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Original Article

Characterization of the transcriptomes of *Atoh1*-induced hair cells in the mouse cochlea

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Abstract: Postnatal mammalian cochlear hair cells (HCs) can be regenerated by direct transdifferentiation or by mitotic regeneration from supporting cells through many pathways, including Atoh1, Wnt, Hedgehog and Notch signaling. However, most new HCs are immature HCs. In this study we used RNA-Seq analysis to compare the differences between the transcriptomes of *Atoh1* overexpression-induced new HCs and the native HCs, and to define the factors that might help to promote the maturation of new HCs. As expected, we found *Atoh1*-induced new HCs had obvious HC characteristics as demonstrated by the expression of HC markers such as *Pou4f3* and Myosin VIIA (Myo7a). However, *Atoh1*-induced new HCs had significantly lower expression of genes that are related to HC function such as Slc26a5 (*Prestin*), *Slc17a8* and *Otof*. We found that genes related to HC cell differentiation and maturation (*Kcnma1*, *Myo6*, *Myo7a*, *Grxcr1*, *Gfi1*, *Wnt5a*, *Fgfr1*, *Gfi1*, *Fgf8* etc.) had significantly lower expression levels in new HCs compared to native HCs. In conclusion, we found a set of genes that might regulate the differentiation and maturation of new HCs, and these genes might serve as potential new therapeutic targets for functional HC regeneration and hearing recovery.

Keywords: Inner ears, cochlea, RNA-Seq, gene expression, supporting cells, hair cell regeneration, hair cell maturation

Introduction

In adult mammals, the loss of cochlear hair cells (HCs) causes permanent hearing loss. A series of previous studies confirmed that new cochlear HCs can be regenerated in neonatal and adult mice [1-4], which makes HC regeneration a potential route for the recovery of hearing function. However, most of the newly generated HCs are immature HCs [3, 5]. Thus understanding the gene expression profile differences between the newly generated HCs and mature HCs and promoting the maturation of newly generated HCs is a key priority in the HC regeneration field.

Atoh1 encodes a key transcription factor for the induction of inner ear HCs during the development and regeneration [6]. In the mouse cochlea, *Atoh1* is first expressed in the cochlear prosensory epithelial domain at embryonic day (E)12.5, and its expression is up-regulated in primitive HCs between E13.5 and E14.5 [7]. *Atoh1* expression peaks at E17.5 and then decreases to barely detectable levels by postnatal day (P) 6 [8-10]. Transcriptome analysis has suggested that many genes, including *Pou4f3*, *Gfi1*, and *Myo7a*, are down-stream genes of *Atoh1* during the process of cochlear development [10]. Consequently, the expression of *Atoh1* determines cell fate in the mouse cochlea, and upregulation of *Atoh1* expression is thus a critical step for HC regeneration. *Atoh1* overexpression can robustly generate new HCs from supporting cells (SCs) in the neonatal mouse cochlea, but this HC generation capacity is significantly reduced in the adult mouse cochlea [11-14]. However, the characteristics of the newly generated HCs are different from the native HCs [5]. The regenerated HCs express some of the HC markers, such as *Myo7a*, *Myo6*, and inner hair cell marker *Otof (otoferlin)*, but none of the new HCs express the mature inner

HC marker *Slc17a8* (*Vglut3*, vesicular glutamate transporter 3) or the mature outer HC marker *Slc26a5* [11, 12, 15, 16]. The regenerated HCs also have no mature stereocilia bundles [16]. All of these reports suggest that new HCs do not have normal HC function and are unable to process sound signals. Cochlear supporting cells, including pillar cells (PCs), Deiters' cells (DCs), inner phalangeal cells (IPhCs), inner border cells (IBCs), and supporting cells in greater epithelial ridge (GER) or lesser epithelial ridge (LER), can be converted to new HCs by Atoh1 overexpression [12, 17, 18]. It is valuable to explore the gene expression profile difference between the new HCs and the native HCs, and thus to identify factors that might promote the maturation of newly generated HCs. In previous study, Yamashita et al. performed RNA sequencing of new converted HCs and mature HCs after conditional overexpression of Atoh1 in PCs and DCs using Fgfr3iCreER mice, and found that 51 transcription factors, including Isl1, were differentially expressed among cHCs, SCs and HCs [19]. In this paper, we analyzed the transcriptomes of *Atoh1*-induced cochlear new HCs using Sox2-CreER mice, in which many SCs including IBCs, IPhCs and other SCs in GER can be converted to new HCs by Atoh1 overexpression. We separated the native HCs from the *Atoh1*-induced new HCs and analyzed their transcriptomes by RNA-Seq. We found that there were significant differences in the gene expression profiles between the *Atoh1*-induced new HCs and the native HCs in cochlear sensory epithelium. Gene ontology (GO) analysis showed that the genes that were significantly more expressed in the native HCs included a variety of genes associated with generation of neurons, cell survival and hearing function, while these genes were expressed lower in the new HCs. In summary, we found a set of genes that might influence the maturation and survival of newly generated HCs, and these genes might serve as potential new therapeutic targets for functional HC regeneration and hearing recovery.

Methodology

Mouse models

We used transgenic mice in the C57BL/6J background. *Sox2*-CreER mice (JAX number 008875) and Rosa26-tdTomato mice (JAX number 007914) were ordered from the Jackson Laboratory. *Atoh1*-eGFP (enhanced green fluorescent protein) mice were provided by Jane Johnson (University of Texas Southwestern Medical Center, Dallas, TX, USA), and CAG-loxP-stop-loxP-*Atoh1*-HA$^+$ mice (*Atoh1*-HA$^+$ mice) were the kind gift of St. Jude Children's Research Hospital. Postnatal day (P) 0 was defined as the day of birth. Both male and female mice were used for all experiments. This study was carried out in strict accordance with the "Guiding Directive for Humane treatment of Laboratory Animals" issued by the Chinese National Ministry of Science and Technology in September, 2006. All animal experiments were approved by the Shanghai Medical Experimental Animal Administrative Committee (Permit Number: 2009-0082), and all efforts were made to minimize suffering and reduce the number of animals used.

Tamoxifen administration

Tamoxifen (Sigma-Aldrich) diluted in corn oil was injected intraperitoneally at the late stage of P3 at 0.20 mg/g bodyweight, and the control group was injected only with corn oil.

Fluorescence-activated cell sorting (FACS)

Sox2-CreER$^+$/*Atoh1*-HA$^+$/*Atoh1*-eGFP$^+$/tdTomato$^+$ mice were sacrificed at P7, and the cochlear epithelium of middle and basal turn was dissected and trypsinized with pre-warmed 0.25% trypsin/EDTA (Invitrogen) at 37°C for 5 min. Soybean trypsin inhibitor (Worthington Biochem) was added to terminate the reaction followed by mechanical trituration with blunt tips and pipetting up and down ~ 100 times. Suspended cells were percolated through a 40 μm cell strainer (BD Biosciences) before FACS. The native HCs were labeled with enhanced green fluorescent protein (eGFP$^+$ cells), new HCs were co-labeled with enhanced green fluorescent protein and tdTomato red fluorescent protein (eGFP$^+$/tdTomato$^+$ cells), and SCs were labeled with tdTomato red fluorescent protein (tdTomato$^+$ cells). The native HCs, new HCs, and SCs were sorted on a BD FACS Aria III (BD Biosciences) using the tdTomato and GFP channels.

