# Three-dimensional mouse embryonic stem cell culture for generating inner ear organoids

Karl R. Koehler[1,2,3] and Eri Hashino[1,2,3]

[1]*Medical Neuroscience Graduate Program,* [2]*Stark Neurosciences Research Institute, and*

[3]*Departments of Otolaryngology-Head and Neck Surgery, Indiana University School of*

*Medicine, Indianapolis, IN 46202.*

## ABSTRACT

This protocol describes a culture system in which inner ear sensory tissue is produced from mouse embryonic stem cells under chemically defined conditions. This model is amenable to basic and translational investigations into inner ear biology and regeneration. In this protocol, mouse embryonic stem cells are aggregated in 96-well plates in medium containing extracellular matrix proteins to promote epithelialization. During the first 14 days a series of precisely timed protein and small molecule treatments sequentially induce epithelia that represent the mouse embryonic nonneural ectoderm, preplacodal ectoderm and otic vesicle epithelia. Ultimately, these tissues develop into cysts with a pseudostratified epithelium containing inner ear hair cells and supporting cells after 16-20 days. Concurrently, sensory-like neurons generate synapse-like structures with the derived hair cells. We have designated the stem cell-derived epithelia harboring hair cells, supporting cells and sensory-like neurons inner ear organoids. This method provides a reproducible and scalable means to generate inner ear sensory tissue *in vitro.*

**INTRODUCTION**

The proper development of organs requires the synchronized organization and coordinated differentiation of large cell populations. To date, a full appreciation of the molecular and cellular signaling mechanisms that govern cellular behaviors and cell fate decisions remains elusive. Culturing embryonic stem (ES) cells as a floating cell aggregate in three-dimensional (3D) culture has emerged as an effective method to generate complex organs *in vitro*; a controllable and readily accessible environment to investigate the mechanisms underlying organogenesis[1,2]. This approach has been used to generate mouse and human cortical tissue, neural retina, liver buds, spermatozoa, intestinal epithelium, anterior pituitary gland tissue, and inner ear sensory epithelium[3-13]. Moreover, in a 3D culture variant using tissue specific stem cells, the villi of the small intestine and liver tissue can be reconstituted from a single adult stem cell[14,15]. The key advantage of 3D culture over a monolayer (2D) culture is that the differentiating cells are free to self-organize into epithelia. In a 2D culture, by contrast, the cells adhere to a culture plate; thus, they are restricted to growing on a flat shape. The 3D environment allows cells to develop more naturally, as they would in the embryo.

In this protocol we describe a 3D culture method for the generation of inner ear sensory epithelia containing hair cells, which display some functional properties of native vestibular hair cells and are innervated by sensory-like neurons (**Figure 1**). To aid the reader with performing the method, we place special emphasis on the proper timing of treatments and the various morphological transformations that take place throughout the differentiation. Additionally, we provide instructions on how to prepare the derived tissue for immunohistochemical and electrophysiological analyses.

**Induction of the cranial placodes and inner ear during embryogenesis**

The conditions described here for inner ear induction were selected on the basis of information gained from studies of mouse, chick, zebrafish, and *Xenopus* ear development. In general, vertebrate inner ear induction begins with the bifurcation of the definitive ectoderm into the nonneural and neural ectoderm. This occurs at approximately embryonic day (E) 6.5-7.5 in mice[16-19]. Studies in *Xenopus,* zebrafish and mouse ES cells have shown that bone morphogenetic protein (BMP) signaling is critical for ectodermal specification; high BMP signaling induces nonneural ectoderm in the lateral ectoderm, while low BMP signaling promotes neural plate formation at the midline[20-25]. At around E7.5-8, the preplacodal ectoderm develops in the head region of the embryo at the border between the nonneural and neural ectoderm, referred to as the preplacodal region (PPR). In vertebrates, attenuation of BMP signaling and concurrent fibroblast growth factor (FGF) signaling is necessary for PPR formation[22,26-31]. From approximately E8-9, varying inductive cues along the anterior-posterior axis act on the PPR to generate cranial placodes[28,32,33]. A defining characteristic of each placode—the adenohypophyseal (anterior pituitary gland), olfactory, lens, trigeminal, epibranchial and otic placodes—is a thicker pseudostratified morphology relative to the surrounding nonneural ectoderm[28,32]. Pax proteins can be used to distinguish placodes along the anterior-posterior axis: Pax6 is expressed in anterior placodes (adenohypophyseal, olfactory, and lens), Pax3 is expressed in the trigeminal placode, and Pax8 is expressed in posterior placodes (otic and epibranchial)[34-36]. The otic and epibranchial placodes arise from a broad Pax8$^+$ region known as the otic-epibranchial placode domain (OEPD)[36]. In mice, the OEPD is specified by FGF signaling, attributable to Fgf3, Fgf8, and Fgf10 proteins emanating from the underlying mesenchyme and hindbrain region of the neural tube[37,38]. Further definition of the OEPD entails

Wnt activity to specify the otic placode, whereas prolonged FGF signaling and additional BMP signaling specifies the epibranchial placodes[39-41]. Otic specification is denoted by an increase in expression of Pax2 protein[39,42]. From E8-8.5, the otic placode invaginates into the periotic mesenchyme to form the otic pit. The otic vesicle (also known as the otocyst) is formed when the otic pit pinches off from the surface ectoderm. The nonneural ectoderm surrounding the invaginating otic placode gives rise to epidermis on the surface of the embryo overlaying the development inner ear[39].

The nascent otic vesicle is patterned into a nonsensory and prosensory region by various inductive cues from the surrounding mesenchyme and neural tube[43]. The prosensory region can be characterized by expression of Pax2, Pax8, Sox2, and Jag1[43,44]. From E9-10, epithelial cells in the prosensory region either delaminate to become inner ear sensory neurons or remain in the epithelium to contribute to the sensory epithelia of the vestibular or cochlear organs[45]. During sensory epithelium development, Pax2 and Pax8 are downregulated, but Sox2 and Jag1 expression are maintained[43]. By E12.5 in the vestibule, the sensory epithelia contain supporting cells (CyclinD1$^+$ Sox2$^+$) and hair cells (Sox2$^+$ Myo7a$^+$ Brn3c$^+$ Calretinin$^+$)[46-48].

**Development of the protocol**

Previous studies have shown that mouse pluripotent stem (PS) cells can be dissociated and aggregated into uniformly shaped spheroids in U-bottomed 96-well plates[5-7,49]. Culturing aggregates in a serum-free medium containing knockout serum replacement (KSR) promotes development of a neural epithelium. KSR is a defined medium-supplement optimized to grow pluripotent stem cells (Patent no. WO 98/30679). The efficient differentiation of mouse ES cells into neural ectoderm in medium containing KSR is thought to be due to the default pathway of neural ectodermal development[49,50]. KSR does not contain any factors important for

mesendoderm (Activin/Nodal/BMP proteins) or nonneural (BMP proteins) induction. Modifying

the signaling factors presented to the neural epithelium can induce specific regions of the

forebrain, midbrain and hindbrain. Until recently, developing neuroepithelia within these

cultures took the form of dispersed epithelial vesicles embedded in unorganized tissue. In a

critical evolution of the method, Eiraku and colleagues demonstrated that extracellular matrix

proteins (i.e. in the form of Matrigel) could be added to the culture medium to promote the

development of a basement membrane on the surface of the aggregate[5]. With the structural

support, the entire surface layer of cells self-organizes into a neural epithelium after 5 days in

culture. These aggregates spontaneously developed into optic cups, which gave rise to complete

neural retinas in about 18-20 days. This work provided proof-of-principle that organogenesis of

complex sensory organs could occur *in vitro*.

       The aforementioned studies were successful at deriving mature neural ectoderm tissues;

however, the cranial placodes—including the otic placode—are derived from the nonneural

ectoderm. Suga and coworkers recently demonstrated that by increasing the size of the

aggregate, a nonneural ectoderm layer spontaneously arises on the surface of the cell aggregates

leaving a neural layer underneath[9]. Mimicking *in vivo* nonneural induction, their data suggest

that endogenous BMP signaling is likely mediating this process in 3D culture; however, the

precise signaling mechanisms are unclear. Using a sonic hedgehog agonist they demonstrated

sequential induction of adenohypophyseal placodes, vesicles, and functional anterior pituitary

tissue from the nonneural epithelium. This result revealed that placode morphogenesis could be

recapitulated in 3D culture. Specifically, placodal invagination and vesicle formation, two

critical morphological steps in both anterior pituitary and inner ear development, were well

preserved in the culture system.

In our recent study[3], we garnered greater control over the nonneural induction process by treating smaller, Matrigel-conditioned, aggregates with BMP4. The timing of BMP4 treatment (day 3) was critical; the surface layer of cells needed time to organize into an epithelium and commit to an ectodermal fate. If we waited until day 5, cells on the surface layer became committed to a neural epithelium fate that could not be reversed by BMP4 treatment. We also demonstrated that TGFß signaling must be inhibited for proper nonneural induction in 3D culture. We treated aggregates with a combination of BMP4 and a TGFß inhibitor, SB-431542 (SB). TGFß inhibition led to a reduction in the number of Brachyury$^+$ mesendoderm-like cells following BMP4 treatment. This effect is consistent with the well-known role of TGFß signaling in mesoderm induction, as well as previous demonstrations that TGFß inhibition promotes ectoderm differentiation from ES cells[51-53]. From embryo studies, we knew that BMP signaling—although necessary for nonneural induction—needed to be attenuated for preplacodal induction[22,28,32]. By our estimation, 24-48 hours elapses between nonneural and preplacodal induction in the mouse embryo; thus, we added a BMP inhibitor, LDN-193189 (LDN), to the medium between days 4 and 5 after BMP/SB treatment. FGF signaling is also important for preplacodal induction. When we performed a combined treatment of FGF2 and LDN (FGF/LDN), the outer-epithelium developed a placode-like morphology and expressed several marker genes of the preplacodal ectoderm, including Six1, AP2, and Gata3[3].

At day 6 of differentiation, patches of cells in the outer epithelium began to express Pax8, a sign of OEPD development. This level of specificity was unexpected because BMP inhibition and FGF signaling are necessary for development of the entire PPR not just the OEPD[28]. One explanation may be that FGF2 and insulin found in the knockout serum replacement have a synergistic caudalizing effect on the tissue; this phenomenon was previously observed while

deriving caudal portions of the brain in 3D culture[54]. We placed aggregates with Pax8-positive

cells in a minimal medium known to support self-organizing tissues, to test if OEPD epithelium

developed into otic vesicles. Under these conditions, the OEPD epithelium gave rise to vesicles

containing Pax2$^+$ Pax8$^+$ Sox2$^+$ and Jag1$^+$ cells, reminiscent of prosensory cells in the otic

vesicle[44,55,56]. By treating aggregates with a potent Wnt inhibitor we discovered that endogenous

Wnt activity was involved in the process of vesicle production; Wnt inhibitor-treated aggregates

produced few otic vesicles. With no additional treatments, aside from medium changes, cells in

the epithelia of otic vesicles developed into hair cells and supporting cells. The derived hair cells

expressed Myo7a, Brn3c, Calretinin, Sox2, and Pax2, denoting a vestibular hair cell

identity[47,48,57,58]. This conclusion was further reinforced by the presence of pointed stereocilia

bundles and elongated kinocilia with the length of greater than 10µm[3,59]. Moreover, the hair cells

were spatially organized into clusters seen in the vestibular end organs, rather than in rows of

hair cells in the organ of Corti of the cochlea. Quantitative analysis revealed that ~1500 hair cells

developed in each cell aggregate by day 20 of differentiation.

We also consistently observed the development of sensory neuron-like cells that arose in

discrete groups alongside the vestibular sensory epithelia. We observed ßIII-tubulin (TUJ1) and

neurofilament-positive cells extending their processes toward hair cells and analysis of synaptic

proteins confirmed the presence of synapse-associated proteins on the basal end of hair cells.

CtBP2-positive puncta, whose number increased over time in culture, suggested the development

of ribbon synapses in stem cell-derived hair cells. More extensive analysis, such as transmission

electron microscopy and electrophysiological recording from button afferents, will be needed to

confirm whether the synapse-like structures truly represent functional ribbon synapses.

