Cellular/Molecular

Characterization of Transcriptomes of Cochlear Inner and Outer Hair Cells

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Inner hair cells (IHCs) and outer hair cells (OHCs) are the two types of sensory receptor cells that are critical for hearing in the mammalian cochlea. IHCs and OHCs have different morphology and function. The genetic mechanisms that define their morphological and functional specializations are essentially unknown. The transcriptome reflects the genes that are being actively expressed in a cell and holds the key to understanding the molecular mechanisms of the biological properties of the cell. Using DNA microarray, we examined the transcriptome of 2000 individually collected IHCs and OHCs from adult mouse cochleae. We show that 16,647 and 17,711 transcripts are expressed in IHCs and OHCs, respectively. Of those genes, ~73% are known genes, 22% are uncharacterized sequences, and 5.0% are noncoding RNAs in both populations. A total of 16,117 transcripts are expressed in both populations. Uniquely and differentially expressed genes account for <15% of all genes in either cell type. The top 10 differentially expressed genes include *Slc17a8*, *Dnajc5b*, *Slc1a3*, *Atp2a3*, *Osbpl6*, *Slc7a14*, *Bcl2*, *Bin1*, *Prkd1*, and *Map4k4* in IHCs and *Slc26a5*, *C1ql1*, *Strc*, *Dnm3*, *Plbd1*, *Lbh*, *Olfm1*, *Plce1*, *Tectb*, and *Ankrd22* in OHCs. We analyzed commonly and differentially expressed genes with the focus on genes related to hair cell specializations in the apical, basolateral, and synaptic membranes. Eighty-three percent of the known deafness-related genes are expressed in hair cells. We also analyzed genes involved in cell-cycle regulation. Our dataset holds an extraordinary trove of information about the molecular mechanisms underlying hair cell morphology, function, pathology, and cell-cycle control.

Key words: **DNA microarray; inner hair cells; mouse; outer hair cells; transcriptome**

Introduction

The transcriptome is the set of all RNA molecules, including mRNAs, rRNAs, tRNAs, and other noncoding RNAs produced in one or a population of cells. Unlike the genome, which is the same for nearly every cell in multicellular organisms, the transcriptome is cell specific and reflects the genes that are being actively expressed at any given time (Velculescu et al., 1997; Okazaki et al., 2002). Because different patterns of gene expression underlie phenotypic differences seen among different cells and tissues, analysis of transcriptomes is of fundamental importance for understanding the genetic mechanisms that control differentiation, proliferation, senescence, metabolism, morphology, and function of a cell or tissue under normal and pathological conditions (Okazaki et al., 2002).

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Hair cells in all vertebrates are polarized neuroepithelial cells that serve as the sensory receptors for the acoustical, vestibular, and lateral-line organs (Fettiplace and Hackney, 2006). Hair cells transduce mechanical stimuli into electrical activity (Hudspeth and Corey, 1977; Hudspeth, 1989). Mechanoelectrical transduction is mediated by the hair bundle, an array of modified microvilli or stereocilia arranged in a staircase on the apical surface of the hair cell (Fettiplace and Hackney, 2006). In addition to the stereocilia specialization critical for mechanoelectrical transduction in the apical membrane, all hair cells also have specializations in the basolateral and synaptic membranes that are responsible for electrical activities and synaptic transmission.

There are two types of hair cells in the mammalian cochlea: (1) inner hair cells (IHCs); and (2) outer hair cells (OHCs). IHCs and OHCs are different in morphology and function (Dallos, 1992). IHCs, receiving predominantly afferent innervation (Spoendlin, 1970), are considered to be the true sensory receptor and transmit information to the brain. OHCs, innervated predominantly by efferent fibers (Spoendlin, 1970), serve as the effector cell that boosts input to IHCs by a receptor potential-driven somatic motility (Brownell et al., 1985; Zheng et al., 2000; Liberman et al., 2002; Dallos et al., 2008). The genetic mechanisms that define morphological and functional specializations of the two types of hair cells are essentially unknown. We took advantage of the distinct morphology of these two sensory receptor cell types to isolate pure pristine populations and examine transcriptomes using GeneChip microarray analysis. The analysis and comparison

of the two populations allow us to determine what genes are expressed in both populations and what genes are uniquely and differentially expressed in each population. While identification of the common genes is important to identify genes that are responsible for shared structure and function (such as apical specialization and mechanotransduction), identification of uniquely or differentially expressed genes reveals a molecular genetic basis for the unique morphology and function of IHCs and OHCs. This dataset is expected to serve not only as a highly valuable resource for unraveling the molecular mechanisms of the biological properties of the two types of hair cells but also for assisting the auditory research community in identifying and exploring the functions of deafness-related genes.

Materials and Methods

Dissection and isolation of hair cells. CBA/J mice (25–30 d old) of either sex were used. The basilar membrane together with the organ of Corti was isolated and transferred to an enzymatic digestion medium in a small Petri dish. The enzymatic digestion medium contained 1 ml of L-15 medium and 1 mg of Collagenase IV (Sigma). After 5 min incubation at room temperature, the tissue was transferred to a small plastic chamber (0.8 ml in volume) containing enzyme-free culture medium (Leibovitz's L-15 medium, 7.35 pH, and 300 mOsm). Hair cells were separated after gentle trituration of the tissue with a small pipette. The chamber containing the hair cells was then mounted onto the stage of an inverted microscope (Olympus) equipped with a video camera.

Collection of isolated hair cells. To collect solitary hair cells, two accessory pipettes with a diameter of 30 μm were used to pick up and transfer IHCs and OHCs. The pickup pipette was fabricated from 1.5 mm thin-walled glass tubing pulled by a two-stage electrode puller. The pipettes are mounted in two separate electrode holders mounted on two Leitz 3-D micromanipulators. By moving the pickup pipette and the stage of the microscope, cells were positioned near the tip of the pipette. The suction port of the pipette holder was connected to a micrometer-driven syringe to provide positive or negative pressure to draw in or expel the cells. After 4–5 cells had been drawn into the pipette, the pipette was lifted out from the bath and quickly transferred to a microcentrifuge tube containing 50 μl of RNA*later* (Thermo Fisher Scientific). Cells were expelled from the pipette by applying positive pressure. The tube was replaced in the ice bucket to prevent degradation of RNAs during cell collection. To obtain highly specific IHCs and OHCs, several steps were taken to avoid contamination by each other and by other cell types. First, we identified the cells being collected. IHCs and OHCs have unique features and are easily identifiable based on their gross morphology (He et al., 2000). The shape of IHCs is commonly described as similar to a flask. The cuticular plate is set at an angle to the main axis of the cell. The OHCs are long and cylindrical. Because of their morphological appearance, IHCs and OHCs are not difficult to recognize. Any ambiguous hair cells were not used. Second, we collected only solitary hair cells that were not attached to any other cell types. Third, we were particularly careful about the suction pressure applied to the pipette to avoid drawing unwanted cells into the pipette. We withdrew the suction pipette (to deposit hair cells) only when the pressure was balanced and no more fluid or cells were being drawn into the pipette.