RNA-Seq and statistical data analysis

HCs purified by FACS were pooled to isolate the total RNA using the AllPrep DNA/RNA/Protein

Mini Kit (QIAGEN). The sequencing library was prepared with the SMART-Seq v4 Ultra Low Input RNA Kit and the Illumina mRNA-Seq Sample Prep Kit. Library quality was analyzed using an Agilent Bioanalyzer. Sequencing was performed using the Illumina HiSeq2500 Platform, and 150-bp paired-end reads were generated. Raw reads were trimmed using Trimmomatic and mapped to the mouse reference genome (mm10) using TopHat2 [20]. Cufflinks2.2.1 was used to assemble the transcripts and measure transcript abundances. Gene differential expression analysis was performed between the two groups using Cufflinks with p value < 0.05. Scatterplot was used to present FPKM values of all differentially expressed genes and the rest. DAVID (Database for Annotation, Visualization and Integrated Discovery) version 6.7 software (http://david.abcc.ncifcrf.gov/home.jsp) was used to determine the functional annotation of significant genes in the datasets as described previously [21].

Immunofluorescence staining

Immunofluorescence staining was performed to confirm the properties of the FACS sorted cells. Myo7a was used as a marker of HCs. Briefly, after fixation, the cells or tissues were blocked with 10% normal donkey serum in 10 mM phosphate-buffered saline (PBS, pH 7.4) and 1% Triton X-100 for 1 h at room temperature and then incubated with primary antibody (rabbit polyclonal anti-Myo7a, Proteus Biosciences) overnight at 4°C. The next day, the samples were incubated for 1 h at 37°C with Alexa 647-conjugated donkey secondary antibody (Invitrogen). Nuclei were stained with DAPI (Sigma-Aldrich). The images were acquired with a Leica SP8 confocal fluorescence microscope (Leica).

RNA extraction and quantitative PCR (qPCR)

qPCR was performed to determine the purity of the FACS-sorted cells and to confirm the RNA-Seq data. Total RNA was extracted from purified cells using an RNeasy Plus Micro kit (Qiagen), and cDNA was generated using Superscript III Reverse Transcriptase (Invitrogen). qPCR was performed with the Master SYBR Green Kit (Applied Biosystems) on a three-step real-time PCR system (Applied Biosystems). Each sample was analyzed in triplicate. The relative quantification of gene expression was determined by the CT method using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase as the internal control [22]. One-way analysis of variance was performed to compare the expression levels of the samples collected by FACS. The primers used for qPCR are listed in Supplementary Table 1.

Results

Overexpression of Atoh1 in SCs generates new HCs in the neonatal mouse cochlea

Sox2-CreER$^+$/*Atoh1*-HA$^+$/*Atoh1*-eGFP$^+$/tdTomato$^+$ mice were used to obtain *Atoh1*-induced new HCs. Considering the Sox2-CreER activity was also detected in many of cochlear hair cells especially in apical turn when tamoxifen were injected at P1 [23], we injected tamoxifen intraperitoneally at P3 and harvested the middle and basal cochlear turns at P7 (**Figure 1A**). *Atoh1* overexpression in cochlear SCs generated many new HCs in the cochleae [24, 25]. In our results, the eGFP$^+$ cells are native HCs that were generated during the development of the inner ear, while the eGFP$^+$/tdTomato$^+$ cells are *Atoh1*-induced newly generated HCs that originated from Sox2$^+$ SCs. The HC marker Myo7a was expressed both in eGFP$^+$/tdTomato$^+$ new HCs and native HCs (**Figure 1B**).

Flow cytometry isolated newly generated HCs and native HCs

To compare the gene expression profiles between newly generated HCs and native HCs, we isolated these two HC populations via flow cytometry (**Figure 2A-D**). As described above, the new HCs were labeled with both eGFP and tdTomato, the native HCs were labeled only with eGFP, and the Sox2$^+$ SCs were labeled only with tdTomato (**Figure 2E**). Cells isolated by FACS were immunostained with Myo7a to confirm their HC identity. As shown in **Figure 2E**, the eGFP$^+$ cells were Myo7a$^+$ and tdTomato$^-$, indicating they were native HCs. The eGFP$^+$/tdTomato$^+$ cells were Myo7a$^+$, indicating that they were newly generated cells originating from Sox2$^+$ SCs. To validate the properties of the newly generated HCs, we performed qPCR using primers for the HC marker *Pou4f3* and the SC marker *Sox2* (**Figure 2F**). There was no significant difference in the expression of

