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Auditory hair cell precursors immortalized from the mammalian inner ear

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Mammalian auditory hair cells are few in number, experimentally inaccessible, and do not proliferate postnatally or *in vitro*. Immortal cell lines with the potential to differentiate into auditory hair cells would substantially facilitate auditory research, drug development, and the isolation of critical molecules involved in hair cell biology. We have established two conditionally immortal cell lines that express at least five characteristic hair cell markers. These markers are the transcription factor Brn3.1, the α9 subunit of the acetylcholine receptor, the stereociliary protein fimbrin and the myosins VI and VIIA. These hair cell precursors permit functional studies of cochlear genes and in the longer term they will provide the means to explore therapeutic methods of stimulating auditory hair cell regeneration.

Keywords: hair cell precursors; hair cell differentiation; cochlear cell lines; α9AChR; Brn3.1

1. INTRODUCTION

Conditionally immortal cell lines from mammalian auditory sensory epithelia should provide important experimental tools in the study of the development, function and regeneration of hearing (Barald *et al*. 1997; Holley & Lawlor 1997). The mammalian cochlea possesses just a few thousand sensory hair cells (Echteler *et al*. 1994), for which there is no demonstrable regeneration after embryonic development (Kelley *et al*. 1995; Corwin & Oberholtzer 1997; Zine & Ribaupierre 1998). Progressive hearing loss is common and most forms of deafness are irreversible. A low level of regeneration has been observed in mammalian vestibular epithelia (Forge *et al*. 1993; Warchol *et al*. 1993), for which attempts to establish cell lines have already been made (Holley *et al*. 1997; Zheng *et al*. 1998).

Although the discovery of genes relating to hair cell function and development will be accelerated by studies on mechanosensory epithelia from the zebrafish (Corwin & Oberholtzer 1997; Nicolson *et al*. 1998), the chick (Corwin & Oberholtzer 1997) and from various studies of murine and human hereditary deafness (Petit 1996), it is essential to study their function in mammalian cells. Some genes have been studied by expression in non-cochlear cells (Elgoyhen *et al*. 1994; Milton *et al*. 1996; Morris *et al*. 1996), but their interactions with other cellular elements and signalling pathways may be significantly different (Lewis *et al*. 1997). Furthermore, we will require suitable *in vitro* preparations to identify those genes that define the specific characteristics of mammalian epithelia, particularly the organ of Corti whose specialized mechanism of mechanical tuning distinguishes it from all other mechanosensory epithelia (Dallos *et al*. 1996).

Constrained by cell numbers and the absence of cell division *in vitro*, we aimed to derive conditionally immortalized hair cell progenitors from the H-2Kb-*ts*A58 transgenic mouse (Jat *et al*. 1991; Holley & Lawlor 1997). This mouse carries a conditionally expressed, temperature-sensitive immortalizing gene that perpetuates cell division, preventing terminal differentiation. It has been used successfully to derive epithelial cell lines from various tissues (Hopfer *et al*. 1996), and from the otocyst at stage E9, the ninth day of embryonic development (Barald *et al*. 1997). The main advantage of this approach over conventional gene transfection is that the immortalizing gene should be stably incorporated into most cell types, including those in the developing mechanosensory epithelia, before their isolation. This minimizes the risk of selecting against important cell types before their immortalization.

The immortalizing gene, a thermolabile mutant of the tumour antigen (T-Ag) from the SV40 virus, is probably most effective if activated before the final mitotic division prior to cell differentiation (Ikram *et al*. 1994; Gonos *et al*. 1996). In the mouse, most cochlear hair cells pass through this stage by E14 (Ruben 1967; Kelley *et al*. 1993) and there is no evidence that precursors or stem cells remain in the cochlea after that time. Thus we decided to select sensory epithelial cells from tissue at stage E13 (Holley & Lawlor 1997). The aim was to immortalize sensory cell precursors before they were fully differentiated, minimizing possible artefacts caused by having to dedifferentiate and redifferentiate a mature cell, thus establishing a cell line that would resemble as closely as possible the normal process of development and differentiation. To identify hair cells *in vitro*, we screened epithelial

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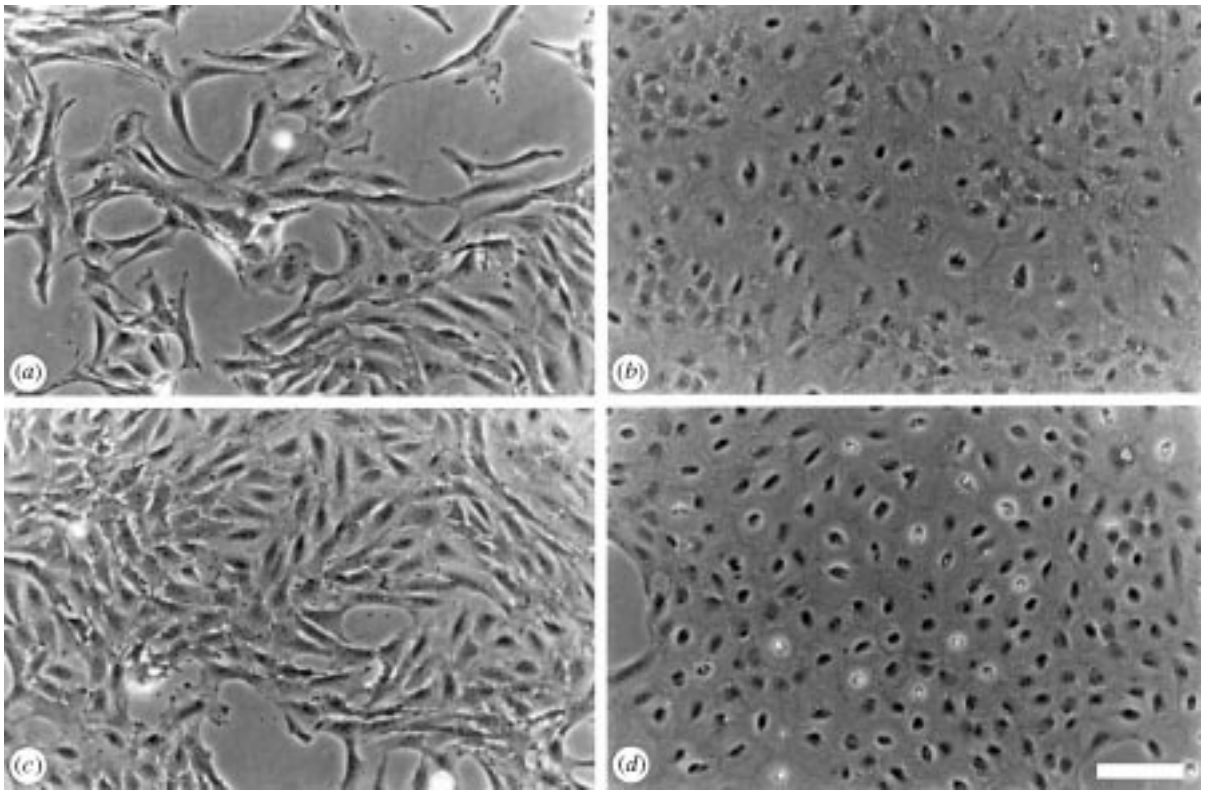

