterize the miRNA expression profile of microdissected auditory epithelia from Corti’s Organ of new born and adult rats. The study identified 16 differentially expressed miRNAs and GO analysis reveals the importance of TGFβ signaling in the hair cell regeneration.

Materials and Methods

Animals

Care and use of the animals in this study were approved by the Institutional Animal Care and Use Committees of Chinese PLA General Hospital

RNA Isolation

The basilar membrane from new born and adult Sprague Dawley rats were dissected from the cochlea in cold phosphate buffered saline (PBS; 10 mM Na2HP04, 1.7 mM KH2PO4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and stored in RNAlater (Ambion, Austin, TX) until use. Small RNAs (<200 nucleotides) were obtained using the mirVana RNA Isolation kit (Ambion, Austin, TX) according to the product manual. The quality and quantity of each RNA preparation were determined using a Model 2100 Agilent BioAnalyzer and Nanodrop ND–1000 spectrophotometer.

Microarray analyses

miRNA expression profiling was performed with RNA from 3 newborn and 3 adult SD rats utilizing the TaqMan Array Rodent MicroRNA Panel (Applied Biosystems, Foster City, CA) using 50 ng of RNA per port for a total of 400 ng. This array contained 365 miRNA targets as well as endogenous controls. Normalization was performed with the small nuclear RNAs (snRNAs) U44 and U48. These snRNAs are stably expressed reference genes suitable for use as normalizers in TaqMan assays. Gene expression levels were quantified using the ABI Prism 7900HT Sequence detection system (Applied Biosystems).

GO analysis

GO analysis was applied to analyze the main function of the differential expression genes according to the Gene Ontology which is the key functional classification of NCBI. Generally, Fisher’s exact test and χ² test were used to classify the GO category, and the false discovery rate (FDR) was calculated to correct the P–value, the smaller the FDR, the smaller the error in judging the p–value. The FDR was defined as \( FDR = \frac{N_i}{N} \), where \( N_i \) refers to the number of Fisher’s test P–values less than \( \chi^2 \) test P–values. We computed P–values for the GOs of all the differential genes. Enrichment provided a measure of the significance of the function: as the enrichment increases, the corresponding function is more specific, which helps finding those GOs with more concrete function description in the experiment. Within the significant category, the enrichment Re was given by: \( Re = \frac{(n_j / n) / (N_j / N)}{n / n} \) where \( n_j / n \) was the number of differential genes within the particular category, \( n \) was the total number of genes within the same category, \( N_j / N \) was the number of differential genes in the entire microarray, and \( N \) was the total number of genes in the microarray.

Pathway Analysis

Similarly, Pathway analysis was used to find out the significant pathway of the differential genes according to KEGG, Biocarta and Reatome. Still, we turned to the Fisher’s exact test and \( \chi^2 \) test to select the significant pathway, and the threshold of significance was defined by P–value and FDR. The enrichment Re was calculated like the equation above. The relationship of the miRNA and genes were counted by their differential expression values, and according to the interactions of miRNA and genes in Sanger MicroRNA database to build the MicroRNA–Gene–Network. The adjacency matrix of miRNA
and genes $A=\{a_{i,j}\}$ was made by the attribute relationships among genes and miRNA, and $a_{i,j}$ represented the relation weigh of gene $i$ and miRNA $j$. In the MicroRNA–Gene–Network, the circle represents gene and the shape of square represents miRNA, and their relationship is represented by one edge. The center of the network is represented by degree. Degree means the contribution one miRNA has to the genes around or the contribution one gene has to the miRNAs around. The key miRNA and gene in the network always have the biggest degrees.

**Results**

**miRNA expression profile analysis**

To gain insights into the role of microRNA alteration that may be associated with the proliferative ability of basilar membrane from new born and adult SD rats, we examined the global expression of mature miRNA using TaqMan® Rodent MicroRNA Arrays V2.0. The miRNA expression profiling identified 18 differentially expressed miRNAs using Two Class Diff method (Table 1). There were 16 miRNAs showing increased expression in mature rats compared to new born rats with increased folding ranging from 17 to 600 folds. Expression levels of 2 miRNAs were reduced in mature rats (rno–miR–29c and rno–miR–29a).

