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Supplemental Information

Generation of innervated cochlear organoid recapitulates early development of auditory unit

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SUPPLEMENTAL FIGURES

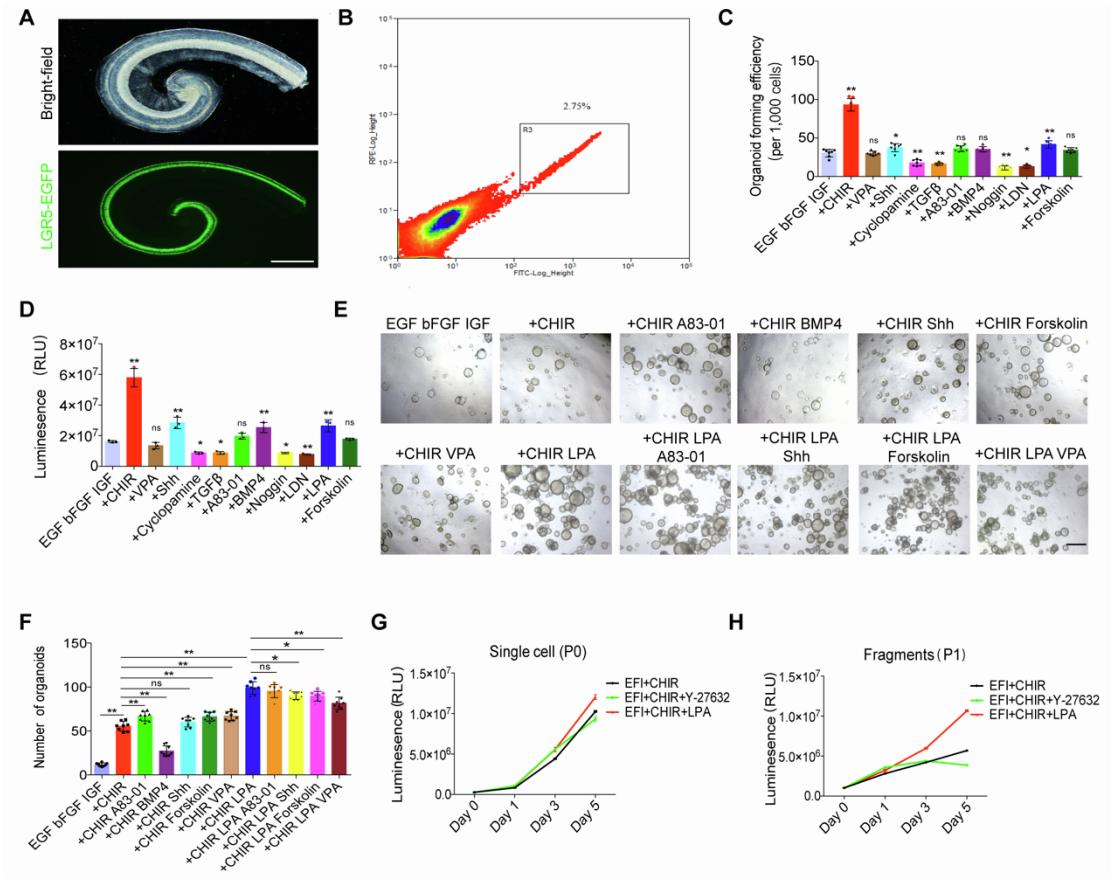

Figure S1. Screening the optimal factors for the inner ear organoid formation. (A) Bright-field and fluorescence microscopy images of the organ of Corti from a PD1 LGR5-EGFP mouse. Scale bar, 500 μm. (B) Isolate LGR5+ cochlear progenitor cells from P1 LGR5-EGFP cochleae by FACS gating strategy. (C) The efficiency of organoid formation under the indicated conditions (n = 3 independent experiments). The data are shown as means ± SEM; *P < 0.05, **P < 0.01, or nonsignificant (ns) P ≥ 0.05 (one-way ANOVA followed by Tukey's multiple comparisons test). (D) The viability of cultured organoids under the indicated conditions (n = 7 independent experiments). The data are presented as means ± SEM; *P < 0.05, **P < 0.01, or nonsignificant (ns) *P* ≥ 0.05 (one-way ANOVA followed by Tukey's multiple comparisons test). (E) Representative bright-field images of cultured P1 organoids under various combinations of growth factors. Scale bar, 500 μm. (F) The number of organoids cultured in different combinations from E (n = 8 independent experiments). The data are presented as means ± SEM; *P < 0.05, **P < 0.01, or nonsignificant (ns) P ≥ 0.05 (one-way ANOVA followed by Tukey's multiple comparisons test). (G) Growth curves of organoids from sorted single cells under the indicated conditions over 5 days (n = 6 independent experiments). (H) Growth curves of organoids from P1 fragments under the indicated conditions over 5 days (n = 6 independent experiments).