Figure 1. Phase-contrast images of cell lines UB/OC-1 and UB/OC-2. UB/OC-1 under proliferative conditions at 33 °C (*a*) and under differentiating conditions at 39 °C (*b*). UB/OC-2 under proliferative conditions at 33 °C (*c*) and under differentiating conditions at 39 °C (*d*). Scale bar is 100 μm.

cells for a number of markers. The transcription factor Brn3.1 is expressed specifically in hair cells within the inner ear from about stage E14 (Ryan 1997), is essential for their differentiation (Erkman *et al*. 1996; Xiang *et al*. 1997), and generates hearing impairments in humans when mutated (Vahava *et al*. 1998). Hair cells subsequently express the α9 subunit of the nicotinic acetylcholine receptor (α9AChR) (Elgoyhen *et al*. 1994; Glowatski *et al*. 1995; Housley & Ryan 1997), a novel receptor isoform characteristic of hair cells. Fimbrin is an actin cross-linking protein that, within the cochlea, occurs only in the hair cell stereocilia (Pack & Slepecky 1995). The myosin VI and VIIA isoforms are specifically expressed by hair cells within the inner ear (Hasson *et al*. 1997). Myosin VI is normally excluded from the stereocilia, being located primarily in the cuticular plate, and to a lesser extent, in the cell body (Hasson *et al*. 1997). Myosin VIIa is also found in the entire hair cell body, but includes a uniform distribution along the stereocilia (Hasson *et al*. 1997). Mutations in the *Myo7a* gene are responsible for syndromic and non-syndromic deafness both in mice (Gibson *et al*. 1995) and in humans (Weil *et al*. 1995; Liu *et al*. 1997), whereas mutations of *Myo6* produce genetic deafness in mice (Avraham *et al*. 1995).

2. MATERIALS AND METHODS

(a) *Tissue dissection and cell culture*

We activated the immortalizing gene in organotypic cultures of auditory sensory epithelia at E13, the thirteenth day of embryonic development, before the hair cells had started to differentiate after their last mitoses. Heterozygous H-2Kb-*ts*A58 transgenic mice were produced by time-mating homozygous males with wild-type C57/Bl6 females. All animals were killed by cervical dislocation in accordance with UK Home Office regulations. Embryos were removed at E13 and primary cultures of the developing organs of Corti were established in minimal essential medium (MEM) with Earle's salts (Gibco BRL), glutamax and 10% foetal calf serum (FCS). For expression of the immortalizing gene, cells were cultured at 33 °C with 50 U ml^{-1} γ-interferon (γIF). After one week, cells were dissociated with 0.25% trypsin and replated. The proportion of epithelial cells was increased by selective adhesion and cells were then cloned by limiting dilution (Freshney 1994). Subsequent cultures were dissociated with trypsin and passaged onto uncoated plastic dishes as they approached confluence. To inactivate the gene, cells were cultured at 39 °C without γIF for 14 d.

(b) *RT-PCR*

Total RNA was extracted from cells at 33 °C and 39 °C. Primers employed for the detection of the different transcripts corresponded to mouse sequences with the exception of α9, which was from rat. Primers were: GAPDH, position 248 (5′ AACGGGAAGCCCATCACC 3′) and 672 (5′ CAGCCTTGGCAGCACCAG 3′); α9, positions 754 (5′ CCTTACCCAGATGTCACCTTCACTC 3′) and 1466 (5′ AACACCATAGCAAAGAAAATCCACA 3′); Brn3.1, positions 205 (5′ CCATGCGCCGAGTTTGTCTCC 3′) and 639 (5′ CTCCACATCGCTGAGACACGC 3′); myosin VI, positions 2343 (5′ACTTCCAAGATTG GATCCGAGGT 3′) and 3576 (5′ GTCGTTTCATGTCA ATCTCCTGC 3′); and myosin VIIa, positions 468 (5′ GCTGTATTATCAGCGGGGAG 3′) and 856 (5′ CTGGT GATG-

