Transfection of cochlear explants by electroporation

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Abstract

The sensory epithelium of the mammalian inner ear, also referred to as the organ of Corti, is a remarkable structure comprised of highly ordered rows of mechanosensory hair cells and non-sensory supporting cells located within the coiled cochlea. A more complete understanding of the cellular and molecular factors that mediate the development of this structure is of interest to a broad group of scientists including developmental biologists and clinical researchers interested in understanding inner ear pathologies. However, the relatively small size of the cochlea and its location within the skull have slowed the pace of discovery. Here we describe an in vitro explant culture technique that can be coupled with gene transfer via electroporation to study the effects of altering gene expression during development of the organ of Corti. While the protocol is largely focused on embryonic cochlea, the same basic protocol can be used on cochleae from mice as old as P5.

Unit Introduction

One of the major challenges in hearing research is the relatively small size and inaccessibility of the mammalian inner ear. The auditory sensory epithelium, also referred to as the organ of Corti, is embedded within the temporal bone of the skull. In an effort to circumvent these limitations, we developed a technique for the isolation and establishment of cochlear explants from embryonic mice between the ages of embryonic day 12 (E12) and E18.5. This technique was modified from a procedure originally described by Sobkowicz et al. (1975) for the isolation of the cochlea from early postnatal mice. In addition, following isolation of the cochlea, square wave electroporation can be used to force expression of DNA plasmids in individual cells within the cochlear duct. The protocols described here include dissection and isolation of the embryonic mouse cochlea, gene transfer by electroporation, and subsequent maintenance and analysis of cochlear explant cultures. Note: The described protocol must be approved by an appropriate institutional animal care and use board.

Materials

Sylgard-charcoal coated glass petri dishes, 60 mm and 100 mm

Matrigel

DMEM (purchased from Invitrogen #12430)

Mattek culture dishes, No. 0 coverslips

Pregnant mouse at desired gestational stage

70% ethanol

Dissection instruments

Scissors

No. 5 Forceps

Dissecting microscope

HBSS/HEPES, cold

100 ml of 10x HBSS (purchased from Invitrogen #14065)

5 ml of 1M HEPES

895 ml of H_2O

Adjust pH to 7.2 – 7.3

Filter sterilize

Cochlear explant culture media

9 mls of DMEM

1 ml of fetal bovine serum

100 μl of 100x N2 supplements

10 μl of 10 mg/ml ciprofloxin

Tissue-culture dishes, 60 mm or 100 mm

Minutien Pins

Plasmid DNA, expression vector of choice, at 1.5 mg/ml in water

Electroporation equipment

Electrodes

Electroporator (ECM-830, BTX)

OS-30 (Dow-Corning)

Prepare Sylgard-coated dissection dishes

1. Mix Sylgard base component with powdered charcoal to desired black level.
2. Mix in curing agent and pour Sylgard into Petri dishes to desired depth.
3. Place Petri dishes under a vacuum of 10-20 mm mercury for 30 minutes to overnight to remove trapped bubbles.
4. Must be done at least 2 days in advance.
5. Sterilize prior to using either by autoclave or 70% ethanol

Prepare Matrigel-coated Mattek dishes

6. Add 5 mls of cold (4°C) sterile DMEM to a 300 µl cold sterile aliquot of Matrigel in a 15 ml conical tube.
7. Mix by inverting the tube.
8. Add 100-200 µl of Matrigel-DMEM mixture to the center of each Mattek dish. Use just enough to cover the bottom of the well created by the coverslip.
9. Place dishes in incubator at 37°C for at least 30 minutes before use.

Dissect embryonic inner ears

10. Anesthetize the pregnant mouse with CO_2.
11. Wipe down the abdomen of mouse with 70% ethanol, and carefully open the abdominal and thoracic cavities.
12. Euthanize the animal by creating a double-pneumothorax of the diaphragm.