RNA extraction and purification. Total RNA, including small RNAs (more than ~18 nt), from ~2000 IHCs and 2000 OHCs separately suspended in RNA*later* were extracted and purified using the Qiagen miRNeasy Mini Kit. On-column DNase digestion was performed to further eliminate DNA contamination in the collected RNA. Quality and quantity of RNA were determined using an Agilent 2100 BioAnalyzer. Separate IHC and OHC RNAs were split into three for separate technical replicates.

GeneChip microarray. Gene expression profiles were determined by GeneChip Mouse Gene 2.0 ST Arrays using ~3–5 ng of total RNA, including small RNAs, obtained from separate IHC and OHC cell populations. The amount of total RNAs obtained from our samples was well above the required amount specified by the manufacturer of the kit. Amplification and synthesis of cDNA were completed using the NuGEN Ovation Pico WTA System V2. Separate IHC and OHC RNAs from each cell population were split into three arrays for technical replicates. The transcriptome profile of the two cell populations was determined by GeneChip microarray analysis (Affymetrix). Synthesis of cDNA, hybridization to chips, and washes were performed according to the protocol of the manufacturer. GeneChips were scanned at 3 μm density with a GeneArray Scanner (Affymetrix). Images were inspected to ensure that all chips had low background but bright hybridization signals. Mean fluorescence signal intensity for each probe was quartile normalized. The average of three mean signals for each gene probe was normalized to that for an added control oligonucleotide. Each gene probe was assessed for expression based on a Wilcoxon's rank-sum test of the gene probe set signals compared with the distribution of signals from the background. The whole-transcript arrays included probes to measure expression of mRNA and long intergenic noncoding RNA transcripts. A total of 41,345 mouse RefSeq transcripts were included in the microarray, according to information provided by the manufacturer.

Gene expression levels and threshold definition. The 18,240 transcriptional units had fluorescent intensity readings that varied from 1.99 to 5021.96 for IHCs and 2.18 to 4843.62 for OHCs. The 2524-fold difference between the lowest and highest intensity readings reflects the huge dynamic range of gene expression in hair cells, which is also a strong indication of high sensitivity and quality of the technique and samples. Because the expression of every transcriptional unit was measured by signal intensity (a numerical value), we defined the baseline intensity level at 10.90 for both populations. This baseline was selected according to published developmental and differential expression profiles of some of the known genes (i.e., *Otof*, *Atoh1*, *Slc26a5*, *Chrna10*, *Chrna9*, and *Strc*) in IHCs and OHCs and determined using a range (10.6–11.5) of intensity values based on a minimal intensity value of 2.2^3 and a doubling of the median values below 12.0. An intensity of 10.9 was the minimum intensity value at which neither a loss nor gain of analysis-ready candidate molecules was found.

Analysis. All the CEL files from the Mouse Gene 2.0 chips were normalized using Expression console (Affymetrix). These data were exported into CCL Main Workbench (CLC Bio) to perform analysis and statistics. Means and SDs were calculated. Paired *t* tests were done by comparing average intensity values for each transcript from three repeats. $p \leq 0.05$ was considered statistically significant. Annotation of the probe set was done using the file for the Mouse Gene 2.0 ST Array, Analyses (Affymetrix) imported into CLC Main Workbench. Updates to the Affymetrix clf data, which provided the annotation for each probe, were done by hand annotation. Identification of differentially expressed genes was facilitated using Ingenuity IPA program (www.ingenuity.com). Entrez Gene, HGNC (HUGO Gene Nomenclature Committee), OMIM (Online Mendelian Inheritance in Men), and Ensembl databases were used for verification, reference, and analyses.

Results

Because the gene expression profiles are highly influenced by development and aging, we chose CBA/J mice aged between 25 and 30 d after birth. The auditory system at this stage is already mature, and age-related hearing loss does not occur until at least 18 months after birth in this mouse strain (Zhang et al., 2013). Therefore, the transcriptome at this age should reflect gene expression minimally influenced by either development or aging. Two thousand IHCs and 2000 OHCs were individually collected from 30 mice. Examples of an isolated IHC and OHC are presented in Figure 1. Total RNA preparations from each cell population were divided to provide three technical replicates for analysis. The expression profile of the two cell populations was determined by microarray analysis. The whole-transcript arrays include probes to measure expression of mRNA and long intergenic noncoding RNA transcripts. A total of 41,345 mouse RefSeq transcripts were included in the microarray, and 18,240 transcripts were examined after excluding control sequences and

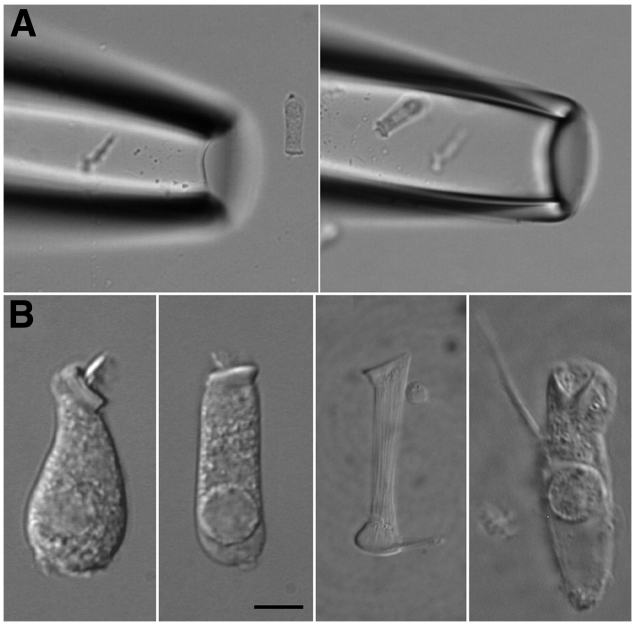

Figure 1. Micrographs of isolated hair cells and supporting cells and a pickup pipette used to collect isolated hair cells. ***A***, Images of a pickup pipette before and after an isolated OHC was drawn into the pipette. ***B***, Images (from left to right) of an isolated IHC, OHC, pillar cell, and Deiters' cell from adult mice. Scale bar, 5 μm for all images in panel B.

judiciously setting a cutoff for background. Of those, 16,647 and 17,711 transcripts were considered "expressed" in IHCs and OHCs, respectively, and 16,117 transcripts were expressed in both cell populations. Among the 16,647 transcripts expressed in IHCs, 12,103 (72.7%) are known genes, 3709 (22.3%) are uncharacterized genes, and 835 (5.0%) are noncoding RNAs. Uncharacterized genes refer to those genes whose encoded proteins have not been assigned an experimentally demonstrated function or biological role. A noncoding RNA refers to a functional RNA molecule that does not encode a protein. Noncoding RNA genes include highly abundant and functionally important RNAs, such as tRNA and rRNA, as well as RNAs, such as small nucleolar RNAs, microRNAs, small nuclear RNAs, piwi-interacting RNAs, and the long noncoding RNAs (Mattick and Makunin, 2006). For OHCs, 12,587 (71.0%) are known genes, 4184 (23.6%) are uncharacterized genes, and 949 (5.4%) are noncoding RNAs. The raw data of microarray together with the searchable dataset (in Excel format) can be downloaded from the National Center for Biotechnology Information–Gene Expression Omnibus (GEO; GEO submissions number GSE56866).

