Characterization of cochlear transcription, translation and energy extraction in aging and noise-induced pathology

by

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M.S.E., University of Connecticut (2008)
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Submitted to the Harvard–MIT Program in Health Sciences and Technology
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Abstract

Success in otologic practice is currently limited by the diagnostic tools and treatment options available to address an individual’s specific presentation of hearing loss. This limitation results from insufficient characterization of the inner ear’s biochemical environment as well as physical hurdles associated with accessing inner ear tissues. The encapsulation of the hearing organ within a bony shell and delicate nature of its tissues make standard tissue biopsy techniques impossible and leave many imaging methods impractical.

This thesis sought to approach these clinical limitations in two ways: (1) performing novel transcriptional and translational characterizations of inner ear tissues and (2) development of a novel technique to access and communicate diagnostic information from within the inner ear.

The first part of this thesis employs whole transcriptome shotgun sequencing to study murine inner ear transcriptional activity in young, healthy animals as well as changes associated with organ aging and noise-induced auditory neuropathy, an important mechanism of hearing impairment in humans. Knowledge of the inner ear’s transcriptional behavior (Part I) is coupled with novel translational insights provided by high-throughput tandem mass-spectrometry (Part III) studies of human inner ear fluids obtained from healthy and pathologic populations. These studies illuminate homeostatic mechanisms employed by the highly specialized inner ear tissues, providing a critical knowledge-base for inner ear scientists and pharmacologists, and identify important expression-level changes which occur during the onset and progression of inner ear pathologies. While these high-throughput studies offer the powerful ability to gain a wealth of knowledge into which genes are active within the inner ear, functional assessment of the specific role these genes play must be assessed in a more focused manner. Phenotypic characterization of mice with specific genetic mutations (Part II) has been performed to provide critical insight into the specific role Fgf23 plays in development and maintenance of the auditory system.

The second arm of this thesis seeks to provide clinical practicality to the above work by developing a method to safely access the inner ear environment to gather
and communicate diagnostic information (Part IV). A guinea pig model was utilized to develop an approach to insert microelectrodes into the fluid spaces of the inner ear in order to harness and monitor the natural electrochemical gradient of the organ. The useful energy extracted from this “biological battery” was used to power a combined microchip/radio transmitter capable of performing voltage-sensing operations within endolymph and wirelessly relaying this information to an external receiver. This study was the first to utilize a mammalian electrochemical potential to power an electronic device. By performing this task while preserving the integrity of the hearing organ this work provides the first, critical proof-of-concept demonstration toward clinically-applicable sensing and therapeutic devices powered by the inner ear. Further refinement of this technique into a long-term, fully-implantable device will enable previously impossible longitudinal studies of organ behavior in awake, behaving subjects and the incorporation of sensing modalities into current inner ear prostheses to monitor biochemical changes and maximize patient benefits.

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Workflow diagram of perilymph analysis outlining specimen collection, grouping, preparation, and analysis. RP-HPLC MS/MS: reversed-phase high performance liquid chromatography tandem mass spectrometry.

Distribution of 271 proteins identified at high confidence within four samples of human perilymph.

Anatomy and physiology of the inner ear. Schematic of a mammalian ear including the external, middle, and inner ear, which includes the cochlea and vestibular end organs (a). Cross section of a typical cochlear half turn highlighting the endolymphatic space (yellow) bordered by tight junctions (red), the stria vascularis (green) and hair cells (blue), which are contacted by primary auditory neurons (orange) (b).
Schematic of the endoelectronics chip and equivalent circuit model of the endocochlear potential and inner ear tissues. To generate and maintain EP, perilymphatic K⁺ enters fibrocytes (F) via Na⁺/K⁺-ATPase and Na-K-Cl-cotransporter. Gap junction networks connect F to strial basal (SB) and intermediate (SI) cells, allowing ions to enter the intra-strial space via KCNJ10 channels. Strial marginal (SM) cells uptake K⁺ against a significant concentration gradient and release K⁺ into endolymph. K⁺ returns to perilymph either by entering hair cells (HC) apically during mechano-electrical transduction of sound and exiting basolaterally via KCNQ4 channels, or via leakage current through the surrounding tissues (solid red lines represent tight-junction networks). With energy from the EP, a boost converter was used to trickle-charge capacitor C_{DD}, which supplies power to an integrated wireless transmitter. Switch S₁ in the converter was used to temporarily store energy in the inductor (L), and switch S₂ was used to transfer the stored energy onto C_{DD} at the higher required voltage. Once C_{DD} had stored sufficient energy, the transmitter entered its active mode and wirelessly transmitted a packet to a nearby receiver, after which it returned to a low-leakage standby mode and the boost converter began to replenish C_{DD}. A start-up rectifier was used with an external wireless power source to initialize the system.
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Photograph of the experimental setup, including the anesthetized guinea pig, the electrodes and the endoelectronics chip.

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Chapter 1

Introduction

Hearing loss is clinically classified into two categories. The sensation of hearing results when sound-induced vibrations of the tympanic membrane and middle-ear ossicles are transmitted to the fluids of the inner ear, inducing stimulation of sensory hair cells and excitation of the auditory nerve. Hearing loss occurs when a condition arises which results in the reduction of sound perception. Epidemiology suggests that hearing loss is present in nearly 17% of American adults and approximately 2–3 newborns in 1000[115]. Hearing loss is broadly classified into two categories: (1) conductive hearing loss (CHL), in which the mechanical transfer of sound energy from the air to the fluid of the inner ear is impeded, and (2) sensorineural hearing loss (SNHL), in which tissue pathology in the inner ear or central auditory pathways hamper signal transduction or neural conduction. SNHL can be the result of damage to almost any of the inner ears approximately 30 different cell types, as well as the ascending auditory nerve, referred to as the spiral ganglion (SG). The SG is a bundle of 35,000–50,000 nerve cells[17] conducting sensory information from inner hair cells (IHCs) to the cochlear nucleus (CN), located within the brain stem.

Diagnosis of underlying pathology is a significant hurdle in the understanding and treatment of SNHL. Diagnosis and treatment of CHL has become fairly routine within otological practice. Direct visualization of the middle ear space coupled with air/bone conduction testing, mobility tympanometry, and modern imaging
techniques provide a wealth of diagnostic information, sufficient to illuminate the underlying pathology. Insight into specific pathology has fostered development of treatment strategies tailored to an individual's condition and allowed clinical intervention in cases of CHL to achieve remarkable success.

Unfortunately, treatment of SNHL has not achieved the same level of clinical proficiency. While SNHL can be routinely differentiated from CHL, determining the specific pathology invoking an individual patient's SNHL remains beyond current diagnostic capabilities. No methods currently exist to directly and individually evaluate the health of the inner ear's many tissues. Thus, physicians are blinded to the underlying pathologic condition in most cases of non-genetic SNHL. This limitation results in a frequent diagnosis of idiopathic SNHL leaving the patient and physician with no clear prognosis and general, often ineffective, treatment options.

Current diagnostics assess hearing performance not tissue health. Many measures of auditory function have been developed and formalized in a quest to gain insight into the health of the cochlea. The most commonly used test of hearing function is the pure-tone audiogram (PTA)[67]. This test is capable of determining psychophysical detection thresholds for a series of pure tones, a useful measure of sensitivity of the auditory organ. However the results of this test provide little insight into the type and extent of pathology within the cochlea. The spread of basilar membrane excitation can result in tone detection originating at off-frequency places[69], masking the severity of trauma at the characteristic place. Also, because this test is a measure of total organ performance, insult to one tissue type can appear identical to a differing pathology as long as they effect performance in similar ways[66]. More critically, recent evidence suggests that a clinically normal PTA can occur despite significant loss of SG fibers[86].

These deficits have spurred the development of a companion test to the PTA based on word recognition scores[68, 69, 66]. This test uses the results of a PTA to predict the information transmission capacity of the auditory system. An ideal filter with frequency characteristics identical to a patients PTA is coupled with the Speech
Intelligibility Index to predict the theoretical maximum amount of speech information that can be conveyed at a specified sound level. This prediction is compared with a patient’s actual performance on a word recognition task. If the two values agree across a range of sound intensities the PTA is interpreted to accurately portray the health of the cochlea at each frequency location. If these two measures do not agree (with the actual performance falling below the predicted value) it may indicate that regions of the auditory periphery are heavily damaged and incapable of contributing to the neural encoding of sound. In this case, off-frequency detection is responsible for portions of the PTA. Alternatively, depleted neuronal populations may be limiting the total information carrying capacity of auditory nerve. While capable of providing improved estimates of functional frequency ranges within the cochlea, and whether or not a patient is a good candidate for hearing aids or cochlear implantation, this test is unable to implicate individual tissues in the pathology.

Non-psychophysical tests have also been developed in order to assess performance at different points within the auditory pathway. This approach is favorable because it is decoupled from a patient’s perception and compartmentalizes the auditory system into smaller blocks which may be assessed independently. Auditory brainstem responses (ABRs)[3, 84, 104] and oto-acoustic emissions (OAEs)[81, 142] provide insight into function at the levels of the auditory nuclei and cochlear amplifier respectively, but neither can pin-point individual cell types in most cases of pathology.

Significance. The over-arching aim of this thesis is to obtain greater understanding of the cochlear environment, transcriptionally (Part I), genetically (Part II) and proteomically (Part III). These characterizations address the current clinical limitation by building the foundations for diagnostics capable of assessing tissue health via biochemical markers, to complement physiological and psychophysical assessments. Diagnostic markers capable of providing insight into inner ear pathology may enable the formulation of individualized treatment strategies (“personalized medicine”) tailored to each patient’s specific pathology. The need for such a tool is immediately apparent from the large number of patients who have unsatisfactory results with
general treatment options. Furthermore, a diagnostic platform is critical for successful implementation of preservative and restorative therapies that may emerge from ongoing research in inner ear development and regeneration[173]. Positive patient outcomes will only result if specific disease states are known and targeted, making a diagnostic tool absolutely critical to treatment success.

Part IV seeks to begin the ‘bench-to-bedside’ transition of cochlear biochemical diagnostic markers by developing a technique to gather and communicate diagnostic information from the inner ear environment. As noted above, diagnostic access to the inner ear has previously been prohibited by its fragile nature and inclosure within the otic capsule and temporal bone. However, ongoing development of sophisticated surgical methods and auditory protheses have enabled successful, invasive manipulation which was previously unthinkable. Building upon these successes, it now seems plausible, with a well developed technique, to access the inner ear fluids for insight into auditory pathology. With this goal, we utilize a guinea pig model to develop an approach to insert microelectrodes into cochlear endolymph and perilymph in order to harness and monitor the natural electrochemical gradient of the organ. The useful energy extracted from this “biological battery” is used to power a combined microchip/radio transmitter capable of performing voltage-sensing operations within endolymph and wirelessly relaying this information to an external receiver. This study is the first to utilize a mammalian electrochemical potential to power an electronic device. By performing this task while preserving the integrity of the hearing organ this work provides the first, critical proof-of-concept demonstration toward clinically-applicable sensing and therapeutic devices located within (and powered by) the inner ear. Further refinement of this technique into a long-term, fully-implantable device capable of sensing biochemical targets will enable previously impossible longitudinal studies of organ behavior in awake, behaving subjects and the incorporation of sensing modalities into current inner ear prostheses (cochlear implants, stapes prostheses, etc.) to monitor biochemical changes and maximize patient benefits.

Beyond diagnostic relevance, the knowledge gained in this investigation is useful to basic auditory scientists and inner ear pharmacologists. Characterization of the inner
ear transcriptome and perilymphatic proteome may provide insight into the molecular mechanisms that function to maintain the inner ear's unique environment[151]. Knowledge of the fluid content will also allow better understanding and prediction of protein-drug interactions, aiding in assessment of pharmacological efficacy and drug delivery within the highly specialized organ[135, 156].
Part I

Inner Ear Transcriptomics
Chapter 2

Count analysis and experimental design strategies for minimal-resource RNA-Seq experiments

Abstract

Utilizing the recent development and refinement of next-generation sequencing platforms, short-read sequencing of RNA (RNA-Seq) has become a standard tool for assessing transcriptional activity of tissues. These analyses are often undertaken with the goal to infer treatment induced differences in RNA expression. In order to detect transcript-level differential expression (DE), many statistical analysis tools, termed DE callers, have been developed. These algorithms utilize a variety of parametric and non-parametric approaches (each with their own strengths and weaknesses) to define null-distributions for read counts and test the hypothesis of differential expression.

While it is broadly recognized that biological replication is a critical part of ideal experimental design, in practice, cost and resource limitations often prevent this ideal from being realized. This leaves researchers struggling to make the most informed decisions from non-ideal, but still useful, experimental data. This work sought to aid researchers working with these types of datasets by (1) identifying the most effective ways to implement two DE callers, DESeq and NOISeq, on non-replicated RNA-Seq datasets, and (2) investigating how two experimental design strategies (multi-level studies and sample pooling) can improve DE caller performance.

We demonstrate that the performance of the NOISeq differential expression caller can be improved by careful selection of normalization method and model parameters.
Optimization between DESeq and NOISeq was observed to be dataset dependent, with DESeq being recommended for datasets with a coefficient of variation (CV) \( \leq 0.2 \) and NOISeq for \( 0.2 < CV < 0.3 \); neither caller makes reliable DE calls when \( CV \geq 0.3 \). We demonstrated that DESeq performance can be improved by including multiple treatment conditions into the dispersion estimation step for datasets with moderate biological variability and small numbers of differentially expressed genes. Also, pooling of biological replicates into one sample is an effective procedure to improve the performance of DESeq in situations with moderate to high biological variability and resource limitations that encourage minimizing the number of distinct samples processed by the sequencer.

2.1 Introduction

As next-generation sequencing (NGS) technologies become increasingly popular in biological sciences, one of the most common applications to emerge is the characterization and quantification of RNA transcription\[100, 108, 111, 158\]. The use of NGS to perform short-read analysis for RNA sequencing (RNA-Seq) is rapidly replacing microarrays as the industry standard due to potential for de novo discovery, single-base resolution, ability to identify alternative splicing, start and end points, higher sensitivity, higher dynamic range and lower variance between technical replicates\[26, 58, 62, 90, 100, 111, 116, 119, 158\].

While RNA-Seq experiments are more cost-efficient than microarray technologies (in terms of expenditure per transcript assessed), the expense of an RNA-Seq experiment is still quite significant. To help researchers maximize experimental efficiency, multiple studies have addressed design and analysis strategies to maximize power for detecting differential expression between two conditions. The typical trade-off in these analyses is whether to prioritize sequencing depth or more replicates. In studies of both synthetic and real-world datasets\[14, 50, 133, 161\] in which differential expression (DE) comparisons were made between two conditions, the general consensus is that sequencing beyond 15-20 million reads per sample offers diminishing returns. Adding additional biological replicates is a more effective use of limited resources, supporting the general belief that replication is the key to confident statistical inference (see Anders, et al.\[7\] for an excellent resource on RNA-Seq background, experimental
However, despite the advantages and cost-reductions associated with current multiplexing capabilities, performing well replicated studies is still an endeavor that may not always be within a researcher's financial or material means. Examples of material hurdles may be a study utilizing extremely unique samples (ex. human fluids obtained only during rare surgeries) or samples that are so small in volume that they need to be pooled across replicates to have enough to analyze. Alternatively, in multi-treatment, multi-level studies, the number of unique condition states may make performing a well replicated study financially infeasible. Fortunately, while not ideally replicated, the incorporation of so many distinct samples in many of these cases can be leveraged to improve model parameter estimation and determination of differential expression.

In order to improve the quality of DE inferences obtained from minimal-resource RNA-Seq studies, we have investigated how two DE callers, DESeq[6, 7] and NOISeq[161] (both available through Bioconductor, http://www.bioconductor.org), perform when provided with synthetic, non-replicated datasets that mimic many possible experimental designs. These DE callers were selected due to their uncommon ability to handle non-replicated data and their differing, but powerful, approaches to read count modeling that allow for adequate handling of the over-dispersion, generated by biological variability, that earlier poisson-based strategies lacked[13]. (DESeq2[98], a significant revision to DESeq, was recently released however changes in dispersion estimation strategies prevent its effective use in non-replicated studies). Previous studies comparing DESeq and NOISeq performance on synthetic and real-world datasets suggested that each method has its advantages and the best results were achieved by combining the results of both callers[183]. Furthermore, study of how each caller performed when replicates were included or removed from the analysis found that DESeq behaves very conservatively without replicates while NOISeq is much more aggressive and error prone.

In this study, we first sought to characterize how model parameters and normalization approaches impact the performance of NOISeq. Once appropriate analysis
parameters were identified, we studied how dataset variance and the percentage of differentially expressed genes effect how accurately DESeq and NOISeq perform. Using these results, we define guidelines for how to best select a DE caller for non-replicated studies. Furthermore, while each platform is capable of utilizing biological replicates, only DESeq is capable of incorporating more that 2 conditions into an analysis. In multi-level designs, this allows DESeq to utilize more count information to determine gene-wise dispersion estimates than NOISeq. The impact of this ability was studied by characterizing when inclusion of multiple treatment conditions (in the dispersion estimation step) improved or hindered DE caller performance. Finally, the impact of pooling multiple biological replicates into one sample for RNA sequencing was studied and found to have a significant beneficial effect on the performance of the DESeq algorithm.

2.2 Models & Methods

2.2.1 Construction of simulated data sets

Simulated count datasets were generated by first determining baseline expression values ($\lambda_i$) for each gene, $i$, by random generation from a gamma distribution with parameters: shape = 0.15 and scale = 1160 (as suggested in [183]). For datasets with differential expression, a defined percentage of the total number of genes was randomly assigned to be either up- or down-regulated. The fold-change, $FC_{i,k}$ (for each condition, $k$), associated with each differentially expressed (DE) gene was defined randomly from an exponential distribution with $\lambda = 1$ (up-regulated: $FC_{i,k} = 1 + X_{\lambda, i}$, down-regulated: $FC_{i,k} = 1/(1 + X_{\lambda, i})$)[133]. Non-DE genes and control conditions have $FC_{i,k} = 1$. DE modified expression values for treatment conditions, and non-modified expression values for control conditions ($\lambda_{i,j,k}$, where $j$ identifies each unique biological replicate of condition $k$), were then generated for each gene and replicate via sampling from a gamma distribution with parameters: shape = $C_{var}^2$ and scale = $\lambda_i \times FC_{i,k}$ (where $C_{var}$ is the target mean coefficient of variation between biological
replicates). Each transcript was assigned a length \((L_i)\) via random sampling from an empirically observed length distribution. Length modified expression values were defined as \(\lambda_{i,j,k}^{LM} = \lambda_{i,j,k} L_i / \text{median}(L)\). Target library size \((S_{lib}, \text{in millions})\) was implemented with a final scaling: \(\lambda_{i,j,k}^{LS} = 10^6 \lambda_{i,j,k}^{LM} \gamma_{i,j,k,S_{lib}} / \sum_i \lambda_{i,j,k}^{LM}\), where \(\gamma_{i,j,k,S_{lib}}\) is a library size variability coefficient drawn from \(\mathcal{N}(S_{lib}, (1 + 0.5(S_{lib}/10 - 1)))\). The final step in dataset construction was to generate simulated counts \((N_{i,j,k})\) from the expression values of each biological replicate of each condition. A poisson distribution with \(\lambda = \lambda_{i,j,k}^{LS}\) was utilized to account for the nature of the sampling in the RNA-Seq read generation process.

### 2.2.2 Differential expression callers

DESeq and NOISeq were downloaded from Bioconductor (http://www.bioconductor.org) and run in the R environment (http://www.r-project.org, v. 3.0.2). DESeq is a parametric method that utilizes the negative binomial distribution for count modeling, while NOISeq, a non-parametric approach, creates empirical distributions of gene-wise fold-change and absolute differences in order to assess differential expression between conditions. Count library normalization within DESeq employs a custom, hard-coded approach termed size factor normalization, while NOISeq allows the user to define their own normalization strategy (or utilize one of four built-in approaches). In order to perform analyses without replication in DESeq, gene-wise dispersion estimates had to be calculated using the “blind” method parameter. This mode groups all samples, independent of condition, into one quasi-condition for the purposes of dispersion estimation. NOISeq normalization strategies and parameter values were defined as specified in each section of the analysis.
2.3 Results & Discussion

2.3.1 NOISEq: Normalization strategies and Parameter space analysis

The mean NOISEq sensitivity and FDR which result from implementation of 5 different normalization strategies (reads per kilobase per million (RPKM)[108], reads per million (RPM), size factor[6], trimmed mean of M-values (TMM)[132] and upper quartile (UQUA)[27]) are presented in Figure 2-1 as a function of mean biological CV (mean \( N_{lib} = 30 \text{ million} \)). Results are presented for two different percentages of differentially expressed genes, 2.5% and 10%. While size factor normalization (utilized by the DESeq package) provides substantial improvements in sensitivity in all conditions, the increased sensitivity comes at the cost of large increases in FDR when \( CV > 0.2 \). The RPM normalization strategy provides modest improvements in sensitivity, particularly in datasets with greater biological variability, and does so while also controlling FDR to similar, or lower, levels as the remaining 3 strategies, hence providing the best overall performance of any normalization approach with the NOISEq package.