13. Remove uterus and place in a dish of cold HBSS/HEPES. Move dish to laminar flow clean bench; all the following steps should be performed using sterile technique on the clean bench.
14. Using sterile forceps, remove embryos from uterus and transfer to a new dish of cold HBSS/HEPES.
15. Remove heads from embryos and transfer to a new dish of cold HBSS/HEPES.
16. Remove the skin and open the dorsal portion of the skull along the midline (see Figure 1A). Remove the brain. Transfer the bases of the skulls to a new dish of cold HBSS/HEPES.
17. Remove the inner ears from the developing temporal bone (Figure 1B), and transfer to a new dish of cold HBSS/HEPES.
18. Transfer inner ears to be dissected to a Sylgard dissection dish with cold HBSS/HEPES. Identify the vestibular portion of the ear, the wider of the two ends (Figure 1C).
19. Immobilize the inner ears by placing minutien pines through the vestibular portion into the Sylgard dish. Pin the ears with their concave (ventral) side toward the surface of the dish (Figure 1D).

Dissect cochleae

20. Identify the base of the cochlear spiral (Figure 2A). Using No. 5 forceps with fine tips, make an incision in the developing cartilage, parallel to the spiral (Figure 2B).

21. Remove the cartilage overlying the cochlea (Figure 2C).
22. Starting at the base, remove the top (ventral) half of the cochlear duct to expose the developing sensory epithelium located on the lower (dorsal) side of the duct (Figure 2E, F). The two halves should separate easily. At embryonic days 13 to 14 (E13-E14), the top half will usually come off in one piece.
23. Using closed forceps, separate the cochlea from the underlying cartilage. Leave as much neuronal and mesenchymal tissue attached as possible. If necessary, pinch through the base with forceps to lift the cochlea cleanly away from the rest of the inner ear (dashed line, Figure 2F).

Electroporation

24. Transfer one dissected cochlear epithelium to a 10 µl drop of plasmid DNA solution in HBSS/HEPES or water on a Sylgard-coated dish.
25. Position the cochlea with the lumenal side of the epithelium facing up. Tilt the cochlea so that it is at an angle of approximately 45° to the plane of the Sylgard dish.
26. Place the electrodes on either side of the cochlea. The negative electrode should be located adjacent to the lumenal surface of the sensory epithelium while the positive electrode should be located on the basement membrane side of the cochlea (Figure 2I). The tips of the electrodes should be completely submerged in the DNA solution.
27. Electroporate the cochlea using an ECM-830 (BTX) or equivalent square wave electroporator with the following settings:

a. 27 Volts

b. 30 msec pulse duration

c. 9 – 10 pulses per cochlea

28. Add 50 μl of cochlear culture media to the drop of DNA with the electroporated cochlea.

29. Continue electroporating cochleae, transferring one at a time into separate drops of DNA.

Plating cochleae

30. Allow at least a 5 minute recovery time following electroporation.

31. Transfer cochleae to Matrigel-coated Mattek dishes, 1 or 2 cochleae per dish.

32. Remove DMEM/Matrigel solution and replace with cochlear explant culture media, 150 μl per dish.

33. Position each cochlea on the Matrigel-coated coverslip with the lumenal surface of the epithelium facing up. Be sure that each explant is completely submerged and no portion is in contact with the surface of the culture media.

34. Incubate for desired length of time, usually 2 to 6 days.

35. After immunostaining or other desired analysis, coverslips may be removed from Mattek dishes by soaking the bottom of the dish in OS-30 for 30 minutes at room temperature.

Commentary

The protocol described here provides a relatively straight forward procedure for the isolation and maintenance of cochlear explant cultures. The organ of Corti is characterized by a striking cellular pattern that includes four ordered rows of hair cells and six ordered rows of associated non-sensory supporting cells (reviewed in Kelley, 2006). The formation of this structure and the specification of a normal complement of both hair cells and supporting cells are essential for normal auditory function. However, the present understanding of the factors that regulate the formation of the organ of Corti is limited. Considering that loss of hair cells and/or supporting cells is the leading cause of both congenital and acquired hearing impairment, a greater understanding of the molecular and genetic pathways that specify these cell types could provide valuable insights regarding the creation of regenerative strategies.

