Generation of inner ear organoids containing functional hair cells from human pluripotent stem cells

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The derivation of human inner ear tissue from pluripotent stem cells would enable in vitro screening of drug candidates for the treatment of hearing and balance dysfunction and may provide a source of cells for cell-based therapies of the inner ear. Here we report a method for differentiating human pluripotent stem cells to inner ear organoids that harbor functional hair cells. Using a three-dimensional culture system, we modulate TGF, BMP, FGF, and WNT signaling to generate multiple otic-vesicle-like structures from a single stem-cell aggregate. Over 2 months, the vesicles develop into inner ear organoids with sensory epithelia that are innervated by sensory neurons. Additionally, using CRISPR–Cas9, we generate an ATOH1-2A-eGFP cell line to detect hair cell induction and demonstrate that derived hair cells exhibit electrophysiological properties similar to those of native sensory hair cells. Our culture system should facilitate the study of human inner ear development and research on therapies for diseases of the inner ear.

The human inner ear contains ~75,000 sensory hair cells that detect sound and movement via mechanosensitive stereocilia bundles1,2. Genetic mutations or environmental insults, such as loud noises and otoxic drugs, can cause irreparable damage to these hair cells, leading to hearing loss or dizziness3–4. We have described the generation of inner ear organoids from mouse pluripotent stem cells (mPSCs) using timed manipulation of the TGFβ, BMP, FGF, and WNT signaling pathways in a three-dimensional (3D) culture system5,6. We found that the mouse inner ear organoids contain sensory hair cells that are structurally and functionally similar to native vestibular hair cells in the mouse inner ear7. Our studies supported a working model of otic induction signaling cascades in which BMP signaling activation and TGFβ inhibition initially specify non-neural ectoderm, and subsequent BMP inhibition and FGF activation induce a pre-otic fate8,9. Despite several recent attempts, a developmentally faithful approach for deriving functional hair cells from human PSCs (hPSCs) has yet to be described10–15.

To generate human inner ear tissue from hPSCs, we began by establishing a timeline of in vitro human inner ear organogenesis (Fig. 1a,b). The inner ear arises from the ectoderm layer and, in humans, produces the first terminally differentiated hair cells by ~52 d post conception16. Inner ear induction from epiblast pluripotent cells begins at ~12 d post conception with the formation of the ectoderm epithelium. This epithelium then splits into the non-neural ectoderm (also known as surface ectoderm), which ultimately produces the inner ear and the epidermis of the skin, and the neuroectoderm (Fig. 1a,b). Thus, in our initial experiments we sought to establish a chemically defined 3D culture system for targeted derivation of non-neural ectoderm epithelia, from which we could derive inner ear organoids (Fig. 1a–c).

We first confirmed that dissociated human embryonic stem cells (hESCs; WA25 cell line) aggregate well in E8 medium containing a ROCK inhibitor, Y-27632, and display superior uniformity and cell survival compared to cells aggregated in a chemically defined differentiation medium (hereafter, CDM; Supplementary Fig. 1 and Supplementary Table 1). Following a 2-day incubation in E8 medium, we transferred aggregates to CDM containing a low concentration of Matrigel and FGF-2 to stimulate epithelization and ectoderm differentiation on the aggregate surface. We previously showed that a combination of BMP4 and the TGFβ inhibitor SB431542 (hereafter, SB) can promote non-neural induction from mouse PSCs. We found that combining 10 ng/ml BMP4 and 10 μM SB (dual SB/BMP4 treatment referred to as SBB) induced not only non-neural marker genes, such as TFFAP2A and DLX3, but also the extraembryonic marker CDX2 (Fig. 1d and Supplementary Fig. 2)17. In contrast, our experiments with human cells here showed that SB treatment alone led to an increase in TFFAP2A and DLX3 expression (Fig. 1d). 100% of SB-treated aggregates generated TFFAP2A+E-cadherin (ECAD)+ epithelium with sensory epithelia that are innervated by sensory neurons. Additionally, using CRISPR–Cas9, we generate an ATOH1-2A-eGFP cell line to detect hair cell induction and demonstrate that derived hair cells exhibit electrophysiological properties similar to those of native sensory hair cells. Our culture system should facilitate the study of human inner ear development and research on therapies for diseases of the inner ear.