After identifying the preferred normalization strategy, the next task was to define the analysis parameters which resulted in the best performance on non-replicated data sets. The main model parameters which require specification are the size \( TR_s \) and number \( TR_n \) of the simulated technical replicates which NOISEq constructs for each experimental condition. The authors of NOISEq[161] suggest that \( TR_s \) is best set to 0.2 while \( TR_n \) is best \( > 5 \), however, no insight into how dataset properties may impact optimal parameter definition is given.

In order to better illuminate the impact of these parameters on model results, we have undertaken a study of how changing these values impacts model sensitivity and FDR (Fig. 2-2). This figure demonstrate how different \( TR_s \) (horizontal panels) and \( TR_n \) (colored lines) values effect model performance as a function of mean \( N_{lib} \) \( (n = 15 \text{ for each point, } CV = 0.3, DE_{frac} = 0.15) \). It is immediately evident that larger values of \( TR_s \) produce lower sensitivities. Larger \( N_{lib} \) sizes also tend to result
Figure 2-1: NOISeq sensitivity (top row) and FDR (bottom row) as a function of mean biological variance (CV) for two different percentages of differential expression when utilizing 5 different library normalization methods (colored lines). Each point is the mean of 15 simulations for which $TR_s$ and $TR_n$ were set to 0.2 and 5, respectively. Error bars designate standard deviation.

in lower sensitivity. At small values of $TR_s$, the impacts of $N_{lib}$ and $TR_n$ are the most pronounced. Namely, when $TR_s$ is small, model performance is improved with larger values of $TR_n$ and smaller values of $N_{lib}$. This relationship is weakened with increasing $N_{lib}$ and nearly abolished with increasing $TR_s$. NOISeq FDR is largely insensitive to any of the three parameters and all cases fail to control error to the designated value, $\alpha = 0.05$.

### 2.3.2 NOISeq vs DESeq

In order to maximize useful insight derived from low-resource RNA-Seq experiments, we sought to compare the two main DE callers currently capable of analyzing non-replicated data-sets, NOISeq and DESeq. Utilizing the parameters defined above for NOISeq, we studied how each caller performs on simulated datasets with varying size, variance and degree of differential expression. The results from DESeq (Fig. 2-3) and
Figure 2-2: Effect of varying $TR_s$ and $TR_n$ on NOISeq sensitivity (top row) and FDR (bottom row) as a function of mean $N_{lib}$. Each point is the mean of 15 simulations for which the biological $CV = 0.3$ and the portion of differentially expressed genes is 15%. Error bars designate standard deviation.

NOISeq (Fig. 2-4) demonstrate significantly different behavior between the models, suggesting that careful consideration should be made while selecting the caller to use for a given experimental dataset.

The sensitivity of DESeq (Fig. 2-3, top row) is observed to be highly dependent on $CV$, with increasing $CV$ yielding significant declines in performance. Increases in library size yield slight improvements in caller performance and DESeq performs best when a smaller portion of genes are differentially expressed. Throughout all conditions, DESeq is draconian in its control of FDR and keeps it well below the targeted value of $\alpha = 0.05$. At its best, DESeq manages to identify $\sim 15\%$ of differentially expressed genes. This is significantly higher than the $\sim 5\%$ identified by NOISeq, which also demonstrate completely inadequately control of FDR at $CV > 0.25$.

Interestingly, NOISeq demonstrates complementary strengths when compared to DESeq. Caller performance declines slightly with increasing library size and NOISeq is most sensitive at moderate $CV$ values. The percentage of differentially expressed genes has no significant impact on sensitivity, but smaller percentages result in unac-
Figure 2-3: Impact of dataset properties on DESeq sensitivity (top row) and FDR (bottom row) as a function of mean $N_{\text{lib}}$. Each point is the mean of 15 simulations. Error bars designate standard deviation.

exceptably high FDRs when $CV$ is larger (although FDR is nearly adequately controlled for 15% DE).

In order to better understand the basis of this divergent caller behavior, the relationship between fold-change due to differential expression, fold-change due to biological variation and ability of each caller to correctly identify differentially expressed genes was studied (Fig. 2-5). At small mean $CV$ values, DESeq has an improved ability to correctly identify DE genes even when biological variability acts against treatment effect. This is demonstrated by the significant portion of identified DE genes which fall in the [-1,1] and [1,-1] graphical quadrants, where effects combine destructively. Conversely, at low $CV$ values, NOISeq has a much smaller percentage of correctly identified DE genes in the destructive quadrants. As mean biological $CV$ increases, dispersion estimates within DESeq also grow and make it so treatment effects need to be reinforced by biological variation to achieve statistical significance. When mean biological variation approaches the same magnitude as treatment effect ($CV \sim 0.3$), the model is no longer adequate to identify differential expression. Conversely, NOISeq, due to its non-parametric data-driven distributions,
Figure 2-4: Impact of dataset properties on NOISEq sensitivity (top row) and FDR (bottom row) as a function of mean $N_{lib}$. Each point is the mean of 15 simulations for which $TR_s$ and $TR_n$ were set to 0.2 and 5, respectively. Error bars designate standard deviation.

maintains the ability to identify DE genes even as the $CV$ grows. At every value of $CV$ NOISEq predominantly identifies DE genes when there is reinforcing biological variation (quadrants [1,1] and [-1,-1]). Indeed NOISEq appears to rely quite heavily on biological variation to help shift DE counts even further from controls. This explains why NOISEq sensitivity increases as $CV$ grows from low to moderate values, as the benefits of reinforcing biological variation grow. However, this behavior has a functional upper limit as observed by the unacceptably rapid growth in FDR in Figure 2-4.

2.3.3 DESeq: Inclusion of multiple treatment conditions

In biological studies, it is common to have more than two levels associated with a particular experimental factor. This experimental design provides the possibility to incorporate additional data into gene-wise dispersion estimates, which, in some cases, may improve their accuracy. When these multi-treatment derived estimates are utilized in a differential expression model between two conditions of interest, they
Figure 2-5: Impact of biological variance on the ability of DESeq (top row) and NOISeq (bottom row) to correctly identify differentially expressed genes in datasets with varying biological CV (horizontal panels). Each panel presents each DE gene from 5 simulations where the mean $N_{lib} = 30$ million reads, 10% of genes were differentially expressed and $TR_s$ and $TR_n$ were set to 0.2 and 5, respectively for the NOISeq analyses.

would likely generate conservative results as the variance associated with differential expression between the multiple conditions will cause dispersion inflation. The trade-off is improved accuracy for the (assumed) larger percentage of genes that have stable expression across conditions (decreasing the likelihood of type-I error), against overly large dispersion estimates for the small percentage of DE genes (increasing the likelihood of type-II error).

To assess when inclusion of multiple treatment levels improves DESeq performance, comparisons were made when 1-5 treatment conditions (in addition to the control) were included in the dispersion estimation step (Fig. 2-6, NOISeq is not currently capable of taking advantage of such designs). This analysis was performed on datasets with varying mean biological CV and fractions of DE genes. In the lowest CV conditions, the utility of including multiple treatment conditions is seen to vary depending on the number of DE genes. When a small number of genes are differentially expressed, inclusion of multiple treatment groups improved DE caller
performance, but at high DE percentages including multiple treatment conditions reduces caller performance. Furthermore, the transition point, where inclusion of multiple treatment conditions switches between beneficial and inhibitory is seen to shift as a function of mean biological $CV$. As $CV$ increases, the inclusion of multiple treatment groups continues to improve caller performance even as the number of DE genes grows higher and higher. FDR is well controlled in every situation with the exception of a few cases when $CV = 0.3$. Summarized, comparisons between libraries with low mean biological $CV$ but large numbers of DE genes are best analyzed in a purely pair-wise fashion. However, studies in which the $CV$ is larger of the number of DE genes is small can be improved by incorporating multiple treatment conditions into the dispersion estimation step of DESeq.

![Graph showing impact of including multiple treatment conditions](image)

Figure 2-6: Impact of including multiple treatment conditions, in addition to the control group, on DESeq sensitivity (top row) and FDR (bottom row) as a function of the number of genes differentially expressed in each treatment group. Each point is the mean of 15 simulations, mean $N_{lib} = 30$ million reads. Error bars designate standard deviation.

The above insight is only useful if a researcher has insight into the mean biological $CV$ and number of DE genes within their data. Calculating the mean $CV$ is relatively straightforward but in most experimental situations there is no $a$ priori information
about the number of DE genes. In order to provide insight into the actual number of DE genes in an experiment (and therefore to better utilize Fig. 2-6), the relationship between the number of significant DE calls made by DESeq and the number of DE genes actually within a two-condition dataset was explored as a function of mean biological CV (Fig. 2-7). At small values of mean biological CV (< 0.15) there is a clear, linear relationship between the number of DE calls and actual DE genes until the caller saturates at a value dependent upon mean CV (≈ 350 DE calls for CV = 0.1 and ≈ 150 when CV = 0.15). As CV grows the correlation diminishes and fails completely by CV = 0.25.

Figure 2-7: Relationship between the number of genes differentially expressed in a dataset that meet inclusion criteria and the number of significant differential expression calls made by DESeq for multiple biological CV conditions. At low values of CV there is a linear relation through small numbers of DE calls until saturation is reached. With large CV values no useful relationship can be defined. Mean \( N_{lib} \) = 30 million reads for each point.

Combining the results from Figures 2-6 & 2-7, it is possible to make informed decisions about the benefits of including multiple treatment conditions in the dispersion estimation process. Using knowledge of the mean biological CV, calculated directly from the count data, and the number of DE calls made by DESeq for a simple pairwise comparison, it may be possible to use Figure 2-7 to estimate the actual number of DE genes resulting from treatment. Using this estimate and mean CV, Figure 2-6
can then be used to determine whether or not utilizing multiple treatment conditions for dispersion estimation will improve caller performance.

2.3.4 Pooled Samples

In some instances, such as small mRNA sample size or the need to assess many conditions in one quick analysis, it may become logistically advantageous to pool mRNA from multiple biological replicates into one sample for sequencing. In order to assess the impact that this pooling has on dataset variability and DE caller performance, we constructed synthetic datasets which contained between 1 and 10 "pooled" biological replicates for both treatment and control conditions. Pooled samples were generated by averaging the $\lambda_{i,j,k}^{LS}$ values for each condition, $k$, and using the mean value to generate an single $N_{i,k}$ for each condition. This is similar to combining multiple replicates worth of mRNA into a single tube prior to library construction and sequencing. A comparison of how multiple DE callers perform on each of these datasets is presented in Figure 2-8 (mean $N_{rib} = 50$ million reads, NOISeq parameters: $TR_s = 0.2$ and $TR_n = 5$).

Interestingly, only DESeq showed the ability to capitalize on the added information available in pooled samples. At moderate mean biological CV values ($CV = 0.3$), increasing the number of pooled replicates dramatically improves DESeq's sensitivity (with no observable change in FDR) but slightly worsens NOISeq's performance. At larger CV values, DESeq shows similar but dampened behavior while NOISeq shows minor decrements in sensitivity but tremendous suppression of FDR with the inclusion of more replicates. This behavior can be better understood by observing the effect of replicate pooling on the effective biological CV (Fig. 2-9). Pooling multiple replicates has an averaging effect such that sample count values are more tightly distributed about the baseline expression value (in effect they display reduced biological variation). This behavior is shown dramatically in Figure 2-9 for two different mean biological CV values (between replicates) as a function of the number of pooled samples. Pooling of up to 10 biological replicates is observed to produce effective CV values that are less than half of the actual biological levels.
Figure 2-8: The impact of pooling mRNA from multiple biological replicates into a single sample on the sensitivity (top row) and FDR (bottom row) of multiple differential expression callers (colored lines) at two levels of biological variance (CV) and % differential expression (vertical panels). Each point is the mean of 15 simulations, mean $N_{lib} = 50$ million reads. Error bars designate standard deviation.

The dramatic reductions in effective dataset CV explain both the observed improvement in DESeq performance and the reductions in NOISeq performance. As noted above, NOISeq benefits greatly when moderate biological variation reinforces DE, leading to increased count differences. The effective reduction in CV resulting from sample pooling serves to reduce the magnitude of this reinforcement by biological variation. Pooling does, however, significantly reduce the FDR in cases of large biological CV. As demonstrated in Figure 2-3, DESeq performs best when biological variation is minimized, hence the effective reduction from pooling leads to much higher caller sensitivities. The Poisson-Bayes model[13] for DE detection performs poorly in all situations and struggles to make any significant detections after $p-$values are BH corrected. This result is particularly interesting because this model is believed to accurately model the variance associated with technical replication but lack the ability to properly handle biological replication. Since sample pooling, in essence, reduces biological variability, one would expect that this more straightfor-
ward model would perform better as the degree of pooling increases. However, that result is not observed in this experiment due largely to the inability of the model to define $p$-values capable of withstanding rigorous multiple hypothesis correction. The fraction of DE genes in the dataset has minimal impact on the effects of pooling for any caller.

Figure 2-9: The effect of sample pooling on the observed mean variation between biological replicates ($CV$) as a function of the number of pooled biological replicates for each treatment condition. Each point is the mean of 15 simulations, mean $N_{16} = 50$ million reads. Error bars designate standard deviation (too small to visualize).

2.4 Conclusion

Performance of the NOISeq differential expression caller on a given experimental dataset can be improved by careful selection of normalization method and parameters associated with the simulated replicates. In experiments with low biological variability, $CV \leq 0.2$, the size factor normalization strategy works best. However, FDR grows rapidly with this strategy as $CV$ increases and reads per million becomes a more effective procedure. The NOISeq parameter governing modeled replicate size was observed to produce the best results with small values ($TR_s = 0.1 - 0.2$) while a
large number of simulated replicates ($TR_n = 5 - 8$) was beneficial.

Comparison of the two most reported differential expression callers which are capable of analyzing non-replicated experiments demonstrated dataset dependent optimization. DESeq is recommended for experiments with $CV \leq 0.2$ while NOISeq appears to perform better for $CV > 0.2$. Neither caller makes reliable DE calls when $CV \geq 0.3$.

In certain cases, DESeq performance can be improved by including multiple treatment conditions into the dispersion estimation step. Whether or not this is beneficial is a function of the mean $CV$ as well as the number of DE genes in the data sets. The first value can be approximated in a straightforward manner from the count libraries and the second may sometimes be estimated from the results of a preliminary DESeq analysis. Inclusion of multiple treatment conditions is recommended for datasets with moderate biological variability and small numbers of differentially expressed genes.

Pooling of biological replicates into one sample is an effective procedure to improve the performance of DESeq in situations with moderate to high biological variability and resource limitations that encourage minimizing the number of distinct samples processed by the sequencer. This procedure is not as powerful as maintaining separate replicate identity during sequencing but constitutes a significant improvement over analyzing only one sample per condition.
Chapter 3

Changes in cochlear transcriptional activity associated with noise-induced primary neuronal degeneration

Abstract

Recent studies on the long-term impact of noise exposure have produced startling revelations concerning the health of the auditory nerve. Acoustic exposures previously believed to cause only temporary reductions in hearing sensitivity have been shown, in rodent models, to cause delayed death of the neurons that compose the auditory nerve. This phenomenon, noise-induced death of the auditory nerve without previous loss of sensory or supporting tissues, is known as noise-induced primary auditory neuropathy, and has important clinical implications. While pure-tone audiograms appear normal, the reduction in total neurons (effective information channels) leads to reduced ability to effectively process complex acoustic stimuli.

To gain insight into the genetic mechanisms underlying the slowly progressive degeneration of spiral ganglion neurons we used high-throughput RNA sequencing to characterize the transcriptional activity within micro-dissected murine spiral ganglia and sensory epithelia at 24 hours, 2 weeks, 2 months and 16 months after exposure to neuropathic and non-neuropathic noise. Transcripts differentially expressed as a result of neuropathic exposure were identified using DESeq analysis software. Annotation, network and transcription factor enrichment studies were performed, using IPA, to uncover the biological significance of differential expression and identify target transcripts for future studies. We demonstrate that many genes typically associated
with immune cell maturation and activity are differentially regulated in response to neuropathic exposure, but that the origin of this differential expression is within the cells of the spiral ganglion, suggesting that the peripheral nervous system may be presenting immune system-like functionality in response to neuropathic trauma.

3.1 Introduction

Hearing loss affects 17% of Americans older than 20 years and 47% of those 75 or older[115]. Of these, an estimated 26 million individuals have high-frequency hearing loss resulting from traumatic noise exposure. Compounding upon the observed decline of hearing organ performance with age (presbycusis), recent work has unexpectedly demonstrated that exposure to moderate intensity noise, previously believed to cause only temporary threshold shifts (TTS), can initiate permanent and irreversible degeneration of the auditory nerve, characterized by declines in spiral ganglion (SG) neuron counts[86]. This phenomenon, noise-induced death of the auditory nerve without the accompanying loss of sensory hair cells or supporting tissues, is known as noise-induced primary auditory neuropathy (NI-PAN). NI-PAN is clinically important but has gone unrecognized until recently because pure-tone audiograms, the main diagnostic hearing tests, appear normal in affected individuals. Diminished neural populations are still capable of relaying information for sensitive detection of tones in quiet, but the reduction in total neurons (effective information channels) leads to reduced ability to process complex acoustic stimulation[86]. This impairment is detectable clinically in the form of depressed word recognition scores[68, 69, 66] and is commonly observed in people who can communicate robustly in quiet environments but are severely impaired in noisy settings. In animal models, NI-PAN is physiologically characterized by TTS and a decrease in the wave I amplitude of the auditory brainstem response (ABR) but no accompanying decrement in distortion product otoacoustic emission (DPOAE) magnitudes. The ABR is an electrical waveform produced by the summed neural response to a tone presentation. As sensory information travels from the periphery to the brain, surface electrodes can detect a characteristic waveform with multiple peaks created to the firing of several auditory nuclei. The
first peak of this waveform, wave I, results from the summed firing of the SG neurons, therefore the peak-to-peak amplitude of wave I provides a metric of the relative size of the functional neural population. DPOAEs, the second primary physiologic hearing test used in animal work, assesses the functionality of the middle ear and cochlear amplifier by measuring the magnitude of a distortion tone generated within the ear in response to stimulation with two pure-tones. Because the tissues responsible for DPOAE generation occur upstream of the SG neurons within the hearing pathway, DPOAE magnitudes recover to normal levels after NI-PAN inducing trauma.

An important feature of NI-PAN is the long time course over which neural death occurs. While decrements in neural response waveforms appear immediately after exposure (only observable once TTS has cleared), SG cell bodies do not begin to degrade for many months in mice \cite{86} (equivalent to years in humans). To understand molecular mechanisms underlying this slow neurodegeneration, we have studied genome-wide transcriptional changes in cochleae exposed to neuropathic noise, using \textit{Whole Transcriptome Shotgun Sequencing}, more commonly referred to as RNA-Seq. As controls, we used age-matched unexposed cochleae and cochleae exposed to non-neuropathic noise. By studying transcriptional changes from 24 hours to 16 months after noise exposure, we have identified novel genetic and immunological mechanisms underlying NI-PAN. Our results suggest future therapeutic approaches to ameliorate communication disorders induced by long-term neural decline.

\section*{3.2 Methods}

\subsection*{3.2.1 Animal Models}

Six week old, male CBA/CaJ mice were exposed to 8-16 kHz noise for 2 hours at neuropathic 97 dB SPL to cause NI-PAN, characterized by a TTS (Fig. 3-1) accompanied by a statistically significant decrement in ABR wave I amplitude (Fig. 3-2), or at non-neuropathic 94 dB SPL (characterized by a TTS with no permanent ABR wave I reduction). ABR and DPOAE responses were measured at 6 hours, 2 weeks,
4 weeks, 10 months and 16 months after exposure. Quantification of neuronal loss via histological counting of functional ribbon synapses within IHCs has confirmed neural damage (Fig. 3-4). All assessments were compared against unexposed male littermates. All procedures were performed under protocols approved by the MEEI Animal Care Committee.

![Graph showing ABR and DPOAE threshold shifts](image)

**Figure 3-1:** ABR (left column) and DPOAE (right column) threshold shifts resulting from 100 dB SPL (top row), 97 dB SPL (neuropathic exposure, center row) and 94 dB SPL (non-neuropathic exposure, bottom row). Both 97 and 94 dB SPL induce TTS while 100 dB SPL causes a permanent threshold shift.