As discussed, the development of this in vitro technique was necessitated by the small size of the cochlea (there are only 2000 to 2500 hair cells in a mature mouse cochlea), and its rather inaccessible location. Based on our experience, explants can be established beginning at any time point between E12 and the early postnatal period. Prior to E12, the cochlear duct has not extended sufficiently to be isolated, while beyond about post-natal day 5 (P5) ossification of the bony portion of the cochlear duct makes dissection considerably more challenging. We have compared the development of cochlear explants with development in vivo and have found a good correlation between in vivo and in vitro. For instance, explants established on E13 and maintained for seven days develop a

cellular pattern of inner and outer hair cells that is largely comparable with the organ of Corti in vivo at the same developmental time point.

The recapitulation of cell fate and patterning in cochlear explants in vitro provides a useful assay for examination of the effects of different soluble factors and cell permeable antagonists. However, in order to examine the effects of modulation of specific gene function within the developing cochlea, we wanted to develop a method for efficient gene transfer. While virally-mediated gene transfer techniques have been used successfully to express foreign genes in developing hair cells (Luebke et al., 2001; Holt, 2002; Stone et al., 2005; DiPasquale et al., 2005), the preparation time required to generate viral vectors is not conducive to the screening of multiple candidate genes. Therefore, after determining that lipid micelle-based transfection reagents, such as FuGene or Dotap, would not effectively transfect cells in cochlear explants, we developed the electroporation protocol described here (Woods et al, 2004; Jones et al., 2006). The use of electric fields to facilitate transfer of small molecules, dyes, or DNA into living cells was first demonstrated in the early 1980s (Neumann et al., 1982), and then used extensively for the transfection of embryonic stem cells. More recently, the applications for electroporation have been expanded to include both in vivo and in vitro approaches, including recent clinical trials (reviewed in Anwer, 2008). In brief, rapid pulsed low voltage charges are used to generate an electric field surrounding individual cells. The transient charge increase causes two changes in cell membranes. First, membranes become more permeable, apparently as a result of reorganization of the polar headgroups within the lipid bilayer, leading to a weakening of the hydration layer (Stulen, 1981;

Lopez et al., 1988). Second, micropores of approximately 1 nm in size are believed to form that can coalesce to form pores as large as 400 nm (reviewed in Mir, 2008). Delayed addition tests using fluorescent dyes suggest that membranes remain permeable for up to 30 minutes following charge application (reviewed in Rols, 2008). However, similar tests using DNA vectors indicate that DNA must be present at the time of charge application. This result suggests that charge mediated DNA transfer does not occur via direct permeablization or through micropores. Instead, it has been suggested that DNA transfer may occur as a result of charge mediated fusion of DNA with the plasma membrane followed by subsequent internalization through endocytosis. This hypothesis is supported by the demonstration that the efficiency of DNA expression can be increased by complexing DNA expression vectors with lipid micelles prior to electroporation (Chernomordik et al., 1990; Rocha et al., 2002). Moreover, DNA transfer also depends on electrophoretic movement of negatively charged DNA molecules towards the positive pole such that transfection efficiency is much higher in cells facing the cathode. However, regardless of the specific mechanism of transfer, the relative simplicity of electroporation, combined with a lack of immunological side effects, has resulted in a rapid expansion of this technique for both in vivo and in vitro applications. Ongoing research suggests that variations in the timing and size of the electric field may lead to higher efficiencies of transfer in terms of both number of cells transfected and overall level of expression, while decreasing cell damage and death.