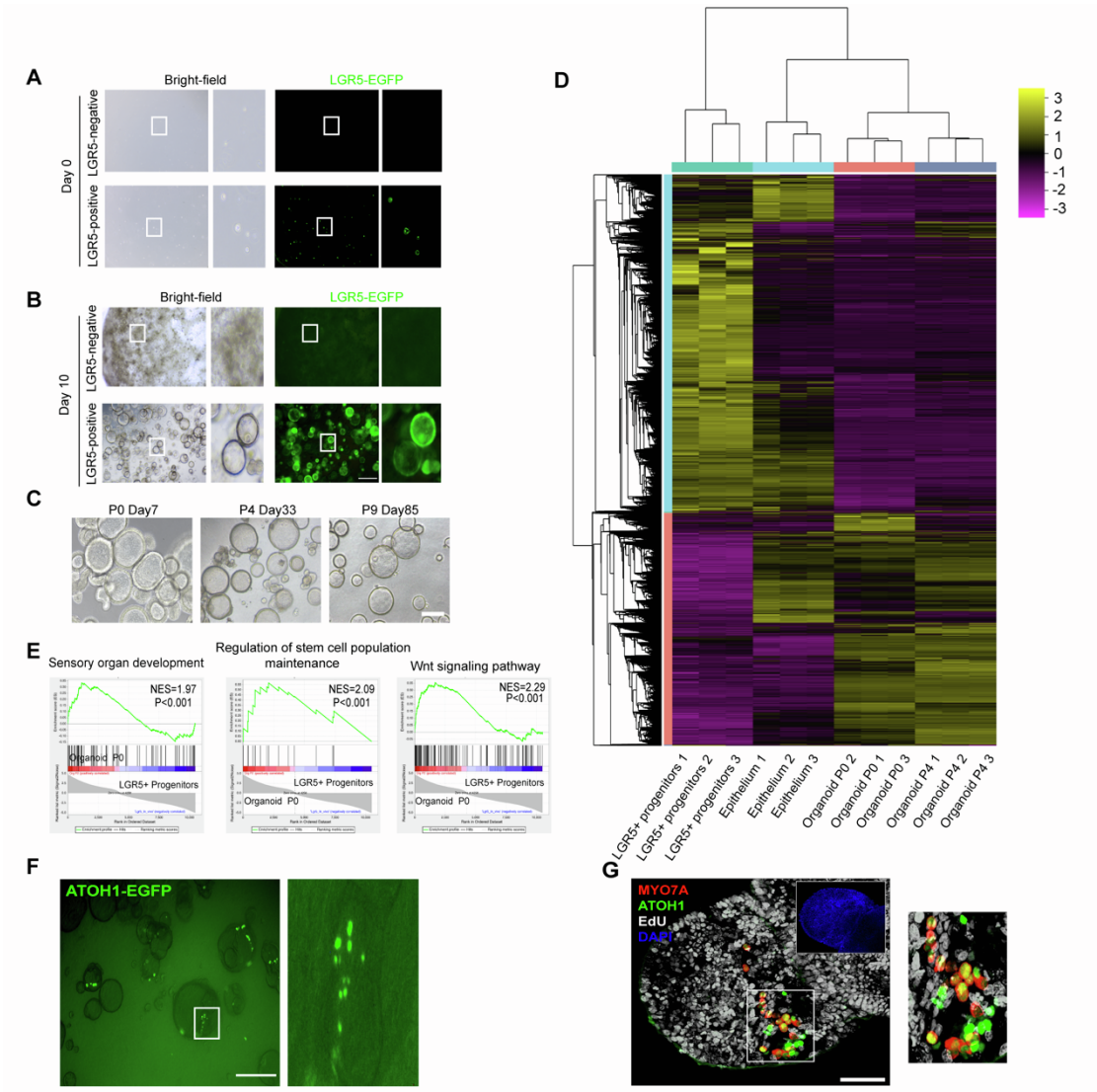

Figure S2. The transcriptome of long-term cultured cochlear organoids. (A, B) Fluorescence and bright-field images of sorted LGR5+ and LGR5– cells cultured in expansion medium at day 0 (A) and day 10 (B). Scale bar, 500 μm. (C) Bright-field images of P0, P7, and P9 organoids. Scale bar, 200 μm. (D) Heatmap showing differentially expressed genes between organoids at passage 0 and primary LGR5+ progenitor cells in the indicated cell types (n = 3). (E) GSEA of differentially expressed genes in organoids at passage 0 versus primary Lgr5+ progenitor cells (n = 3). (F) Fluorescence images showing ATOH1-EGFP expression in P0 organoids cultured in expansion medium. Scale bar, 250 μm. (G) Immunofluorescence analysis of P0 organoids showing the expression of the HC markers ATOH1 and MYO7A, with proliferative cells stained by EdU. Scale bar, 50 μm.