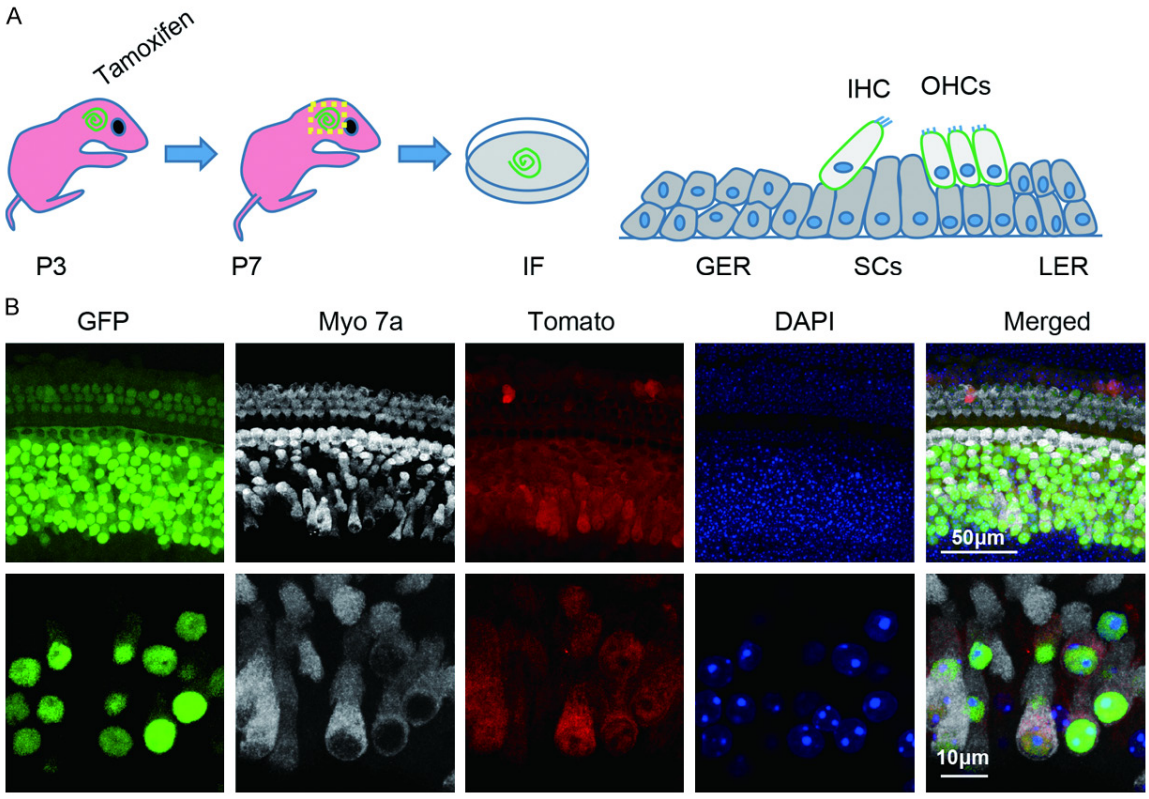


Figure 1. Increased expression of *Atoh1* in SCs induced new cochlear HCs in neonatal mouse. A. Diagram of the organ of Corti and scheme of Atoh1 overexpression. Cochlear hair cells are surrounded by SCs. Tamoxifen was injected intraperitoneally at the late stage of P3 and sensory epithelium was harvested at P7. B. In experimental mouse (*Sox2*-CreER/*Atoh1*-OE/*Atoh1*-nGFP/tdTomato mouse), the new HCs were labelled with green fluorescent protein(GFP) and tdTomato red fluorescent protein, native HCs were labeled with green fluorescent protein, SCs were labeled with tdTomato red fluorescent protein. OHC, outer hair cell; IHCs, inner hair cells; SCs, supporting cells; GER, greater epithelial ridge; LER, lesser epithelial ridge; P, postnatal; IF, immunofluorescence. Scale bar, 50 μm or 10 μm.

Pou4f3 and *Sox2* between the native HCs and the new HCs, confirming that the isolated eGFP$^+$/tdTomato$^+$ cells had lost supporting cells identification and been converted to new HCs. The eGFP$^+$/tdTomato$^+$ new HCs had significantly lower expression of the mature outer HC marker *Slc26a5*, which encodes the Prestin protein, when compared with eGFP$^+$ native HCs (**Figure 2F**). Together, these results confirmed that the eGFP$^+$/tdTomato$^+$ cells induced by upregulation of *Atoh1* were newly generated HCs that were different from the native HCs.

Analysis of RNA-Seq results

Transcriptomes of native HCs and new HCs were analyzed by RNA-Seq. Isolated native HCs and new HCs from at least six mice were used for extraction of total RNA, and two biological replicates were sequenced for analysis. GO analysis was performed on differentially expressed genes using DAVID. A total of 47-83 million paired reads were obtained for each sample, with at least 48% of the reads mapping correctly to the reference genome. To analyze the differences between native HCs and new HCs, we examined the fragments per kilobase of transcript per million mapped reads (FPKM) values in each sample (**Figure 3A**). The cyan dots represent the highly differentially expressed genes in the eGFP$^+$/tdTomato$^+$ double-positive new HCs, and the red dots represent the highly differentially expressed genes in the native HCs. These results demonstrated that the newly generated HCs were quite different from the native HCs.

We then checked the RNA expression levels of specific cell markers for cochlear HCs and SCs in the newly generated eGFP$^+$/tdTomato$^+$ HCs

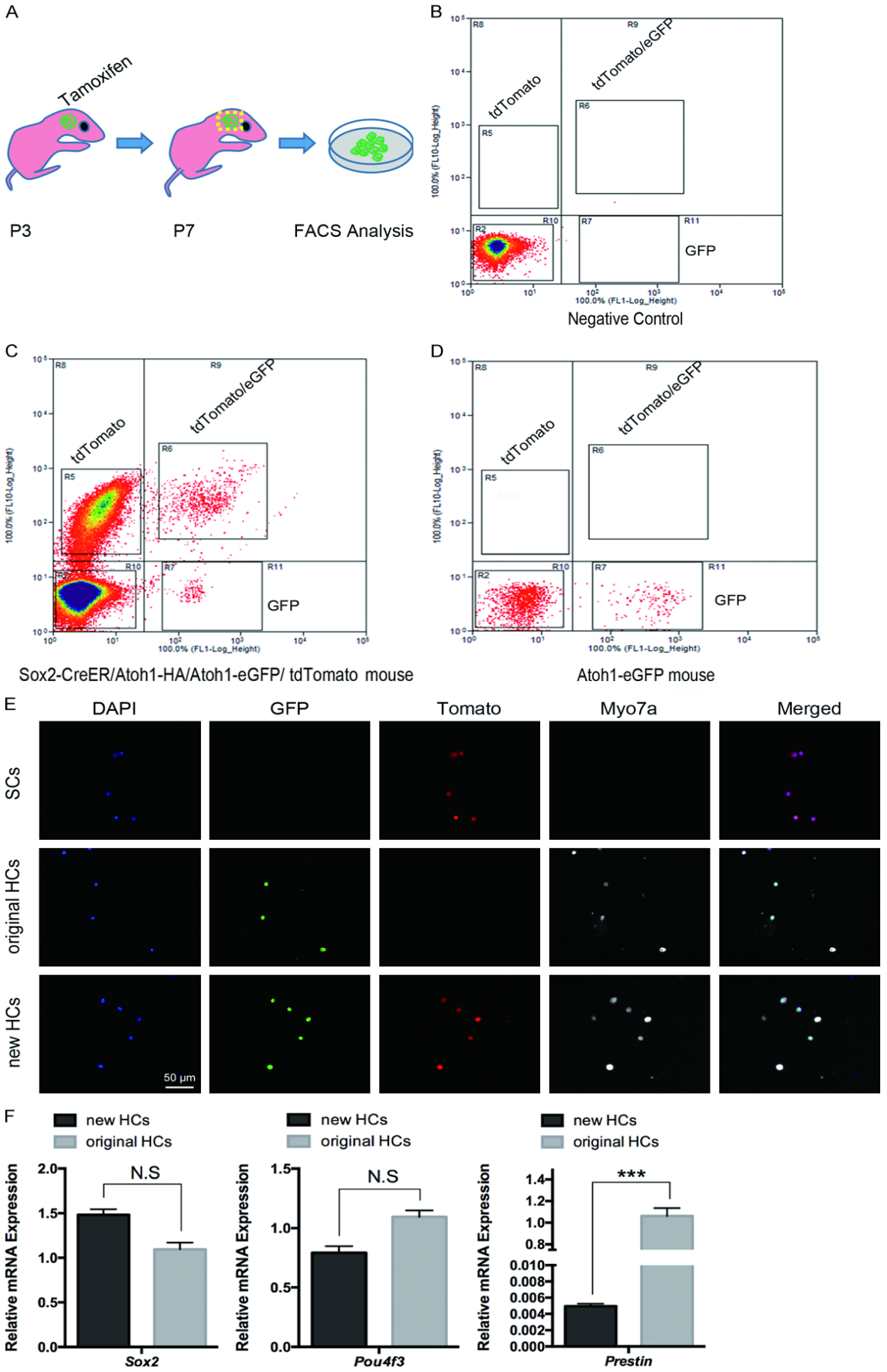

A
Tamoxifen
P3
P7
FACS Analysis
B
tdTomato
tdTomato/eGFP
GFP
Negative Control
C
Sox2-CreER/Atoh1-HA/Atoh1-eGFP/ tdTomato mouse
D
Atoh1-eGFP mouse
E
DAPI
GFP
Tomato
Myo7a
Merged
SCs
original HCs
new HCs
50 μm
F
new HCs
original HCs
Relative mRNA Expression
N.S
N.S
***
Sox2
Pou4f3
Prestin

