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Using injectoporation to deliver genes to mechanosensory hair cells

Wei Xiong[1,2], **Thomas Wagner**[1], **Linxuan Yan**[1], **Nicolas Grillet**[1,2], and **Ulrich Müller**[1]

[1]Department of Molecular and Cellular Neuroscience, The Dorris Neuroscience Center, The Scripps Research Institute, La Jolla, California, USA.

Abstract

Mechanosensation, the transduction of mechanical force into electrochemical signals, allows organisms to detect touch and sound, to register movement and gravity, and to sense changes in cell volume and shape. the hair cells of the mammalian inner ear are the mechanosensors for the detection of sound and head movement. the analysis of gene function in hair cells has been hampered by the lack of an efficient gene transfer method. Here we describe a method termed injectoporation that combines tissue microinjection with electroporation to express cDNAs and shRNAs in mouse cochlear hair cells. Injectoporation allows for gene transfer into dozens of hair cells, and it is compatible with the analysis of hair cell function using imaging approaches and electrophysiology. Tissue dissection and injectoporation can be carried out within a few hours, and the tissue can be cultured for days for subsequent functional analyses.

INTRODUCTION

Organisms contain specialized cell types that are crucial for perceiving mechanical stimuli. Sensory nerve endings and support cells in the skin transmit tactile stimuli[1]. Muscle spindles and Golgi tendon organs sense muscle tension[2]. Hair cells in the mammalian cochlea and vestibule sense sound-induced vibrations and head movements, respectively[3]. For decades, hair cells have been important models for the study of the mechanisms that regulate mechanotransduction in vertebrates. The mechanically sensitive organelle of a hair cell consists of rows of stereocilia that form a tightly connected bundle at the apical hair-cell surface[4]. Mechanotransduction channels are localized in close proximity to the tips of stereocilia, and they are gated by tip-link filaments that connect the stereocilia within a hair bundle[3]. Hearing impairment is the most common form of sensory impairment in humans, and >100 genetic loci have been linked to the disease. The vast majority of the affected genes are expressed in hair cells[5–7], but the mechanisms by which they regulate hair cell function are still poorly defined. This is partly because of the fact that it has been

Correspondence should be addressed to W.X. (wei_xiong@outlook.com) or U.M. (umueller@scripps.edu).
[2]Present addresses: School of Life Sciences, Tsinghua University, Beijing, China (W.X.); Department of Otolaryngology, Stanford University, Stanford, California, USA (N.G.).

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exceptionally difficult to combine gene transfer into auditory hair cells with the subsequent analysis of their function by physiological or imaging approaches. We therefore developed an efficient gene delivery method for hair cells that relies on simple plasmid vectors. We have recently demonstrated the utility of this approach, which we term injectoporation because it combines tissue microinjection with electroporation, for the study of gene function in hair cells[8]. Here we describe an optimized version of this method, provide methodological details, and discuss potential problems and limitations of the injectoporation procedure.

Applications of the protocol

We have demonstrated that injectoporation is an efficient method for the transfer of small shRNAs and cDNAs of variable size into hair cells[8]. The longest cDNA that we have successfully expressed contains an open reading frame of 10,065 bp encoding the mouse cadherin-23 (CDH23) protein[9–12]. After gene transfer, hair cells can be cultured for at least 5 d without obvious effects on hair bundle morphology. Injectoporation is compatible with the analysis of the distribution of ectopically expressed proteins using immunofluorescence microscopy. The expression of shRNA constructs, truncated proteins or dominant negative/constitutively active constructs allows gain-of-function and loss-of-function studies to obtain insights into the molecular mechanisms that regulate hair cell development and function. Hair cell development can be analyzed by immunofluorescence microscopy and electron microscopy to reveal morphological details. Mechanotransduction can be studied by imaging approaches following the injectoporation of genetically encoded Ca^{2+} sensors (e.g., GCaMP3; refs. 8,13) or by electrophysiological techniques. cDNAs can also be expressed in hair cells from mutant mice to test for functional rescue and to carry out structure-function analysis to identify important protein domains[8]. Given the versatility of injectoporation, we anticipate that this technique will lead to the rapid functional annotation of many previously uncharacterized genes linked to hearing loss, as well as of genes that have been shown to be expressed in hair cells using microarray or proteomics approaches[14–16]. The protocol can also be modified for use with other genetically encoded indicators such as the recently described voltage indicator[17]. Injectoporation may well be suited for the introduction of proteins into hair cells or for the use of membrane permeant–targeting peptide methods[18] to achieve rapid and reversible knockdown of endogenous proteins. As such, the system can be readily adapted to meet the needs of different researchers. We anticipate that modifications of this protocol will be useful for the study of other cell types that are difficult to transfect, including mechanosensory cells in other tissues such as Merkel cells in the skin[19].

Comparison with other methods

Several gene transfer methods have been previously developed for mechanosensory hair cells. Each of them has advantages and limitations. Adenoviral vectors[20–22] and adeno-associated virus (AAV)[23–25] are particularly useful for *in vivo* gene transfer into hair cells. For example, AAV has been used as a vector to restore vesicular glutamate transporter 3 (VGLUT3) expression in VGLUT3-deficient hair cells and to rescue the associated hearing defects[26]. Adenovirus vectors have been used successfully to study gene function in cultured hair cells, for example, to express transmembrane channel-like 1 (TMC1) and TMC2 proteins[27,28]. However, virus production is time consuming, and viral coat proteins may

cause immune responses that adversely affect cell function[29]. There are also limits to the size of the transgene that can be accommodated by the viral backbone. Gene size in particular is limiting for AAV vectors, which typically can accommodate inserts of up to 5 kb, although larger inserts may be tolerated by some serotypes[30]. Biolistic ('gene-gun') transfection has been applied to hair cells, but it is inefficient, leads to cellular damage and is not compatible with physiological measurements[31]. Even with an improved biolistic configuration only a few cells per sensory epithelium are transfected[32]. As an alternative, electroporation methods have been developed, but they have been successfully applied only to inner ear tissue to study developmental processes[33,34]. An elegant electroporation method has also been used for gene transfer into the otic vesicle, but the technique is difficult to carry out and it is most useful for the study of hair cell function in situations in which the expression of transgenes or shRNAs throughout embryogenesis does not affect developmental processes[35]. The injectoporation method that we describe here circumvents many of these technological limitations and permits the interrogation of the molecular mechanisms that regulate hair cell development and function, as well as the pathophysiological mechanisms that cause disease.

Experimental design

Organotypic cochlear cultures

Figure 1a shows a diagram of the inner ear and a cross-section of the cochlea highlighting specific features such as the hair cells, stria vascularis, basilar membrane and Reissner's membrane. The mechanosensory hair cells are fragile, and special care is required during dissection. We use a six-step procedure to obtain healthy organotypic cultures (Fig. 1b–h). The inner ear from animals between postnatal day (P) 0 and P8 is dissected from the skull (Fig. 1b) and the bony capsule of the cochlea is removed with forceps (Fig. 1c). The cochlear duct is microdissected from the rest of the tissue and transferred to the lid of a 35-mm Petri dish (Fig. 1d). An incision is made along the length of the cochlear duct between Reissner's membrane and the stria vascularis (Fig. 1e). The cochlear duct is cut into a basal, a middle and an apical piece (Fig. 1f). During the entire procedure, the tissue is covered with solution to prevent mechanical damage to the hair bundles caused by shear stress at the liquid-to-air interface. The tissue fragments settle to the bottom of the Petri dish and attach to its surface (Fig. 1g) without the need for any special coating. Higher-magnification views allow for the visualization of the organ of Corti, Reissner's membrane and the stria vascularis (Fig. 1h).

Gene delivery into hair cells by injectoporation

Figure 2 shows a diagram and pictures of the injection and electroporation configuration, in which the microinjection pipette and electroporation electrodes are arranged opposite to each other at nearly 180°. The movement of the pipette and electrodes is controlled by micromanipulators, and it is visualized on an upright microscope.

We reasoned that electroporation methods for hair cells might have been difficult to carry out because of the problems of plasmid access to the cell bodies of hair cells, which are coupled apically to support cells by specialized tight junctions and adherens junctions[36–38]

(Figs. 1a and 3a). Therefore, plasmid solution that is added to the apical surface of hair cells mostly gains access to the hair bundles. Access to hair cells from the basal surface of the tissue is also limited by the cell bodies of support cells and the basilar membrane that shield the cell bodies of hair cells from the fluid-filled space of the scala tympani (Figs. 1a and 3a). We therefore inject the plasmid into the space surrounding the hair cell bodies, followed by electroporation (Fig. 3b).

