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Generating inner ear organoids containing putative cochlear hair cells from human pluripotent stem cells

Minjin Jeong1, Molly O’Reilly2, Nerissa K. Kirkwood2, Jumana Al-Aama3, Majlinda Lako1, Corne J. Kros2 and Lyle Armstrong1

Abstract
In view of the prevalence of sensorineural hearing defects in an ageing population, the development of protocols to generate cochlear hair cells and their associated sensory neurons as tools to further our understanding of inner ear development are highly desirable. We report herein a robust protocol for the generation of both vestibular and cochlear hair cells from human pluripotent stem cells which represents an advance over currently available methods that have been reported to generate vestibular hair cells only. Generating otic organoids from human pluripotent stem cells using a three-dimensional culture system, we show formation of both types of sensory hair cells bearing stereociliary bundles with active mechano-sensory ion channels. These cells share many morphological characteristics with their in vivo counterparts during embryonic development of the cochlear and vestibular organs and moreover demonstrate electrophysiological activity detected through single-cell patch clamping. Collectively these data represent an advance in our ability to generate cells of an otic lineage and will be useful for building models of the sensory regions of the cochlea and vestibule.

Introduction
Achieving the functions of the vertebrate inner ear requires a complex arrangement of cells that arise during embryonic development in a precisely orchestrated spatiotemporal manner. A principal cause of hearing loss is the death and/or dysfunction of the cells present in the organ of Corti1–4 which cannot regenerate post-partum in mammals meaning loss of individual cell types is irreversible5. This condition, known as sensorineural hearing loss, is a global healthcare challenge with 600 million persons worldwide affected6. Presbycusis, the age-related decline in hearing capacity is possibly the most prevalent neurodegenerative disease of ageing7 however chronic noise exposure and xenobiotic toxicity are significant contributing factors to hearing loss worldwide. The induction of human inner ear tissue from pluripotent stem cells could be applicable not only to modelling of sensorineural hearing loss but also for the generation of clinically useful sensory cells. Despite reports that progenitor cells capable of differentiating into cochlear hair cells may be isolated from neonatal mouse cochleae8 and putative differentiation of mesenchymal stem cells into hair progenitor cells9, the only cells that reliably differentiate into cells of an otic phenotype are pluripotent stem cells10–15. Most protocols have employed two-dimensional differentiation methods which are less likely to recapitulate inner ear development, therefore protocols that mimic the developmental progression towards inner ear construction are more likely to succeed in producing structures containing the desired cell types.
Recent work shows that pluripotent stem cells generate self-organising otic placode-like structures under 3D minimal culture conditions generating cells of the vestibular sensory epithelia, namely hair cells, neurons and supporting epithelial cells. To date, these protocols have not generated cells of a cochlear hair cell phenotype. Herein, we present a novel method that results in the conversion of hESC and hiPSC into 3D “organoids” containing otocyst-like structures comprising all the cell types normally present in the cochlea and vestibule.

**Results**

**Adaptation of existing protocols for the generation of 3D otic organoids**

We took advantage of a published protocol which utilised 3D culture conditions and stage-specific growth factor addition to generate otic organoids containing mecano-sensory hair cells. We combined these conditions (Figure S1A) with forced aggregation of cells in U-shaped lipidure-coated plates (3000 cells/well) to direct differentiation of hESC however, this did not generate stable organoids (Figure S1B). Further modifications included substitution of GMEM for DMEM/F12 (Figure S1C) and increasing cell number per well in line with other literature protocols (Figure S1D), however only a concentration of 2-mercaptoethanol of 0.1 mM (Figure S2) was found to generate otic placode-like structures by day 32 of differentiation. Moreover, prior culture of hESC and hiPSC on mitotically inactivated mouse embryonic fibroblast feeder layers (MEFs) is essential for generation of otic organoids containing more mature cochlear cell types. The key points of this protocol are summarised as follows:

- Co-culture of hESC/hiPSC with MEF feeder layers prior to generation of embryoid bodies (EBs)
- Association of 9000 cells per well in 96-well lipidure-coated low adhesion plates to generate EBs
- Inclusion of the Rho-Kinase inhibitor Y-27632 (20 μM) and 0.1 mM 2-mercaptoethanol until differentiation day 8
- Addition of 1% matrigel to the differentiation medium between differentiation days 8 and 10.