Based on our results, cochlear explants between the ages of E13 and P0 can be effectively transfected by electroporation. While cochlear explants can be established at E12,

electroporation of explants younger than E13 causes too much damage to the tissue to allow useful analysis. As discussed above, the orientation of the explant relative to the transfecting electrodes directly determines which cell types are transfected. Transfection of epithelial cells located in the floor of the cochlear duct is achieved by orienting the explant such that the lumenal surface of the epithelium is facing the negative electrode. A limited number of transfected cells can initially be seen approximately 12 to 18 hours following electroporation, and the number of identifiable transfected cells continues to increase over the course of a seven day experiment. Strong expression of transfected plasmids also continues for the duration of each experiment, usually not more than eight days (Figure 3). For reasons that we cannot readily explain, transfection efficiency is not uniform across the mediolateral axis of the duct. Typically, there is a greater number of transfected cells located in Kolliker's organ, a transient epithelium located medial to the sensory epithelium. Lower and more variable numbers of transfected cells are found in the developing sensory epithelium and in epithelial cells located lateral to the sensory epithelium (a region referred to as the lesser epithelial ridge (LER))(Figure 3A,B). Moreover, transfection efficiency in the sensory epithelium decreases with developmental age such transfected cells are rarely observed in this region in explants transfected at P0. The bases for these changes are not known, but may be related to the formation of dense actin and/or microtubule meshworks in the lumenal surfaces of both developing hair cells and supporting cells. Finally, the promoter of the expression vector chosen also affects the distribution of transfected cell types. In our experience, the human cytomegalovirus immediate early promoter (CMV) yields robust expression in Kölliker's organ, but very few cells transfected cells are found in the sensory epithelium (Figure 3A). Use of the

composite CMV/ chicken β-actin CAG promoter typically results in a higher percentage of transfected cells within the sensory epithelium (Figure 3D, F).

To determine whether application of the electroporating voltage leads to cell death or alters cell fate, we have assayed for changes in cell survival and cell fate in explants transfected with a GFP-reporter construct. Results of cell death analysis indicate only a minor increase in the level of cell death in electroporated explants (Jones et al., 2006). Similarly, analysis of the cell fates adopted by GFP-transfected cells in the sensory epithelium indicates that approximately 50 to 55% of transfected cells develop as hair cells while the remaining transfected cells develop as supporting cells. These results are consistent with the ratio of hair cells to supporting cells in a normal epithelium, suggesting that electroporation does not directly influence cell fate.

In contrast with expression of GFP alone, we have demonstrated that cell fate in both the sensory and non-sensory regions of cochlear explants can be influenced by electroporation. Forced expression of the basic helix-loop-helix gene Atoh1 induces a hair cell fate at greater than 95% efficiency in both the sensory epithelium and in Kolliker's organ (Zheng and Gao, 2000; Jones et al., 2006)(Figure 3C-E). In contrast, forced expression of Id3, Sox2 or Prox1 acts to inhibit hair cell fate within the sensory epithelium (Jones et al., 2006; Dabdoub et al., 2008)(Figure 3F).

