Transcriptomic Analysis of the Developing and Adult Mouse Cochlear Sensory Epithelia

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Abstract

The adult mammalian cochlea lacks regenerative ability and the irreversible degeneration of cochlear sensory hair cells leads to permanent hearing loss. Previous data show that early postnatal cochlea harbors stem/progenitor-like cells and shows a limited regenerative/repair capacity. These properties are progressively lost later during the postnatal development. Little is known about the genes and pathways that are potentially involved in this difference of the regenerative/repair potentialities between early postnatal and adult mammalian cochlear sensory epithelia (CSE). The goal of our study is to investigate the transcriptomic profiles of these two stages. We used Mouse Genome 430 2.0 microarray to perform an extensive analysis of the genes expressed in mouse postnatal day-3 (P3) and adult CSE. Statistical analysis of microarray data was performed using SAM (Significance Analysis of Microarrays) software. We identified 5644 statistically significant differentially expressed transcripts with a fold change (FC) ≥ 2 and a False Discovery Rate (FDR) ≤ 0.05. The P3 CSE signature included 3,102 transcripts, among which were known genes in the cochlea, but also new transcripts such as, Hmgaa2 (high mobility group AT-hook 2) and Nrarp (Notch-regulated ankyrin repeat protein). The adult CSE overexpressed 2,542 transcripts including new transcripts, such as Prl (Prolactin) and Ar (Androgen receptor), that previously were not known to be expressed in the adult cochlea. Our comparative study revealed important genes and pathways differentially expressed between the developing and adult CSE. The identification of new candidate genes would be useful as potential markers of the maintenance or the loss of stem cells and regenerative/repair ability during mammalian cochlear development.

Introduction

Cochlear sensory epithelium (CSE) contains the auditory receptors referred to as hair cells (HCs) that are essential for hearing [1]. These sensory cells can be damaged as a consequence of acoustic trauma, ototoxic drugs, or simply with aging. Although, HCs in mammals are produced only during embryonic development and not able to regenerate when lost during the postnatal period of maturation [2], some studies using in vitro assay suggested a limited non-proliferative regeneration/repair capacity within the ototoxic-damaged explants derived from the early postnatal CSE [3–5]. It has been also suggested from studies using transgenic and knock-out mice [6–10] that some proliferative potential, although restricted under normal in vivo conditions, is retained in the early postnatal CSE. In addition, recent studies demonstrated the presence of stem/progenitor cells within the postnatal-P3 mouse CSE and their mitotic capacity to form clonal spheres when maintained under appropriate in vitro conditions [11–14]. Nevertheless, this stem cell population is progressively exhausted during later postnatal development [12]. Recently, we also showed that the supporting cells in the mouse postnatal CSE express many stem/progenitor markers which were down regulated in the adult, that could be correlated to the loss of stem/progenitor cells within the adult mammalian cochlea [14]. Thus, comparison of expression profiles between P3 and adult mouse CSE is hypothesized to identify differentially regulated genes involved in stem/progenitor cell maintenance and the capacity of this sensory epithelium for regeneration/repair.

DNA microarray is a powerful technology that currently permits comparison of gene expression at the whole-genome scale [15]. Gene expression profiling using microarrays has been applied in the inner ear during the past decade, especially in bird, fish and rodent species. Gene expression analysis within the bird inner ear was especially investigated in order to understand the molecular mechanisms that control the regeneration capacity [16,17], and also to gain insights on the genetic programs that control inner ear development [18]. In zebrafish, microarrays were applied to investigate the specific transcriptome of HCs [19]. Recently, one transcriptional analysis on zebrafish inner ear after acoustic trauma revealed growth hormone, as critically involved in the post-trauma regeneration process [20]. In mammals, gene expression profiling was investigated in order to identify tissue specific genes and/or to examine changes in gene expression under several conditions [21–25]. Regarding the gene expression changes in the mammalian cochlea during maturation, only a limited number of studies have been performed. Chen and...
Corey [26], used GeneChip arrays (i.e., oligonucleotide array set covering 15,000 known genes and 21,000 EST clusters) to explore the gene expression patterns in a whole cochlea between two developmental stages (i.e., P2 and P3) reporting a differential gene expression that correlates with the onset of cochlear function. In our study, we compared, for the first time, the whole genome expression profiles between the postnatal P3 and adult cochlear stages, using mouse chip Affymetrix 430.02.

The aim of this study is to explore changes in genes and pathways underlying the known difference in the stem/progenitor cells maintenance and in the capacity of regeneration/repair between P3 and adult CSE.

Materials and Methods

Ethics Statement
All animal work was conducted according to the Guide to the Care and Use of Laboratory Animals [27] and all procedures were approved by ethics Committees of the INNERM (Institut National de la Santé et de la Recherche Medicale) and CNRS (Centre National de la Recherche Scientifique).