### 3.2.2 Cochlear Microdissection and RNA Sequencing

Animals were exposed at 6 weeks of age and RNA harvested 24 hours, 2 weeks, 2 months and 16 months after exposure. For each time point and exposure group, basal cochlear mRNA was collected and pooled from 10 male mice (20 ears). The basal turns were specifically targeted because they form the high-frequency sensitive portion of the auditory organ, the portion which shows more pronounced decline with
Figure 3-2: ABR wave I peak-to-peak amplitude growth functions recorded at 6 hours, 2 weeks, 4 weeks, 10 months and 16 months after exposure to 97 (top row), 94 (center row) and 100 dB SPL (bottom row) noise for 2 hours. The left column presents the growth function in response to 11.3 kHz tones and the right column presents the response to 32 kHz tones. ABR amplitudes are significantly diminished at both frequencies in the 100 dB SPL exposure case and statistically normal in the 94 dB SPL condition. In the 97 dB SPL condition, ABR amplitudes are normal at the 11.3 kHz location but ~ 30% decrements are seen in the response amplitude at the 32 kHz location immediately following noise exposure. At later time points, the difference between the exposed and unexposed animals diminishes; this is due to more rapid age-associated decline of signal response in the control population.

age and the acoustic exposure parameters used. Cochleae were surgically excised from the skull and microdissected in RNAlater (Ambion). The otic capsule and lateral wall were removed, leaving behind the Organ of Corti (OoC) and modiolus, of which the apical turns were removed to leave only the basal (high-frequency) region (Fig. 3-
Figure 3-3: DPOAE growth functions recorded at 6 hours, 2 weeks, 4 weeks, 10 months and 16 months after exposure to 97 (top row), 94 (center row) and 100 dB SPL (bottom row) noise for 2 hours. The left column presents the growth function in response to 11.3 kHz tones and the right column presents the response to 32 kHz tones. The 100 dB SPL condition significantly reduces response magnitude but the 94 and 97 dB SPL exposures cause no permanent reductions.

5). Total cell RNA was purified using RNeasy spin columns (Qiagen)(process same as [152]). Cochlear mRNA was purified using a polyA selection, converted into a normalized cDNA library and sequenced on an Illumina platform (as described in [40]). Reads were aligned with the mouse genome and corrected for intron splicing using the Bowtie[92] and TopHat[165] algorithms.
Figure 3-4: Average number of ribbon synapses observed on the inner hair cells at the 11 kHz (left column) and 32 kHz (right column) characteristic places following exposure to 97 (top row) and 94 dB SPL noise (bottom row). While the 94 dB SPL exposure caused not significant losses relative to unexposed control mice, the 97 dB SPL exposure induced ~30% declines in the number of functional synapses at the 32 kHz location. Smaller declines were also observed in the 11 kHz location at later time points.

Figure 3-5: Schematic representation of cochlear microdissection prior to RNA collection. Cochleae were dissected from the skull base (A). The otic capsule was removed (B), followed by removal of the tissues of the lateral wall (C). Finally, the apical turns were removed (D), leaving only the basal Organ of Corti and modiolus.

### 3.2.3 Differential Expression Analysis

The differential expression caller *DESeq*[6, 8] was utilized to identify genes with altered expression in response to sound. By implementing the negative binomial dis-
tribution and sharing dispersion information across genes, this package is capable of inferring differential expression between samples in multi-factor, multi-condition experiments with minimal biological replication.

To account for unequal library sizes, the count data were normalized using the size factor technique native to the `DESeq` platform. This method determines the median normalized count ratio for each sample and uses that to balance the sample read distributions. This technique has been shown to be more robust than normalizing by library size because total RNA-Seq counts can be highly influenced by a small number of transcripts[6].

While lack of biological replication is not uncommon in RNA-Seq analyses[23], particular care must be taken to maximize confidence in model-based inferences. Lack of replication constrains the method that can be used to predict gene-wise variance, and thus impacts model inferences of differential expression. Dispersion estimates had to be calculated blind to sample condition, pooling multiple samples into a single quasi-state and estimating dispersions (combined technical, biological and experimental) from the entire sample population. Since multiple sample conditions are being grouped this method is prone to inflating dispersion estimates and making conservative predictions of differential expression. However, because the number of differentially expressed genes is expected to be small relative to the total number, this inflation should not be substantial.

After dispersion estimation, the data set was filtered to remove any identifier that did not have at least 10 reads within a single sample. This step removes targets for which there is insufficient evidence to make a confident determination of differential expression. To identify genes differentially regulated due to neuropathic exposure at each time point, the count libraries for each corresponding pair of neuropathic and non-neuropathic samples was passed to `DESeq`’s negative binomial model along with the gene-wise dispersion estimates. P-values returned by the model were adjusted using the Benjamini-Hochberg method to control the false discovery rate, $\alpha = 0.05$. 

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3.2.4 Enrichment Discovery and Comparison to Previous Work

Genes determined to be differentially expressed between exposure groups were analyzed for enrichment of annotation terms, molecular networks, pathways and transcription factors using Ingenuity Pathway Analysis (IPA, Ingenuity® Systems, www.ingenuity.com). IPA is a bio-molecular interactions database, curated from the literature, that detects annotation enrichment within submitted target lists via the Fisher Exact test. It is also capable of identifying pathways and constructing molecular networks based on input genes and assessing the significance of their up- or down-regulation based on the fold-changes of the constituent members. Nodal molecules and highly connected secreted molecules from high scoring IPA networks ($p$-value $\leq 10^{-15}$) were identified to expand the list of potential biomarkers and key molecular players. Differentially expressed genes identified in this study of NI-PAN trauma were compared with gene lists curated from the literature for PTS and TTS exposure models to highlight key similarities and differences in the ear's response to insult.

3.2.5 qPCR

qPCR validation of target genes, identified through RNA-Seq, was carried out on an Applied Biosystems StepOnePlus real-time PCR system. mRNA samples from neuropathically and non-neuropathically exposed mice at each of the 4 time points used in the RNA-Seq analysis were also studied with qPCR. Primers were acquired from Life Technologies (Grand Island, NY) for the following genes: *ApoE* (Mm01307193_g1), *Ccl2* (Mm00441242_m1), *Cebpb* (Mm00843434_s1), *Cebpe* (Mm02030363_s1), *Cxcl10* (Mm00445235_m1), *Dbp* (Mm00497539_m1), *Elane* (Mm01168928_g1), *Gata1* (Mm01352636_m1), *Mmp8* (Mm00439509_m1), *Mmp9* (Mm00442991_m1), *Nr4a1* (Mm01300401_m1) and *18s* (Hs99999901_s1).

3.2.6 Immunohistochemistry

Exposed mice were deeply anesthetized with ketamine (100 mg/kg) and xylazine(20 mg/kg) and intracardially perfused with 4% paraformaldehyde (PFA). Cochleae were
exposed, the round and oval windows punctured and intracochlearly perfused before extraction and fixation for 2 hours in 4% PFA on a shaker. Cochleae were decalcified in EDTA for 3 days on a shaker, dehydrated with an alcohol ladder, embedded in paraffin and cut in 10μm sections. Cochlear sections were de-paraffinized in Histoclear (2X - 6 mins), re-hydrated with an alcohol ladder (100%, 95%, 75%, 50%, 2X - 1 min), exposed to DAKO target retrieval solution (Dako North America, Carpinteria, CA) at 95°C for 30 min for heat-mediated antigen retrieval, and allowed to cool for 20 min before rinsing in dH2O and sterile PBS. Slides were blocked for 24 hours on a shaker at 4°C in 5% NHS with 1% Triton-X in sterile PBS. Primary antibodies (rabbit anti-CEBPE: Santa Cruz Biotech. [sc-158, 1:25]; rabbit anti-PPBP: Bioss [bs-2550R, 1:200]; rabbit anti-S100A9: Novus Biologicals [NB110-89726, 1:100]; rabbit anti-IBA1: Wako [019-19741, 1:2000]) were applied for 24 hours at 4°C. Following washing in sterile PBS (3X - 10 mins) on the shaker, secondary antibodies were applied for 1 hour at room temperature (donkey anti-rabbit: Jackson ImmunoResearch [711-545-152, 1:200]; peroxidase-conjugated goat anti-rabbit: Jackson ImmunoResearch [111-035-003, 1:200]). For non-fluorescence staining, slides were washed and DAB solution was applied for 10 minutes. Slides were washed again and rinsed in dH2O and cover-slipped with Vecta mounting media.

3.3 Results

The library size of each sample is presented in Table 3.1. Because of the longitudinal nature of the experiments, the 24 hr, 2 wk and 2 mo samples were multiplexed together in 1 lane of the sequencer (analysis block 1) while the 16 mo samples were multiplexed together in a different sequencer run (analysis block 2). Reads were mapped onto 30,385 identifiers within the mouse genome. After normalization and filtering with the 10 count minimum, 19,953 genomic identifiers were analyzed for differential expression.

Due to the large discrepancies in library size between the two analysis blocks, gene-wise dispersion estimates were calculated for each block and passed along with
the respective count data to the negative binomial model for pairwise comparisons between neuropathic and non-neuropathic samples. The 4 comparisons identified 160 transcripts that were differentially regulated due to neuropathic exposure at at least one time point. Plots of log₂ fold change between exposure conditions versus mean normalized counts are presented in Figure 4-1 for each time point. The distribution across time points was: 24 hours after exposure - 23 significant transcripts, 2 weeks after exposure - 112, 2 months after exposure - 24 and 16 months after exposure - 72 transcripts. Five transcripts (Ear1, Ear2, Ear3, Prg2 and S100a9) were differentially expressed at all time points. Importantly, the bulk of differential expression at 2 weeks after exposure is positive and results from expression increases in the neuropathic sample, while at 16 months after exposure the differential expression is largely negative and results from expression increases in the non-neuropathic sample. Thirty-six (50%) of the transcripts differentially expressed 16 months after exposure were also differentially expressed 2 weeks after exposure, indicating that many of the changes occurring soon after neuropathic exposure are also occurring after non-neuropathic exposure, but on a significantly delayed time-scale.

### 3.3.1 Sample Similarity

To assess sample level similarity, a variance stabilizing transformation [6, 8] was applied to the normalized count data to generate a more homoscedastic data set. Principal component analysis (PCA), also known as eigenvalue decomposition, was performed on the complete set of transcripts in the analysis (Fig. 3-7, top panel) and on a reduced set containing the 160 differentially regulated transcripts (bottom panel). PCA enacts an orthogonal transformation on the dataset to define linearly uncorrelated variables, principal components, of which the first principal component
Figure 3-6: Plot of mean normalized count value versus the log₂ fold change between exposure conditions for 24 hr (top left), 2 wk (top right), 2 mo (bottom left) and 16 mo (bottom right) after noise exposure. Fold change is calculated as neuropathic relative to non-neuropathic exposure. Transcripts determined to be differentially expressed are denoted by black markers.

explains the largest source of variance in the data. In the full dataset, the first principal component is dominated by time after noise exposure while exposure condition dominates the second principal component. However, when only differentially expressed transcripts are analysed, the primary source of variance switches and exposure condition dominates the first principal component with time after exposure dominating the second. This confirms that exposure-induced differences are dominant within the differentially expressed transcript list and that DESeq is performing as expected. An interesting exception to this schema is the close similarity between the 2 weeks after exposure neuropathic and the 16 months after exposure non-neuropathic samples. This similarity was noted above, as a significant number of genes are shared between the two time points. Furthermore, of these shared genes, they are predominately up-regulated in the neuropathic 2 week sample and also up-regulated in the non-neuropathic 16 month sample, reinforcing the concept that neuropathic and non-neuropathic exposure conditions have overlapping expression changes but on vastly different time-scales.
Figure 3-7: Principal component analysis of RNA-Seq samples using all transcripts (top) and only the transcripts determined to be differentially expressed in response to neuropathic exposure (bottom). Exposure condition is the dominant explanatory variable when only differentially expressed genes are utilized.

3.3.2 Expression Profile Clustering and Transcription Factor Enrichment

Genes determined to be differentially expressed due to exposure condition were grouped, based on their fold-change expression profiles, using expectation-maximization (EM) clustering. EM clustering was utilized because its use of multivariate normal distributions to model expression clusters and its capability to autonomously identify the optimum number, size, shape and volume of expression clusters, via maximization of the Bayesian information criteria (BIC), produced higher quality clustering results with less user oversight than a k-means algorithm. The 160 transcripts identified in the exposure analysis were clustered into 7 temporal expression patterns – the profiles for each cluster are presented in the top row of Figure 4-2. These clusters reinforce the behavior seen in Figure 4-1; the dominant pattern of expression is up-regulation at 2 weeks after exposure and down-regulation at 16 month after exposure (however, this is largely due to increases in expression levels in the non-neuropathic group).

Transcription factors (TFs) relevant to each expression cluster were identified in two ways. First, Ingenuity Pathway Analysis (IPA) was used to identify TFs among the differentially expressed genes. Secondly, an IPA Core analysis was performed on each expression cluster to find enriched upstream regulators within each cluster of
similarly varying genes. The expression profiles of each identified TF are presented in the bottom row of Figure 4-2, with each TF presented below its identifying differential expression cluster. Potential regulatory TFs for each cluster were assessed by comparing expression profiles and those identified as potentially regulatory are listed in Table 3.2. Of the 13 TFs of interest, 5 occur in deafness loci with unidentified causative genes and 3 are known to play critical roles in the auditory system.

![Figure 3-8](image)

Figure 3-8: RNA-Seq determined log₂ fold changes (neuropathic relative to non-neuropathic noise) vs time after exposure for the 7 expression clusters determined by EM analysis (top row). Expression profiles of native and enriched transcription factors associated with each cluster are presented in the bottom row.

### 3.3.3 Overlap with Previous Rodent Studies

Previous studies, using a variety of different noise parameters, have identified several genes involved in the cochlear response to noise traumas that cause TTS or PTS in rodents. Although differential expression is influenced by specific noise parameters, we compared our list of differentially expressed genes resulting from NI-PAN with genes confidently implicated in PTS and TTS to gain insight into similarities and differences among these conditions. Comparing the 160 genes identified in this study with 161 transcripts implicated in PTS and 84 genes implicated in TTS, curated from the literature, we find 2 genes common to PTS and NI-PAN (*Ccl2* and *Elane*), 1 gene common to TTS and NI-PAN (*Gp5*), and 3 genes common to PTS, TTS and NI-PAN.
<table>
<thead>
<tr>
<th>TF</th>
<th>Cluster</th>
<th>Hearing Importance</th>
<th>Deafness locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cebpe</td>
<td>1,4,6</td>
<td>?</td>
<td>DFNA53</td>
</tr>
<tr>
<td>Gata1</td>
<td>1,4</td>
<td>?</td>
<td>-</td>
</tr>
<tr>
<td>Hmgb2</td>
<td>4</td>
<td>?</td>
<td>DFNA24</td>
</tr>
<tr>
<td>Nfe2</td>
<td>4</td>
<td>?</td>
<td>-</td>
</tr>
<tr>
<td>Spib</td>
<td>4</td>
<td>?</td>
<td>-</td>
</tr>
<tr>
<td>Irf7</td>
<td>6</td>
<td>?</td>
<td>DFNA32</td>
</tr>
<tr>
<td>Cebpb</td>
<td>7</td>
<td>Regulates Prestin[63]</td>
<td>DFN6B5</td>
</tr>
<tr>
<td>Churc1</td>
<td>7</td>
<td>?</td>
<td>-</td>
</tr>
<tr>
<td>Dnttip1</td>
<td>7</td>
<td>?</td>
<td>DFN6B5</td>
</tr>
<tr>
<td>Hdac2</td>
<td>7</td>
<td>Role in avian supp. cells[150]</td>
<td>-</td>
</tr>
<tr>
<td>Med30</td>
<td>7</td>
<td>?</td>
<td>-</td>
</tr>
<tr>
<td>Nr4a1</td>
<td>7</td>
<td>Response to noise trauma[38]</td>
<td>-</td>
</tr>
<tr>
<td>Taf5</td>
<td>7</td>
<td>?</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.2: Enriched and differentially expressed transcription factors with potentially important regulatory roles in the ear’s response to neuropathic exposure.

(Cebpb, Cxcl10 and Nr4a1).

### 3.3.4 Biological Significance of Differential Expression

IPA core analyses were conducted a second time on the 160 differentially expressed transcripts, this time analyzing all differentially expressed transcripts together, at each time point, thus providing functional insight into the temporal evolution of expression changes. The IPA *Comparison Analysis* tool was used to compare the results of each core analysis to better characterize the temporal nature of neuropathically induced changes (Fig. 4-3). The predicted biological consequences of expression changes are overwhelmingly dominated by functions involved in the maturation, movement and activity of immune cells (leukocytes, phagocytes, myeloid cells, neutrophils, eosinophils, granulocytes, etc.). These functions are predominantly up-regulated 2 weeks after exposure to neuropathic noise and are significantly down-regulated, relative to non-neuropathic specimens, 16 months after exposure. The exceptions to this pattern are related to response to infection and pathways involved in organismal death. The top enriched toxicology functions include several renal pathologies, which is interesting in light of the similarities between the kidney and inner ear. These functional predictions strongly implicate immune cell invasion and phagocytosis or
the activation of pathways typically associated with immune cells within inner ear tissues.

IPA analysis of the differentially expressed genes demonstrated exceptionally strong enrichment of 5 TFs (*MKL2, MKL1, SRF, CEBPE, CEBPA*). None of these TFs have
been implicated in hearing. However, \textit{MKL1} and \textit{MKL2} are known to be regulated via Wnt signaling and require the presence of \textit{SRF} to bind DNA\cite{34, 141}. \textit{SRF} occurs within the DFNA21 locus and \textit{MKL1} within DFNB40. \textit{CEBPE} is located within the DFNA53 locus. The \textit{Cebpe} promoter sequence was studied using TFSEARCH\cite{4}; 42 high-scoring sites were identified including one highly conserved \textit{Gata-1} binding site. \textit{Gata-1} has an expression profile which is very similar to \textit{Cepbe} and has binding sites in a many of the differentially expressed genes. No auditory phenotype has been linked to \textit{Gata-1} and the KO mouse is embryonic lethal\cite{59}. IPA was also used to identify nodal network molecules (Tab. 3.3), which may play critical biological roles, and highly connected extracellular molecules (Tab. 3.4), which may be useful biomarkers of network activity.

3.3.5 Validation and Localization

From the above analyses, 9 transcripts of interest were selected for qPCR validation (Fig. 4-5, error bars present standard error of the means, \( n \geq 3 \)). Of the 9 genes tested, 4 (\textit{Ccl2, Cebpe, Cxcl10} and \textit{Nr4a1}) were confirmed to be differentially expressed due to noise exposure at at least one time point (\( p\)-value < 0.05). This validation rate is lower than has been observed in well-replicated RNA-Seq experiments (> 80\%\cite{12, 62}), but is quite high given the non-replicated design, owning to the quasi-replication effect of pooling of 20 ears into each sequenced sample. Overall, this provides increased confidence in the RNA-Seq results but still encourages caution when interpreting any non-replicated data-set.

To determine if macrophage invasion of the cochlea is responsible for the observed changes in transcriptional activity, immunohistochemistry was performed on murine cochlear sections using an antibody against the macrophage marker, IBA1 (Fig. 4-7). At 24 hours post exposure (Fig. 4-7A-C), specimen from all three exposure groups have positive staining within the stria vascularis and in the region of type-IV fibrocytes. However, the two exposed conditions have additional positive immunoreactivity within the region of spiral ganglion cell bodies and modiolus, which the unexposed ears lack. Furthermore, light, but positive staining is uniquely observed in neuro-
Table 3.3: Nodal molecules identified in significant IPA networks ($p$-value $\leq 10^{-15}$).

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Time After Exp.</th>
<th>Diff.</th>
<th>Expressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccl2</td>
<td>24 hr</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>Mmp9</td>
<td>24 hr, 2 wk</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>Erk1/2</td>
<td>24 hr, 2 wk</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Collagens</td>
<td>2 wk</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>Cebpb</td>
<td>2 wk</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>II1</td>
<td>2 wk</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Akt</td>
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<td>N</td>
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</tr>
<tr>
<td>Srf</td>
<td>2 wk</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Mkld</td>
<td>2 wk</td>
<td>N</td>
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</tr>
<tr>
<td>Ca2</td>
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<td>N</td>
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</tr>
<tr>
<td>App</td>
<td>2 wk</td>
<td>N</td>
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</tr>
<tr>
<td>Ubc</td>
<td>2 wk</td>
<td>N</td>
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</tr>
<tr>
<td>Nfkb</td>
<td>2 wk, 16 mo</td>
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</tr>
<tr>
<td>P38mapk</td>
<td>2 wk</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Tnf</td>
<td>2 mo</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Cxcl10</td>
<td>2 mo</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>Erk1/2</td>
<td>2 mo</td>
<td>N</td>
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<td>Hgb3</td>
<td>16 mo</td>
<td>Y</td>
<td></td>
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<tr>
<td>Cybb</td>
<td>16 mo</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>Mmp8</td>
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Cluster specific nodes

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<tr>
<td>beta estradiol</td>
<td>6</td>
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</table>

Table 3.3: Nodal molecules identified in significant IPA networks ($p$-value $\leq 10^{-15}$).

