Video Article

Dissection of Adult Mouse Utricle and Adenovirus-mediated Supporting-cell Infection

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Abstract

Hearing loss and balance disturbances are often caused by death of mechanosensory hair cells, which are the receptor cells of the inner ear. Since there is no cell line that satisfactorily represents mammalian hair cells, research on hair cells relies on primary organ cultures. The best-characterized ***in vitro*** model system of mature mammalian hair cells utilizes organ cultures of utricles from adult mice (**Figure 1**) [1-6]. The utricle is a vestibular organ, and the hair cells of the utricle are similar in both structure and function to the hair cells in the auditory organ, the organ of Corti. The adult mouse utricle preparation represents a mature sensory epithelium for studies of the molecular signals that regulate the survival, homeostasis, and death of these cells.

Mammalian cochlear hair cells are terminally differentiated and are not regenerated when they are lost. In non-mammalian vertebrates, auditory or vestibular hair cell death is followed by robust regeneration which restores hearing and balance functions [7, 8]. Hair cell regeneration is mediated by glia-like supporting cells, which contact the basolateral surfaces of hair cells in the sensory epithelium [9, 10]. Supporting cells are also important mediators of hair cell survival and death [11]. We have recently developed a technique for infection of supporting cells in cultured utricles using adenovirus. Using adenovirus type 5 (dE1/E3) to deliver a transgene containing GFP under the control of the CMV promoter, we find that adenovirus specifically and efficiently infects supporting cells. Supporting cell infection efficiency is approximately 25-50%, and hair cells are not infected (**Figure 2**). Importantly, we find that adenoviral infection of supporting cells does not result in toxicity to hair cells or supporting cells, as cell counts in Ad-GFP infected utricles are equivalent to those in non-infected utricles (**Figure 3**). Thus adenovirus-mediated gene expression in supporting cells of cultured utricles provides a powerful tool to study the roles of supporting cells as mediators of hair cell survival, death, and regeneration.

Video Link

The video component of this article can be found at https://www.jove.com/video/3734/

Protocol

1. Utricle Dissection and Culture

1. Euthanize an adult (4 weeks of age or older) mouse using an approved protocol and decapitate.
2. Snip the external auditory canal on both sides of the head and pull the skin forward towards the nose. Bisect the head from back to front and remove the brain from both sides to reveal the bony labyrinth.
3. Trim the skull away from the bony cochlea and transfer the bony labyrinth (including the bulla) to a tissue culture hood equipped with a dissecting microscope (Note: Adenovirus work should be performed in a Class II biological safety cabinet).
4. In the hood, transfer each cochlea to a 35mm tissue culture dish containing sterile dissecting media (M199, Gibco/Invitrogen #12350) (**Figure 1A**).
5. If the auditory bulla is still intact (it is possible to remove the bulla during the gross dissection by inserting your thumbnail between the bulla and the apex of the cochlea), use two forceps (one #3 and one #5) to break the bulla (**Figure 1B**).
6. Identify the landmarks of the bony cochlea preparation: apex, base, ossicles, oval and round windows, VIIIth nerve root, semicircular canals (**Figure 1 C, D**).
7. Place the inner ear in the dish with the medial side facing up, such that the oval and round windows and apex are on the bottom of the dish and the VIIIth nerve root is facing up. Hold the preparation using a #3 forceps in the region of the semicircular canals (**Figure 1D**).
8. Using a scalpel equipped with a #11 blade, cut off the apex of the cochlea just apical to the nerve root (**Figure 1D**).
9. Turn the preparation so that the cut portion is facing up. Use the anterior semicircular canal as a handle to stabilize the preparation (**Figure 1E**).