To determine what genes are expressed in IHCs and OHCs, we analyzed the transcripts in each population. Figure 2 shows the expression levels for the 200 most abundant transcripts in IHCs. For comparison, expression levels for the same transcripts in OHCs and abundance rankings for these transcripts in OHCs are also illustrated. Figure 3 similarly shows the 200 most abundant transcripts in OHCs compared with the same transcripts in IHCs. As shown in both figures, the vast majority of the known genes and the uncharacterized transcripts richly expressed in one population are also abundantly expressed in the other.

We analyzed the genes that are differentially and uniquely expressed in IHCs and OHCs, because these genes may underlie unique structures and functions of the cells of each cell type. We compared the expression levels of all the transcripts in OHCs with those of IHCs. Figure 4*A* presents an overall picture of expressed transcripts in each population. Differentially expressed genes were categorized as those whose expression levels were above background and at least 1.6-fold different between the two cell types. Uniquely expressed genes were those whose expression levels were below background in only one cell type. In IHCs, there are 1645 differential expressed genes, with 1296 being significantly different ($p \leq 0.05$). OHCs exhibited 836 differentially expressed genes, with 198 being significantly different. Figure 4, *B* and *C*, illustrates the top 150 differentially expressed genes in IHCs and OHCs. Notably, differential expression of *Slc26a5*, *Chrna10*, *Chrna9*, *Strc*, *Kcnq4*, and *Ocm* in OHCs and *Otof* and *Slc17a8* in IHCs has been demonstrated previously (Elgoyhen et al., 1994, 2001; Sakaguchi et al., 1998; Kubisch et al., 1999; Yasunaga et al., 1999; Zheng et al., 2000; Verpy et al., 2001, 2008; Ruel et al., 2008). Interestingly, *Myo3* was differentially expressed in adult IHCs (7.4-fold difference between IHCs and OHCs), although stronger expression in OHCs was detected in P0 mice (Walsh et al., 2002). The comparison also identified a significant number of genes whose functions in hair cells have not been characterized. These include *Slc1a3*, *Slc7a14*, and *Bcl2* in IHCs and *Lbh* and *Aqp11* in OHCs. *Slc1a3* and *Slc7a14* encode proteins that are associated with transport of glutamate–aspartate (Rothstein et al., 1994). *Bcl2* encodes B-cell leukemia/lymphoma 2 protein, which regulates apoptosis. *Lbh* is a transcription factor that is important for the development of limb bud and heart (Briegel and Joyner, 2001). *Aqp11* encodes aquaporin 11, an integral membrane protein that forms pores in the membrane and regulates water balance. There are 529 and 1596 uniquely expressed genes in IHCs and OHCs, respectively. Figure 5, *A* and *B*, shows the top 150 uniquely expressed genes in IHCs and OHCs. As shown, most are expressed at relatively low levels, and approximately half are uncharacterized genes and, of the known genes, half represents noncoding RNAs.

Hair cells have specializations in the apical, basolateral, and synaptic membranes that include the stereocilia bundle, mechanotransduction apparatus, voltage-dependent ion channels, neurotransmitter vesicles, and cholinergic receptors. We analyzed genes encoding proteins that are associated with these specializations using categories based on HGNC Gene Families/Groupings Nomenclature. A recent study using mass spectrometry identified >1100 proteins present in stereocilia bundles isolated from chicken vestibular hair cells (Shin et al., 2013). Figure 6*A* shows IHC and OHC expression levels for 66 transcripts encoding stereocilia-associated proteins compared with the concentration of each protein reported previously in vestibular hair cell stereocilia (Shin et al., 2013). As shown, 62 of these genes are expressed in both types of cochlear hair cells. Only four such genes, *Pde1c*, *Ush2a*, *Pip4k2b*, and *Myo3b*, are below background. Most abundantly expressed transcripts have relatively high levels of protein expression, except for *Lmo7*, *Ush1c*, *Myo3a*, *Pcdh15*, *Myo15*, and *Triobp*, whose protein levels were relatively low. Two transcripts, *Ywhaq* and *Atp2b2*, were highly expressed in IHCs and OHCs, but no proteins were detected by mass spectrometry (Shin et al., 2013). Also included are expression values for 22 additional genes that encode proteins for myosin, spectrin, whirlin, villin, and fascin. These proteins are likely to be associated with the structure and function of stereocilia.

All hair cells contain mechanotransduction channels in the stereocilia. Recent evidence suggests that TMC1 (transmembrane channel-like 1) and TMC2 might be part of the mechanotransduction channels (Kawashima et al., 2011; Kim et al., 2013; Pan et al., 2013). As shown in Figure 6*B*, *Tmc1* and *Tmc4* are expressed at relatively high levels in both IHCs and OHCs. *Tmc6* and *Tmc8* were detected with low expression in both cell types. As