Pathic specimens in the region where spiral ganglion fibers exit Rosenthal’s canal (Fig. 4-7A). By 2 weeks post exposure, macrophages appear to vacate the region near the end of Rosenthal’s canal in the neuropathic group and both noise-exposed groups present similar staining in the spiral ligament, modiolus and region surrounding SG cell bodies (Fig. 4-7D). This staining is largely consistent as far out at 16 months post exposure with the exception of decreased signal in the region of type-IV fibrocytes (Fig. 4-7E).
Table 3.4: Highly connected extracellular molecules identified within significant IPA networks ($p$-value $\leq 10^{-15}$).

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<tr>
<th>Molecule</th>
<th>Time After Exp.</th>
<th>Diff. Expressed</th>
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<td>Cc12</td>
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<td>Y</td>
</tr>
<tr>
<td>Mmp9</td>
<td>24 hr, 2 wk</td>
<td>Y</td>
</tr>
<tr>
<td>Elane</td>
<td>24 hr</td>
<td>Y</td>
</tr>
<tr>
<td>Mmp8</td>
<td>2 wk</td>
<td>Y</td>
</tr>
<tr>
<td>Colla1</td>
<td>2 wk</td>
<td>Y</td>
</tr>
<tr>
<td>IgG</td>
<td>2 wk</td>
<td>N</td>
</tr>
<tr>
<td>Tnf</td>
<td>2 mo, 16 mo</td>
<td>N</td>
</tr>
<tr>
<td>Epo</td>
<td>2 mo</td>
<td>N</td>
</tr>
<tr>
<td>Ppbp</td>
<td>2 mo</td>
<td>Y</td>
</tr>
<tr>
<td>Cxcl10</td>
<td>2 mo</td>
<td>Y</td>
</tr>
<tr>
<td>Vegf</td>
<td>2 mo</td>
<td>N</td>
</tr>
<tr>
<td>C3</td>
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</tr>
<tr>
<td>Hp</td>
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<tr>
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</tr>
<tr>
<td>Il4</td>
<td>16 mo</td>
<td>N</td>
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</table>

Figure 3-10: qPCR measured fold changes in gene expression (neuropathic exposure relative to non-neuropathic). 4 of 9 tested genes were validated as differentially expressed ($p$-value $< 0.05$, marked with asterisks). Error bars present standard error of the means.

To test robustness of the RNA-Seq data, three additional targets were selected for cochlear immunolocalization from our list of differentially expressed transcripts because they occur within known deafness loci (CEBPE: DFNA53; PPBP: DFNA24; S100A9: DFNA7, DFNA49). All three target proteins are expressed by SG cells.
Figure 3-11: Cochlear immunostaining for the macrophage marker IBA1. (A) At 24 hours post exposure to neuropathic noise positive staining is observed in the regions of type-IV fibrocytes, stria vascularis, SGNs (inset D), modiolus and exit of Rosenthal’s canal (inset). (B) Tissues from mice exposed to non-neuropathic noise demonstrate similar staining, but no reactivity was observed within Rosenthal’s canal. (C) Unexposed specimen have staining restricted to the stria and spiral ligament. (D) At two weeks post exposure and (E) 16 months post exposure both exposed groups have similar staining patterns. (F) Negative control (processed without primary antibody) demonstrates that latent peroxidase activity is not responsible for the observed staining.

(Fig. 3-12A-C), validating our neuropathic model. In addition, S100A9 is also expressed in the inner sulcus and Organ of Corti.

### 3.4 Discussion

The observed localization of CEBPE, PPBP and S100A9 to the area of the SG neuronal cell bodies and schwann cells, reinforces our belief that the model is primarily a neuronal phenotype and that observed differential expression is being dominated by expressional changes in the SG. Furthermore, of the four deafness loci inclusive
of these genes, both DFNA7[49] and DFNA53[178] present phenotypes reminiscent of progressive damage associated with accumulated noise trauma in that they are progressive, post-lingual, high-frequency hearing losses that eventually spread to all frequencies. DFNA24[64] is a pre-lingual, non-progressive high-frequency hearing loss while DFNA49[107] is a progressive, post-lingual, mid-frequency loss.

All three of our immunolocalized targets have known roles in the immune system. PPBP is a chemokine with a critical role in neutrophil activation[30] and S100A9 is part of a complex that controls myeloid cell function and acts upstream of TNFα[171]. CEBPE, which repeatedly appeared in the TF search and its differential expression was validated with qPCR, also plays a critical immune cell role. In myeloid cells, Cebpe is pro-apoptotic (via down-regulation of anti-apoptotic Bcl-2 and Bcl-x), it halts cell cycle progression and is essential for differentiation and maturation of granulocyte progenitors[112]. Cebpe knockout mice can not generate functional neutrophils and eosinophils[177] (unfortunately, their hearing has not been studied).

While there appear to be changes in macrophage localization in response to noise exposure, the very minor differences between the responses to neuropathic and non-neuropathic stimuli do not support the hypothesis that immune cell invasion of the ear is responsible for the RNA-Seq observations. This damage mechanism therefore appears different than the many documented cases of immune cell invasion of the ear in response to more damaging noise exposures[57, 73, 136, 137]. Combined with
the clear CEBPE, PPBP and S100A9 reactivity in cells of the SG, these results suggest that part of the peripheral nervous system is taking on an immune cell like role. The ability of neural tissues to adopt immune system roles has been observed in the literature and it is becoming increasingly apparent that the nervous system can utilize pathways canonically associated with the immune system to regulate its own tissues[22, 89, 138, 139]. Interestingly, complement component 3 (C3), a critical component of developmentally phagocytic microglia, and the α-subunit of its receptor – integrin α-M (Itgam) – were found to be differentially expressed in our study. The β-subunit of C3’s receptor – integrin β-2 (Itgb2) – has a similar fold change expression profile but did not reach statistical significance.

To gain insight into the mechanism by which neuronal cell death is progressing, we analyzed the RNA-Seq fold change expression profiles of genes which have previously been associated with distinct cell death pathways. Genes associated with autophagy, paraptosis and multiple aspects of apoptosis were identified from the literature[29, 185] and their expression profiles are presented in Figure 4-10. Several genes important in apoptosis appear to have exposure dependent expression changes while genes important in autophagy and paraptosis have expression profiles that are largely independent of exposure condition. This suggests that some genes involved in apoptotic death may be playing a role, however, none of these changes were found to be statistically significant by DESeq so more evidence is needed to clearly identify the mechanism of cell death.

We present the first description of the dynamic changes in genome-wide transcriptional activity of the murine cochlea for up to 16 months following NI-PAN inducing noise exposure during adolescence. Enriched pathways and transcriptional regulators have been identified, some of which have known roles in the auditory system while others have not been described in the ear. Many of these previously undocumented TFs occur within known deafness loci and are particularly interesting due to the master roles they may play in cochlear neuropathology and immune function. Immunostaining results suggest that, while many of the transcripts identified are canonically associated with the immune system, they localize to cells of the spiral ganglion. These
Figure 3-13: RNA-Seq determined log$_2$ fold changes in expression (neuropathic relative to non-neuropathic noise) of genes associated with different cell death pathways as a function of time after exposure. Members of the apoptosis pathway showed the most variability; however, none of these fold changes were statistically significant.

Findings have important implications for understanding the ear’s response to damage and will help inform future development of targeted therapies to prevent or reverse neuro-degeneration.
Chapter 4

Changes in cochlear transcriptional activity associated with aging

Abstract

Hearing loss effects 17% of Americans older than 20 years and increases in prevalence as a function of age to effect 47% of those 75 or older. This decline of auditory organ performance with age is clinically termed presbycusis, a condition which imposes a tremendous communication burden on, and severely impacts the quality of life for millions of individuals. The increased incidence with age results from many factors, including: accumulation of traumatic exposures to noise, and long-term deterioration of an organ that does not spontaneously regenerate.

To gain insight into the genetic mechanisms underlying the slowly progressive loss of hearing associated with aging, we used high-throughput RNA sequencing to characterize the transcriptional activity within micro-dissected spiral ganglia and sensory epithelia in 6 weeks old, 8 weeks, 15 weeks and 17.5 months old mice. Transcripts differentially expressed as a result of aging were identified using DESeq analysis software. Annotation, network and transcription factor enrichment studies were performed, using Ingenuity Pathway Analysis (IPA), to uncover the biological significance of differential expression and identify target transcripts for future biomarker and therapeutic studies. We demonstrate that many genes typically associated with immunological roles are differentially regulated within the ear as it ages, but that the origin of this differential expression is not invading immune cells. Furthermore, we found substantial overlap between genes which respond to noise trauma and genes which alter expression due to aging.
4.1 Introduction

Hearing loss affects 17\% of Americans older than 20 years and increases in prevalence as a function of age to affect 47\% of those 75 or older\[115\]. The decline of auditory organ performance with age, independent of damage incurred via acute trauma, is clinically termed *presbycusis*; a condition which imposes a tremendous communication burden on and severely impacts the quality of life for millions of individuals. Cells of the adult mammalian inner ear do not spontaneously regenerate. For the auditory system to remain functional, the delicate mechanosensory structures must therefore be robust against prolonged accumulation of damage.

To make progress in clinical intervention, understanding the biological mechanisms involved in organ homeostasis and aging is critical to successfully develop treatment strategies to address this debilitating pathology. We sought to identify relevant genetic changes by performing transcriptional characterizations of the cells of the murine cochlear sensory epithelia and spiral ganglion longitudinally from adolescence to old-age. Whole-transcriptome shotgun sequencing (RNA-Seq) and bioinformatic tools were utilized to identify differentially expressed genes throughout the time-course of aging and attribute biological significance to these expression changes. To the best of our understanding, RNA-Seq had not been undertaken in the mature and aging ears of healthy mice. While a great deal of research has been focused on the transcriptional characterization of developing auditory structures (see review\[70\]), the field still lacks genome-wide understanding of transcriptional behavior important to the mature and aging organ. By studying transcriptional changes from adolescence to old age, we have identified novel genes underlying organ decline. Some genes previously associated with response to noise trauma have also been found to play roles in inner ear aging and may offer therapeutic targets to combat communication disorders.
4.2 Methods

4.2.1 RNA Collection and Differential Expression Analysis

For each of the 4 age samples, 6 weeks old, 8 weeks, 15 weeks and 17.5 months (chosen to match the post-exposure times within the NI-PAN study), the basal turns of 10 male CBA/CaJ mice (20 ears) were micro-dissected as described previously (Chapter 3). The high-frequency portion of the cochlea was targeted because it shows more pronounced decline with age and the acoustic exposure parameters used in the NI-PAN study. All procedures were performed under protocols approved by the MEEI Animal Care Committee.

Sequencing, read alignment and count analysis were performed as described previously (Chapter 3). Briefly samples were sequenced on an Illumina platform and reads were aligned with the mouse genome using Bowtie[92] and TopHat[92]. DESeq[6, 8], a differential expression caller available through Bioconductor, was used to test for differential expression between age conditions. In order to account for the lack of separately sequenced replicates (RNA samples from biological replicates were pooled prior to sequencing), dispersion estimates were calculated blind to sample condition, with all 4 samples pooled into one quasi-state. This causes DESeq to behave conservatively, as condition pooling inflates dispersion estimates for genes with true differential expression. However, the small number of differentially expressed genes, relative to the total, should minimize this effect. The library data was filtered to remove any transcript which did not have at least 10 reads within a single sample. DESeq determined significance values of differential expression were adjusted for multiple hypothesis testing using the Benjamini-Hochberg (BH) correction to control the false discovery rate, $\alpha = 0.05$.

4.2.2 Enrichment Discovery and Comparison to Previous Work

Transcripts found by DESeq to have statistically significant changes in expression between time points were uploaded to Ingenuity Pathway Analysis (IPA, Ingenuity®)
Systems, www.ingenuity.com) to investigate the biological significance of the observed
differential expression. Enriched annotation terms, molecular networks, pathways and
transcription factors (TFs) as well as nodal molecules and highly connected secreted
molecules were identified to generate a list of potential biomarkers and key molecular
players. Transcripts identified in this study of inner ear aging were compared with
gene lists curated from the literature for PTS and TTS exposure models as well
as the transcripts identified in our previous work studying a model of noise-induce
primary auditory neuropathy (Chapter 3) in order to identify common pathways
across multiple modes of auditory degeneration.

4.2.3 qPCR

mRNA samples from 6 weeks old, 8 weeks, 15 weeks and 17.5 months old mice were
used for qPCR analysis. Eight different target genes, seven of which were identi-
fied as differentially expressed by RNA-Seq, were selected for expression validation
(Cebpe was not determined to be differentially expressed by RNA-Seq, but is impli-
cated in the TF study and was the most interesting target from the NI-PAN work
(Chapter 3)). qPCR analysis was performed on an Applied Biosystems StepOnePlus
real-time PCR system. Primers were procured from Life Technologies (Grand Is-
land, NY) for the following genes: Apoe (Mm01307193_g1), Cebpe (Mm02030363_s1),
Dbp (Mm00497539_m1), E2f2 (Mm00624964_m1), Elane (Mm01168928_g1), Gata1
(Mm01352636_m1), Mmp2 (Mm00439498_m1) and Nr4a1 (Mm01300401_m1).

4.2.4 Immunohistochemistry and In situ Hybridization

Aged mice were perfused, and their cochleae fixed in 4% paraformaldehyde (PFA),
as described previously (Chapter 3). After decalcification and dehydration, cochleae
were embedded in paraffin and cut in 10\(\mu\)m sections. Slides were de-paraffinized
in Histoclear (2X - 6 mins), re-hydrated with an alcohol ladder (100%, 95%, 75%,
50%, 2X - 1 min), exposed to DAKO target retrieval solution (Dako North America,
Carpinteria, CA) at 95°C for 30 min for heat-mediated antigen retrieval, and allowed
to cool for 20 min before rinsing in dH$_2$O and sterile PBS. Slides were blocked for 24 hours on a shaker at 4°C in 5% NHS with 1% Triton-X in sterile PBS. Primary antibodies (rabbit anti-IBA1: Wako [019-19741, 1:2000, 24 hours at room temp]; goat anti-NR4A1: Santa Cruz Biotech. [sc-7013, 1:100, 24 hours at 4°C]; rabbit anti-MMP2: Novus Biologicals [NB200-193, 1:500, 24 hours at 4°C]) were applied. Following washing in sterile PBS (3X - 10 mins) on the shaker, a secondary antibodies were applied for 1 hour at room temperature (peroxidase-conjugated goat anti-rabbit: Jackson ImmunoResearch [111-035-003, 1:200]; donkey anti-goat: Invitrogen [A21432, 1:1000]; donkey anti-rabbit: Invitrogen [A21206, 1:1000]). For non-fluorescence staining, slides were washed and DAB solution was applied for 10 minutes. Slides were washed a final time, rinsed in dH$_2$O and cover-slipped with Vecta mounting media. In situ hybridization for Ngp and Prg2 was performed on cochlear sections as previously described[80]. The probe against Ngp spanned nucleotides 23-326 of the mRNA product and the probe against Prg2 spanned nucleotides 42-387. The first and last 20 bases of each segment were used as primer sequences.

4.3 Results

The 4 cDNA libraries were multiplexed together and run in one lane on the Illumina sequencer. The number of reads obtained from each sample was: 6 weeks old (21,280,796), 8 weeks (20,069,482), 15 weeks (19,075,453) and 17.5 months (15,413,683). Reads were mapped to the mouse genome and associated with 30,085 identifiers. After filtering with the 10 read minimum and normalization, 19,158 transcripts were analyzed by DESeq for differential expression. A single set of gene-wise dispersion estimates was calculated from the entire analysis using all 4 samples (sharing method parameter = “blind”) and passed along with the normalized count values to the negative binomial model for differential expression analysis. Three separate comparisons were performed, one for each of the 8 weeks old, 15 weeks and 17.5 months old samples; each was referenced to the 6 weeks old sample for differential expression determination.
DESeq analysis determined 409 transcripts to be differentially expressed due to aging at at least one time point. Plots of \( \log_2 \) fold change between age points versus the mean normalized counts demonstrate that changes largely involve reduced expression over time, with comparatively few genes experiencing up-regulation (Fig. 4-1). The distribution of differential identifications across time points was: 8 weeks old - 163 significant transcripts, 15 weeks old - 300 and 17.5 months old - 206 transcripts. Seventy-six transcripts where found to have significant differential expression in all three comparisons while an additional 53 were shared between the 8 weeks and 15 weeks old comparisons, 47 between 15 weeks and 17.5 months, and 8 between 8 weeks and 17.5 months old.

Figure 4-1: Plots of mean normalized count value versus the \( \log_2 \) fold change relative to the 6 weeks old sample (8 weeks (left), 15 weeks (center) and 17.5 months (right)). Genes determined to be differentially expressed are denoted by black markers. In all three comparisons, the majority of differential expression is observed to be down-regulation.

4.3.1 Expression Profile Clustering and Transcription Factor Enrichment

Expectation-maximization (EM) clustering was utilized to group differentially expressed transcripts based on their temporal fold change profiles. Clustering in this mannor may provide useful enrichment for genes involved in pathways together or
regulated by similar controllers. Using the Bayesian Information Criteria (BIC), the EM algorithm identified 10 different expression clusters within the 409 differentially expressed transcripts (Fig. 4-2, top row). Each cluster was studied individually within IPA to identify potential upstream regulators. The expression profiles of native transcription factors (TFs) (TFs differentially expressed themselves) and enriched TFs (TFs with known regulatory control over several cluster genes) are presented below each cluster (Fig. 4-2, bottom row). TF expression profiles were compared to the mean cluster profile to identify potentially regulatory interactions. TFs with similar (or mirrored) temporal expression patterns were identified as potentially important and are listed in Table 4.1. Twenty-seven TFs were identified in this analysis, 10 of which reside genomically in non-syndromic deafness loci with unidentified causative genes, 1 is known to cause non-syndromic hearing loss and 9 which have known roles in the auditory system. Eight of these age-related TFs were also identified as potentially important in our matched study of a NI-PAN exposure model (Chapter 3).

Figure 4-2: Plot of log₂ fold change between age points for the 10 expression clusters identified by EM analysis (top row). Native and enriched TFs associated with each cluster are presented in the bottom row.

4.3.2 Biological Significance of Differential Expression

To gain insight into the temporal nature and biological significance of differential expression, IPA core analyses were performed on all 409 differentially expressed genes
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</table>

* TF also identified in noise exposure study (Chapter 3)

Table 4.1: Transcription factors identified, or enriched for, within the differential expression clusters.

at each time point. Core Analysis results were compared using the Comparison Analysis tool to better characterize the temporal nature of the biological impacts. The predicted biological impacts of differential expression largely effect development, movement and function of the hematological system, with less common influences on tissue development, morphology and death (Fig. 4-3). Most effected functions show self-similar behavior over time, either constantly activated or inhibited. Widespread inhibition across all time points is observed for functions relevant to activation and movement of cells of the immune system. Conversely, significant activation is observed
across time points of pathways involved in the quantity of these cells. The most significantly effected biological function observed within the dataset is the strong activation of organismal death that is observed in 17.5 month old mice.

Figure 4-3: Heatmap of activation and inhibition, relative to expression at 6 weeks old, of bio-functions and diseases with highly significant activation Z-scores (|Z-score| \geq 2.5 in at least one comparison) determined by IPA. The activation Z-score is a metric which combines standard enrichment p-values with fold change directionality to more accurately predict down-stream effects of differential expression, |Z-score| \geq 2 are considered significant[85].

IPA-generated networks, with highly significant enrichment within the differential
transcript list ($p$-value $\leq 10^{-15}$), were studied to identify central molecular nodes (Tab. 4.2) and highly connected extracellular and secreted molecules (Tab. 4.3). Nodal molecules are of interest because they may be important regulatory or therapeutic targets, while extracellular molecules may provide useful diagnostic information as biomarkers. Thirty-nine key nodal molecules were identified within highly significant IPA networks along with 17 extracellular molecules. Several molecules were common to both lists and some where also identified within the TF search.