![Schematic overview of the 90-day protocol used to generate otic organoids containing putative cochlear/vestibular hair cells and sensory neurons.](image)

**Fig. 1 Generating otic organoids.** a Schematic overview of the 90-day protocol used to generate otic organoids containing putative cochlear/vestibular hair cells and sensory neurons. GMEM Eagle’s Minimal Essential Medium, KOSR knockout serum replacement, NEAA nonessential amino acids, SP sodium pyruvate, P/S penicillin/streptomycin, BME 2-mercaptoethanol, RI Y-27632, BMP BMP4, SB SB-431542, LDN LDN-193189, FGF FGF2. b, c Representative examples of 3D otic vesicles (indicated by white arrows) obtained between days 16 and 36 from hESC (H9) (b) and from hiPSC (SB-Ad3) (c). Pictures shown are representative of at least four independent experiments (n = 4). Scale bars represent 50 μm.
Characterisation of human pluripotent stem cell-derived pro-sensory otic vesicles

Using our in-house protocol (Fig. 1a), we generated 3D organoids with vesicular structures (Fig. 1b, c) which were apparent from day 16 of differentiation, but became more numerous with time. By differentiation day 20, each organoid contained 1.5 ± 0.5 (±s.d., n = 32, 4 experiments) otic pit and otic vesicles from hESC and 1.6 ± 0.7 (±s.d., n = 32, 4 experiments) from hiPSC. The size range of vesicular structures was 100–200 µm.

Putative otic lineage cells in such vesicles were identified by double immunostaining with pro-sensory otic vesicle markers. In the E9.5 mouse, pro-sensory otic vesicles are defined by expression of Pax2, Pax8, Ecad, Sox2, Jag1, cyclin D1, and Myo7a, but in equivalent human embryos (approximately 22 days post conception) the pro-sensory domains are only recognised by SOX2 expression. Accordingly, we observed E-cadherin (ECAD)/SOX2-positive otic vesicles (Fig. 2b) corroborated by quantitative RT-PCR showing peak E-cadherin expression at differentiation days 20 and 36 (Fig. 3). Few cells within these otic vesicles expressed PAX2 (Fig. 2c) and SOX9 (Fig. 2d). Extra-vesicular PAX2 expression was also noted (Fig. 2c) and we speculate these might be precursors of neurons that form in the 3D otic organoids. It is not clear that areas of cells expressing the above genes correspond to pro-sensory otic vesicles since no cells with sensory phenotype expression (such as MYO7A) are present at this stage and were not observed in day 20 organoids (data not shown). SOX2 expression quantified
using Image J software on stained vesicle sections indicates both hESC (H9) and hiPSC (SB-Ad3) generated similar numbers of SOX2 expressing cells (25.27 ± 3.07% and 23.30 ± 1.78%; data representative of 8–16 organoids per experiment, $n = 3$), respectively.

By day 36, putative pro-sensory vesicles expanded to 300–450 μm. At this stage, otic vesicles were observed in 87.5 ± 45.1% from hESC and 30.6 ± 27.7% from hiPSC of organoids examined (24–36 organoids, $n = 3$). These pro-sensory vesicles show partial co-expression of PAX2/SOX2 (Fig. 2e) and SOX9/SOX2 (Fig. 2f) but are distinguished from day 20 vesicles by expression of F-ACTIN and the stereocilia marker ESPIN (Fig. 2g). Image J quantification indicates the percentages of SOX2 expressing cells present in day 36 vesicles to be 19.17 ± 1.49% (hESC) and 24.64 ± 6.67% (hiPSC; data representative of 16 organoids per experiment, $n = 3$). ESPIN expression at this time point is interesting since expression of this marker begins at 7 weeks of gestation in humans. The cells of the neuroepithelial layer that give rise to the vestibular sensory epithelium have a single micro kinocilium surrounded by microvilli. A subset of these cells differentiates into vestibular hair cells which present several larger stereocilia. TEM analysis of day 36 otic vesicles showed immature kinocilia (Fig. 2h) and microvilli (Fig. 2i). Similar observations were obtained by immunostaining of otic vesicles obtained from hiPSC (Figure S3B–G).