Figure 3b shows a diagram of the positioning of the microinjection pipette, which depends on the cell type to be targeted. For gene transfer into outer hair cells (OHCs), the microinjection pipette is inserted between the second and third row of OHCs that are in parallel with the pipette (Fig. 3b, top; Fig. 3c). For injection into inner hair cells (IHCs), the IHC row and microinjection pipette are oriented perpendicular to each other (Fig. 3b, bottom; Fig. 3d). For each explant, the pipette is inserted only at one location. After injection, electrical pulses are applied to the tissue before removal of the microinjection pipette. Subsequently, the tissue is transferred to a humidified CO_2 incubator and maintained at 37 °C.

To monitor gene transfer into hair cells, we use vectors to express fluorescence proteins such as EGFP. Stereociliary proteins such as espin[39–41] are tagged with mCherry to highlight specific cellular structures (Fig. 3e). To quantify the efficiency of gene transfer into hair cells, we cut the cochlear tissue into an apical, middle and basal piece, and we injectoporate the tissue at varying ages with an EGFP expression vector. We determine the average number of EGFP-expressing cells in each explant after 1 d *in vitro* (Fig. 3f).

Evaluation of hair bundle morphology

Injectoporation can be combined with the analysis of hair bundle morphology by scanning electron microscopy (SEM)[8,42]. After injectoporation, EGFP fluorescence is used as a guide to identify injectoporated hair cells; histological ‘landmarks’ are introduced by scarring the tissues with suction pipettes (Fig. 4a,b). The cochlear tissue is then processed for SEM (Fig. 4c–f), and the injectoporated cells are identified by their relative position to the landmarks.

Analysis of protein distribution in hair cells

To evaluate the extent to which injectoporation is compatible with the analysis of the subcellular localization of ectopically expressed proteins in hair cells, cDNAs for stereociliary proteins such as Usher syndrome type 1G protein, SANS (scaffold protein containing ankyrin repeats and SAM domain), which is localized near stereocilia tips[43,44], and espin, which is localized throughout the hair bundle[39–41], are fused in frame to fluorescence proteins such as EGFP. The expression vectors encoding these fusion proteins are then injectoporated in OHCs. Cochlear tissue is cultured and collected for observation by fluorescence microscopy. We have successfully carried out this procedure by injectoporating hair cells between P0 and P8 and by culturing them for up to 5 d (Fig. 4g–j and data not shown).

Ca^{2+} imaging and electrophysiology to measure mechanotransduction

Mechanotransduction channels in hair cells are nonselective cation channels[45]. Mechanotransduction can therefore be monitored using Ca^{2+} imaging. However, membrane-permeable Ca^{2+} dyes have been of limited use because dyes such as Fura-2AM do not penetrate hair cells easily, and the loaded hair cells burst[46]. So far, only membrane-impermeable Ca^{2+} dyes have been successfully applied to image hair cells, but they need to be dialyzed into hair cells using whole-cell patch clamping[46,47]. Injectoporation offers the alternative to introduce genetically encoded Ca^{2+} indicators such as GCaMP3 (ref. 13) into hair cells (Fig. 5). We injectoporate hair cells between P0 and P4 with GCaMP3, culture them for one or several days and activate mechanotransduction channels with a fluid jet (Fig. 5b,c). As controls, mechanically activated Ca^{2+} responses are examined in the presence of blockers of the transducer channel (Fig. 5d; lanthanum, 100 μM; curarine, 400 μM; amiloride, 500 μM and dihydrostreptomycin, 200 μM; refs. 48–51). In parallel, we compare voltage-gated membrane currents of hair cells to determine basic membrane properties of the injectoporated cells (Fig. 5e). To measure mechanotransduction currents directly, we use whole-cell recordings while stimulating the hair bundle with a stiff glass probe (Fig. 5f).

Perturbation of gene function using shRNA and cDNA expression

The study of *CDH23* gene function is shown as an example application. During development, CDH23 is a component of the extracellular linkages that provide cohesion within the hair bundle[52–54]. In mature hair cells, CDH23 is a component of tip links[54,55], which gate mechanotransduction channels in hair cells[56,57]. We injectoporate P1 hair cells to coexpress GCaMP3, as well as an shRNA construct targeting CDH23. The effectiveness of the shRNAs to knock down CDH23 is first validated in heterologous cells (not shown). 3 d later, hair bundles are stimulated with a stiff probe or with a fluid jet to evaluate mechanotransduction currents using electrophysiology or Ca^{2+} imaging, respectively (Fig. 6). To evaluate gene knockdown, the cells are fixed and stained with CDH23-specific antibodies (Fig. 6a–d). Conversely, we express full-length CDH23 cDNAs in hair cells from CDH23-deficient Ames waltzer^{v2J} mice (Pcdh15^{av-2J} (ref. 10) to test for functional rescue. Notably, the CDH23 cDNA is >10 kb (refs. 9–11), but nevertheless functional rescue can be achieved (Fig. 6g,h).

MATERIALS

REAGENTS

- Experimental animals are chosen as required (e.g., C57BL/6 mice between E17–P6 in this protocol) **! CAUTION** All animal procedures must be carried out by following the relevant institutional guidelines and regulations.
- HBSS, no calcium, no magnesium, no phenol red (Gibco, cat. no. 14175095)
- 10× HBSS, no calcium, no magnesium, no phenol red (Gibco, cat. no. 14185052)
- DMEM/F12 (DMEM: nutrient mixture F-12), (Gibco, cat. no. 11330-057)
- DMEM/F12, no phenol red (Gibco, cat. no. 21041-025)

- FBS (Gemini, cat. no. 100–106)
- Ampicillin (BioPioneer, cat. no. c0029)
- Plasmids, as required. In this protocol, we use pEGFP-N1 (Clontech) and GCaMP3 (Addgene, cat. no. 22692) as indicators; other expression constructs to encode hair bundle proteins include CDH23 (Kazmierczak *et al*.[55]), espin-mCherry (generated by exchanging EGFP with mCherry within p-EGFP-C2-small-espin[40]) and SANS-EGFP (generated by RT-PCR from inner ear tissue and fused at their C terminus in frame to EGFP using the pEGFP-C1 vector (Clontech)). In general, plasmids using a cytomegalovirus (*CMV*) promoter have been useful for expressing cDNAs in hair cells
- shRNA plasmids, as required (e.g., shRNAs against CDH23, Sigma-Aldrich, Mission shRNA)
- Endo-free maxiprep kit (Qiagen)
- NaCl (Sigma-Aldrich, cat. no. S3104)
- NaH_2PO_4 (Sigma-Aldrich, cat. no. S3139)
- KCl (Sigma-Aldrich, cat. no. P9541)
- $CaCl_2$ (Sigma-Aldrich, cat. no. C5670)
- $MgCl_2$ (Sigma-Aldrich, cat. no. M8266)
- Glucose (Sigma-Aldrich, cat. no. G8270)
- EGTA (Sigma-Aldrich, cat. no. E3899)
- Mg-ATP (Sigma-Aldrich, cat. no. A9187)
- Na-GTP (Sigma-Aldrich, cat. no. 51120)
- H-HEPES (Sigma-Aldrich, cat. no. H4034)
- NaOH (Sigma-Aldrich, cat. no. S5881)
- Formaldehyde, 16% (vol/vol) aqueous solution (EMS, cat. no. 15710)
- Glutaraldehyde, 25% (vol/vol) aqueous solution (EMS, cat. no. 16100)
- PBS powder (BioPioneer, cat. no. MB1001)
- BSA (Sigma-Aldrich, cat. no. A3059)
- Triton X-100 (Sigma-Aldrich, cat. no. T8532)
- Dako pen (Dako, cat. no. 2012-12)
- Primary antibodies, as required. We use chicken anti-GFP at a 1 to 500 (vol/vol) dilution (Abcam, cat. no. ab13970) and customized rabbit anti-CDH23 at a 1 to 500 (vol/vol) dilution (Kazmierczak *et al*.[55]).
- Phalloidin, as required. We use Alexa Fluor 488 phalloidin (Molecular Probes, cat. no. A12379) and Alexa Fluor 647 Phalloidin (Molecular Probes, cat. no. A22287)

- Secondary antibodies, as required. We use Alexa Fluor 488 goat anti-chicken IgG (Invitrogen, cat. no. A11039, 1:2,000 dilution) and Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen, cat. no. A11036, 1:2,000 dilution)
- Mounting medium, e.g., ProLong Gold (Life Technologies)
- Nail polish, transparent and fast-dry (any brand)
- Paraformaldehyde
- Distilled, deionized water
- Ethanol, 70% (vol/vol)

