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Characterizing human vestibular sensory epithelia for experimental studies: new hair bundles on old tissue and implications for therapeutic interventions in ageing

Ruth R. Taylor [a,*], Daniel J. Jagger [a], Shakeel R. Saeed [a,b], Patrick Axon [c], Neil Donnelly [c], James Tysome [c], David Moffatt [c], Richard Irving [d], Peter Monksfield [d], Chris Coulson [d], Simon R. Freeman [e,f], Simon K. Lloyd [e,f], Andrew Forge [a]

[a] UCL Ear Institute, London, UK
[b] Royal National Throat Nose and Ear Hospital, UCLH NHS Foundation Trust, London, UK
[c] Addenbrooke's Hospital, Cambridge University NHS Foundation Trust, Cambridge, UK
[d] Queen Elizabeth Hospital, University Hospitals Birmingham NHS Foundation Trust, Queen Elizabeth Medical Centre, Birmingham, UK
[e] Manchester Royal Infirmary, Central Manchester University Hospitals NHS Trust, Manchester, UK
[f] Salford Royal Infirmary, Salford Royal NHS Foundation Trust, Salford, UK

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ABSTRACT

Balance disequilibrium is a significant contributor to falls in the elderly. The most common cause of balance dysfunction is loss of sensory cells from the vestibular sensory epithelia of the inner ear. However, inaccessibility of inner ear tissue in humans severely restricts possibilities for experimental manipulation to develop therapies to ameliorate this loss. We provide a structural and functional analysis of human vestibular sensory epithelia harvested at trans-labyrinthine surgery. We demonstrate the viability of the tissue and labeling with specific markers of hair cell function and of ion homeostasis in the epithelium. Samples obtained from the oldest patients revealed a significant loss of hair cells across the tissue surface, but we found immature hair bundles present in epithelia harvested from patients >60 years of age. These results suggest that the environment of the human vestibular sensory epithelium could be responsive to stimulation of developmental pathways to enhance hair cell regeneration, as has been demonstrated successfully in the vestibular organs of adult mice.

1. Introduction

Inner ear disorders, deafness, and balance disequilibrium are among the most common disabling conditions; indeed, it could be argued that the inner ear is responsible for a greater incidence of disability than any other organ system in the body. Such disorders become increasingly prevalent with age. More than 40% of those >50 years old have some degree of clinically significant hearing loss and this percentage increases dramatically to 70% in those >70 years of age (http://www.actiononhearingloss.org.uk/your-hearing/about-deafness-and-hearing-loss/statistics.aspx). Dizziness is the most common reason for visits to a general practitioner among those >60 years old, and balance disequilibrium is a significant contributor to falls in the elderly (Davis, 2009; Department of Health, UK, 1999; Gates et al., 2008; Herdman et al., 2000; Jarvinen et al., 2008; Macias et al., 2005; Pothula et al., 2004). The most common cause of hearing impairment and balance dysfunction is the loss of the mechanosensory "hair" cells from the sensory epithelia of the cochlea, the hearing organ (Brown et al., 1989; Hawkins, 1973; Kujawa and Liberman, 2006; Ohlemiller, 2004; Prosen et al., 1981), and the vestibular system, which subserves balance (Baloh et al., 2001; Rauch et al., 2001; Wright, 1983). In nonmammalian vertebrates, birds, reptiles, amphibians, and fish, lost hair cells are replaced spontaneously by new ones (Adler and Raphael, 1996; Corwin and Cotanche, 1988; Cotanche, 1987; Ryals and Rubel, 1988; Stone and Cotanche, 2007; Taylor and Forge, 2005). These arise from the nonsensory supporting cells that surround each hair cell. There is no regeneration of hair cells in the mammalian cochlea, so auditory deficits are permanent. Regeneration of hair cells in the vestibular system of mammals has been reported (Forge et al., 1993, 1998; Kawamoto et al., 2009; Warchol et al., 1993), but the capacity to do so spontaneously is limited severely, so that vestibular functional deficits resulting from hair cell loss are also permanent. It is also not known whether the

* Corresponding author at: UCL Ear Institute, 332, Gray's Inn Road, London WC1X 8EE UK. Tel.: +44 20 7979 8954; fax: +44 20 7679 8990.
E-mail address: ruth.r.taylor@ucl.ac.uk (R.R. Taylor).

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capacity to regenerate hair cells is retained in humans, or whether it declines with age.

Studies of the inner ears of animals have revealed pathologic processes that lead to hair cell death (Baker et al., 2014; Cheng et al., 2005; Esterberg et al., 2013; Forge and Li, 2000; Schacht et al., 2012). From such understanding, possibilities for therapeutic interventions to protect hair cells from lethal damage are being investigated. The potential for replacing lost hair cells with new ones, either through inducing endogenous regenerative mechanisms similar to those that occur spontaneously in nonmammalian vertebrates, or via exogenous processes such as application of precursors derived from stem cells, has also been suggested. Although it is assumed generally that cellular and molecular mechanisms observed in the inner ear tissues from animals are applicable to human tissue, both scientifically and for translational purposes, this requires validation. In humans, the inner ear is encased within the temporal bone, reputed to be the hardest bone in the body, at the base of the skull. The consequent inaccessibility of human inner ear tissue limits severely possibilities for their direct experimental manipulation.