Figure 2. Screening new hair cells and native cochlear hair cells by flow cytometry. A. Scheme to perform flow cytometry. Tamoxifen was injected intraperitoneally at the late stage of P3 and sensory epithelium was harvested at P7. B. Wild type mice were used as negative control. C. Three groups of cells were obtained in experimental mice. D. *Atoh1*-eGFP mice were used as positive control. E. Immunofluorescent staining was used to detect the purity of sorted cells. F. qPCR was performed to validate the purity of sorted cells. FACS, fluorescence activated cell sorting. Scale bar, 50 μm.

and the native eGFP$^+$ HCs. We found no significant difference in the expression of SC markers (*Sox2*, *Jag1*, and *Prox1*) and the most commonly used early HC markers (*Pou4f3*, *Myo15*, and *Myo3a*) between these two groups, confirming that the eGFP$^+$/tdTomato$^+$ cells had a HCs identity and were not SCs anymore. However, some HC markers, including *Myo7a*, *Myo6*, *Pvalb*, *Gfi1*, *Slc26a5*, *Slc17a8*, and *Otof*, were expressed at significantly lower levels in the new HCs compared to the native HCs (**Figure 3B**). Considering that *Slc26a5* and *Slc17a8* are used as markers of mature and functional cochlear HCs [26-28], these results demonstrated that *Atoh1*-induced new HCs would be immature and lack sensory function.

Genes enriched in newly generated HCs and native HCs

We next determined the genes that were abundantly expressed in the newly generated HCs and native HCs, respectively. **Figure 4** shows the 200 most highly expressed genes in the native HCs and newly generated HCs. The numbers at the right of each row indicate the rankings of the expression level in the other group. We found the top 200 most highly expressed genes in the native HCs were expressed at lower levels in the new HCs, which might be related to HC maturation and function, while the top 200 most highly expressed genes in the new HCs were also highly expressed in the native HCs, except for 28 genes (*Rps23*, *Epyc*, *Rps9*, *Prdx2*, *Psmb6*, *Vmo1*, *Rplp0*, *S100a13*, *Id1*, *Tmsb10*, *Npy*, *Rpl8*, *Gm1673*, *Id3*, *Uchl1*, *S100a10*, *Anapc13*, *Hes6*, *Cib1*, *Saa3*, *Hint2*, *Chmp2a*, *Enho*, *Pgp*, *Psmb3*, *Snrpg*, *Fabp7*, and *Polr2j*).

Differentially expressed genes between the newly generated HCs and the native HCs

Of the 23,798 genes obtained from RNA-Seq of newly generated HCs and native HCs, 1,070 were differentially expressed between the two groups, including 32 genes that were exclusively expressed in the new HCs and 188 genes that were exclusively expressed in the native HCs.

Figure 5A shows the top 100 differentially expressed genes (fold change > 1.5, $P < 0.05$) that were highly enriched in native HCs and the total 52 differentially expressed genes (fold change > 1.5, $P < 0.05$) that were highly enriched in newly generated HCs. The black numbers at the right of each row represented the gene expression fold change.

Figure 5B showed the top 20 genes (fold change > 1.5, $P < 0.05$) that were uniquely expressed in native HCs or new HCs, respectively. We found that the genes that were uniquely expressed in the native HCs seemed to have closer connection with HC function while most of the genes that were uniquely expressed in new HCs have not yet been studied in the inner ear.

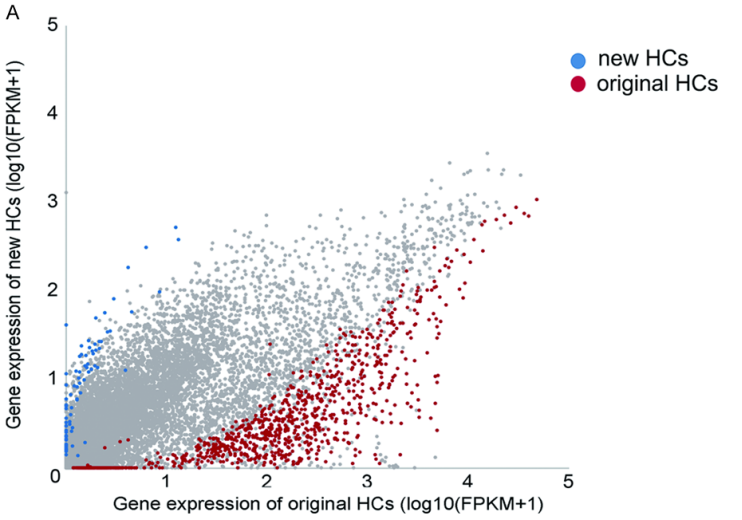
Analysis of differentially expressed genes related to HC maturation and function

Among the top 100 differentially expressed genes (**Figure 5A**) that were highly enriched in the native HCs, *Pkd2* [29], *Mtr* [30], and *Sik3* [31] are related to auditory function and inner ear development. Moreover, three genes related to calcium channel activity, *Orai2* [32], *Erc2* [33], and *Tmem203* [34], were significantly more highly expressed in the native HCs compared with the newly generated HCs, but the functions of these genes in the inner ear have not been previously reported.

Among the top 52 differentially expressed genes (**Figure 5A**) that were highly enriched in the new HCs, only two genes, *Oc90* [35] and *Misp* [36], were reported previously to have a function in the inner ear.

Among the genes that were uniquely expressed in the native HCs (**Figure 5B**), only *Hist2h2ab* and *Jam2* have been previously reported to play a role in the inner ear. The histone gene *Hist2h2ab* was detected in HC stereocilia bundles, but the precise function was not established [37]. Among the genes that were unique-

A



B

Non-differentially expressed HC and SC markers (RNA-Seq data)

	gene	sample_1	sample_2	value_1	value_2	p_value
1	*Sox2*	original HCs	new HCs	61.4579	21.8346	0.51235
2	*Prox1*	original HCs	new HCs	0.0458458	0.0110288	1
3	*Jag1*	original HCs	new HCs	3.5283	5.02626	0.7617
4	*Atoh1*	original HCs	new HCs	790.74	83.3876	0.18585
5	*Pou4f3*	original HCs	new HCs	16.7178	16.1277	0.9756
6	*Myo15*	original HCs	new HCs	0.977894	1.01663	0.97405
7	*Myo3a*	original HCs	new HCs	0.170306	0.78245	0.30215