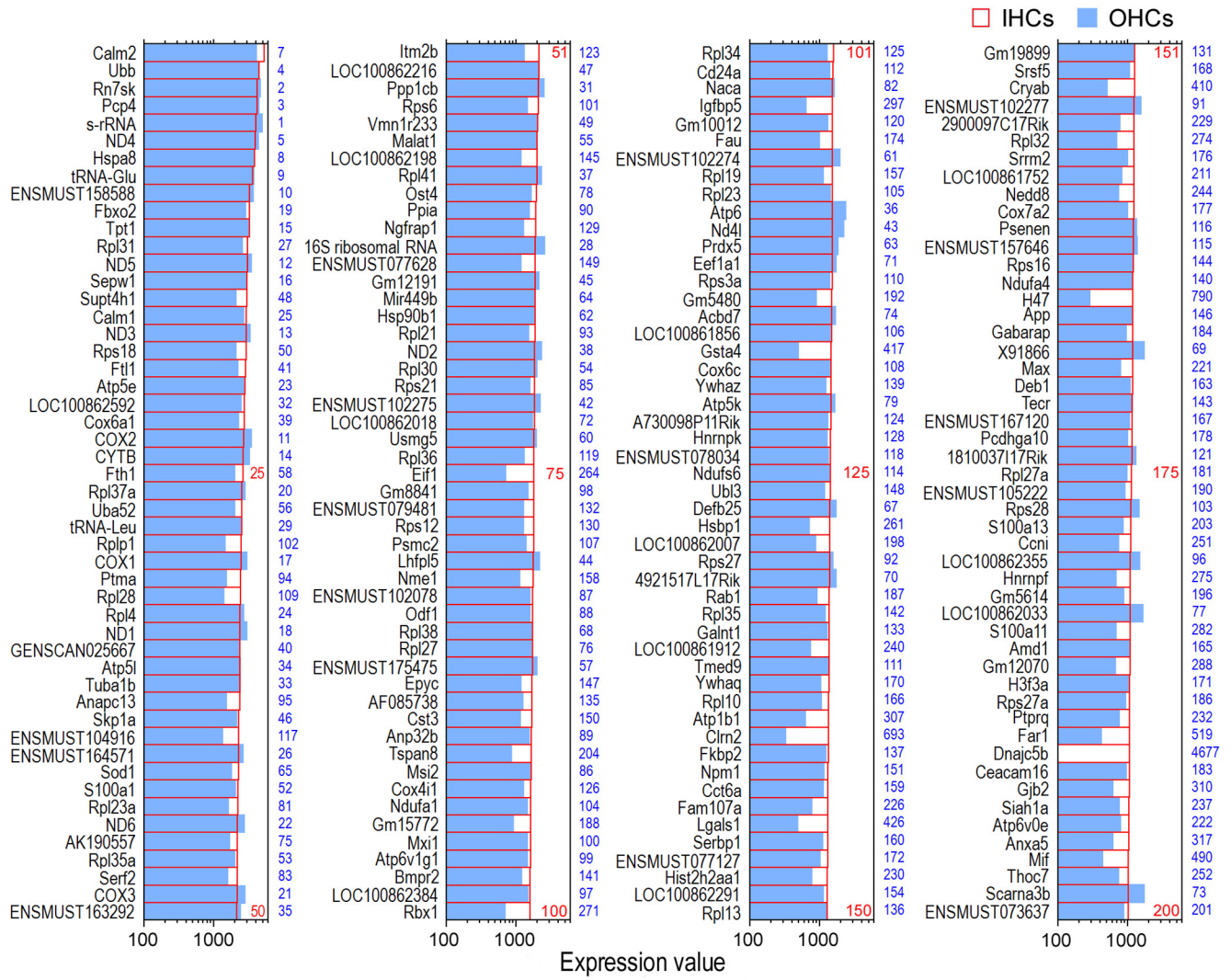

Figure 2. Expression levels of the top 200 genes in IHCs in descending order. The expression value of the same genes in OHCs is also presented. Numbers in red signify the abundance rank of the genes in IHCs; the numbers in blue on the right represent the ranking of the same genes in OHCs. In this and all subsequent figures, ENSMUST identification numbers are truncated to show the last six digits.

expected, *Tmc2* expression was not detected in adult hair cells (Pan et al., 2013). We also examined genes that encode stretch-activated ion channels in hair cells, because they are another group of mechanotransducers that conduct ionic currents by responding to mechanical stimulation (Sachs, 2010). Eight members of the TRP family were expressed in both populations of hair cells, with *Trpm3* and *Trpm7* having relatively high expression.

Hair cells also contain various types of ion channels in the basolateral and synaptic membranes (Kros, 1996). These channels are responsible for establishing and maintaining membrane potential, shaping receptor potentials and trigging neurotransmitter release, and regulating cell volume. Figure 6*B* lists genes that encode various types of Cl^-, Ca^{2+}, Na^+, and K^+ channels. Several genes are expressed at relatively high levels, and a majority of these are differentially expressed. *Cacna1d*, *Cacnb2*, *Cacng5*, *Kcnb2*, *Kcnj13*, *Kcnn2*, and *Kcnq4* are differentially expressed in IHCs, whereas *Ano3*, *Ano4*, *Cacnb4*, *Scn1b*, *Kcnab1*, *Kcnh8*, and *Kcnma1* are differentially expressed in OHCs.

IHCs and OHCs contain afferent and efferent synapses (Glowatzki and Fuchs, 2000, 2002; Weisz et al., 2009; Safieddine et al., 2012). We examined genes that encode proteins associated with neurotransmitter vesicle transport and release, as well as nicotinic cholinergic receptors. As shown in Figure 6*B*, eight subunits of nicotinic cholinergic receptors are expressed in both types of hair cells. However, *Chrna10* and *Chrna9* are highly and differentially expressed in OHCs. In contrast, *Slc17a8*, *Slc7a14*, and *Slc1a3* are highly and differentially expressed in IHCs. Genes that are related to SNARE proteins and synaptotagmins are expressed in both cell types, with higher expression in IHCs in most cases.

Transcription regulators/factors are involved in the regulation of gene expression. We also analyzed transcription factors in the two hair cell populations. The 10 most abundantly expressed transcription factors in IHCs and OHCs include *Eny2*, *Etv5*, *Fif1*, *Gata3*, *Hsbp1*, *Mtpn*, *Sox2*, *Tceb2*, *Zbtb20*, and *Zfp36*. However, the most differentially expressed transcription factor in OHCs is *Lbh*, which exhibited a 13-fold difference ($p < 0.001$).

Approximately 23% of transcripts detected are uncharacterized sequences. Although their biological roles remain to be determined, the high level of expression of these sequences suggests that they are important for hair cell structure and functions. Noncoding RNAs are involved in nearly every aspect of gene expression, which includes transcriptional regulation, splicing,