Culture of mouse embryonic stem cells (mESCs)
The undifferentiated mESCs (CGR8 line kindly provided by Bernard Binetruy, Aix-Marseille University, France) were expanded in the absence of feeder cells in DMEM culture medium (Gibco by Life Technologies) supplemented with LIF (leukemia inhibitory factor) on gelatin-coated plates. When the propagated cells were confluent at 80-90% (about 5-7 days), they were passaged using 0.25% trypsin-EDTA (Gibco by Life Technologies). The undifferentiated and untreated cells used for immunohistochemistry were harvested from passage 2 cell cultures and fixed in paraformaldehyde 4% in Phosphate Buffer Solution (PBS) for 20 min at room temperature. After several rinses they were processed for immunohistochemistry with HMGAA2 antiserum following the same immunostaining protocol used for cochlear tissue sections.

Cochlear tissue preparation and Immunohistochemistry

For postnatal day-3 (P3) cochlear tissue, the animals were decapitated and their cochleas were removed and immersed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C overnight. Next, the tissue was washed in PBS, and incubated on ice in 10%, sucrose solution for 0.5 h, and after, the tissue was washed in PBS, and incubated on ice in 10%, sucrose solution for 0.5 h, and then incubated in 20% sucrose at 4°C overnight. The tissue was then incubated in 1:1 Cryo-OCT Compound (VWR, France) and 20% sucrose at 4°C overnight. Then, the tissue was washed in 100% OCT for 10 min and then incubated in 20% sucrose at 4°C overnight. The tissue was then washed in PBS, and incubated on ice in 10%, sucrose solution for 0.5 h, and then incubated in 20% sucrose at 4°C overnight. The tissue was then incubated in 1:1 Cryo-OCT Compound (VWR, France) and 20% sucrose at 4°C overnight. Then, the tissue was washed in 100% OCT for 10 min and then fixed in cryosectioned in 10 um sections, and subsequently stored at -20°C.

For labeling experiments, the tissue cryosections were incubated in PBS supplemented with 4% normal BSA plus 0.3% Triton X-100 (Sigma, France) for 2 h. Next the cryosections were incubated in a solution containing 1:200 diluted anti-rabbit antisem with HMGAA2 antiserum following the same immunostaining protocol used for cochlear tissue sections.

Sample Collection and RNA Extraction

RNA samples used in this study were extracted from CSE dissected from postnatal day three (P3) and eight-week-old adult Swiss Webster mice. We used this mouse strain because it keeps normal hearing beyond eight weeks [28]. P3 mice were sacrificed by decapitation. For adult stage, cervical dislocation was performed before decapitation. The heads were hernected along the sagital midline and the brain and the cochlea were dissected from the temporal bones. After removing the otic capsule, the modiolus, the spiral ligament and the stria vascularis from the cochlea, the CSE were collected and immediately placed in RNA later solution (Ambion). For each stage (P3/adult), three independent dissection experiments were carried out separately in order to obtain three biological replicates. In each experiment, 40 CSE were extracted from 20 mice and pooled for RNA extraction. Thus, three independent RNA samples were obtained for each stage. The total RNA from each biological sample was extracted using RNAeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. After several washings and DNase treatment, elution was carried out in 20 μL of DNase-free water. RNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). RNA integrity and quality were evaluated with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). RNA samples were stored at -80°C until microarray hybridization.

Microarray Hybridization

The Affymetrix 3’ IVT express protocol was used to prepare cRNA (one-cycle amplification) with a starting concentration of 200 ng of total RNA. First-strand cDNA was synthesized using an oligo-dT primer that incorporates a T7 promoter sequence. cDNA was then amplified by in vitro transcription (IVT) with T7 RNA polymerase. 12 μg of Biotinylated and fragmented cRNA were used for hybridization with Mouse Genome 430 2.0 gene chip (Affymetrix) that contains more than 45,000 transcripts covering the whole mouse genome. Subsequent washing and staining of the arrays were performed using the GeneChip Affymetrix station protocol. A total of six chips (three chips for P3 samples and three chips for adult samples) were used for microarray hybridization experiments. The microarray data were obtained in accordance with the minimal informations about microarray experiment (MIAME) recommendations (PMID: 11726920). All data are accessible at the gene expression Omnibus (GEO) repository https://www.ncbi.nlm.nih.gov/geo through the provisional access series number GSE32963.

Data Analysis and Visualization

Scanned GeneChip images were processed using the Affymetrix GCOS 1.4 software. Microarray data were analyzed using the Affymetrix Expression Console™ software and normalization was performed with the MAS.5.0 algorithm to obtain the signal intensity and the detection call (present, marginal, or absent) for each probe set. To compare the gene expression profiles of the six CSE samples according to the P3 and adult stages, we first filtered the samples based on the “detection call”. This “call” can either be “present” when the perfect match probes are significantly more hybridized that the mismatch probe, p<0.4) “marginal” (for p≥0.04 and ≤0.06) or absent (p>0.06). Probe sets were used when they were present in at least 4 samples out of 6. A statistical technique called Significance Analysis Microarrays (SAM) software [29] was used with a fold change of 2 and a false discovery rate (FDR) of <5%. SAM allowed the identification of genes whose expression varied significantly among the P3 and adult groups. Hierarchical clustering was carried out with CLUSTER and TREEVIEW software [30]. This algorithm is based closely on the average-linkage method of Sokal and Michener [31] based on correlation coefficient.