### 4.3.3 Overlap with Previous Rodent Studies of Noise Trauma

Previous studies, using a variety of different noise parameters, have identified several genes involved in the cochlear response to noise traumas that cause TTS or PTS in rodents. We compared our list of differentially expressed genes due to aging with genes confidently implicated in PTS and TTS to gain insight into similarities and differences among these conditions. Comparing the 409 transcripts identified in this study with 161 genes implicated in PTS and 84 genes implicated in TTS, curated from the previously published literature, we find 2 genes common to PTS and aging ($Elane$ and $Gpnmb$), 3 genes common to TTS and aging ($Dbp$, $Dnajb9$ and $Otos$), and 1 gene common to PTS, TTS and aging ($Nr4a1$).

Comparing our list of 409 transcripts differentially expressed as a result of age with the 160 differentially expressed as a result of NI-PAN (Chapter 3) yielded a much richer overlap of variable transcripts. In total, 82 transcripts were identified by both NI-PAN and age studies, 78 uniquely responded to NI-PAN and 327 to aging. When membership of the clusters from both the age and NI-PAN studies are compared (Fig. 4-4), 4 similar cluster-paris are evident. Age cluster 2 and NI-PAN cluster 1 are the most similar, sharing 17 transcripts ($> 50\%$ of the age cluster transcripts are shared), while the pairs (5,1), (5,4) and (6,7) share 10, 9 and 11 transcripts, respectively. The dominant biological functions that IPA associated with Age cluster 2 and NI-PAN cluster 1 were highly similar and enriched for movement and activation of immune cells (both phagocytes and those of the myeloid lineage). Interestingly, both clusters also contained a significant number of transcripts associated with damage to
the respiratory epithelia. The dominant biological function shared by cluster pair (5,1) was activation of myeloid cells, cluster pairs (5,4) and (6,7) did not have any specific, shared functional implications – although pair (6,7) did each have implications in nervous system development.

Figure 4-4: Heatmap of cluster similarity between differential expression clusters identified due to aging and NI-PAN inducing noise trauma. Darker colors indicate more shared transcripts within clusters and the numbers in parentheses indicate cluster size.

4.3.4 Validation and Localization

From the above analyses, 8 transcripts of interest were selected for qPCR validation (Fig. 4-5, error bars present standard error of the means, n ≥ 3). Unfortunately, several 6 week old samples were contaminated, resulting in n = 1, so fold change values were calculated relative to the 8 week old expression values. Of the 8 genes tested, 5 (Dbp, Elane, Gata1, Mmp2 and Nr4a1) were confirmed to be differentially expressed due to aging at at least one time point (p-value < 0.05). Cebpe was confirmed as not differentially expressed, in agreement with the RNA-Seq result, but had been included in the qPCR analysis due to its central role in the NI-PAN study. This validation rate is encouragingly high given the non-replicated design, approaching rates which are observed in well-replicated RNA-Seq experiments (> 80%[12, 62], and increases confidence in the RNA-Seq results. However, despite this observed benefit of pooling 20 ears into each sequenced sample, caution is encouraged when interpreting any non-replicated data-set.
Figure 4-5: qPCR determined fold changes in gene expression (relative to the 8 week old sample). qPCR confirmed that Dbp, Elane, Gata1, Mmp2 and Nr4a1 were differentially expressed (p-value < 0.05, marked with asterisks). Error bars denote standard error of the mean.

Immunohistochemistry was performed on cochlear sections to determine the localization of differentially expressed gene products and to determine if immune cell activity within the ear was likely responsible for the observed expression changes. Immunostaining was performed with antibodies against NR4A1 and MMP2, proteins whose mRNA was validated as differentially expressed by qPCR, and IBA1, a macrophage marker useful for the visualization of immune cells.

Specific NR4A1 reactivity was observed within the nuclei of most cell types within the cochlea, including cells of the Organ of Corti (OC), spiral ligament (SL) and spiral ganglion neurons (SGNs) (Fig. 4-6). While staining remains nuclear at all ages for cells of the OC and SL (not shown), localization within SGNs changes with age. At young ages, 6 and 8 weeks old, NR4A1 is located in the nuclei of SGNs (Fig. 4-6A,B). By 15 weeks old (Fig. 4-6C) this localization begins to transition to punctate cytoplasmic staining and by 17.5 months this transition is complete (Fig. 4-6D). Staining of cochlea exposed to neuropathic noise (Fig. 4-6 top row) shows similar behavior at all time points with the exception of 6 weeks old (24 hours post exposure, Fig. 4-6A insets I-II). Instead of the nuclear staining observed in the unexposed animals, staining in exposed animals is diffuse throughout the cell.

Neither MMP2 (Fig. ) or IBA1 (Fig. 4-7) show significant changes in cochlear lo-
calization with animal age. MMP2 is observed to be highly expressed in the cytoplasm of SGNs, interdental cells, OC and SL. IBA1 immunostaining revealed macrophages occurring naturally within the stria vascularis and in the region of type-IV fibrocytes at all time points. Macrophages were also observed within the modiolus (in the region of spiral ganglion fibers) in 15 week old specimen (Fig. 4-7 C).

![Figure 4-6: Immunostaining for NR4A1 in the cochleae of mice harvested at 6 weeks old (left column, A), 8 weeks old (mid-left column, B), 15 weeks old (mid-right column, C), and 17.5 months old (right column, D). In unexposed cochlea (bottom row) specific NR4A1 reactivity is observed within the nuclei of spiral ganglion neurons at 6 and 8 weeks of age (A, B, inset IV). At 15 weeks old, nuclear staining is still present but punctate cytoplasmic staining is also observable (C). By 17.5 months of age, punctate cytoplasmic staining is dominant (D, inset V). Staining of cochlea exposed to neuropathic noise (top row) shows similar behavior at all time points with the exception of 6 weeks old (24 hours post exposure, insets I-II). Instead of the nuclear staining observed in the unexposed animals, staining in exposed animals is diffuse throughout the cell.](image)

Two transcripts, Ngp and Prg2, determined by RNA-Seq to be differentially expressed in all age comparisons, were selected for in situ hybridization experiments
Figure 4-7: Immunostaining for IBA1 (a macrophage marker) in the cochleae of unexposed mice harvested at 6 weeks old (A), 8 weeks old (B), 15 weeks old (C), 17.5 months old (D) and 6 weeks old negative control (E). Positive immunostaining for macrophages was observed within the stria vascularis and in the region of type-IV fibrocytes at all time points. Positive staining was also observed in the modiolus at 15 weeks old (inset C). No reactivity was observed when the primary antibody was not applied (E).

Figure 4-8: Immunostaining for MMP2 in the cochleae of mice harvested at 6 weeks old (left column, A), 8 weeks old (mid-left column, B), 15 weeks old (mid-right column, C), and 17.5 months old (right column, D). MMP2 is observed to be highly expressed in the cytoplasm of SGNs, interdental cells, OC and SL and has no obvious changes in expression localization or intensity with either age or neuropathic exposure (top row).

(Fig. 4-9). Strong positive staining for both transcripts is observed in the neuronal cell bodies of the SG, providing strong evidence for a neural phenotype with age.
Figure 4-9: *In situ* hybridization staining for *Ngp* (left column, A-B) and *Prg2* (right column, C-D). Positive staining for both transcripts (A,C) is observed within the neuronal cell bodies of the SG. Anti-sense controls (B,D) for both transcripts had no observable staining.

### 4.4 Discussion

To gain insight into which mechanisms may be dominating cellular death within the inner ear we studied the RNA-Seq determined fold change expression profiles of genes as apoptotic associated with distinct cell death pathways[29, 185] (Fig. 4-10). Several genes important in apoptosis appear to have age dependent expression changes while genes important in autophagy and paraptosis have expression profiles that are less variable with age. This suggests that apoptotic death may be playing a role, however, only two genes had statistically significant changes in expression; the apoptosis inhibitor *Birc5* (also called *Survivin*), which was substantially down-regulated with age and *Nr4a1* (a TF with a role in the intrinsic pathway), which also shows down-regulation with age.

*Nr4a1* encodes an intracellular TF with many aliases, including nuclear receptor subfamily 4 group A member 1 (NR4A1), nerve growth factor IB (NGFIB), TR3 and NUR77. It is a member of the *Nur* nuclear receptor family with opposing mitogenic and apoptotic effects, depending on sub-cellular localization. When NR4A1 localizes to the nucleus, it hetero-dimerizes with other receptors to form a TF with mitogenic effects[56, 125]. Upon apoptotic stimulation, NR4A1 translocates to the cytoplasm where it induces cytochrome c release from mitochondria[93]. This pro-apoptotic role
Figure 4-10: RNA-Seq determined log₂ fold changes (relative to expression at 6 weeks old) of genes associated with different cell death pathways as a function of time after exposure. Members of the apoptosis pathway showed the most variability; however, only two genes had a statistically significant change in expression (black lines), intrinsic member \( Nr4a1 \) and the inhibitor \( Birc5 \).

is independent of transcriptional regulation, functioning directly at the mitochondria and capable of occurring without an intact DNA-binding domain. Interestingly, to localize to mitochondria and induce apoptosis, NR4A1 must interact with the canonically anti-apoptotic BCL2[95] (Bcl-2 is known to be down-regulated by CEBPE, which was important in the NI-PAN exposure response). This interaction causes the Bcl-2 homology domain 3 (BH3) to be exposed, activating its pro-apoptotic function.

In macrophages, NR4A1 also plays a pro-inflammatory role within the nucleus, mediated by NF-\( \kappa \)B, by transcriptional activation of genes involved in the inflammation response (including responses to physical stimuli such as vibrations[102, 123]).

\( Nr4a1 \) has previously been shown to be up-regulated within the ear following noise exposure[38]. In addition to higher levels of the transcript, the sub-cellular localization of the protein was observed to shift from the nuclei to the cytoplasm, following the stereotypical pro-apoptotic behavior. This response was observed in nearly every cell type located within the Organ of Corti, spiral ligament and SG. Our previous study on NI-PAN inducing exposure (Chapter 3) also found \( Nr4a1 \) to be up-regulated early after exposure, both by RNA-Seq and qPCR.

In order to better understand this link between age- and noise-related differential
expression, immunostaining of cochleae exposed to neuropathic noise was performed
(Fig. 4-6, top row) in conjunction with unexposed tissues. While NR4A1 localization
appeared identical across exposure groups for the three oldest time points (Fig. 4-6B-D), a critical difference is observed at 6 weeks of age (Fig. 4-6A, which corresponds to
24 hours post neuropathic exposure). At this time point unexposed cochlea demon-
strates nuclear staining in the SGNs, but exposed cochlea present diffuse cytoplasmic
staining. This is similar to the responses observed previously to much more damag-
ing exposures[38], however this shift in localization is limited only to SGNs in the
NI-PAN model, reinforcing our belief that this exposure is inducing a dominantly
neural phenotype. This translocation reverses within two weeks of exposure, and
importantly, localization is seen to shift to punctate staining within the cytoplasm in
both exposure conditions as the animals age. This indicates that NR4A1 translocates
to the mitochondria (where it is pro-apoptotic) as a natural part of aging in SGNs.

We present the first RNA-Seq study of the dynamic changes in genome-wide tran-
scriptional activity of the murine cochlea as it ages from adolescence to old-age.
Transcripts that were differentially expressed due to aging have been identified and
probed for enriched biological functions and transcriptional regulators. Several of
the identified TFs have known roles in the auditory system, but the majority have
not been directly linked to inner ear function; additionally, several occur in deafness
loci with unknown causative genes. Immunostaining results suggest that immune
cell invasion of the ear is not responsible for the observed expression changes, de-
spite the fact that many of the transcripts identified are canonically associated with
the immune system, and in situ hybridization experiments localized two of the lon-
gitudinally differentially expressed genes to the SG. These findings have important
implications for understanding the inner ear’s aging process and the homeostatic
mechanisms necessary for preservation of this remarkable sensory organ.
Cluster specific nodes

<table>
<thead>
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<tr>
<td>2</td>
<td><em>Camp</em></td>
</tr>
<tr>
<td>4</td>
<td><em>Tp53</em></td>
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<td><em>Ctnnb1</em></td>
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<tr>
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<td><em>Pdgf bb</em></td>
</tr>
<tr>
<td>Several</td>
<td><em>beta estradiol</em></td>
</tr>
<tr>
<td>Several</td>
<td><em>Insulin</em></td>
</tr>
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</table>

Table 4.2: Nodal molecules identified within IPA generated networks constructed from age-induced differentially expressed genes. Molecules in bold were determined to be differentially expressed.
<table>
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</tr>
<tr>
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<td>8 wk, 15 wk, 17.5 mo</td>
</tr>
<tr>
<td>IgG</td>
<td>8 wk</td>
</tr>
<tr>
<td>Vegf</td>
<td>8 wk, 15 wk, 17.5 mo</td>
</tr>
<tr>
<td>Tgf beta</td>
<td>8 wk, 15 wk</td>
</tr>
<tr>
<td>II12</td>
<td>8 wk</td>
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<tr>
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<tr>
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<td>Insulin</td>
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<td>15 wk</td>
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<td>Apoe</td>
<td>15 wk</td>
</tr>
<tr>
<td>Epo</td>
<td>15 wk</td>
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<tr>
<td>Tnf</td>
<td>15 wk, 17.5 mo</td>
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<td>17.5 mo</td>
</tr>
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<td>17.5 mo</td>
</tr>
<tr>
<td>Ifng</td>
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</table>

Table 4.3: Highly connected extracellular molecules identified within significant IPA networks generated from differentially expressed genes at each age point. Molecules in bold were determined to be differentially expressed.
Part II

Auditory Phenotype of $Fgf_{23}$ Mutant Mice
Chapter 5

FGF23 deficiency leads to mixed hearing loss and middle ear malformation in mice

Abstract

Fibroblast growth factor 23 (FGF23) is a circulating hormone important in phosphate homeostasis. Abnormal serum levels of FGF23 result in systemic pathologies in humans and mice, including renal phosphate wasting diseases and hyperphosphatemia. We sought to uncover the role FGF23 plays in the auditory system due to shared molecular mechanisms and genetic pathways between ear and kidney development, the critical roles multiple FGFs play in auditory development and the known hearing phenotype in mice deficient in klotho (KL), a critical co-factor for FGF23 signaling.

Using functional assessments of hearing, we demonstrate that Fgf23−/− mice are profoundly deaf. Fgf23+/− mice have moderate hearing loss above 20 kHz, consistent with mixed conductive and sensorineural pathology of both middle and inner ear origin. Histology and high-voltage X-ray computed tomography of Fgf23−/− mice demonstrate dysplastic bulla and ossicles; Fgf23+/− mice have near-normal morphology. The cochleae of mutant mice appear nearly normal on gross and microscopic inspection. In wild type mice, FGF23 is ubiquitously expressed throughout the cochlea.

Measurements from Fgf23−/− mice do not match the auditory phenotype of Kl−/− mice, suggesting that loss of FGF23 activity impacts the auditory system via mechanisms at least partially independent of KL. Given the extensive middle ear malformations and the overlap of initiation of FGF23 activity and Eustachian tube development, this work suggests a possible role for FGF23 in otitis media.
5.1 Introduction

Fibroblast growth factor 23 (FGF23) is a circulating hormone, typically secreted by osteoblasts and osteocytes, which regulates renal phosphate handling[21, 149, 179]. Phosphate wasting diseases (including tumor-induced osteomalacia, X-linked and autosomal-dominant hypophosphatemic rickets, all of which may present with hearing phenotypes) occur with elevated serum levels of FGF23[15, 42, 52]. Reduced FGF23 activity leads to decreased urinary excretion of phosphate[20], as in familial tumoral calcinosis. Mice deficient in Fgf23 exhibit severe hyperphosphatemia, hypervitaminosis D, hypercalcemia, ectopic calcifications, growth retardation, organ atrophy, infertility and shortened life expectancy[97, 145, 144, 149].

This study sought to characterize the murine auditory phenotype due to FGF23 deficiency because other members of the FGF superfamily are critical for normal development of the auditory system in humans and animal models[5, 126, 140, 170], kidney and inner ear development rely on shared molecular mechanisms and genetic pathways[77, 164] and both organs are susceptible to toxicity from aminoglycoside antibiotics and loop diuretics[164, 75, 176]. Additionally, deficiency in klotho (KL), a critical co-factor for FGF23 mediated signaling[88, 168], results in hearing loss[31, 78]. Alpha-KL is a single-pass transmembrane protein that increases FGF receptor (FGFR) affinity for FGF23[88, 168] and decreases affinity to other FGFs. KL expression has been reported in several cochlear cell types[78, 160], but the sensory epithelia of Kl−/− mice appear normal[31]. The skeletal phenotypes present in Fgf23−/− mice are also present in Kl−/− mice and double knockouts. Bioactive FGF23 injection results in phenotypic rescue of Fgf23−/− mice but not in KL knockouts[113]. A vitamin D-deficient diet rescues the auditory and systemic Kl−/− phenotypes[31, 166]. Injection of just the C-terminal tail of FGF23, capable of binding the FGFR1c-Klotho complex but not regulating phosphate handling, can reduce phosphate wasting via receptor competition in both normal and FGF23 over-expresser mice[61].

It is currently unclear if the Kl−/− auditory phenotype is due to altered FGF23
signaling because KL and FGF23 can exhibit functions independent of each other [87, 117]. However, the leading hypothesis is that hearing loss results from demineralization of the auditory ossicles, resulting from hypervitaminosis D[31] induced by loss of renal FGF23 activity. If correct, hearing loss should be seen in Fgf23−/− mice and should be conductive in nature. To understand the relevance of FGF23 in auditory function, we studied Fgf23−/− and Fgf23+/− mice using physiologic, histologic and radiologic tools. We demonstrate that FGF23 is critical for normal development of the middle ear and for function of both the middle and inner ear. We also demonstrate a connection between FGF23 deficiency and predilection to otitis media. This finding has diagnostic and therapeutic implications as the genetic basis of human otitis media is poorly understood[32] and otitis media is the most common cause for pediatric antibiotic and surgical intervention[24].

5.2 Materials & Methods

For this study, we utilized a previously developed Fgf23 null mouse (Fgf23−/−)[149, 113, 180] in which the entire Fgf23 gene has been replaced with the lacZ gene. Male, 6-week old mice were generated via the breeding of Fgf23+/− mice. Genotypes were confirmed with PCR. All studies were approved by the Institutional Animal Care and Use Committee at Harvard Medical School.

5.2.1 Audiometric testing

Physiologic performance of the auditory systems of Fgf23−/−, Fgf23+/−, and Fgf23+/+ littermates was assessed using auditory brainstem response (ABR), which assess organ and neural function, and distortion product oto-acoustic emission (DPOAE) measurements, which assess middle ear function and cochlear amplification. ABR waveforms were recorded from anesthetized mice (ketamine 0.1 mg/g and xylazine 0.02 mg/g) via sub-dermal electrodes placed at the ipsilateral pinna and vertex (grounded at the junction of the tail and torso). A brief tone was presented into the ipsilateral ear canal and the resulting electrode waveform was recorded and averaged (512 presenta-
Tones were presented at frequencies from 5.66–45.25 kHz in half-octave steps and intensities ranging 15–80 dB SPL in 5 dB steps. Threshold was defined as the first intensity at which recognizable and repeatable peaks became observable in the averaged recording. ABR wave I amplitude (measured peak-to-peak from the local maxima of $P_I$ to the local minima of $N_I$) and latency were determined for each frequency and intensity using the ABR Peak Analysis software (Bradley Buran, Eaton-Peabody Laboratory). Amplitude and latency values were calculated for the middle 5 frequency points due to increased noise in the highest and lowest measurements. Latency values are presented in dB SL (sensation level), as opposed to dB SPL like amplitude measurements. At high enough stimulation intensities ($\geq 60$ dB SPL tones) ABR amplitudes have a greater dependance on absolute intensity due to growth of excitation and cochlear saturation.

DPOAEs were measured in anesthetized mice via simultaneous presentation of two tones (denoted $f_1$ and $f_2$, the frequency of $f_2 = 1.2f_1$ Hz and intensity of $f_2 = f_1 - 10$ dB SPL) into the ipsilateral ear canal while concurrently recording ear canal sound pressure. The energy of the $2f_1 - f_2$ Hz distortion tone, generated by non-linear cochlear amplification of the input tones, was monitored and threshold defined as the $f_2$ tone intensity which generates a distortion tone greater than 0 dB SPL (noise floor $\sim -10$ dB SPL).

Differences between mutant and wildtype threshold, magnitude and latency values were tested for statistical significance using Welch’s t test. ABR threshold, amplitude and latency data are from N=7, 3 and 3 animals from the $Fgf^{23-/-}$, $Fgf^{23+/-}$, and $Fgf^{23+/-}$ genotypes respectively. DPOAE thresholds were measured from N=5, 4 and 9 ears of each genotype.