EQUIPMENT

- Dissection tools, as required; e.g., rough and fine scissors, a pair of fine forceps and a curette (FST)
- Culture dishes, e.g., diameters 35 mm and 10 cm (e.g., Greiner CellStar)
- Dissection scope (e.g., Nikon SMZ1000)
- Humidified incubator (37 °C, 5% CO_2, e.g., Fisher Scientific Isotemp)
- Desktop centrifuge (e.g., Eppendorf 5417)
- Borosilicate glass capillary (e.g., Sutter, cat. no. BF150-117-10)
- Pipette puller (horizontal or vertical, e.g., Sutter P-97)
- Micro-forger (Narishige, no. MF-830)
- Anti-vibration table and Faraday cage (e.g., TMC)
- Pressurized gas tank with N_2 and regulators (any brand)
- Upright microscope (e.g., Olympus BX51WI) with a low-power objective (e.g., UPLFLN 4×, Olympus) and a high-power water-immersion objective (e.g., LUMplanFl/IR 60×, Olympus)
- Micromanipulators and patch-clamp stage holder (e.g., Sutter MP-285)
- Platinum wires, 0.016-inch diameter (e.g., Surepure Chemetals, 99.95% pure)
- Electroporation pipette holder (e.g., Narishige H-1)
- Microinjection pipette holder (e.g., Warner MP series)
- Two-channel switchers, Luers and tubing (e.g., Value Plastics)
- Square-wave electroporator (e.g., BTX, cat. no. ECM 830)
- CCD camera (e.g., Q-imaging ROLERA-RX)
- Illuminator (e.g., Sutter DG-4)
- Fluid-jet dispense instrument (e.g., Parker Picospritzer)
- Compressed N_2 (any brand)

- Imaging software (e.g., ImageJ, US National Institutes of Health (NIH)/ University of California San Francisco (UCSF) Micro-Manager)
- Peristaltic pump (e.g., WPI Peri-Star)
- Patch-clamp amplifier system (e.g., HEKA EPC10)
- Data analysis software (e.g., Wavemetrics Igor Pro)
- Piezoelectric stack actuator (e.g., Physik Instrument P-885)
- Deconvolution or confocal microscopy system (e.g., DeltaVision, Applied Precision)
- Round cover glass (e.g., Fisher Scientific, cat. no. 15CIR-2)
- Critical point dryer (e.g., Tousimis Atosamidri-815)
- Scanning electron microscope (e.g., Hitachi S-4800-ll field emission scanning electron microscope)

REAGENT SETUP

▲ **CRITICAL** All solutions, except where indicated, can be stored at 4 °C for up to 3 months.

Dissection solution—Dissection solution is HBSS containing 0.1 mM $CaCl_2$ and 1.5 μg/ml ampicillin.

Electroporation medium—Add 1.5 μg/ml ampicillin to DMEM/F12.

Culture medium—Culture medium is DMEM/F12 containing 1% (vol/vol) heat-inactivated FBS and 1.5 μg/ml ampicillin.

Plasmid solution for electroporation—Prepare the plasmids using endotoxin-free solutions. Dilute the plasmid with endotoxin-free water to a concentration of 3–5 μg/μl to prepare a stock solution. Store the stock solution at −20 °C. For the final working solution, dilute the indicator plasmid (e.g., GCaMP3 or shRNA) to 0.5 μg/μl and cDNA constructs to 1 μg/μl. Plasmids encoding cDNAs can be injected at varying concentrations, but we see consistently good expression with the indicated plasmid concentration of 1 μg/μl. Plasmids for GCaMP3 and shRNAs can be injected at varying concentrations, but we observed reliable expression without toxic effects at the indicated concentration of 0.5 μg/μl. Concentrations might vary for different shRNA constructs, which should routinely be tested for gene knockdown in heterologous cells before use in hair cells. The osmolarity of the working solution is adjusted to 330–350 mOsm with 10× HBSS. The working solution can be stored at 4 °C for up to 1 week.

External recording solution—External recording solution is 144 mM NaCl, 0.7 mM NaH_2PO_4, 5.8 mM KCl, 1.3 mM $CaCl_2$, 0.9 mM $MgCl_2$, 5.6 mM glucose and 10 mM H-HEPES (pH 7.4).

Internal recording solution—Internal recording solution is 140 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 2 mM Mg-ATP, 0.3 mM Na-GTP and 10 mM H-HEPES (pH 7.2). Add Mg-ATP and Na-GTP before use.

PBS—Dissolve 1 bag of PBS powder in 1,000 ml of ddH_2O.

Fixation solution—Fixation solution is 4% (vol/vol) paraformaldehyde in PBS. This solution is freshly made.

Wash solution—Wash solution is 0.5% (vol/vol) Triton X-100 in PBS.

Blocking and permeabilizing solution—Blocking and permeabilizing solution is 4% (wt/vol) BSA and 0.5% (vol/vol) Triton X-100 in PBS. This solution is freshly made.

Immunostaining solution—Immunostaining solution is 1% BSA and 0.5% (vol/vol) Triton X-100 in PBS. This solution is freshly made.

Fixative for SEM—Fixative for SEM is 2.5% (vol/vol) glutaraldehyde, 4% (vol/vol) formaldehyde, 0.05 mM HEPES buffer, pH 7.2, 10 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.9% (wt/vol) NaCl. This is freshly made.

Washing buffer for SEM—Washing buffer for SEM is 0.05 mM HEPES buffer (pH 7.2), 10 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.9% (wt/vol) NaCl.

EQUIPMENT SETUP

Preparation of electroporation electrodes and injectoporation setup—Fix a pair of platinum wires on the electroporation electrode holder. Bend the two platinum wires into an ‘L’ shape; the distance between the two wires should be adjusted to 2–3 mm (Fig. 2c–e). Mount the electroporation electrode holder on the left-hand micromanipulator. Mount the microinjection pipette holder (Warner MP series) on the right-hand micromanipulator (Fig. 2a,b). We recommend assembling the two micromanipulators to face each other. The inlet of the microinjection pipette is connected to a two-channel switcher and to a 1-ml syringe through plastic tubing (Fig. 2a). After injectoporation and subsequent culture, the tissue is ready for functional analyses including Ca^{2+} imaging or electrophysiology, or for immunohistochemistry and SEM analysis.

Preparation of microinjection pipettes and suction pipettes—We use Sutter glass capillaries to produce micropipettes. The RAMP value is 477, as measured by our Sutter P-97 micropipette puller. The parameters for pulling microinjection pipettes are as follows: 520 (HEAT), 0 (PULL), 40 (VELOCITY) and 250 (TIME). The microinjection pipettes have a diameter of 2–3 μm, and they are freshly made before injectoporation. Before imaging and electrophysiology, the tectorial membrane is removed using a suction pipette (Fig. 5a). Suction pipettes are produced by breaking the tips of regular microinjection pipettes to 5–10 μm diameter, which are visualized by a micro-forger.

Ca^{2+} imaging and fluid-jet stimulation—The microscope needs to be equipped for Ca^{2+} imaging[58]. GCaMP3 is expressed in hair cells for monitoring Ca^{2+} signals elicited by hair bundle deflection. The Micro-manager software is used to drive the illuminator and camera[59]. 120 fluorescence images of Ca^{2+} responses are acquired at a sample rate of two per second. The hair bundle is stimulated with a fluid jet that uses the Parker Picospritzer connected to a nitrogen tank to apply air pressure to the microinjection system. Microinjection pipettes are used as fluid-jet pipettes. The pipette is back-filled with the external solution and mounted in the microinjection pipette holder. With the air pressurized, adjust the air regulator on the Picospritzer to have a reading of 10 p.s.i. on the pressure gauge. During image acquisition, provide stimuli of 0.1-, 0.3- and 0.5-s duration at 80-s intervals by operating the knobs on the front panel of the Picospritzer.

Whole-cell recording and stiff-probe stimulation—Set up the electrophysiology and mechanical stimulation systems as described[60,61]. In our case, injectoporated and cultured cochlear OHCs are used for electrophysiological analyses. As mentioned before, the dish lid is used for culturing cochlear tissues. The dish lid also serves as the recording chamber. The right-hand micromanipulator is used to place the patch-clamp headstage and recording pipette. We use HEKA EPC10 to record whole-cell currents of hair cells. The left-hand micromanipulator holds the mechanical stimulation probe set. Briefly, the stimulation probe is made by fire-polishing a patch pipette to a round tip with a diameter of 4–6 μm. The stimulation probe is fixed onto the piezoelectric actuator that is mounted on a pipette-holder and positioned at an angle of 15°. Use the analog-out channel of the EPC10 to send voltage steps to drive a 10× homemade amplifier that controls the movements of the piezoelectric stack actuator. The displacement dynamics of the piezoelectric actuator need to be calibrated if it is used for the first time. A stage micrometer is used to measure the ratio of displacement to the input voltage. Set the driving voltages by referring to the calibration results. Igor software is used to analyze the electrophysiological data. For step-by-step instructions for hair-cell recordings, see the PROCEDURE below.