Quantitative Real Time PCR

We performed qRT-PCR to validate the expression of a set of selected genes. Primers were designed with Primer Explorer 2.0 software or the online Roche software (www. universalprobelibrary.com, Roche). The GAPDH gene was used as endogenous control in order to normalize data. Each RNA sample was converted to cDNA using Random hexanucleotide primers and M-MLV reverse transcriptase (Invitrogen). The PCR reaction mix was prepared by adding QuantiTect SYBR Green PCR Master Mix at 1x, adequate primer at 0.5 μM and cDNA diluted at 1/25 to RNA free water. Amplification was performed using the Light Cycler 480 system (Roche) under the following conditions: an initial denaturation step at 95°C for 15 min followed by 45 three segment cycles of amplification (95°C/15 sec, 55°C/20 sec and 72°C/25 sec). For each target gene, three reactions were done against the endogenous control on the same run. The results were read using the Light Cycler 480 Software (Roche) and then analyzed using the delta Ct ΔCt method [32]. The list of primer sequences is shown in Table S1.

Statistical Analysis

We used Mann Whitney test from GraphPad Software to analyze the RT-per data. The differences among cochlear samples were considered significant when the p-value was ≤0.05.

Gene Ontology Enrichment Analysis

The gene ontology (GO) enrichment analysis, the biological processes and networks of the differentially expressed genes were generated by Ingenuity Pathway Analysis (IPA) tools (www. ingenuity.com). The 46 genes (Tables 1 and 2) were imported into IPA and each gene identifier was overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these genes were then generated based on their connectivity. A network score was calculated based on the hyper-geometric distribution and calculated with the right-tailed Fisher’s exact test (P value ≤0.05 was considered significant). The over-representation of functional
categories, biological processes and canonical pathways of the Q-PCR validated genes is confirmed by the gene annotation web tool “DAVID”, Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov/).

Results

Cochleae were removed from P3 and adult mice. The stria vascularis, Reissner’s membrane, spiral ligament and a major part of the spiral ganglion were removed. The remaining cochlear tissue is designated in this study as micro-dissected cochlear sensory epithelia (CSE). It consists of two types of sensory hair cells (inner and outer) and four types of supporting cells (Dieter’s, Hensen’s, Claudius’, inner and outer pillar cells), in addition to supporting cell subtypes (border and inner phalangeal cells) within the inner hair cell area. In the P3 cochlea, micro-dissected CSE samples included also thickened areas formed by tall columnar cell mounds, i.e. the greater epithelial ridge (GER) and the lesser epithelial ridge (LER). During development the GER cells progressively recede to form the inner sulcus (IS). In the adult cochlea, the micro-dissected CSE samples consists of two types of hair cells (inner and outer) and four types of supporting cells (Dieter’s, Hensen’s, Claudius’, inner and outer pillar cells), in addition to the IS and interdental cells within the limbus zone (Fig. 1). In all cases, the tissue micro-dissections were performed by the same approach and by the same investigator and special care was taken to keep, as much as possible, all parameters constant.

Identification of Differentially Expressed Genes between P3 and Adult CSE

We were interested in the changes in gene regulation within the CSE that occurred between P3 and adult stages. Using the SAM analysis, we identified a total of 5644 transcripts with a False Discovery Rate (FDR) ≤0.05 that significantly distinguished the P3 and adult CSE sample groups. Among those transcripts, 3102 are up-regulated in P3 (i.e., P3 molecular signature), in contrast the remaining 2542 are preferentially regulated in the adult (i.e., adult molecular signature). The number of transcripts that are specific for a given category of a CSE sample indicates that there is a significant variation across the two categories of CSE as demonstrated also by the hierarchical clustering which shows a clear segregation of the CSE samples based on this list of 5644 transcripts (Fig. 2). The six samples that we analyzed are arranged into two major clusters as shown on the top of the dendrogram (Fig. 2). Every three CSE samples corresponding to the same stage form a main cluster together. This result indicates that the triplicate samples for the same condition are concordant, supporting the reliability of our microarray assay. However, within the P3 samples cluster, the samples 2 and 3 are more related to each other than sample 1. In addition, the samples 1 and 3 in adult samples cluster are more linked than sample 2. These differences could be related to variations of the sample collection during the dissection procedure.

Validation of the Microarray Results

Among SAM transcripts, we selected 38 genes to validate by Q-PCR. These genes were chosen based either on their high fold change and/or their potential functions. These genes are named “Top 20 P3 up-regulated genes (Top up P3)” (FC ranged from 3.16 to 131.29) and “Top 26 up-regulated adult genes (Top up adult)” (FC ranged from 2.36 to 460.06), and listed in Tables 1 and 2 respectively. Both top up P3 and top up adult lists contain known and previously characterized genes.