### 5.2.2 Histology and Immunohistochemistry

Histologic sections were prepared to assess the microscopic anatomy of the inner ear. Mice were anesthetized using ketamine (0.1 mg/g) and xylazine (0.02 mg/g) before intra-cardiac perfusion. 4% paraformaldehyde (PFA) was used for paraffin embedding and 1.5% PFA/2.5% gluteraldehyde for araldite work. A ventral approach was
performed to expose the bulla and tympanic membrane for photography and extraction. The round and oval windows were perforated and intra-cochlear perfusion was performed to improve tissue preservation. Cochleae were extracted and submerged in fixative for 2 hours at room temperature. Samples for paraffin embedding were decalcified in EDTA for 2 days and stained with hematoxylin and eosin. Cochleae for araldite embedding were incubated in 1% osmium tetroxide for 1 hour and decalcified in EDTA with 1% gluteraldehyde for 3-4 days at room temperature.

Slides for FGF23 immunostaining were deparaffinized in xylene and rehydrated with an alcohol ladder (100%, 95%, 75% and 50%). Sections were immersed in 3% H$_2$O$_2$ in methanol, then blocked in 5% goat serum for 30 minutes, and incubated with rat anti-FGF23 primary antibody (AMGEN, 1:200) overnight at 4°C. Biotinylated goat anti-rat secondary antibody (Vector Laboratories, BA-9401, 1:200) was applied for 1 hour at room temperature followed by HRP substrate (BD Pharmingen, 92121) and developed with DAB for 2 minutes (Vector Laboratories, SK4100, 1:10). Slides were counterstained with hematoxylin (VWR, 95057-844).

5.2.3 $\mu$CT analysis

Gross morphology of the bulla, ossicles and cochlea of $Fgf23^{-/-}$ and $Fgf23^{+/+}$ mice were studied using high-voltage X-ray computed tomography ($\mu$CT). Mice were anesthetized and intra-cardially fixed as above. Soft tissues were removed from the head to expose the skull. The cranium and hard palate were carefully dissected, leaving only the portion of the skull base between the bullae and extending forward to contain the bony ear-canals. The tympanic membrane of each ear was perforated and samples were fixed overnight in 4% PFA.

$\mu$CT scans were performed on a Scanco Medical $\mu$CT35 System (Scanco Medical, Brüttisellen, Switzerland). Scans were conducted in 70% ethanol with parameters: voxel size - 7 $\mu$m, X-ray tube potential - 55 kVp, X-ray intensity - 0.145 mA and integration time - 600 ms (as detailed in [180]).

Raw $\mu$CT data were imported into Voxar 3D 6.3 (Toshiba Medical Visualization Systems, Japan). For the 2D reformats, the raw data for each ear were separated and
then brought up on a clinical imaging work station viewing console, where the raw data were displayed in three orthogonal planes. The data were first reformatted into a dataset of 7 μm thick images rendered in a plane parallel to the posterior semicircular canal (PSCC) and sagittal to the head. An orthogonal coronal data set was then made in a plane perpendicular to the PSCC and coronal to the head. For the 3D reconstructions, the raw data for each ear were displayed using 3D volume rendering with preset window level settings that were optimized for middle ear analysis (settings provided by the software developer). Regions of interest were manually segmented to exclude the walls of the middle ear and to isolate the ossicles.

The sagittal and coronal images, and segmented 3D reconstructions from the Fgf23−/− samples were reviewed and compared with the Fgf23+/+ specimens with regard to morphology of the ossicles, contour of the incudomalleal joint, pneumatization of the petrous apex, pneumatization of the mastoid, Eustachian tube patency and CT homogeneity of the bulla, otic capsule and ossicles.

5.3 Results

5.3.1 Anatomic Findings

Morphological assessments of middle and inner ear anatomy revealed that bullae from Fgf23−/− mice appear cloudy and lack the precise refinement in shape that is characteristic in Fgf23+/+ mice (Fig. 5-1A). The auditory ossicles are similarly a cloudy white hue and show significant dysplasia, consistent with abnormal bone remodeling (Fig. 5-1B). In contrast, the cochleae and vestibular organs of all genotypes appear similar, although Fgf23−/− cochleae are slightly smaller and whiter (Fig. 5-2). The bullae of Fgf23+/− mice have normal shape but small, white, cloudy patches. The ossicles appear slightly dysplastic. In no cases did the Fgf23+/− morphological phenotype approach the severity of the Fgf23−/− mice. Middle ear effusions and signs of otitis media were observed in 4 of 5 Fgf23−/− and 1 of 3 Fgf23+/− mice. Our finding of severe middle-ear phenotypes in Fgf23−/− mice are consistent with the phenotype
described in other bones, including severe axial and appendicular skeletal malformations, characterized by nodules, rachitic lesions and narrowed growth plates[149]. Others have not observed obvious abnormality in the *Fgf23*+/− genotype[144].

Figure 5-1: Morphological and histological comparisons of the middle and inner ears. (A) The bullae of *Fgf23*−/− mice (bottom row) appear white and cloudy with less structural refinement than both *Fgf23*+/+ (top row) and *Fgf23*+/− mice (middle row), indicating incomplete ossification. (B) The auditory ossicles are dysplastic in *Fgf23*−/− mice. (C) H&E stained, paraffin sections demonstrate increased vascularization of the bony labyrinth in *Fgf23*−/− mice (arrowheads). (D) The highly-organized laminar structure of the otic capsule, bordering the spiral ligament, is lost in the *Fgf23*−/− genotype. Lines in A and B are orienting lines from the microscope objective.

The membranous labyrinth of the inner ear appears anatomically normal, healthy and properly organized in *Fgf23*−/− mice (Fig. 5-3). However, inspection of the bony labyrinth in *Fgf23*−/− mice uncovers phenotypic differences. The modiolus appears
Figure 5-2: Morphological comparisons of the otic capsule from (A) $Fgf23^{+/+}$, (B) $Fgf23^{+-}$ and (C) $Fgf23^{-/-}$ mice. All genotypes appear similar but the $Fgf23^{-/-}$ cochlea is slightly smaller and whiter. Black lines are orienting lines from the microscope objective.

Immunostaining of cochlear slides revealed the widespread presence of FGF23 throughout most cell types of the inner ear (Fig. 5-4). Immunoreactivity was observed within cells of the spiral ligament, stria vascularis, Organ of Corti, spiral limbus and within the spiral ganglion neuronal cell bodies as well as osteocytes in $Fgf23^{+/+}$ (Fig. 5-4A) and $Fgf23^{+-}$ mice (not shown). The strongest staining was observed in the stria vascularis and Organ of Corti. No immunoreactivity was observed in $Fgf23^{-/-}$ mice (Fig. 5-4B), or in $Fgf23^{+/+}$ mice processed without the primary antibody (Fig. 5-4C), indicating the antibodies are reacting specifically.

### 5.3.2 $\mu$CT Reconstruction

Comparison of 2D $\mu$CT sections from $Fgf23^{-/-}$ and $Fgf23^{+/+}$ mice (N=2,2) uncovered several bony phenotypes of the ossicles and mastoid bone. The malleal head appears dysplastic in $Fgf23^{-/-}$ specimens (Fig. 5-5C), as does the incus, either abnormally notched or heart-shaped, and the stapes demonstrates thickening of the crura and footplate (Fig. 5-5B). The incudomalleal joint appears abnormally contoured in the $Fgf23^{-/-}$ genotype, rough, irregular and notched, compared to the smoothly con-
Figure 5-3: Mid-modiolar cochlear section from a $Fgf23^{-/-}$ mouse. (A) Neural populations and gross anatomical structure appear normal. (B) The stria vascularis (StVas), spiral ligament (SpLig), spiral limbus (SpLim), tectorial membrane (TM) and Reissner’s membrane (RM) are similar to $Fgf23^{+/+}$ mice. (C) Inner hair cells (IHC), Outer hair cells (OHC), supporting cells (SC) and basilar membrane (BM) are morphologically normal. Sections were embedded in araldite and osmium stained.
Figure 5-4: FGF23 immunohistochemistry in Fgf23+/+ and Fgf23−/− mice. (A) Specific staining was observed in the cells of the spiral ligament, stria vascularis, Organ of Corti, spiral limbus and within the spiral ganglion neuronal cell bodies in Fgf23+/+ mice (similar patterns were observed in Fgf23+/− mice). Inset images are zoomed in views of the boxed regions. (B) No immunoreactivity was observed in Fgf23−/− mice. (C) Similarly, no staining was observed in negative controls, from Fgf23+/+ mice which were processed without the primary antibody.

toured, tight fitting and angular interface of the Fgf23+/+ mice (Fig. 5-5C). The mastoid (Fig. 5-5C) and petrous apex (Fig. 5-5B) are substantially under-pneumatized. The bony portion of the Eustachian tube is patent and similar to Fgf23+/+ mice (Fig. 5-5C). The incus and malleal head appear heterogeneously lucent when compared to the homogeneously dense formations found in the Fgf23+/+ mice (Fig. 5-5C). Similarly, the bulla, otic capsule and vestibular compartments are characterized by lower density and poor lamellar organization (Fig. 5-5A).

Three-dimensional reconstruction of the ossicles highlights the lack of structural refinement in Fgf23−/− mice (Fig. 5-5D). Regions with particularly noticeable dysplasias include the incudal pocket, manubrium and separation of the malleal ridge and head. The lucency of the Fgf23−/− bones, versus the diffusely dense Fgf23+/+ counterparts (noted above), is reflected in the unsharpness of the borders of both 3D reconstructed Fgf23−/− ossicular chains. This results from poor μCT contrast with surroundings due to lower ossicular density.
Figure 5-5: 2D and 3D µCT reconstructions of bullae from $Fgf23^{+/+}$ and $Fgf23^{-/-}$ mice. Each row contains reconstructions from one ear (top row: $Fgf23^{+/+}$, bottom row: $Fgf23^{-/-}$). (A) The otic capsule and bulla show loss of structural refinement and decreased density (arrowheads). (B) In $Fgf23^{-/-}$ mice the footplate of the stapes demonstrate thickening (arrowheads). (C) The incus and incudomalleal joint are dysplastic (arrowheads) and the mastoid is under-pneumatized (asterisks). (D) The borders of 3D reconstructed $Fgf23^{+/+}$ ossicles are sharp and well-defined while those of $Fgf23^{-/-}$ ossicles are blurry due to poor contrast with surroundings, resulting from decreased bone density in the mutant. $Fgf23^{-/-}$ ossicles demonstrate reduced structural refinement, particularly in the malleus and incus.

5.3.3 Audiometric Assessment

ABR measurements (Fig. 5-6A) demonstrate that $Fgf23^{-/-}$ mice have profound hearing loss across all frequencies compared to $Fgf23^{+/+}$ littermates ($p \leq 0.001$). $Fgf23^{+/+}$ mice have normal hearing below 20 kHz and losses of up to 25 dB above that frequency. DPOAE measurements demonstrate similar trends (Fig. 5-6B): $Fgf23^{-/-}$ mice have nearly complete hearing loss while $Fgf23^{+/+}$ mice demonstrate 40 dB of loss at the highest frequencies and have normal hearing below 30 kHz.

Because ABR threshold measurements are known to be insensitive to certain types of neural damage[86], ABR wave I amplitude and latency were also analyzed. Changes in the amplitude of ABR wave I can be more sensitive than threshold measurements to certain forms of auditory pathology and signal neuronal malfunction months before
Figure 5-6: Auditory measurements from Fgf23 mutant mice. (A) ABR and (B) DPOAE thresholds demonstrate profound hearing loss at all frequencies in Fgf23−/− mice, and moderate hearing loss at high frequencies in Fgf23+/− mice. (C) ABR wave I amplitudes appear slightly depressed in Fgf23+/− mice but are not statistically differentiable (except at 32 kHz where threshold differences impact magnitude (p ≤ 0.01)). ABR amplitudes in Fgf23−/− mice are significantly reduced compared to the other genotypes (p ≤ 0.001). (D) Threshold adjusted ABR wave I latency measurements demonstrate significant increases in latency in both Fgf23−/− and Fgf23+/− genotypes when compared to Fgf23+/+ littermates (p ≤ 0.01). Error bars present standard error of the mean.

Histological observation of neuronal loss[86]. Figure 5-6C presents the peak-to-peak amplitude of ABR wave I as a function of tone frequency. For each frequency and genotype, the data represents the average amplitude of response to the 60, 70 and 80 dB SPL tone presentations. Wave I amplitudes are statistically similar between the Fgf23+/+ and Fgf23+/− groups at all frequencies except 32 kHz. At this frequency, the ~20 dB loss detected by threshold measurements (Fig. 5-6A) is affecting a significant reduction in amplitude (p ≤ 0.01). ABRs were only consistently measured from Fgf23−/− mice at 80 dB SPL for each frequency (due to extremely high thresholds) and were significantly reduced across the frequency range (p ≤ 0.001).

Threshold-adjusted ABR wave I latency values are presented in Figure 5-6D. Unlike amplitude, it is customary to present latency values for tone levels relative to each animal’s hearing threshold (dB SL) rather than absolute level (dB SPL)[94]. For each frequency, the data represent the average response latency to tones presented at 5, 10 and 15 dB SL. Both Fgf23−/− and Fgf23+/− mice have increased wave I latencies, indicating slower neural signaling. The observed increase is most significant at high frequencies and more pronounced in the Fgf23−/− genotype.

These results demonstrate profound hearing loss in Fgf23−/− mice, resulting in
large part from conductive deficiencies due to ossicular dysplasia. $Fgf^{23+/-}$ mice demonstrate high-frequency hearing loss, of mixed conductive and sensorineural origin. Significant DPOAE threshold shifts at high-frequencies provide evidence for a conductive component to the loss, while the lower onset frequency of the ABR threshold shifts indicates a sensorineural component. ABR wave I latency data also support the presence of a sensorineural pathology. The near equivalence of ABR amplitudes in $Fgf^{23+/-}$ and $Fgf^{23+/-}$ mice suggests that the functional neural populations are of similar size (in agreement with histology), but the increased latency in $Fgf^{23+/-}$ mice indicates a retrocochlear phenotype as a result of the mutation[94]. The increased latency is particularly interesting as no previous study has identified a heterozygote phenotype.

5.4 Discussion

Our findings of significant conductive impairments in $Fgf^{23-/-}$ mice coupled with a sensorineural component indicate that the mechanisms of hearing loss due to FGF23 and KL deficiency are different, albeit possibly overlapping. KL expression has previously been demonstrated in the cochlea, specifically in the stria vascularis, spiral ligament, outer hair cells, inner hair cells and spiral ganglion cells[78, 160], potentially enabling tissue specific FGF23 sensitivity. Our immunostaining results demonstrate that FGF23 is even more widely present throughout the ear than KL, and suggest that FGF23-KL interaction may be occurring in many of the inner ear's critical tissues. $Kl^{-/-}$ mice have been shown to exhibit hearing loss; 14-18 dB threshold shifts have been detected in ABRs elicited by clicks as well as tones at 4, 8, 16 and 32 kHz in 3 week old 129/SvJ mice[31]. Another study, more directly comparable to our study due to the common background strain and similar animal age, observed an ~18 dB shift in ABR thresholds for 8 kHz tones in 6–14 week old C57/Bl6 mice[78]. The authors also analyzed non-threshold adjusted ABR wave I latency in response to 100 dB SPL tones and found a significant delay of ~0.4 ms in $Kl^{-/-}$ mice.

The ~50 dB ABR threshold shift we observed across all frequencies in $Fgf^{23-/-}$
mice is much more severe than the $Kl^{-/-}$ phenotypes. Conversely, the observed ABR wave I latency delays were more severe in $Kl^{-/-}$ mice than the $\sim 0.2$ ms delays at higher frequencies in $Fgf23^{-/-}$ mice (we observed no significant delay at 8 kHz). However, because the results from $Kl^{-/-}$ mice were non-threshold adjusted, these metrics are not directly comparable. ABR latency decreases as tone intensity (in dB SL) increases; therefore the latency difference observed in $Kl^{-/-}$ mice may have a significant contribution owing to the $\sim 18$ dB threshold shift (the significance of this effect is reduced because tones are well above threshold for both groups). The threshold-adjusted metric we utilized, where ABR latencies are measured just above animal threshold, prevents sensitivity differences from influencing the latency and allows the result to be more demonstrative of actual differences in the rate of signal transduction and neural conduction.

Unfortunately, neither of the above studies of Klotho mutations presented hearing results from $Kl^{+/-}$ mice; thus comparison with the unexpected $Fgf23^{+/-}$ phenotype is not possible. However, the mixed hearing loss associated with the $Fgf23^{+/-}$ phenotype is intriguing, particularly the presence of significant threshold-adjusted latency delays at frequencies below DPOAE threshold shifts, strongly indicating a neural phenotype. Overall, this divergence of $Fgf23$ and $Kl$ phenotypes is made more interesting by recent research which has suggested a calcineurin-mediated FGF23 signaling pathway[117], and activation of calcineurin has previously been shown to contribute to noise induced hearing loss[106, 167].

Our results raise questions concerning the prevalence of hearing loss among individuals with FGF23 deficiencies. No auditory phenotype has been reported in FGF23-mediated familial tumoral calcinosis, which results from a missense mutation in $FGF23$. However, diseases with known elevations in serum concentrations of FGF23 co-present with hearing loss[44]. Our findings demonstrate that FGF23 deficiency is sufficient to induce profound sensory impairment in mice. The lack of such an obvious phenotype in humans suggests important differences between species, potentially stemming from robust protection against hypervitaminosis D in humans[31]. Alternatively, a putative auditory phenotype in humans may be less severe, and therefore
less studied, than the debilitating systemic phenotypes.

Our findings also suggest a potential role for FGF23 in otitis media, a clinically important disease that afflicts 75-90% of Americans at least once before 3 years of age[115, 121]. Otitis media is estimated to cost the national healthcare system more than $4 billion annually[24], and in extreme cases can result in long-term speech and learning impairments[121]. Currently, genetic risk factors are poorly understood[32], but the prevalence of pediatric cases is commonly believed to be related to the ongoing maturation of the Eustachian tube[33], which undergoes significant development in the first decade of life. Interestingly, hyperphosphatemia in Fgf23−/− mice (which precedes other observed symptoms) first appears at P10[144]. It is believed that a FGF23-independent mechanism of phosphate homeostasis must be functioning prior to this age. Our observations support this suggestion as structures which are nearly mature by P10 show very little dysplasia while structures undergoing continued maturation demonstrate more drastic phenotypes. At P10, the ossicles of wildtype mice are still surrounded by mesenchyme, indicating incomplete development, while the remainder of the inner ear space has begun to clear the mesenchymal tissue[45]. In humans with reduced FGF23 activity, familial tumoral calcinosis, hyperphosphatemia may present as early as 21 months old[16], indicating that FGF23 is important in phosphate handling when most episodes of otitis media develop. Given the potential temporal and mechanistic overlap of initiation of FGF23 activity and Eustachian tube development, this work suggests a possible role for FGF23 in the genetic predisposition to otitis media, a link that warrants further investigation.
Part III

Inner Ear Proteomics
Chapter 6

The proteome of human perilymph[99]

Abstract

Current diagnostic tools limit a clinician's ability to discriminate between many possible causes of sensorineural hearing loss. This constraint leads to the frequent diagnosis of the idiopathic condition, leaving patients without a clear prognosis and only general treatment options. As a first step toward developing new diagnostic tools and improving patient care, we report the first use of liquid chromatography-tandem mass-spectrometry (LC-MS/MS) to map the proteome of human perilymph. Using LC-MS/MS, we analyzed four samples, two collected from patients with vestibular schwannoma (VS) and two from patients undergoing cochlear implantation (CI). For each cohort, one sample contained pooled specimens collected from five patients and the second contained a specimen obtained from a single patient. Of the 271 proteins identified with high confidence among the samples, 71 proteins were common in every sample, and used to conservatively define the proteome of human perilymph. Comparison to human cerebrospinal fluid and blood plasma, as well as murine perilymph, showed significant similarity in protein content across fluids; however, a quantitative comparison was not possible. Fifteen candidate biomarkers of VS were identified by comparing VS and CI samples. This list will be used in future investigations targeted at discriminating between VS tumors associated with good versus poor hearing.

6.1 Introduction

Hearing is initiated when sound-induced vibrations of the eardrum and middle-ear ossicles are transmitted to the fluids of the inner ear, leading to stimulation of sensory

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hair cells and excitation of the auditory nerve. Hearing loss is broadly classified into two categories: (1) conductive hearing loss (CHL), in which mechanical energy transfer from the air to the inner ear is impeded, and (2) sensorineural hearing loss (SNHL), in which tissue pathology in the inner ear or central auditory pathways hamper signal transduction or neural conduction. SNHL can be the result of damage to almost any of the inner ears approximately 30 different cell types, as well as the nerves connecting the cochlea to the brain. Although SNHL can be routinely differentiated from CHL, diagnosing the specific pathology invoking an individual patient’s SNHL is a major challenge. No method currently exists to directly evaluate the inner ear, leaving the clinician blind to the underlying pathologic condition in SNHL. Indirect measures, such as audiograms, word recognition scores[66], auditory brainstem responses and otoacoustic emissions[3] can provide some insight, but are unable to make a specific determination.

