Supporting Materials

Materials and Methods

Generation of the advanced-passage cell cultures

Generation of the primary culture: To generate primary cell cultures, we dissected utricles from embryonic day 14 white leghorn chicken eggs and removed the otoconia. Then we used a sapphire microscalpel to trim away all of the non-sensory epithelium and the outer edges of the utricle’s hair cell epithelium. We separated the remaining sheet of pure sensory epithelium from the stroma and the utricular nerve fibers using 0.5 mg/ml thermolysin (Sigma) at 37 °C for 15 min and lifted the sensory epithelium from the stroma using the tip of a 30 ga needle. At this point, a second trimming was performed using a sapphire microscalpel to cut away all the edges from the sheet, so that only the central part was collected. We pooled the pure central-most sheets of sensory epithelium from 16 utricles and dissociated them in 0.05% trypsin-EDTA (Invitrogen) for 10 minutes at 37 °C followed by gentle trituation. We stopped the enzymatic digestion by adding 10% FBS (Hyclone) in DMEM/F-12 (Invitrogen), followed by centrifugation (200g for 5 min) and a second trituration. This produced a suspension of single cells with a few cell clumps. After the cell number was determined using a hemocytometer, the cells were plated at ~8000 cells/cm² in several wells of a Falcon 24-well plate (Becton Dickinson) in DMEM/F-12 with 10% FBS at 37°C in a 5% CO₂ atmosphere. Four independent primary cell cultures were generated from 64 ears following these procedures.

Cell expansion: Cells were passaged when they reached ~70% confluence, every ~7 days. We used 0.05% trypsin (Worthington) to passage the cells and we stopped the reaction by adding 10% FBS in DMEM/F-12 and centrifuging at 200g for 5 min. Cells were counted
using a hemocytometer, plated into additional wells at the original density of ~8000 cells/cm² and gradually expanded into larger wells and flasks during subsequent passages up to passage 19. We used some of the cells from passages for immunocytochemistry and froze cells from various passages, thawing and expanding them for later use. After passage 6, we used some samples of the cells for suspension cultures.

**Sphere generation**

We used two methods to culture cells in liquid suspension beginning at passage 6, which was reached ~6 weeks after the start of each primary culture. First, we cultured 500 µl suspensions of ~32,000 dissociated cells/ml in 24-well plates with gentle agitation in a serum-free medium containing DMEM/F-12, B27 (Invitrogen), N2 (Invitrogen) with FGF₂ (20 ng/ml, R & D system), and heparan sulfate proteoglycan (HSPG, 20 ng/ml, Sigma). More recently, we have cultured suspensions of 4000 cells in each of many 20 µl hanging drops containing the same serum-free medium. We observed the formation of spheres using phase and differential interference contrast microscopy.

**Proliferation assays**

The expansion of cultures passaged on 2-D substrates in serum-containing medium was evaluated by counting cells using a hemocytometer. Doubling times were calculated as $C_1 = C_0 \times 2^{(t_1-t_0)/td}$. $C_1$ = cell number at time point t1, $C_0$ = cell number at time point t0, and td = the doubling time of the cell population.
We determined the incidence of S-phase entry in twenty-four 2-D cultures and compared that to the incidence in thirty-six suspension cultures (6 replicates for each time points, on day 2, 4, 6, 8, 12 and 16 as shown in Fig. 1F). For the 2-D cultures the cells were plated on glass cover slips in 10% FBS+DMEM/F-12 for 4 hr. Then, the medium was replaced with serum-free media containing DMEM/F-12, B27, N2 with FGF2 and HSPG. The 36 hanging drops were cultured in parallel using the same serum-free medium. We added BrdU at 3 µg/ml (Sigma) to the cultures for 24 hr before fixation. Cultures were fixed on day 2, 4, 6, 8, 12, and 16 in 4% paraformaldehyde in 0.1 M phosphate buffer for 20 minutes at RT. We did not assay BrdU incorporation for the 2-D condition after day 8, because all the 2-D cultures reached 80-100% confluence by that point.

**Immunocytochemistry:** We incubated the BrdU-treated samples in DNase I (500 unit/ml, Sigma) for 1 hr at 37°C, followed by 10% normal donkey serum for 30 minutes. Then the samples were incubated overnight at 4°C in anti-BrdU (1:50, BD) and/or another primary antibody in 0.2% Triton X-100 in 0.1 M phosphate buffer followed by 2 hr incubation in the appropriate Cy2, Cy3, or Cy5 secondary antibody (Jackson ImmunoResearch Lab). DNA was counterstained with 4’, 6-diamidino-2-phenylindole, dihydrochloride (DAPI, 10 µg/ml, Invitrogen) and F-actin was stained with Alexa 488/568/647 phalloidin (5 unit/ml, Invitrogen) at RT. The specimens were mounted in Slow fade (Invitrogen) and images were captured using a Zeiss LSM 510 laser scanning confocal microscope or Zeiss Axiovert or Axioskop Epifluorescence microscopes equipped with cooled CCD cameras. The BrdU-positive and DAPI-stained nuclei were counted using MetaMorph software (Molecular Devices) or Volocity software (Improvision) after complete 3-D reconstruction from the confocal images of the spheres.
Mesenchymal-To-Epithelial transition assay