10. Using #5 forceps, remove the cochlea at the modiolus down to the base. At the point where the hook region of the cochlea turns downward, use a fine probe (half of a broken #55 forceps) to lift the last bony shelf of the osseous spiral lamina (**Figure 1F**), revealing the saccule. Just beneath the saccule is the stapes footplate, which is an important landmark. The utricle is immediately adjacent to the stapes footplate **(Figure 1G)**, and it is surrounded by bone. Use the probe to chip the bone away enough to reveal about 1/3 of the utricle. Carefully remove the utricle using #55 forceps (**Figure 1H**). This procedure will usually result in removal of the pigmented roof epithelium as the utricle is pulled from the bony preparation. However, if the utricle is removed with the roof epithelium intact, the roof can be removed using #55 forceps.
11. Utricles for adenovirus infection (or live imaging experiments) must have the otoconia removed during the dissection (prior to infection). In this case, use a luer-lock syringe that is filled with dissecting media and fitted with a small-bore needle (usually 26-28 gauge). Hold the utricle at the edge using #55 forceps (**Figure 1I**). Bring the needle tip close to the utricle with the bevel of the needle pointing down. Use a stream of dissecting media to blow off the otoconia (**Figure 1J**). Alternatively, otoconia can be removed by brushing them off using an eyelash tool (Ted Pella, Inc. #113). Utricles that are not going to undergo adenoviral infection are normally cultured free-floating in 24-well tissue culture plates with the otoconia intact (see below for post-fix otoconia removal, which is easier).
12. Once all utricles are dissected, change the dissecting media to sterile culture media: DMEM F12 (Gibco #11320) supplemented with 5% FBS and 50 U/ml penicillin G (Sigma).
13. Utricles are cultured at 37 °C and 5% CO_2. If cultures are to be maintained for more than 48 hours, half of the culture media should be changed every other day. Cultures can be maintained for a week or longer.

2. Adenovirus Infection of Supporting Cells

Important: Working with adenovirus requires Biosafety Level 2 (BSL2) procedures and certification. Check with your Institutional Biosafety Officer for guidance and training on BSL2 procedures.

1. Dissect utricles as above in dissection media (no serum). Remove otoconia as above.
2. When ready to infect, transfer 1 utricle (hair cells up) into each well of a Nunc mini-tray (Fisher #12-565-68) containing 15 µL **serum-free** DMEM F12 (this is important because serum can inactivate the virus). This transfer is easier with a 1.5 mm microcurette (#10082-15 from Fine Science Tools).
3. Our Ad-GFP is serotype 5 with the viral E1 and E3 genes deleted and the human CMV promoter driving the transgene (GFP). This virus was obtained from Vector BioLabs (Philadelphia, PA vectorbiolabs.com). Stock viruses are provided at titers of 1.0-4.0×10^{10} PFU/ml. Add 0.5-2 µl of stock virus to each well containing a utricle (**Figure 1L**). The actual amount used varies depending on the virus and stock titer. We usually infect each utricle with 1.0-4.0×10^{7} PFU.
4. Culture the utricles in virus-containing media at 37 °C/5% CO_2 for 2 hours. After 2 hours, transfer the utricles back to the 24-well tissue culture plate containing culture media with serum. Culture the utricles overnight at 37 °C/5% CO_2.
5. Utricles can be used the next day for live imaging experiments, or they can be fixed for hair cell immunochemistry (see below). Viral transgene expression increases over time, so if transgene expression is low at 24 hours post-infection it may beneficial to culture cells an additional 24 hours in serum-containing media. If you see hair cell toxicity, reduce the amount of virus used.

3. Utricle Fixation, Otoconia Removal, and Immunochemistry

1. At the end of the culture period, fix utricles in 4% paraformaldehyde for either 3 hours at room temp (on a rocking platform) or overnight at 4 °C. Wash utricles (3 times, 15 min each) in 0.1 M (1X) Sorensen's phosphate buffer pH 7.4. These steps are performed in a 24-well tissue culture plate.
2. Otoconia removal: *Note: Otoconia must be removed prior to adenovirus infection (see step 1.11 above). However, utricles that are not to be infected can be cultured and fixed with the otoconia still intact. In this case, use the steps described here to remove the otoconia after fixation.* Inspect utricles for any remaining pigmented roof epithelium. Carefully remove any remaining roof epithelium using two #55 forceps. To remove the otoconia, replace Sorensen's phosphate buffer with 750-1000 µl Cal-Ex decalcifier (Fisher #CS510-1D). Leave Cal-Ex on utricles for 1 minute and 40 seconds (do not exceed 2 minutes). Replace Cal-Ex with 0.1 M Sorensen's phosphate buffer and wash (5 times, 5 min each).
3. Incubate utricles in sodium borohydride (Sigma #452882, 1% in deionized water) for 10 mins. Wash utricles in 0.1 M Sorensen's phosphate buffer (5 times, 5 min each).
4. Place utricles in Blocking Solution (PBS + 2% bovine serum albumin + 0.8% normal goat serum + 0.4% Triton X-100) for 3 hours at room temperature on a rocking platform.
5. Add primary antibodies and incubate overnight at 4 °C. Anti-Myosin 7a (either Proteus Biosciences #25-6790 or #MYO7A 138-1 from the Developmental Studies Hybridoma Bank) at 1:100 in blocking solution labels all hair cells. To separately label striolar and non-striolar hair cells, we have used a double-label protocol with monoclonal anti-calmodulin (Sigma #C 3545; 1:150) and polyclonal anti-calbindin (Chemicon #AB1778, Temecula, CA, USA; 1:200). Secondary antibodies (usually Alexa fluor conjugated secondary antibodies from Invitrogen, Carlsbad, CA, USA) are diluted 1:500 in blocking solution and incubated for 4 hours in the dark at room temperature on a rocking platform.
6. Mount utricles on glass slides using Fluoromount-G (Southern Biotech, Birmingham, AL, USA).