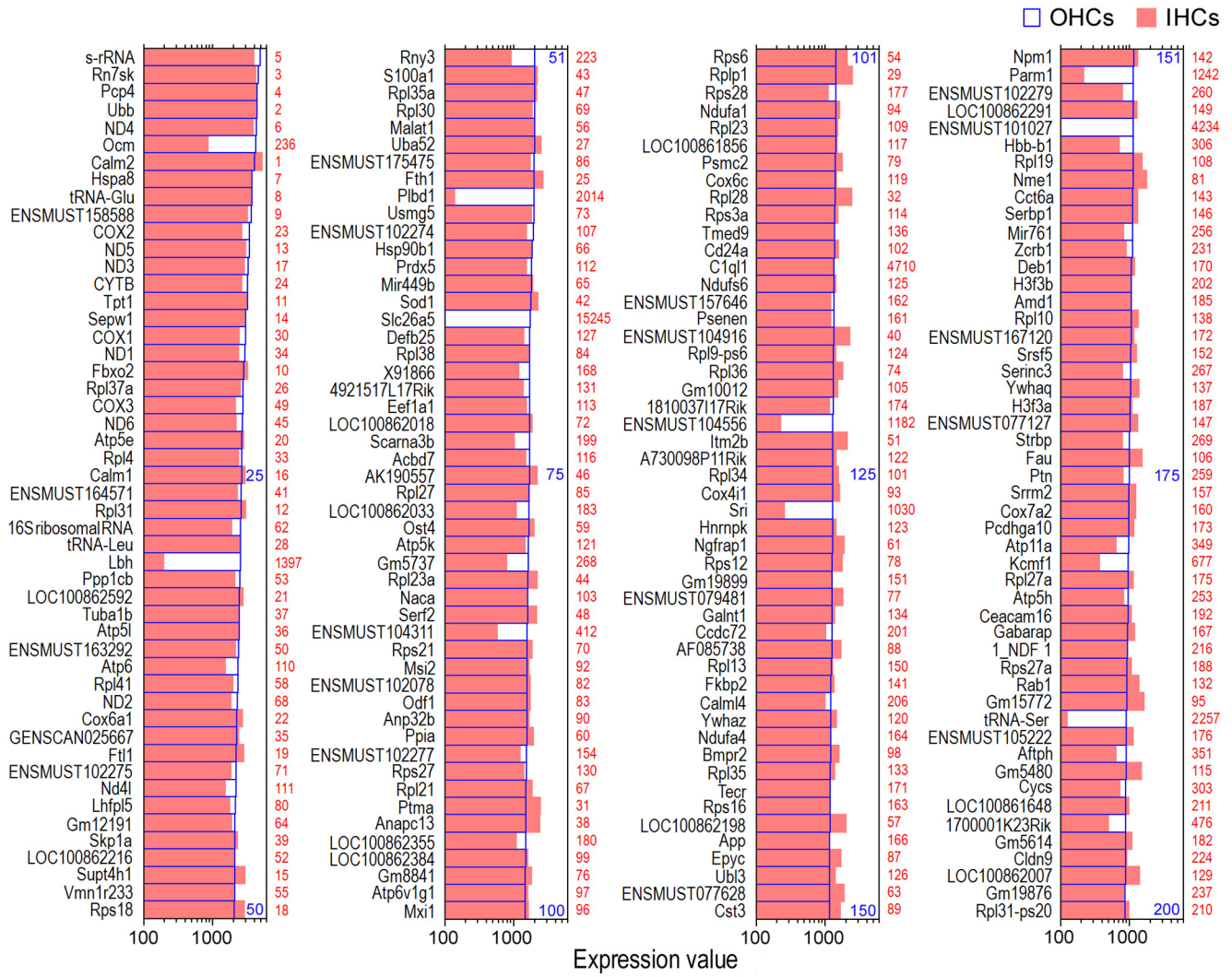

Figure 3. Expression levels of the top 200 genes in OHCs. The expression value of the same genes in IHCs is also presented for comparison. Numbers in blue signify the abundance rank of the genes in OHCs; the numbers in red on the right represent the ranking of the same genes in IHCs.

translation, and posttranscriptional regulation. A number of tRNAs and rRNAs (such as *Rn7sk*, *s-rRNA*, *tRNA-Glu*, and *tRNA-Leu*) were among the most abundantly expressed in both populations of hair cells. Some precursor microRNAs were also indicated in the analyses but may not necessarily be reflective of mature microRNAs.

We also analyzed genes that are important for cell-cycle regulation in both types of hair cells. Mammalian cochlear hair cells and supporting cells are postmitotic; consequently, damaged hair cells cannot be spontaneously replaced, leading to permanent deafness. However, lower vertebrates, such as birds and fish, can spontaneously regenerate lost hair cells from the underlying supporting cells through proliferation and transdifferentiation (Stone and Cotanche, 2007). Thus, analyses of cell-cycle regulation genes in hair cells could provide genetic information pinpointing differences between mammalian hair cells and lower vertebrate hair cells and illuminating a road map for targeted manipulation of genes to induce hair cell regeneration in mammals. It is estimated that up to 1000 cell-cycle-regulated genes might exist in the mammalian cell (Forrest et al., 2003). We examined the expression of 85 genes known to be involved in cell-cycle regulation and commonly assayed in a cell-cycle PCR array. These genes include those that promote or inhibit progression of the cell cycle, the transitions between each of the phases, DNA replication, checkpoints, and arrest. Among those 85 genes, 67 genes were expressed in hair cells. As shown in Figure 7*A*, *Atr*, *Bcl2*, *Casp3*, *Ccnc*, *Cdkn1a*, *Cdkn1b*, *E2f3*, *Msh2*, *Nbn*, *Skp2*, and *Stmn1* are differentially expressed in IHCs, whereas *Cdc7*, *Dst*, *Mdm2*, *Nek2*, and *Rbl2* are differentially expressed in OHCs. Nineteen genes, including *Birc5*, *Brca1*, and *Brca2*, were not detected in either population of hair cells.

Finally, we examined the expression of deafness-related genes in hair cells. Mutations or deficiencies affecting ~120 genes have been linked to inherited syndromic or nonsyndromic hearing loss (Morton and Nance, 2006; Smith et al., 2014). We analyzed the expression of 101 known deafness genes, excluding X-chromosome-linked and mitochondrial genes. Figure 7*B* shows expression levels of the 101 deafness genes in IHCs and OHCs. Among these, 82 and 85 genes were expressed in IHCs and OHCs, respectively. Many genes showed various degrees of differential expression.

Discussion

With the completion of the full human and mouse genome sequences, increasing attention has focused on identifying the com-