PROCEDURE

Dissection and culture ● TIMING ~60 min

1| Disinfect the dissection tools with 70% (vol/vol) ethanol. Wipe the dissection scope and surgery area with 70% (vol/vol) ethanol.

2| Prepare Petri dishes for dissection and culture. Fill the dissection dishes with cold dissection solution. Fill the culture dishes with culture medium.

▲ **CRITICAL STEP** We recommend using the 35-mm Petri-dish lids for culture. This allows successful positioning of the electrodes for electroporation and subsequent electrophysiology.

3| Decapitate the mouse after anesthesia. Spray the head with 70% (vol/vol) ethanol. Open the skull, remove the brain and expose the inner ears. Transfer the inner ears into the dissection solution (Fig. 1b, cochlea part shown in brackets).

4| Use forceps to remove the bony capsule of the cochlea (Fig. 1c, cochlear coil shown in brackets). Lift the cochlear tissue off from the modiolus. Transfer the cochlear tissue to the lid of a 35-mm Petri dish containing electroporation medium (Fig. 1d).

▲ **CRITICAL STEP** Use the microcurette to transfer tissues.

5| Rip a seam between Reissner's membrane and the stria vascularis (Fig. 1e). Cut the cochlea into three fragments of approximately equal size (apical, middle and basal coils; Fig. 1f). Attach the cochlear fragments to the bottom of the culture dish (Fig. 1g,h).

▲ **CRITICAL STEP** It is not necessary to remove the tectorial membrane at this step. Perform dissection as fast as possible.

? **TROUBLESHOOTING**

6| Place the culture dishes into a 10-cm Petri dish. Transfer the 10-cm dish to the incubator (37 °C, 5% CO_2). After 4–6 h, the cochlear fragments are ready for injectoporation.

▲ **CRITICAL STEP** Use the 10-cm Petri dish as a humidifying chamber. It can hold three 35-mm Petri-dish lids.

Injectoporation ● TIMING ~60 min

7| Prepare the plasmid working solution as mentioned in Reagent Setup.

▲ **CRITICAL STEP** Spin the working solution at 18,000*g* for 10 min at 4 °C to keep the injection pipettes from clogging.

8| Spray the electroporation electrodes with 70% (vol/vol) ethanol. Wipe the high-power objective with 70% (vol/vol) ethanol. Let them dry in air.

▲ **CRITICAL STEP** Disinfect the high-power objective and the electroporation electrode to prevent the tissue culture from possible contamination.

9| Use the pipette puller to produce microinjection pipettes with a tip diameter of 2–3 μm.

▲ **CRITICAL STEP** The diameter is crucial. A microinjection pipette with larger tip diameter might destroy the tissue, whereas a microinjection pipette with smaller tip diameter might be easily clogged.

10| Take the culture dish from the incubator and place the dish on the recording stage of the upright microscope. Determine the region of hair cells to be transfected by examining hair-cell morphology with a high-power objective. Switch back to the low-power objective. Use the left-hand micromanipulator to position the electroporation electrodes so that the cochlear fragment is between the two electrodes (Fig. 2c–e).

▲ **CRITICAL STEP** Choose hair cells in tissue that is flattened out well. Unsuccessful dissection can introduce damage to hair cells. Avoid the region with unhealthy cochlear hair

cells, which can be recognized by the abnormal morphology of the rows of stereocilia and the presence of necrotic tissue.

11| Use a 2-μl pipette tip to back-fill the microinjection pipette with 1 μl of plasmid solution. Mount the microinjection pipette in the pipette holder. Apply ~0.1 ml of air pressure to the microinjection pipette through the 1-ml syringe, and hold the air pressure by turning the two-channel switcher to the OFF position. Use the right-hand micromanipulator to move the microinjection pipette near the top of the hair cells (Fig. 2b–e). Visualize by high-power objective and move the microinjection pipette to penetrate the apical surface of the sensory epithelium.

▲ **CRITICAL STEP** For OHCs, the microinjection pipette is in parallel with the hair-cell row and the injection site is between the second and third row (Fig. 3b, top; Fig. 3c). For IHCs, the microinjection pipette is placed perpendicular to the row of IHCs and the injection site is next to the hair cell bodies (Fig. 3b, bottom; Fig. 3d).

? **TROUBLESHOOTING**

12| Lift up the high-power objective. Perfuse the tissue for 10 s by constantly applying 0.1 ml of positive air pressure to the microinjection pipette. Apply electroporation. Many bubbles emerge during electroporation. Withdraw the microinjection pipette.

▲ **CRITICAL STEP** We recommend an electroporation protocol of three pulses at a 60-V amplitude, a 15-ms duration and a 1-s interval. Keep constant positive air pressure on the microinjection pipette to maintain a relatively high concentration of plasmid solution during injectoporation. Injectoporate once per cochlear fragment.

13| For multiple cochlear tissues to be transfected, repeat Steps 10–12. The microinjection pipette can be used again if there is no clog.

14| After electroporation, change half of the solution to the culture medium. Add 1 ml of sterile fluid between the 35-mm Petri dishes to maintain the moisture. Transfer the dishes back to the humidified CO_2 incubator. Loosely cover the 10-cm dish in the incubator. For prolonged culturing, replace half of the culture medium every other day with fresh culture medium.

▲ **CRITICAL STEP** Change the medium after electroporation, as the electroporation can change the pH of the solution. Prepare culture medium ahead of time to equilibrate the medium to room temperature (RT, 20–24 °C).

? **TROUBLESHOOTING**

Morphological or physiological analysis

15| Proceed to functional studies as early as 6–8 h after injectoporation. Explants can be cultured for at least 5 d. Follow option A for immunostaining, option B for calcium imaging, option C for electrophysiology and option D for SEM.

A. Immunostaining ● TIMING 2–3 d

i. Carry out all procedures in the culture dish. Fix the cochlear tissue for 30 min with 4% (vol/vol) paraformaldehyde solution. Remove the tectorial membrane. Wash the tissue three times (10 min) in PBS on a shaker.

■ **PAUSE POINT** The fixed tissue can be stored at 4 °C for up to 1 week.

ii. Remove PBS from the dish. Use the Dako pen to draw a circle of 20 mm in diameter around the tissues. Add 100 μl of blocking and permeabilizing solution in the circled region. Bathe the tissue for 1 h at RT on a shaker.

iii. Prepare the primary antibody in immunostaining solution with proper dilution. Add the primary antibody solution to the tissue. Incubate the tissue on a shaker overnight at 4 °C.

iv. Rinse the tissue several times with wash solution. Dilute the secondary antibody in the immunostaining solution. Incubate the tissue in the secondary antibody solution for 2 h at RT.

v. Rinse the tissue several times with wash solution. Embed the tissues with a drop of mounting medium. Cover the tissues with a 15-mm round cover-glass slip.

vi. Wait overnight to let the cover glass flatten out the tissue. Use nail polish to seal the cover glass. Acquire fluorescence images.

? **TROUBLESHOOTING**

B. Calcium imaging ● TIMING 30–120 min

i. Use DMEM/F12 without phenol red as the culture medium when Ca^{2+} imaging is applied for functional analysis.

ii. Turn on the required instruments, such as the microscope, illuminator, camera and computer. Open the imaging software to make sure that all hardware is online. Check the air flow of the tubing system for fluid-jet stimulation. Prepare fluid-jet electrodes and suction pipettes.

iii. Use the culture dish as the imaging chamber. Place the dish on the stage of the microscope. Use suction pipettes to remove the tectorial membrane and cell debris (Fig. 5a).

? **TROUBLESHOOTING**

iv. Find a hair cell expressing GCaMP3 by monitoring baseline fluorescence. Set the exposure time to assure sufficient capacity of the camera to monitor the dynamic range of the Ca^{2+} signal. Back-fill a fluid-jet pipette with external solution. Position the fluid-jet electrode tip close to the hair bundle.