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BMP inhibitor LDN-193189 (hereafter, LDN; dual LDN/SB treatment referred to as LSB). As previously shown in hESC monolayer cultures, LSB treatment of WA25 aggregates upregulated neuroectoderm markers, such as PAX6 and N-cadherin (NCAD), and abolished TFP2A and ECAD expression, suggesting that endogenous BMP signals drive non-neural conversion (Fig. 1f and Supplementary Fig. 4). To further validate our approach, we treated human induced PSCs (hiPSCs) (mND2-0) with SB and found, contrary to our results with WA25 hESCs, that SB-only conditions generated PAX6+ neuroectodermal and TFP2A+ECAD– neural crest-like cells (Supplementary Fig. 5). We reasoned that variation in endogenous BMP levels may underlie the different outcomes, and the BMP concentration may have to be fine-tuned for each cell line. Accordingly, a low concentration of BMP4 (2.5 ng/ml) in addition to SB (i.e., SBB) resulted in generation of TFP2A+ECAD+ neural epithelium from mND2-0 hiPSCs (Fig. 1g and Supplementary Fig. 5). With either the SB or SBB approaches, the resulting epithelia closely resembled non-neural epithelia with mPSCs5,6. In contrast to our mouse culture, non-neural conversion occurs without off-target induction of Brachyury (BRA)5 mesendoderm cells (Supplementary Fig. 6). The following data were generated using primarily the SB approach on WA25 hESCs and were subsequently confirmed using the SBB approach on mND2-0 hiPSCs.

Next, we attempted to convert the non-neural epithelium into otic placode epithelium before keratinocyte commitment. Human cranial placodes arise at ~18–24 d post conception; thus, assuming hPSCs represent cells at ~12 d post conception, otic placodes would develop in our culture within the first 6–12 d of differentiation with proper signaling modulation (Fig. 1b). Drawing on our previous finding that FGF activation and BMP inhibition are essential for pre-platece and otic induction from mPSC cultures, we treated day 4 SB-treated aggregates with a combination of FGF-2 and LDN (hereafter, SBFL). With SBFL treatment, the outer epithelium thickened relative to SB-treated samples and expressed a combination of posterior plaque markers, such as PAX8, SOX2, TFP2A, ECAD, and NCAD, indicating a phenotype similar to the otic-epibranchial progenitor domain (OEPD) from which the otic placode arises (Fig. 1h–k and Supplementary Fig. 7). When allowed to undergo self-guided differentiation in a minimal medium, we found that SBFL aggregates generated BRN3a+TU1+ sensory-like neurons during days 10–30 (Supplementary Fig. 8). Since both the epibranchial placodes and the otic vesicles produce sensory neurons, we investigated which tissue type had developed. We did not detect expression of the otic marker PAX2 nor did we observe in SBFL-treated aggregates any vesicles, whose presence would signify otic induction (data not shown). Thus, we concluded that SBFL treatment may be sufficient to induce epibranchial neurons, yet failed to initiate otic induction.

To promote PAX2 expression and vesicle formation, we tested various signaling modulators (Supplementary Fig. 9). Although none of the conditions we tested had a detectable effect on PAX2 gene expression using qPCR analysis, extensive immunostaining drew our attention to a small population of PAX2+PAX8+ECAD+ cells in the epithelia of aggregates of control samples on day 12, reminiscent of the otic placodes in vivo (Fig. 1–n). Considering that extracellular matrix might provide structural support for vesicle formation, we transferred day 12 aggregates to Matrigel droplets in a minimal medium (Fig. 2a). In these cultures, we observed radial production of migratory cells but no vesicle-like structures or PAX2+ cells (Fig. 2b and Supplementary Fig. 10). WNT activation seems to be essential for otic, but not epibranchial development in vitro18–22. After Matrigel-embedded WA25 cell aggregates were treated with CHIR99021 (CHIR), a GSK3β inhibitor that activates the WNT pathway23, 90.9±5.2% (±s.d.) of the aggregates on days 12–16 (n = 84, seven experiments) showed epithelial protrusions reminiscent of the otic pits that precede vesicle development in vivo (Fig. 2c). These structures were PAX2+PAX8+SOX2+SOX10+JAG1+, confirming their otic identity (Fig. 2d–h and Supplementary Fig. 10). The pits were accompanied by migrating TFP2A+SLUG+SOX10+ cranial neural-crest-like cells that formed a mesenchyme around the otic pits, similar to the peri-otic mesenchyme in vivo (Fig. 2c–f and Supplementary Fig. 10).