Table 1. The 20 selected P3 up-regulated genes.

| Probeset     | UniGene ID | Gene Title   | Gene Symbol | Fold change P3/Adult |
|--------------|------------|--------------|-------------|---------------------|
| 1452403_a_at | Mm.41776   | otoconin 90  | Oc90        | 131.29              |
| 1415832_at   | Mm.2679    | angiotensin II receptor, type 2 | Agtr2 | 107.91               |
| 1419632_at   | Mm.42209   | tectorin alpha | Tecta | 73.56               |
| 1430912_a_at | Mm.42139   | tectorin beta | Tectb | 59.68               |
| 1448326_a_at | Mm.34997   | cellular retinoic acid binding protein 1 | Crabp1 | 57.98               |
| 1421556_at   | Mm.159128  | serine (or cysteine) peptidase inhibitor, clade A, member 3A | Serpin3aA | 48.49               |
| 1416776_at   | Mm.9114    | crystallin, mu | Crym | 45.35               |
| 1434921_at   | Mm.287100  | nuclear receptor subfamily 2, group E, member 1 | Nr2e1 | 39.33               |
| 1422851_at   | Mm.157190  | high mobility group AT-hook 2 | Hmga2 | 26.75               |
| 1456883_at   | Mm.39821   | storkhead box 1 | Stox1 | 26.46               |
| 1417359_at   | Mm.7386    | microfibrillar-associated protein 2 | Mfap2 | 20.79               |
| 1424010_at   | Mm.272278  | microfibrillar-associated protein 4 | Mfap4 | 16.85               |
| 1421106_at   | Mm.22398   | jagged 1 | Jag1 | 7.02               |
| 1423116_at   | Mm.137268  | hairy and enhancer of split 5 (Drosophila) | Hes5 | 5.76               |
| 1419697_at   | Mm.65396   | SRY-box containing gene 2 | Sox2 | 5.42               |
| 1449822_at   | Mm.57229   | atonal homolog 1 (Drosophila) | Atoh1 | 5.07               |
| 1441350_at   | Mm.4947    | fibroblast growth factor 3 | Fgf3 | 4.29               |
| 1415999_at   | Mm.29581   | hairy/enhancer-of-split related with YRPW motif 1 | Hey1 | 3.87               |
| 1417985_at   | Mm.46539   | notch-regulated ankyrin repeat protein | Narp | 3.67               |
| 1450922_a_at | Mm.18213   | transforming growth factor, beta 2 | Tgfβ2 | 3.16               |

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expressed in the inner ear including Oc90 (x131); Tecta (x73); Tectb (x59), Jag1 (x7) and Hes5 (x6) for up P3 list and GFAP (x120), Kcnj16 (x21) and Gh (x89) for up adult list. In addition, we identified new genes not previously known to be expressed in the mammalian cochlea such as, Hmga2 (x26) and Nrarp (x3) from the up P3 list, and Prl (x460;) and Ar (x3) from the up adult list. For all analyzed genes, the Q-PCR results showed the same variation of gene expression changes as revealed by the microarray approach. However, the fold change values were observed to be mainly greater in Q-PCR when compared to those obtained from the microarrays. The differential expression revealed by Q-PCR was significant for 17 of 18 up P3 analyzed genes (94%) (p-value, 0.05). In the case of adult up-regulated genes, the changes in the expression levels were significant for 15 of 20 genes (p-value, 0.05) (75%) (Fig. 3).

In addition, to confirm the changes in expression at the protein level (Fig. 4), we performed immunocytochemistry for a newly identified gene (i.e., Hmga2) known to be important for chromatin remanient and pluripotency in stem cells [33]. We observed up-regulation of Hmga2 within the P3 CSE in a variety of supporting cell subtypes (i.e., Dieters’ cells, border cells, inner and outer pillar cells and Hensen’s cells). The hair cells showed a relatively weak Hmga2 immunoreaction as compared to that of the surrounding supporting cells.

Functional Annotation of Selected Genes Differentially Expressed between P3 and Adult CSE

IPA was used to access to functional properties of the selected genes up-regulated in P3 and adult CSE. (Tables 1 and 2), confirmed by Q-PCR. Only significant functions (p<0.001) were reported. The selected P3 up-regulated genes were associated with multiple functions including auditory and vestibular system development, nervous system development, cellular growth/proliferation, and embryonic development (Fig. S1A) and signaling pathways such as, Notch signaling, embryonic stem cell pluripo-
tency and Wnt/βcatenin signaling (Fig. S1B). Among these pathways, four are above threshold for significance (p<0.05).

Inversely, the adult CSE up-regulated genes were related to biological functions including cell-to-cell interactions, DNA replication, cell cycle and cell death (Fig. S1C). For the pathways, the 26 selected up-regulated adult genes are mainly associated to NF-kB signaling, glucorticoid receptor signaling, FGF signaling and actin cytoskeleton signaling (Fig. S1D). DAVID (Database for Annotation, Visualization and Integrated Discovery) analysis gave mainly similar results as reported by IPA function and pathway analysis (data not shown). Gene lists from comparisons showing significant differences in gene expression were submitted to DAVID (www.david.abcc.ncifcrf.gov). DAVID provides exploratory visualization tools that promote discovery through functional
classification while simultaneously remaining linked to rich sources of biological annotation.