**Comparison with other methods**

Several methods for generating inner ear cells from PS cells have been developed in recent years[60-63]. Of note, Oshima and colleagues provided the first demonstration that functional inner ear hair cells could be derived from ES and iPS cells[61]. The hair cells they derived had stereocilia and a kinocilium with similar architecture to native vestibular hair cells, expressed many hair cell-associated markers, and were shown to be mechanosensitive. They used FBS, insulin-like growth factor-1, Dkk1 (a Wnt signaling inhibitor) and SIS3 (a Smad3 inhibitor) followed by FGF2 treatment to stimulate otic progenitor development in stem cell aggregates. In order for these otic progenitors to develop into hair cell-like cells, however, they had to be plated on a mitotically inactivated layer of chicken utricle cells[61]. The need for exogenous tissue may limit the routine use of this method. Moreover, the efficiency of hair cell induction was low, accounting for only ~1-2% of the total population of cells. Additional methods utilized a monolayer culture to derive hair cells; thus, limiting the ability to compare the morphology of stem cell-derived and *in vivo* tissues. Moreover, stereocilia bundles with a kinocilium have not been observed in a completely monolayer format (i.e. without exogenous supporting/stromal cells). Notably, Ouji et al. recently reported two methods to produce Myo7a-positive cells from stem cells in a monolayer format[62,64]. They observed F-actin-positive protrusions from the cells reminiscent of hair bundles; however, individual stereocilia were not visible and they did not report whether a kinocilium was present. Consequently, it is unclear if the hair cell-like cells can attain the proper morphology under the conditions described by Ouji and collegues[62,64]. The method described here is advantageous because it does not require exogenous tissue, undefined media components, and generates inner ear sensory epithelia that appear to be structurally and physiologically comparable to native vestibular sensory epithelium. The average number of

~1,500 hair cells produced per aggregate—based on analysis of four separate experiments—is significant considering that each adult mouse inner ear contains ~30,000 hair cells (i.e. in both the vestibule and cochlea combined)[65]. Thus, ~20 aggregates provide a comparable number of hair cells to the entire inner ear.

**Future applications**

Investigating the signaling mechanisms and gene regulatory networks underlying nonneural, preplacodal and inner ear induction in mouse embryos is challenging; mouse embryos are inaccessible *in vivo* and cannot be maintained *ex vivo* for more than 2 days. Moreover, it is difficult to collect the large amount of embryonic tissue needed to perform biochemical analyses such as chromatin immunoprecipitation. The described 3D culture system addresses these issues because it is both accessible and the derived tissues are highly representative of their embryonic counterparts. We routinely perform experiments involving 200-400 aggregates, yet the method can be easily scaled to produce more aggregates. For larger scale experiments, a researcher should be able to simultaneously maintain 20 or more 96-wells plates totaling ~2000 aggregates. Further increases in scale may be attainable with automation. Besides studying early otic placode induction, this culture system can be used to evaluate hair cell and inner ear sensory neuron development, as well as hair cell-sensory neuron synaptogenesis. Additionally, recent advances in gene targeting technology may also be applied to this culture model to knockout and knock-in genes to study inner ear phenotypes[66,67]. The 20-30 day, all *in vitro,* format is a quick and cost effective alternative to generating an entire mouse line if inner ear analysis is all that is desired. The 3D culture system may also be suitable for mid- to high-throughput drug screening to uncover novel compounds with regenerative or toxic effects on inner ear sensory epithelia; however, several of the limitations discussed in the next section will need to be addressed to

make the system more amenable to high-throughput applications. In addition, translation of our findings to human ES cells or iPS cells will be critical for potential applications in cellular therapies for vestibular disorders or hearing loss.

**Limitations**

Heterogeneity is both an advantage and a limitation of this 3D culture system. Unlike a monolayer format, cells in an aggregate receive varying concentrations of signaling cues depending on their spatial location within the aggregate. This often leads to unsynchronized differentiation of cells located at different depths of the aggregate; cells in the surface layer differentiate faster than those in the core. In our system, BMP4 signaling induces a nonneural ectoderm surface layer as well as Brachyury$^+$ mesendodermal cells in the central core of the aggregate. BMP4 is known to induce mesendodermal cells from PS cells. Cells in the central core express the pluripotency gene Nanog through day 3 of differentiation; thus, BMP4 likely diffuses through the surface layer of the aggregates to act on these pluripotent cells. This cell layer gives rise to mesenchyme-like cells that eventually cover the surface of the aggregates and give rise to muscle, cartilage and adipose[3]. The presence of these cell types does not appear to encumber, and may play an essential role in, otic induction. Indeed, the periotic mesenchyme *in vivo* is an integral component of inner ear morphogenesis[68]. Due to this heterogeneity, however, the method would benefit from the use of florescent reporter PS cell lines to help identify key phenotypic changes. Specifically, PS cells containing fluorescently tagged marker genes for nonneural, preplacodal, otic prosensory cells, hair cells, supporting cells and inner ear neurons would be highly amenable to this method. Most notably, incorporation of reporter cell lines would facilitate florescence-activated cell sorting (FACS) analysis of key inner ear cell types. Another limitation is that the method only produces vestibular sensory organ-like structures.

Further modifications to the protocol will be needed to investigate induction and differentiation of cochlear cell types.

**Experimental design**

**ES cell culture in LIF-2i medium (prior to start of procedure).** We maintain our ES cells in Leukemia inhibitory factor (LIF)-2i medium because we have observed considerably less spontaneous differentiation under these conditions compared to fetal bovine serum or fetal calf serum (FBS/FCS)-based culture media. LIF-2i medium has been shown to restrict ES cells to a naïve state of pluripotency; therefore, it ensures a homogenous population of undifferentiated cells[69]. If acclimating ES cells grown under different conditions (e.g. in media containing FBS/FCS) to LIF-2i medium is required, we recommend passaging the cells 5-10 times prior to beginning a differentiation experiment to ensure that the cell population is homogeneous. We typically passage the cells 2-3 times after thawing before beginning a differentiation experiment. ES cells should be ~80% confluent before passaging or starting a differentiation experiment.

**Nonneural and preplacedal induction (steps 1-31).** To start ectodermal differentiation, ES cells are dissociated and distributed, 3,000 cells per well, onto 96-well plates. The medium contains 1.5% knockout serum replacement which has been previously shown to be optimal for generating neural retinas[5]. This concentration also appears to be optimal for preplacodal induction, as higher concentrations seem to prohibit proper epithelium development. Matrigel, which contains extracellular matrix proteins such as Laminin and Entactin, should be added on day 1 at a final concentration of 2% (vol/vol; **Fig. 2**). We recommend using the growth factor-reduced Matrigel. If a more defined medium composition is desired, a recent report suggests that a purified Laminin/Entactin complex can be used to replace Matrigel[10]. Likewise, we have used

Geltrex (Gibco; cat. no. A1413202) with comparable results to Matrigel. With an optimal

composition of extracellular matrix proteins, an epithelium should appear by day 3 (**Fig. 2c**), at

which point BMP4 and the TGFß inhibitor SB-431542 (SB) are added to the culture medium.

When attempting the protocol for the first time, we recommend performing several

control treatments. At the least, set aside 8 aggregates (i.e. one vertical column) for each of these

treatments: untreated control, BMP, SB, BMP/SB, BMP/SB-FGF, BMP/SB-LDN, and BMP/SB-

FGF/LDN. We suspect that the optimal timing of treatments may vary slightly between ES cell

lines. In our experience, the timing of FGF/LDN treatment is critical and should be varied

between days 4 and 5 to optimize the culture. In our previous publication[3], we used a powder

form of LDN diluted in DMSO at a working concentration of 100 nM LDN; however, lot-to-lot

variability was a recurrent issue. We now recommend using a pre-diluted version of LDN at a

higher working concentration of 1 µM. The outer-epithelium of BMP/SB-FGF/LDN treated

aggregates should thicken and express Pax8 by days 6-8 (**Fig. 3 and 4**).

**Otic prosensory vesicle formation and hair cell induction (steps 32-29).** We describe two

formats for long-term culture: 96- and 24-well formats. Although more labor-intensive, the 96-

well format may be advantageous for analyses requiring the tracking of individual aggregates for

the duration of the culture. To initiate self-organized development of otic vesicles, day 8

aggregates are transferred into a minimal medium containing DMEM/F12 and N2 supplement.

The inner core of mesodermal and neural cells should migrate out to cover the surface of the

aggregate (**Fig. 5**). Pax2^{+} Pax8^{+} vesicles should evaginate from the OEPD epithelium into this

layer of cells between days 8-12 (**Fig. 6a, b, d**). By days 12-14, otic vesicles should express the

prosensory markers Sox2 and Jag1 (**Fig. 6c**). Vesicles should also express Myo7a in a diffuse

pattern similar to the developing otic vesicle *in vivo*[3].

Hair cells, denoted by an elongated morphology and Myo7a, Brn3c, Sox2, Pax2, and Calretinin expression, should arise within the otic vesicle epithelia between days 14-16 (**Fig. 6e-h**). The luminal surface of the sensory epithelia should have strong F-actin bands indicative of cell-cell tight junctions (**Fig. 6f**). Supporting cell nuclei are positioned basal to the hair cell nuclei in the epithelium and can be identified by expression of Sox2 and CyclinD1 (**Fig. 6g, k, l**)[3].

**Inner ear organoid formation (step 40).** Between days 12-20, some vesicles become visible along the outer edge of the aggregates. We recommend monitoring these vesicles daily to see that they grow in size over time. By day 20-24, ~10-20% of aggregates should have produced vesicles that are 200-1500 µm in diameter, protrude from the aggregate surface, and contain sensory regions with numerous hair cells and nonsensory regions without hair cells (hereafter these structures will be referred to as inner ear organoids; **Fig. 6i-l and 7**). Protruding organoid formation appears to be highly sensitive to culture technique, proper manipulation and careful preparation of media throughout the protocol. Moreover, organoids are observed only on the outer surface of aggregates, suggesting that proper orientation of an otic vesicle is a necessary prerequisite to later organoid development. For instance, in the 24-well culture format, we have never seen organoid development at the interface of two conjoined aggregates; thus, we only culture 1-3 aggregates per well to maximize the surface area available for organoids to emerge. We recommend using protruding organoid development as a benchmark for proper execution of the protocol and health of the culture. We typically culture aggregates for no more than 30 days, however, longer culture is feasible. For long-term culture (>30 days), we recommend changing half of the medium daily and limiting the amount of time the plates remain outside of the incubator to ensure optimal health of the tissue.

**Endpoint analysis (step 41).** We describe how to fix aggregates for immunohistochemical analysis (options A and B) and how to prepare samples for electrophysiological recordings (option C). We describe an approach for preparing aggregates for immunostaining in a cryosection (option A) and wholemount format (option B) that is suitable for fixing samples at any time point during the procedure. Due to their small size, it is important to carefully handle aggregates harvested between days 1-8 throughout the fixation and embedding process; aggregates are easily lost during this process. Imaging the aggregates in a wholemount format can be challenging due to the need to rotate the aggregate to reveal structures of interest. We have developed a custom imaging chamber system that is effective at preparing aggregates for imaging (**Supplementary Fig. 1**). Additionally, we describe an adapted version of a protocol used to isolate and prepare rodent utricles for electrophysiology experiments (option C). This technique can be used to isolate, dissect and position organoids for single-cell electrophysiological analyses (**Fig. 7**). For this method there is an optional dispase treatment that helps to break up the mesenchymal cells in the aggregate and greatly shortens the time it takes to liberate organoids from the aggregate. Dispase may disrupt certain structural components of the hair cell stereocilia; thus, depending on the endpoint analysis, it may be necessary to omit dispase treatment. Note that labeling the hair cells with a styryl dye such as FM1-43 or using a cell line with a hair cell-specific fluorescent reporter could improve the success of this dissection. Following dissection, we have cultured isolated epithelium for up to 5-6 hours while performing electrophysiology. If long-term isolated culture of epithelia is desired, based on previous studies of utricle explants, it is likely that dissected epithelia could be maintained for 10 or more days under the right culture conditions[70].