Figure Legends

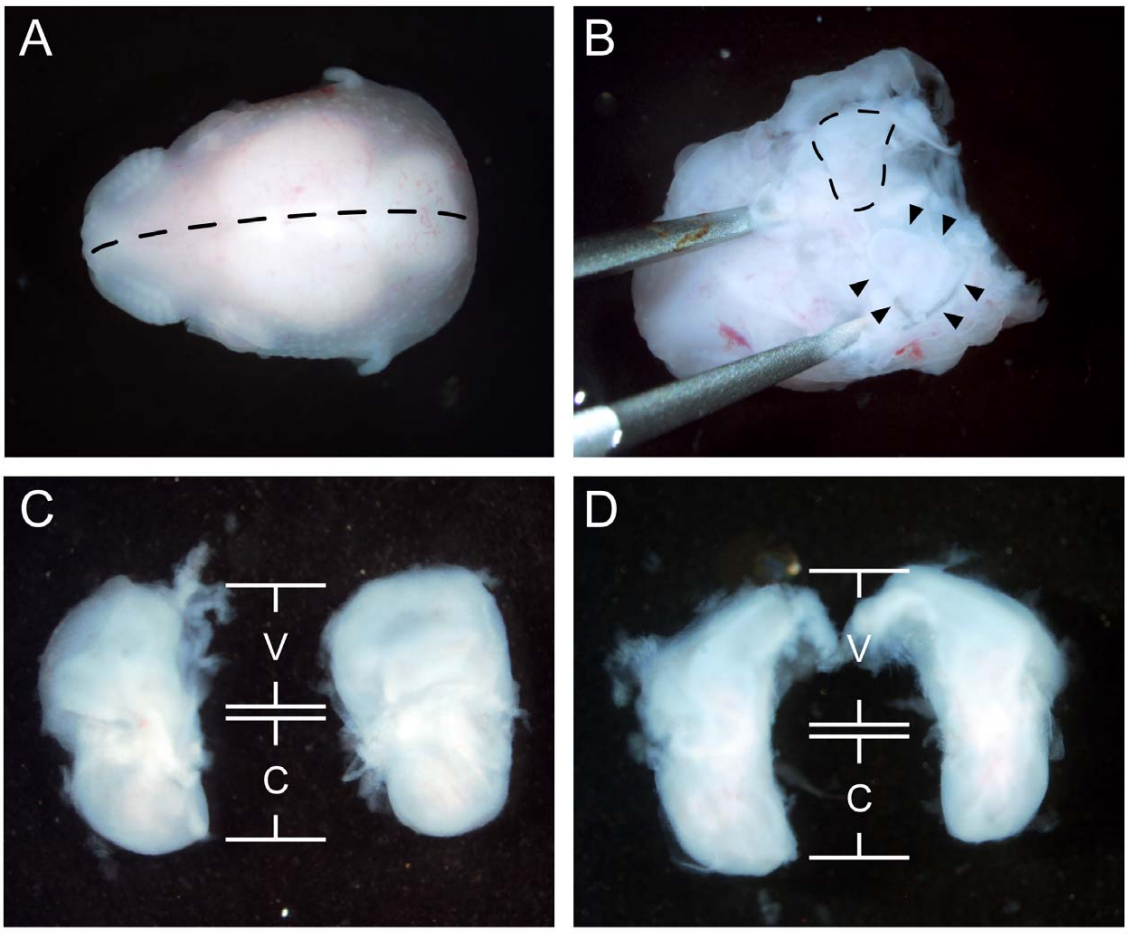
Figure 1. Isolation of developing bony labyrinth of the inner ear. A. Dorsal view of the head of a mouse at E14.5. Dotted line indicates dorsal midline. The skull should be opened along this line followed by removal of the brain. B. Once the brain has been removed, the developing bony labyrinth of the inner ear (outlined) can be visualized in the temporal bone located in the ventral floor of the skull (arrow). The bony labyrinth can be isolated by dissecting around its borders. C. Ventral view of the isolated bony labyrinths. Cochlear and vestibular regions are indicated. D. Anterior view of the bony labyrinths oriented as in C, indicating the natural curvature between the cochlear and vestibular regions.