We cultured the aggregates in stationary droplets until day 18 and then transferred them to a 24-well plate on an orbital shaker or a spinner flask for further self-organized maturation. Both formats produced similar results. At 20–30 d in culture, vesicles remained visible through the surface of 71.7±23.3% (±s.d.) WA25 cell aggregates examined (n = 37, three experiments; Supplementary Fig. 11). In each aggregate we immunostained, we found multiple otic vesicles surrounding a central core epithelium that expressed the basal keratinoocyte markers TFP2A and KRT5 (n = 15, three experiments; Fig. 2g–i and Supplementary Video 1). As late as day 35, we observed vesicles and otic-placode-like epithelia that appeared to be partially attached or incorporated into the epidermal epithelium (Fig. 2h). In addition, older vesicles (>30 d) expressed the ubiquitin ligase FBXO22, which is highly specific to developing inner ear epithelia in mice (Fig. 2i24,25. Each aggregate we analyzed on days 30–40 contained SLUG+ mesenchymal cells and cartilaginous masses with S100+SOX9+ chondrocytes, which also stained positive for Alcian blue (n = 7, three experiments; Supplementary Fig. 10). These tissues likely arose from the cranial neural-crest-like cells seen during vesicle formation on days 12–18. Thus, our culture system appears to have derived multiple cranial tissues associated with the anatomic region surrounding the inner ear.

After 40–60 d of incubation, vesicles with complex multi-chambered morphologies were visible through the aggregate surface (Fig. 2) and Supplementary Video 2. Notably, a subset of vesicles in aggregates derived from either WA25 or mND2-0 developed epithelia with cells expressing multiple hair cell markers, including MYO7A, PCP4, ANXA4, SOX2, and CALB2 (Fig. 2k–q and Supplementary Fig. 12). The sensory-like epithelia also contained SOX2+SOX10+SPARCL1+ cells, reminiscent of supporting cells in the mammalian utricle26. The luminal cells in these epithelia had elongated morphologies with F-actin-rich apical junctions characteristic of inner ear sensory epithelia (Fig. 2l–o). The cells expressing hair cell markers also had F-actin-rich and espin (ESPN) apical stereocilia bundles protruding into the vesicle lumen that were associated with an acetylated-alpha-Tubulin (TUBA4A)+ kinocilium (Fig. 2m–p,r). Together, these findings confirm that the hPSC-derived otic vesicles generated inner ear organoids with sensory epithelia containing hair cells and supporting cells. Moreover, the hair bundle morphology and the densely packed clusters of hair cells were reminiscent of vestibular end organs.