**MATERIALS**

**REAGENTS**

- Mouse ES cells acclimated to growth in LIF-2i medium and at 80% confluency. We recommend the use of any well-established mouse pluripotent stem cell line with this protocol. We primarily use a feeder-free ES cell line derived from R1 mice; however, this protocol has been tested with a mouse iPS cell line and several transgenic ES cell lines with comparable results.

- GMEM (Gibco, cat. no. 11710-035)

- Advanced DMEM/F12 (Gibco, cat. no. 12491-015)

- Sodium Pyruvate, 100 mM (Stemcell Technologies, cat. no. 07000)

- Non Essential Amino Acids, 10 mM (Stemcell Technologies, cat. no. 07600)

- Penicillin-Streptomycin (Stemcell Technologies, cat. no. 07500)

- Knockout Serum Replacement (KSR; Gibco, cat. no. 10828-010) **CRITICAL** KSR is light sensitive. Store in the dark at -20ºC per the manufactures recommendation.

- 2-Mercaptoethanol (Gibco, cat. no. 21985-023)

- Normocin (Invivogen, cat. no. ant-nr-1)

- N2 Supplement (Gibco, cat. no. 17502-048)

- GlutaMax (Gibco, cat. no. 35050-079)

- Matrigel: We use the growth factor-reduced and LDEV-free Matrigel from BD Biosciences (cat. no. 354230). Each lot of Matrigel has a different total protein concentration, so we advise contacting BD Biosciences to order specific lots or lots with similar protein concentrations. We have used concentrations in the range of 8.7-9.2 mg/mL successfully with this protocol.

- Recombinant BMP4 (Stemgent, cat. no. 03-0007)

- FGF-2 (Peprotech, cat. no. 100-18B)

- SB-431542 in solution (Stemgent, cat. no. 04-0010-05)

- LDN-193189 in solution (Stemgent, cat. no. 04-0074-02) **CRITICAL** LDN-193189 is light
  sensitive. Store in the dark at -20ºC per the manufactures recommendation.

- Neurobasal Medium (Gibco, cat. no. 21103-049)

- B27 Supplement minus Vitamin A (Gibco, cat. no. 12587-010)

- PD-0325901 in solution (Stemgent, cat. no. 04-0006-02)

- CHIR99021 in solution (Stemgent, cat. no. 04-0004-02)

- 0.1% Gelatin (Stemcell Technologies, cat. no. 07903)

- 0.25% Trypsin-EDTA (Gibco, cat. no. 25200-072)

- Phosphate Buffered Saline (PBS; Gibco, cat. no. 10010-023)

- ESGRO® Leukemia Inhibitory Factor (LIF; Millipore, cat. no. ESG1106)

- Dimethyl Sulfoxide (DMSO; Sigma, cat. no. D8418)

- Paraformaldehyde (PFA, Electron Microscopy Sciences, cat. no. 15710)

- MDX4-4210 platinum silicone elastomer A & B kit or equivalent silicone elastomer (Dow
  Corning)

- Vacuum grease (Fisher Scientific, cat. no. S41718)

- Normal goat serum (Vector Laboratories, cat. no. S-1000)

- Urea (Sigma, cat. no. U5378)

- Glycerol (Sigma, cat. no. G5516)

- Triton X-100 (Sigma, cat. no. T8787)

- Antibodies required for desired characterization (**Table 1**)

**EQUIPMENT**

- Cell culture dish (60 mm; BD Falcon, cat. no. 353002)

- Bacterial dish (100 mm; Fisher Scientific, cat. no. 0875712)

- U-bottom 96-well plate (Lipidure-coat; Gel Company, cat. no. LCU96)

- 24-well plate (Lipidure-coat; Gel Company, cat. no. LCMD24)

- Safe-Lock centrifuge tubes (1.5 and 2 mL; Eppendorf, cat. no. 022363204, 022363352)

- Transfer pipets (Fisherbrand, cat. no. 13-711-7M)

- Polystyrene round bottom centrifuge test tubes with cell strainer tops (5 mL; BD Falcon, cat. no. 352235)

- Reagent Reservoirs (VistaLab, cat. no. 21-381-27F)

- Tissue-Tek Cryomolds (Electron Microscopy Sciences, cat. no. 62534-10)

- Olympus FV1000 multiphoton microscope or equivalent inverted confocal microscope equipped with a long-working distance objective (20-25X)

- Silicone isolators with adhesive (20 mm diameter x 2.0 mm deep or equivalent; Electron Microscopy Sciences, cat. no. 70336-44)

- Cover glasses (24 x 50-1 and 25 x 25 or equivalent; Fisher Scientific, cat. no. 12-544-14 and 12-548-C)

- 15 mm diameter round cover glasses (thickness no. 0; Assistent, cat. no. 01105509)

- Dumont #5 forceps (Electron Microscopy Sciences, cat. no. 72701-1)

- Tungsten needles (FST, cat. no. 10130-05, 10130-10)

- Minutien Pins (FST, cat. no. 26001-10)

- Biosafety cabinet

- $CO_2$ incubator

- Hotplate

- Water bath

- Desiccator

- Cryostat

**REAGENT SETUP**

**Human recombinant BMP4 stock solution (100 ng/µL)** In the biosafety cabinet, add 100 µL of sterile 4 mM HCl to 10 µg of BMP4; vortex the solution and spin down in a tabletop centrifuge. Store BMP4 solution in 5 µL aliquots at -20ºC for 6 months or at -80ºC for 1 year.

**Human recombinant FGF-2 stock solution (200 ng/µL)** In the biosafety cabinet, add 250 µL of sterile PBS or 5mM Tris (pH 7.6) to 50 µg of FGF-2; vortex the solution and spin down in a tabletop centrifuge. Store FGF-2 solution in 6 µL aliquots at -20ºC for 6 months or at -80ºC for 1 year.

**LIF-2i ES cell maintenance medium** To prepare 100 mL basal LIF-2i medium, combine 48.75 mL DMEM/F12, 48.75 mL Neurobasal medium, 1 mL B27 supplement, 500 µL N2 supplement, and 1 mL GlutaMax in a sterile 250 mL bottle. This medium can be used for 3-4 weeks. Prepare complete LIF-2i medium immediately before use by adding 100 µL Penicillin-Streptomycin, 10 µL LIF, 1 µL PD-0325901, and 3 µL CHIR99021 to 10 mL of the basal LIF-2i medium. Complete LIF-2i medium can be used for up to 1 week at 4ºC. **CRITICAL STEP** It is important to use B27 that does not contain Vitamin A for ES cell culture. Vitamin A can be converted into retinoic acid in culture and cause spontaneous neural induction. Serial passaging can eliminate any neuronal cells that may arise while adapting ES cells to LIF-2i medium.

**Ectodermal differentiation medium** To prepare 100 mL ectodermal differentiation medium, combine 95.5 mL GMEM, 1.5 mL KSR, 1 mL sodium pyruvate, 1 mL non-essential amino

acids, 1 mL Penicillin-Streptomycin, and 180 µL 2-Mercaptoethanol in a sterile 250 mL bottle.

Complete ectodermal differentiation medium should be used for the duration of one experiment

or 1 week at 4ºC. Adjust the volume of medium made up according to the number of 96-well

plates being seeded with ES cells. We typically make 40-50 mL for two 96-well plates and use

the same medium for the Matrigel, BMP/SB and FGF/LDN treatments.

**Maturation medium** To prepare 100 mL maturation medium, combine 97.9 mL Advanced

DMEM/F12, 1 mL N2 supplement, 1 mL GlutaMax, 100 µL Normocin in a sterile 250 mL

bottle. Complete maturation medium can be used for up to 2 weeks if stored at 4ºC. **CRITICAL**

**STEP** During the maturation phase of culture, Penicillin-Streptomycin should be replaced with

Normocin which does not contain aminoglycosides. Aminoglycosides such as streptomycin are

toxic to inner ear hair cells[71]. Ampicillin would be another suitable alternative.

**ScaleA2 Solution** To prepare 1 L of ScaleA2 solution, dissolve 240.24 g (4 M final

concentration) of urea powder in 750 mL of Milli-Q water. Add 1 mL of Triton X-100 (0.1%

vol/vol final concentration) and 100 g of glycerol (10% wt/vol final concentration). Add Milli-Q

water to a final of volume of 1 L. Store at room temperature (15-25ºC) for at least 1 year.

**Partially cured MDX4-4210 silicone elastomer** In a bacterial culture dish, stir together part A

and B of the MDX4-4210 silicone elastomer at a 1:5 ratio using a handheld P1000 tip. The

mixture should have a viscous paste-like consistency when properly mixed. Use the P1000 tip to

trowel approximately 500 µL of the mixture into 1.5 mL Eppendorf tubes. Aliquots can be stored

at -20ºC indefinitely until needed.

**EQUIPMENT SETUP**

**Wide-mouth P1000 tips** Use scissors to cut approximately 2 mm off the ends of P1000 tips. We

typically cut the tips of an entire box of 96 tips and then autoclave the box to sterilize.

**Whole-aggregate imaging chamber** This is a modified version of a previously described imaging chamber[72]. Here we have optimized the chamber to allow for imaging through either side of the chamber (**Suppl. Fig. 1**). Remove the adhesive backing from a 1-2 mm deep silicone isolator and place firmly onto a 24 x 50 cover glass. Using forceps remove the protective cover from the top of the silicone isolator. The chamber is now ready to be loaded with a sample. After loading the sample, place a 25 x 25 cover glass on top of the silicone isolator to complete the chamber. If desired forceps can be used to separate the top cover glass in order to remove the sample. Chambers can be reused several times if the silicone isolator is gently cleaned with water between uses and kept free of debris. Multiple samples can be loaded into one chamber or multi-chamber silicone isolators can be used to separate samples.

**Cover glass sensory epithelium holder** Cut one minutien pin in half to obtain two properly sized minutien pins for making the holder. Use a handheld P1000 tip to place a droplet of silicone elastomer onto a 15 mm cover glass in an off-center position. Use no. 5 forceps to embed one end of the minutien pins into the silicone droplet so that the other ends converge on the center of the cover glass. Position the pins so that 500-1500 µm gap is present between each end; the epithelium will be held in place between these two ends. Epithelia sizes vary, so we recommend making a variety of epithelium holders with different sized gaps. Use forceps to flatten the pins against the cover glass. Place the epithelium holder directly on the surface of a >100ºC hotplate for 5-10 minutes to cure the silicone elastomer. Holders can be stored at room temperature in a bacterial culture dish indefinitely. Before use, place a droplet of 10% Matrigel in DMEM/F12 on the holder and incubate at 37ºC for 1-hour.

**PROCEDURE**

**ES cell maintenance and passaging**

**TIMING 30 min**

**1** In the biosafety cabinet, coat a 60 mm culture dish with 2 mL of 0.1% gelatin. Leave for 20 minutes and in the mean time proceed to step 2.

**2** Warm at least 1 mL of 0.25% Trypsin-EDTA and 20 mL of LIF-2i medium in a 37ºC water bath.

**3** To begin dissociation of the 80% confluent ES cells, aspirate the spent LIF-2i medium and wash once with PBS.

**4** Add 400 µL of 0.25% Trypsin-EDTA and incubate at RT for ~1-2 min. After 30 seconds, shake the plate horizontally to break up the cells. Under a microscope, confirm that the cells have rounded and are detached from the surface of the plate.

**5** Collect the dissociated cells into 1 mL of LIF-2i medium and transfer the cells to a 2 mL microcentrifuge tube.

**6** Triturate the cells using a P1000 tip to break up clumps into single-cells. **CRITICAL STEP** Care should be taken to not introduce air bubbles into the medium.

**7** Centrifuge for 2-5 minutes at 200 g.

**8** While the cell suspension is in the centrifuge, aspirate the 0.1% gelatin from the 60 mm plate from step 1. Leave the plate for ~5 minutes to dry.

**9** Discard the supernatant and resuspend the cells from step 7 in 1 mL LIF-2i Medium.

**10** Plate the cells on the dried gelation coated plate (from step 8). Select plating density based on when you next wish to split the cells. Density should be between 1:10 (for next passage in ~2 days) and 1:50 (for next passage in ~4 days). Incubate cells at 37ºC in 5.0% $CO_2$ until ready for passage.