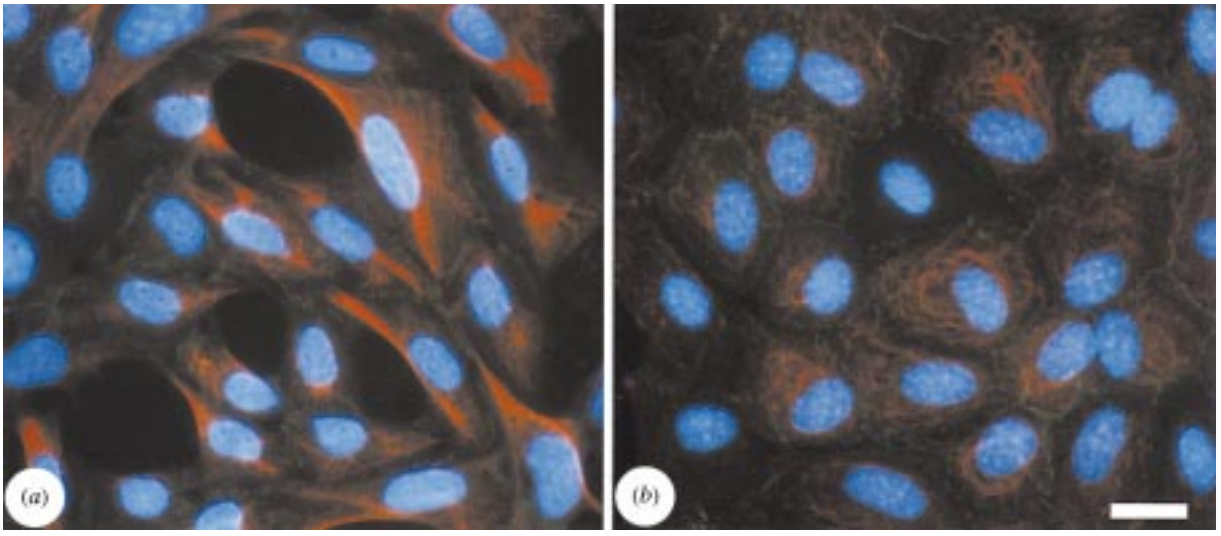

Figure 2. Immunofluorescence images of cell line UB/OC-2 labelled with anti-cytokeratin (TRITC) and anti-cadherin (FITC) antibodies. The nuclei are stained blue with DAPI. Both epithelial markers are detected under proliferative (*a*) as well as under differentiating conditions (*b*). However, cell boundaries are more evident under differentiating conditions. Note the reorganization of cytokeratin filaments under differentiating conditions. Scale bar is 25 µm.

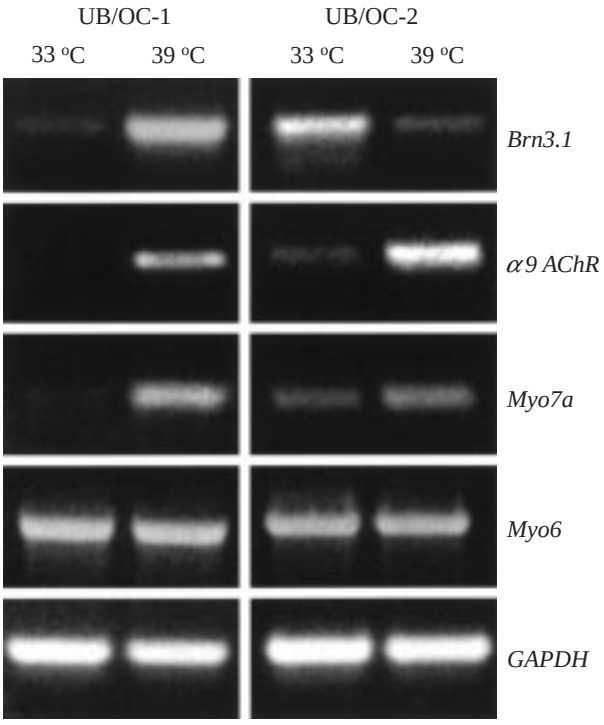

Figure 3. Detection of expression of *Brn3.1*, *α9AChR*, *Myo7a* and *Myo6* at 33 °C and after 14 days at 39 °C by RT-PCR. Bottom row, *GAPDH* used as a normalizing control. The amplified products were analysed on 1.2% agarose gels stained with ethidium bromide.

CAGTTACCCATG 3′). PCRs were performed under conditions that maintained the amplifications within the comparable, exponential phase determined by previous kinetic analysis. The identities of the PCR products were confirmed by sequencing and by restriction enzyme digestion.

(c) *Immunolabelling*

Cultures were fixed with 4% paraformaldehyde. The anti-Brn3.1 antibody was a rabbit polyclonal, characterized by Xiang *et al*. (1995) and obtained from BAbCo, California. The secondary antibody was visualized with the ELF-AP amplification kit (Molecular Probes, Oregon). The anti-fimbrin antibody was a rabbit polyclonal, a gift from Dr P. Matsudaira, Massachusetts Institute for Technology, Cambridge, USA. Whole rabbit serum was used as a control. The monoclonal anti-pan cytokeratin and the polyclonal anti-pan cadherin antibodies were from Sigma (C-2562 and C-3678, respectively). Cytokeratin, cadherin and fimbrin were visualized with either FITC or TRITC-conjugated secondary antibodies.