To facilitate live-cell imaging and electrophysiological experiments, we engineered an hESC reporter cell line to label nascent hair cells with enhanced green fluorescent protein (eGFP). We used the CRISPR–Cas9 system to insert a 2A-eGFP gene cassette at the stop codon of ATOH1, which is highly expressed during hair cell induction and early maturation (Fig. 3a and Supplementary Fig. 13)7. We verified inner ear organoid induction from two clones containing the bi-allelic insertion of the 2A-eGFP cassette (hereafter, ATOH1–2A–eGFP cells), using the inner ear organoid induction protocol. As early as day 39, we observed eGFP+ hair-cell-like cells emerging in inner ear...
Figure 1 Step-wise induction of otic-placode-like epithelia. (a) Overview of mammalian ectoderm development in the otic placode cranial region. (b) Timeline for key events of human otic induction. Day 0 on the timeline indicates the approximate stage of development represented by hPSC ~12 d post conception. (c) Differentiation strategy for non-neural ectoderm (NNE), otic-epibranchial progenitor domain (OEPD), and otic placode induction. Potentially optional or cell-line-dependent treatments are denoted in parentheses. (d) qPCR analysis on day 2 of differentiation of WA25 cell aggregates treated with DMSO (control), 10 µM SB, or 10 µM SB + 10 ng/ml BMP4, denoted as SBB. Gene expression was normalized to undifferentiated hESCs; FC, fold change; n = 3 biological samples, two technical repeats; *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant; box limits: upper/lower quartile; center line: median; whiskers: max./min. (e,f) Representative TFAP2A, ECAD, and PAX6 expression in WA25 aggregate treated with 10 µM SB (n = 15 aggregates, three experiments) or with 200 nM LDN + 10 µM SB for 6 d (n = 6 aggregates, three experiments). (g) Representative image of an SB-treated WA25 aggregate on day 8: live (h) and immunostained with PAX8 and TFAP2A antibodies (i). When comparing morphology in h and i, note that the outer epithelium crumples into the aggregate core during the cryosectioning process. (j,k) Representative image of an SB-treated WA25 aggregate on day 8 after treatment with 50 ng/ml FGF-2 and 200 nM LDN (SBFL) on day 4: live (j) and immunostained with PAX8 and TFAP2A antibodies (k). (l–n) WA25 SBFL-treated aggregates on day 12. The outer epithelium contains PAX8+ECAD+ cells (l) and occasional patches of PAX8+PAX2+ otic-placode-like cells (m,n). The specimens shown were treated with 25 µl of additional CDM on day 8. Images are representative of specimens obtained from at least three separate experiments. Scale bars, 100 µm (e–m), 50 µm (n).
organoids (Supplementary Fig. 13). The individual organoids often contained multiple discrete patches with hundreds of eGFP+ cells (Fig. 3b–d and Supplementary Video 3). Immunostaining with hair cell markers, such as BRN3C and ESPN (present on 99.4% of eGFP+ hair-cell-like cells), confirmed the hair cell identity of eGFP+ cells (Fig. 3c,f). Occasionally, we observed eGFP expression in disorganized clusters of neuron-like cells, which were clearly distinct from hair cells (~20% of aggregates, Supplementary Fig. 14). On days 60–100, 19.7 ± 7.0% (± s.d.) of aggregates contained at least one organoid with eGFP+ hair cells (n = 167, 7 experiments). The seemingly low efficiency of hair cell induction may be due to our inability to detect organoids deep within the aggregates, or it could indicate that the endogenous signals required for sensory epithilia formation vary from aggregate to aggregate. Every WA25 and mND2-0 aggregate examined
on days 60–100 contained organoids with PAX8+/PAX2+/SOX10+ non-sensory epithelia (n = 21, five experiments; Supplementary Fig. 12e), suggesting that although inner ear induction was highly reproducible, non-sensory or immature otic epithelium were preferentially induced. Using 3D reconstructions, we estimated that there were 68–779 hair cells per organoid (mean = 266, n = 12 organoids, four experiments). The stem-cell-derived hair cells could be maintained for >150 d in floating culture and retained hair bundles even after dissection and sub-culturing (Fig. 3b–f; Supplementary Videos 3–4). In mice, Atoh1 is downregulated as hair cells mature; however, we did not detect any noticeable changes in eGFP expression on day 100–150 organoids, suggesting that maturation may be delayed or ATOH1 expression is more prolonged in human inner ear organogenesis.

Next, we examined whether hPSC-derived hair cells function similarly to native mammalian hair cells. Using aggregates generated from ATOH1-2A-eGFP cells, we dissected and flat-mounted inner ear organoids during differentiation days 63–70. The cells had large outwardly rectifying currents (Fig. 4a–c), typical of type II hair cells of human vestibular organs27, mouse vestibular organs28, and mouse organoid hair cells2. The amplitude of the currents ranged from several hundred picoAmps to several nanoAmps, presumably depending on the maturity of the cell. Small, rapidly activating, rapidly inactivating outward currents were also present in 6 of 14 hPSC-derived organoid hair cells, eGFP is transcribed along with ATOH1. (i.e., SVideo 4). Scale bars, 100 µm (c), 50 µm (b), 25 µm (e), 5 µm (d, f).

those of rodent vestibular cells. Negative resting potentials in mouse vestibular hair cells are maintained by prominent inward rectifier currents, which are carried by Kir2.1 channels30. Inward rectifier currents develop early in all rodent vestibular hair cells and mouse organoid hair cells but were absent in human organoid cells and native human vestibular cells27. The constricted lumen morphology seen in most organoids >60-d old and in all of the organoids used for recording made hair bundle deflection and mechanotransduction analysis inaccessible (Fig. 3b–e and Supplementary Video 3). Nonetheless, to our knowledge there have been no previous recordings of voltage-dependent currents and membrane response (Fig. 4a–h) from hPSC-derived human hair cells. Furthermore, the profile of current expression strongly suggests that the hPSC-derived organoid cells, like mouse inner ear organoid cells, have adopted a vestibular type II hair cell phenotype.

