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Transfection of mouse 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. This unit describes 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.

Key terms for indexing

hair cell; hearing; cell fate

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 express DNA plasmids in individual cells within the cochlear duct. The protocol described here includes dissection and isolation of the embryonic mouse cochlea, gene transfer by electroporation, and subsequent maintenance and analysis of cochlear explant cultures.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

Materials

Sylgard-charcoal coated glass petri dishes, 60 mm and 100 mm (see recipe)

Matrigel (BD Biosciences)

DMEM (Invitrogen, #12430)

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Culture dishes, No. 0 coverslips (MatTek)

Pregnant mouse at desired gestational stage

70% ethanol

Dissection instruments

Scissors

No. 5 Forceps

Dissecting microscope

HBSS/HEPES, cold

100 ml of 10x HBSS (Invitrogen, #14065)

5 ml of 1M HEPES (Invitrogen, #15630)

895 ml of H₂O

Adjust pH to 7.2 – 7.3

Filter sterilize

Cochlear explant culture media

9 mls of DMEM (Invitrogen, #12430)

1 ml of fetal bovine serum

100 µl of 100x N2 supplement (Invitrogen, #17502-048)

10 µl of 10 mg/ml ciprofloxin

Tissue-culture dishes, 60 mm or 100 mm

Minutien Pins (Fine Science Tools)

Plasmid DNA, expression vector of choice, at 1.5 mg/ml in water (details of plasmid selection criteria are included in the commentary).

Electroporation equipment

Electrodes

Electroporator (ECM-830, BTX)

OS-30 solvent (Dow-Corning)

Prepare Matrigel-coated Mattek dishes

1. Add 5 mls of cold (4°C) sterile DMEM to a 300 µl cold sterile aliquot of Matrigel in a 15 ml conical tube.
2. Mix by inverting the tube.
3. 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.
4. Store dishes in incubator at 37°C for at least 30 min and up to 3 days before use.

Dissect embryonic inner ears

5. Euthanize an anesthetized pregnant mouse using the appropriate approved protocol.

6. Wipe down the abdomen of mouse with 70% ethanol, and carefully open the abdominal cavity with scissors.
7. 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.
8. Using sterile forceps, remove embryos from uterus and transfer to a new dish of cold HBSS/HEPES.
9. Remove heads from embryos by pinching through neck with forceps, and transfer to a new dish of cold HBSS/HEPES.
10. Remove the skin and open the dorsal portion of the skull along the midline (see Figure 1A). Remove the brain. Transfer the opened skulls to a new dish of cold HBSS/HEPES.
11. Remove the inner ears from the developing temporal bone (Figure 1B), and transfer to a new dish of cold HBSS/HEPES.
12. Transfer inner ears to a Sylgard dissection dish with cold HBSS/HEPES. Identify the vestibular portion of the ear, the wider of the two ends (Figure 1C).
13. Immobilize the inner ears by placing Minutien pins 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

14. 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).
15. Remove the cartilage overlying the cochlea (Figure 2C, D). Be sure to confirm that the cochlear duct is not adhered to the underside of the piece of cartilage prior to removal.
16. 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.
17. Using closed forceps, separate the entire cochlea duct 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). Two views of the isolated cochlea are shown in Figure 2G, H.

Electroporation

18. Transfer one dissected cochlear epithelium to a 10 μ l drop of plasmid DNA solution at 1.5 mg/ml in HBSS/HEPES or water on a Sylgard-coated dish.
19. Position the cochlea with the luminal (ventral) 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.
20. Place the electrodes on either side of the cochlea. The negative electrode should be located adjacent to the luminal 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.

21. 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
22. Add 50 μ l of cochlear culture media to the drop of DNA with the electroporated cochlea.
23. Continue electroporating cochleae, transferring one at a time into separate drops of DNA.

Plating cochleae

24. Allow at least a 5 minute recovery time following electroporation.
25. Transfer cochleae to Matrigel-coated Mattek dishes, 1 or 2 cochleae per dish.
26. Remove DMEM/Matrigel solution and replace with cochlear explant culture media, 150 μ l per dish.
27. Position each cochlea on the Matrigel-coated coverslip with the luminal 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.
28. Incubate for desired length of time, usually 2 to 6 days, depending on the desired developmental stage for analysis. Six days *in vitro* is approximately equal to a mouse developmental stage of P0. If incubating explants for more than 3 days, change the culture media at least once during the incubation.
29. After immunostaining or other desired analysis, coverslips may be removed from Mattek dishes by soaking the bottom of the dish in OS-30 solvent for 45 minutes at room temperature.
30. After 6 days *in vitro*, the rows of hair cells should be clearly identifiable when stained with an appropriate antibody such as anti-Myosin VI (See Figure 3) or anti-Myosin VIIa.