**Microarray–based GO analysis**

According to the threshold of GOs, regulation by miRNAs is considered significant if the $P$-value and FDR are $<0.001$ and $<0.05$, respectively. The high–enrichment GOs targeted by over–expressed miRNAs are negative regulation of epithelial cell differentiation, common–partner SMAD protein phosphorylation, mesenchymal–epithelial cell signaling, regulation of transforming growth factor beta2 production, etc (Figure 1). In contrast, significant GOs corresponding to under–expressed miRNAs appear to be protein heterotrimerization, negative regulation of phosphatidylinositol biosynthetic process and regulation of mitosis. Among these, the maximum–enriched–GO relating to TGFβ2 and SMAD signaling suggests that they play an important role in the proliferation potential of SE.

Signaling pathways regulated by differentially expressed miRNAs. Functional analysis of miRNAs by KEGG revealed that 19 signal transduction pathways were up–regulated and 14 were down–regulated (Figure 2). The up–regulated signaling pathways, including Wnt, TGF–β and MAPK, have been shown to participate in the activation of stem cells. A wide variety of cellular processes, including regulation of actin cytoskeleton, MAPK and GnRH signaling pathways also feature the functions of significant signaling pathways.

<table>
<thead>
<tr>
<th>miRNA ID</th>
<th>Fold change($2^{ΔΔCT}$)</th>
<th>style</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>rno–miR–296–star</td>
<td>601.8687077</td>
<td>up</td>
<td>AGGGCCGCCUUCAAGUCUUGU</td>
</tr>
<tr>
<td>rno–miR–183</td>
<td>311.2671462</td>
<td>up</td>
<td>UAUGGCAUAGGUAGAAUUCACU</td>
</tr>
<tr>
<td>rno–miR–130b</td>
<td>73.88701805</td>
<td>up</td>
<td>CAGUGCAUGUAAGCGACGGCAU</td>
</tr>
<tr>
<td>rno–miR–298</td>
<td>73.54126053</td>
<td>up</td>
<td>GAGCAAGGAGGGCGUUCUUCGCGC</td>
</tr>
<tr>
<td>rno–miR–199a–5p</td>
<td>39.0786033</td>
<td>up</td>
<td>CCACUGUUGCAGACUCCGUUCU</td>
</tr>
<tr>
<td>rno–miR–106b–star</td>
<td>38.46729557</td>
<td>up</td>
<td>CCCACUGUUGGUACUUGCCUC</td>
</tr>
<tr>
<td>rno–miR–323</td>
<td>37.0342188</td>
<td>up</td>
<td>CACAUUCAAGCGACGGCGCUC</td>
</tr>
<tr>
<td>rno–miR–301b</td>
<td>36.10785966</td>
<td>up</td>
<td>CAGUGCAUGUAAGCGACGGCAU</td>
</tr>
<tr>
<td>rno–miR–342–5p</td>
<td>19.28817847</td>
<td>up</td>
<td>AGGGGUGCACAUUGCAUGAG</td>
</tr>
<tr>
<td>rno–miR–19a</td>
<td>18.83546833</td>
<td>up</td>
<td>UGGGCAAAUCUAAGCAGUCAGA</td>
</tr>
<tr>
<td>rno–miR–18a</td>
<td>18.76863728</td>
<td>up</td>
<td>UAGGGUGCAUCUGUGCAUGAG</td>
</tr>
<tr>
<td>rno–miR–487b</td>
<td>18.75103727</td>
<td>up</td>
<td>AAUGGCAACAGGGCAUCCACUU</td>
</tr>
<tr>
<td>rno–miR–20a</td>
<td>18.57354895</td>
<td>up</td>
<td>UAAAGGCGUAAUAGCGAGGUAG</td>
</tr>
<tr>
<td>rno–miR–344a–3p</td>
<td>18.35504823</td>
<td>up</td>
<td>UGACUAGCGAAAGCGUGACGGC</td>
</tr>
<tr>
<td>rno–miR–431</td>
<td>17.97658408</td>
<td>up</td>
<td>UGGUGCACGGCGGUGCAGGCA</td>
</tr>
<tr>
<td>rno–miR–301a</td>
<td>17.94604464</td>
<td>up</td>
<td>CAGUGCAUAGUAAGCGACGGCA</td>
</tr>
<tr>
<td>rno–miR–29c</td>
<td>0.071923249</td>
<td>down</td>
<td>UAGGCAACGAAAGCGUAGGUA</td>
</tr>
<tr>
<td>rno–miR–29a</td>
<td>0.035852454</td>
<td>down</td>
<td>UAGGCAACGAAAGCGUAGGUA</td>
</tr>
</tbody>
</table>