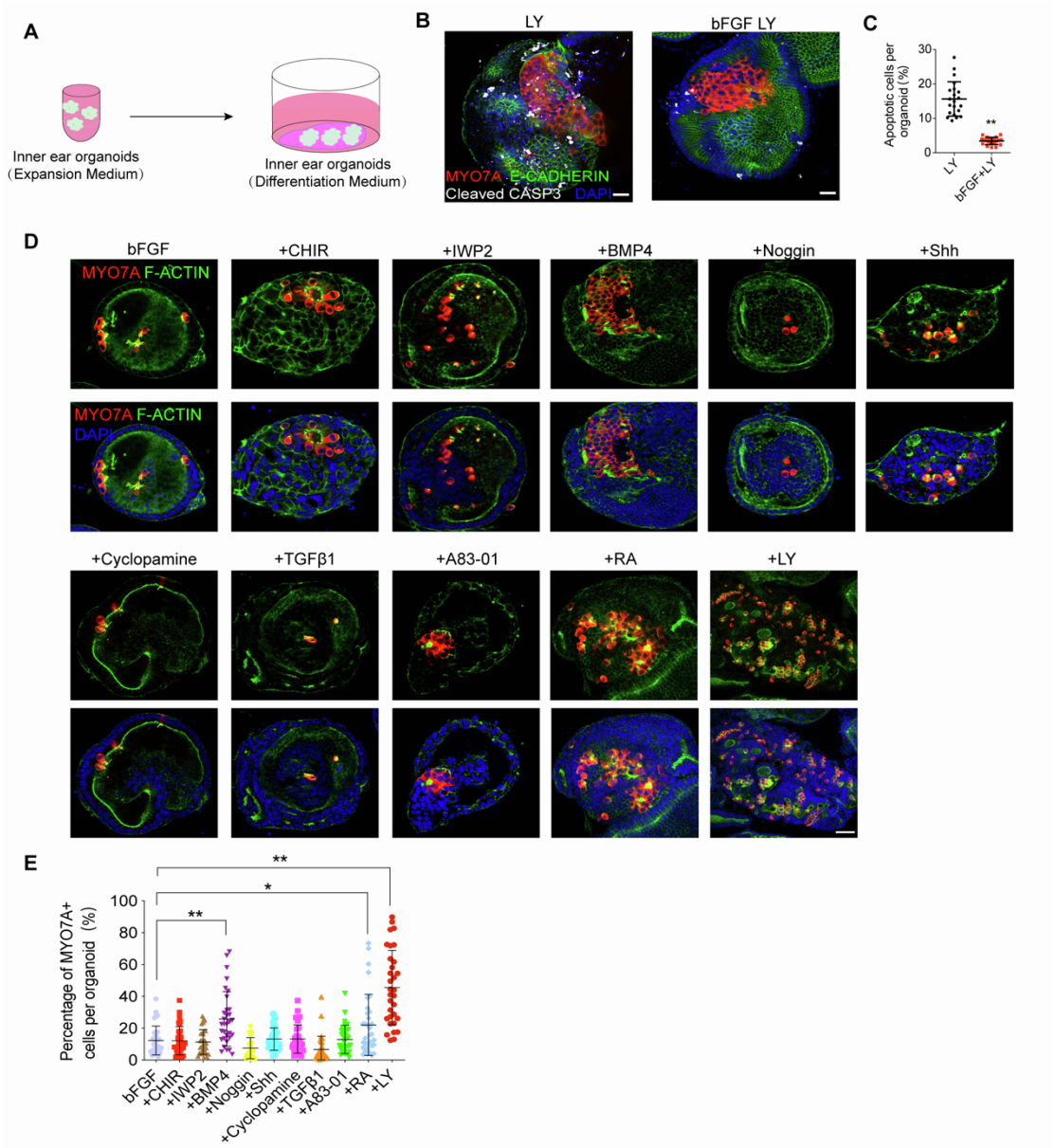

Figure S3. Screening conditions for differentiating organoids into hair cells. (A) Schematic of cochlear organoid differentiation. (B) Immunofluorescence analysis of MYO7A, E-CADHERIN, and Cleaved CASP3 expression of differentiated day 14 organoids in the indicated medium. Scale bar, 50 μm. (C) Quantify the percentage of CASP3+ apoptotic cells per organoid in the indicated medium from B. 21 organoids at each condition, three independent experiments. The data are presented as means ± SEM; ** P < 0.01 (unpaired two-tailed Student's t-test). (D) Immunofluorescence analysis of MYO7A and F-ACTIN expression of differentiated day 14 organoids in the indicated medium. Scale bar, 50 μm. (E) Quantification of the percentage of MYO7A+ cells in HC organoids. 32-33 organoids at each condition, three independent experiments. The data are presented as means ± SEM; *P < 0.05, **P < 0.01 (one-way ANOVA followed by Tukey's multiple comparisons test).

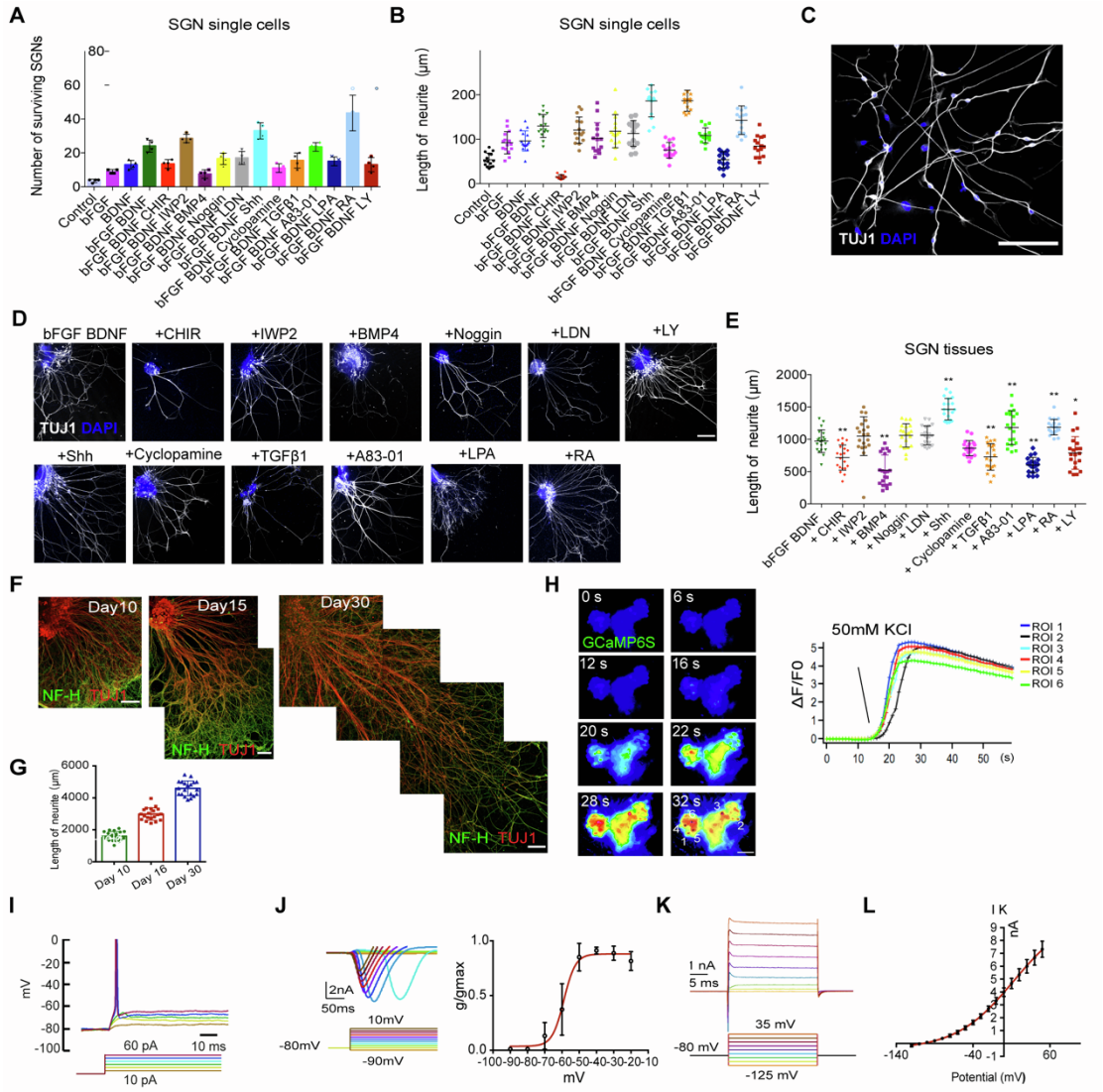

Figure S4. Screening conditions for SGN growth and Functional SGNs in co-cultures. (A) Quantify the number of SGNs cultured in different media for 7 days from dissociated single SGNs (n = 4 independent experiments). (B) Quantify the length of SGN neurites cultured in various media for 7 days from dissociated single SGNs. 15 SGNs at each stage, three independent experiments. (C) Representative fluorescence images of cultured SGNs stained with TUJ1 in the medium containing bFGF, BDNF, and Shh. Scale bar, 100 μm. (D) Immunofluorescence analysis of cultured SGN tissues in the indicated conditions at day 7 showing the staining of TUJ1. Scale bar, 300 μm. (E) Quantification of the length of SGN neurites in different media from D. 20 SGNs at each stage, three independent experiments. The data are presented as means ± SEM; *P < 0.05, **P < 0.01 (one-way ANOVA followed by Tukey's multiple comparisons test). (F) Immunofluorescence analysis of SGNs in the 3D culture at day 10, day 15, and day 30. TUJ1 staining of SGN bodies and neurites and NF-H staining of SGN neurites. Scale bar, 200 μm. (G) Quantification of the length of SGN neurites at different times from F. 20 SGNs at each stage, three independent experiments. (H) Representative image of calcium imaging in cultured day 30 SGNs and the single-cell tracings of calcium transients in the region of interest (ROI). Time is shown in seconds (s). Scale bar, 100 μm. (I) Representative images of the fired action potentials of cultured SGNs evoked by step current injections. (J) Examples of Na^+ currents of cultured SGNs elicited by voltage steps. Depolarizing voltage pulses from –90 mV to +10 mV in 10 mV steps were applied, n = 4. (K) Outward K^+ currents of cultured SGNs were elicited in response to hyperpolarizing and depolarizing voltage steps. (L) Steady-state current-voltage curves of the currents were shown in D (n = 7).