Finally, we examined neurogenesis during inner ear organ formation. We observed the first sign of TUJ1+BRN3A+ neurons in aggregates during days 20–30 after the start of differentiation (Supplementary Fig. 15). Immunostaining for TUJ1 and neurofilaments (light chain, NEFL, and heavy chain, NEFH) during days 60–75 revealed a mix of unipolar and bipolar neurons surrounding inner ear organoids (Fig. 4i) and Supplementary Video 5). Inner ear ganglia neurons are bipolar, whereas epibranchial and neural crest ganglia neurons typically mature from a bipolar to a unipolar morphology31,32; thus, the cells observed here may be derived from placode or neural crest. We also observed S100β+ cells, reminiscent of myelinating Schwann cells, associated with NEFL+ soma and processes (Fig. 4k and Supplementary Fig. 15). In each sensory epithelium we analyzed, we found neurites infiltrating the epithelium and contacting hair cells (Fig. 4j–l, n = 15 sensory epithelia, six separate experiments). A subset of hair cells displayed CTBP2+ puncta, indicating putative ribbon-synapse-like structures (Fig. 4lm and Supplementary Fig. 15). Moreover, the CTBP2+ puncta were typically localized near synaptophysin (SYP)+ puncta, a post-synaptic
Figure 4 hESC-derived hair cells have similar electrophysiological properties as native hair cells and form synapse-like contacts with sensory neurons. (a) Family of outward rectifier potassium currents recorded from a human organoid hair cell (d64), evoked by the series of voltage steps shown below. (b) The outward currents had an activation range that was well-fitted by a Boltzmann equation (line) with voltage of half-maximal activation of −31 mV. (c) Mean ± s.e.) maximal current-voltage relationships for seven human organoid hair cells (d64–d67) and eight mouse utricle type II hair cells. For current–voltage relations, we averaged data from hair cells with large currents over 2 nA. (d) Family of rapidly activating, rapidly inactivating, inward currents evoked by the depolarizing steps shown below. (e) Family of slowly activating, non-inactivating inward currents (d64) evoked by hyperpolarizing steps, shown below. (f) Activation curve for the current family shown in e, fitted by a Boltzmann equation (line) with a voltage of half-maximal activation of −71 mV. (g) Family of membrane responses (d64) recorded in current-clamp mode, evoked by the current injection protocol shown below. (h) Membrane response (d65) to three cycles of a 5-Hz sine wave stimulus (below). (i) A representative 3D projection of eGFP+ hair cells with ESPN+ hair bundles surrounded by clusters of sensory-like neurons in a wholemount immunostained sample. Insets 1 and 2 show the two neuron morphologies observed: unipolar and bipolar (asterisks indicate neurites from other neurons). Inset 3 shows hair cell morphology and NEFH+ neurites in the sensory epithelium (asterisks indicate neuronal processes in the epithelium). Panel i is an image from Supplementary Video 5 (i.e., SVideo 5). (j) Representative confocal image of a cryosection with NEFL+ neurons innervating an organoid sensory epithelium (dotted region of interest highlighted in inset). (k) Representative cryosection showing that S100β+ Schwann-like cells associate with neuronal soma and appear to myelinate NEFL+ neuronal processes (dotted region of interest highlighted in inset). (l) Representative cryosection showing that NEFL+ neuronal processes infiltrate the epithelium and are closely associated with CTBP2+ puncta at the base of eGFP+ hair cells. (m) Representative cryosection showing that CTBP2+ puncta are co-localized with SYP+ puncta (putative synapses are highlighted in insets 1-4). (n) Summary of neurogenesis analysis. Images are representative of specimens obtained from at least three separate experiments. Scale bars, 100 µm (i), 25 µm (j, k), 10 µm (l, m), 5 µm (i insets).
Together, these findings suggest that the human inner ear organoids assemble a sensorineural circuit between hair cells and sensory neurons, similar to in vivo organs and mouse inner ear organoids (Fig 4n)6,7,31. However, it remains to be determined whether the neurons innervating hair cells are authentic vestibular afferent neurons and whether the putative synapses are functional.