▲ **CRITICAL STEP** Keep a distance of ~5 μm between the fluid-jet electrode tip and the hair bundle (Fig. 5b, inset). Too close or too far does not generate a reproducible Ca^{2+} response.

v. Perform this step only if it is required. For phamacological tests, add agonist/antagonist into the recording solution and fluid jet before Ca^{2+} imaging.

vi. Choose the region of interest in the imaging software. Adjust the focus plane to the cuticular plate. Start multi-image acquisitions. Stimulate the hair bundle with the fluid jet during acquisition.

▲ **CRITICAL STEP** Use three sequential fluid-jet pulses of 0.1-, 0.3- and 0.5-s duration. Healthy OHCs provide a Ca^{2+} signal with graded intensities (Fig. 5b).

? **TROUBLESHOOTING**

C. Electrophysiology ● TIMING 30–120 min

i. Use culture dish lids as recording chambers. Before recordings, replace the culture medium with external recording solution. Keep the explants for 10 min in the incubator for recovery. Turn on the illuminator if fluorescent hair cells are to be recorded. Perform hair-cell recordings at RT.

ii. Use the pipette puller and micro-forger to make patch pipettes with a resistance of 3–5 MOhm. The parameters are the same as those used to make microinjection pipettes, except that the VELOCITY is changed from 40 to 55. Prepare suction pipettes with a tip diameter of 5–10 μm, as mentioned above.

iii. If a stimulation probe has already been prepared, skip this step. Otherwise, polish the stimulation probe from a patch pipette to a tip diameter of 4–6 μm. Mount the stimulation probe on the piezo actuator.

iv. Transfer the culture dishes containing cochlear tissues onto the stage of the upright microscope. Perfuse the dish with external recording solution using the peristaltic pump.

v. Use the high-power objective to visualize the cochlear tissue. Remove the tectorial membrane with a suction pipette (Fig. 5a). Locate a transfected OHC (indicated by fluorescence). Move the stimulation probe carefully and position it around the hair bundle.

▲ **CRITICAL STEP** Remove the tectorial membrane and cell debris with suction pipettes before recordings.

vi. Use a suction pipette to make a hole around the hair cell to be recorded to gain access to the cell body. Back-fill a patch-clamp pipette with internal recording solution and mount it into the pipette holder. Whole-cell patch-clamp the hair cell by sealing the lateral wall of the cell body. Set the holding potential to −70 mV. Precisely position the stimulation probe to make sure that it is in contact with the hair bundle. Measure mechanotransduction currents by replacing the stimulation probe from −400 to 1,000 nm at 200-nm steps.

? **TROUBLESHOOTING**

D. Scanning electron microscopy ● TIMING 2–3 d

i. Visualize injectoporated cochlear tissue under the fluorescence microscope with a high-power objective (Fig. 4a). Find EGFP-expressing cells. Make incisions as landmarks on the cochlear tissue with suction pipettes (Fig. 4b). These landmarks help in the identification of the EGFP-expressing cells. Take pictures that contain the EGFP-expressing cells and landmarks. Repeat all these steps if there are multiple cochlear tissues.

ii. Fix the tissues for 2 h at RT with freshly made fixative and remove the tectorial membrane. Dehydrate the samples, and process the tissues to the critical drying point using an Atosamidri-815 (Tousimis) or similar. Mount the tissue with carbon tape and coat it with iridium.

iii. Image the samples with a Hitachi S-4800-ll field emission scanning electron microscope or similar. The transfected cells are identified using the incision marks on the tissue.

? **TROUBLESHOOTING**

Step 5: damaged hair bundles in cultured hair cells—During dissection and transfer, the cochlear tissue has to be kept immersed in solution to prevent damage to the hair bundles. Avoid touching the hair cells, especially the hair bundles.

Step 11: damaged hair cells during injectoporation—The microinjection must be very gentle but effective. Position the tip of the injection pipette along the plane of the hair cell bodies. You will see the hair cell bodies float up when the microinjection pipette enters the tissue. If you are not sure of the pipette position, move the microinjection pipette back and forth and find the right plane. Do not give excessive air pressure, as it can destroy the tissue. If the electrode is clogged, change to a new one.

Step 14: poor gene transfer efficiency—Very few hair cells may be injectoporated. If some supporting cells are transfected, it indicates that your electroporation step is good but the microinjection step might not be successful. The microinjection pipette might be positioned too low and within the supporting cell layer.

Step 15A(vi): dim staining—If you are not used to doing immunostaining in a dish, the tissue can be transferred to a 96-well plate. However, be careful to mount the cochlear tissue in the upside-up orientation. Always use fresh solutions, especially the fixation solution. Follow the instructions to prepare the antibody solution. Higher antibody concentrations might increase the background. If exogenous fusion protein is expressed and the fluorescent signal is dim, coexpression of indicator constructs such as EGFP will help locate the transfected hair cells.

Step 15B(iii, vi): weak ca^{2+} responses—After injectoporation and subsequent culture, the tectorial membrane sometimes is assembled again on the top of hair cells. Use a suction

pipette to remove the tectorial membrane, dead cells and debris. Incomplete cleaning will cause minimal detection of Ca^{2+} signal. We recommend three sequential fluid-jet stimuli of increasing duration (e.g., 0.1, 0.3 and 0.5 s) at 80-s intervals. Only hair cells with graded Ca^{2+} signals are suitable for analysis. The Ca^{2+} responses induced by the 0.3-s fluid-jet stimulations are used for quantifications.

Step 15C(vi): low rate of whole-cell sealing—The hair cells in cultured cochlea behave somewhat differently from those in acutely dissociated cochlea. You may need more practice to get used to whole-cell patch clamp–cultured hair cells. Avoid hair cells expressing very bright fluorescence.

● **TIMING—**Steps 1–6, dissection and culture: ~60 min

Steps 7–14, injectoporation: ~60 min

Step 15A, immunostaining: 2–3 d

Step 15B, calcium imaging: 30–120 min

Step 15C, electrophysiology: 30–120 min

Step 15D, scanning electron microscopy: 2–3 d

ANTICIPATE RESULTS

In this section, we describe some typical results that we have obtained using this protocol.

Transfection of hair cells

By following this protocol, EGFP and espin-mCherry can be delivered into cochlear hair cells with high efficiency. Robust EGFP signals are observed in the cell bodies of hair cells, and espin-mCherry is detected in the hair bundles (Fig. 3e) within 4 h after injectoporation regardless of the age of explants. The signal is detectable for at least 5 d after injectoporation. Hair cells can be efficiently injectoporated at embryonic ages, as well as postnatal ages between P0 and P8 (Fig. 3f, data not shown). By controlling the position of the microinjection pipette, genes can also be transferred into various supporting cell types (Supplementary Fig. 1).

Quantification of gene-transferred hair cells

The number of transfected hair cells is relatively high (Fig. 3f). For instance, nearly 30 hair cells can be transfected at P2 in the apical cochlear turn. The injectoporation efficiency decreased with increasing age of the cochlear tissue. Hair cells in the apical cochlea are electroporated more efficiently than those in the basal part, with a gradient in between.

Morphology of hair bundles

Hair bundle morphology is not detectably altered in most of the untransfected and transfected hair cells during the culture period (Fig. 4c–f).

Localization of stereociliary proteins

Consistent with the reported expression pattern of the endogenous proteins[40,62,63], SANS-EGFP is localized toward sterociliary tips (Fig. 4g,h), whereas espin-EGFP labels the entire hair bundle (Fig. 4i,j).

Ca^{2+} imaging of mechanotransduction responses

Upon mechanical stimulation of hair bundles with a fluid jet, fluorescence intensity increases robustly in GCaMP3-expressing OHCs (Fig. 5b). The maximal amplitude of the stimulus-evoked signal increased from P0 to P4 (Fig. 5c), which is in agreement with the observation that transducer currents increase in amplitude during hair cell maturation[64]. Little increase in fluorescence intensity is observed in the presence of blockers of the transducer channel (Fig. 5d).

Patch-clamp recording of membrane currents

The I-V curves are indistinguishable between injectoporated hair cells expressing EGFP and noninjectoporated control hair cells in nearby tissue (Fig. 5e). Injectoporated and control OHCs display rapidly activating transducer currents, which subsequently adapted (Fig. 5f). The amplitudes of saturated transducer currents at maximal deflection (>1μm) evoked with a stiff probe are comparable in injectoporated and control cells (Fig. 5f), but they are ~10% smaller than those obtained in acute preparations[8,42,64,65]. The current displacement plots reveal no differences between control and injectoporated cells (Fig. 5f).

Perturbation of gene function using shRNA

CDH23 expression is nearly abolished in cells expressing GCaMP3 and shCDH23, but not in neighboring GCaMP3-negative cells or in cells electroporated with a control shScramble construct (Fig. 6a–d, data not shown). Transducer currents and Ca^{2+} signals are nearly abolished in injectoporated cells expressing shCDH23 but not in control cells (Fig. 6e,f).