Maturation and characterisation of human pluripotent stem-cell derived otic vesicles

By day 60, 29.2 ± 8.3% of organoids from hESC and 30.6 ± 4.8% from hiPSC had otic vesicles (data representative of 36–48 organoids, $n = 4$) and expressed ATOH1 (Fig. 4a) and MYO7A (Fig. 4b, c) in cells adjacent to the lumen of putative otic vesicles. ATOH1 expression was observed in 62.85 ± 2.82% (hESC) and 48.11 ± 4.32% (hiPSC) of cells present. Quantitative RT-PCR showed peak ATOH1 expression at day 36 with stepwise increased expression of MYO7A from days 20–78 (Fig. 3). Some MYO7A expressing cells co-expressed SOX9 (Fig. 4b), but not SOX2 (Fig. 4c). The SOX2 expressing
cells were found in a distinct layer from MYO7A, immediately below that adjacent to the lumen (Fig. 4c) and may be putative supporting epithelial cells. The numbers of SOX2-expressing cells in day 60 otic vesicles decreased slightly (19.01 ± 8.37% (hESC) and 12.67 ± 1.09% (hiPSC; data representative of 16 organoids per experiment, n = 3). Also apparent was the expression of proteins that indicate early innervation of prospective hair cells such as β-III-tubulin (TUBB3, Fig. 4d, e), Neurofilament (NFM, Fig. 4f, g), Synaptophysin (SY, Fig. 4h, i) and Glutamine synthetase (GS, Fig. 4a, j). TUBB3 was present in cells projecting towards the putative sensor-neural epithelium expressing ESPIN (Fig. 4d) and PAX2 (Fig. 4e). Similarly, NFM staining together with ESPIN (Fig. 4f) and ATOH1 (necessary for hair-cell specification and differentiation)24 (Fig. 4g) suggested the presence of bipolar neurons, which are typical of inner ear ganglion neurons near the putative otic vesicles. Synaptophysin (SY), a post-synaptic marker of ribbon synapses25, was present in or near cells projecting into the putative sensory epithelium expressing ESPIN (Fig. 4h) and PAX2 (Fig. 4i). In vivo, glutamine synthetase GS is expressed by satellite glial cells which envelope the spiral ganglion neurons to maintain valid signal transmission26. We found strong GS expression in the epithelial cell layer adjacent to the otic vesicle lumen expressing ATOH1 (Fig. 4a) and PAX2 (Fig. 4j). These data indicate possible synaptic connections between putative neurons and hair cells. TEM suggested the presence of hair cells (Fig. 4k) with putative afferent nerve endings beneath the hair cells and a possible presynaptic body at their base (Fig. 4l). TEM images of day 60 organoids showed only a few short microvilli per cell (Fig. 4m, n), suggesting hair cell immaturity. Similar observations were obtained by
immunostaining (Figure S4A–I) and TEM (Figure S4J, K) of hiPSC derived otic vesicles.