**TROUBLESHOOTING**

**ES cell differentiation: Initiating ectodermal induction (two 96-well plates)**

**TIMING 45 min**

**11** When cells are 80% confluent, aspirate the LIF-2i medium and wash three times with PBS at RT. **CRITICAL STEP** It is important to remove all trace of LIF-2i medium, as signaling factors within the LIF-2i can influence differentiation.

**12** Add 400 µL of 0.25% Trypsin-EDTA and incubate at RT or 37ºC for ~1-2 min. After 30 seconds, shake the plate horizontally to break up the cells. Under a microscope, confirm that the cells have rounded and are detached from the surface of the plate.

**13** Collect the dissociated cells into 1 mL of Ectodermal differentiation medium and transfer them into a 2 mL microcentrifuge tube.

**14** Break the cell clumps into single-cells by pipetting with a P1000 tip. Pellet the cells, by centrifugation at 200 g for 2-5 minutes.

**15** Completely remove the supernatant and resuspend the cell pellet in 1 mL Ectodermal differentiation medium.

**16** Forcefully pipet 1 mL of fresh Ectodermal differentiation medium through a cell-strainer-top test tube to prime the strainer. Pipet the 1 mL of ES cell suspension drop-wise onto the cell strainer. Next, pipet 1 mL of fresh Ectodermal differentiation medium drop-wise onto the cell strainer. There should be 3 mL in the test tube. **CRITICAL STEP** This step is important for ensuring that the cells are completely dissociated into single cells.

**17** Mix the cell suspension by pipetting with a P1000 tip and determine the concentration of cells using a hemocytometer.

**18** Dilute the appropriate volume of cell suspension in 22 mL of fresh Ectodermal

differentiation medium to acquire a final concentration of 30,000 cells per mL (i.e. $6.6 \times 10^5$

total cells). For example, if the cell suspension contains $1 \times 10^6$ cells per mL, dilute 0.66 mL

of cell suspension ($6.6 \times 10^5$ divided by $1 \times 10^6$) in 21.34 mL of Ectodermal differentiation

medium. Invert several times to mix.

**19** Pour the cell suspension into a reservoir and aliquot 100 µL of cell suspension into each well

of two 96-well plates using a multichannel pipette.

**20** Place the plates in a 37ºC incubator with 5.0% $CO_2$ for 24 hours.


**Differentiation day 1: Addition of Matrigel**

**TIMING 30 min**

**21** Mix 10.56 mL of Ectodermal differentiation medium with 440 µL of Matrigel to make 11

mL of complete medium. **CRITICAL STEP** Keep the medium and Matrigel on ice or in a

4ºC refrigerator immediately prior to mixing. Matrigel will become gelatinous at

temperatures above 15ºC. It is important to work quickly and hold the medium up to a light

to see that the Matrigel is properly mixed.

**22** Warm Ectodermal differentiation medium containing Matrigel in 37ºC water bath.

**23** Holding the multichannel pipette at an angle, carefully remove 50 µL of media from each

well of the plate from step 20 so that the cell aggregate at the bottom of the well remains

undisturbed.

**24** Add 50 µL of the Ectodermal differentiation medium containing Matrigel to each well. Note

that the final concentration of Matrigel is 2% v/v.

**25** Mix by pipetting 4-6 times. Return plates to the incubator for 48 hours.

**Differentiation day 3: Addition of BMP4 and SB-431542 (BMP/SB)**

**TIMING 30 min**

**26** Prepare an appropriate amount of Ectodermal differentiation medium for each experimental

condition. Typically 6 mL is sufficient for two 96-well plates.

**27** Add BMP4 and SB-431542 to the Ectodermal differentiation medium at a 5X concentration.

For 6 mL, add 3 µL of BMP4 and 3 µL of SB-431542 (i.e. 0.5 µL per 1 mL).

**28** Add 25 µL of Ectodermal differentiation medium containing BMP/SB to each well of the

plate from step 26. Each well now contains 125 µL of medium with a final concentration of

10 ng/mL BMP4 and 1 µM SB-431542. Incubate cells for a further 24-48 hours.

**Differentiation day 4-5: Addition of FGF-2 and LDN-193189 (FGF/LDN)**

**TIMING 30 min**

**29** Prepare an appropriate amount of Ectodermal differentiation medium for each experimental

condition. Typically 6 mL is sufficient for two 96-well plates.

**30** Add FGF-2 and LDN-193189 to the Ectodermal differentiation medium at a 6X

concentration. For 6 mL, add 4.5 µL of FGF-2 (i.e. 0.75 µL per 1 mL) and 3.6 µL of LDN-

193189 (i.e. 0.6 µL per 1 mL).

**31** Add 25 µL of Ectodermal differentiation medium containing FGF/LDN to each well of the

plates from step 28. Each well now contains 150 µL of medium with a final concentration of

25 ng/mL FGF-2 and 1 µM LDN-193189. Continue to incubate cells for 3-4 days.

**Differentiation day 8: Transition to long-term culture**

**TIMING 45 min**

**32** Using a P1000 pipette tip, transfer the aggregates from each well into a 15 mL conical tube. Use different 15 mL tubes for different conditions if necessary.

**33** Allow the aggregates to settle at the bottom of the tube for 1 minute and then carefully aspirate the media.

**34** Add at least 5 mL pre-warmed DMEM/F12 to wash the aggregates.

**35** Repeat steps 33 and 34 at least 3 times.

**36** Remove excess DMEM/F12.

**37** Re-suspend the aggregates in complete Maturation medium containing 1% Matrigel. Transfer the aggregates to a 100 mm bacterial dish using a wide-mouth P1000. Add an appropriate volume of additional Maturation medium to allow 1 mL containing 1-3 aggregates to be distributed into individual wells of a 24-well plate. **CRITICAL STEP** Use an additional 1-2 mL of Maturation medium to account for medium spreading out across the surface of the plate.

**38** Using a wide-mouth P1000 tip, pipet 1-3 aggregates in 1 mL into wells of a 24-well plate. Aggregates in the bacterial plate should be visible without a microscope.

Alternatively, single aggregates can be transferred to a new 96-well plate in 150-200 µL of Maturation medium containing Matrigel.

**39** Incubate cells for a further 22 days (24 well plates) or 12 days (96 well plates). For 24-well plates, beginning on day 10, replace half of the medium with Maturation medium that does not contain Matrigel every other day (i.e. day 12, 14, 16, etc.). Half of the medium should be replaced in 96-well plates every day beginning on day 9.

**40** Under these conditions, organoids can be cultured for up to 30 days (24 well plates) or 20 days (96 well plates). Longer culture durations will likely require different media compositions or culture formats.

**TROUBLESHOOTING**


**End point analysis**

41 Aggregates can be fixed at any point during the procedure for imaging as a cryosection (option A) or as a whole mount (option B). Option C describes how to prepare sensory epithelia for electrophysiological recording, which can be performed following the appearance of sensory epithelia with hair cells (days 16-30).

    A) **Preparation of aggregates for imaging – cryosections**

**TIMING 1 week**

  **i.** Pipet aggregates into a 2 mL tube using a P1000 tip (during days 1-6 or a wide-mouth P1000 (after day 6).

 **ii.** Aspirate the medium and immediately add 4% PFA in PBS to the aggregates.

**iii.** Incubate the aggregates in 4% PFA overnight at 4ºC to fix the tissue.

**iv.** Wash the aggregates at least 3 times with PBS. **PAUSE POINT** Fixed aggregates can be stored for several months in PBS at 4ºC. For long-term storage, we recommend storing the aggregates in PBS containing 0.02% sodium azide to discourage bacterial growth.

 **v.** Incubate the aggregates in a series of sucrose-PBS solutions (i.e. 10% sucrose, 20% sucrose, and 30% sucrose) for 30 minutes each with rocking. **PAUSE POINT** Sucrose treated aggregates can be stored at 4ºC for up to 1 week.

**vi.** Pipet the aggregates into a cryomold and carefully remove the sucrose solution using a P20 pipette. Position the aggregates in the center of the cryomold.

**vii.** Slowly add tissue-freezing medium along the outer-walls of the cryomold. Do not allow the aggregates to float away from the bottom of the cryomold.

**viii.** Place the cryomolds in a vacuum desiccator for 30 minutes to remove bubbles from the tissue-freezing medium.

**ix.** Freeze the samples on dry ice for 30 minutes or until the tissue-freezing medium is opaque. **PAUSE POINT** Frozen tissue blocks can be stored at -80ºC in a sealed container or zip-locked bags for several years.

**x.** Cryosection tissue blocks into 10-15 µm thick sections on a cryostat.

**xi.** Perform immunohistochemistry according to a standard protocol using the antibodies in table

**TROUBLESHOOTING**


**B) Preparation of aggregates for imaging – wholemount**

**TIMING 2 week**

**i.** Perform steps 41-44.

**ii.** Incubate the aggregates in PBS containing 10% normal goat serum, and 0.1% Triton X-100 at room temperature overnight on shaker. **CRITICAL STEP** Goat serum can be replaced with bovine serum albumin or normal horse serum depending on the antibodies being used.

**iii.** Remove the blocking solution and suspend the aggregates in PBS containing 3% normal goat serum, 0.1% Triton X-100, and primary antibodies. Incubate at room temperature for 2-3 days on a shaker.

iv. Wash 3 times with PBS containing 0.1% Triton X-100 for 1 hour each at room temperature on a shaker.

v. Incubate the aggregates in PBS containing 3% normal goat serum, 0.1% Triton X-100, and secondary antibodies at room temperature for 2-3 days on a shaker. **CRITICAL STEP** Wrap the samples in foil to protect the fluorophores from photobleaching during this and subsequent steps.

vi. Repeat step 55.

vii. Incubate aggregates in scaleA2 solution for 4-5 days prior to imaging. **CRITICAL STEP** ScaleA2 solution will cause slight enlargement of the tissue.

viii. To prepare for imaging, transfer ~250-500 µL (depending on the volume of the chamber) of scaleA2 solution containing an aggregate to an imaging chamber.

ix. Affix the top coverslip slowly. Some liquid will escape. A small air bubble will not be detrimental to later imaging.

x. While imaging, flip the imaging chamber to image the specimen from multiple orientations.


C) **Preparation of sensory epithelia for electrophysiological recordings**

**TIMING 1-2 hours**

i. Between days 20-30, identify an aggregate that has a protruding organoid with a long-axis diameter greater than 750 µm.

ii. Under a dissecting scope, use two tungsten needles to tease the organoid out of the cell aggregate. Attempt to clean mesenchymal and neuronal cells from the surface of the organoid using the tungsten needles. Try not to puncture the organoid while dissecting. **CRITICAL STEP** It is difficult to assess the morphology of the portion of the organoid embedded in the

aggregate. As this region most often harbors hair cells, extreme care should be taken not to destroy this portion of epithelium. Begin dissecting from the center of the aggregate out toward the surface. Puncture and anchor the center of the aggregate with one needle while teasing outward with the other (**Fig. 7a, left**).

iii. Use an inverted light-microscope to examine the organoid. Note the apparent thickness of the epithelium from multiple orientations. Regions of the epithelium containing hair cells appear thicker (~30 µm) than regions devoid of hair cells (~15 µm).

iv. Back under the dissecting scope, use the tungsten needles to puncture the organoid in a region not containing hair cells. Use one needle to anchor the tissue while pulling away rapidly with the other to create incisions in the epithelium. Attempt to make two incisions in an X-pattern opposite the region that likely contains hair cells (**Fig. 7a, middle**).

v. Use the tungsten needles to flatten the epithelium in a four-leafed clover pattern. The inside surface of the organoid (i.e. the apical end of the hair cells) should be facing up.

vi. Submerge an epithelium holder in the medium near the flattened epithelium.

vii. Use a pipet tip to gently position the flatten epithelium onto the center of the epithelium holder.

viii. Use fine forceps to pull up one minutien pin and a tungsten needle to position the epithelium underneath (**Fig. 7a, right**).

ix. With one end of the epithelium secured, repeat **viii** for the other end.

x. Lightly blow medium across the secured epithelium with a P200 pipette to test the stability of the epithelium preparation.

xi. The epithelium is now ready to be transferred to an electrophysiology rig for further experimentation.