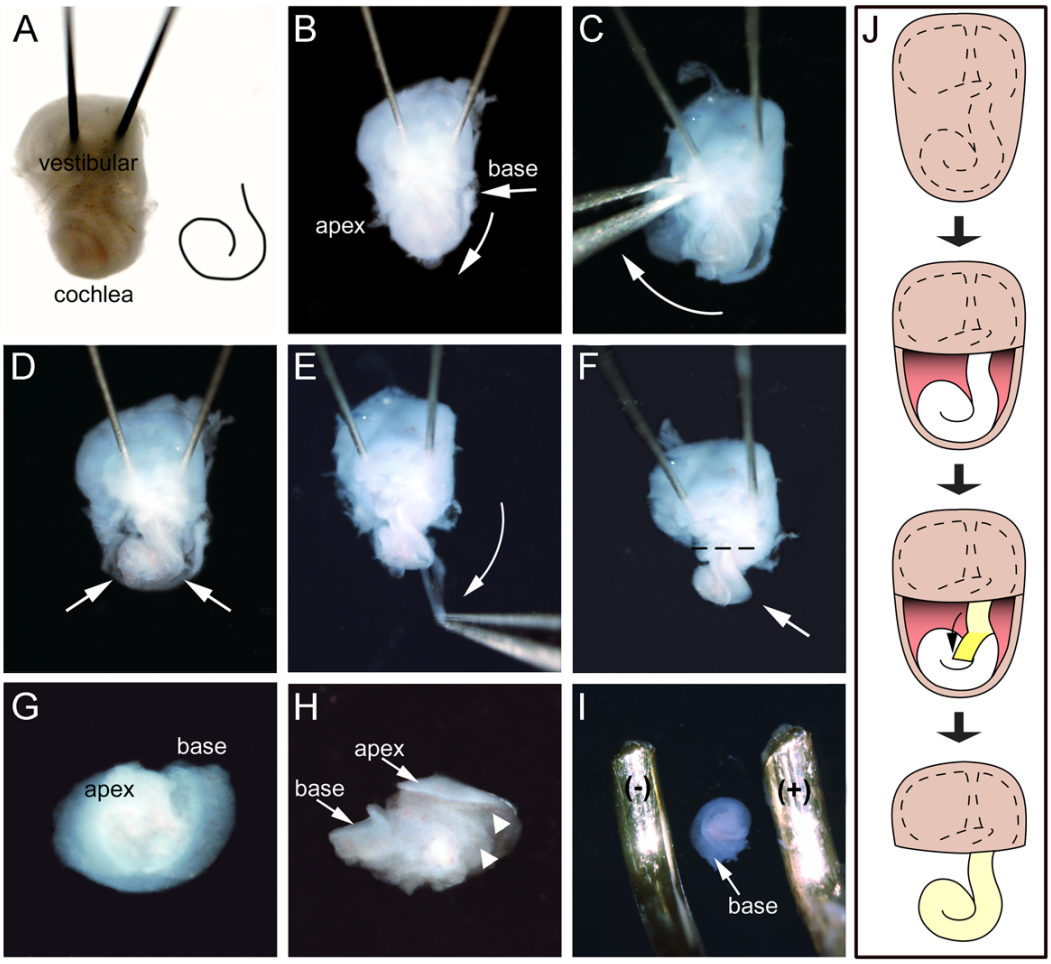
Figure 2. Isolation of the developing cochlear duct and sensory epithelium. A. The bony labyrinth should be oriented with the ventral side up and immobilized by placing two minutien pins through the vestibular region. Once immobilized, it will be possible to identify the base of the cochlear duct through the bony labyrinth. The line on the right illustrates the shape of the cochlear spiral. B. Fine forceps should be used to make an opening that runs parallel to the duct (arrow). C. Use forceps to continue to increase the size of the opening in the bony labyrinth by working along the outside edge of the cochlea (arrow). D. Once the ventral surface of the bony labyrinth of the cochlea has been removed, the developing cochlear duct can be visualized (arrows). E. To expose the developing sensory epithelium of the cochlea, carefully remove the upper (ventral) half of the duct using fine forceps (arrow). Following removal of the upper half of the duct, the remainder of the bony labyrinth of the cochlea can also be removed. F. At this

point the developing sensory epithelium (organ of Corti) is completely exposed (arrow). Next, separate the cochlea from the vestibular region of the ear by using fine forceps to cut along the dotted line. G. Ventral view of the isolated cochlear spiral with basal and apical ends indicated. H. In a side view the epithelium is present as a spiral that extends from the base to the apex (arrows). The lower region of the cochlea is comprised of mesenchymal derivatives (note small blood islands, arrowheads) and developing spiral ganglion neurons. I. For electroporation, the cochlea should be oriented between the electrodes with the base located closer to the negative electrode (indicated in the image).