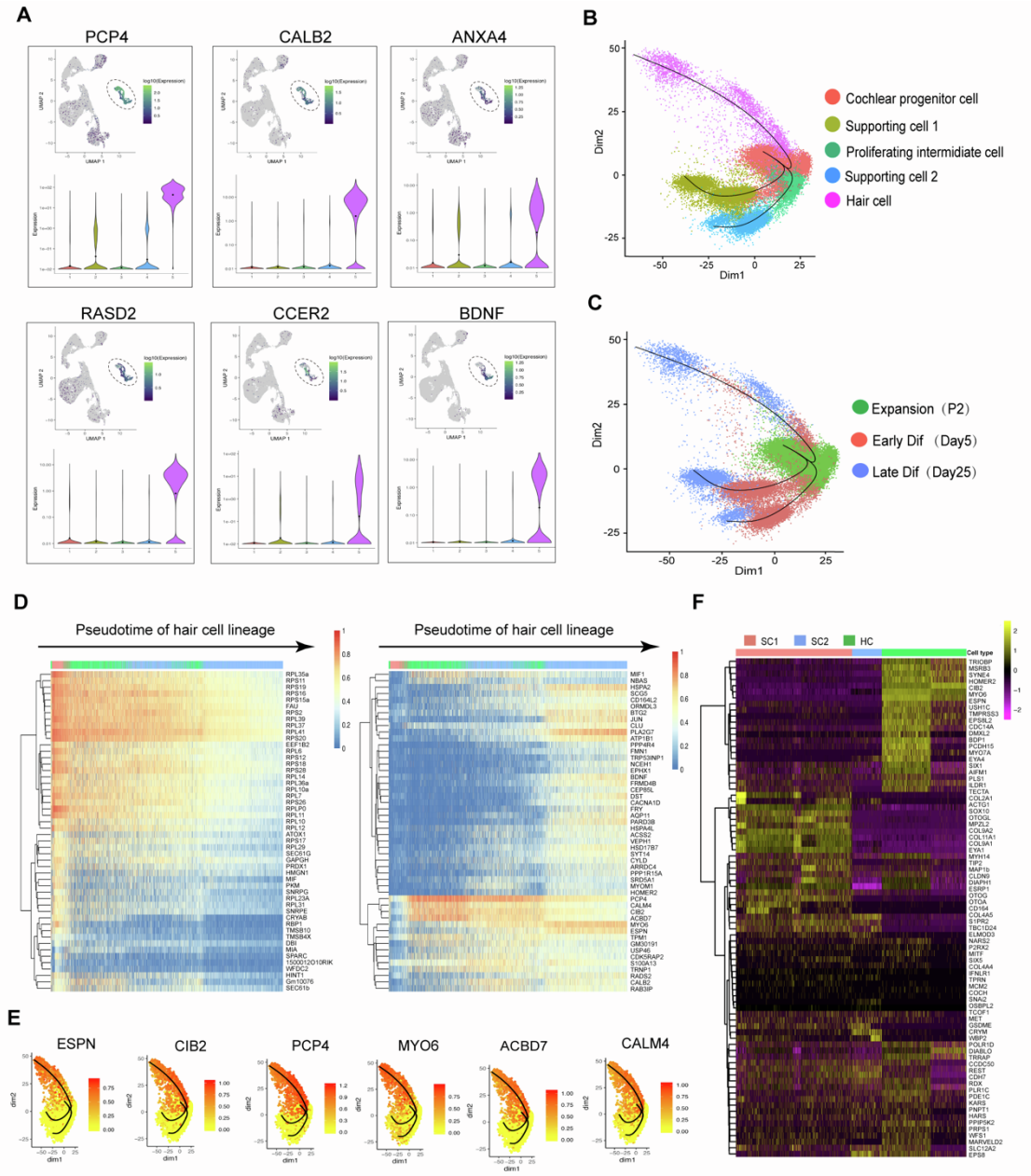

Figure S5. ScRNA-Seq analysis of HC genes during organoid development. (A) UMAP plots and violin plots show HC-related gene expression. (B, C) Pseudotime study of the five main clusters at different stages using Slingshot identified putative lineage relationships between the clusters based on gene expression similarities. Three branches were identified during organoid differentiation. (D) Heatmaps displaying the top 50 differentially expressed genes along the HC trajectory. (E) The visualizations show the lineage reconstruction color-coded by the dynamic expression of six selected genes over pseudotime. (F) Heatmap showing the expression of deafness-related genes in the SCs and HCs from differentiated day 25 organoids.