Reagents and Solutions

Sylgard-coated dissection dishes

At least 2 days in advance of use, mix Sylgard base component with powdered charcoal until an opaque black color is achieved. Mix in curing agent and pour Sylgard into Petri dishes until half full. Place Petri dishes under a vacuum of 10–20 mm mercury for 30 minutes to overnight to remove trapped bubbles. Allow to completely cure (approximately 24 hours) before using. Sterilize prior to using either by autoclave or covering the surface of the dish with 70% ethanol for 15–20 min. Pour off 70% ethanol and allow the dish to air dry in the laminar flow clean bench prior to use.

Commentary

Background Information

The protocol described in this unit provides a relatively straightforward 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.

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 on the development of the cochlea. 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 in this unit (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 in order to be transduced into the cells. This result suggests that charge-mediated DNA transfer does not occur via direct permeabilization 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.

Critical Parameters

Age of embryonic explants

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 the authors' experience, mouse cochlear 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 not attempted electroporation in cochlear explants from other species. We have compared the development of cochlear explants with development in vivo and have found a correlation between in vivo and in vitro progression. For instance, explants established on E13 and maintained for six days develop a cellular pattern of inner and outer hair cells that is comparable with the organ of Corti in vivo at the same developmental time point, approximately P0. Based on the authors' results, cochlear explants between the ages of E13 and P0 can be effectively transfected by electroporation. While non-electroporated cochlear explants can be established at E12, electroporation of explants younger than E13 causes too much damage to the tissue to allow useful analysis.

Orientation of explant during electroporation

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 luminal surface of the epithelium is facing the negative electrode. 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.

Plasmid Promoter

Finally, the promoter of the expression vector chosen also affects the distribution of transfected cell types. In the authors' experience, the human cytomegalovirus immediate early promoter (CMV) yields robust expression in Kölliker's organ, but very few 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).

Anticipated results

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 7-day experiment. Strong expression of transfected plasmids also continues for the duration of each experiment, usually not more than 8 days (Figure 3). For reasons that are not entirely understood, transfection efficiency is not uniform across the mediolateral axis of the duct. Typically, there is a greater number of transfected cells located in Kölliker'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 that transfected cells are rarely observed in this region in explants dissected and 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 luminal surfaces of both developing hair cells and supporting cells.

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 no increase in the number of cells undergoing cell death in electroporated cochlear explants as compared with non-electroporated control explants (J. Jones and M. Kelley, unpublished observation). 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 the process of electroporation itself, or transfection of GFP, does not 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 of specific developmentally related genes. 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).

Troubleshooting

Two common difficulties are an unacceptably high level of cell death, resulting in disrupted cochlear development, and poor transfection efficiency. Both problems can be caused by improper spacing of the electrodes relative to the explant. If the electrodes are too close, the explant is overly damaged by the electroporation. If the electrodes are too far away, very few cells will be transfected. See Figure 2I for the correct placement of electrodes. Removal of too much of the underlying mesenchymal and neuronal tissue from the cochlear epithelium can also result in a fragile explant that may be excessively damaged by electroporation. Transfection efficiency can be affected by two additional factors. High quality plasmid DNA is critical for optimal transfection efficiency, as is a DNA concentration of 1–2 mg/ml. Damage to the electrodes, such as flaking of the gold plating, will also lower transfection rates.

Time considerations

For an experienced investigator, allow 3 to 4 hours for dissection, electroporation, and plating of cochlear explants from an average-sized mouse litter. As each embryo yields two explants, inexperienced investigators may want to share litters of embryos with another investigator. Once the inner ears are isolated from the skull, the protocol may be paused for short periods of time (15 to 20 min), by keeping cochleae at subsequent stages of dissection in cold HBSS/HEPES on ice, but it is best to have all explants plated within 4 hours of the initial euthanasia of the pregnant mouse.

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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 (indicated by arrowheads). C. Ventral view of the isolated bony labyrinths. Cochlear (C) and vestibular (V) regions are indicated. D. Anterior view of the bony labyrinths oriented as in C, illustrating the natural curvature between the cochlear (C) and vestibular (V) 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 minuten 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 drawing on the right illustrates the shape of the cochlear spiral. B. Beginning in the base, use fine forceps to make an opening (straight arrow) and extend it parallel to the duct (curved 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). J. Schematic diagram depicting the main dissection steps: isolation of the inner ear, removal of overlying cartilage, opening the cochlear duct, and isolation of the cochlear sensory epithelium.

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 (red) while transfected cells are labeled with anti-GFP (green). 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 (green). 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 (green), are predominantly inhibited from developing as hair cells.