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Research report

Assessment of differential gene expression in vestibular epithelial cell types using microarray analysis

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Abstract

Current global gene expression techniques allow the evaluation and comparison of the expression of thousands of genes in a single experiment, providing a tremendous amount of information. However, the data generated by these techniques are context-dependent, and minor differences in the individual biological samples, methodologies for RNA acquisition, amplification, hybridization protocol and gene chip preparation, as well as hardware and analysis software, lead to poor correlation between the results. One of the significant difficulties presently faced is the standardization of the protocols for the meaningful comparison of results. In the inner ear, the acquisition of RNA from individual cell populations remains a challenge due to the high density of the different cell types and the paucity of tissue. Consequently, laser capture microdissection was used to selectively collect individual cells and regions of cells from cristae ampullares followed by extraction of total RNA and amplification to amounts sufficient for high throughput analysis. To demonstrate hair cell-specific gene expression, myosin VIIA, calmodulin and α9 nicotinic acetylcholine receptor subunit mRNAs were amplified using reverse transcription–polymerase chain reaction (RT-PCR). To demonstrate supporting cell-specific gene expression, cyclin-dependent kinase inhibitor p27kip1 mRNA was amplified using RT-PCR. Subsequent experiments with α9 RT-PCR demonstrated phenotypic differences between type I and type II hair cells, with expression only in type II hair cells. Using the laser capture microdissection technique, microarray expression profiling demonstrated 408 genes with more than a five-fold difference in expression between the hair cells and supporting cells, of these 175 were well annotated. There were 97 annotated genes with greater than a five-fold expression difference in the hair cells relative to the supporting cells, and 78 annotated genes with greater than a five-fold expression difference in the supporting cells relative to the hair cells.

Theme: Sensory systems
Topic: Auditory, vestibular and lateral line: periphery

Keywords: Hair cell; Inner ear; Gene expression; Microarray; Supporting cell; Vestibular

1. Introduction

The Human Genome Project has been the most significant undertaking in recent molecular biology. This project has focused on enumerating the basic components of human biology (such as genes, genomes and proteomes) and describing rudimentary aspects of behavior in an attempt to deconstruct the biological processes into their molecular components [9]. Such essential information will provide the basis for mapping gene activity into physiological processes, thereby shifting the focus from lists of genes to pathways, networks, molecular machines, organelles and eventually the cell itself as a unit of work. The development of microarray and other high throughput technologies has made it possible to measure the relative abundance of mRNA from thousands of genes per experiment. Currently, a significant effort is being devoted to the creation of public databases for storing and mining gene expression data

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obtained through these experiments (GenBank, DNA Data Bank of Japan and European Molecular Biology Laboratory databases and formats). Recording data in these repositories has become a condition for publication in several, if not most journals [35]. The Gene Ontology Consortium and other groups are working on defining the standards for the description of eukaryotic genes and the function and location of their gene products. A key caveat is that the data is highly context-dependent, and experimental information must be provided for the data to be truly useful. Essential information includes the number and size of the starting histological samples, the amount of RNA in each cell type, the number of cells harvested, the use or omission of RNA amplification procedures, the method and efficacy of labeling probes, protocols for hybridization, characteristics of the microarray chip, the sensitivity of the image acquisition hardware, the bioinformatics package employed and the interpretation of results. Therefore, the creation of standards at every step is essential for meaningful data generation, interpretation and comparison across experiments. The present study is aimed at establishing the standards for RNA acquisition for gene expression studies in the inner ear of the rat and subsequent transcriptome analysis using microarray gene expression profiling.

In the inner ear, gene expression profiling at a cellular and histological level poses multiple significant challenges. The cells in the vestibular and auditory sensory epithelia are densely packed, making the acquisition of homogeneous hair and supporting cell populations a challenge. Different methodologies have been devised to approach this problem. A common solution in other systems has been the extraction of total RNA from cultures of pure cell populations. In the inner ear, sensory epithelia, in vitro propagation of dissociated cells that retain the phenotype and transcriptome of the mature sensory epithelia cells has not been accomplished. Previous gene expression studies in the inner ear of multiple species have analyzed total RNA extracted from whole end organs [5,6,15,18,26,27,34,40], and thus evaluated a combination of several cell types, including hair and supporting cells from the epithelia and cells from the underlying stroma (fibroblasts, endothelial cells, blood cells). In other studies, the sensory epithelia was removed from the end organ by a 60 minute incubation in solution containing thermolysin followed by mechanical separation of hair cells and supporting cells from the stroma [14]. RNA, from 50,000 epithelial cells, was then extracted and amplified prior to analysis. While this method allows for the isolation of sensory epithelial cells, the long thermolysin incubation time and the mechanical trauma are likely to trigger cell repair and cell death programs, and thus make it difficult to elucidate the patterns of gene expression in the "normal" tissue. To overcome this problem, methods for amplifying nanogram to microgram quantities of RNA have been developed [41].

Laser capture microdissection (LCM) has been used extensively for the procurement of targeted cell types from specific microscopic regions of tissue sections. With this method, a tissue section is covered with a transparent plastic film and the cells of interest are identified under light microscopy. An infrared laser beam is used to melt the film onto the cells, causing them to adhere. The film is then removed while the surrounding tissue remains on the glass slide. While this technique has been used successfully elsewhere for gene expression studies [4], to our knowledge, there are no published applications for use in the inner ear. Herein, we report the laser microcapture acquisition of individual cell types from the inner ear sensory epithelia and subsequent transcriptome analysis in the cell type of interest using microarray expression profiling.

2. Materials and methods

This study was performed in accordance with the United States Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, the *NIH Guide for the Care and Use of Laboratory Animals*, and the Animal Welfare Act (7 U.S.C. et seq.); the animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Medical College of Wisconsin.

2.1. Capture of hair and supporting cell populations

Young adult Brown Norway rats (*Rattus norvegicus*) were anesthetized with sodium pentobarbital and decapitated. The temporal bones were removed and the individual cristae ampullaris were dissected under a dissecting microscope. Individual cristae were quickly placed in Tissue-Tek OCT cryoembedding media (Sakura Finetek, Torrance, CA), and frozen in liquid nitrogen-chilled isopentane for 15 s. The tissue was sectioned at 10 μm thickness with a Microm HM500 cryotome (Microm, Walldorf, Germany), melted onto sialinated microscope slides and stored at −80 °C until use. Approximately 40 sections were obtained per crista and three cristae were utilized for each experiment. All experiments were performed in duplicate or triplicate. At the time of preparation, tissue sections were stained according to the protocol outlined in the Arcturus Histogene staining kit manual (Arcturus Engineering, Mountain View, CA). Briefly, slides were incubated sequentially in the following RNAse free solutions: 70% ethanol for 30 s, double distilled water for 30 s, dye for 20 s, double distilled water for 30 s, and serial ethanol 70%, 95% and 100% for 30 s each, followed by one min incubation in xylene. Slides were then allowed to air dry for 15 min and transferred to the Arcturus Pixcell II LCM system. Laser capture microdissection was done following one of two different protocols. In the first protocol, a laser spot diameter of 15 μm and laser beam pulse settings of 75 mV and 250 ms were selected. Under these settings, the more superficial layer of the cristae ampullaris sensory epithelium encompassing types I and II hair cells, and the supporting cell cytoplasm interspersed