**TIMING**

Steps 1-10, Maintenance and passaging ES cells: 30 min

Steps 11-20, ES cell differentiation: Initiating ectodermal induction (two 96-well plates): 45 min

Steps 21-25, Differentiation day 1: Addition of Matrigel: 30 min

Steps 26-28, Differentiation day 3: Addition of BMP4 and SB-431542 (BMP/SB): 30 min

Steps 29-31, Differentiation day 4-5: Addition of FGF-2 and LDN-193189 (FGF/LDN): 30 min

Steps 32-40, Differentiation day 8: Transition to long-term culture: 45 min

Steps 1-40, Total time to generate inner ear organoids: 20-30 days

Step 41 option A, Preparation of aggregates for imaging - cryosections: 1 week

Step 41 option B, Preparation of aggregates for imaging - wholemount: 1 week

Step 41 option C, Preparation of sensory epithelia for electrophysiological recordings: 1-2 hours

**TROUBLESHOOTING**

Troubleshooting advice can be found in **Table 2**.

**ANTICIPATED RESULTS**

For reproducible results we have found that it is absolutely critical to use fresh medium and well-preserved small molecule and recombinant protein stocks for each experiment. In addition, to reduce the time for performing each manipulation of the aggregates, we advise only running 2-3 96-well plates per experiment until the protocol is mastered. With experience, the various morphological changes can be readily identified under a light microscope.

With proper dissociation, counting, and pipetting we have found that cell aggregates are 250-270 µm in diameter 24 hours after plating (i.e. at the time of Matrigel addition). As a quality control measure, we check to see that there is minimal debris floating around the aggregates (i.e. denoting cellular death) and that the average aggregate diameter across all 96-well plates has a standard deviation of 5 µm or less (**Fig. 2a**). Following another 48 hours of culture (d3), the aggregates should roughly double in size to 480-500 µm in diameter and a translucent epithelium should be visible on the surface of the aggregate (**Fig. 2c**). Our previous data suggest that this epithelium is representative of the definitive ectoderm because it expresses E-cadherin and does not express the neural marker Sox1, the mesendodermal marker Brachyury or the pluripotency marker Nanog[3]. Following BMP/SB treatment on day 3, AP2 expression can be seen in the outer-epithelium beginning on day 4. If the FGF/LDN treatment timing is optimal, on day 6 of differentiation, the outer-epithelium should appear to thicken (~30 µm apparent thickness) relative to the epithelium of aggregates treated with BMP/SB alone (~15 µm apparent thickness). We also observe a distinctive ruffling of the outer-epithelium (**Fig. 3**). Over the next 24-36 hours these morphological characteristics become more apparent and should be quite obvious if the experiment was performed correctly. Between days 6-8, patches of Pax8^{+} E-cadherin (Ecad)[+] epithelium are visible in the outer-epithelium indicating the development of OEPD tissue (**Fig 4**).

Upon transfer to maturation medium (d8-11), the aggregates lose discernable morphological features as the inner core cells migrate to the surface and cover the outer-epithelium (**Fig. 5a**). By day 12, however, in properly treated and manipulated aggregates, the core of the aggregate should become partially translucent, indicating the evacuation of the inner core cells; the size of each aggregate should be ~1.5-2 mm. It is common for aggregates to conjoin to one another after day 12 of the culture if not cultured individually. Also, by day 12,

vesicles should be visible by looking alone the edges of the aggregates or at the translucent central region of the aggregate (**Fig. 6a-d**). To have confidence that an experiment was performed correctly, we recommend checking to see that at least 10% of the aggregates have vesicles overtly visible using a light microscope by day 12-14. Immunohistochemistry should be performed to assess the true number of vesicles generated, which should be in the range of 15-25 vesicles per aggregate. On day 12, vesicles display a wide range of intraluminal diameters from 1-100 µm. Two organoid variants should develop during days 14-30: embedded and protruding (**Fig. 6e-l, 7b-d**). The majority organoids containing hair cells will remain embedded in the tissue for the duration of the culture. These are difficult to see with the naked eye; however, protruding organoids are readily apparent. In total, we normally see that 3-6 organoids develop from the original group of vesicles per aggregate by day 20. Protruding organoids should be visible in ~15% of the aggregates.

**Authors' contributions** K.R.K. designed the protocol, made figures and wrote the manuscript. E.H. edited the manuscript.

TL1RR025759).

**Corresponding authors** Correspondence and requests for materials should be addressed to EH

(ehashino@iupui.edu) or KRK (krkoehle@iupui.edu).

## REFERENCES

1. Sasai, Y. Cytosystems dynamics in self-organization of tissue architecture. *Nature* **493,** 318–326 (2013).
2. Sasai, Y., Eiraku, M. & Suga, H. In vitro organogenesis in three dimensions: self-organising stem cells. *Development (Cambridge, England)* **139,** 4111–4121 (2012).
3. Koehler, K. R., Mikosz, A. M., Molosh, A. I., Patel, D. & Hashino, E. Generation of inner ear sensory epithelia from pluripotent stem cells in 3D culture. *Nature* **500,** 217–221 (2013).
4. Gonzalez-Cordero, A. *et al.* Photoreceptor precursors derived from three-dimensional embryonic stem cell cultures integrate and mature within adult degenerate retina. *Nature Biotechnology* **31,** 741–747 (2013).
5. Eiraku, M. *et al.* Self-organizing optic-cup morphogenesis in three-dimensional culture. *Nature* **472,** 51–56 (2011).
6. Eiraku, M. *et al.* Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. *Cell Stem Cell* **3,** 519–532 (2008).
7. Eiraku, M. & Sasai, Y. Mouse embryonic stem cell culture for generation of three-dimensional retinal and cortical tissues. *Nature Protocols* **7,** 69–79 (2012).
8. Nakano, T. *et al.* Self-Formation of Optic Cups and Storable Stratified Neural Retina from Human ESCs. *Cell Stem Cell* **10,** 771–785 (2012).
9. Suga, H. *et al.* Self-formation of functional adenohypophysis in three-dimensional culture. *Nature* **480,** 57–62 (2011).
10. Nasu, M. *et al.* Robust Formation and Maintenance of Continuous Stratified Cortical Neuroepithelium by Laminin-Containing Matrix in Mouse ES Cell Culture. *PLoS ONE* **7,** e53024 (2012).
11. Lancaster, M. A. *et al.* Cerebral organoids model human brain development and microcephaly. *Nature* **501,** 373–379 (2013).
12. Spence, J. R. *et al.* Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. *Nature* **470,** 105–109 (2010).
13. McCracken, K. W., Howell, J. C., Wells, J. M. & Spence, J. R. Generating human intestinal tissue from pluripotent stem cells in vitro. *Nature Protocols* **6,** 1920–1928 (2011).

14. Sato, T. *et al.* Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459,** 262–265 (2009).

15. Huch, M. *et al.* In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. *Nature* **494,** 247–250 (2013).

16. Li, L. *et al.* Location of transient ectodermal progenitor potential in mouse development. *Development* **140,** 4533–4543 (2013).

17. Cajal, M. *et al.* Clonal and molecular analysis of the prospective anterior neural boundary in the mouse embryo. *Development* **139,** 423–436 (2012).

18. Beddington, S. P. An autoradiographic analysis of the potency of embryonic ectoderm in the 8th day postimplantation mouse embryo. *J Embryol Exp Morphol* **64,** 87–104 (1981).

19. Beddington, R. S. An autoradiographic analysis of tissue potency in different regions of the embryonic ectoderm during gastrulation in the mouse. *J Embryol Exp Morphol* **69,** 265–285 (1982).

20. Wilson, P. A. & Hemmati-Brivanlou, A. Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature* **376,** 331–333 (1995).

21. Wilson, P. A., Lagna, G., Suzuki, A. & Hemmati-Brivanlou, A. Concentration-dependent patterning of the Xenopus ectoderm by BMP4 and its signal transducer Smad1. *Development (Cambridge, England)* **124,** 3177–3184 (1997).

22. Kwon, H.-J., Bhat, N., Sweet, E. M., Cornell, R. A. & Riley, B. B. Identification of early requirements for preplacodal ectoderm and sensory organ development. *PLoS genetics* **6,** e1001133 (2010).

23. Barth, K. A. *et al.* Bmp activity establishes a gradient of positional information throughout the entire neural plate. *Development (Cambridge, England)* **126,** 4977–4987 (1999).

24. Neave, B., Holder, N. & Patient, R. A graded response to BMP-4 spatially coordinates patterning of the mesoderm and ectoderm in the zebrafish. *Mechanisms of development* **62,** 183–195 (1997).

25. Harvey, N. T. *et al.* Response to BMP4 signalling during ES cell differentiation defines intermediates of the ectoderm lineage. *Journal of cell science* **123,** 1796–1804 (2010).

26. Pieper, M., Ahrens, K., Rink, E., Peter, A. & Schlosser, G. Differential distribution of competence for panplacodal and neural crest induction to non-neural and neural ectoderm. *Development (Cambridge, England)* **139,** 1175–1187 (2012).

27. Reichert, S., Randall, R. A. & Hill, C. S. A BMP regulatory network controls ectodermal cell fate decisions at the neural plate border. *Development (Cambridge, England)* **140,** 4435–4444 (2013).

28. Grocott, T., Tambalo, M. & Streit, A. The peripheral sensory nervous system in the vertebrate head: A gene regulatory perspective. *Developmental biology* **370,** 3–23 (2012).

29. Ahrens, K. & Schlosser, G. Tissues and signals involved in the induction of placodal Six1 expression in Xenopus laevis. *Developmental biology* **288,** 40–59 (2005).

30. Brugmann, S. A., Pandur, P. D., Kenyon, K. L., Pignoni, F. & Moody, S. A. Six1 promotes a placodal fate within the lateral neurogenic ectoderm by functioning as both a transcriptional activator and repressor. *Development (Cambridge, England)* **131,** 5871–5881 (2004).

31. Litsiou, A., Hanson, S. & Streit, A. A balance of FGF, BMP and WNT signalling positions the future placode territory in the head. *Development (Cambridge, England)* **132,** 4051–4062 (2005).

32. Schlosser, G. Induction and specification of cranial placodes. *Developmental biology* **294,**

303–351 (2006).

33. Patthey, C. & Gunhaga, L. Specification and regionalisation of the neural plate border. *The European journal of neuroscience* **34,** 1516–1528 (2011).

34. Bhattacharyya, S. & Bronner-Fraser, M. Competence, specification and commitment to an olfactory placode fate. *Development (Cambridge, England)* **135,** 4165–4177 (2008).

35. Baker, C. V., Stark, M. R., Marcelle, C. & Bronner-Fraser, M. Competence, specification and induction of Pax-3 in the trigeminal placode. *Development (Cambridge, England)* **126,** 147–156 (1999).

36. Ladher, R. K., O'Neill, P. & Begbie, J. From shared lineage to distinct functions: the development of the inner ear and epibranchial placodes. *Development (Cambridge, England)* **137,** 1777–1785 (2010).

37. Wright, T. J. & Mansour, S. L. Fgf3 and Fgf10 are required for mouse otic placode induction. *Development (Cambridge, England)* **130,** 3379–3390 (2003).

38. Urness, L. D., Paxton, C. N., Wang, X., Schoenwolf, G. C. & Mansour, S. L. FGF signaling regulates otic placode induction and refinement by controlling both ectodermal target genes and hindbrain Wnt8a. *Developmental biology* **340,** 595–604 (2010).

39. Ohyama, T., Mohamed, O. A., Taketo, M. M., Dufort, D. & Groves, A. K. Wnt signals mediate a fate decision between otic placode and epidermis. *Development (Cambridge, England)* **133,** 865–875 (2006).

40. Riccomagno, M. M., Takada, S. & Epstein, D. J. Wnt-dependent regulation of inner ear morphogenesis is balanced by the opposing and supporting roles of Shh. *Genes & Development* **19,** 1612–1623 (2005).

41. Freter, S., Muta, Y., Mak, S.-S., Rinkwitz, S. & Ladher, R. K. Progressive restriction of otic fate: the role of FGF and Wnt in resolving inner ear potential. *Development (Cambridge, England)* **135,** 3415–3424 (2008).