Differentially expressed HC markers (RNA-Seq data)

	gene	sample_1	sample_2	value_1	value_2	p_value
1	*Myo6*	original HCs	new HCs	283.612	14.6254	0.0316
2	*Myo7a*	original HCs	new HCs	158.93	1.76779	0.01465
3	*Pvalb*	original HCs	new HCs	9074.06	123.237	0.00775
4	*Gfi1*	original HCs	new HCs	86.2088	2.98232	0.0487
5	*Slc26a5*	original HCs	new HCs	95.8455	3.76969	0.0123
6	*Slc17a8*	original HCs	new HCs	46.7475	0.453706	0.014
7	*Otof*	original HCs	new HCs	1944.66	12.3006	0.0023

Figure 3. Differentially expressed genes in native HCs and new HCs. A. All differentially expressed genes in native HCs and new HCs (fold change > 1.5, P < 0.05). The red dot represents the expression level of transcripts from native HCs, and the cyan dot represents the expression level of the same transcript from new HCs. B. The expression levels of HC and SC markers.

ly expressed in newly generated HCs (**Figure 5B**), *Cldn24* [38], *Myl3* [39], and *Myf6* (*Mrf4*) [40] have been reported to be expressed in the inner ear, but their functions in HCs remain unknown.

We then analyzed the cell signaling genes involved in the development of cochlear HCs. The Notch signaling pathway plays multiple roles in early cell fate decisions and in cochlear HC development [25, 41], and there was no significant difference in the expression of Notch pathway genes (*Jag2*, *Jag1*, *Notch1*, *Hes1*, *Dll1*, and *Dll3*) between the native HCs and new HCs.

The Hedgehog signaling pathway regulates cell proliferation and cochlear HC formation in the early differentiation process[42], and there was no significant difference in the expression of Hedgehog pathway genes (*Shh*, *Smo*, *Ptch1*, and *Gli1*) between the two groups. The Wnt pathway is vital to the differentiation and regeneration of inner ear HCs [43, 44], and we found that the genes participating in the canonical Wnt pathway (*Ctnnb1*, *Axin2*, *Lgr6*, *Lgr5*, and *Lef1*) exhibited no differences in expression between the two groups, while the other Wnt pathway components (*Wnt5a*, *Wnt4*, *Wnt9a*, and *Tcf4*) and Wnt-targeted genes (*Pdgfra* and *Dpp10*) had significantly lower expression in new HCs compared with the native HCs (P < 0.05 for all) (**Figure 6A**, **6B**).

To identify the gene enrichment in the new HCs and native HCs, GO analysis was performed. Compared with native HCs, the genes related to inner ear development and morphogenesis were expressed at lower levels in the new HCs (fold enrichment > 1.5, P < 0.05), including *Kcnma1*, *Myo6*, *Myo7a*, *Grxcr1*, and *Gfi1* that are related to the GO term "auditory receptor cell differentiation" (GO:0042491) and *Wnt5a*, *Fgfr1*, *Fgf8*, *Myo6*, *Myo7a*, *Atp8a2*, *Tbx1*, *Gfi1*, and *Col11a1* that are related to the GO term "inner ear morphogenesis" (GO:0042472). These genes included the common markers of HCs *Myo6*, *Myo7a*, and *Gfi1* and the inner ear marker *Fgf8*.

Transcription factors (TFs) include a large number of genes that determine the cell fate during the processes of development and differentiation. We performed comparative analyses of TF genes and found that 109 TF genes (GO:0006351, P < 0.01) had significantly lower expression in new HCs compared with native HCs. The functions of most of the TFs in the for-

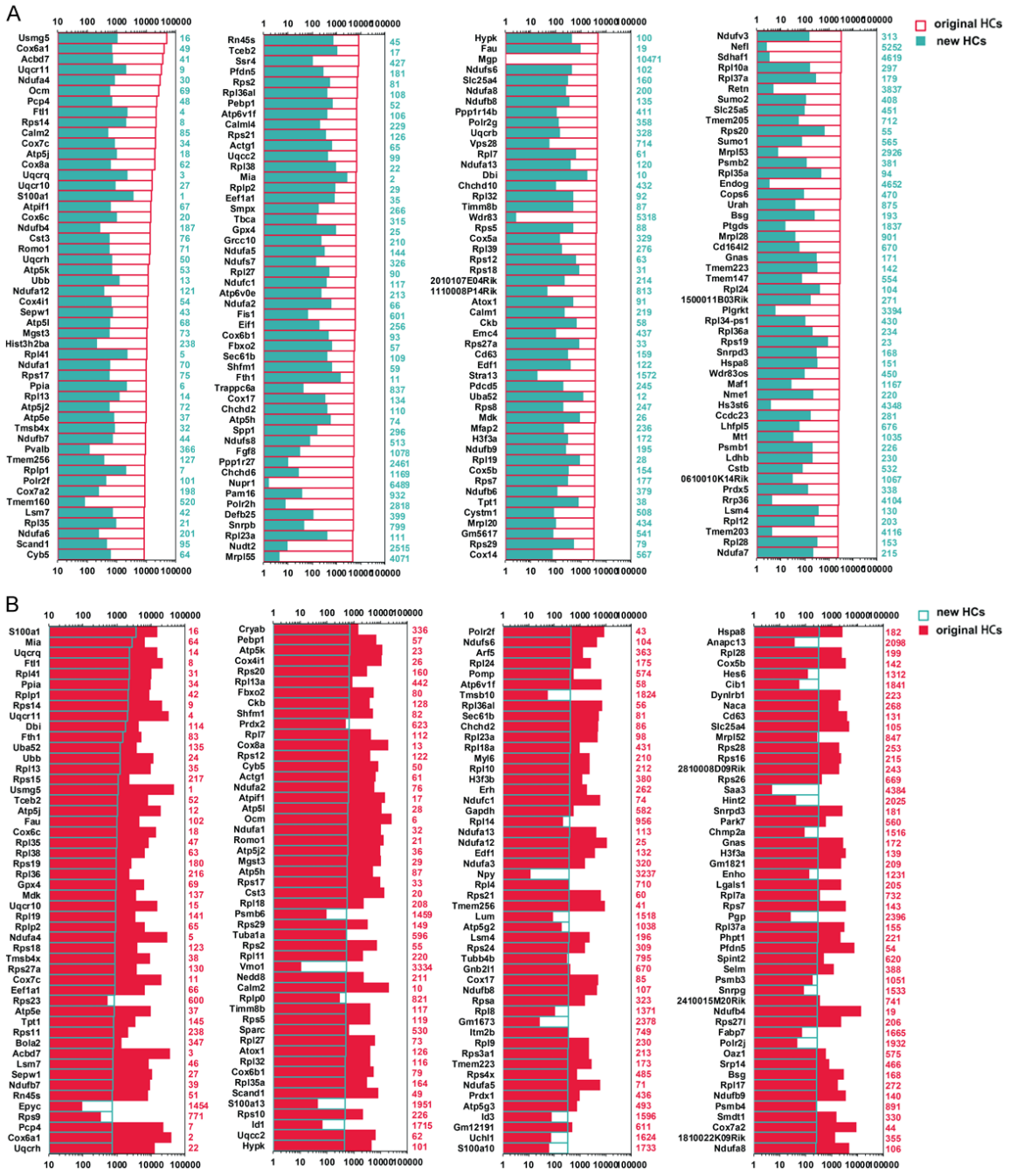


Figure 4. Top 200 genes expressed in native HCs and new HCs. A. The top 200 genes expressed in native HCs in decreasing order. The cyan numbers on the right side of each panel represent the ranking of the same genes in new HCs. B. Top 200 genes in new HCs in decreasing order. The red numbers on the right side of each panel represent the ranking of the same genes in native HCs.