Potential Networks Associated with P3 Specific Genes

IPA network analysis on the top 20 up P3 genes identified three significant networks. The Figure 5 illustrates the number one ranked network. Most of selected up P3 genes, 17 of 20, interact with each other within this network. These genes are mostly related to cochlea and nervous system development and function. Notch signaling pathway appears notably in this network. It is represented by Hes5, Hey1, Jag1, Atoh1 and, also the new identified Notch signaling transcript in the CSE (i.e., Nrarp). The network shows interactions within Notch pathway and also between Notch players and other genes known in the early development of cochlea (i.e., Sox2, Tgfb and Fgf3). Hmga2, a newly identified gene in P3 mouse CSE, is involved in stem cell pluripotency in other tissues, appears among the genes within this network. However, it does not exhibit direct interactions with known cochlear stem/progenitor cells markers (i.e., Sox2, Jag1).

In order to gain insights on the potential function of Hmga2 in the P3 CSE, we explored the interactions between up-regulated genes included in the 1000 P3 list by using the IPA software. Network with 1000 genes showed more interactions as compared to 20 genes. The network involving Hmga2 is illustrated in Figure 6. Most of the genes forming this network are associated with epigenetic modifications of DNA, such as HDAC2 and Smarce. These interactions are concordant with a role of Hmga2 as a DNA binding protein that alters the architecture of chromatin to enhance gene transcription. The network (Fig. 6) shows two direct interactions for Hmga2: Let7microRNA-Hmga2 and Hmga2-HDAC2 interactions. Interestingly, both MirLet7 and HDAC2 have been shown to have a role in newt and bird inner ear regeneration [34,35].

Potential Networks Associated with Adult Specific Genes

Network analysis assembled three significant networks from selected adult up-regulated genes. We present here the highest score network (Fig. 7). This network shows interactions between the most regulated genes in the adult CSE, the Prl (FC>160) and other up-regulated adult genes from the selected list such as Gh, Ar and Stat1.

Of interest, the Gh exhibits a greater than 89 FC increase in adult CSE. As shown in (Figs. S2, S3), both Prl and Gh signaling pathways act via Jak2-Stat pathway involving Stat transcription factors. This may explain the interactions of Prl and Gh with Stat genes in the network shown in Figure 7. Interestingly, Stat1 has been demonstrated to be involved in cisplatin-mediated apoptosis of HCs within the cochlea, attenuated by the use of siRNA against Stat1 [36]. The up-regulation of Stat1 (FC>3) in adult CSE suggests that it may have a role in age-related HC death in the adult cochlea.

Within the network (Fig. 7), both Prl and Gh displayed interactions with the Ar, which previously was not reported to be expressed in the mammalian cochlea. Also, in the same network, both Prl and Gh interact with the Ap1 complex. Ap1 is a dimer formed by c-Jun and c-Fos, that acts as a transcription factor in several functions such as, apoptosis and proliferation. In the cochlea, the up-regulation of Ap1 has been suggested to be involved in HC death after either noise trauma or ototoxic drug exposure [37,38].
Figure 2. Hierarchical clustering. The expression signature of P3 and adult CSE were visualized by hierarchical clustering on the 5,644 significantly regulated probe sets. The six samples are arranged in columns and transcripts are arranged in rows. The transcripts segregate into two main major clusters. As observed in dendrogram, a first branch indicates a clear segregation between the two stages (All the replicate samples of the P3 group self-cluster into one branch. Adult samples self-cluster into another branch into which all the samples self-cluster). In each stage, a tree represents relationship among samples whose branch lengths reflect the degree of similarity between the samples according to gene expression profile (The samples with similar expression patterns are adjacent). Genes up-regulated in and down-regulated in each group were color-coded in red and green respectively. Cluster I regroups the P3 up-regulated transcripts (i.e., Jag1, Hes5, Sox2). Cluster II was a group of transcripts up-regulated in the adult cochlea samples (i.e., Prl, Gh, Gfap).

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Discussion

In this study, we report for the first time a large-scale analysis of changes in gene expression of CSE dissected from P3 and adult mice. The results of this approach may provide directions for future investigations into the understanding of the known difference in the ability for regeneration/repair between the early postnatal/developing and adult cochleae. We detected a number of genes that were known in the P3 (i.e., Jag1, Hey1, Sox2) and adult (i.e., Gfap, Kcnj16, Gh). In addition, we detected unreported genes in the P3 (i.e., Nrarp, Hmga2) and adult (i.e., Ar and Prl) mouse cochleae. These genes have been shown to be linked into known networks and pathways not previously implicated in the mammalian cochlea. We will then focus principally on these new genes regulated between P3 and adult CSE and their respective networks to illustrate the gene expression regulation.