**Table 1** Differential miRNA expression of mature to newborn rat
Discussion

MicroRNA has recently been an area of interest for a variety of human diseases. The disability of inner ear hair cells proliferation contributes to hear loss in aged population. In the present study, we compared the miRNA expression profile in the basilar membrane of inner ear between new born and adult rats. Our results reveal that several miRNAs are differentially expressed between the two different phenotypic cells and probably contribute to the loss of proliferation potentials of basilar membrane cells in adult rats. To the best of our knowledge, these preliminary results provide novel insights for the first time into the functional significance of miRNAs in basilar membrane cells development.

Given that embryonic HCs and SCs proliferation within the sensory epithelia culminates between embryonic day 13 (E13) and E15 in mammals and stops after birth, the fundamental mechanisms probably exist behind the loss of proliferation potential in embryonic HCs and SCs after birth. One of the possibilities is that coordinated and tightly controlled gene expression programs orchestrate the development process. miRNA as key regulators may play important roles during this phenotypic transition, further adding another layer of complexity to the regulatory network for basilar membrane proliferation. Our miRNA microarray data suggest that the expression profile of miRNAs in the rat inner ear appears to be well established by P0, consistent with the fact that early inner ear develop-
ment and cell fate specification mostly occur embryonically 9.

Our profiling data identify two distinct expression patterns of miRNA between new born and adult rat basilar membranes. Such difference appears to be associated with basilar membrane proliferation. The miRNA expression profiling in this study identified 18 differentially expressed miRNAs, with 16 increased and 2 decreased in mature rats compared to new born rats. miRNAs with increased expression in adult basilar membrane included miR-296, mi-130b, miR-183, etc. miR-296 has been demonstrated to modulate the pluripotency of embryonic SCs by repressing the expression of Oct4, Sox2, and Nanog 10. In vertebrates, the expression domain of conserved miRNA-183 (miR-183) family members appears to be restricted to ciliated neurosensory epithelial cells and certain cranial and spinal ganglia. In zebrafish the miRNAs are detected in the eye, nose epithelium, sensory hair cells of the ear and neuromasts 11, and injection of miR-183 and miR-200 family members in zebrafish embryos have been demonstrated to impact development and affect neuromast migration 12. Additionally, expression of miR-183 family members in mouse eye and sensory hair cells of the ear have been previously demonstrated 13.

Our findings provide a useful assay for the differentially expressed miRNAs in new born and adult rat organ of Corti. GO analysis and Signaling pathway analysis revealed the important roles of miRNA in the regulation of Wnt and TGF-β signaling. Further study focusing on this relationship will be of important.

Acknowledgments

This work was supported by grants from the Major State Basic Research Development Program of China (973 Program) (#2011CBA01000), the National Basic Research Program of China (973 Program) (#2012CB967900), and the National Natural Science Foundation of China (NSFC) (#81000483), New Star Project of Science and Technology of Beijing (2010B083) to Z.H.H.

Reference


(Received May 3, 2012)