mation of inner ear HCs remain unknown except for five TF genes (*Ctnnd1*, *Tbx1*, *Kdm1a*, *Lmo4*, and *Gfi1*). *Kdm1a*, which encodes lysine-specific demethylase 1, has an important role in HC regeneration and cell differentiation of inner ear progenitors [45, 46]. *Lmo4* and *Tbx1* are transcriptional regulators that mediate morphogenesis during inner ear development. *Ctnnd1*, which encodes p120-catenin, regulates convergent extension and uniform orientation of sensory HCs during cochlear development [47]. The expression of other TFs that are

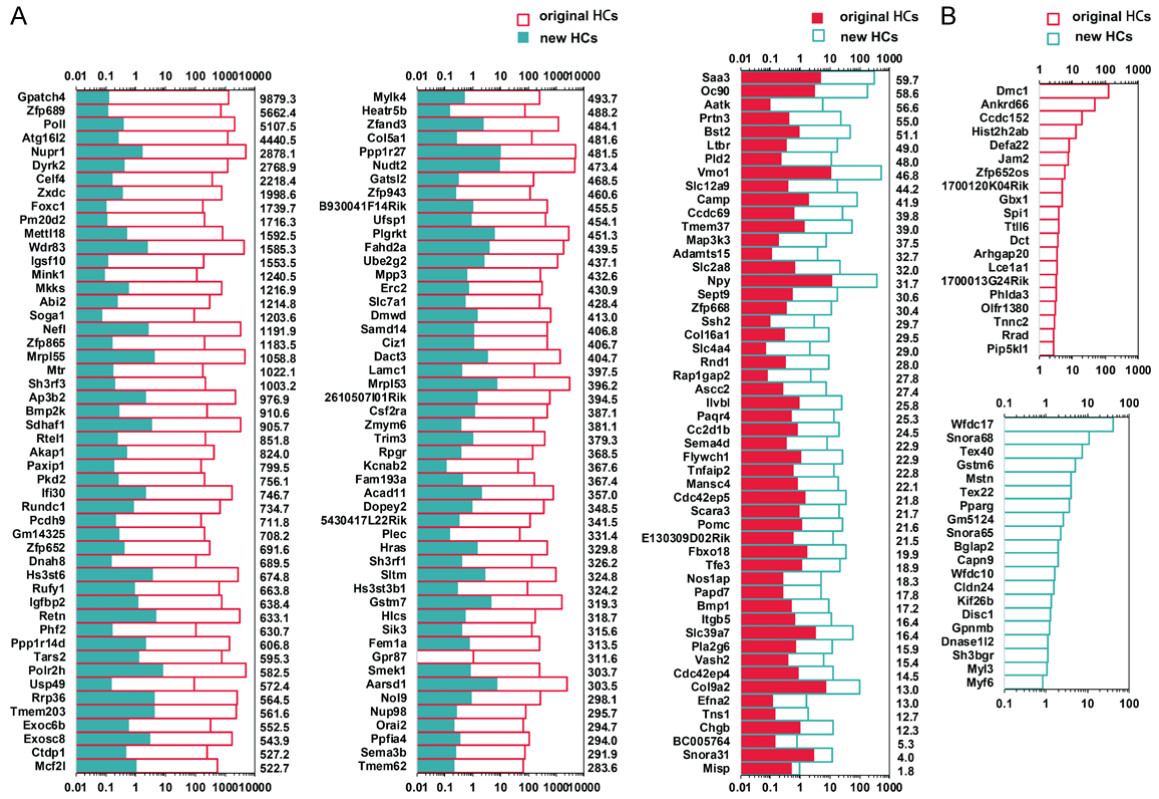


Figure 5. Top differentially expressed genes highly enriched in native HCs and new HCs. A. Top 100 genes differentially expressed in native HCs and the black numbers on the right side of each panel represent the gene expression fold change in native HCs compared to new HCs. Total 52 differentially expressed genes highly enriched in new HCs and the black numbers on the right side of each panel represent the gene expression fold change in new HCs compared to native HCs. B. The top 20 genes exclusively expressed in native HCs or new HCs.

essential for the early differentiation of HCs, including *Brn3.1*, *Six1*, *Sox4*, and *Sox11*, showed no significant difference between the new HCs and the native HCs.

Mechanoelectrical transduction of sound waves requires intact HC bundles and transduction channels, and GO analysis showed that the genes related to the sensory perception of sound (GO:0007605), including *Kcnma1*, *Fgfr1*, *Clrn1*, *Myo6*, *Myo7a*, *Grxcr1*, *Tbx1*, *Tmprss3*, *Slc17a8*, *Tjp1*, *Cdkn2D*, *Otof*, *Col11a1*, *Dnm1*, and *Fbxo11*, showed significantly reduced expression in new HCs compared to native HCs ($P < 0.05$).

Sensory HCs become post-mitotic during embryogenesis and remain quiescent throughout the remainder of their life. The post-mitotic state is essential for the maturation and normal hearing function of cochlear HCs, and the maintenance of the post-mitotic state is associated with cyclin-dependent kinase inhibitors [48]. We analyzed the cell cycle genes in new HCs and native HCs and found that several negative cell cycle regulators (GO:0030308), including *Hyal1*, *St7l*, *Dab2ip*, *Nf2*, *Ddx3x*, *D7ertd443e*, *Dact3*, *Phb*, *Cdkn2D*, *Bmpr2*, *Rrad*, and *Adam15*, had lower expression in new HCs compared with native HCs, especially *Cdkn2D* (*p19Ink4d*). *Cdkn2D* is a cyclin-dependent kinase inhibitor gene that is expressed in postnatal cochlear HCs and participates in maintaining the post-mitotic state of differentiated cochlear HCs [49]. The expression level of other important cell cycle regulators for HC development, including *Cdkn1B* (*p27Kip1*) and *Cdkn1a* (*p21Cip*), showed no significant difference between new HCs and native HCs.