By day 90, otic vesicles appeared more mature (Fig. 5) and were observed in 14.6 ± 4.2% from hESC and 22.2 ± 9.6% from hiPSC of organoids examined (36–48 organoids, n = 3). Published data suggest hiPSC differentiation only to hair cells of vestibular phenotype; hence we wished to determine if this also applied to our modified differentiation protocol. Four distinct populations of hair cells exist in the inner ear; type I and type II in the vestibular organs and inner (IHCs) and outer cochlear hair cells (OHCs). Expression of Sox2 and Pax2 may distinguish vestibular from cochlear hair cells in mouse and chicken27,28. We detected SOX2 expression in only a few putative hair cells (Fig. 5a), thus we analysed other potential markers to distinguish hair cell types. Oncomodulin (Ocm), specific to the cochlear OHCs of adult rats, mice and guinea pigs29, is a possible marker for putative OHCs. Ocm is present in the hair cell body of mouse type I vestibular hair cells30; however, immunohistochemical location of the protein in our otic vesicles appears restricted to the cell membrane adjacent to the lumen (Fig. 5b) supporting an OHC phenotype. Otopetrin1 (Otop1) is specific to peripheral supporting cells of vestibular epithelia in mouse31,32. Some hESC/hiPSC derived hair cell-like cells marked by MYO7A expression (15.8 ± 2.43% in hESC and 12.9 ± 4.59% in hiPSC; 16 organoids per experiment, n = 3) also expressed OTOP1 near the apical surface of the sensory epithelium (Fig. 5c). In addition, calbindin2 (CB2) uniquely labels type II vestibular hair cells in mice16; however CB2 expression was undetected in the otic organoids (Fig. 5d). The expression of OTOP1 without CB2 suggests the presence of vestibular supporting cells but not type II vestibular hair cells. It has been reported that human IHCs and OHCs express PARVALBUMIN (PV);33 however whether PARVALBUMIN is localised to vestibular hair cells is unknown. Expression of PRESTIN uniquely labels human OHC basolateral plasma membrane33. On day 90, most hESC-derived ESPIN\(^+\) hair-cell-like cells expressed PARVALBUMIN (PV)33 and some expressed PRESTIN (Fig. 5f) suggesting an OHC phenotype. Similar results were obtained using the hiPSC line (Figure S5A–F). Using 3D reconstructions, we estimated there were 46 ± 19 putative hair cells defined by MYO7A expression and putative 33 ± 18 outer hair cells defined by MYO7A and PRESTIN expression per hESC-derived otic vesicle. Similarly we estimated there were 71 ± 33 putative hair cells and 37 ± 14 outer hair cells (26 organoids, 3 experiments) per hiPSC-derived otic vesicles. This was corroborated by quantitative RT-PCR analysis showing hair cell markers (Espin, Prestin, Otopetrin 1, Parvalbumin) to be expressed at

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**Fig. 5 Immunohistological analysis of cochlear and vestibular hair cell markers at day 90.** MYO7A\(^+\)SOX2\(^-\) cells show the possibility of cochlear hair cells. a Presence of putative outer cochlear hair cells is indicated by expression of OCM\(^+\)MYO7A\(^+\) (b) while OCM\(^-\)MYO7A\(^+\) cells may be cochlear hair cells or type II vestibular hair cells. OTOP1\(^+\) cells are possible vestibular supporting cells (c) however, no cells with type II hair cell marker CB2 could be detected (d). Expression of PV cells indicates cochlear hair cells which also demonstrate ESPIN\(^+\) stereocilia (e) and PRESTIN\(^+\)MYO7A\(^+\) cells (f) are supportive of an outer hair cell phenotype. Data are representative of 16 organoids from at least three separate experiments (n = 3). Scale bars present 50 \(\mu\)m (d), 40 \(\mu\)m (e), 20 \(\mu\)m (a–c, f).
levels comparable or higher to a foetal brain sample at day 78 of differentiation (Fig. 3).

We observed cylindrical cells typical of type II vestibular or cochlear OHCs (Figure S6A and B) and cells with the more bulbous morphology of type I vestibular or cochlear IHCs (Figure S6C). More mature stereocilia-like structures were apparent (Figure S6D–F). The hair bundle length had increased from day 60 (~1–3 μm) and the longest stereocilia were 5–6 μm. At this stage, hair bundles were structurally similar to adult hair cells in vivo with cilia thickening towards the tip (Figure S6D) and arrangement of stereocilia into bundles (Figure S6D – G) some of which had the stereocilia arranged in a “stepwise” size order (Figure S6E) with rootlets extending into the cell body (Figure S6F). Moreover, cross-sections of possible stereocilia (Figure S6G) and kinocilia (Figure S6H) displayed the characteristic configuration of nine microtubule doublets surrounding two central microtubules. Thin connections were observed between some stereocilia (Figure S6I) suggesting the formation of tip links which correlates reasonably well with the earliest appearance of such structures at gestation week 14–15. Putative nerve endings beneath hair cell-like cells were observed (Figure S6) with possible nerve endings filled with numerous and heterogeneous vesicles contacting potential hair cells (Figure S6K, L). In addition to button-like nerve terminals, a possible calyx terminal (enveloping the cell in a cup-like fashion) which contacts with type I hair cells was also observed (Figure S6M).