(d) *Electrophysiology*

Experiments were carried out at 20–25 °C with conventional whole-cell recording techniques. Cells were continually superfused with fresh MEM at 200–500 µl min^{-1}. Drugs were applied by pressure from fused micropipettes forming a multi-barrelled array 50–100 µm from the cell. Acetylcholine chloride, strychnine hydrochloride and nicotine hydrogen tartrate were prepared as concentrated stock solutions in H_2O and diluted in MEM. Patch electrodes (typical resistance 2–4 MΩ) contained (in mM): KCl, 144; $MgCl_2$, 4; EGTA, 5; Na_2ATP, 2; Na_2HPO_4, 8; NaH_2PO_4, 2; pH 7.3 (KOH). All drugs were obtained from Sigma unless otherwise stated. Recordings were obtained using an EPC 7 patch-clamp amplifier (List-Medical, Germany) and stored on magnetic tape. Voltage ramps were generated using computer software (Cambridge Electronic Design, UK). Data were sampled at 5 kHz and low-pass filtered at 1 kHz.

3. RESULTS

(a) *Selection of sensory epithelial cells*

Cytokeratin is normally expressed in all cochlear epithelial cells during embryonic development, but in hair cells it is progressively downregulated and becomes undetectable by immunofluorescence by about P4, the fourth postnatal day (Kuijpers *et al*. 1992). Cells cloned by limiting dilution were selected for their epithelial morphology and for the expression of mRNA for the α9 subunit of the acetylcholine receptor (α9AChR). In this

way we established two cell lines, UB/OC-1 and UB/OC-2, named after the University of Bristol and the organ of Corti (figure 1). Both proliferate at 33 °C in the presence of γIF, the permissive conditions for expression of T-Ag. At 39 °C without γIF, immunolabelling revealed that T-Ag was downregulated, proliferation decreased markedly and the cells formed regular, confluent, epithelial-like monolayers.

In UB/OC-1 only 10% of cells were labelled with pan-cytokeratin antibodies at 33 °C, but none were labelled after 14 days at 39 °C. In UB/OC-2 the pattern was quite different with 92% of cells labelling at 33 °C and 80% at 39 °C. In both cell lines after 14 days at 39 °C the cells formed polygonal intercellular junctions that labelled with antibodies against the junctional protein cadherin (figure 2). Cells did not become labelled with antibodies specific for neurofilaments. Cytokeratin expression is often unstable in epithelial cells *in vitro*, even in those for which it is characteristic of the fully differentiated phenotype (Steinberg & Defendi 1985). This precludes any link that we might make between cytokeratin expression and presence of differentiating hair cells.

(b) *Expression of Brn3.1*

Brn3.1 can be considered a definitive marker for hair cells in tissue derived from the developing organ of Corti and it was clearly expressed in both cell lines. Its mRNA was barely detectable in UB/OC-1 at 33 °C, but expression had been upregulated after 14 days at 39 °C (figure 3). In UB/OC-2 it was already expressed at 33 °C, but appeared to be downregulated at 39 °C. The cellular distribution of the protein was monitored by immunofluorescence (figure 4). The nuclei of a few cells from UB/OC-1 labelled weakly at 33 °C, but every cell was unambiguously labelled at 39 °C. In UB/OC-2 all cell nuclei were labelled under both conditions. On Western blots representing each condition, the antibody labelled a protein band with an M_r of about 35 000, the expected mobility for Brn3.1, and the relative intensities for each condition correlated directly with the results from RT-PCR (data not shown). This pattern has remained stable for UB/OC-1 throughout at least 50 passages, equivalent to more than 200 population doublings, and for UB/OC-2 throughout 30 passages, equivalent to about 150 population doublings.

Brn3.1 is normally expressed in the brain, the retina and the trigeminal and dorsal root ganglia (Ninkina *et al*. 1993), but within the inner ear it is only expressed in hair cells (Erkman *et al*. 1996; Ryan 1997; Xiang *et al*. 1997). Thus we conclude that all cells in UB/OC-1 and UB/OC-2 are able to differentiate into hair cells. The differential pattern of expression of Brn3.1 in the two cell lines suggests that it is not an artefact either of the immortalizing gene or of the culture conditions. Downregulation in UB/OC-2 at 39 °C implies that Brn3.1 may normally be downregulated to a plateau level in adult mice after an initial peak of expression during embryonic development, but there are presently no quantitative data from experiments *in vivo*.

(c) *Expression of α9AChR*

According to analysis by RT-PCR, mRNA for the α9AChR was upregulated in both cell lines at 39 °C (figure 3), although some expression was detected in UB/OC-2 at 33 °C. Cells had low input conductances at a holding potential of −40 mV (UB/OC-1, 1.95 ± 0.47 nS (mean ± s.e.m.), n = 10; UB/OC-2, 2.14 ± 0.21 nS, n = 10). In general, there was little evidence for expression of voltage-activated currents with time-dependent activation kinetics. However, in some cells from UB/OC-2 at 39 °C there were ionic currents with time-dependent activation (Jagger *et al*. 1998). The zero current potential (V_z), equivalent to the resting membrane potential measured with a microelectrode, was typically −20 mV, but variable between cells. Cell capacitances were also variable (UB/OC-1, 9.5 ± 4.6 pF (mean ± s.e.m.), n = 10; UB/OC-2, 17.3 ± 2.3 pF, n = 10). There was no evidence for cell–cell coupling.

Acetylcholine activated inward currents in 11 out of 35 cells from UB/OC-1 at 39 °C and in 17 out of 34 cells from UB/OC-2 at 33 °C. There were no responses in cells from UB/OC-1 at 33 °C (n = 19), consistent with the PCR results. The pharmacological profile of the response was characteristic of α9AChRs, as the currents were reversibly inhibited by 10 μM strychnine (UB/OC-1 at 39 °C, n = 2; UB/OC-2 at 33 °C, n = 2) (Seguela *et al*. 1993; Elgoyhen *et al*. 1994) and were not activated by nicotine. Nicotine reversibly inhibited the effects of 10 μM ACh (figure 5*a*). The inward current activated by ACh had a reversal potential positive to 0 mV (figure 5*b*). The observed range (0 mV to +15 mV) suggests that cations including Ca^{2+} contribute to the α9 current.