42. Groves, A. K. & Bronner-Fraser, M. Competence, specification and commitment in otic placode induction. *Development (Cambridge, England)* **127,** 3489–3499 (2000).

43. Groves, A. K. & Fekete, D. M. Shaping sound in space: the regulation of inner ear patterning. *Development (Cambridge, England)* **139,** 245–257 (2011).

44. Neves, J., Parada, C., Chamizo, M. & Giraldez, F. Jagged 1 regulates the restriction of Sox2 expression in the developing chicken inner ear: a mechanism for sensory organ specification. *Development (Cambridge, England)* **138,** 735–744 (2011).

45. Appler, J. M. & Goodrich, L. V. Connecting the ear to the brain: Molecular mechanisms of auditory circuit assembly. *Progress in neurobiology* **93,** 488–508 (2011).

46. Laine, H., Sulg, M., Kirjavainen, A. & Pirvola, U. Cell cycle regulation in the inner ear sensory epithelia: role of cyclin D1 and cyclin-dependent kinase inhibitors. *Developmental biology* **337,** 134–146 (2010).

47. Oesterle, E. C., Campbell, S., Taylor, R. R., Forge, A. & Hume, C. R. Sox2 and Jagged1 Expression in Normal and Drug-Damaged Adult Mouse Inner Ear. *Journal of the Association for Research in Otolaryngology : JARO* **9,** 65–89 (2007).

48. Xiang, M., Gao, W. Q., Hasson, T. & Shin, J. J. Requirement for Brn-3c in maturation and survival, but not in fate determination of inner ear hair cells. *Development (Cambridge, England)* **125,** 3935–3946 (1998).

49. Watanabe, K. *et al.* Directed differentiation of telencephalic precursors from embryonic stem cells. *Nature Neuroscience* **8,** 288–296 (2005).

50. Muñoz-Sanjuán, I. & Brivanlou, A. H. Neural induction, the default model and embryonic

stem cells. *Nature Reviews Neuroscience* **3,** 271–280 (2002).

51. James, D., Levine, A. J., Besser, D. & Hemmati-Brivanlou, A. TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development (Cambridge, England)* **132,** 1273–1282 (2005).

52. Camus, A., Perea-Gomez, A., Moreau, A. & Collignon, J. Absence of Nodal signaling promotes precocious neural differentiation in the mouse embryo. *Developmental biology* **295,** 743–755 (2006).

53. Chambers, S. M. *et al.* Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nature Biotechnology* **27,** 275–280 (2009).

54. Muguruma, K. *et al.* Ontogeny-recapitulating generation and tissue integration of ES cell-derived Purkinje cells. *Nature Neuroscience* **13,** 1171–1180 (2010).

55. Bouchard, M., de Caprona, D., Busslinger, M., Xu, P. & Fritzsch, B. Pax2 and Pax8 cooperate in mouse inner ear morphogenesis and innervation. *BMC developmental biology* **10,** 89 (2010).

56. Christophorou, N. A. D., Mende, M., Lleras-Forero, L., Grocott, T. & Streit, A. Pax2 coordinates epithelial morphogenesis and cell fate in the inner ear. *Developmental biology* **345,** 180–190 (2010).

57. Warchol, M. E. & Richardson, G. P. Expression of the Pax2 transcription factor is associated with vestibular phenotype in the avian inner ear. *Developmental neurobiology* **69,** 191–202 (2009).

58. Lysakowski, A. *et al.* Molecular microdomains in a sensory terminal, the vestibular calyx ending. *Journal of Neuroscience* **31,** 10101–10114 (2011).

59. Li, A., Xue, J. & Peterson, E. H. Architecture of the mouse utricle: macular organization and hair bundle heights. *Journal of neurophysiology* **99,** 718–733 (2008).

60. Li, H., Roblin, G., Liu, H. & Heller, S. Generation of hair cells by stepwise differentiation of embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **100,** 13495–13500 (2003).

61. Oshima, K. *et al.* Mechanosensitive Hair Cell-like Cells from Embryonic and Induced Pluripotent Stem Cells. *Cell* **141,** 704–716 (2010).

62. Ouji, Y., Ishizaka, S., Nakamura-Uchiyama, F. & Yoshikawa, M. In vitro differentiation of mouse embryonic stem cells into inner ear hair cell-like cells using stromal cell conditioned medium. *Cell Death and Disease* **3,** e314 (2012).

63. Chen, W. *et al.* Restoration of auditory evoked responses by human ES-cell-derived otic progenitors. *Nature* **490,** 278–282 (2012).

64. Ouji, Y., Ishizaka, S., Nakamura-Uchiyama, F., Wanaka, A. & Yoshikawa, M. Induction of inner ear hair cell-like cells from Math1-transfected mouse ES cells. *Cell Death and Disease* **4,** e700 (2013).

65. Brigande, J. V. & Heller, S. Quo vadis, hair cell regeneration? *Nature Neuroscience* **12,** 679–685 (2009).

66. Ding, Q. *et al.* A TALEN genome-editing system for generating human stem cell-based disease models. *Cell Stem Cell* **12,** 238–251 (2013).

67. Ding, Q. *et al.* Enhanced Efficiency of Human Pluripotent Stem Cell Genome Editing through Replacing TALENs with CRISPRs. *Cell Stem Cell* **12,** 393–394 (2013).

68. Trowe, M.-O. *et al.* Loss of Sox9 in the periotic mesenchyme affects mesenchymal expansion and differentiation, and epithelial morphogenesis during cochlea development in the mouse. *Developmental biology* **342,** 51–62 (2010).

69.    Ying, Q.-L. *et al.* The ground state of embryonic stem cell self-renewal. *Nature* **453,** 519–523 (2008).
70.    Gaboyard, S. *et al.* Three-dimensional culture of newborn rat utricle using an extracellular matrix promotes formation of a cyst. *Neuroscience* **133,** 253–265 (2005).
71.    Lerner, S. A., Matz, G. J. & Hawkins, J. E. *Aminoglycoside ototoxicity*. (Little Brown and Company, 1981).
72.    Yokomizo, T. *et al.* Whole-mount three-dimensional imaging of internally localized immunostained cells within mouse embryos. *Nature Protocols* **7,** 421–431 (2012).

## FIGURE LEGENDS

**Figure 1: Overview of inner ear induction protocol.** A comparison of cell fate and morphological transitions that take place during *in vivo* and *in vitro* inner ear development. Note that the *in vivo* time points are approximate values derived from mouse developmental studies. Moreover, the cell fate transition model depicted in the left-hand column is a generalization of models derived from studies in various vertebrate species. The preplacodal fate, for instance, has not been as clearly defined in the mouse system as it has in the *Xenopus*, chick and zebrafish models. The right-hand column describes the experimental procedures and step numbers associated with each cell fate transition in the culture. ESC, embryonic stem cells; de, definitive ectoderm; nne, nonneural ectoderm; ne, neural ectoderm; me, mesendoderm; ppe, preplacodal ectoderm; epi, epidermis; oepd, otic-epibranchial placode domain.

**Figure 2: Development of the definitive ectoderm epithelium. a-c,** DIC images of representative aggregates on days 1, 2, and 3 of 3D culture. **d-i**, Following Matrigel addition on day 1, Laminin is incorporated into a basement membrane (**e, f, h, i**) on the surface of the aggregate. An epithelium develops on the aggregate surface by day 3. See Table 1 for a list of antibodies used for characterization. ESC, embryonic stem cells. Scale bars, 100 µm

**Figure 3: Morphology changes following BMP/SB-FGF/LDN treatment. a,** FGF/LDN treatment induces preplacodal ectoderm from nonneural ectoderm. **b, c**, The morphology of the nonneural ectoderm layer on the surface of the aggregates thickens and ruffles in response to FGF/LDN treatment. Scale bars, 100 μm

**Figure 4: Aggregate organization on day 8 of differentiation. a, b,** The epithelium of the OEPD in an E8 mouse embryo and (**c-f**) the outer-epithelium of BMP/SB-FGF/LDN treated aggregates express Pax8 and Ecad. See Table 1 for a list of antibodies used for characterization. Panel **b** was previously published in Supplementary Figure 6 of Koehler et al. Nature 2013. All animal experiments were performed in accordance with the Indiana University Institutional Animal Care and Use Committee (IACUC) guidelines. Scale bars, 100 (**b,c**), 30 (**d-f**), and 10 (**b-inset**) μm.

**Figure 5: Cellular rearrangement between days 8 and 12 of differentiation. a,** Schematic of the stages of rearrangement during days 8, 9-11 and 12-14. **b-e,** Day 12 aggregates that received treatment with BMP/SB (b), BMP/SB-FGF (c), BMP/SB-LDN (d) or BMP/SB-FGF/LDN (e). The numbered indicators denote key characteristics to look for when assessing the state of cellular rearrangement: 1) An outer-layer of translucent epithelium indicates the development of epidermis and improper cellular rearrangement, 2) an outer-layer of opaque tissue indicates rearrangement, 3) the appearance of a translucent central core indicates the proper migration of tissue to the outer-surface of the aggregate. Nascent otic vesicles should be visible in translucent regions similar to #3. No rearrangement is seen in panels b and c, partial rearrangement is seen in

panel d and complete rearrangement in panel e. Panels **a-e** were adapted from Supplementary

Figure 9 of Koehler et al. Nature 2013. Scale bars, 250 µm.


**Figure 6: Hair cell induction and organoid types. a, b,** Otic vesicles can be seen using a

conventional light microscope while aggregates are in culture. **c, d**, Otic vesicles express Jag1,

Sox2, Pax2, and Pax8. **e-l**, There are two organoid variants: embedded (**e-h**) and protruding (i-l).

The sensory epithelia of each inner ear organoids contain CyclinD1 (cD1)$^+$ Sox2$^+$ supporting

cells and Myo7a$^+$ Brn3c$^+$ and Calretinin (Calb2)$^+$ hair cells. **i**, **j**, Protruding organoids are

typically >200 µm in diameter and are visible at low magnification or, occasionally, by the naked

eye. See Table 1 for a list of antibodies used for characterization. Panels **g** and **h** were previously

published in Supplementary Figure 13 of Koehler et al. Nature 2013. Scale bars, 250 (**j**), 100 (**l**),

50 (**k**), 25 (**b-d**) µm.


**Figure 7: Inner ear organoid dissection for electrophysiological analysis. a,** A schematic of

the organoid dissection method described in steps 62-72. The red dots and dotted lines with

arrows refer the placement or motion that should be performed with two tungsten needles. **b-d**,

Representative protruding organoid before dissection. **e-g,** Representative dissected sensory

epithelium in an epithelium holder ready for experimentation. Post-recording immunostaining

reveals the field of Myo7a$^+$ hair cells with F-actin$^+$ stereocilia from which electrophysiological

recordings were taken. See Table 1 for a list of antibodies used for characterization. Panels **b-g**

were previously published in Supplementary Figures 14 and 15 of Koehler et al. Nature 2013.

Scale bars, 250 (**b-f**), 5 (**g**) µm.