Functional characterisation of human pluripotent stem cell-derived otic vesicles

In vivo, mature hair cells exhibit rapid permeability to the styryl pyridinium dye FM1-43FX via mechanotransduction channels located in the stereocilia. We show that staining with this dye is restricted to putative hair cells and beta-III-tubulin expressing putative sensory neurons in 3D otocyst-like structures (Figure 6a–c). Co-immunostaining with otic lineage associated antibodies confirmed FM1-43FX localisation to cells with hair cell (Figure 6b) or sensory neuron phenotype (Figure 6c). FM1-43FX does not penetrate supporting epithelial cells and we observed a corresponding absence of staining in the SOX2 expressing cells (Figure 6d). Again, these data are confirmed by parallel experiments using a hiPSC line (SB-Ad3) (Figure S7A–D).

We examined the electrophysiology of prospective neurons and hair cells by culturing dissected organoids on collagen coated coverslips (because 3D organoids are too dense to patch clamp individual hair cells) (Fig. 1b). Four days after plating, neuronal-like cells appeared at the periphery of the otic vesicles (Figure 7a) from which it was possible to obtain electrophysiological recordings. All cells examined in voltage-clamp displayed a fast-activating, rapidly inactivating inward current, presumably a Na+ current, in response to increasing membrane depolarisation. Rapid inward currents are followed by slower outward K+ currents (Figure 7b). The peak I–V curve derived from the voltage-clamp recordings is predominantly determined by the inward Na+ currents (Figure 7c), which activated positive to about -45 mV and reached a maximum size of ~1.86 nA at -18 mV. A further three cells with similar morphology were tested with a Cs+-based intracellular solution, which blocked the outward K+ currents. In these cells (Cm 8.1 ± 1.1 pF), peak inward Na+ currents averaged -1.35 ± 0.05 nA at -15.3 ± 1.8 mV (all means ± SEM). The expression profile of membrane currents in neuron-like cells closely resembled that of primary auditory neurons in the rat cochlea. Although these data were interesting, it is possible that the neuronal outgrowths do not originate from putative spiral ganglion neurons present in the otic vesicles. To circumvent this problem, we disaggregated dissected otic vesicles to single cells, then plated the cell suspension onto growth factor reduced matrigel coated coverslips. Adherent cells were identified as hair cell-like (Figure 7d) or neuronal-like after 4 days on the basis of morphology and FM1-43FX staining allowing us to record small voltage-gated K+ currents, while Na+ currents were absent. Figure 7e shows the time course and size of the currents for one cell. The I–V curve for this cell, obtained after subtraction of the leak conductance, shows the K+ currents start to activate positive to about -55 mV (Figure 7f). The outward K+ current reached a size of 0.33 nA at -4 mV for this cell, while for another similar cell the size was 0.20 nA (Figure 7f).