The α9AChR is relatively sensitive to ACh, with a dissociation constant (K_D) of 10 μM (Elgoyhen *et al*. 1994), compared to a K_D of 150 μM for α7 (Puchacz *et al*. 1994). In cells from UB/OC-2 the K_D for ACh was 3 μM (figure 5*c*). ACh (1 μM) activated an inward current of at least 20 pA at −40 mV. The sensitivity of α9 measured here from inward current is higher than that found in guinea-pig outer hair cells, where outward current was measured (Housley & Ashmore 1991). This may reflect differences in α9 expression in the two systems or in receptor regulation. In isolated mammalian hair cells, outward currents are thought to arise by the permeation of α9 by Ca^{2+} and the consequent activation of K^+ currents to cause hyperpolarization of the cell (Blanchet *et al*. 1996; Evans 1996). In less than 10% of cells from both cell lines described here an outward current was activated at a holding potential of −40 mV, but only after repetitive ACh application. This current was associated with a negative shift of V_z. The small fraction of cells responding in this way suggests either that channels carrying the outward current are activated only after a threshold internal calcium concentration has been reached or that they are expressed only in those cells which are at later stages of development. The latter case seems likely because the α9AChRs are functionally expressed some six days before the full complement of calcium-activated potassium channels in developing rat cochlear hair cells (Dulon & Lenoir 1996).

The expression of the α9AChR without other cellular currents means that the cells provide a convenient experimental preparation with which to study the physiology and pharmacology of this unusual subunit against its native cell background. Evidence from other cell types suggests that the expression of α9AChRs might

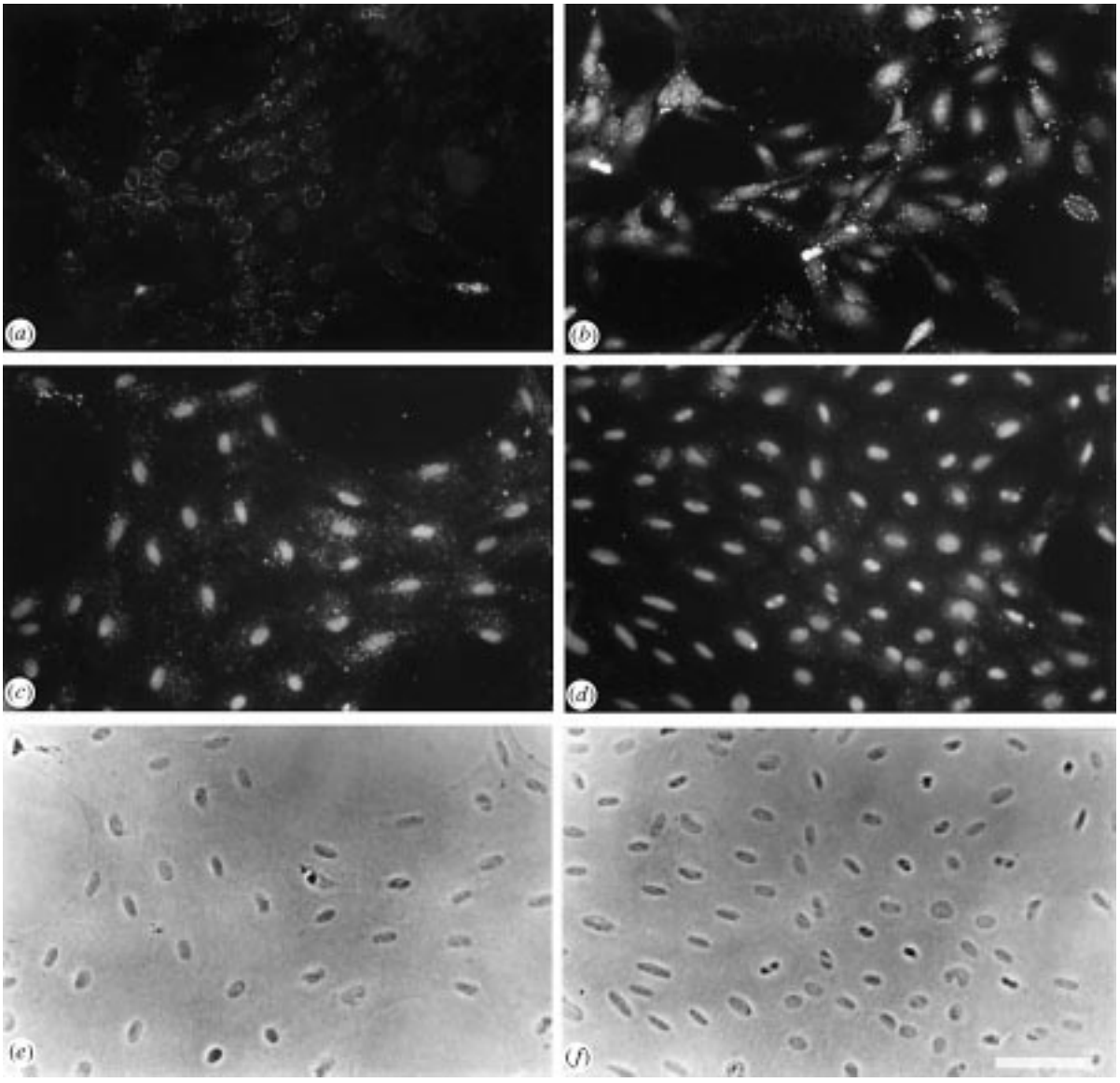

Figure 4. Immunofluorescence and phase contrast images of cultured cell monolayers labelled with an antibody to Brn3.1. Most nuclei in UB/OC-1 were unlabelled for Brn3.1 at 33 °C, but all nuclei were labelled after 14 days at 39 °C. All nuclei were positive in UB/OC-2 under both conditions. (*a*) UB/OC-1 at 33 °C, (*b*) UB/OC-2 at 33 °C, (*c*) UB/OC-1 at 39 °C, (*d*) UB/OC-2 at 39 °C; (*e*) and (*f*) are the same fields as (*c*) and (*d*) but visualized under phase-contrast. Scale bar is 100 μm.

be regulated by Brn3.1 (Milton *et al*. 1996), a possibility that can readily be studied in the cochlear cell lines.