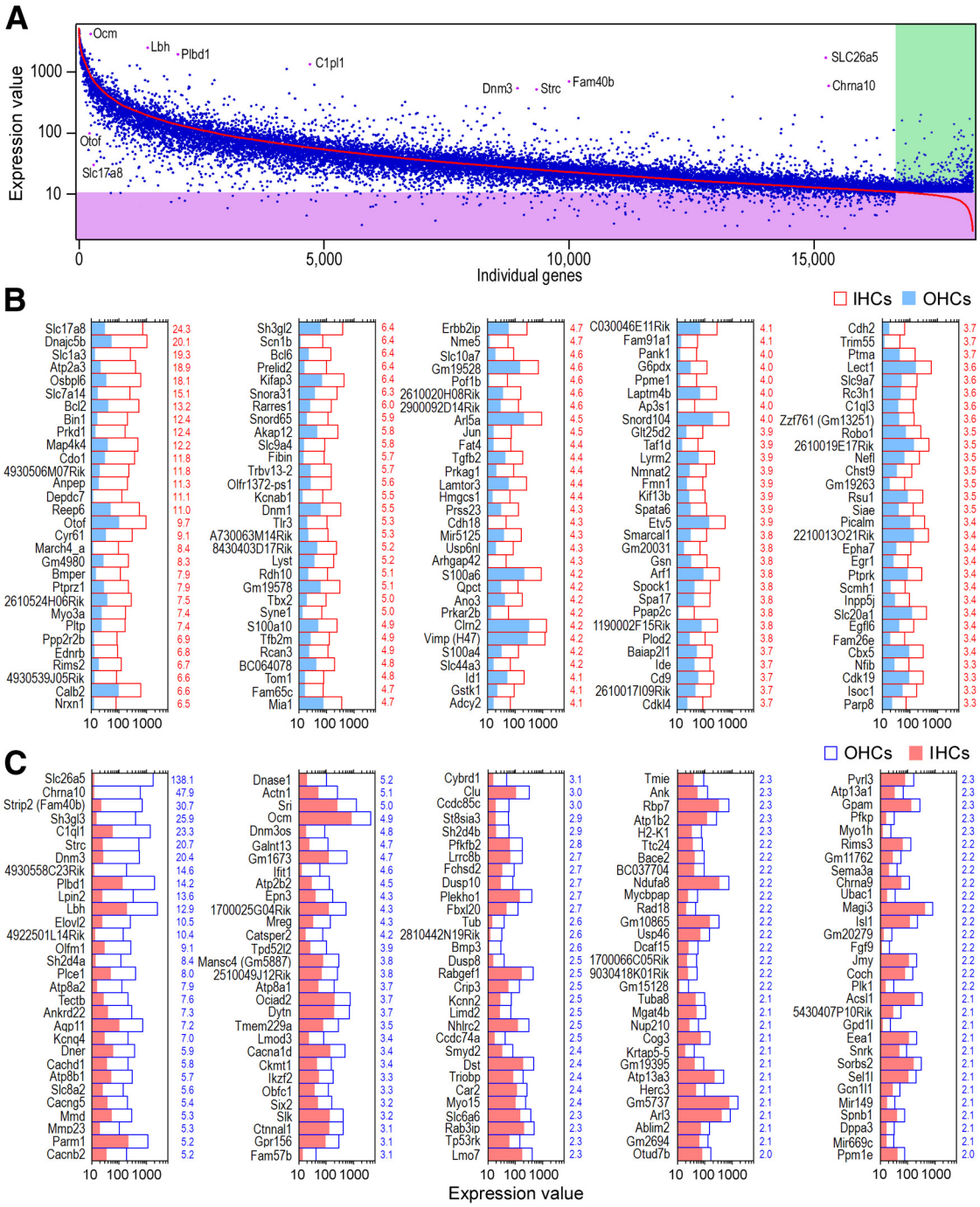

Figure 4. Differentially expressed genes in IHCs and OHCs. ***A***, All differentially expressed genes in IHCs and OHCs. The red line represents the expression level of 18,240 transcripts of IHCs; each blue dot represents the expression level of the same transcripts of OHCs. Genes that are differentially expressed in OHCs are above the red line; genes that are differentially expressed in IHCs are below the red line. Uniquely expressed genes in IHCs and OHCs are highlighted in the light purple and light green areas. Some representative genes that are differentially expressed in IHCs and OHCs are marked by purple dots. ***B***, The 150 most differentially expressed genes in IHCs. The numerical values in red on the right represent the fold difference in expression for IHCs versus OHCs. ***C***, The 150 most differentially expressed genes in OHCs. The numerical values in blue on the right represent the fold difference in expression for OHCs versus IHCs.

plete set of genes and genes expressed in various types of cells in mammals (Okazaki et al., 2002). To date, no transcriptomes of adult mammalian cochlear hair cells have been characterized, although transcriptomes from zebrafish hair cells and neonatal vestibular hair cells were described (McDermott et al., 2007; Hertzano et al., 2011). Our work is the first genome-wide transcriptome study that examines the gene expression profiles of pure populations of adult mouse cochlear IHCs and OHCs. We show the expression of 16,647 and 17,711 transcripts and their abundance in IHCs and OHCs. Despite the fact that a majority of the transcripts are expressed in both types of hair cells, each population contains a considerable number of differentially and uniquely expressed genes that underlie the different biological properties of the two hair cell types.

A significant number of genes are associated with hair cell specializations in the apical, basolateral, and synaptic membranes. Analyses of these genes can facilitate identification of important candidate genes for critical structures in hair cell func-

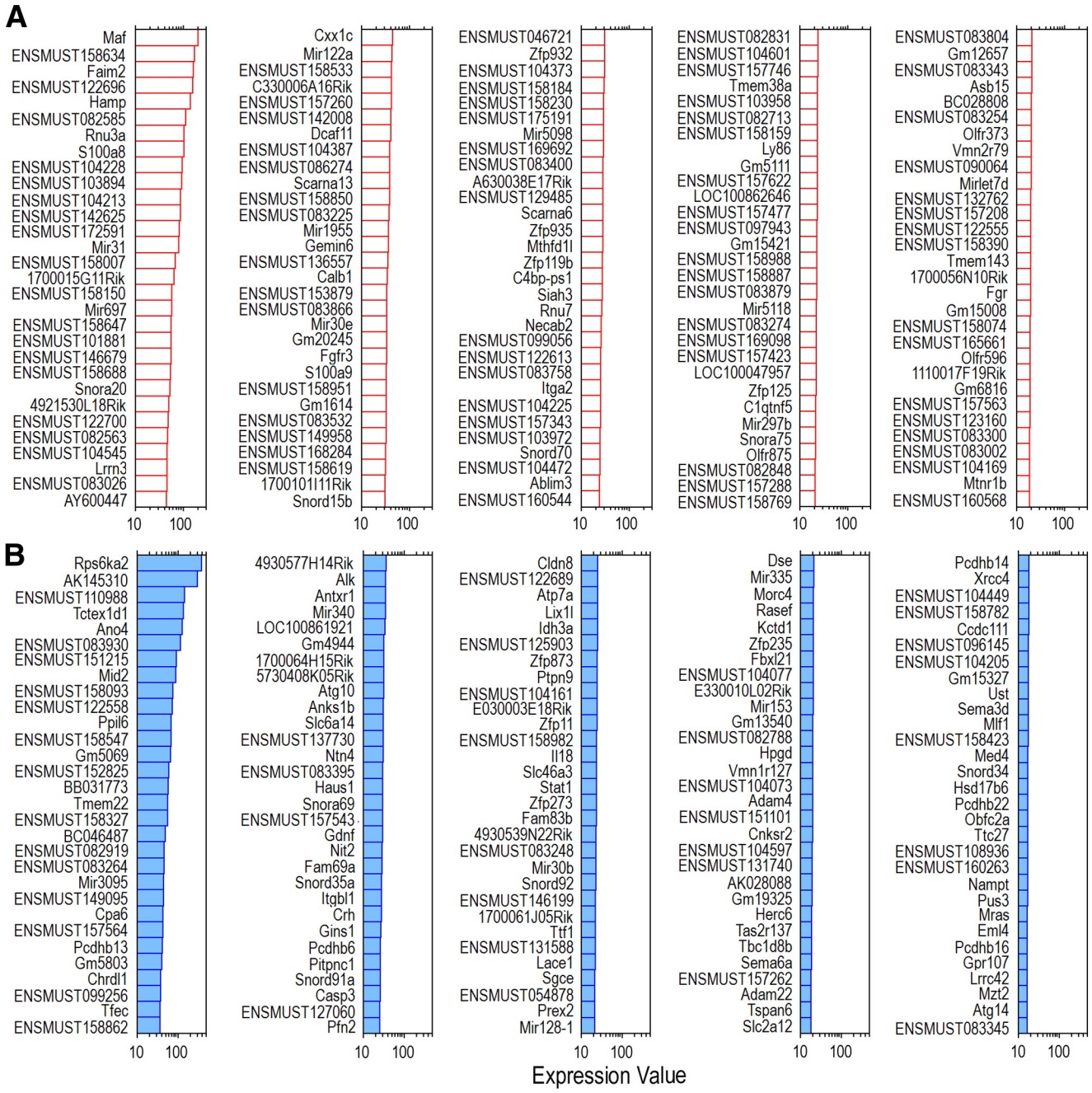

Figure 5. The top 150 uniquely expressed genes in IHCs and OHCs. Uniquely expressed genes are those whose expression levels were below background in only one cell type. ***A***, The expression levels of the top 150 uniquely expressed genes in IHCs. ***B***, The expression levels of the top 150 uniquely expressed genes in OHCs.

tion. Stereocilia contain the transduction apparatus (i.e., tip link and transduction channels) critical for mechanotransduction. The precise molecular identity of the transduction channel is still elusive. TMC1 was suggested recently to be a component of the mechanotransducer channel (Kim et al., 2013; Pan et al., 2013), and mutations in *Tmc1* cause deafness (Steel and Bock, 1980; Kurima et al., 2002; Vreugde et al., 2002). We show that *Tmc1*, *Tmc4*, *Tmc6*, and *Tmc8* are expressed in both types of hair cells. *Tmc1* is expressed at relatively high levels in both populations.