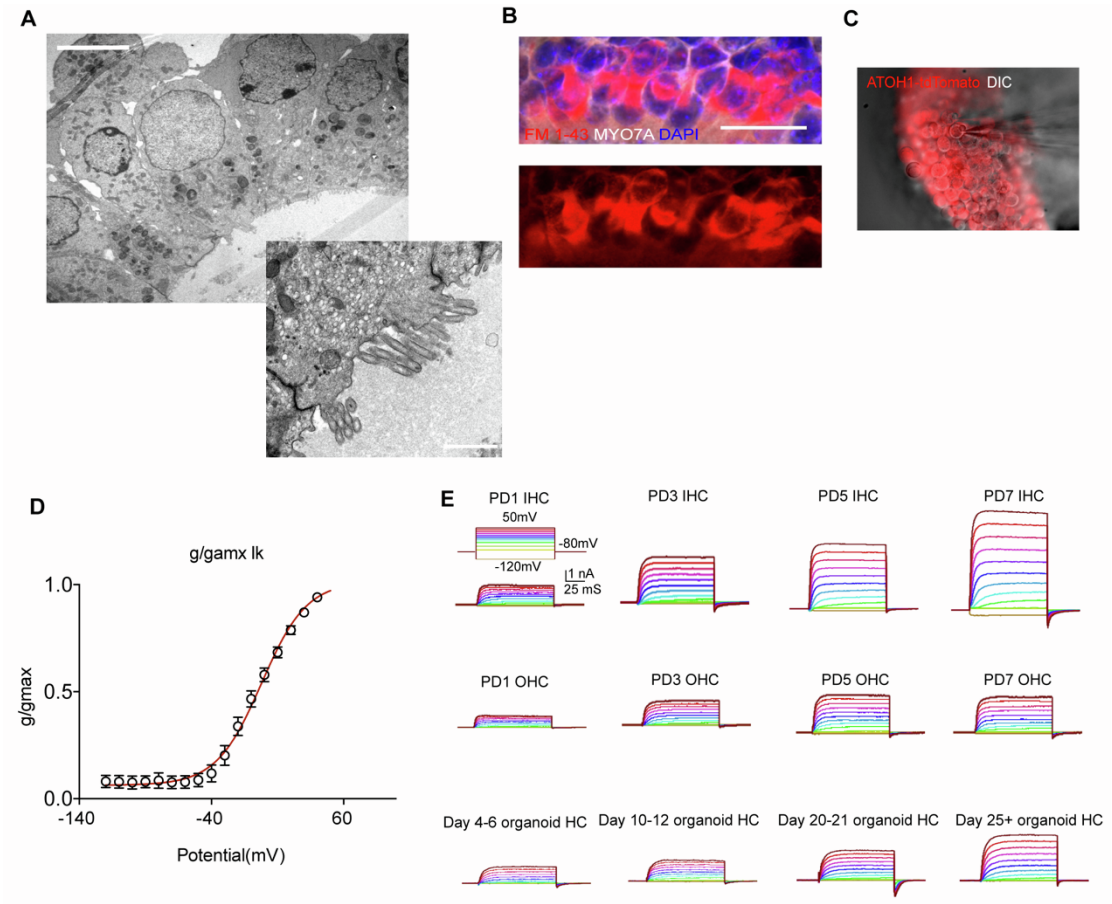

Figure S6. Characterization of differentiated HCs of cochlear organoids. (A) Transmission electron microscopy showing the ultrastructure of day30 organoid slices. Scale bar, 5 μm. Higher magnification of the images showing the stereociliary bundles of organoids. Scale bar, 1 μm. (B) Representative fluorescence analysis showing the uptake of FM1-43 dye by Myo7a+ organoid HCs. Scale bar, 25 μm. (C) Merging of DIC and fluorescence images showing recording pipettes patched to Atoh1-tdTomato+ HCs. Scale bar, 25 μm. (D) K+ currents fitted by a Boltzmann equation (line) with a half-maximal activation voltage of −50 mV in day 25 organoid HCs. (E) Examples of outward K+ currents for native HCs and organoid HCs. The stages of the HCs are indicated.

SUPPLEMENTAL TABLES

Supplemental Table 1. Primers for genotyping

Gene	Forward Primer	Reverse primer
tdTomato WT	5'-AAGGGAGCTGCAGTGGAGTA-3'	5'-CCGAAAATCTGTGGGAAGTC-3'
tdTomato Mut	5'-CTGTTCCTGTACGGCATGG-3'	5'-GGCATTAAAGCAGCGTATCC-3'
LGR5CreER WT	5'-CTGCTCTCTGCTCCCAGTCT -3'	5' - ATACCCCATCCCTTT TGAGC -3'
LGR5 CreER Mut	5'-CTGCTCTCTGCTCCCAGTCT -3'	5' - GAACTTCAGGGTCAG CTTGC -3'
POU4F3-DTR WT	5'-CACTTGGAGCGCGGAGAGCTAG-3'	5'-CCGACGGCAGCAGCTTCATGGTC-3'
POU4F3-DTR Mut	5' -GTCAAAAAAGTGCCTTAGAG -3'	5'-CCGACGGCAGCAGCTTCATGGTC-3'
ATOH1-EGFP WT	5'-GCGGTCTGGCAGTAAAAACTATC-3'	5'-GTGAAACAGCATTGCTGTCACTT-3'
ATOH1-EGFP Mut	5' -AAGTTCATCTGCACCACCG-3'	5' -TCCTTGAAGAAGATGGTGCG-3'
ATOH1 CreER WT	5'-CTAGGCCACAGAATTGAAAGATCT-3'	5'-GTAGGTGGAAATTCTAGCATCATCC-3'
ATOH1 CreER Mut	5'-GCGGTCTGGCAGTAAAAACTATC-3'	5'-GTGAAACAGCATTGCTGTCACTT-3'

Supplemental Table 2. List of antibodies and reagents

Antibodies	Supplier	Identifier
Rabbit anti-KI67	Abcam	Cat# ab15580, RRID:AB_443209
Chick anti-GFP	Abcam	Cat# ab13970, RRID:AB_300798
Chick anti-NF-H	Abcam	Cat# ab4680, RRID:AB_304560
Mouse anti-CTBP2	BD Biosciences	Cat# 612044, RRID:AB_399431
Rabbit anti-YAP	Cell Signaling Technology	Cat# 15117, RRID:AB_2798714
Cleaved CASPASE-3	Cell Signaling Technology	Cat# 9661, RRID:AB_2341188
Rabbit anti-MYO7A	Proteus BioSciences	Cat# a25-6790, RRID:AB_2314838
Mouse anti-TUJ1	Millipore	Cat# MAB5564, RRID:AB_570921
Rabbit anti-ESPIN	Novus	Cat# NBP1-90588, RRID:AB_11015490
Goat anti-PTRESIN	Santa Cruz Biotechnology	Cat# sc-22692, RRID:AB_2302038
Mouse anti-β-CATENIN	Santa Cruz Biotechnology	Cat# sc-7963, RRID:AB_626807
Goat anti-SOX2	Santa Cruz Biotechnology	Cat# SC17319, RRID:AB_661259
Rabbit anti-SOX9	Sigma	Cat# HPA001758, RRID: AB_1080067
Rabbit anti-VGLUT3	Synaptic Systems	Cat# 135 203, RRID:AB_887886
Anti-E-cadherin, Alexa Fluor 488	Thermo Fisher Scientific	Cat# 53-3249-82, RRID:AB_10671003
Mouse anti-PSD95	UC Davis/NIH NeuroMab Facility	Cat# K28/43, RRID:AB_2877189
Chemicals and recombinant proteins		
Matrigel	Corning	354230
Cell recovery solution	Corning	354253
EGF	Peprotech	315-09
bFGF	Peprotech	450-33
IGF	Peprotech	250-19
Shh	Peprotech	315-22