(d) ***Expression of myosin VI and myosin VIIa***

Both cell lines express the actin-based motors myosin VI and myosin VIIa. In UB/OC-1, expression of the *Myo7a* transcript was detected with RT-PCR, and was upregulated at 39 °C (figure 3). In UB/OC-2, *Myo7a* was also upregulated at 39 °C, although the level of expression at 33 °C was higher than that in UBOC-1. *Myo6* mRNA was detected at steady levels in both cell lines under both conditions.

(e) ***Expression of fimbrin***

Within the cochlea, fimbrin is specific for hair cells and is responsible for cross-linking the parallel arrays of actin bundles within the stereocilia (Drenckhahn *et al*. 1991; Pack & Slepecky 1995). UB/OC-1 and UB/OC-2 express fimbrin under both conditions studied, as determined by Western blots. The antibody labelled a unique band with an M_r of 68 000, the expected value for fimbrin (not shown). Immunofluorescent labelling reveals that it co-localizes with actin bundles, particularly in focal contacts (figure 6).

4. DISCUSSION: DIFFERENTIATION OF HAIR CELLS

To understand the differentiation of the two cell lines in relation to normal hair cells *in vivo*, we have constructed, from published information, a diagram of likely cell lineages in the organ of Corti (figure 7). Mechanosensory epithelia are composed of two principal cell types, namely the sensory hair cells and their adjacent supporting cells. There is convincing evidence from mammals and non-mammals that hair cells and supporting cells share a common progenitor (Forge *et al*.

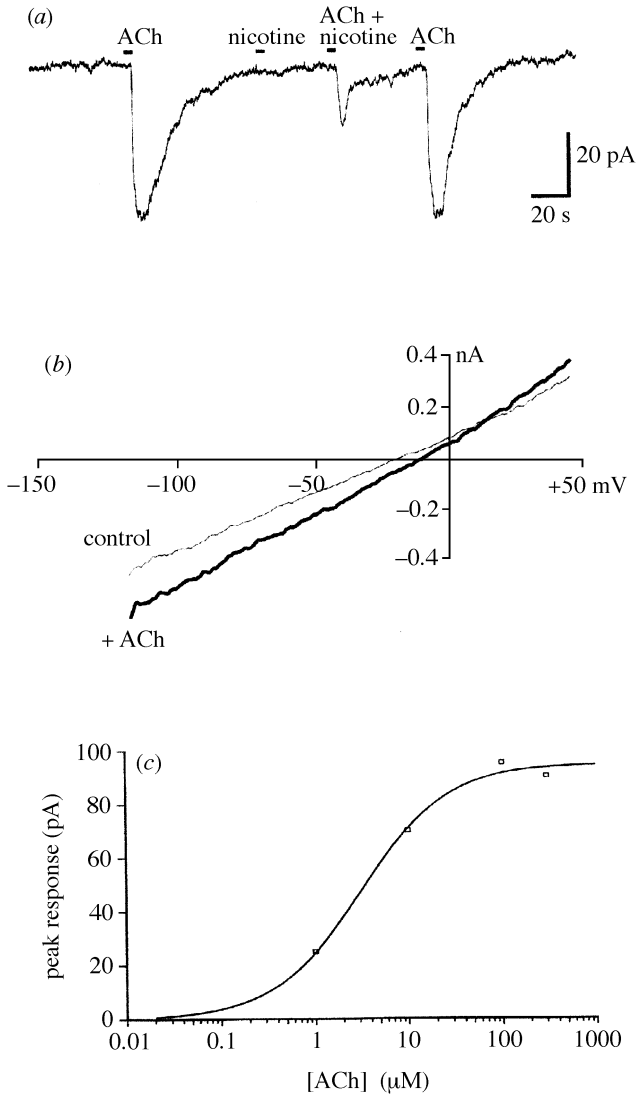

Figure 5. Receptor currents in response to application of acetylcholine (ACh). (*a*) ACh (10 μM) activated an inward current. Currents were recorded from UB/OC-2 cells, previously cultured at 33 °C, in the whole-cell patch configuration, holding potential −40 mV. Nicotine (100 μM) applied to cells from UB/OC-1 ($n=6$) and UB/OC-2 ($n=5$) did not activate an inward current and reversibly inhibited the effects of 10 μM Ach. The delay in the ACh response and the slow washout time were due to the slow perfusion rate. (*b*) Current–voltage characteristics of ACh-activated current. Whole-cell current recorded from UB/OC-1 cells previously cultured at 39 °C, holding potential −40 mV, in response to command voltage ramp (170 mV s^{-1}). A 5-s application of ACh (10 μM) activated an inward current with a reversal potential around +11 mV. V_z depolarized by 7 mV during application. (*c*) Dose–response curve of a single UB/OC-2 cell, previously cultured at 33 °C, for different ACh concentrations. Smooth line fitted by a Hill equation with $K_D=3.1$ μM and coefficient 0.9 using a Levenberg–Marquadt algorithm. In four cells tested from UB/OC-2, the K_D for ACh was between 1 μM and 10 μM. Experiments were conducted at room temperature.