Discussion

We report an optimised protocol for generating putative cochlear hair cells from hESC and hiPSC. Within days 16–20, putative otic vesicles started to form from epithelium situated on the surface of the cell aggregates. Approximately 20 days later, ESPIN-positive microvilli appeared within possible pro-sensory otic vesicles progressing toMYO7A expressing hair-cell-like cells appeared and in parallel, neuronal-like cells developed alongside otocysts containing primitive hair cells by day 60. On day 90, otic organoids included PRESTIN positive potential OHCs and amphora-shaped potential vestibular type I hair cells. Differing propensities of the single hESC and hiPSC lines used in this study to generate otic vesicles containing more mature cell types only became apparent at differentiation day 90. This may reflect differences in the genetic background of the individual pluripotent stem cell lines, or an intrinsic difference between embryonic and induced pluripotent stem cells, however it is difficult to make this conclusion until the differentiation protocol is applied to broader range of pluripotent stem cell lines.
Quantitative RT-PCR analysis, immunohistochemistry, TEM, and the capability to take up FM1-43FX support the development of both vestibular and cochlear hair cell phenotypes in addition to possible innervation by putative neurons. Our electrophysiological analysis indicates some similarity to immature hair cells from embryonic mice but this does not allow us to confirm their identity as either cochlear or vestibular hair cells. That notwithstanding, the modest differences between our protocol and that of the Hashino group may be sufficient to induce the formation of hair cells with a cochlear phenotype. They key modifications employed in our protocol are the...
prior culture of hESC and hiPSC on MEFs and the concentration of 2-mercaptoethanol needed to ensure robust conversion of embryoid bodies into otic organoids. While the requirement for a precise level of 2-mercaptoethanol can be understood in terms of modulating concentrations of oxygen free radicals such that survival of some cell types may be favoured, the need for MEF co-culture is less easily rationalised. Culture in matrigel/mTeSR1 is known to cause lineage-specific priming of hESC that enhances neural differentiation at the expense of other differentiation pathways such as haematopoiesis. Exposure of mTeSR1 primed hESC to MEFs and/or MEF conditioned hESC medium reverses this preference so it is possible that a similar mechanism operates in the requirement for MEF co-culture prior to otic differentiation observed in this study. A key question arising from previous studies is the mechanism by which differentiation into vestibular hair cells becomes the “default” phenotype. Vestibular hair cells appear earlier during development so perhaps preference for vestibular differentiation reflects this timescale in vitro, but given that the 3D organoids from our study and others have been maintained for up to 150 days in culture, this seems a lesser possibility. Sonic hedgehog (Shh) is required for terminal differentiation of cochlear hair cells. This process seems to require Shh synthesis by the developing spiral ganglion neurons, but expression levels decrease from a basal to apical direction in the developing cochlea and this pattern matches that of hair cell differentiation in the cochlear duct. Thus temporal expression levels of Shh may differ in 3D organoids produced using our protocol or this may even be a consequence of the different pluripotent stem cell lines used in our study. Mechano-sensory cell specification is mediated by FGF20 signalling and this may be specific to the cochlea since formation of vestibular structures is unaffected in Fgf20 knockout mice. A role for FGF signalling in otic development is known and the ligands involved in otic placode induction have been identified as FGF3 and FGF10. In our study, organoids were exposed to FGF2 on days 5–8 to promote otic

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for the cells on MEF is knockout-Dulbecco’s modified Eagle’s medium (DMEM), 1 mM L-glutamine, 100 mM nonessential amino acids (NEAA), 20% knockout serum replacement (KOSR, Gibco; www.lifetechnologies.com), 1% penicillin–streptomycin (Gibco) and 8 ng/ml bFGF (Invitrogen; www.lifetechnologies.com). The medium was changed daily.