4. Representative Results

Utricles cultured using this method retain full complements of both hair cells and supporting cells (**Figure 2**). Hair cells in healthy cultures show round nuclear profiles surrounded by thin cytoplasmic regions that are myosin 7a-positive (**Figure 2**, top panel). Supporting cells (labeled with anti-Sox2) are smaller and more densely packed than hair cells (**Figure** 2, lower panel). Adenovirus infects 25-50% of the supporting cells in the utricle, and no hair cells are infected (**Figure 2**, Ad-GFP panels). It should be noted that the optimal working titer of each adenovirus must be determined empirically, since hair cell death is possible if the viral titer is too high. In addition, regions of mechanical damage (caused during the dissection) will take up large amounts of virus. These regions of mechanical damage are easily distinguishable by a continuous line of cells

with very high levels of GFP expression and missing hair cells. This is in contrast to the scattered GFP expression in supporting cells of an undamaged culture (**Figure 2**).

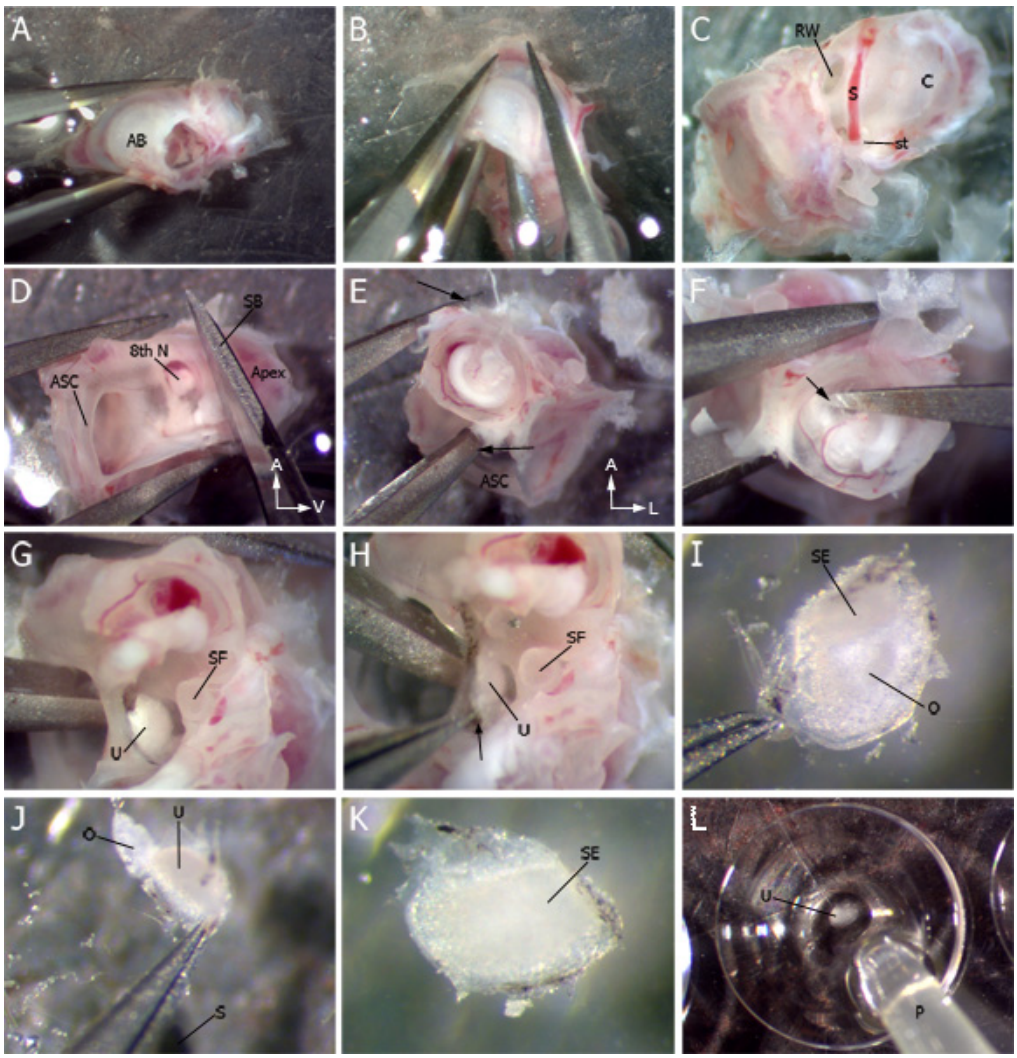

Figure 1. Utricle Dissection. A: Auditory bulla (AB). **B:** The bulla is broken using two sturdy forceps. **C:** Bony cochlea (*RW = round window, S = stapedial artery, C = cochlea, ST = stapes*). **D:** A scalpel blade (SB) is used to cut off the apex of the cochlea apical to the VIIIth cranial nerve (8^{th} N). *ASC = anterior semicircular canal*. **E:** The preparation is held firmly using a #3 forceps with one fork inserted into the anterior semicircular canal (ASC) and the other on the outermost bone (arrows). The membranous labyrinth is removed using forceps. **F:** With the membranous labyrinth removed, the basal turn of the osseous spiral lamina is visible near the hook region. A fine probe is inserted beneath this bony shelf to lift the shelf up and remove it (arrow). **G:** After removal of the saccule, utricle (U) is visible immediately adjacent to the stapes footplate (SF). **H:** Utricle (U) is removed using a #55 forceps (arrow). *SF = stapes footplate*. **I:** Utricle with otoconia (O) partially removed and sensory epithelium (SE) visible underneath. **J:** Otoconia (O) are removed from the utricle using a stream of media delivered by a syringe fitted with a 26-gauge needle. The shadow of the needle (S) is visible at the bottom of the image. **K:** Utricle with otoconia removed and sensory epithelium (SE) visible. **L:** Adenoviral infection: each utricle (U) is placed in an individual well of a mini-tray. Supporting cells are infected with adenovirus by pipetting virus into the well (P = Pipet tip).