In summary, the highly differentially expressed genes in the native HCs were associated with inner ear development and hearing function, including genes involved in calcium channel activity, HC stereocilia bundles, several cell signaling genes and TFs related to HC develop-

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Differentially expressed genes of HC differentiation and maturation (RNA-Seq data)

	gene	sample_1	sample_2	value_1	value_2	p_value
1	*Tbx1*	original HCs	new HCs	2.23913	0	0.0015
2	*Kcnma1*	original HCs	new HCs	94.144	0.390009	0.00865
3	*Grxcr1*	original HCs	new HCs	846.971	5.30554	0.01285
4	*Otof*	original HCs	new HCs	1944.66	12.3006	0.0023
5	*Ctnnd1*	original HCs	new HCs	398.253	1.71811	0.0009
6	*Fgfr1*	original HCs	new HCs	245.685	2.28855	0.02005
7	*Myo6*	original HCs	new HCs	283.612	14.6254	0.0316
8	*Pvalb*	original HCs	new HCs	9074.06	123.237	0.00775
9	*Cdkn2d*	original HCs	new HCs	1354.89	34.6703	0.04475
10	*Slc17a8*	original HCs	new HCs	46.7475	0.453706	0.014
11	*Fgf8*	original HCs	new HCs	5068.04	32.0087	0.01655
12	*Gfi1*	original HCs	new HCs	86.2088	2.98232	0.0487
13	*Ctnnb1*	original HCs	new HCs	91.15	39.025	0.51135
14	Axin2	original HCs	new HCs	0.0652884	0.0431113	1
15	*Lgr6*	original HCs	new HCs	0.143992	0.83359	0.21565
16	*Lgr5*	original HCs	new HCs	3.08176	7.58996	0.67695
17	*Lef1*	original HCs	new HCs	0.11393	0.139675	1
18	*Wnt9a*	original HCs	new HCs	28.6511	0.300362	0.03845
19	*Tcf4*	original HCs	new HCs	107.625	3.03355	0.0111
20	*Wnt4*	original HCs	new HCs	14.3399	0.389041	0.04175
21	*Wnt5a*	original HCs	new HCs	351.46	2.66101	0.0019
22	*Pdgfra*	original HCs	new HCs	14.5099	0.448152	0.04155
23	*Dpp10*	original HCs	new HCs	18.8757	0.118717	0.047

B

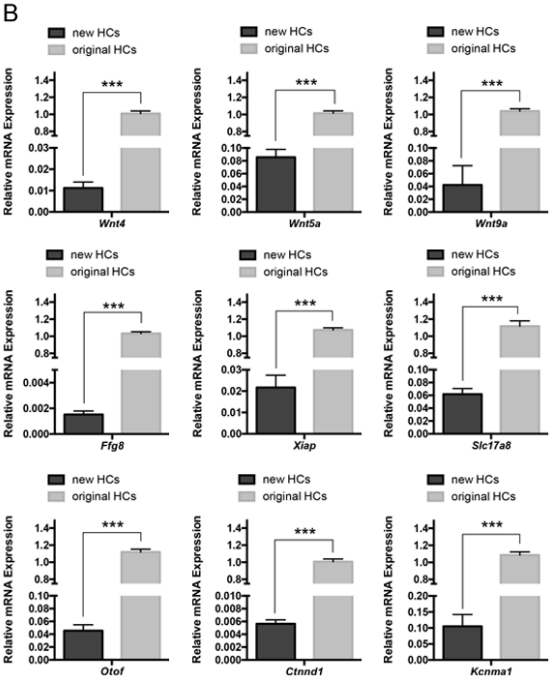

Figure 6. The results of RNA-Seq assay and qPCR assay. A. The differentially expressed genes related with HC differentiation and maturation from RNA-Seq data. B. The qPCR results of the differentially expressed genes.

ment. These results suggest that the reduced expression of these genes might be responsible for the inability of newly generated HCs to mature into functional HCs.

Gene ontology analysis of the differentially expressed genes in new HCs and native HCs

In order to obtain a comprehensive view of the genes that were differentially expressed in the new HCs and native HCs, GO analysis was performed to categorize the different functional gene clusters in both groups (**Figure 7A**). We found that the genes associated with HC maturation and hearing function were highly enriched in the native HCs, including negative regulation of cell growth (GO:0030308), auditory receptor cell differentiation (GO:0042491), generation of neurons (GO:0048699), positive regulation of synapse maturation (GO:0090129), negative regulation of NF-kappaB import into nucleus (GO:0042347) and positive regulation of response to cytokine stimulus (GO:0060760). However, the highly enriched genes in new HCs were associated with negative regulation of cytokine production (GO:0001818), positive regulation of cell migration (GO:0030335) and regulation of cell shape (GO:0008360).

STRING protein-protein interaction analysis was also performed to assemble the predicted networks of the differentially expressed genes (fold change > 1.5, $P < 0.05$) in native HCs and new HCs. **Figure 7B** shows the complex network of genes that are involved in hearing function.

Discussion

Many approaches have been taken to trigger or enhance HC regeneration, including manipulating genes, TFs, and signaling pathways [50, 51]. *Atoh1* overexpression in cochlear SCs is considered as one of the most powerful methods for triggering HC regeneration because of its crucial role in HC specification and HC differentiation in mice [12, 15]. Unfortunately, most of newly generated HCs are immature. In this work we discussed the differentially expressed genes between new HCs and native HCs including the genes associated with development, cell channel activity, cell signals, transcription factors, mechanoelectrical transduction and the cell survival genes, in order to understand the distinction between *Atoh1*-induced HCs and native HCs.

Although most of genes that were uniquely expressed in the native HCs have not been previously reported to play a role in the inner ear, some genes appeared to participate in HC development and auditory function such as