BMP4	Peprotech	315-27
Noggin	Peprotech	250-38
TGFβ1	R&D	P04202
Retinoic acid	Sigma	R2625
pVc	Sigma	49752
A83-01	Sigma	SML0788
Valproic acid	Sigma	PHR1061
CHIR99021	Sigma	SML1046
IWP-2	Sigma	I0536
LPA	Sigma	L7260
Y-27632	Sigma	Y0503
Forskolin	Sigma	S2449
HEPES	Sigma	PHR1428
NAC	Sigma	A9165
L-Glutamate	Sigma	G8415
NaCl	Sigma	S5886
KCl	Sigma	P5405
NaH_2PO_4	Sigma	5.43840
$CaCl_2$	Sigma	C5670
$MgCl_2$	Sigma	M4880
Dextrose	Sigma	D9434
KCH_3SO_3	Sigma	83000
EGTA	Sigma	E3889
TEA-Cl	Sigma	T2265
CsCl	Sigma	289329
Na-GTP	Sigma	G8877
Verteporfin	Sigma	SML0534
PD0325901	Sigma	PZ0162
Mg-ATP	Sigma	A9187
Cyclopamine	Selleck	S1146
SB525334	Selleck	S1476
LDN193189	Selleck	S2618
LY411575	Selleck	S2714
616452	Selleck	S7223
F-actin Readyprobes	Thermo Fisher Scientific	R37112
EdU, Alexa Fluor 647	Thermo Fisher Scientific	C10635
DMEM/F-12, GlutaMAX	Thermo Fisher Scientific	10565042
DAPI	Thermo Fisher Scientific	D1306
N-2 Supplement	Thermo Fisher Scientific	A13707
B-27 Supplement	Thermo Fisher Scientific	17504044
Penicillin-Streptomycin	Thermo Fisher Scientific	15070063
Trypsin	Thermo Fisher Scientific	25200056
Soybean Trypsin Inhibitor	Thermo Fisher Scientific	17075029
TrypLE	Thermo Fisher Scientific	12605010
FM1-43	Thermo Fisher Scientific	T35356

Supplemental Table 3. Primers for real-time PCR

Gene	Forward Primer	Reverse primer
LGR5	5'-CAGCCTCAAAGTGCTTATGCT-3'	5'-GTGGCACGTAACTGATGTGG-3'
MYO6	5'-TGTTAAGGCAGGTTCCTTGAAG-3'	5'-ACACCAGCTACAACTCGAAAC-3'
MYO7A	5' -CATCCGCCAGTACACCAACAA-3'	5' -TCCCCGCTGATAATACAGCAC-3'
POU4F3	5' -CGACGCCACCTACCATACC-3'	5' -CCCTGATGTACCGCGTGAT-3'
ACBD7	5' -ATGTCCTTGCAGGCTGATTTT-3'	5' -TGTAGAGCCCGTAGAGTTCCT-3'
CCER2	5' -AGGACCCACAAGTCTGGGG-3'	5' -CCTCCCTCTGGATGTTGCT-3'
PVALB	5' -ATCAAGAAGGCGATAGGAGCC-3'	5' -GGCCAGAAGCGTCTTTGTT-3'

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Culture of cochlear epithelia and isolation of mouse primary cochlear progenitor cells

The cochleae were dissected from postnatal day 0–1 mice and quickly placed in cold phosphate buffered saline (Hyclone). The modiolus and the stria vascularis were carefully removed to separate the sensory epithelia. The tissues were mounted on the 2 mg/ml laminin-coated glass Petri dishes for cochlear epithelial culture. After the tissues were fully attached to the dishes, the culture medium was added. To isolate cochlear progenitor cells, the tissues were incubated with 0.125% trypsin (Thermo Fisher Scientific) for 12–15 min at 37°C. An equal volume of trypsin neutralizer solution (Thermo Fisher Scientific) was added to stop the enzymatic reaction. Dissociated cells were washed with DMEM/F-12 (Thermo Fisher Scientific) twice and filtered through a 40 μm cell strainer. Cells were then resuspended in FACS buffer (2% serum, 0.2% bovine serum albumin (BSA) in DMEM/F-12 (Dulbecco's modified Eagle medium)). For sensory epithelium isolation, cells were blocked for 10 min on ice in FACS buffer before incubation with the anti-E-CADHERIN 488-conjugated antibody for 20 min. For cochlear progenitor cell isolation, cells were dissociated from LGR5-EGFP mice and then sorted using a MoFlo® SX FACS cytometer (Beckman Coulter).

Primary cochlear progenitor cell culture and expansion

Typically, sorted cells were resuspended in an expansion culture medium with a mixture of ice-cold Matrigel (Corning, 354230) at a ratio of 1:10 (vol: vol) then seeded in non-attachment plates. The expansion culture medium was based on DMEM/F-12 supplemented with penicillin/streptomycin, N2, and B27 (all from Thermo Fisher Scientific), 1.25 mM N-acetylcysteine (NAC) (Sigma), 50 ng/ml EGF (Peprotech), 50 ng/ml bFGF (Peprotech), 50 ng/ml IGF (Peprotech), 3 μM CHIR99021 (Sigma), and 10 μM Y-27632 (Sigma) on the first day. The cells were then washed with DMEM/F12 and centrifuged for 6 min at 1,500 rpm at 4°C, and the culture medium containing Y-27632 was exchanged for a medium containing 2.5 μM LPA (Sigma). Every 3–4 days, the organoids were removed from the Matrigel and culture medium by centrifugation and transferred into a new matrix. Organoids were passaged by mechanically pipetting into tiny fragments in a 1:6–1:8 split ratio every 8–10 days.

Organoid-forming efficiency and viability assays

To exclude dead cells, sorted cells were labeled by propidium iodide (Sigma) and counted using a hemocytometer. A total of 100 μl single-cell suspension (~1,000 cells) mixed with 10 μl Matrigel were plated into an ultra-low attachment U-bottom 96-well plate (corning), with each culture condition tested in triplicate. After 10 days, the number of organoids was counted. The CellTiter-Glo® 3D Cell Viability Assay (Promega) was used to test the organoid cell viability. After washing with cold PBS, the organoids were collected by centrifugation and then lysed with a CellTiter-Glo 3D kit for 15 min at room temperature. The luminescence was measured by an automatic microplate reader (Molecular Devices). EdU was supplied at a concentration of 5 μM for 4 h before fixation for labeling proliferative cells. For single-cell organoid formation, cultured cells were imaged using a Lionheart™ FX Automated Live Cell Imager and Gen5 3.03 software.