In the past decade, accumulating evidence has suggested essential roles for Notch and Wnt/b-catenin signaling in the vertebrates inner ear development [39,40]. Our gene analysis revealed more than 3-fold change in the expression of Nrarp (Notch-regulated ankyrin repeat protein), an element of a negative feedback system that attenuates Notch pathway-mediated signaling [41] that has not been previously reported in the inner ear. An increase in the Nrarp transcription has been observed following the induction of Notch signaling, suggesting its involvement in the Notch signaling inhibitory feedback loop [42]. The expression of the Nrarp in the P3 CSE suggests that alternate Notch signaling pathways are operating to pattern the developing CSE in a context dependent manner. Further work is needed to elucidate the specific role of this Notch1 regulatory molecule in the mammalian cochlea that may help to shed more light on the diverse roles of the Notch signaling during inner ear development [43].

The stem cell pluripotency is amongst the most significant pathways related to P3 CSE. This pathway is principally represented by Sox2, Tgfb2 and Hmga2 in our selected up P3 list. The Hmga2 encodes a small chromatin-associated protein that cooperates with other factors to regulate gene expression [44]. During embryogenesis, the expression of Hmga2 is initially at a maximum level throughout the whole embryo, then is subsequently restricted to mesenchymal derivatives only, with later...
expression becoming undetectable in adult tissues [45]. It has been reported that Hmga2 is under the time-dependent regulation of the microRNA let-7 [46]. The let-7 miRNA family was among the first group of miRNA suggested to regulate ‘stemness’ by repressing self-renewal in both normal development and cancer [47]. Interestingly, Let-7 miRNA members have been suggested as potential regulators of the dedifferentiation in lens and inner ear HC regeneration of the newt [34]. In the same manner, in teleost fish a robust regenerative response to retinal injury relies on Müller glia dedifferentiation into a cycling population of progenitor cells through let-7 miRNA down-regulation [48]. Hmga2 expression is present in human and mouse pluripotent embryonic stem (ES) cells indicating a critical role of these proteins during development and growth [49]. Furthermore, it has been found that Hmga2 expression in human ES cells is closely correlated to the expression of pluripotency specific genes such as, Utf1, Sox2 and Oct4. In addition, it has been shown that Hmga2 promotes neural stem cell self-renewal in young but not old mice by reducing the expression of two negative regulators of the cell cycle, i.e. p16(Ink4a)/p19(Arf) [50]. Interestingly, our microarray showing an up-regulation of Hmga2 in the P3 CSE supports the previously observation as to the persistence of multipotent stem cells in the developing P3 cochlea and their lack in the adult cochlea of the mice [12]. In addition, we have previously demonstrated the expression of a battery of stem/progenitor markers within the P3 CSE and their down-regulation from the adult CSE [14]. The up-regulation of Hmga2 in the P3 cochlea is another stem cell marker that may reaffirm the persistence of multipotent stem cells in the P3 CSE cochlea and their lack in the cochleae of adult mice. The IPA network for P3 CSE upregulated genes (Fig. 6) indicates that Hmga2 directly interacts with HDAC2 (histone deacetylase 2). Interestingly, the HDAC has been demonstrated to regulate supporting cell proliferation during the regenerative proliferation in the avian utricular sensory epithelium.

Figure 4. Immunohistochemistry analysis of Hmga2. Photomicrographs of: (A–C) Cross-sections through the middle turn of the P3 cochlea and (D–F) undifferentiated CGR8 mouse embryonic stem (ES) cells growing in feeder free (leukemia inhibitory factor containing medium) cell culture used as positive control for Hmga2 expression. This positive control was not carried out simultaneously and was only included as a supplemental reference tool. In the P3 cochlea, the expression of the Hmga2 protein (red label) is mostly detected in the supporting cells within the IHC (BC) and OHC (DC) areas, in addition to pillar cells (PC) and Hensen’s cells (HeC). The OHCs are weakly labelled with the Hmga2 antiserum. The ES cells and cross-sections are counterstained with DAPI (shown in blue). IHC: inner hair cells; OHC: outer hair cells; BC: border cells; DC: Deiters’ cells; GER: greater epithelial ridge; LER: lesser epithelial ridge. Scale bars = 25 µm in (A–C) and 50 µm in (D–F).

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In another study with the mouse cochlea, HDAC was hypothesized to have a role in aminoglycoside antibiotic-induced HC death since its expression showed a transient increase after gentamicin administration [51]. In another hand, analysis of the selected adult up-regulated genes indicated that the biological functions are mostly related to cell death, polypeptide hormones, cellular growth, and inflammatory/immune response. Of interest, among the 26 selected up adult genes, microarray analysis and Q-PCR validation revealed two secreting pituitary hormones, i.e, growth hormone and prolactin (Gh/Prl). The Prl was the highest expressed gene in the adult CSE with a FC of 460. Previous findings have suggested a potential role of Prl in inner ear function. For example, a hyperprolactenemia has been reported in patients presenting with an inner ear dysfunction [52,53]. These observations were followed by a study demonstrating that hyperprolactenemia induced by a long-term estrogen treatment in guinea pig led to conductive hearing loss probably linked to an inner ear ionic homeostasis defect [54]. A recent study showed that hormone replacement therapy involving estrogen and progestin can promote hearing loss supporting the previous data [55]. Our gene expression data are consistent with these findings revealed for the first time that the Prl transcripts are expressed in the adult CSE supporting a role for Prl hormone in auditory function.