hESC and hiPSC on MEF were dissociated with Accutase (Gibco; www.lifetechnologies.com) for 3 min, resuspended in differentiation medium and plated 100 μl per well (9000 cells/well) on 96-well low-cell-adhesion U-bottom plates (Lipidure Coat, NOF; www.nofamerica.com). Differentiation medium was G-MEM (Life Tech; www.lifetechnologies.com) supplemented with 1.5% KOSR (Invitrogen; www.lifetechnologies.com), 0.1 mM NEAA, 1 mM sodium pyruvate, 1 mM penicillin/streptomycin and 0.1 mM 2-mercaptoethanol (Life Tech). On day 1, half of the medium in each well was exchanged for fresh differentiation medium containing Growth Factor Reduced Matrigel (2% (v/v) final concentration, BD; www.bd.com). On day 3 of the protocol, BMP4 (10 ng/ml, Gibco) and SB-431542 (1 μM, TOCRIS; www.tocris.com) were added to each well at 5× concentration in 25 μl of fresh media. On days 5, FGF2 (25 ng/ml, Gibco) and LDN-193189 (1 μM, STEMGENT; www.stemgent.com) were added to each well at 6X concentration in 25 μl of fresh media. The concentration of Y-27632 (Chemdex) was maintained at 20 μM throughout days 0–8. The concentration of Matrigel was maintained at 2% (v/v) throughout days 1–8. On day 8 of differentiation, organoids were transferred to 6 or 12 well plates (Lipidure Coat, NOF) in N2 medium containing 1% (v/v) Matrigel. N2 medium contained Advanced DMEM/F12 (Gibco), 1X N2 Supplement (Life Tech), 50 μg/ml Normocin (Invitrogen) and 1 mM GlutaMAX. After 48 h the medium was changed completely with new N2 medium. Beginning on day 10, half of the medium was changed every other day during long-term floating culture for up to 90 days.

Quantitative RT-PCR (qRT-PCR)

RNA was isolated using the ReliaPrep® RNA Cell Miniprep System (Promega; https://www.promega.co.uk). Single-stranded complementary DNA was synthesised from 1 μg of the RNA samples using the GoScript Reverse Transcription System (Promega). The qRT-PCR was performed with the GoTaq qPCR Master Mix (Promega) in a QuantStudio™ 7 Flex real-time PCR system (Life Technologies). The reaction parameters were as follows: 95°C for 2 min to denature the cDNA and primers, 50 cycles of 95°C for 15 s followed by annealing/extend 60°C for 60 s. A comparative Ct method was used to calculate the levels of relative expression, whereby the Ct was normalised to the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This calculation
gave the ΔCt value, which was then normalised to a reference sample (i.e. a positive control), giving the ΔΔCt. The fold change was calculated using the following formula: $2^{-\Delta\Delta C_t}$. Foetal brain (BioChain; https://www.biochain.com/) was used for positive control.

Primers used: ECAD forward: AGCGGTGTGTA CTGTGAAGG, reverse: CTCTCTCCGCTCT TCCT; PAX2 forward: GAGCGGATTCTCCCGGCAAC, reverse: GTCAGAGGGGACGATGTG; ATOH1 forward: CCTCCAGCAAACAGGTGAAT, reverse: TT GTTGAAGCAGGGATAACAT; MYO7A forward: GA GTCAAGGCTTCTCAGCTT, reverse: GTGACCAG GGCCCAATCTC, ESPIN forward: CAGAGTGCA GACAAAGACAA, reverse: GCAGCGTAGTGGATAG

**Immunohistochemistry**

Organoids were fixed with 4% paraformaldehyde. The fixed specimens were cryopreserved with a treatment of 30% sucrose and then embedded in tissue freezing medium OCT. Frozen tissue blocks were sectioned into 12 μm cryosections. For immunostaining, a 1% bovine serum albumin and 0.3% Triton X-100 solution was used for cryosections. For immunostaining, a 1% bovine serum albumin and 0.3% Triton X-100 solution was used for cryosections. The following antibodies were used: anti-SOX9 (rabbit, Millipore, 1:200, Polyclonal), anti-SOX2 (mouse, R&D Systems, 1:62.5, Clone 245610), anti-PAX2 (Rabbit, Thermo Fisher, 1:200, Polyclonal), anti-ECADHERIN (mouse, BD, 1:200, Clone 67A4), anti-ESPIN (rabbit, Novus Biologicals, 1:200, Polyclonal), anti-FACTIN (mouse, Abcam, 1:400, Clone 4E3.4d), anti-SYNAPTOPHYSIN (mouse, SIGMA, 1:200, Clone SVP-38), anti-ATOH1 (rabbit, Abcam, 1:200, Polyclonal), anti-GLUTAMINE SYNTHETASE (mouse, Millipore, 1:200, Clone GS-6), anti-TUBB3 (mouse, Covance, 1:500, Clone TUJ1), anti-MYO7A (rabbit, Abcam, 1:500, Polyclonal), anti-MYO7A (mouse, Santa Cruz, 1:100, Clone C-5), anti-NEUROFILAMENT (mouse, Invitrogen, 1:20, Clone RMdo-20), anti-CB2 (rabbit, Cayman Chemical, 1:50, Polyclonal), anti-OCM (rabbit, Novus Biologicals, 1:100, Polyclonal), anti-OTOP1 (rabbit, Novus Biologicals, 1:66.7, Polyclonal), anti-PRESTIN (rabbit, Thermo Fisher, 1:200, Polyclonal), anti-PARVALBUMIN (mouse, Thermo Fisher, 1:2000, Clone PARV-19). All antibodies were validated using embryonic tissues representative of the otic vesicle and foetal brain. Primary antibodies were omitted in control immunohistochemistry staining.