Organoid-SGN explant co-culture

For organoid differentiation, a glass Petri dish was coated with 2 mg/ml laminin solution (diluted in PBS) overnight. The organoids were subsequently transferred onto the glass dish. For culturing SGN explants, the cochlear modiolus was dissected from PD2 mice, cut into pieces without the peripheral tissue, and then plated onto the laminin-coated glass dish. To establish the periphery auditory circuit model, the SGN tissues were placed flat on the dish with the fibers facing towards the organoids, ensuring that the distance between the organoids and SGN tissues was approximately 100–300 μm. After fully adhering to the plate, 60 μl Matrigel was added to cover the organoids and SGN tissues. The differentiation medium was DMEM/F-12 supplemented with penicillin/streptomycin, N2, and B27 (all from Thermo Fisher Scientific), 1.25 mM NAC (Sigma), 10 ng/ml bFGF (Poprotech), 100 ng/ml BDNF (Poprotech), 100 ng/ml Shh (Poprotech), 0.5 μM A83-01 (Sigma), 1 μM RA (Sigma), and 5 μM LY411575 (Sigma). After 5 days of culture, the medium was exchanged to LY411575-free medium on the basis of other ingredients. After Matrigel solidification at 37°C for 30 min, the differentiation medium was added. During culture, the medium was replaced every 2–3 days.

Immunofluorescence analysis and quantification

The cultured organoids in the expansion medium were collected by centrifugation and fixed with 4% paraformaldehyde (PFA) (Sigma) on ice for 30 minutes and then centrifuged at 600 rpm for 5 minutes. After washing with PBS three times, the organoids were attached to the microscope slides by drying. For differentiated organoids, cultured SGN explants and cochlear organoid-SGN co-cultures were fixed with 4% PFA on ice overnight, then washed with PBS three times. Subsequently, the samples were permeabilized and blocked in PBS containing 1% Triton X-100 (Sigma) and 5% fetal bovine serum (Sigma) for 3 h at room temperature (RT). After incubating with primary antibodies diluted in PBS + 0.1% Triton X-100 in a humidified chamber overnight and rinsing with PBS, the samples were incubated with Alexa Fluor-conjugated secondary antibodies (1:400 diluted in PBS + 1% Triton X-100, Invitrogen) for 6 h at RT. For F-ACTIN staining, the samples were washed three times, and an Alexa 555-conjugated F-actin probe (1: 1,000 dilution, Thermo Fisher Scientific) was incubated with the samples for 15 min at RT. The Click-iT cell proliferation kit (Invitrogen) was used to visualize the EdU signal. The antibodies and relevant reagents are listed in Supplementary Table 2. After three washes in PBS, the nuclei of the samples were stained with DAPI (Sigma) for 10 min at RT. *Z*-stacks of optical sections were captured on an Sp8 confocal microscope (Leica) and processed with Fiji. Quantification of the percentage of positively stained cells was manually determined with Fiji.

In situ hybridization assays

Cochleae were isolated from P1 mice and fixed in 4% PFA for 6 h at room temperature. After washing with PBS, the samples were incubated with a gradient of sucrose solutions (15%, 20%, and 30%) and embedded in an Optimal Cutting Temperature compound (Sakura Finetek) for 2 h before flash freezing in a liquid nitrogen bath. Serial sections of the inner ear vestibular organ, cochlea, and spiral ganglion were cut at 15 μm thickness with a cryostat (Leica CM 1950). Superfrost-plus microscope slides (Thermo Scientific) were used for in situ hybridization staining of the cryosections. All samples and cryosections were stored at –80°C for no longer than 3 months before use.

RNAscope® Fluorescent Multiplex Reagents Kit, RNAscope® H2O2 and Protease Reagents Kit, RNAscope® Wash Buffer Reagents Kit, and gene-specific probes (CCER2; ACBD7) were purchased from Advanced Cell Diagnostics. In situ hybridization for CCER2 and ACBD7 was performed according to the manufacturer's protocol. The cryosections were heated at 60°C for 2 h, followed by gradient ethanol dehydration (50%, 70%, 100%, 5 min each). Sections were then immersed in H_2O_2 for 10 min at RT and pretreated with Target Retrieval Reagent (Advanced Cell Diagnostics) at 95°C for 5 min, cleared in 100% ethanol, air dried, and incubated with Protease III for 30 min at 40°C. For probe hybridization, sections were incubated with CCER2 and ACBD7 probes separately for 2 h at 40°C, and the signals were serially amplified by AMP1 to AMP3 incubation. After the addition of horseradish peroxidase (HRP), the probe signals were visualized by Opal 570 dye incubation for 30 min at 40°C, followed by HRP blocker treatment. Subsequent immunofluorescence staining was conducted using standard protocols. Images were captured on an Sp8 confocal microscope and analyzed using Fiji software.

Electron microscopy

Cochlear organoids at differentiated day6, day12, and day30 were fixed in 2.5% glutaraldehyde and 2% PFA diluted in 0.1 M phosphate buffer (PB) overnight. For scanning electron microscopy, samples were dehydrated in an ethanol gradient after washing in PB. The samples were then washed with solution A (100% ethanol + n-pentyl acetate, 2:1) and solution B (100% ethanol + n-pentyl acetate, 1:2) for 15 minutes each and then treated with 100% n-pentyl acetate for 15 min three times. After drying for 2 h, the samples were placed in the ion sputtering apparatus and sprayed with gold. Samples were analyzed using a HITACHI-H500 scanning electron microscope. For transmission electron microscopy, fixed organoids were embedded in epoxy resin (Sigma), then sectioned at a thickness of 50 nm and mounted on copper grids. Sections were stained with 4% uranyl acetate for 5 min and 0.4% lead citrate for 1 min. The ultrastructure of the stained sections was analyzed with a transmission electron microscope (CM120, Philips).

RNA isolation and quantitative real-time PCR analysis

Organoids in cocultures were picked and washed with cold PBS and centrifuged to remove the Matrigel. A RNeasy Micro Kit (Qiagen) was used to extract the total RNA from the cultured organoids, and a PrimeScript™ RT reagent Kit with gDNA Eraser kit (TaKaRa) was used to remove genomic DNA, and cDNA was synthesized according to the manufacturer's instructions. real-time PCR was performed using the TB Green *Premix Ex Taq*™ (Tli RNaseH Plus) kit (TaKaRa) on an Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument. Primers for real-time PCR were designed using NCBI Primer-BLAST and are listed in Supplementary Table 3. Data were normalized to the *Gapdh* housekeeping gene, and $2^{-\Delta\Delta CT}$ values calculated relative to the appropriate control groups are shown.

RNA sequencing and analysis

Organoids cultured in the basic medium (EFG, bFGF, IGF), the basic medium + CHIR, and the basic medium + CHIR + LPA were collected, and passage 0 and passage 4 organoids in the expansion medium were collected. CPCs and cochlear epithelial cells from PD1 mice were sorted by Lgr5-EGFP and anti-E-cadherin 488-conjugated antibody staining, respectively. The RNA-sequencing libraries were generated by the Illumina mRNA-Seq Sample Prep Kit. FASTQ files of paired-end read files were generated using the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing on an Illumina HiSeq2500 150-bp Paired-End Platform. Kallisto and DEseq2 were used to analyze the RNA-seq data. Genes and transcripts were annotated using the RefGene database (NCBI). Briefly, after Benjamini–Hochberg correction for multi-testing, genes with a P-value greater than the false discovery rate were considered to be significantly differentially expressed. Differential gene expression analysis was performed with the DESeq2 package, and heatmaps were generated with the color range scaled to reflect the quantile distribution of the data. Correlation analysis was performed with the Corrplot package.