Clinically, the limitation described above results in a frequent diagnosis of idiopathic SNHL leaving the patient and physician with no clear prognosis and general, and often ineffective, treatment options. A diagnostic tool capable of providing insight into inner ear pathology would enable the formulation of individualized treatment strategies (personalized medicine) tailored to each patient’s specific pathology. The need for such a tool is immediately apparent from the large number of patients who have unsatisfactory results with general treatment options. Furthermore, a diagnostic platform is critical for successful implementation of preservative and restorative therapies that may emerge from ongoing research in inner ear development and regeneration[173]. Positive patient outcomes will only result if specific disease states are known and targeted, making a diagnostic tool absolutely critical to treatment success.

To lay the groundwork for development of a diagnostic platform capable of filling this clinical need, we present the first analysis of the human perilymph proteome using mass spectrometric (MS) techniques. Perilymph, a proximal fluid of the inner ear, bathes spiral ganglion cell bodies of the auditory nerve and nearly all of the tissues vital to sound transduction. Due to its localization, any protein secreted by
a damaged cell or released during an apoptotic or necrotic event will be found in perilymph at higher concentrations than in more peripheral fluids such as blood or cerebrospinal fluid (CSF).

Previous work has demonstrated the utility of perilymph as a diagnostic fluid. Prior to modern imaging technologies, vestibular schwannoma (VS), an important cause of SNHL in clinical practice, was diagnosed via a significant, i.e. >2.5-fold increase in the total protein content of perilymph[147, 148]. This example demonstrates the potential for significant change in perilymphatic protein levels as a result of pathology, encouraging our search for relevant diagnostic information. Identification and validation of a specific set of biomarkers coupled with the development of a refined collection technique, minimizing the risk to hearing, may facilitate the collection and analysis of perilymph for diagnostic purposes in a clinical setting.

The present investigation is, to the best of our knowledge, the first attempt to define the proteome of human perilymph using mass spectrometry. Previous knowledge of the perilymphatic proteome is derived mainly from 2-D gel electrophoresis work aimed at diagnosis of perilymphatic fistula[11, 122, 163, 162]. These early investigations established the presence of over 100 proteins in the fluid, nearly 30 of which were subsequently identified. Our work extends the existing proteomic characterization and compares the protein profile of perilymph to other bodily fluids. We have compared protein content in different pathologic states as a first step towards the discovery of disease biomarkers. The similarity of human and mouse perilymph has also been analyzed to explore the potential applicability of mouse models for discovery of biomarkers relevant for human SNHL.

Beyond the previously mentioned diagnostic possibilities, the knowledge generated in this investigation will be of significant utility to basic auditory scientists and inner ear pharmacologists. Characterization of the protein content of perilymph may provide insight into the molecular mechanisms that function to maintain the inner ears unique environment. Knowledge of the fluid content will also allow better understanding and prediction of protein-drug interactions, aiding in assessment of pharmacological efficacy and drug delivery within the highly specialized organ.
6.2 Methods

6.2.1 Collection of Samples

Perilymph specimens were obtained from 12 patients undergoing clinically indicated surgeries. All patients had profound sensorineural hearing loss with speech discrimination score < 40%, where 100% is normal. Although detailed medical histories are not available, all patients were healthy enough to undergo general anesthesia without complications. All procedures, six translabyrinthine craniotomies for resection of vestibular schwannomas (VS) and six cochlear implantations (CI), involved surgical opening of the cochlea and collection of approximately 1 \( \mu l \) of perilymph prior to it being displaced. Perilymph samples obtained from CI patients generally have protein concentrations near 2 \( \mu g/\mu l \) and those from VS patients 15-30 \( \mu g/\mu l \)\[147, 148\]. The specimens were placed in 0.2 ml phosphate buffered saline (PBS) and immediately stored at -80C. The study was approved by the Institutional Review Board of the Massachusetts Eye and Ear Infirmary. Five VS specimens were a generous gift from Dr. Jose Fayad (House Ear Clinic, Los Angeles).

Utilizing these 12 specimens, four samples were prepared for MS/MS analysis. Sample CI\(_1\) contained the perilymph specimen from one CI patient and CI\(_2\) contained the pooled specimens from the remaining five CI patients (Fig. 6-1). Similarly, sample VS\(_1\) contained the perilymph specimen from one VS patient and VS\(_2\) contained the pooled specimens of the five remaining VS patients. Samples were grouped in this manner to determine if pooling samples would result in more protein identifications per analysis. The CI samples, representing a heterogeneous disease group, served as controls for the VS biomarker search (a homogeneous disease group), as hearing loss in VS may be associated with elevated protein content of perilymph, presumably due to an unknown toxic substance produced by the VS\[110, 153\].

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6.2.2 Mass Spectrometric Analysis

Samples were fractionated using polyacrylamide gel electrophoresis and subjected to in-gel tryptic digestion followed by reversed-phase liquid chromatography in-line with a tandem mass spectrometer (GeLC-MS/MS) (Fig. 6-1). In brief, entire gel lanes were divided into 7-10 sections (VS1: 8, VS2: 10, CI1: 7, CI2: 10) and proteins in each gel section were digested with trypsin[114, 154]. Peptides extracted from each gel section were analyzed by nanoflow reversed-phase high-performance liquid chromatography (HPLC) system (Eksigent) hyphenated with an LTQ–Orbitrap mass spectrometer (Thermo Scientific) (samples CI1 and VS1) or microscale capillary HPLC (Surveyor, Thermo Scientific) hyphenated with an LTQ mass spectrometer (Thermo Scientific) (samples CI2 and VS2). The LC columns (15 cm x 100 m ID, New Objective) were packed in-house (Magic C18, 5 m, 100 , Michrom BioResources). Samples were analyzed with a 60-minute linear gradient (0-35% acetonitrile with 0.2% formic acid) and data were acquired in a data-dependent fashion, with six MS/MS scans for every full scan spectrum.

All data generated from the gel sections were searched against the IPI-human database (v3.61)[83] using the Paragon Algorithm[143] integrated into the Protein-Pilot search engine (v.3; AB/Sciex). Search parameters were set as follows: sample type, identification; Cys alkylation, iodoacetamide; Instrument, Orbitrap/FT (1-3 ppm) (CI1 and VS1) or LTQ (CI2 and VS2); special factors, gel-based ID; ID focus, none; database, international protein index (IPI) human (v.3.61); detection protein threshold, 99.0%; and search effort, thorough ID. To be considered a valid identification, proteins were required to score above a threshold established by a 1% global false detection rate (corresponding to Unused scores in sample VS1 ≤ 2.01, VS2 ≤ 7.23, CI1 ≤ 2.47, and CI2 ≤ 5.58). This criterion enforced confidence intervals exceeding 99% for each identification. Additionally, each valid protein identification was required to have a minimum of two high confidence (≥95%) peptide identifications attributed to it.

Quantitative comparison of protein expression was accomplished via analysis of
Specimen collection: needle stick through round window membrane

Specimen pooling

SDS-PAGE fractionation and gel sectioning

Tryptic digestion

RP-HPLC MS/MS analysis

Bioinformatics: peptide and protein identification

Candidate biomarker discovery: expression and pathway analysis

Figure 6-1: Workflow diagram of perilymph analysis outlining specimen collection, grouping, preparation, and analysis. RP-HPLC MS/MS: reversed-phase high performance liquid chromatography tandem mass spectrometry.

spectral count data using the statistical framework QSPEC[39]. QSPEC employs hierarchical Bayes estimation of a generalized linear mixed effects model to identify proteins with differential expression across data sets. A protein was considered differentially expressed across disease state if QSPEC returned a Bayes factor larger than 10 and the fold change exceeded 1.5.

QSPEC analysis of a 2 disease state data set, each with 2 replicates, would typically couple the spectral count data of all 4 samples into one analysis to maximize
the amount of information utilized and increase the statistical power. However, the differences in sample preparation and instrumentation (described previously) make the optimal analytic approach ambiguous. To gain maximal insight into the data, three different implementations of the QSPEC package were tested and the results compared. The three analysis paradigms studied were: (1) spectral counts from all 4 samples were coupled into a single 2-disease-state/2-replicate analysis, (2) spectral counts were summed within each disease state, prior to analysis, to generate a single 2-disease-state/1-replicate data set, and (3) two 2-disease-state/1-replicate QSPEC analyses were conducted and the results were combined using a rudimentary scoring metric to determine expression characteristics. Samples prepared/analyzed similarly were directly compared such that the first QSPEC analysis compared samples CI1 and VS1 and the second compared CI2 and VS2.

These three paradigms have different benefits and drawbacks. Method 1 is generally the most favorable as it utilizes all of the spectral count data generated in the 4 MS/MS analyses along with the spectra distribution characteristics of each sample. However, this method can fail when replicates have significantly different statistical properties.

Method 2 includes all of the available spectral data into one analysis but fails to utilize information contained within the individual statistical properties of each sample. The creators of QSPEC analyzed algorithm performance with summed vs. distinct replicates and demonstrated, with replicates having similar statistical distributions, that this type of combination yielded satisfactorily equivalent results to method 1[39].

Method 3 has the benefit of utilizing all of the available spectral and sample distribution information but suffers because the data is divided between two separate analyses. To merge the results of method (3), a simple scoring metric was employed. Proteins found to be over-expressed in the VS sample of either analysis were given a score of +1, those up-regulated in the CI samples a -1, and those without differential expression were given a score of 0. Scores were summed across the two analyses resulting in individual protein scores ranging from -2 to +2.
6.3 Results

A total of 271 proteins were identified at high confidence within the four perilymph samples (Fig. 6-2). The number of proteins identified (MS/MS spectra generated) per sample was: CI_1: 225 (35,027), VS_1: 167 (21,272), CI_2: 106 (112,465), VS_2: 90 (111,127). As none of the samples can be considered clinically normal, we began our analysis with the conservative requirement that a protein must be present in all four samples to be considered part of the core proteome. This analysis generated a list of 71 proteins identified with high confidence in all four samples (Tab. A.1). Comparison of this list to previous work[163], using 2-D gel electrophoresis of perilymph obtained during stapedectomy procedures, shows strong similarity. Our list of 71 proteins contains 92% of those previously identified, and adds another 46 to the knowledgebase.

Figure 6-2: Distribution of 271 proteins identified at high confidence within four samples of human perilymph.

If the ‘normal’ proteome inclusion requirement is loosened to include proteins identified in only three of the four samples the number of identifications increases to 99 (lower panel of Tab. A.1). This number is similar to the number of protein spots separated by Thalmann et al.[163] and includes 100% of the proteins previously identified. Such a reduction of inclusion criteria may be warranted based on the low levels of reproducibility observed in MS analyses (< 60% similarity across repeated trials[181]), suggesting the requirement that a protein be present in all four samples.
is overly stringent.

6.3.1 Similarity to CSF, blood plasma, and mouse perilymph

Comparing the 71 common protein identifications with published proteomes of human cerebrospinal fluid (CSF) and blood plasma, we note a strong similarity across the fluids (Tab. A.1). The CSF database downloaded from the Max-Planck Unified Proteome Database (MAPU)[182, 186] contained 56 of 71 of the proteins we identified in perilymph while the plasma database obtained from Plasma Proteome Institute (PPI)[10, 9] contained 56 of 71. In each case, the majority of the non-common proteins are suspected contaminants (keratins, carbonic anhydrases). This similarity among human fluids was expected as animal models of the fluids contain similar protein complements. However, the quantity of individual proteins and the total protein content varies widely[162].

Comparison of our perilymph dataset with the proteome of mouse perilymph published by Swan et al.[156] revealed moderate similarity across the two fluids (Tab. A.1). Orthologues of 31 of the 52 proteins identified in that investigation were found in our list of human perilymphatic proteins (orthology determined using OrthoDB[172]).

6.3.2 Quantitative comparison of CI and VS samples

The first iteration of QSPEC analysis, employing method 1, resulted in the identification of 104 proteins with differential expression between CI and VS samples. As mentioned above, this method was favorable due to its utilization of all available spectral count data in one analysis. However, the high degree of dissimilarity in spectral distribution properties, owing to the large difference in the number of spectra acquired by each MS/MS platform, resulted in poor model behavior and unreliable Bayes factors. The normalizing constant \( N_j \) employed in the generalized linear mixed effects model within QSPEC can adequately account for small differences in total spectral counts between replicates, but in this comparison the difference was large (>4 fold).
At the protein level this causes heterogeneous counts within and across disease states. This results in poor model fit and inflation of the Bayes factor due to increased model flexibility when the differential expression term is included. Such false identifications can be partly filtered by only considering fold changes in excess of 50%[39] as valid, as this reduces the chances of over-fitting, but the extreme deviation in these data sets may render such methods insufficient.

QSPEC analysis using method 2 resulted in identification of 324 differentially-expressed proteins. Summing counts within disease state had the benefit of generating two data sets with similar statistical properties (resulting in better stability of the model), but did so at the cost of information about each samples spectral distribution statistics. This informational loss induced the large discrepancy between the results of methods 1 and 2 and caused very different model behavior than QSPECs authors experienced with their statistically more similar replicates[39]. To illustrate a common conflict, consider the protein hornerin (HRNR). HRNR was identified based on 22 spectra in sample CI 1 and was not found in any other sample. Using method 1, HRNR was not determined to be differentially expressed because the zero spectra from CI 2 suppressed the Bayes factor below significance. However in method 2, QSPEC compared 22 spectra from the CI samples vs. 0 spectra from the VS samples and determined it to be differentially expressed. Furthermore, because the number of spectra associated with samples CI 2 and VS 2 was significantly greater than CI 1 and VS 1, this analysis paradigm gave heavy weighting to the second set of samples.

The final QSPEC analysis, method 3, identified 25 proteins scoring ±2 (indicating up-regulation in both VS or CI samples respectively) and 271 proteins scoring ±1 (up-regulated in one VS or CI sample while the other analysis did not reach significance). In addition to the hundreds of proteins which scored ‘0’ because neither analysis reached significance, twenty proteins scored ‘0’ due to conflicting expression patterns across the two sample groups. Despite the drawback of splitting the data, this approach worked well as each analysis compared data sets with similar statistical parameters and took full advantage of information imbedded within sample spectral distributions. This resulted in improved model stability and more confident determin-
Table 6.1: Candidate biomarkers of human vestibular schwannomas associated with poor hearing — identified via uniqueness criteria and spectral count analysis

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
<th>Entrez Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRYM</td>
<td>μ-Crystallin</td>
<td>1428</td>
</tr>
<tr>
<td>FN1</td>
<td>Fibronectin 1</td>
<td>2335</td>
</tr>
<tr>
<td>KRT10</td>
<td>Keratin 10</td>
<td>3858</td>
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<td>Apolipoprotein C-III</td>
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<td>Versican</td>
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<td>Dermcidin</td>
<td>117159</td>
</tr>
<tr>
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<td>Serpin peptidase inhibitor, clade B, member 12</td>
<td>89777</td>
</tr>
<tr>
<td>CTSD</td>
<td>Cathepsin D</td>
<td>1509</td>
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<td>Superoxide dismutase 3, extracellular</td>
<td>6649</td>
</tr>
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<td>Parkinson disease protein 7</td>
<td>11315</td>
</tr>
<tr>
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<td>Serpin peptidase inhibitor, clade F, member 1</td>
<td>5176</td>
</tr>
<tr>
<td>CHI3L1</td>
<td>Cartilage glycoprotein 39</td>
<td>1116</td>
</tr>
<tr>
<td>LRP2</td>
<td>Low density lipoprotein receptor-related protein 2</td>
<td>4036</td>
</tr>
</tbody>
</table>

*italics: found only in VS samples; bold: scored 2 in QSPEC analysis*

...nations of differential expression. As such, the results of method 3 were selected for further analysis.

It is important to note that all of the spectra generated in the MS/MS analysis of the 4 samples, with no limitations on spectra confidence, were analyzed by QSPEC. In cases where a protein was evidenced by many low-confidence (but no high-confidence) spectra this resulted in a QSPEC report of differential expression, even though the protein in question did not meet requirements for a confident identification.

When the results of method 3 are filtered to remove these non-significant returns, isoform conflicts and immunological proteins, 3 proteins remained that scored ±2 (CRYM, FN1, and KRT10) and 65 that scored ±1. Of these 68 proteins, 14 were selected as particularly interesting due to their differential expression and biological function, Table 6.1.
6.4 Discussion

Interestingly, while the total protein content of the VS samples, as determined by SDS-PAGE analysis, was greater than the CI samples (due to higher protein concentration in perilymph from VS patients, data not shown), more identifications were made within the latter group based on our search criteria (combined VS samples: 171; combined CI samples: 237). This difference may be real, indicating decreased complexity of the VS perilymph proteome due to reduced protein secretion (possibly because of tissue loss), or may be an artifactual result from limitations of the dynamic range of detection. Tumor induced up-regulation of a small number of proteins may have suppressed the signal of lower abundance proteins, thus impeding their detection. Separate analysis of each section of the electrophoresis gel likely reduced, but did not eliminate, the potential signal suppression due to high abundance proteins.

Comparison across samples demonstrates that more proteins were identified in each of the samples derived from the specimen of one patient (CI\textsubscript{1} and VS\textsubscript{1}) than in the samples derived from the specimens of five patients (CI\textsubscript{2} and VS\textsubscript{2}). This disparity may have a similar cause as above in that pooling of samples led to suppression of the signal from low abundance proteins. However, the higher resolution and mass accuracy of the LTQ/Orbitrap MS used to analyze samples CI\textsubscript{1} and VS\textsubscript{1} is the most likely reason for the observed increase in identifications. In the context of the experiment, this discontinuity would likely serve to reduce the number of identifications common across all samples and individual disease groups.

Beyond the obvious limitations induced by the use of pathologic specimens, additional evidence for the need to perform a deeper characterization of perilymph comes from the comparison with published proteomes of human CSF and blood plasma. Both databases are significantly larger than the currently observed perilymph proteome, as each is composed of several hundred proteins. However, these body fluids are plentiful and large volumes in the ml-range are available in contrast to perilymph, of which only single digit \(\mu\)l-amounts can be obtained. Thus, multidimensional separation strategies that are optimized for minimized sample losses would be highly
advantageous for the comprehensive analysis of the perilymph proteome.

Direct and quantitative comparison of perilymph with CSF and/or plasma, similar to previous work with mice[156], would improve our understanding of human perilymph as a functional fluid. Such work would also be useful to assess the possibility that the similarity between perilymph and plasma is due to blood contamination of the perilymph sample.

It is worth noting that the list of 71 common proteins contains several that may not be of perilymphatic origin. The robust presence of carbonic anhydrases (common in red blood cells), multiple keratins and hemoglobins suggest that samples have suffered contamination from blood and cellular debris. However, carbonic anhydrase is known to be abundant in the cochlea, comprising about 1% of the protein of the membranous lateral wall[47]. In this analysis, we tried to minimize blood contamination by using visibly transparent samples of perilymph and excluding obviously pink specimens. Standardized methodologies must be carefully implemented in the future to limit sources of contamination and establish whether these proteins are in fact typical components of perilymph.

6.4.1 Candidate Biomarkers of VS

Exact mechanisms of hearing loss associated with VS are not known as some VS induce hearing loss while others do not, a phenomenon that shows poor correlation with tumor size or growth[48, 55, 110]. Evidence suggests that these tumors cause hearing loss not only by compressing the auditory nerve but also by secreting a substance toxic to the cochlear nerve or inner ear[153, 155]. In line with this hypothesis, we compared the VS and CI samples to generate an initial list of candidate biomarkers for VS associated with poor hearing (all samples were collected from patients with severe to profound deafness in the affected ear). Candidates were identified using uniqueness criterion, i.e. identified within both VS but neither CI sample, and differential expression analysis using the QSPEC algorithm for spectral counting.

Two proteins where identified in both VS samples but neither CI sample, Fig. 2. The first, \( \mu \)-crystallin (CRYM, encoded by CRYM) is also known as NADPH-
regulated thyroid hormone binding protein. CRYM is found in the cytoplasm where it binds and promotes transcriptional activity of the thyroid hormone triiodothyronine (T3)[76]. Expression occurs within many cells of the spiral ligament[169] and mutation results in autosomal dominant deafness through changes in intracellular localization and the loss of its T3 binding ability, potentially leading to impaired K+ recycling[1, 118].