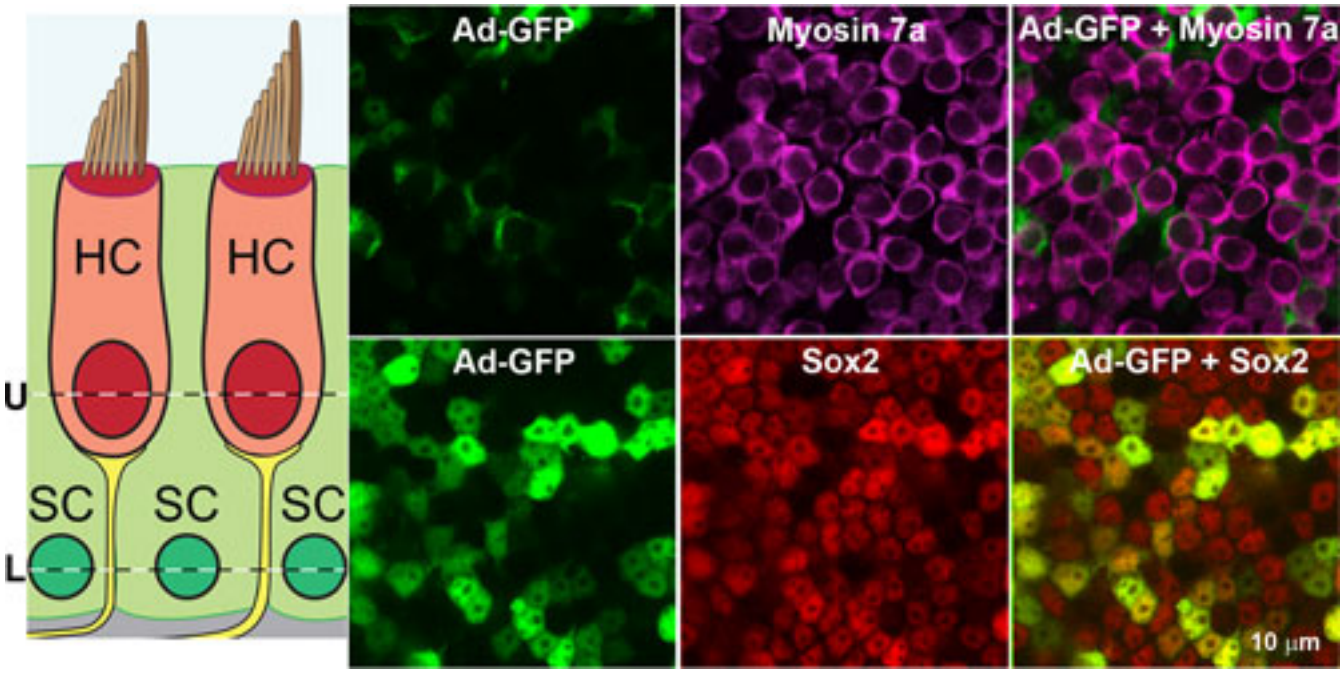

Figure 2. Adenovirus-mediated infection of supporting cells. Utricles were infected with adenovirus driving expression of green fluorescent protein (Ad-GFP). Shown are confocal images of the hair cell layer and the supporting cell layer in the same area of one utricle. Hair cells are labeled with an antibody against Myosin 7a (magenta). Supporting cells are labeled with an antibody against Sox2 (red). Schematic shows the structure of the utricle sensory epithelium and the locations of the hair cells (HC) and supporting cells (SC). Locations of confocal (optical) sections shown in the upper (U) and lower (L) panels are indicated by dashed lines in the schematic. Upper panels: In confocal images taken at the level of the hair cell nuclei, Ad-GFP signal appears in the spaces between hair cells and does not overlap with the hair cell marker Myosin 7a. Lower panels: In confocal sections at the level of the supporting cell nuclei, Ad-GFP signal colocalizes with the supporting cell marker Sox-2. Ad-GFP infection results in GFP expression in supporting cells only, and no hair cells are infected.

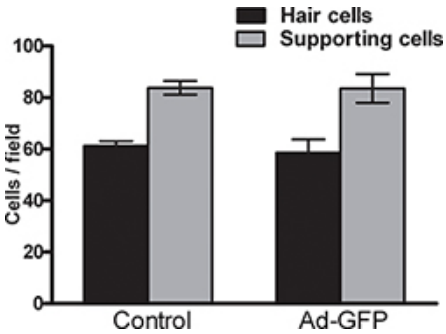

Figure 3. Adenoviral infection does not result in death of hair cells or supporting cells. Utricles were infected with Ad-GFP at 4×10^8 PFU/ml. Utricles were labeled with antibodies against Myosin 7a (hair cell marker) and Sox2 (supporting cell marker). Hair cell and supporting cell counts were equal for control utricles and Ad-GFP infected utricles.

Discussion