## TABLES

**Table 1 - Antibodies needed for tissue characterization**

| Useful marker for | Antibody | Host | Supplier | Catalog No. | Dilution |
|---|---|---|---|---|---|
| PSC[a] | Nanog | Rabbit | Abcam | AB80892 | 1:100 |
| MES[b] | Brachyury | Goat | Santa Cruz | SC17745 | 1:20 |
| NE[d], MES | N-cadherin | Mouse | BD Biosciences | 610920 | 1:100 |
| NE | Sox1 | Rabbit | Cell Signaling | 4194 | 1:100 |
| PSC, DE[c], NNE[e], PPE[f], EPI[g], OTV[h], SE[i] | E-cadherin | Mouse | BD Biosciences | 610181 | 1:250 |
| NNE, PPE | AP2 | Mouse | DSHB | 3B5 | 1:50 |
| PSC, DE, OTV, SE | Sox2 | Mouse | BD Biosciences | 561469 | 1:100 |
| NNE, PPE, OTV | GATA3 | Goat | R&D Systems | AF2605 | 1:50 |
| PPE | Six1 | Rabbit | Sigma | HPA001893 | 1:100 |
| OTV, NE | Pax8 | Rabbit | Abcam | AB97477 | 1:100 |
| OTV, HC (nuclei) | Pax2 | Rabbit | Invitrogen | 716000 | 1:100 |
| Anterior PPE, NE | Pax6 | Rabbit | Abcam | AB5790 | 1:200 |
| Intermediate PPE | Pax3 | Mouse | DSHB | N/A | 1:100 |
| EPI (nuclei) | p63 | Mouse | Santa Cruz | SC8431 | 1:50 |
| EPI (cytosol) | Cytokeratin-5 | Rabbit | Sigma | SAB4501651 | 1:100 |
| OTV, SE | p27$^{kip1}$ | Mouse | BD Biosciences | 610241 | 1:100 |
| OTV, SE | Jagged-1 | Rabbit | LSBio | LSC138530 | 1:50 |
| Epithelium (Apical) | aPKC | Rabbit | Santa Cruz | SC208 | 1:50 |
| Epithelium (Basal) | Laminin-β1 | Rat | Abcam | AB44941 | 1:50 |
| HC (nuclei) | Brn3c | Mouse | Santa Cruz | SC81980 | 1:50 |

| HC (cytosol) | Myo7a | Rabbit | Proteus | 256790 | 1:100 |
| HC cuticle/kinocilia | α-Tubulin | Mouse | Sigma | T6793 | 1:100 |
| Neurons | ßIII-Tubulin (TuJ1) | Mouse | Covance | MMS-435P | 1:500 |
| SN[k], HC | Calretinin (Calb2) | Mouse | Millipore | MAB1568 | 1:200 |
| SN | Brn3a | Mouse | Millipore | MAB1585 | 1:20 |
| SN | Islet1 (39.4D5) | Mouse | DSHB | 39.4D5 | 1:5 |
| SN | Neurofilament (L) | Rabbit | Millipore | AB1983 | 1:100 |
| Synapse | Synaptophysin | Rabbit | Invitrogen | 180130 | 1:100 |
| Synapse | Rab3 | Mouse | BD Biosciences | 610379 | 1:50 |
| Synapse | Snap25 | Mouse | BD Biosciences | 610366 | 1:100 |
| Ribbon Synapse | Ctbp2 | Mouse | BD Biosciences | 612044 | 1:50 |

[a]PSC, pluripotent stem cell; [b]MES, mesendoderm; [c]DE, definitive ectoderm; [d]NE, neural ectoderm; [e]NNE, nonneural ectoderm; [f]PPE, preplacodal ectoderm; [g]EPI, epidermis; [h]OTV, otic vesicle; [i]SE, sensory epithelium; [j]HC, hair cells; [k]SN, sensory neurons
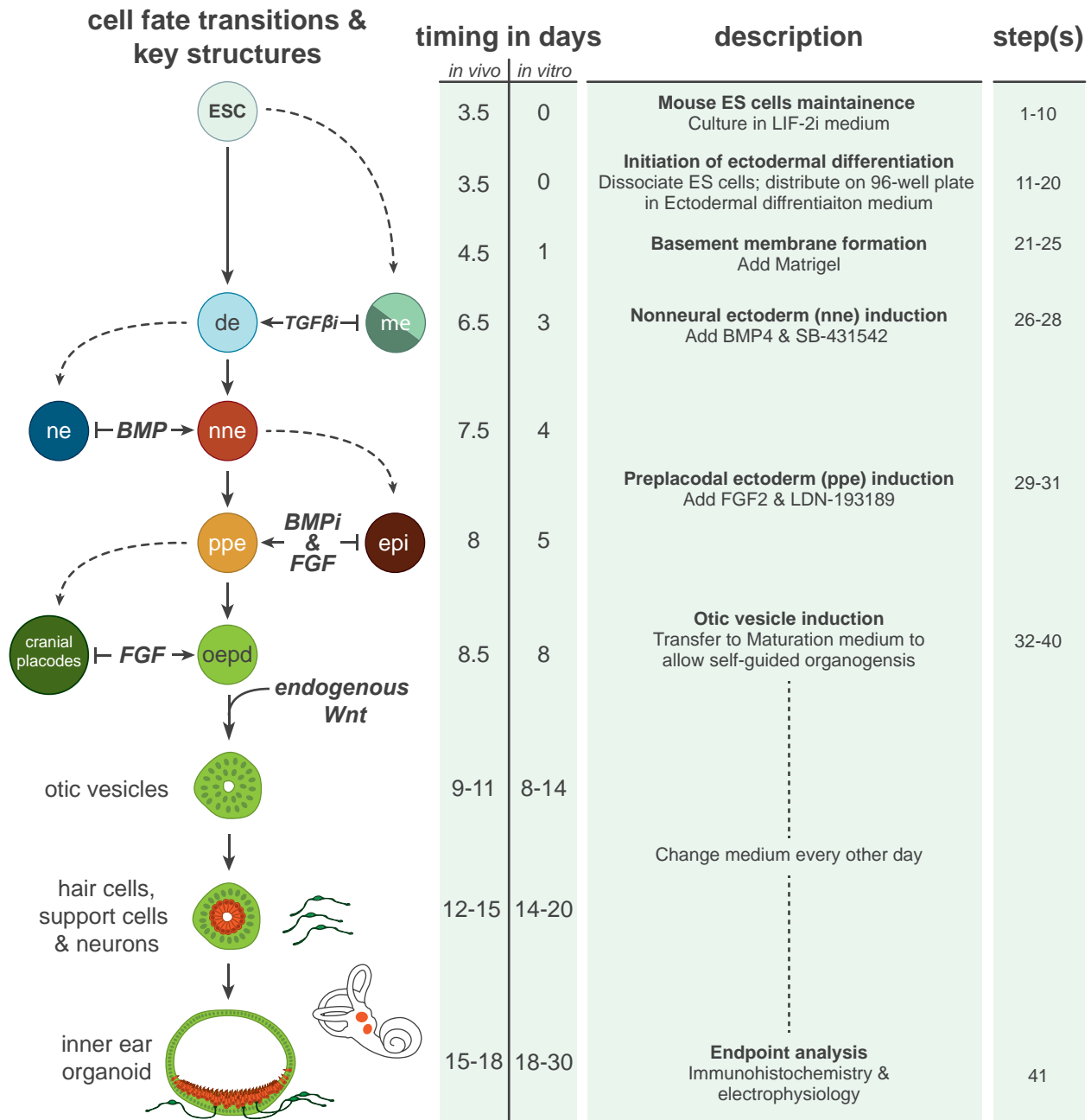
## Table 2 - Troubleshooting

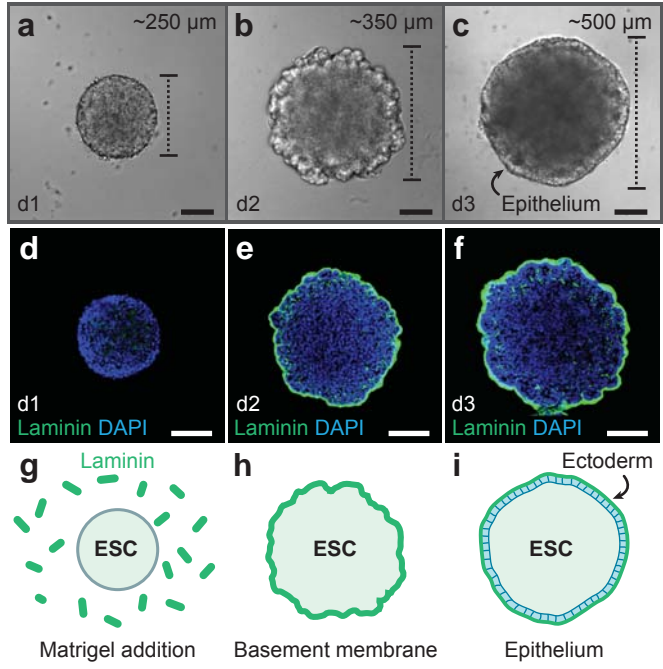| Steps | Problem | Possible Reason | Solution |
|---|---|---|---|
| 1-10 | ES cells do not attach to the plate | Insufficient gelatin coating | Re-plate the cells on a new plate with fresh gelatin coating |
| | | Cells are not yet adapted to LIF-2i medium | Perform several passages |
| 11-28 | Epithelium does not form or thicken over time in control samples | Ectodermal medium was not made properly | Check reagent expiration dates; Make new Ectodermal medium |

| | | Matrigel was not mixed into medium properly | Make sure that Matrigel is added to Ectoderm differentiation medium during preparation at 4ºC. |
| 29-31 | Epithelium does not thicken and/or ruffle | One of the small molecules or proteins has expired or is not present at an optimal concentration | Test each treatment separately and replace small molecule or protein stocks |
| | | Matrigel concentration is not optimal | Reduce/Increase Matrigel concentration |
| 29-31 | The aggregates themselves or the medium color in wells on the edge of the plate look different from the rest of the plate | Disproportionate evaporative loss in the edge wells | Check that incubator is properly humidified |
| | | | Only analyze aggregates from interior wells |
| 32-40 | Cellular rearrangement does not occur during days 8-12 | Cell aggregates were transferred to floating culture too early/late | Transfer aggregates at a different time point between days 7-8.5 |
| 32-40 | No Pax2, Pax8, Sox2, Ecad+ vesicles develop by day 12 | Improper cellular rearrangement | Transfer aggregates at a different time point between days 7-8.5 |
| | | Carry-over of signaling molecules/proteins from day 0-8 culture | Wash aggregates with DMEM/F12 at least 3 times prior culture in maturation medium |
| | | | Check that proper morphology is observed during days 0-8 |
| | | Aggregates are damaged during transfer to maturation medium | Use wide-mouth or cut P1000 tips or a sterile transfer pipet to manipulate cell aggregates |
| 29-40 | Excessive development of beating myocytes, cartilaginous masses and adipose apparent between days 14-20 | LDN-193189 concentration was suboptimal; insufficient suppression of mesenchyme development | Increase the concentration of LDN-193189 (1 µM should be sufficient) |
| 32-40 | Large translucent organoids do not develop by day 18-20 | Too many aggregates were cultured together | Reduce the number of aggregates in each well on 24-well plates |

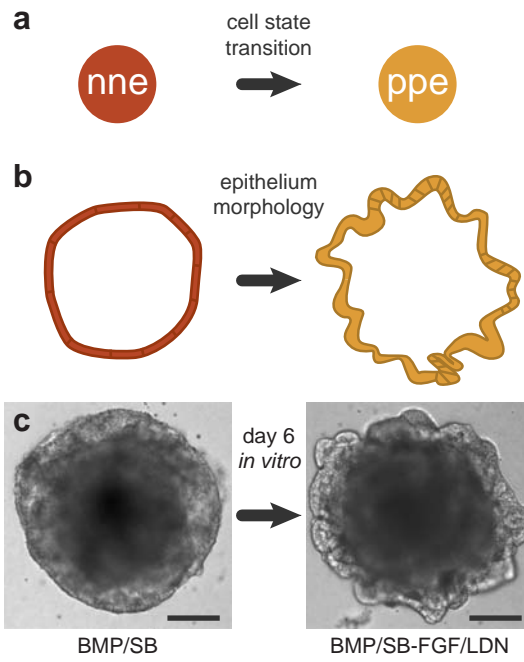| | | Matrigel concentration is too high during floating culture | Reduce Matrigel concentration to 0-0.5% at the start of floating culture |
|---|---|---|---|
| 41-51 | No hair cells observed in cyrosectioned organoids | Incomplete analysis | Ensure that the entire organoid is analyzed; cyrosections through nonsensory regions give the appearance of no hair cells |