1993; Warchol *et al*. 1993; Kelley *et al*. 1995; Fekete 1996; Jones & Corwin 1996). During the final mitotic divisions, which occur in the mouse at E12–E14, pluripotent progenitors give rise to non-sensory precursors (NSP) and prosensory precursors (PSP). The fate of NSPs is

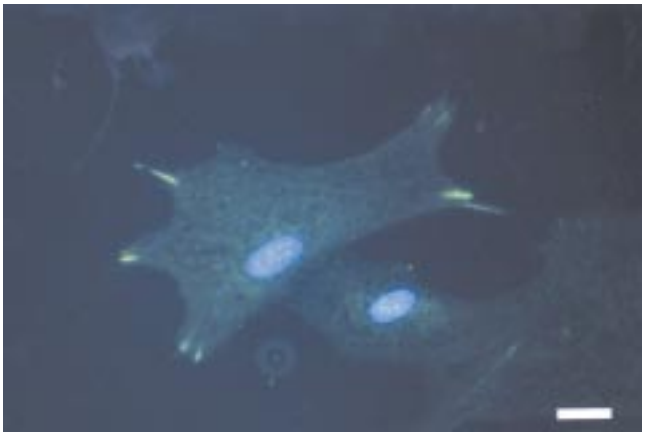

Figure 6. Immunofluorescence labelling of UB/OC-2 at 39 °C with an anti-fimbrin antibody (FITC). Nuclei are stained blue with DAPI. Fimbrin localizes primarily to focal contacts. Scale bar is 25 μm.

unclear, but they could either give rise to the lateral sulcus cells or be removed by apoptosis. The existence of PSPs was proposed by Kelley *et al*. (1993) after observing that exposure to retinoic acid during a critical, post-mitotic window, produces supernumerary hair cells as well as supporting cells. The PSP may thus represent an uncommitted precursor that can differentiate, without undergoing mitosis, into either a committed hair cell precursor (CHCP) or a committed supporting cell precursor (CSCP). These committed precursors will not fully differentiate until the second week of postnatal development. The exact timing and mechanism of commitment to either phenotype has not been discovered, but the transcription factor Brn3.1, one of the earliest hair cell markers, may be responsible for a substantial part of the differentiated phenotype.

The expression of markers at 33 °C relates to the stage that the original cell was in when the immortalization took place. The relatively low expression of Brn3.1, the α9AChR and myosin VIIa in UB/OC-1 compared with UB/OC-2 at 33 °C suggests that the former may have been immortalized at an earlier stage of differentiation. This difference is possible for several reasons. First, the cells were derived from a critical embryonic stage when Brn3.1 is initially expressed. Second, hair cells normally differentiate progressively along the organ of Corti from base to apex with a delay of 2–3 days (Ruben 1967; Nishida *et al*. 1998) and the two cell lines may have been derived from different locations along this gradient. Third, there may have been some temporal variation in development between pups from which the primary cultures were taken.

Given that all cells in UB/OC-2 expressed Brn3.1 at 33 °C we conclude that they represent committed hair cell precursors (figure 7). This implies that the T-Ag has induced proliferation of what are normally post-mitotic cells without causing regression of the phenotype at that stage of differentiation. Because UB/OC-1 did not express Brn3.1 at 33 °C we predicted that it could differentiate asymmetrically to produce both hair cells and supporting cells at 39 °C. The result that all cells expressed Brn3.1 at 39 °C was thus surprising. The simplest interpretation is