Functional rescue using cDNA expression

The morphological hair bundle defects caused by the CDH23 mutation are rescued after expressing full-length CDH23 cDNA in hair cells from CDH23-deficient Ames waltzer^{v2J} mice (Fig. 6g). The mechanotransducer responses are restored (Fig. 6h). Injectoporation is therefore suitable to express even large cDNAs in hair cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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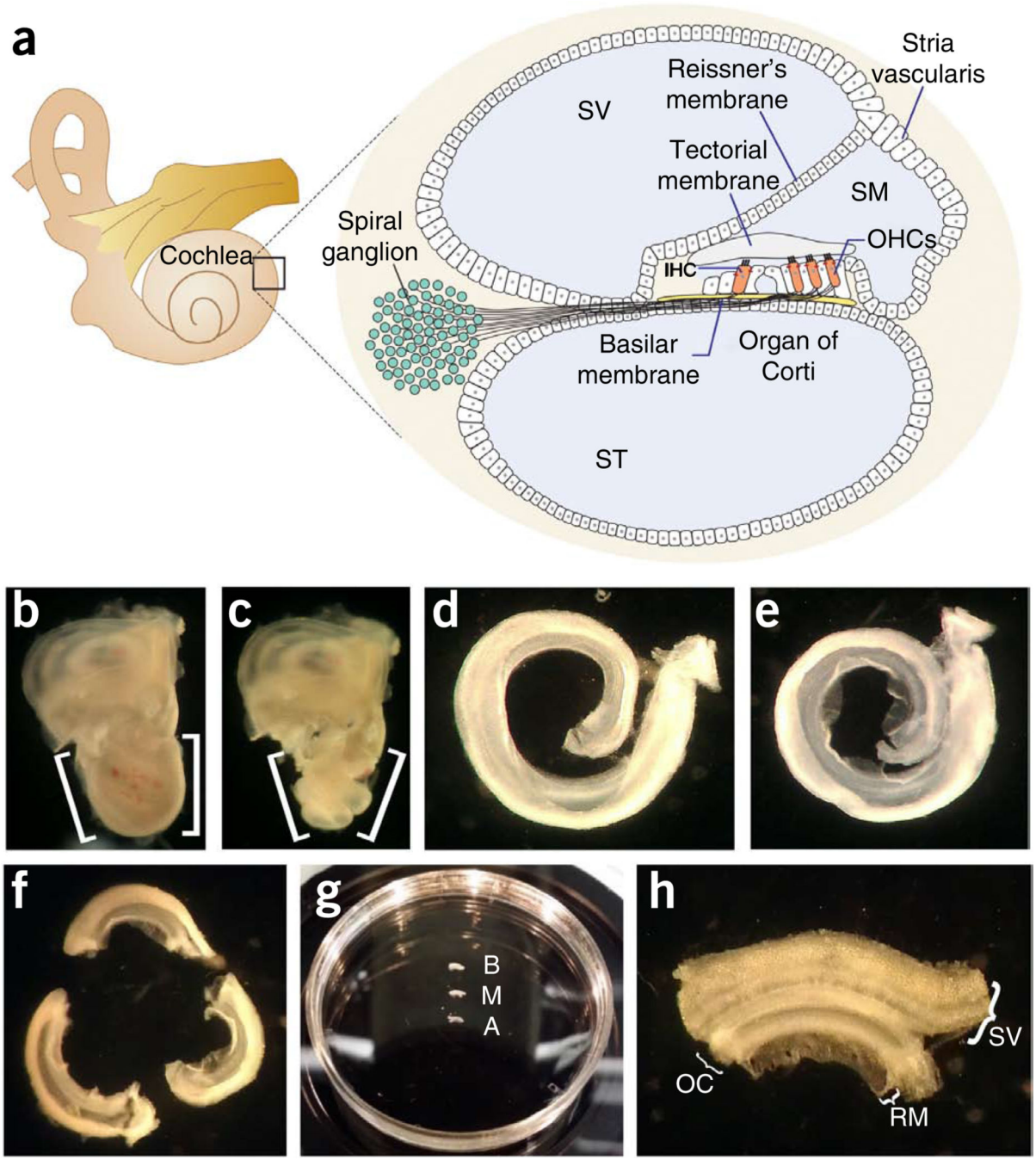

Figure 1.

Tissue dissection. (**a**) Diagram of the cochlea with a cross-section on the right (SV, scala vestibule; SM, scala media and ST, scala tympani). (**b–h**) Images of the dissection procedure. (**b**) The inner ear tissue was removed from the skull. The part containing the cochlea is indicated by white brackets. (**c**) Exposed cochlear duct (white brackets) after removal of the surrounding bony capsule. (**d**) The cochlear duct detached from the inner ear. (**e**) The cochlear duct is cut open between Reissner's membrane and stria vascularis. (**f**) The cochlear duct is cut into apical, middle and basal fragments. (**g**) The cochlear tissues

attaches to the culture dish (B, basal; M, middle and A, apical). (**h**) A close-up view of attached cochlear fragment (SV, stria vascularis; OC, organ of Corti and RM, Reissner's membrane).

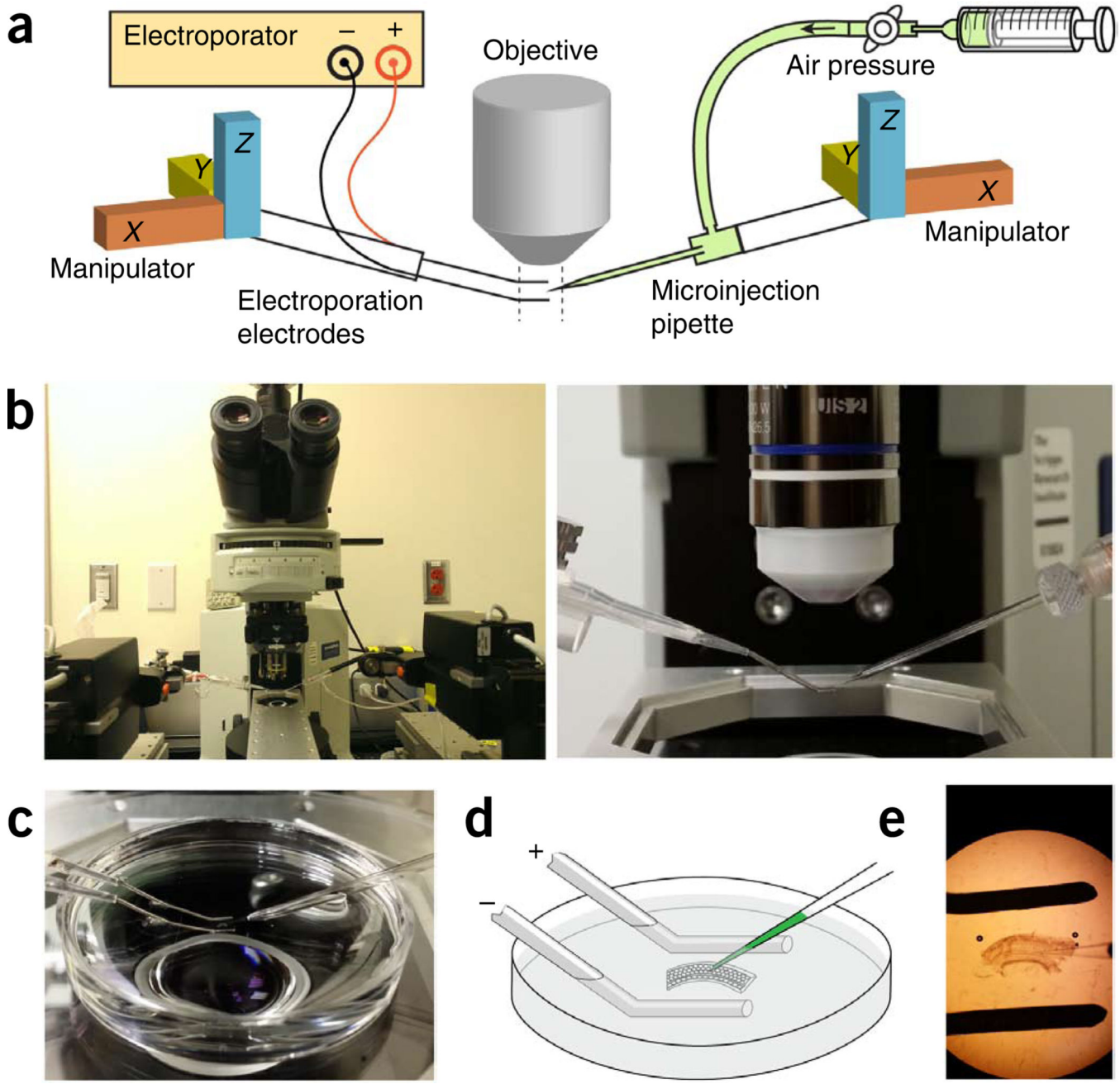

Figure 2.