The second protein found within both VS samples and neither CI sample was low density lipoprotein-related protein 2 (LRP2, encoded by LRP2, also known as megalin). LRP2 is a trans-membrane receptor protein found primarily in absorptive epithelial cells, including those of the ear and kidney[35, 51, 82, 131]. LRP2 can bind a wide variety of structurally dissimilar ligands and is a key player in mediating endocytosis of many substances including lipoproteins, sterols, vitamin binding proteins, and hormones. Mutations in LRP2 result in Donnai-Barrow and facio-oculo-acoustico-renal (FOAR) syndromes[79], both of which present with sensorineural hearing loss. Surprisingly, LRP2 was not found to be differentially expressed by QSPEC because its spectral count in each VS sample was small. Despite this, LRP2 has been included in the list of potential candidates because of its detection pattern; LRP2 will be carefully validated in future work.

Analysis of the remaining proteins identified in Table 6.1 shows enrichment for many serine protease inhibitors. This class of molecules plays a critical role in regulating the inflammation response. Similarly, PARK7 and SOD3 both play active roles in regulating oxidative stress while CTSD and VCAN have been implicated in both cancer and neurological disorders. These molecules therefore appear particularly relevant to our interest in illuminating mechanisms of inner ear damage and will be investigated in future work.

### 6.5 Conclusion

Analysis of human perilymph specimens has provided novel insight into the protein content of the inner ear fluid microenvironment. However, the specimens originate
from clinically diseased ears, and thus may not represent the complete proteome of normal perilymph. Using mass spectrometry, we have more than doubled the known proteome to include over 70 proteins. This number is less than the approximately 100 proteins previously separated using 2D gel electrophoresis[163], although the gap may be narrowed by loosening the stringency of our inclusion criteria, as supported by known limitations of MS analysis. The proteins in perilymph are found to be similar to CSF and plasma, but quantitative comparison is not possible at this time. Human perilymph proteins are also similar to those in mouse perilymph, lending potential support to the use of a mouse model as a vehicle for biomarker discovery. Finally, a list of 15 candidate biomarkers of VS is generated, with some candidates having known roles in hearing and deafness, and other candidates showing significant up-regulation as determined via spectral count analysis with QSPEC.
Part IV

Inner Ear Energetics
Chapter 7

Energy extraction from the biologic battery in the inner ear[103]

Abstract

The endocochlear potential (EP) is a battery-like electrochemical gradient found in and actively maintained by the inner ear[71, 19]. The EP has never before been used as a power source for electronic devices. Here we demonstrate, for the first time, that it is possible to operate an electronic system using a mammalian EP as the source of energy into the system. This was achieved by designing an anatomically-sized, ultra-low quiescent-power energy harvester integrated with a wireless sensor. Although other forms of in-vivo or electrochemical energy harvesting have been previously shown[72, 65, 130, 36, 128, 74], none are currently suitable to power implanted electronic devices in the vicinity of the ear, eye, and brain of mammals. With future research in electrode design, we envision using this biologic battery to power chemical sensors or drug-delivery actuators, enabling transformative opportunities for new diagnostics and therapies in hearing research and related fields.

7.1 Introduction

Implantable electronics for medical applications typically require large energy reservoirs to operate reliably over long periods of time. In both human healthcare and animal studies, anatomy often limits the size of implantable batteries, requiring sur-
gical re-implantation or cumbersome external wireless power sources for long-term operation. Harvesting energy from nearby energy sources is an alternative approach to extend implant life, and with sufficient available energy, to allow the implant to operate autonomously. Since humans and animals already generate and consume a vast amount of power\[127\], biologically-based energy harvesting is a potentially well suited solution to power implanted devices. However, some biologically-derived energy sources such as heat differentials\[74\] and muscle movements\[46\] are not suitable for mammalian implantable applications due to the requirements of externally worn apparatuses. While promising, previously described in-vivo energy sources, such as kinetic vibrations\[128\] and biofuel cells\[72, 65, 130, 36, 28\], are inherently intermittent, are not generated by mammals, or are presently too difficult to extract in anatomically-sized devices.

Figure 7-1: Anatomy and physiology of the inner ear. Schematic of a mammalian ear including the external, middle, and inner ear, which includes the cochlea and vestibular end organs (a). Cross section of a typical cochlear half turn highlighting the endolymphatic space (yellow) bordered by tight junctions (red), the stria vascularis (green) and hair cells (blue), which are contacted by primary auditory neurons (orange) (b).

At 70–100 mV, the EP is the largest positive direct current (DC) electrochemical potential in mammals, and is the main driving force for cochlear mechanotransduction of sound pressure wave to neurotransmitter release and excitation of the auditory
nerve. Although the EP is well understood and modeled[159, 43, 71], it has never before been harvested to drive electronics. Its inherent stability over mammalian lifetime[91] makes it a potential candidate for powering long-term energy-autonomous implantable devices. The EP is found within endolymph — a specialized inner ear fluid with a uniquely higher concentration of potassium than is found intracellularly. Endolymph is separated from the surrounding extracellular spaces bathed in perilymph, which is similar in ionic composition to the cerebrospinal fluid, by a complex network of tight junctions (Fig. 7-1). The EP effectively acts as a biologic battery whose potential is actively stabilized by a specialized arrangement of potassium channels, pumps and cotransporters (Fig. 7-2) in cells of the stria vascularis, a specialized structure that borders the endolymphatic space.

Any electronic system that interfaces and extracts power from the EP should not interfere with normal function of the cochlea. Total current flow through the guinea pig cochlea (including both sensory and non-sensory cells), whose anatomy and physiology is similar to that of humans, has been previously determined to be 0.8–1.6 $\mu$A/mm of cochlear wedge[184]. Given the 18 mm length of the guinea pig cochlea[54], currents generated by the stria vascularis on the order of 14–28 $\mu$A occur in the guinea pig physiologically, and place an upper limit on extractable electric current from the cochlea.

### 7.2 Results and Discussion

In practice, the maximum current extractable from the EP by an implanted energy harvester is constrained well below this limit by high impedance electrodes, which are designed to have sharp tips such that, when inserted into the EP space, collateral cell damage is minimized. The input power to the implantable energy harvester, which we refer to as the endoelectronics chip, is given as: $P = \frac{V_{IN}^2}{R_{in,eff}} = \frac{V_{EP}}{(R_{in,eff} + R_{elec})^2} R_{in,eff}$ where $V_{EP}$ is the endocochlear potential, $R_{elec}$ and $R_{in,eff}$ are the impedances of the glass electrodes and the endoelectronics chip, respectively, and $V_{IN} = \frac{V_{EP} R_{in,eff}}{(R_{in,eff} + R_{elec})}$ is the effective input voltage to
To generate and maintain EP, perilymphatic K$^+$ enters fibrocytes (F) via Na$^+$/K$^+$-ATPase and Na-K-Cl–cotransporter. Gap junction networks connect F to strial basal (SB) and intermediate (SI) cells, allowing ions to enter the intra-strial space via KCNJ10 channels. Strial marginal (SM) cells uptake K$^+$ against a significant concentration gradient and release K$^+$ into endolymph. K$^+$ returns to perilymph either by entering hair cells (HC) apically during mechano-electrical transduction of sound and exiting basolaterally via KCNQ4 channels, or via leakage current through the surrounding tissues (solid red lines represent tight-junction networks). With energy from the EP, a boost converter was used to trickle-charge capacitor $C_{DD}$, which supplies power to an integrated wireless transmitter. Switch $S_1$ in the converter was used to temporarily store energy in the inductor (L), and switch $S_2$ was used to transfer the stored energy onto $C_{DD}$ at the higher required voltage. Once $C_{DD}$ had stored sufficient energy, the transmitter entered its active mode and wirelessly transmitted a packet to a nearby receiver, after which it returned to a low-leakage standby mode and the boost converter began to replenish $C_{DD}$. A start-up rectifier was used with an external wireless power source to initialize the system.

To maximize the extractable power given electrode impedance constraints, $R_{in, eff}$ needs to be configured close to $R_{elec}$. For traditionally used glass microelectrodes, the tradeoff between electrode impedance and bluntness reaches a point of diminishing returns at $R_{elec} = 0.4–1.1$ MΩ. This results in extractable power ranging from 1.1–6.3 nW with $V_{IN}$ in the range of 30–55 mV.
The low EP voltage, in conjunction with low extractable power, poses two significant challenges for harvesting net positive energy. First, transistor-based electronics require hundreds of millivolts to start-up without requiring expensive semiconductor post processing [37, 157]. Second, the maximum extractable power levels are at least an order of magnitude less than the quiescent power of the most efficient energy harvesting and sensing circuits in the literature [174, 146, 129]. For continuous, self-sustaining operation, quiescent power should be well below the maximal extractable power.

We overcame the challenges of energy harvesting from the EP with a custom integrated circuit featuring a wireless kick-start energy receiver and ultra-low-quiescent-current energy-buffering power electronics. The low-voltage turn-on issue was addressed by delivering an initial one-time wireless start-up charging packet of less than two seconds between an external radio frequency (RF) source and an on-board 3 × 4 mm² loop antenna. This charging packet was converted from RF power to DC in order to charge a 200 nF energy buffer capacitor, \( C_{DD} \) (Fig. 7-2), up to a maximum of 1.4 V. At such a voltage, \( C_{DD} \) contained up to 200 nJ, which was sufficient to operate the system for at most six minutes before the stored energy was completely exhausted (given a total system power consumption of 573 pW as discussed below).

To sustain the charge on \( C_{DD} \) once the external RF source was removed, a boost converter harvested the low-voltage EP energy by transferring and converting this energy to a higher voltage suitable for buffering energy onto \( C_{DD} \). For system sustainability, the quiescent power consumption of the circuits that control the boost converter should be much less than the available output power. We implemented a continuously running timer and driver circuits that consumed 527 pW at 0.9 V, controlling the boost converter and activating a load circuit every 40–360 seconds. The boost converter achieved an efficiency of 53.4%. After including the timer and driver overhead, 60–2840 pW of net positive power was extracted and available for the load circuit.

In any implanted sensing system, communication is essential to convey sensed information to the external world. Here, we integrated a wireless radio transmitter
that, through the use of extensive leakage power reduction techniques[134], consumed a standby power of 46 pW at 0.9 V. To minimize the implants physical volume, the transmitter shared the on-board antenna with the wireless kick-start circuit. The output data rate was set by an integrated ring oscillator, and could operate at instantaneous data rates programmable from 100 kbps to 10 Mbps up to a 1 m distance. Combined, the active energy consumption, packet length, and standby power dictate the maximum frequency of packet transmissions. With the given power budget, the transmitter spent approximately 0.0001% of its time in active mode.

The chip was fabricated in a standard complementary metal-oxide-semiconductor (CMOS) process with a minimum feature size of 0.18 µm, and occupied a total volume of $2.4 \times 2.4 \times 0.2$ mm$^3$. The chip was wirebonded to a printed circuit board prototype (Fig. 7-3a) that is currently sufficiently small to be implantable in the human mastoid cavity, or in the bulla cavity of cats, gerbils, or chinchillas (Fig. 7-1)[101].

We operated the chip for up to five hours using the EP of an anesthetized Hartley Albino *Cavia porcellus* (guinea pig) as the source of energy into the system, following an initial wireless kick-start. For feasibility studies, the system was operated externally, with the electrode tips inserted into the cochlea and the electrode shafts connected to the endoelectronics chip located externally (Fig. 7-4). Figure 7-3b shows the $C_{DD}$ system supply voltage, $V_{DD}$, which replenished and persisted throughout a 20 minute measurement (Fig. 7-5 shows the results from a five hour experiment). Since the system operated longer than the energy contained in the initial kick-start otherwise permitted, these results demonstrate, for the first time, an energy harvesting system powered primarily from a mammalian electrochemical potential. As the boost converters average input impedance was configured to approximately equal the electrode impedance, the converter extracted close to the maximal possible power from the EP given physical constraints of electrodes. As a result, $V_{DD}$ slowly fluctuated as the animals EP, and therefore the available energy into the system varied.

On a smaller time scale, as shown in Fig. 7-3c for three different experiments, $V_{DD}$ periodically dropped by 36–48 mV during active-mode wireless transmissions. Following the voltage drop, the boost converter continued to harvest energy from the
Figure 7-3: Physical implementation and measurement results of the endoelectronics system. Photograph of the prototype endoelectronics board on a human index figure (a). Transient waveform of $V_{DD}$ over the course of 20 minutes when operating with a guinea pig EP as the only source of energy into the system (apart from the initial wireless kick-start) (b). Transient waveform of $V_{DD}$ for three different experiments on three different animals. A 300 second time scale was used to show $C_{DD}$ charging from the boost converter harvesting EP energy, as well as rapid periodic discharging from wireless transmissions (c). Measurement of $V_{IN}$, which is a measurement of EP following a voltage drop across the electrode impedance. The black line is measured by an external device powered by a battery, while the light grey line is the $V_{IN}$ estimate derived directly from the instantaneous data rate of wirelessly received data after a one-time normalization calibration (d). Compound action potential thresholds measured before and after the current extraction experiment shown in sub-panel b (e).

EP by trickle-charging capacitor $C_{DD}$, thereby recovering energy spent during the transmission.

We used energy harvested from the EP to not only power a wireless transmitter, but also to sense relative changes in the EP after the voltage drop seen in the electrode resistance (i.e., $V_{IN}$). Because the instantaneous radio data rate was generated internally by a ring oscillator whose frequency varied with $V_{DD}$ (which in turn varied with the EP and therefore $V_{IN}$), the wirelessly received data bits were inherently encoded with EP information. Thus, by monitoring the output data rate we demonstrated a self-powered system that is also capable of continuously monitoring relative changes in the EP (Fig. 7-3d). Trends in sensed $V_{IN} = V_{EP}R_{in,eff}/(R_{in,eff} + R_{elec})$, powered
by the EP, were within a 0.45 mV RMS error of direct measurements by an external battery-powered device over a 2.5 hour experiment.

To test whether the system affected hearing, compound action potential thresholds were measured before and after electrode insertion (Fig. 7-6), and before and
Figure 7-6: Audiogram data before and after the positive electrode is inserted into endolymph from perilymph in an anesthetized guinea pig. In both tests, the negative electrode is located in perilymph. Within the measurement tolerances, the result shows minimal-to-no hearing loss after electrode insertion across all frequencies.

after current draw (Fig. 7-3e) in two different anesthetized guinea pigs, respectively. Because the cochlea is tonotopically organized, with the high frequencies encoded at the cochlear base, and low frequencies encoded at the cochlear apex, cochlear function was tested along its length by using tone pips of different frequencies. Insertion of the beveled glass microelectrodes did not adversely affect hearing (Fig. 7-6). Likewise, current draw during the experiment shown in Fig. 7-3b had essentially no effect on hearing for frequencies up to 16 kHz (Fig. 7-3e). A small degradation in threshold was observed at a frequency of 23 kHz, which is encoded close to the round window (near the cochlear base). This degradation is likely due to the electrode tip, which was designed to be $\sim 2\mu m$ in diameter after beveling so to allow low electrode impedance, typically $\sim 800$ k$\Omega$, which is required for energy extraction from the EP. At such sizes, electrodes can cause local physical trauma to the cells lining the basilar membrane, and thereby allow leakage of ions across the endolymph-perilymph barrier, as well as leakage of ions from the fluid-filled electrodes into cochlear fluids. In contrast, electrodes that have been used to stably record the EP for many hours have tips $< 1\mu m$ in diameter, and impedances greater than 5 M$\Omega$[105]. Taken together, our data point
that major improvements in electrode design are required to allow eventual long-term energy extraction from the EP. In the meantime, our prototype system demonstrates feasibility with minimal effect on hearing in a short run.

With future miniaturization of low-impedance electrode tip, and with refinements in minimally invasive surgical approaches to endolymph, (e.g. via Reissners membrane[18]), our system would be useful in animal studies with far-reaching implications for biotechnology and medicine. For example, since small rodents are commonly used in hearing research, the proposed energy harvesting system could enable miniaturized and fully implantable sensing systems with minimal risk of malfunction from external infections or trauma. We envision implementing chemical and other electronic sensors and actuators, not only in the cochlea and the nearby vestibular end organs of the inner ear (Fig. 7-1), but also in the adjacent structures such as the temporal lobe of the brain, facial nerve, and carotid artery, all of which are within millimeters of the cochlea.

In the future, energy extraction from the cochlea may be applicable to humans. Surgical insertion of devices, such as cochlear implants and stapes prostheses, into the human inner ear is already possible with minimal risks of damaging normal or residual hearing[109, 53]. Although the risk of hearing loss is higher when inserting an electrode into endolymph than perilymph, there are many forms of deafness where the EP is normal, such as deafness due to loss of sensory cells, cochlear neurons, or supporting cells. In such cases, the benefit of a battery-less system for sensing of key molecules in the inner ear may outweigh the potential risk to the EP during electrode insertion into endolymph. Although we have identified and overcome key challenges in developing the components of the system necessary to harvest net positive energy from the inner-ear for hours at a time, important future work is required in electrode miniaturization and surgical approaches in order to advance from demonstrating feasibility, to integrating all components into a safe, fully-implantable system for long-term operation.
7.3 Methods

Surgical procedures were performed at the Massachusetts Eye and Ear Infirmary with the approval of the institutional Animal Care Committee (protocol # 09-09-026). Animals were anesthetized and experiments were conducted in a heated, acoustically insulated chamber. Most animals had spontaneous, natural breathing. Mechanical ventilation was only used if respiration faltered due to anesthesia. The auricle and neighboring musculature were reflected ventrally to expose the external auditory meatus and bulla. The lateral wall of the bulla, up to the caudal edge of the tympanic ring, was removed to allow visualization of and access to the round window. Pulled glass microelectrodes, mounted on micromanipulators, were advanced through the round window to access the fluid spaces of the inner ear. The negative electrode was inserted into the perilymph-filled scala tympani, and the positive electrode was inserted through the basilar membrane and sensory epithelium into the endolymph-filled scala media (Fig. 7-7). Microelectrodes were pulled from Borosil capillary tubing (FHC, Bowdoin, ME). The tips of electrodes were beveled at a 25° angle using a BV-10 Microelectrode Beveler (Sutter Instruments, Novato, CA) to achieve tip diameters of ~2 μm and electrode impedance of ~400-800 kΩ for both electrodes. Electrodes were mounted in half-cell holders containing Ag/AgCl exchange pellets (World Precision Instruments, Sarasota, FL). Electrolyte composition was 2 M KCl. All circuit grounds were with reference to perilymph.

Tone-pip audiograms were constructed by presenting brief tones (3.0 ms duration, 0.5 ms rise/fall times), at half-octave spacing between 2–32 kHz, to the external ear canal of the surgically targeted ear. The resulting compound-action potential was recorded using a metal-wire electrode placed adjacent to the round window. The threshold was defined as the minimum sound intensity required to elicit a response above 10 μV (the noise floor was measured to be approximately 4 μV).

The printed circuit board was manufactured with an FR-4 substrate, and the chip and two capacitors were mounted on the top of the board, while the inductor was mounted on the bottom. The chip was encapsulated by a non-conductive epoxy for
mechanical stability (not shown in Fig. 7-3a for clarity). The system supply voltage was measured during in vivo experiments using a Keithley 2602 sourcemeter, while $V_{IN}$ was measured using an Agilent U1253A set to 1GΩ input impedance. A wireless receiver, built using discrete components, was used to down-convert and record the instantaneous transmitter data rate on a Tektronix TDS3064B oscilloscope. The chip power consumption was measured during characterization experiments using a Keithley 6430 sourcemeter and high-isolation triaxial cables for accurate low-current measurements.

The chip was initialized with a wireless kick-start from an external RF source operating at 2.4 GHz placed several millimeters away for no more than two seconds. Due to on-chip voltage clamps, capacitor $C_{DD}$, which had a capacitance of 200 nF, charged to at most 1.4 V during initialization. At 1.4 V, capacitor $C_{DD}$ was storing $E = 0.5C_{DD}V_{DD}^2 = 200 \text{ nJ}$ of energy. During operation, the boost converter, radio, and peripheral circuits consumed 573 pW (at 0.9 V). At this power consumption, the circuit would theoretically operate for at most 6 minutes before completely exhausting the energy stored on capacitor $C_{DD}$; in practice, time-to-exhaustion is typically much shorter. Taken together, any experiment lasting longer than a few minutes extracted
energy from the EP.
Appendix A

Supplemental Tables
Table A.1: Proteins identified in multiple samples of human perilymph using RP-HPLC MS/MS.

<table>
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<th>Symbol</th>
<th>Name</th>
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<th>CSF</th>
<th>Plasma</th>
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<td>X</td>
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<td>X</td>
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[145] Takashi Shimada, Itaru Urakawa, Yuji Yamazaki, Hisashi Hasegawa, Rieko Hino, Takashi Yoneya, Yasuhiro Takeuchi, Toshiro Fujita, Seiji Fukumoto, and