**FM1-43FX labelling**

The presence of functional mechanosensitive channels was confirmed using a FM1-43FX dye uptake assay. Organoids were incubated in Advanced DMEM/F12 containing FM1-43FX (5 Mm final concentration; Invitrogen) for 1 min and then washed three times in Advanced DMEM/F12. Organoids were dissociated to single cells by 40 min incubation in Accutase then added Advanced DMEM/F12 to inactivate Accutase. The cells were then plated onto slides using the Cytospin method. For nuclear staining, Hoechst dye was used. The cells were imaged to confirm dye uptake and immediately fixed with 4% paraformaldehyde. For some experiments, cells were fixed and stained with otic lineage associated antibodies to confirm the identity of hair cells. Cells were examined using Nikon Ti Eclipse confocal microscope. Fields were chosen at random.

**Electrophysiological recordings**

Organoids were transferred to the microscope chamber and immobilised using a nylon mesh fixed to a stainless steel ring. The chamber was continuously perfused with an extracellular solution containing (in mM): 135 NaCl, 5.8 KCl, 1.3 CaCl2, 0.9 MgCl2, 0.7 NaH2PO4, 5.6 D-glucose, 10 HEPES–NaOH, 2 Sodium pyruvate. MEM amino acids solution (from 50X concentrate), and MEM vitamins solution (from 100X concentrate) were added (Fisher Scientific). The pH was adjusted to 7.48 (osmolality ~308 mmol/kg). The organoids were observed using an upright microscope (Leica DM LFSA) with Nomarski differential interference (DIC) contrast optics, using a ×63 water-immersion objective. Whole-cell patch-clamp recordings were performed at room temperature using an Otopatch (Cairn Research) patch-clamp amplifier. Patch pipettes contained the following (in mM): 131 KCl, 3 MgCl2, 5 Na2ATP, 1 EGTA-KOH, 5 HEPES–KOH, 10 sodium phosphocreatine, pH 7.28 (osmolality ~295 mOsmol/kg). For recording sodium currents in isolation, patch pipettes contained (in mM): 137 CsCl, 2.5 MgCl2, 1 EGTA-CsOH, 2.5 Na2ATP, 10 sodium phosphocreatine, 5 HEPES-CsOH, pH 7.3 (osmolality ~292 mOsmol/kg). Patch pipettes were pulled to give a resistance in the range of 2–3 MΩ, and their tips were coated with surf wax (Mr Zogs SexWax) to minimise the fast capacitive transient.
across the wall of the patch pipette. Immediately before recording, the series resistance ($R_s$) and the membrane capacitance ($C_m$) of the cell were noted.

Data were acquired through a CED Power 1401 data acquisition interface using Signal software (Cambridge Electronic Design) and stored on a computer for off-line analysis using Origin (Origin Lab) data analysis software. For potassium current analysis, current amplitudes were measured from the steady-state current towards the end of the voltage step, before the tail currents emerged. For sodium current analysis, current amplitudes were measured at the peak of the sodium current.

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Conflict of interest
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