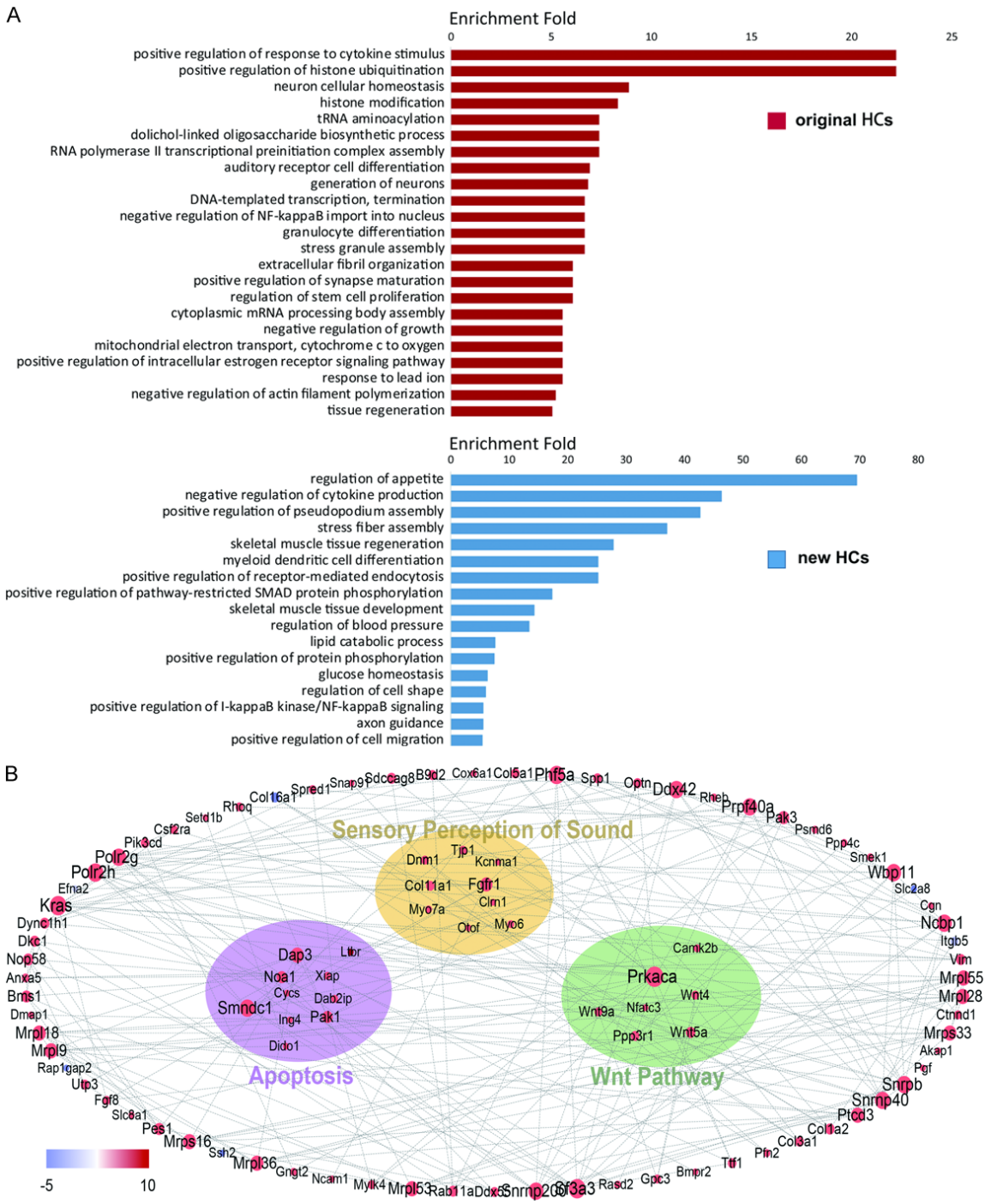

Figure 7. GO analysis of differentially expressed genes in new HCs and native HCs. A. The functional gene groups were enriched in native HCs. B. The functional gene groups were enriched in new HCs. C. The STRING protein interaction analysis of genes that are differentially expressed in native HCs (red) and new HCs (blue).

Hist2h2ab [37]. In addition, *Gbx1* is associated with sensory neuron axon guidance in the developing nervous system [52], and *Ttll6* is associated with microtubule bundle formation and positive regulation of cilium movement in the brain [53, 54]. Finally, *Rrad* (*Rad*) is associated with the regulation of calcium ion transmembrane transport in muscle tissue [55]. Considering that stereocilia, mechanotransduction, calcium ion channels, and synaptic

junctions with spiral ganglion neurons are critical for hearing function in the inner ear [56, 57], all of these genes likely participate in HC development and auditory function. The lack of these genes in the new HCs might be responsible for their immature development state.

All differentially expressed genes related to auditory HC formation ((GO:0042491) and (GO:0042472)) were expressed at significantly lower levels in the new HCs. Except the common HC marker *Myo6*, *Myo7a*, and *Gfi1* and IHC marker *Fgf8*, we found the gene *Fgfr1*, which was critical for the maintenance of sensory progenitors during the development of the auditory sensory epithelium, were also expressed at significantly lower levels in the new HCs, but its role in postnatal HCs remains unclear [58, 59]. The low expression of *Myo6*, *Myo7a*, *Gfi1*, and *Fgf8* in new HCs might also be responsible for their immature state.

The transmission of sound waves requires intact HC stereocilia bundles and transduction channels, however, the genes related to sensory perception of sound (GO:0007605) were expressed at lower levels in new HCs compared to native HCs. The gene *Grxcr1* (glutaredoxin cysteine-rich 1) is associated with actin cytoskeletal architecture in the developing stereocilia of sensory HCs, and the absence of *Grxcr1* causes hearing dysfunction [60]. *Clarin-1* (*Clrn1*) is essential for mechanical transduction and stereocilia bundle integrity, and mutations in *Clarin-1* have been shown to interrupt FM1-43 uptake [61]. *Kcnma1* expression is related to HC BK (Big Potassium) ion channels function and hearing sensitivity, and *Kcnma1* deficits lead to hearing loss [62, 63]. In addition to hair bundles and ion channels, synaptic function is indispensable for sound transduction. The *Otof* and *Slc17a8* genes are essential for the formation of IHC ribbon synapses and contribute to the HC synaptic vesicles that deliver the sound signals to the spiral ganglion neuron [64, 65]. The low expression of auditory function-related genes might prevent the new HCs from maturing, and the up-regulation of these genes might be helpful for functional HC regeneration.

Negative cell cycle genes (GO:0030308) had reduced expression in new HCs compared with native HCs, especially *Cdkn2d* (p19Ink4d). These results suggest that *Atoh1*-induced new HCs have difficulty in exiting the cell cycle, and upregulation of *Cdkn2D* might be a potential way to promote the development and maturation of new HCs.

Conclusion

In this study we showed that up-regulation of *Atoh1* initiated the transdifferentiation process of SCs, and the genes associated with HC morphology and function were up-regulated, and the genes associated with SCs were down-regulated. However, when compared with the native HCs, the genes associated with HC maturation and function were expressed at significantly lower levels in the new HCs, including the hearing function-related genes, mechanoelectrical transduction genes and HC stereocilia bundle genes. Based on our data, future strategies for HC regeneration need to include the new targets to promote the functional development and the maturation of new HCs.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. qPCR primers in this article

	Left 5'-3'	Right 5'-3'
Slc26a5	CCTCGTTGAGCCTTGTTCAG	ACCAACAGAATCATCAACGAGG
Vglut3	GTGGTGCCCATCAGTAAGGT	GCCAGTTGTCCTCCTCTGAC
Otoferlin	TCAGCAACAAGCTGATAGGG	AGCGTGTCGGTCACCTCTAC
Wnt5	AACTGCAGCACAGTGGACAA	GTACGTGAAGGCCGTCTCTC
Wnt4	GCCATCTCTTCAGCAGGTGT	GTCACAGCCACACTTCTCCA
Wnt9a	CTGGAACTGCACCCTGGA	AGATGGCGTAGAGGAAAGCA
Fgf8	TGTTGCACTTGCTGGTTCTC	GCTTGGAGGCAGAGAGTGG
Kcnma1	CCCGAAAGTGTCCATATTGC	ACGCACATGTCACAGAGGTT
Xiap	TGGAAGCCAAGTGAAGACCC	TCTTGCCCCTTCTCATCCAA
Ctnnd1	TGTGGAGAATTGTGTTTGCCT	GAGGAGGCAAACACGCTCTG
Pou4f3	CGACGCCACCTACCATACC	CCCTGATHTACCGCGTGAT
Sox2	GCGGAGTGGAAACTTTTGTCC	CGGGAAGCGTGTACTTATCCTT
GAPDH	CCACAGTCCATGCCATCACT	TCTTCTGGGTGGCAGTGATG