To assay for and localize cells that had adopted a mesenchymal cell phenotype, we used antibodies against the mesenchymal intermediate filament, vimentin (1) and the mesenchymal zinc finger transcriptional factor, slug (2), and fluorescent phalloidin labeling of F-actin organized in stress fibers. To assay for cells that had adopted an epithelial phenotype, we used antibodies against E-cadherin and N-cadherin to recognize those proteins localized to adherens junctions and fluorescent phalloidin labeling of F-actin organized in cortical bands juxtaposed to epithelial cell-cell junctions. The primary antibodies included anti-vimentin #314 (a gift from Dr. Robert Goldman, Northwestern University, Chicago, IL), anti-slug (62.1E6), anti-E-cadherin (7D6) and anti-N-cadherin (6B3, all from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences). To determine whether the epithelial cells of the spheres expressed a basal protein at the sphere's inside surface, we incubated fixed spheres with an antibody specific for avian laminin (3H11, from the Developmental Studies Hybridoma Bank).

Assay for hair cell differentiation and supporting cell identification

We used the following hair cell markers in immunocytochemical assays for hair cell differentiation: anti-myosin VIIa (1:200, Proteus), anti-calretinin (1:2000, Chemicon), anti-parvalbumin3 (1:2000, a gift from Dr. Stefan Heller, Stanford University), anti-HCS-1/otoferlin (1:100, from our laboratory) (3-6). Secondary antibodies included Cy2, Cy3, or Cy5 conjugated donkey anti-mouse, -goat, or-rabbit affinity pure IgG (Jackson ImmunoResarch Laboratory). We used SCA (anti-supporting cell antigen at 1:100, a gift
from Dr. Guy Richardson, University of Sussex, UK), an antibody specific to avian supporting cells, to label supporting cells in the spheres.

**Hair cell bundle identification**

We used scanning electron microscopy, transmission electron microscopy, and immunocytochemical labeling of the stereocilia and the kinocilium to identify the newly generated hair bundles.

*Scanning electron microscopy:* Samples were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.15 M cacodylate buffer at pH 7.4 for 1 hr at RT and postfixed in 1% OsO₄ with 0.8% K₃Fe(CN)₆ in 0.15 M cacodylate at 4°C for 2-4 hrs. Then they were dehydrated in an ethanol series, critical point dried, sputter coated with gold palladium, and examined in a JEOL 6400 scanning electron microscope equipped with an Orion (JEOL) digital image acquisition system.

*Transmission electron microscopy:* Samples were fixed as for scanning electron microscopy, embedded in epoxy, and sectioned on a Leica Ultracut UCT ultramicrotome. Thin sections were collected on formvar coated grids, stained with uranyl acetate and lead citrate, and examined on a JEOL 1230 transmission electron microscope equipped with a SIA-L3C digital camera.

*Hair bundle labeling:* To visualize F-actin in the stereocilia, we incubated specimens in Alexafluor-conjugated phalloidin at 5 unit/ml for 30 minutes. We used anti-acetylated tubulin (1:200, Sigma) to label the single eccentrically localized kinocilium of each hair bundle. We also used anti-HCA (7) (1:200, a gift from Dr. Guy Richardson, University of
Sussex, UK), an antibody specific to a 275 kDa antigen of the hair bundle to label the new bundles.

Counting of hair cells bearing bundles: We used Volocity to reconstruct complete 3-D images of spheres after immunocytochemistry, fluorescent staining with phalloidin and DAPI, and confocal microscopy. We counted the hair cells that were labeled by anti-myosin VIIa and also had phalloidin-labeled hair bundles on their apical surfaces. We counted those hair cells and all the other cells in those spheres using the DAPI labeling of their nuclei and the Volocity software.

Assay of mechanotransduction channel function

As an initial optical assay for mechanotransduction channel function, we incubated 20 spheres in DMEM/F12 containing the fixable form of cationic, styryl pyridinium dye N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridiniumdibromide, FM1-43 (5 µM, Invitrogen) for 10 seconds at RT and then rinsed them for 5 min in three changes of DMEM/F12. We immediately examined the majority of those spheres using a Zeiss LSM 510 confocal microscope. We fixed others in 4% paraformaldehyde in DMEM/F12 for 20 mins at RT and examined them by confocal microscopy later. We used Volocity and Axiovision software (Zeiss) to reconstruct 3-D views of five of the FM1-43-labeled hair cells from the stacks of confocal images that we captured at different focal depths.

Gentamicin block and recovery: To test the specificity of the FM1-43 assay, we incubated five spheres in 1 mM gentamicin for 5 minutes at RT before incubating them with 5 µM FM1-43 for 10 seconds at RT. After three rinses in DMEM/F12 for 5 minutes at RT, we examined the spheres by confocal microscopy and found that the gentamicin
pre-treatment had blocked the entry of FM1-43 into the hair cells. To determine whether the hair cells would label with FM1-43 after reversal of the gentamicin block, we washed out gentamicin in fresh DMEM/F12 for 20 minutes at 37°C in a 5% CO₂ atmosphere, then incubated the spheres in FM1-43 for 10 seconds, rinsed them for five minutes in three changes of DMEM/F12 and examined them by confocal microscopy.

References