Injectoporation setup. (**a**) Diagram of the injectoporation setup. (**b**) Left, image of the injectoporation setup. Note that the electroporator is not shown. Right, horizontal view of the electroporation electrodes and microinjection pipette. (**c–e**) Placement of the cochlear fragment, the electroporation electrodes and the microinjection pipette in a dish lid. (**c**) A photo view. (**d**) A cartoon view. (**e**) A microscopic view.

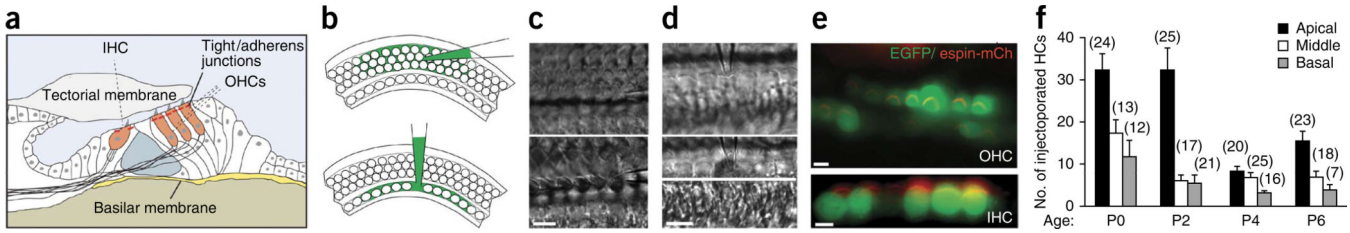

Figure 3.

Diagram of the microinjection procedure and evaluation of the injectoporation efficiency. (**a**) The diagram shows a cross-section of the organ of Corti. (**b**) For gene transfer into OHCs, the microinjection pipette penetrates the surface between the second and third row of OHCs (top); to transfect IHCs, the microinjection pipette penetrates the surface of an IHC and then delivers plasmid to the neighboring IHCs (bottom). The plasmid solution is shown in green. (**c**) Images of the cochlear tissue before (top) and after (bottom) microinjection of OHCs. (**d**) Images of the cochlear tissue before (top) and after (bottom) microinjection of IHCs. (**e**) Cochlear tissues from P4 animals are co-injectoporated with an expression vector for EGFP and espin-mCherry and analyzed after 1 d *in vitro* (DIV). The top image shows OHCs and the lower image shows IHCs. Note EGFP signal in the cell body, and espin-mCherry signal in stereocilia. (**f**) The number of EGFP-expressing P4 hair cells per fragment determined after 1 DIV. The apical, middle and basal fragments of the cochleae were counted separately. P0 (apical: 32.3 ± 3.8; middle: 17.3 ± 3.1 and basal: 11.8 ± 3.7), P2 (apical: 32.3 ± 5.1; middle: 6.2 ± 1.2 and basal: 5.6 ± 1.1), P4 (apical: 8.4 ± 1.0; middle: 6.9 ± 1.0 and basal: 3.1 ± 0.4) and P6 (apical: 15.4 ± 2.3; middle: 7.1 ± 1.2 and basal: 3.9 ± 1.2). The number of analyzed cochlear explants is indicated on the top of each bar. Values are means ± s.e.m. Scale bars, 10 μm (**c,d**) and 5 μm (**e**).

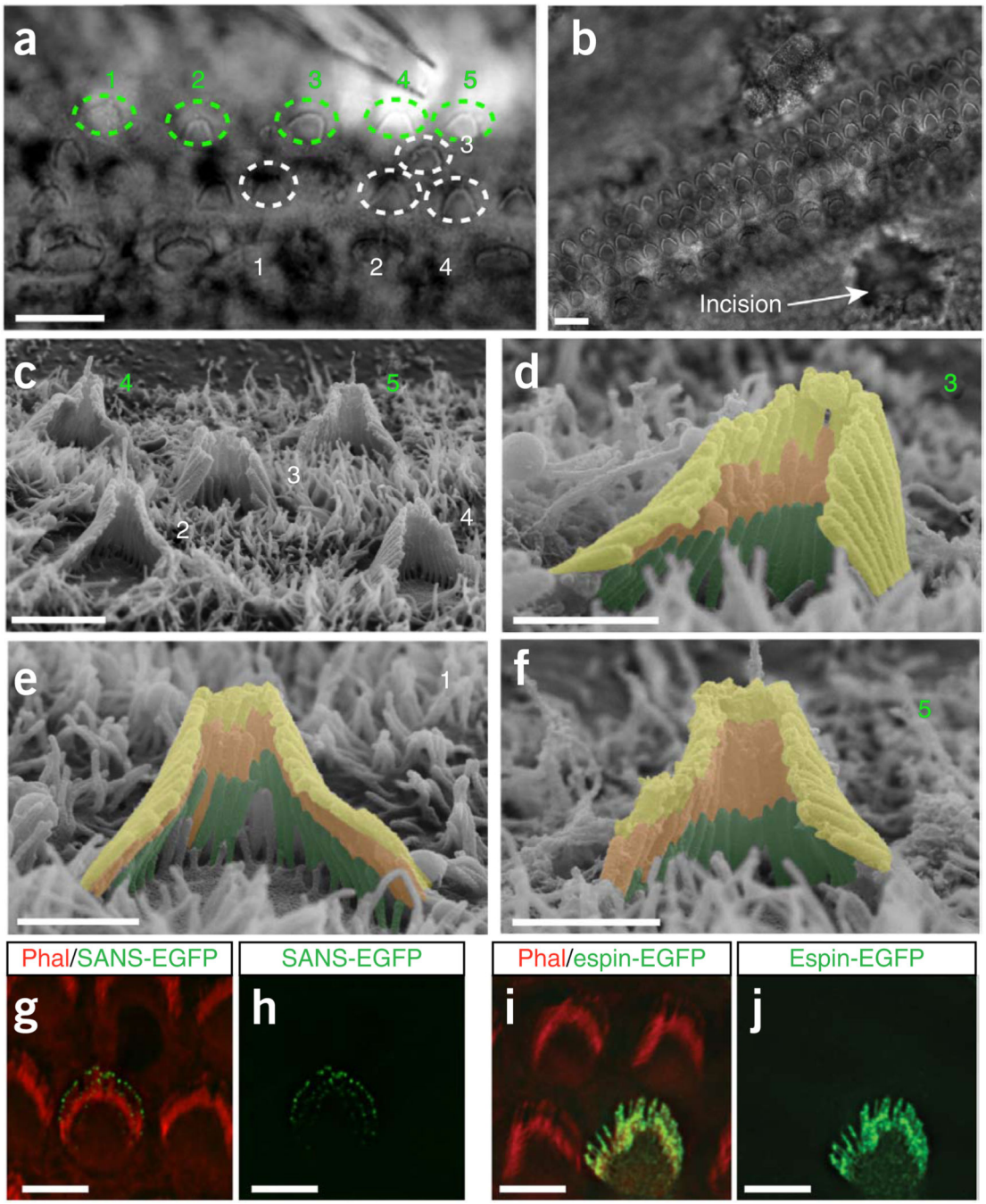

Figure 4.

Histological analysis of injectoporated hair cells. (**a**) The image shows transfected OHCs within cochlear tissue. The tissue was injectoporated at P4 to express EGFP and imaged after 1 d *in vitro* (DIV). Cells expressing EGFP are marked with green circles and numbers. Cells that did not show a clear fluorescence signal and therefore did not efficiently take up or express the EGFP expression vector are indicated with white circles and numbers. Note that a suction pipette is used to remove the cell debris. (**b**) The image shows the cochlear tissue with an incision as a landmark. (**c–f**) SEM images of the same cochlear tissue shown

in **a**. (**c**) Overview of the cells shown in **a**. The stereocilia are false-colored. Hair cells are numbered. Hair bundle morphology is not significantly affected by injectoporation. (**g–j**) OHCs are injectoporated at P4 to express SANS-EGFP (**g,h**) or Espin-EGFP (**i,j**) and analyzed after 1 DIV. SANS-EGFP (green) is localized toward the tips of stereocilia. Espin-EGFP (green) is widely expressed in stereocilia. Stereocilia are visualized with phalloidin (Phal, red). Scale bars, 2 μm (**a,b**), 1 μm (**c–f**) and 5 μm (**g–j**).