Gh was also observed to be among the most significant up-regulated genes in the adult CSE (FC >89). Interestingly, a previous microarray analysis of noise-exposed zebrafish showed that Gh was significantly up-regulated during the process of zebrafish inner ear HC regeneration [20]. In a parallel study, these authors showed that exogenous Gh promotes post-acoustic trauma HC regeneration in the zebrafish ear through the stimulation of cell proliferation [56]. These findings are in contrast with our present data with the mammalian cochlea showing that Gh is endogenously up-regulated in the adult that is known to lack any regenerative capacity [57]. In addition, previous gene expression

**Figure 5. Functional network analysis related to P3 up-regulated genes.** The most significant network assembled by selected P3 up-regulated genes includes 15 genes related to inner ear development most of them are components of the Notch pathway (i.e., Hey1, Hes5, Jag1). Hmg2, a newly identified stem cell gene in the P3 CSE appears also among the interacting genes within the network. Solid lines between nodes indicate direct molecular interaction between connected genes whereas dotted lines indicate an indirect functional interaction. Genes included in the P3 signature are in pink and those not found in the signature are in white.

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profiling following a noise trauma indicated a two-fold decrease in Gh in the adult rat cochlea [58]. They are also in contrast with the up-regulation of Gh after noise exposition in the zebrafish model. It is possible that the relatively simple structure of the ears of non-mammalian vertebrates combined with spontaneous cell cycle re-entry, is likely to enhance the proliferative promoting action of Gh than can occur in the highly specialized mammalian organ of Corti that relies on a small number of post-mitotic, highly specialized cells for hearing sensitivity. However, the converging point between the studies using the zebrafish and rodent models is the important role of GH in the sensory-neural structures of the inner ear that appears to be conserved across vertebrates and therefore warrants further investigation. We also found that the Androgen receptor (Ar) is among the up-regulated genes in the adult CSE (FC >3). The Ar transcripts have been shown previously to be expressed only in the fish inner ear [59]. Our microarray data demonstrate for the first time the expression of Ar gene in the mammalian cochlea. It has been reported that the Prl and Gh enhance the expression of Ar-mRNA in the rat’s prostate cells [60]. This result may explain the sychronic expression of these three transcripts (i.e., Ar, Gh and Prl) in the adult CSE as revealed by our microarray data suggesting that Prl and Gh may be involved in the regulation of Ar-mRNA expression in the adult cochlea. Interestingly, it has been reported in the literature that there is a cross talk between Notch and Ar pathways. Indeed, Hey1, an intracellular effector of the Notch signaling has been shown to act as a specific repressor on Ar expression in mammalian cell lines [61]. Furthermore, gene profiling data showed that the expression of Notch1 and its ligand Jagged1 is regulated by Ar in the prostate [62]. Taken together, these data could explain the down-regulation of Hey1, Notch1, and Jagged1 in the adult CSE where Ar is over-expressed as revealed by our microarray and Q-PCR results.

Overall, we identified a total of 5644 transcripts that significantly (FDR ≤ 0.05) distinguished gene expression profile of the P3 and adult CSE. We focused on two highly up-regulated genes (i.e., Nrarp, Hmga2) in postnatal-P3 and two up-regulated genes (i.e., Ar, Prl) in adult CSE as examples to highlight not previously reported genes that may account for the known difference in repair/regenerative ability of the early postnatal and adult mammalian cochleae.

Our data may constitute the first step toward further studies to understand the functional role of regulated and new detected
genes as related to the development and regeneration pathways in the mammalian cochlea.

**Supporting Information**

**Figure S1** Biological functions and pathways associated with the selected differentially expressed genes. (A) IPA function analysis on the up-regulated P3 genes. (B) IPA pathway analysis on the up-regulated P3 genes. (C) IPA function analysis on the up-regulated adult genes. (D) IPA Pathways analysis on the up-regulated adult genes. The Threshold represents the p-value = 0.05. The ratio (shown as squares on B and D) represents the number of analyzed genes in a given pathway divided by total number of genes that form this pathway.

**Figure S2** Prolactin signaling pathway identified by IPA software. The Prl transduction signal via PRLR shows that Prl may act via Jak2-Stat and/or Ras pathways depending on the function. Jak2-Stat pathway involves Stat1, Stat3 and Stat 5 transcription factors. Ras pathway is activated in cell cycle, proliferation and cell death functions. It involves Ap1 complex in the nucleus formed by c-Jun and c-Fos immediate genes. The pathway figure explains the interactions between Prl, Stat and AP1 shown in the IPA up adult network (Fig. 5).