In conclusion, we have established a robust differentiation system for guiding the development of human inner ear organoids in 3D culture (Supplementary Fig. 16 and Supplementary Table 2 for a comparison to past human otic induction approaches). Our findings support our previous model5,6,22 of in vitro otic induction and underscore the importance of carefully timed GSK3β inhibition for otic placode specification. Notably, the convoluted and multi-chambered morphologies of human inner ear organoids bear a striking resemblance to the inner ear’s membranous labyrinth, which consists of a series of tubes and chambers containing sensory and non-sensory epithelia (Figs 2j–l, 3i and Supplementary Videos 2–5). In addition, much like mouse organoids, the hPSC-derived organoids appear to form vestibular sensory epithelia by default; thus, additional signaling manipulation will be needed to initiate cochlear organogenesis6,7. We expect that this culture system will provide a useful tool for uncovering mechanisms of human inner ear development, studying genetic disease of the inner ear, and developing potential therapies.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

K.R.K. conceived, designed, and led the study, performed experiments, analyzed data, and drafted the manuscript with input from all authors. J.N. generated the ATOH1-2A-eGFP cell line, performed experiments, and wrote the manuscript. E.L.-M. performed experiments, data analysis, J.R.H. designed and analyzed electrophysiology experiments and wrote the manuscript. E.H. designed and oversaw the study and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

hPSC culture. Human PSCs (WA25 hESCs, passages 22–50; mND2-0 hPSCs, passages 28–46) were cultured in Essential 8 (EB) Medium (Invitrogen, cat. no. A1517001) or Essential 8 Flex Medium (EBi) (Invitrogen, cat. no. A2858501) supplemented with 100 µg/ml Normalcin (Invitrogen, cat. no. Ant-nr-1) on recombinant human Vitronectin-N (Invitrogen, cat. no. A14700)−coated 6-well plates according to an established protocol. At 80% confluency or every 4 or 5 d, the cells were passaged at a split ratio of 1:10–1:20 using 0.5 mM Ethylenediaminetetraacetic acid (EDTA) in Dulbecco’s phosphate-buffered saline (DPBS). Both cell lines were acquired from the WiCell Research Institute and arrived with a statement of verification and authenticity. For additional validation and testing information refer to the cell line webpages: https://www.wicell.org/home/stem-cell-lines/catalog-of-stem-cell-lines/wa25.cmsx, https://www.wicell.org/home/stem-cell-lines/catalog-of-stem-cell-lines/mirj?1-md2-0.cmsx. The cell lines were not tested for authenticity upon receipt. Cell lines were determined to be mycoplasma contamination-free using the MycoAlert Mycoplasma Detection Kit (Lonza). Prior to differentiation, pluripotency was assessed using immunocytochemistry to detect stem-cell markers, SOX2, OCT4, and SSEA-1 (Supplementary Figs. 1, 5, and 13). Routine karyotyping was not performed, however, the passage number was minimized to reduce the risk of spontaneous chromosomal duplications or genetic mutations.

hPSC differentiation. To start differentiation, hPSC cells were dissociated with StemPro Accutase (Invitrogen, cat. no. A1110501) and distributed, 5,000 cells per well, onto low-adhesion 96-well V-bottom plates in EB medium containing 20 µM Y-27632 (Stemgent, cat. no. 04-0012-02) and Normalcin. Following a 48-h incubation, the aggregates were transferred to low-adhesion 96-well V-bottom plates in 100 µl of chemically defined medium (CDM) containing 4 ng/ml FGF-2 (Peprotech, cat. no. 100-18B), 10 µM SB-431542 (Stemgent, cat. no. 04-0010-05) and, for some experiments, 2.5 ng/ml BMP4 (Stemgent, cat. no. 03-0007), and 2% growth factor reduced (GFR) Matrigel (Corning, cat. no. 354230) to initiate non-neuronal induction—that is, differentiation day 0. CDM contained a 50:50 mixture of F-12 Nutrient Mixture with GlutaMAX ( Gibco) and Iscove’s Modified Dulbecco’s Medium with GlutaMAX (IMDM; Gibco), additionally supplemented with 0.5% Bovine Serum Albumin (BSA), 1× Chemically Defined Lipid Concentrate (Invitrogen), 7 µg/ml Insulin (Sigma), 15 µg/ml Transferin (Sigma), 450 µM Mono-Thioglycollate, and Normalcin (see Supplementary Table I for detailed formulation). After 4 d of incubation, 25 µl of CDM containing a 250 ng/ml FGF-2 (50 ng/ml final concentration) and 1 µM LDN-193189 (200 nM final concentration; Stemgent, cat. no. 04-0074-02) was added to the pre-existing 100 µl of media in each well. After an additional 4 d (8 d total), 25 µl of CDM was added to the media. For some experiments, CDM containing a 18 µM CHIR99021 (3 µM final concentration; Stemgen, 04-0004-02) was added to the pre-existing 125 µl of media in each well on day 8—we determined that this treatment is optional for inner ear differentiation. Between a given condition and the control group. Refer to Supplementary Table 4 for primer details.