Sensory hair cells are susceptible to death caused by a variety of stresses, including aging, noise trauma, and exposure to ototoxic drugs, including the aminoglycoside antibiotics and the antineoplastic agent cisplatin. In mammals hair cell death results in permanent hearing loss and/or balance disturbance. *In vitro* model systems are critical tools for studies aimed at determining the cellular and molecular mechanisms underlying hair cell death, as well as those aimed at preventing or reversing hair cell death. Unlike cochlear hair cells from adult mammals, hair cells of the utricle survive well in culture. Utricle hair cells are sensitive to death from exposure to the same therapeutic drugs that are toxic to cochlear hair cells, and the cellular mechanisms underlying ototoxic hair cell death and survival are similar for both utricular and cochlear hair cells [12-18]. Moreover, when data obtained in the utricle model system are tested *in vivo,* the utricle preparation has proven to be a reliable predictor of hair cell survival and death in the mature cochlea [12, 18-21]. The mouse utricle preparation also allows us to examine the effects of specific proteins by utilizing utricles from transgenic and knockout animals.

The signals that mediate the survival and death of sensory hair cells under stress are poorly understood. However, emerging evidence suggests that other cell types in the inner ear may play important roles in determining whether hair cells under stress ultimately live or die [11, 22, 23]. The utricle model system can be used to examine these critical cell-cell interactions in a mature mammalian sensory epithelium. Adenoviral infection

provides a method of altering gene expression in supporting cells, thus facilitating studies of the role(s) of supporting cells in hair cell survival, death, phagocytosis, and regeneration.

Disclosures

No conflicts of interest declared.

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