Stretch-activated ion channels are another group of mechanotransducers that conduct ionic currents by responding tk;1to mechanical stimulus in the cell membrane (Sachs, 2010). Stretch-activated currents have been observed previously in OHCs, although their function is unclear (Ding et al., 1991). The channels have three distinct "superfamilies": (1) the ENaC/DEG family; (2) the TRP family; and (3) the K1-selective family. We detected the expression of eight members of the TRP family in both populations. Two members, *Trpm3* and *Trpm7*, have relatively high expression. However, no transcripts of *Trpa1* and two other superfamilies (i.e., ENaC/DEG and the K1-selective families) were detected. The expression of the genes of TMC and stretch-activated channels in both types of hair cells suggests that both could be candidates for the mechanotransduction channels. The expression of several subfamilies of these genes in hair cells suggests that mechanotransduction would not be completely impaired if only one or two subfamilies of genes were mutated.

Our analyses have provided a more comprehensive view of the gene expression for various types of Cl^-, Ca^{2+}, K^+, and

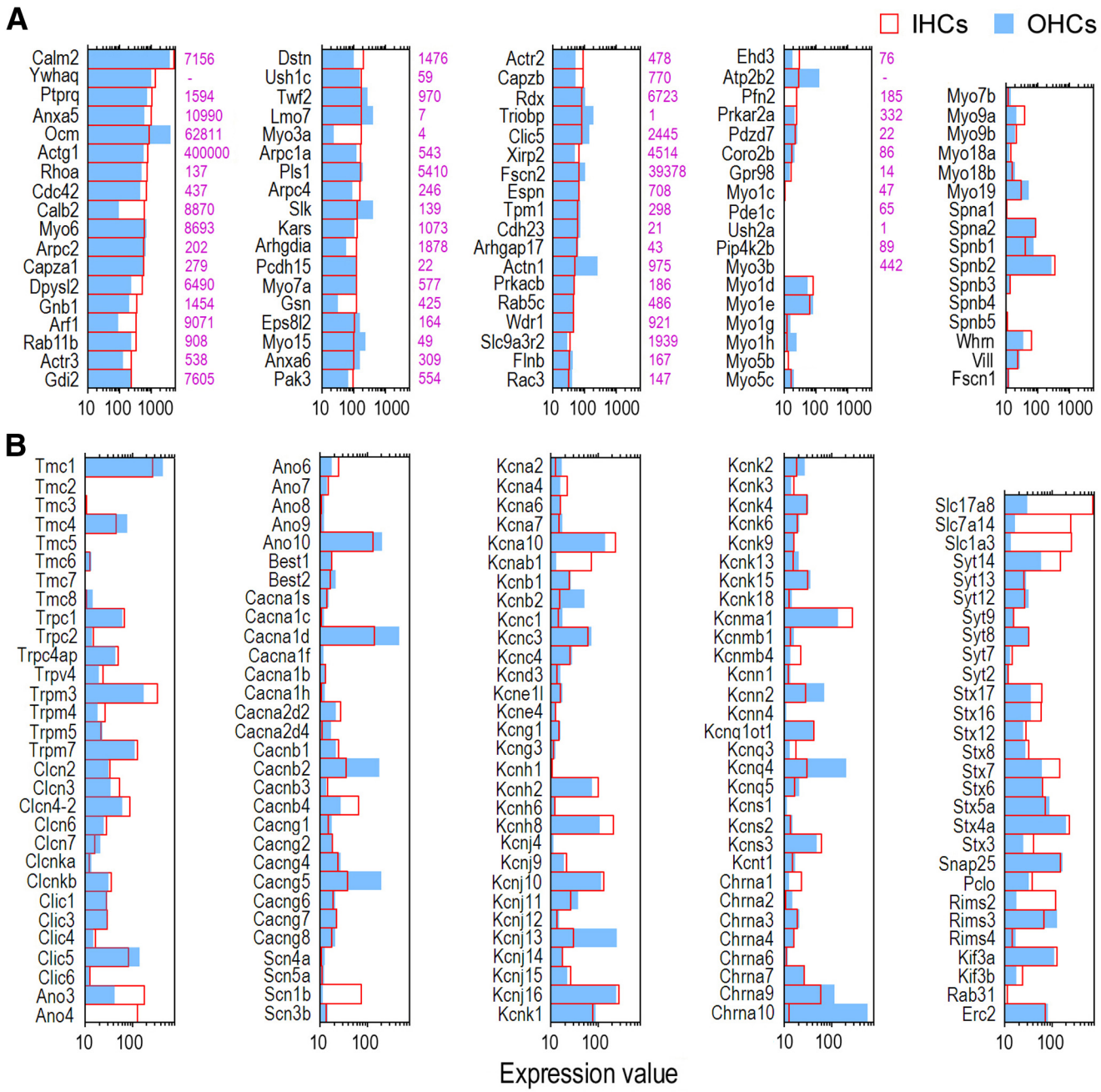

Figure 6. Genes related to stereocilia structure, ion channels, neurotransmitter vesicle release, and ACh receptor. ***A***, Comparison of expression levels for genes that encode proteins associated with stereocilia bundles. Numerical values to the right represent the amount of protein detected in stereocilia by Shin et al. (2013). ***B***, Comparison of expression levels for genes associated with ion channels, neurotransmitter vesicle transport and release, and ACh receptors.

Na^+ channels in hair cells than any previous studies. Past studies using electrophysiology demonstrated the presence of voltage-dependent Ca^{2+}, K^+, and Na^+ channels in adult hair cells (Kros, 1996). The gene expression profiles of ion channels in hair cells appear to be more complex and diverse than has been appreciated previously. Many genes that were not known previously to be expressed in hair cells were detected. We show that most genes that encode various types of ion channels are expressed in both types of hair cells, and most of them show differential expression (Fig. 6*B*). Nineteen of 26 known Ca^{2+} channel genes are expressed in both types of hair cells, and most of them are differentially expressed in OHCs. *Cacna1d*, *Cacnb2*, and *Cacng5* are expressed at relatively high levels. L-type Ca^{2+} channels (e.g., *Cacna1d*) were electrophysiologically demonstrated in adult OHCs (Nakagawa et al., 1991). Only four Na^+ genes were detected in hair cells, and all are expressed at low levels, except *Scn1b*. Voltage-dependent Na^+ currents have been detected in some adult OHCs, although their function in adult cells is unclear (Witt et al., 1994). K^+ channels are the most widely distributed type of ion channel found in virtually all cell types and control a wide variety of cell functions. Fifty-two K^+ channel genes are expressed in both populations of hair cells. *Kcna10*, *Kcnh8*, *Kcnj10*, *Kcnj13*, *Kcnj16*, *Kcnma1*, and *Kcnq4* show relatively higher level of expression in both populations. *Kcnb2*, *Kcnj13*, *Kcnn2*, and *Kcnq4* are differentially expressed in OHCs, whereas *Kcnab1*, *Kcnh8*, and *Kcnma1* are differentially expressed in IHCs.