Quantitative PCR. Analysis was performed as previously described on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) or a Bio-Rad CFX96 quantitative PCR machine (Bio-Rad). Data were normalized to L27 expression (internal control) and the fold change was calculated relative to Ct values from d0 WA25 aggregates using the ΔΔCt method. Unless stated otherwise, data represent at least three separate biological samples from separate experiments. All indicators of statistical significance refer to comparisons between a given condition and the control group. Refer to Supplementary Table 4 for primer details.

Immunohistochemistry. Aggregates were fixed with 4% paraformaldehyde for 20 min at room temperature or at 4 °C overnight. The fixed specimens were cryoprotected with a graded treatment of 15% and 30% sucrose and then embedded in tissue-freezing medium. Frozen tissue blocks were sectioned into 12-µm cryosections on a Leica CM-1860 cryostat. For immunostaining, a 10% goat or horse serum in 0.1% Triton X-100 1× PBS solution was used for blocking, and a 3% goat or horse serum in 0.1% Triton X-100 1× PBS solution was used for primary/secondary antibody incubations. Alexa Fluor conjugated anti-mouse, rabbit, or goat IgG (Invitrogen) were used as secondary antibodies. ProLong Gold Antifade Reagent with DAPI (Thermo Scientific) was used to mount the samples and visualize cellular nuclei. For whole-mount staining, a similar staining paradigm was used; however, the Triton X-100 concentration was increased to 0.5%, and the blocking and primary/secondary incubations were done at 37 °C on a rotating shaker for 24 h and 48 h, respectively. Following each incubation, the samples were subjected to three 1-h washes in 1× PBS containing 0.5% Triton X-100 at 37 °C on a rotating shaker. Whole-mount samples were mounted in Scale54 clearing solution for 1–2 d or Scale54Q (clearing solution for 1–2 h before imaging). Microscopy was performed on a Leica DMi8 inverted microscope, a Nikon TE2000 inverted microscope, or an Olympus FY1000-MPE Confocal/Multiphoton Microscope. 3D reconstruction was performed using the Imaris 8 software package (Bitplane) housed at the Indiana Center for Biological Microscopy. For the segmentation analysis in Supplementary Video 3 and estimates of hair cell number per organoid, eGFP+ cells were processed using the ‘Spots’ module in Imaris. Classification was based on estimated size, quality, and signal intensity. Objects touching the border of the image were excluded. The following build parameters were used to identify eGFP+ cell bodies: estimated XY diameter = 7.00 µm;
estimated Z diameter = 10.00 µm; ‘Quality’ above 20.0; ‘distance to image border X/Y/Z’ above 0.001 µm; ‘intensity center Ch = 1’ above 1.500. These parameters generated estimated, not absolute, counts and were adjusted to provide highly conservative values. For quality control, we performed slice-by-slice analysis of optical sections for three representative organoids. Movies were generated in Imaris from the raw image files and compiled in Adobe Premiere Pro to add titles and text. See Supplementary Table S5 for a list of antibodies.

Electrophysiological recordings. Human organoids were shipped at day 62 in cold Hibernate A medium supplemented with 1x GlutaMax, 1x B27 Supplement (without Vitamin A), 22 mM NaCl, and Normocin. They were replaced back into OMM on day 63 in an incubator at 5% CO2 and 37°C. On recording days, organoids were dissected out using sharp tungsten needles (Fine Science Tools) and pinned to glass coverslips. The eGFP+ signal was used to find areas with hair cells and to target hair cells for recording. Whole-cell patch clamps were performed on the semi-intact tissue with 4- to 5-MΩ glass electrodes. Data were acquired using an Axopatch 200B amplifier (Molecular Devices), filtered at 5000 Hz, then digitized at 20 kHz through a Digidata 1322A converter. The recording pipette solution contained (in mM): 135 KCl, 5 HEPES, 5 EGTA, 2.5 MgCl2, 2.5 K2-ATP, 0.1 CaCl2, adjusted with KOH to pH 7.4, –285 mmol kg⁻¹. The external solution contained: 137 NaCl, 5.8 KCl, 0.7 NaH2PO4, 10 HEPES, 1.3 CaCl2, 0.9 MgCl2, 5.6 Glucose, and was supplemented with vitamins and essential amino acids (Invitrogen), adjusted to pH 7.4 with NaOH, –310 mmol kg⁻¹. Recordings were compensated 40% and cells were held at –66 mV for voltage clamp. Averages are reported ± s.e.m.