Figure 3. Examples of cochlear electroporations. A. Low magnification image of an explant established on E13.5 and maintained for 6 days in vitro. Hair cells are labeled with an antibody against Myosin6 while transfected cells are labeled with anti-GFP. Apex and base of the cochlea are indicated. B. A higher magnification view of a transfected explant treated as in A. Transfected cells are present in Kolliker's organ (KO), the sensory epithelium (SE) and in the lesser epithelial ridge (LER). C. High magnification view of cells transfected with an Atoh1 expression vector. Endogenous hair cells within the SE are labeled with anti-Myosin6 (red). Atoh1-transfected cells located in the SE (arrow) or in KO (arrowheads) have also developed as hair cells. Note that virtually all transfected cells appear yellow as a result of expression of the hair cell marker Myosin6. D. High magnification view of Atoh1-transfected cells within the sensory epithelium. Each transfected cell has developed as a hair cell. E. Cluster of Atoh1-transfected cells located in KO. Actin is labeled in blue. The induction of a group of hair cells leads to an accumulation of actin that is similar to what is observed in the

SE. F. Cells transfected with the inhibitory bHLH, Id3, are predominantly inhibited from developing as hair cells.

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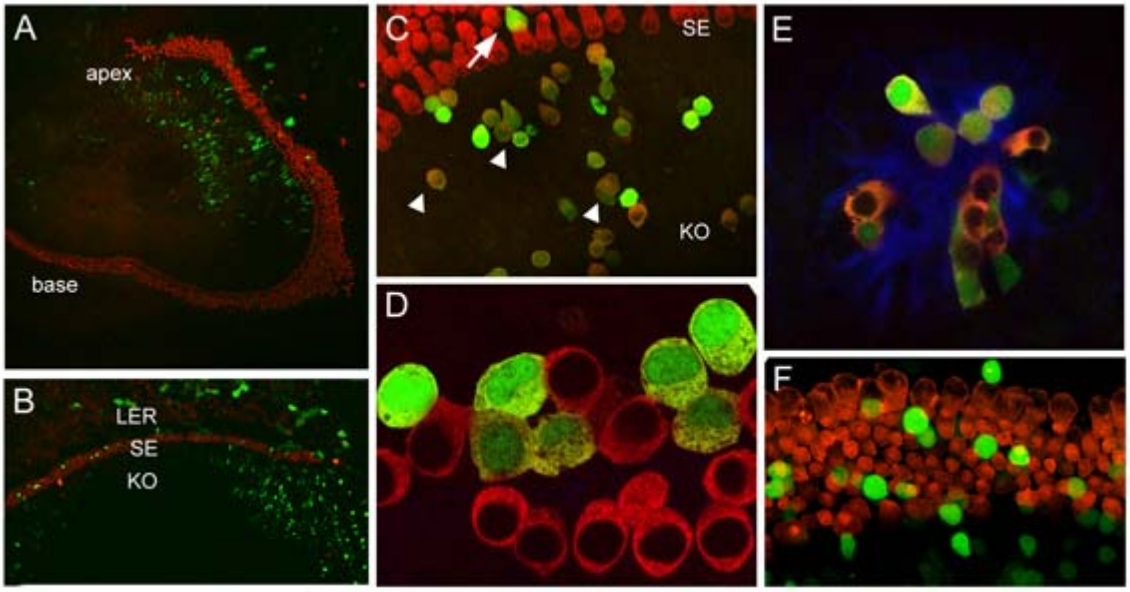
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Acknowledgements: The authors wish to thank Dr. Jennifer Jones for providing the image of Id3 transfection. This work was supported by the Intramural Program at NIDCD.

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