There are some occasions, however, when viable inner ear tissue from humans becomes available for experimentation. During surgery for excision of vestibular schwannomas (also known as acoustic neuromas), the vestibular portion of the inner ear is exposed, removed, and usually discarded, but it can be harvested for study. Mature vestibular sensory tissues from several different animal species have been successfully maintained ex corporeally in organotypic culture for $\geq$4 weeks, thereby enabling direct experimental studies of the tissue (Cunningham, 2006; Li and Forge, 1995), and studies of human inner ear tissue maintained in culture have been reported (Kesser et al., 2008; Warchol et al., 1993). However, few studies have performed long-term cultures, and previous studies have been limited to a small number of samples.

For this study, we established a consortium of surgeons who work at the major centers in England where trans-labyrinthine surgery to remove acoustic neuromas is performed to obtain a consistent supply of relatively large numbers of samples. Ultimately, our aim is to use human vestibular tissue in experimental studies for hair cell protection and regeneration as potential therapies to ameliorate age-related functional deficits. In this study, we assess characteristics of the human vestibular sensory tissue and its maintenance in culture. We report on hair cell pathologies that suggest effects of ageing and the novel observation of the presence of immature hair bundles on human utricular epithelia from elderly people. This work establishes the explanted human utricular macula from trans-labyrinthine surgery as a model in vitro system for translational inner ear studies.

2. Methods

2.1. Harvesting and collecting tissue

Samples were obtained from patients undergoing excision of a vestibular schwannoma (acoustic neuroma) via a trans-labyrinthine approach. Tissue was collected anonymously after obtaining informed consent from the patient, and with the approval of the UK National Health Services (NHS) and University College London (UCL) Ethics committees and the local NHS research and development administrations at the National Hospital for Neurology and Neurosurgery, UCLH NHS Trust, London; Addenbrooke's Hospital, Cambridge; Queen Elizabeth Hospital Birmingham; Manchester Royal Infirmary; and Salford Royal Infirmary. Patient gender and age were recorded. A standardized procedure for accessing and removing the vestibular sensory tissue that could be used by all the surgeons who were involved in the harvesting was developed. Patients ranged between 16 and 81 years old (mean, 50).

Upon removal, tissue was transferred immediately to the medium to be used for long-term maintenance and brought to the laboratory. Samples from London, Cambridge, and Birmingham were collected personally and transported to the laboratory arriving within 20 minutes, approximately 2 and 3.5 hours, respectively, after removal from the patient. Samples from Manchester and Salford were sent by mail (using the Royal Mail Safebox containers), usually arriving within 2 days after the surgery, but sometimes later.

2.2. Maintenance and manipulation of vestibular tissue in explant culture

To culture vestibular tissue after transportation, utricles and cristae were incubated in 0.5 mL of Minimal Essential Medium (MEM) glutamax (Gibco) with 1% *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) and 10% fetal bovine serum (Hyclone) free-floating in a 24-well plate for $\leq$4 weeks at 37°C, 5% CO_2. Ciprofloxacin and amphotericin B were added to the medium to prevent microbial contamination. Medium was changed (50%) on alternate days. To explore the responses of supporting cells to hair cell loss, hair cells were ablated by exposure to 2 mmol/L gentamicin for 48 hours.

2.3. Assessment and processing of tissue

On arrival in the laboratory, tissue was examined for suitability of use. Tissue was transferred to a 35-mm petri dish with fresh medium and examined under a dissecting microscope. Our criteria for use included a clearly defined utricle with intact epithelium on the upper surface. Fanned-out nerve tracts on the underside assisted identification. Undamaged cristae within ampullae were also retained.

To assess the viability of the isolated human vestibular tissue, some samples at random were rinsed in HEPES-buffered Hanks buffered salt solution (HBHBSS) pH 7.3 exposed for not 30 seconds to 20 μmol/L FM1-43 or FM1-43FX (Molecular Probes, Invitrogen, Paisley), a fixable derivative of FM1-43, in serum-free medium, MEM with glutamax, and 1% HEPES. Samples were then washed twice in HBHBSS before examination on an inverted microscope (Nikon) and images acquired using a Hamamatsu Orca camera with Simple PCI software. Brief exposure to FM1-43 labels viable hair cells, but nonsensory cells in the tissue including supporting cells remain unlabeled (Gale et al., 2001).

Tissue was fixed for immunohistochemistry or fluorescent labeling in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 90 minutes. Tissue was then prepared for examination as whole mounts or as cryosections. For whole mounts, after fixation utricles and cristae were rinsed in PBS, permeabilized in 0.5% Triton in PBS for 20 minutes, and placed in blocking solution (10% goat serum in PBS with 0.15% triton X-100). Tissue was incubated in primary antibody overnight at 4°C rinsed thoroughly in several changes of PBS and then incubated with appropriate secondary antibody conjugated to a fluorophore for 90 minutes at room temperature. Samples were mounted onto slides using Vectashield with DAPI (Vector Laboratories) to label nuclei and examined and imaged on either a Zeiss Axiovert or Zeiss LSM 510 confocal microscope. For cryosections, fixed tissue was incubated overnight at 4°C in 30% sucrose solution and embedded in low-temperature-setting agarose, oriented and mounted on specimen support stubs using optical coherence tomography. Sections were cut at 15 μm and collected on polylysine coated slides (Polysine, VWR). Labeling was performed as for whole mounts.