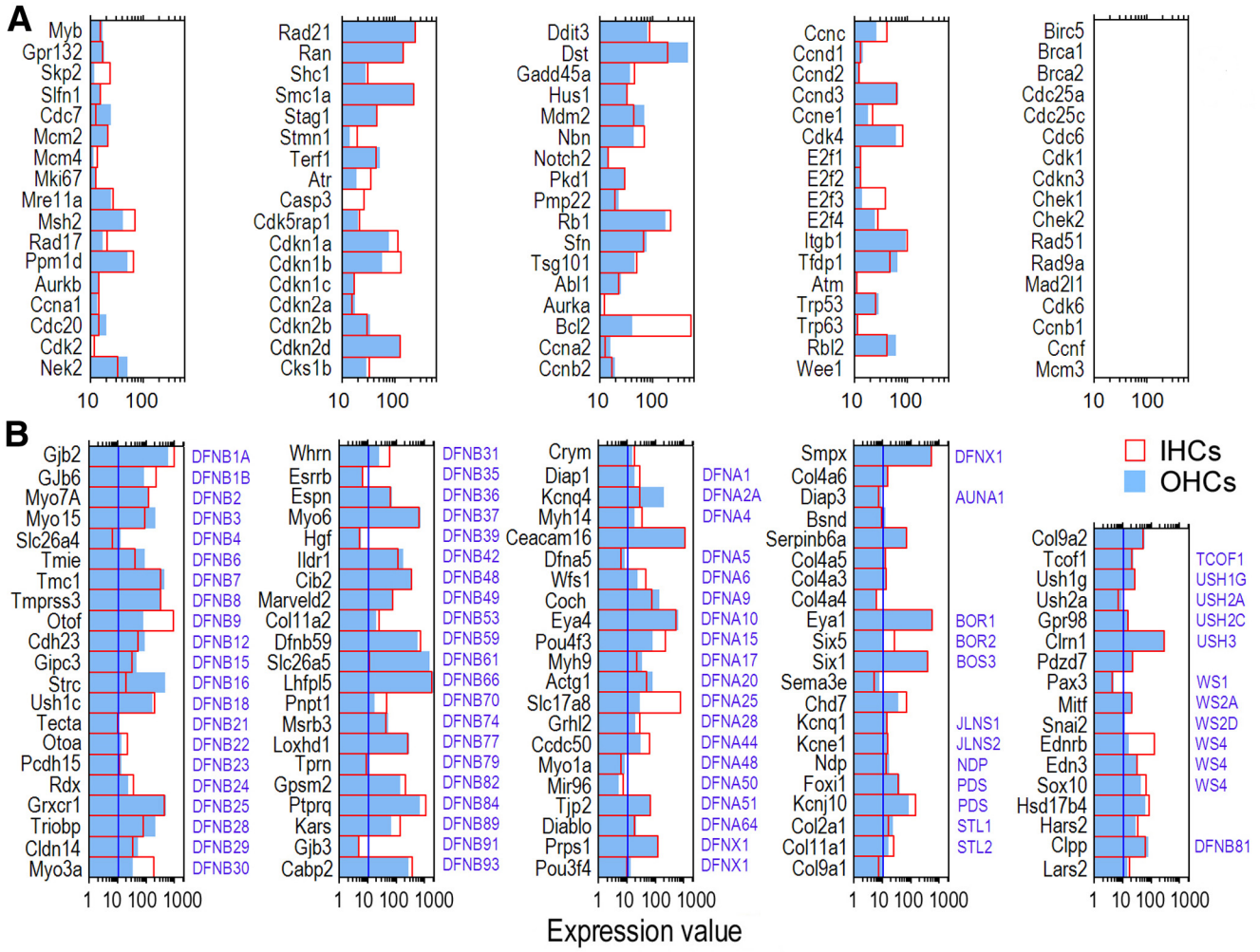

Figure 7. Genes regulating cell cycle and genes related to deafness. ***A***, Expression levels of 85 genes that are important for cell-cycle regulation. ***B***, Comparison of expression levels for 101 known deafness genes. The blue line in each panel marks the baseline for expression. Genes whose expression levels fall below the baseline are not considered to be expressed.

KCNQ4, which may underlie $I_{k,n}$ in hair cells (Marcotti and Kros, 1999), is important for OHC function. Most members of the three different types of Cl^- channels are expressed in both populations, although corresponding physiological confirmation of Cl^- channels has been lacking.

A comprehensive picture of the gene expression profile of many proteins associated with synaptic transmission in both types of hair cells is also provided in our analysis. The expression of genes that are associated with neurotransmitter vesicle transport and fusion has not been demonstrated in hair cells, with the exception of a few genes (i.e., *Chrna9*, *Chrna10*, *Otof*, *Slc17a8*, and *Syne1*; McDermott et al., 2007; Hertzano et al., 2011). Most of these vesicle-related genes show differential expression favoring IHCs. Low-level expressions of these genes are also detected in OHCs. Thus, our data provide the molecular basis for the presence of a functional afferent synapse in OHCs (Weisz et al., 2009). We detected the gene expression of several subunits of cholinergic receptors in both types of hair cells, with significant differential expression of *Chrna10* and *Chrna9* in OHCs. This is in agreement with previous studies (Elgoyhen et al., 1994, 2001) and the innervation pattern of adult OHCs (Spoendlin, 1970).

Transcriptome comparison of IHCs and OHCs is extremely useful for identifying genes underlying unique structures and functions of each type of hair cell. We show that *Slc17a8* and *Otof* are among the genes that are highly and differentially expressed in IHCs, whereas *Slc26a5*, *Chrna10*, *Chrna9*, *Strc*, *Kcnq4*, and *Ocm* are highly and differentially expressed in OHCs. This pattern of differential expression of these genes is remarkably consistent with the division of function between IHCs and OHCs in the mammalian cochlea. The agreement of the differential gene expression patterns between our study and previous studies also validates the high purity of IHC and OHC population used for this study.

DNA microarray and RNA sequencing are the two common techniques for transcriptome studies. Either technique has pros and cons (Shendure, 2008). As with all new technologies, RNA sequencing studies present significant challenges, including sample input requirements and the complex bioinformatics analysis (Malone and Oliver, 2011; Martin and Wang, 2011). Thus, microarray is still a powerful technique for transcriptome analysis with exceptional quantitative accuracy, despite the fact that microarray has limitations in dynamic range and identification of multiple splice variants of the genes and represents a closed assay system, because it relies on the prerequisite of sequence information (Shendure, 2008). We chose to use the microarray technique because the small amount of total RNA from such a refined population of cells was not suitable for conventional mRNA isolation

and RNA sequencing high-throughput sequencing. Furthermore, the microarray data is highly standardized and easy to analyze.

Although single-cell transcriptome analysis using next-generation sequencing technology is gaining popularity (Tang et al., 2009; Sandberg, 2014), our study using a pool of 2000 pristine and homogenous cells has the unparalleled advantage of higher sensitivity for transcript detection and more representative results. Our approach using suction pipette technique has some significant advantages for transcriptome analysis for the following reasons: First, our analyses were based on two separate types of hair cells and thus provide critical information of what genes are uniquely expressed in each hair cell type. Second, we used adult hair cells that best represent functionally mature hair cell transcriptomes. Third, our approach minimizes the probability of contamination by supporting cells, where three important steps were employed to ensure that each population of cells was highly specific based on distinct and identifiable morphology. Finally, cells were maintained in cold solution during collection and protected in RNAlater solution to avoid the potentially rigorous conditions of live cell sorting using flow cytometry.

It is important to point out that the transcriptomes of adult IHCs and OHCs reported here establish a framework for future characterization of all genes expressed in hair cells. Our study also represents a major step forward in bridging the knowledge gap between genes and their functional products that define hair cell biology.

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