**Figure S3** Growth hormone signaling pathway identified by IPA software. Canonical pathway reveals growth hormone-related gene signaling. Canonical pathway analysis with IPA software showing known growth-hormone related pathways including SOCS, STAT1 transcription factor, and PKC genes. The canonical pathway is established by the IPA software from its previous database of publications and may include computationally generated networks from many tissues at any age. Pink-colored genes are up-regulated genes in the adult list.

**Table S1** Primers pairs used for Real time PCR.

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Author Contributions

Conceived and designed the experiments: IS AZ. Performed the experiments: IS SA ES. Analyzed the data: IS SA SM AZ. Wrote the paper: IS SA AZ.

References

1. Hudspeth AJ (1997) How hearing happens. Neuron 19: 947–950.
2. Warchol ME (2011) Sensory regeneration in the vertebrate inner ear: Differences at the levels of cells and species. Hear. Res. 275: 72–79.
3. Zine A, De Ribaupierre F (1998) Replacement of mammalian auditory hair cells. Neuron 9: 263–268.
4. Staecker H, Van De Water TR (1998) Factors controlling hair-cell re-generation in the inner ear. Curr. Opin. Neurobiol. 8: 490–497.
5. Lenoir M, Vago P (1997) Does the organ of Corti attempt to differentiate new hair cells after antibiotic intoxication in rat pups? Int. J. Dev. Neurosci. 15: 487–495.
6. Smeti I, Zindy F, Abdala C, Liu F, Li X, et al. (2003) Progressive hearing loss in mice lacking the cyclin-dependent kinase inhibitor Ink4d. Nat. Cell Biol. 5: 422–426.
7. Sage C, Huang M, Kariini K, Gutierrez G, Vollrath MA, et al. (2005) Proliferation of functional hair cells in vitro in the absence of the retinoblastoma protein. Science 307: 1114–1118.
8. Mantela J, Jiang Z, Ylikoski J, Liitto M, et al. (2007) p19 (Ink4d) and p16 (Ink4a) are involved in the maintenance of cochlear hair cells. J. Neurosci. 27: 1277–1287.
9. Laine H, Doetzhofer A, Manzel A, Ylikoski J, Laiho M, et al. (2007) p19 (Ink4d) and p16 (Ink4a) regulates proliferation and survival of cochlear hair cells. Proc. Natl. Acad. Sci. U.S.A. 104: 11820–11825.

We thank Drs T. Van de Water (University of Miami) for his critical reading and helpful comments on an earlier version of the manuscript.
52. Horner KC, Guieu R, Magnan J, Chays A, Cazals Y (2002) Prolactinoma in some Ménière’s patients–is stress involved? Neuropsychopharmacology 26: 135–138.

53. Falkenius-Schmidt K, Rydmarker S, Horner KC (2005) Hyperprolactinemia in some Menière patients even in the absence of incapacitating vertigo. Hear. Res 203: 154–158.

54. Horner KC, Cazals Y, Guieu R, Lenoir M, Sauze N (2007) Experimental estrogen-induced hyperprolactinemia results in bone-related hearing loss in the guinea pig. Am. J. Physiol. Endocrinol. Metab. 293: E1224–1232.

55. Price K, Zhu X, Guimaraes PF, Vasilyeva ON, Frisina RD (2009) Hormone replacement therapy diminishes hearing in peri-menopausal mice. Hear. Res 252: 29–36.

56. Sun H, Lin C-H, Smith ME (2011) Growth hormone promotes hair cell regeneration in the zebrafish (Danio rerio) inner ear following acoustic trauma. PLoS ONE 6: e28372.

57. Kopecky B, Fritzsch B (2011) Regeneration of Hair Cells. Making Sense of All the Noise. Pharmaceuticals. 4: 884–879.

58. Cho Y, Gong T-WL, Kanicki A, Alschuler RA, Lomax MI (2004) Noise overstimulation induces immediate early genes in the rat cochlea. Brain Res. Mol. Brain Res. 130: 134–141.

59. Forlano PM, Marchatterre M, Deitche DL, Bass AH (2010) Distribution of androgen receptor mRNA expression in vocal, auditory, and neuroendocrine circuits in a teleost fish. J. Comp. Neurol 518: 493–512.

60. Reiter E, Bonnet P, Sente B, Dombrowsicz D, de Leval J, et al. (1992) Growth hormone and prolactin stimulate androgen receptor, insulin-like growth factor-I (IGF-I), and IGF-I receptor levels in the prostate of immature rats. Mol. Cell. Endocrinol 88: 77–87.

61. Belandia B, Powell SM, Garcia-Pedrero JM, Walker MM, Bevan CL, et al. (2005) Hey1, a mediator of notch signaling, is an androgen receptor corepressor. Mol. Cell. Biol 25: 1425–1436.

62. Nantermet PV, Xu J, Yu Y, Hodor P, Holder D, et al. (2004) Identification of genetic pathways activated by the androgen receptor during the induction of proliferation in the ventral prostate gland. J. Biol. Chem 279: 1310–1322.