Generation of ATOH1-2A-eGFP reporter cell line. gRNAs (5′-TCGGAT GAGGCAAGTTAGGA-3′ and 5′-GTCACTGTAAATGGGAATGGG-3′, offset = 0 bp) targeting the stop codon region of ATOH1 promoter (Addgene #48873)38. To construct the donor vector, a 2A-eGFP-PGK-Puro cassette (Addgene #31938)39 flanked by two 1-kb homology arms PCR amplified from extracted WA25 hESC genomic DNA were cloned into a pUC19 backbone. The two gRNA vectors and the donor vector, as well as a vector expressing Cas9n under the control of the CMV promoter (Addgene #41816)40 were transfected into WA25 hESCs with 4D Nucleofector (Lonza) using the P3 Primary Cell 4D-Nucleofector X kit and Program CB-150. After nucleofection, cells were plated in growth medium containing 1× RevitaCell (Thermo Fisher) for improved cell survival rate, and 1 µM of Scrt (Xcessbio) for higher HDR efficiency41. 0.5 µg ml⁻¹ puromycin selection was performed for 10 d starting from 48 h post-nucleofection. The PGK-Puro sub-cassette flanked by two loxP sites was removed from the genome after puromycin selection by nucleofection of a Cre recombinase expressing vector (Addgene #13775). Clonal cell lines were established by low-density seeding (1–3 cells cm⁻²) of dissociated single hESCs followed by isolation of HESC colonies after 5–7 d of expansion. Genotypes of the clonal cell lines were analyzed by PCR amplification followed by gel electrophoresis, and by Sanger sequencing of total PCR amplicons or individual PCR amplicons cloned into TOPO vectors. Cell lines with bi-allelic eGFP integration were used for inner hair cell differentiation. Hair-cell-bearing organoids were identified using an inverted epifluorescent microscope (Leica DMi8). We only counted eGFP⁺ cells observed in an organoid epithelium with defined apical and basal edges that were located toward the surface of the aggregate. Hair-cell-bearing organoids located in the interior of the aggregates were undetectable using this approach. See Supplementary Data for donor plasmid and insertion site sequences.

Statistical analysis. All statistics were performed using GraphPad Prism 7 software. A Shapiro-Wilk normality test was used before analysis to determine that the data had a normal distribution. Statistical significance was determined using a one-way analysis of variance (ANOVA) followed by a Dunnett’s post hoc test for multiple comparisons to a control group (e.g., vehicle treated). A Brown-Forsythe test was used to determine that the variation among sample groups was similar. No statistical test was used to predict sample size, the investigators were not blinded to the treatment groups, and the samples were not randomized.

Representative data and reproducibility. Unless stated otherwise, images are representative of specimens obtained from at least three separate experiments. For immunohistochemical (IHC) analysis of aggregates between days 0–12, we typically sectioned 3–6 aggregates from each condition in each experiment. IHC analyses for later stages of the protocol were performed on at least two aggregates from each condition per experiment. The final culture method was successfully replicated 23 times by four independent investigators (K.R.K., J.N., E.L.M., and J.L.) using the WA25 (wild-type or ATOH1-2A-eGFP) cell line. The method, with noted modifications, was replicated three times using the mND2-0 hiPSC line by K.R.K. A replication was deemed successful by confirming pit formation during days 12–18 and positively identifying hair-cell-bearing inner ear organoids in at least one aggregate on days 50–100 of differentiation. If either of these criteria were not met, an experiment was deemed a failure and excluded from analysis. The investigators observed a failure rate of ~21% (6/29 experiments), which was typically attributed to expiration/mishandling of a critical reagent or technical error.

Data availability. Data are available from the corresponding authors upon reasonable request.

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