Single-cell sequencing and analysis

Organoids from passage 2 (day 8) in expansion medium, differentiated day 5, and differentiated day 25 were collected and incubated with 0.125% trypsin for 25 minutes and dissociated into single cells by mechanical pipetting. Cells were incubated with E-cadherin antibody for 10 minutes and then were stained with DAPI (Sigma) on ice for 5 minutes, and then cells were sorted using a MoFlo® SX FACS cytometer (Beckman Coulter). Sorted single, living cells were resuspended in 40 mL of fresh cell media. According to the manufacturer's recommendations, the single-cell transcriptomic profiling was performed using the Single Cell 3' Reagent v3 kits (10X Genomics). In brief, single cells were loaded on a 10X Genomics Chromium Single Cell instrument to generate single-cell gel beads in the emulsion. We used microfluidics to ensure that an individual gel bead encapsulated a single cell in an oil suspension. Gel beads in the emulsion were lysed, and RNA was reverse transcribed to cDNA followed by Illumina bridge amplification of cDNA. Libraries were sequenced on an Illumina HiSeq 4000.

Raw 10X single-cell RNA-seq data from three time points (expansion, early differentiation, and late differentiation) were demultiplexed, aligned (GRCm38), and feature-counted by Cellranger using the default settings. Downstream analysis was performed with a set of R (3.6) packages. The expression matrices were then filtered to retain cells with at least 500 genes detected and to retain genes detected in more than 1% of all cells in each dataset. The filtered matrices were combined, dimensionally reduced (monocle3 'preprocess_cds'), aligned (monocle3 'align_cds'), and clustered (resolution equals to 1×10^{-6}). The top 100 marker genes were computed for each cluster using the regression-based differential expression testing implemented in monocle3. GO term analysis was performed using ClusterProfiler, and pseudotime analysis for CPC differentiation was performed with Slingshot for the pseudotime trajectory construction and with TradeSeq for the gene expression analysis along the trajectory. The top two principal component coordinates were used as the dimension-reduction input for Slingshot, and default parameters were applied. The top genes associated with pseudotime were calculated with the TradeSeq 'startVsEndTest' function to obtain Wald statistics for each gene in each trajectory. To construct pseudotime trajectory for HC development, Monocle2 was applied. The cells were ordered by density peak clustering based on the genes differentially expressed between clusters. Data visualization for this study was performed using R packages, including monocle3, Seurat V3, TradeSeq, and heatmap.

Optogenetic stimulation and electrophysiological recordings

The AAV DJ carrying the coding sequences for Scarlet and Chrimson driven by the CAG promoter was generated using a virus-free helper system. Titers were calculated by qPCR with WPRE primers (WPRE-F: gactggtattcttaactatgttgctc; WPRE-R: ccaggatttatacaaggaggag). SGN tissues were plated in a 100 µl differentiation medium in a glass Petri dish, and 2 µl AAV was added to each well. After 24 h, the solution was exchanged entirely with a fresh medium.

The ion channel activities of infected SGNs (post-infection day 15) were tested under light stimulation. All optogenetic stimulation of Chrimson was performed with multi-wavelength LEDs mounted on the microscope (Thorlabs, LED4D241). The samples' localization and stimulating cells' selection was made under a C11440-42U30 Hamamatsu Flash 4.0 Scmos camera. Light at 5 mW/mm^2 at 590 nm was used to induce Chrimson-mediated optical spiking.

Whole-cell patch-clamp recordings were performed to measure the electrophysiological properties of cultured SGNs and organoids. To record K^+ channel and action potentials, SGNs, organ of Corti explants, and organoids were placed in extracellular solution containing 135 mM NaCl, 5.8 mM KCl, 0.7 mM NaH_2PO_4, 1.3 mM $CaCl_2$, 0.9 mM $MgCl_2$, 5.6 mM Glucose, 10 mM HEPES, and essential amino acids and vitamins (Sigma), pH 7.4, ~310 mmol/kg. Recording pipettes were filled with intracellular solution containing 135 mM potassium methanesulfonate, 20 mM KCl, 0.5 mM Na-GTP, 3 mM Mg-ATP, 10 mM HEPES, 2 mM EGTA, and essential amino acids and vitamins (Sigma), pH 7.4, ~285 mmol/kg. To record Ca^{2+} currents, samples were bathed in extracellular solution containing 100 mM NaCl, 5.8 mM KCl, 0.7 mM NaH_2PO_4, 1.3 mM $CaCl_2$, 0.9 mM $MgCl_2$, 5.6 mM Glucose, 35 mM TEA-Cl, 10 mM HEPES, and essential amino acids and vitamins (Sigma), pH 7.4, ~310 mmol/kg. Recording pipettes were filled with intracellular solution containing 135 mM cesium methanesulfonate, 10 mM CsCl, 10 mM TEA-Cl, 0.5 mM Na-GTP, 3 mM Mg-ATP, 10 mM HEPES, 2 mM EGTA, and essential amino acids and vitamins (Sigma), pH 7.4, ~285 mmol/kg. Recordings were performed with an Axopatch 200B amplifier (Molecular Devices) at RT. Data were analyzed using pClamp 10 software (Molecular Devices).

Ca^{2+} imaging

Co-cultured SGNs at day 30 were used for Ca^{2+} imaging. SGNs were transfected with AAV2/9-hSyn-Gcamp6s for 24 h and then exchanged with a fresh medium. After 15 days, infected SGNs in the differentiation medium were used to observe Ca^{2+} activity at 488-nm excitation by a Leica DM IL LED inverted microscope. For pharmacological stimulation, time-lapse images were captured before and after treatment with 50 mM KCl solution or 50 µM L-glutamate solution (Sigma) at a 1 frame/sec speed. Fluorescent image stacks were processed and analyzed using Fiji.

Statistical analyses

Statistical differences were calculated using GraphPad Prism 6.0. The results are presented as means ± SEM, and $p < 0.05$ was considered statistically significant. Unpaired two-tailed Student's t-test was performed when two groups were analyzed. To compare multiple groups, one-way ANOVA or two-way ANOVA followed by Dunnett's multiple comparison test was performed.