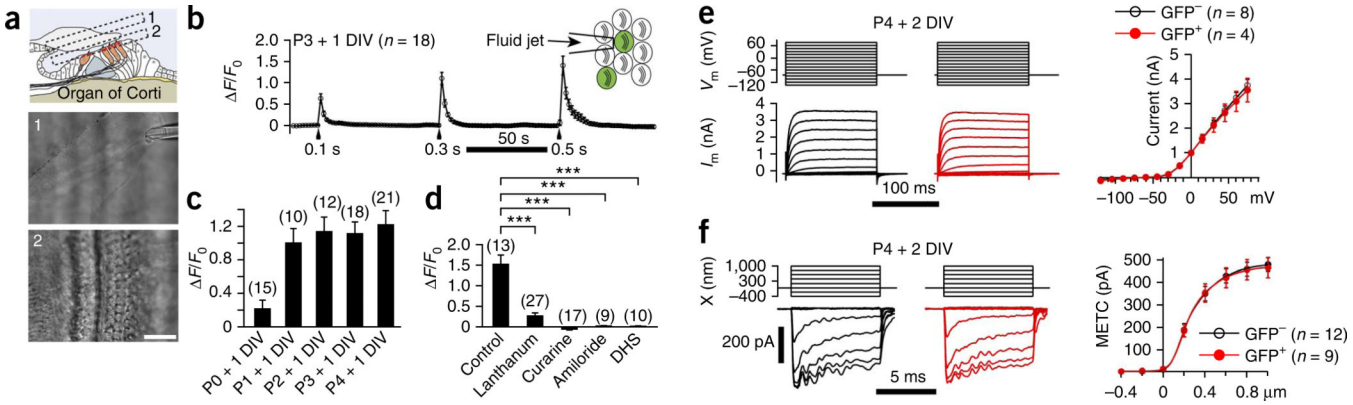

Figure 5.

Physiological analyses of mechanically activated Ca^{2+} responses and membrane currents. (**a**) Removal of tectorial membrane before physiological analyses. Image 1 shows a suction pipette that is used to remove the tectorial membrane. Image 2 shows the layer of hair cells underneath the tectorial membrane. Note the shadow of the suction pipette. (**b**) Mean values of fluid jet–induced Ca^{2+} response in OHCs. OHCs are transfected at P3 to express GCaMP3, and they are maintained for 1 d *in vitro* (DIV). The inset shows the position of the fluid jet relative to the hair bundle. Fluid-jet pulse duration was increased from 0.1 to 0.3 and to 0.5 s. (**c**) Quantitative analysis of GCaMP3 signals in OHC injectoporated between P0 and P4 and maintained for 1 DIV. The amplitude of the second Ca^{2+} response peak was measured. Values of $\Delta F/F_0$ are 0.22 ± 0.10 for P0 + 1 DIV, 1.01 ± 0.17 for P1 + 1 DIV, 1.14 ± 0.17 for P2 + 1 DIV, 1.12 ± 0.14 for P3 + 1 DIV and 1.22 ± 0.17 for P4 + 1 DIV. The total number of analyzed hair cells is indicated in parentheses in **c** and **d**. (**d**) Ca^{2+} response of P4 + 2 DIV wild-type OHCs with/without the indicated pharmacological blockers (control, 1.53 ± 0.22; lanthanum, 100 µM, 0.27 ± 0.06; curarine, 400 µM, −0.07 ± 0.01; amiloride, 500 µM, 0.02 ± 0.00; and DHS, dihydrostreptomycin, 200 µM, 0.01 ± 0.03). (**e**) Representative example showing membrane currents activated by depolarizations in OHCs that are injectoporated at P4 and cultured for 2 DIV. Currents of untransfected OHCs are shown as black traces and EGFP-expressing OHCs are shown as red traces. The 150-ms voltage pulses increased from −120 to 90 mV at 15-mV steps. The currents at the end of each pulse are measured for quantitative analysis. At right, the membrane currents are plotted against voltage. (**f**) Representative examples of mechanotransduction currents (METC) of EGFP-transfected OHCs (red traces) and control untransfected OHCs (black traces). 10-ms hair bundle displacements increasing from −400 to 1,000 nm at 200-nm steps are used. Quantification of currents is shown on the right. Values are mean ± s.e.m. in all panels. Scale bar, 35 µm (**a**). ***$P < 0.001$.

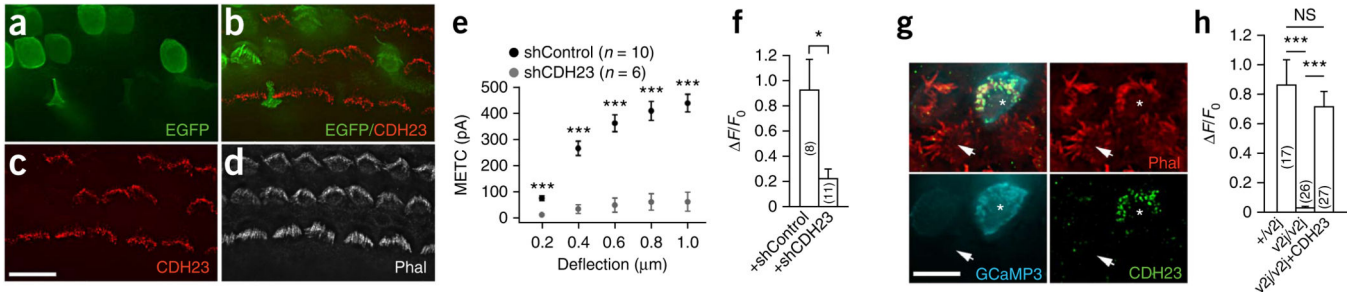

Figure 6.

Analysis of CDH23 functions in OHCs. (**a–d**) OHCs are co-injectoporated at P1 with a plasmid expressing EGFP and an shRNA targeting CDH23 (shCDH23). Knockdown of CDH23 in EGFP-expressing cells is determined after 3 d *in vitro* (DIV) by immunohistochemistry. CDH23 (red) expression is no longer detectable in the stereocilia (white; Phal, phalloidin) of EGFP (green)-expressing OHCs. (**e**) Current displacement plots showing that mechanotransduction currents (METCs) are drastically reduced in OHCs expressing shCDH23 but not a scrambled control shRNA (shControl). The METC values (in pA) of controls are 75.98 ± 9.35 at 0.2 μm, 266.32 ± 26.86 at 0.4 μm, 362.50 ± 32.32 at 0.6 μm, 409.84 ± 36.37 at 0.8 μm and 439.23 ± 33.89 at 1.0 μm, respectively. In shCDH23-treated cells, 12.71 ± 3.33 at 0.2 μm, 35.00 ± 16.79 at 0.4 μm, 49.85 ± 27.04 at 0.6 μm, 61.87 ± 31.27 at 0.8 μm and 62.43 ± 36.50 at 1.0 μm. (**f**) Quantitative analysis of GCaMP3 signals in OHCs that were injectoporated at P1 to express shCDH23 or scrambled control shRNA. The tissues are cultured for 3 DIV and the OHCs are stimulated with a fluid jet with the same protocol as in Figure 5b. The values of $\Delta F/F_0$ are 0.93 ± 0.24 for shControl and 0.23 ± 0.07 for shCDH23. (**g,h**) OHCs from CDH23$^{v2J/v2J}$ mice are co-injectoporated at P1 with expression vectors for GCaMP3 and full-length CDH23 and analyzed after 4 DIV. (**g**) Cochlear whole-mounts are stained with Phal (red) to reveal stereocilia. GCaMP3 (blue) and CDH23 (green) are visualized with specific antibodies. Note that hair bundles in noninjectoporated cells of CDH23$^{v2J/v2J}$ mice are splayed (arrows). Overexpression of CDH23 (asterisks) rescues the morphological defect. (**h**) Mechanotransduction responses as analyzed by imaging GCaMP3 are perturbed in CDH23$^{v2J/v2J}$ mice but rescued by overexpression of CDH23. The values of $\Delta F/F_0$ are 0.87 ± 0.17 for CDH23$^{+/v2J}$ OHCs, 0.03 ± 0.01 for CDH23$^{v2J/v2J}$ OHCs and 0.72 ± 0.11 for CDH23$^{v2J/v2J}$ OHCs expressing CDH23. The number of analyzed hair cells is indicated. Values are mean ± s.e.m. Scale bars, 10 μm (**a–d,g**). ***$P < 0.001$. NS, not significant.