**Supplementary Information**

**Supplementary Figure 1: Aggregate imaging chamber. a,** Typical imaging chamber constructed from two cover glasses and a silicone isolator. One aggregate (sample) is loaded in the chamber. Note the air bubble that forms during the loading process. **b**, Projection drawing of an aggregate imaging chamber. Note that this chamber design is an example and can be easily customized using different sized cover glasses or silicone isolators.
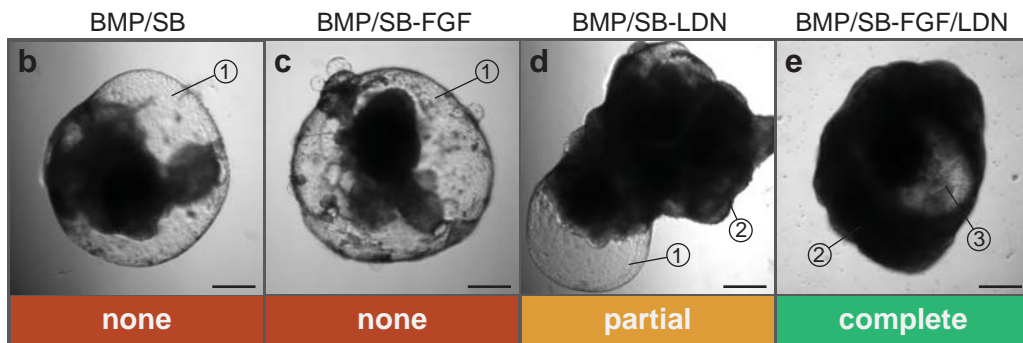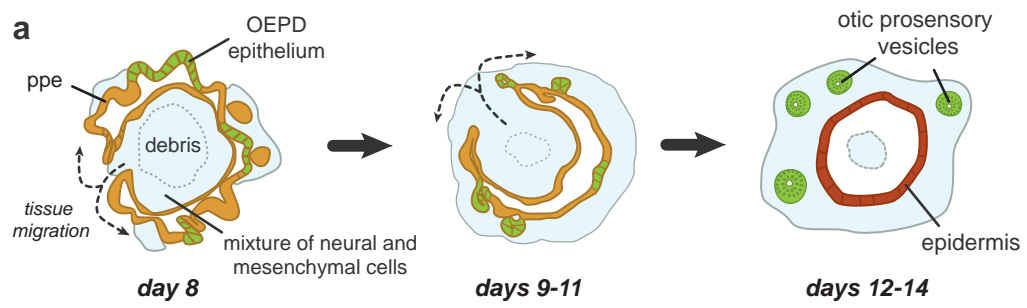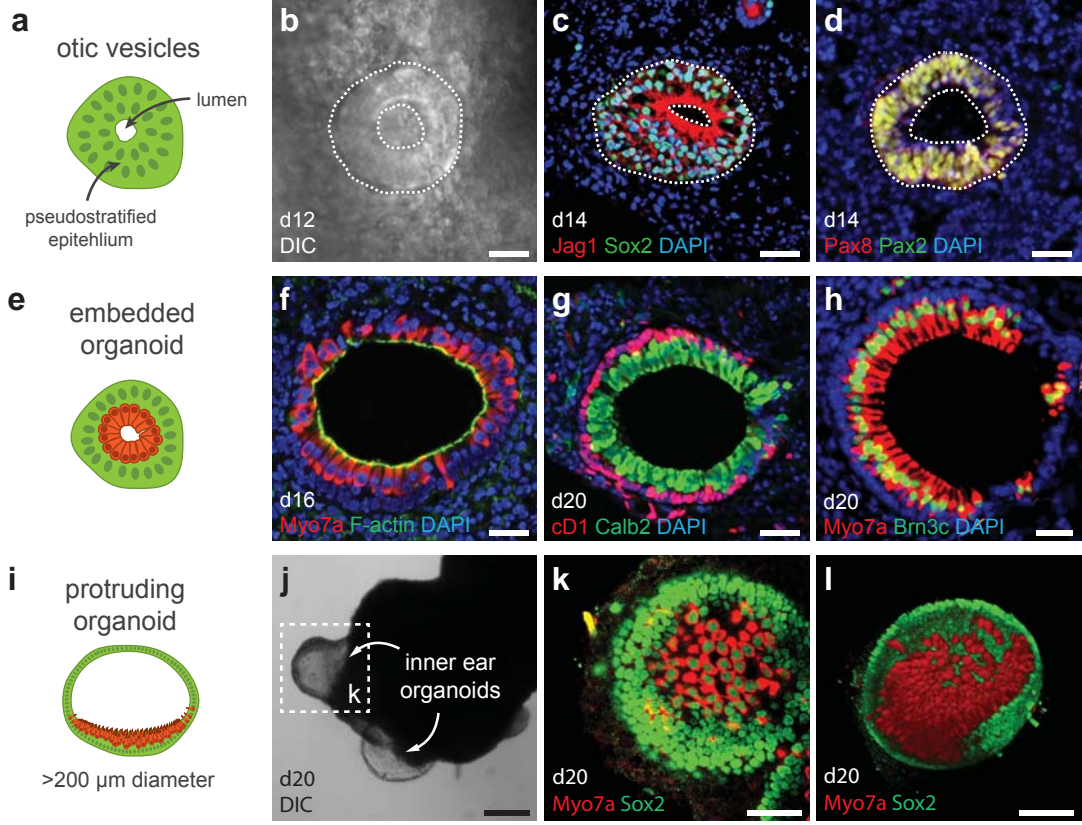
## cell fate transitions & key structures



| | timing in days | | description | step(s) |
|---|---|---|---|---|
| | *in vivo* | *in vitro* | | |
| ESC | 3.5 | 0 | **Mouse ES cells maintainence**<br>Culture in LIF-2i medium | 1-10 |
| | 3.5 | 0 | **Initiation of ectodermal differentiation**<br>Dissociate ES cells; distribute on 96-well plate<br>in Ectodermal diffrentiaiton medium | 11-20 |
| | 4.5 | 1 | **Basement membrane formation**<br>Add Matrigel | 21-25 |
| de ←TGFβi— me | 6.5 | 3 | **Nonneural ectoderm (nne) induction**<br>Add BMP4 & SB-431542 | 26-28 |
| ne ⊣ BMP → nne | 7.5 | 4 | | |
| | 8 | 5 | **Preplacodal ectoderm (ppe) induction**<br>Add FGF2 & LDN-193189 | 29-31 |
| ppe ← BMPi & ⊣ epi<br>FGF | | | | |
| cranial placodes ⊣ FGF → oepd | 8.5 | 8 | **Otic vesicle induction**<br>Transfer to Maturation medium to<br>allow self-guided organogensis | 32-40 |
| *endogenous Wnt* | | | | |
| otic vesicles | 9-11 | 8-14 | | |
| | | | Change medium every other day | |
| hair cells, support cells & neurons | 12-15 | 14-20 | | |
| inner ear organoid | 15-18 | 18-30 | **Endpoint analysis**<br>Immunohistochemistry &<br>electrophysiology | 41 |

| a ~250 µm | b ~350 µm | c ~500 µm |
| d1 | d2 | d3 Epithelium |

| d | e | f |
| d1 | d2 | d3 |
| Laminin DAPI | Laminin DAPI | Laminin DAPI |

g Laminin
ESC
Matrigel addition

h
ESC
Basement membrane

i Ectoderm
ESC
Epithelium

# BMP/SB-FGF/LDN
## treatment

**a**

nne → *cell state transition* → ppe

**b**

→ *epithelium morphology* →

**c**



day 6 *in vitro*

BMP/SB                    BMP/SB-FGF/LDN

**a**

OEPD

E8
embryo

**b**

OEPD

A
↓
P

Pax8
Ecad
DAPI

BMP/SB-FGF/LDN
day 8

**c**

Pax8 Ecad

**d**

d8
Pax8

**e**

Ecad

**f**

Pax8 Ecad DAPI

**a**

ppe

OEPD epithelium

debris

*tissue migration*

mixture of neural and mesenchymal cells

**day 8**

**days 9-11**

otic prosensory vesicles

epidermis

**days 12-14**

| BMP/SB | BMP/SB-FGF | BMP/SB-LDN | BMP/SB-FGF/LDN |

**b** ①

**c** ①

**d** ② ①

**e** ② ③

**none**

**none**

**partial**

**complete**

*state of rearrangment on day 12*

**a** otic vesicles

lumen

pseudostratified
epitehlium

**b** d12
DIC

**c** d14
Jag1 Sox2 DAPI

**d** d14
Pax8 Pax2 DAPI

**e** embedded
organoid

**f** d16
Myo7a F-actin DAPI

**g** d20
cD1 Calb2 DAPI

**h** d20
Myo7a Brn3c DAPI

**i** protruding
organoid

>200 µm diameter

**j** d20
DIC

inner ear
organoids

k

**k** d20
Myo7a Sox2

**l** d20
Myo7a Sox2

**a**

core

mesenchyme/
neurons

● anchor  – – – teaser

non-sensory

sensory

1/4 turn, x2 cuts

sensory epithelium
(apical surface)

Hold down with
Minutien pins

**b** DIC ~1100 µm

**c** Myo7a

**d** non-sensory

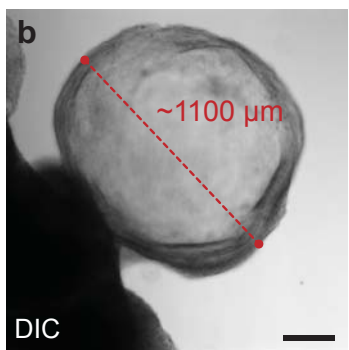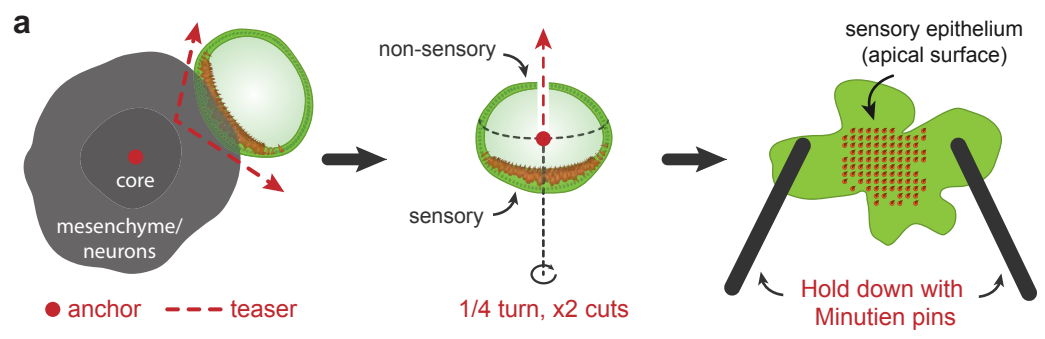sensory

Myo7a Sox2

**e** DIC

**f** recording field

k

Myo7a DAPI

**g** Myo7a F-act
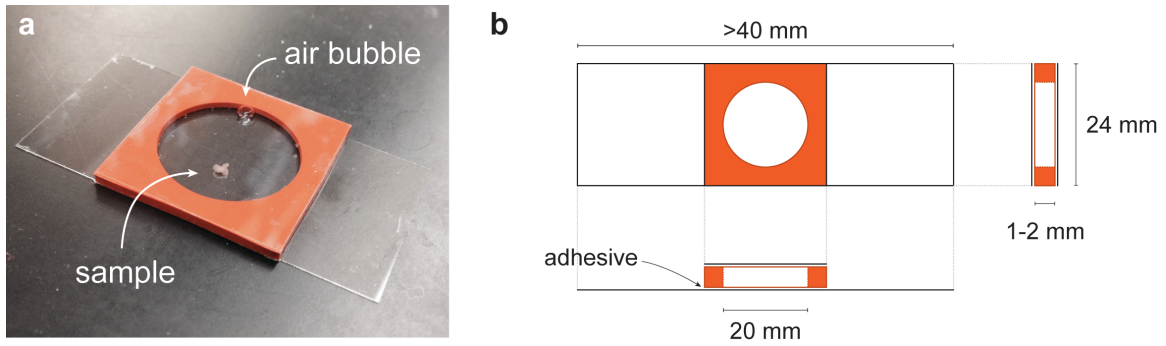
**Aggregate imaging chamber. a,** Typical imaging chamber constructed from two cover glasses and a silicone isolator. One aggregate (sample) is loaded in the chamber. Note the air bubble that forms during the loading process. **b,** Projection drawing of an aggregate imaging chamber. Note that this chamber design is an example and can be easily customized using different sized cover glasses or silicone isolators.