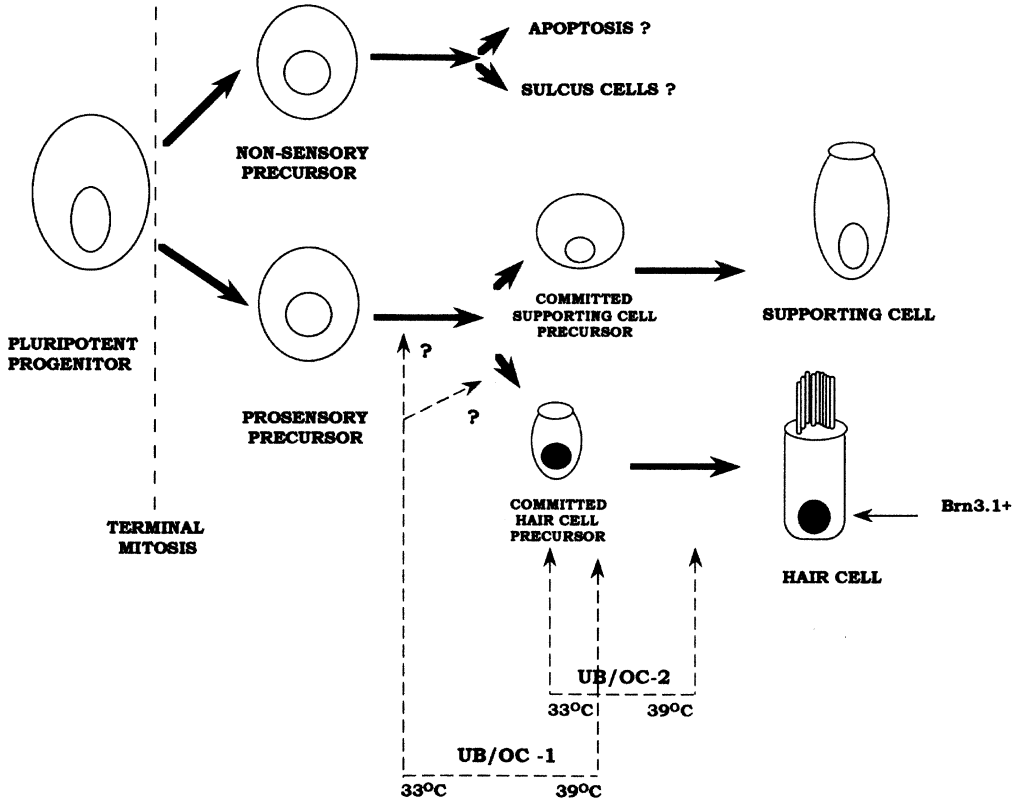

Figure 7. Model of cell lineages in the organ of Corti. A pluripotent progenitor passes through its last mitosis at approximately E14, giving rise to a non-sensory and a prosensory precursor. The prosensory precursor has the potential to differentiate without undergoing mitosis (Kelley *et al*. 1993, 1995) into either a committed hair cell precursor or a committed supporting cell precursor. UB/OC-1 has been immortalized prior to the expression of Brn3.1. Hence, they could represent either cells that are already committed to the hair cell pathway but that have not yet expressed Brn3.1, or the uncommitted prosensory precursor with the potential to differentiate into supporting cells as well as hair cells. UB/OC-2 has been immortalized after the onset of Brn3.1 expression, placing this cell line further ahead in the differentiation pathway.

that the cells were immortalized after commitment to the hair cell fate but before expression of Brn3.1 (figure 7). This implies that Brn3.1, although essential for further differentiation, is not instrumental in effecting commitment. This is consistent with the idea that it acts as a survival factor (Xiang *et al*. 1997) rather than as an inducer, such as the transcription factor MyoD in muscle cells (Weintraub *et al*. 1989) and Cdx2 in gut enterocytes (Suh & Traber 1996). The 'survival factor' hypothesis is also supported by the fact that a deletion in the human gene produces progressive hearing loss beginning between the ages of 18 and 30, rather than a severe deafness from birth (Vahava *et al*. 1998). It is possible, however, that UB/OC-1 retains the potential to differentiate both hair cells and supporting cells under suitable culture conditions. Thus it may correspond to the uncommitted PSPs, implying that the hair cell phenotype is the default pathway for differentiation. It should be possible to test these hypotheses by implantation experiments or by co-culture with other cell types. The cell lines represent an invaluable tool to study the biology and roles of Brn3.1, particularly to elucidate its involvement in hair cell commitment and to explore the effects that different mutations to be found in humans may have over gene expression.

Under current culture conditions the cells do not develop an organized cuticular plate or hair bundle. However, the fact that they express fimbrin and myosins VI and VIIa suggests that the necessary elements for apical differentiation are present. It is reasonable to expect that under suitable conditions and polarizing cues, a fully differentiated hair cell will develop.

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REFERENCES

Avraham, K. B., Hasson, T., Steel, K. P., Kingsley, D. M., Russell, L. B., Mooseker, M. S., Copeland, N. G. & Jenkins, N. A. 1995 The mouse *Snell's waltzer* deafness gene encodes an unconventional myosin required for structural integrity of inner ear hair cells. *Nat. Genet.* **11**, 369–375.

Barald, K. F., Lindberg, K. H., Hardiman, K., Kavka, A. I., Lewis, J. E., Victor, J. C., Gardner, C. A. & Poniatowski, A. 1997 Immortalised cell lines from embryonic avian and murine otocysts: tools for molecular studies of the developing inner ear. *Int. J. Dev. Neurosci.* **15**, 523–540.

Blanchet, C., Erostegui, C., Sugusawa, M. & Dulon, D. 1996 Acetylcholine-induced potassium current of guinea pig outer hair cells: its dependence on a calcium influx through nicotinic-like receptors. *J. Neurosci.* **16**, 2574–2584.

Corwin, J. T. & Oberholtzer, J. C 1997 Fish and chicks: model recipes for hair-cell regeneration? *Neuron* **19**, 951–954.

Dallos, P., Fay, R. & Popper, A. 1996 *The cochlea. Springer handbook of auditory research*, vol. 8. New York: Springer.

Drenckhahn, D., Engel, K., Hofer, D., Merte, C., Tilney, L. G. & Tilney, M. S. 1991 Three different actin filament assemblies occur in every hair cell: each contains a specific actin cross-linking protein. *J. Cell Biol.* **112**, 641–652.

Dulon, D. & Lenoir, M. 1996 Cholinergic responses in developing outer hair cells of the rat cochlea. *Eur. J. Neurosci.* **8**, 1945–1952.

Echteler, S. M., Fay, R. R. & Popper, A. N. 1994 Structure of the mammalian cochlea. In *Comparative hearing: mammals. Springer handbook of auditory research*, vol. 4 (ed. R. R. Fay & A. N. Popper), pp. 134–171. New York: Springer.

Elgoyhen, A. B., Johnson, D. S., Boulter, J., Vetter, D. E. & Heinemann, S. 1994 α9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. *Cell* **79**, 705–715.

Erkman, L., McEvilly, R. J., Luo, L., Ryan, A. K., Hooshmand, F., O'Connell, S. M., Keithley, E. M., Rappaport, D. H., Ryan, A. F. & Rosenfeld, M. G. 1996 Role of transcription factors Brn-3.1 and Brn-3.2 in auditory and visual system development. *Nature* **381**, 603–606.

Evans, M. G. 1996 Acetylcholine activates two currents in guinea-pig outer hair cells. *J. Physiol.* **491**, 563–578.

Fekete, D. M. 1996 Cell fate specification in the inner ear. *Curr. Opin. Neurobiol.* **6**, 533–541.

Forge, A., Li, L., Corwin, J. T. & Neville, G. 1993 Ultrastructural evidence for hair cell regeneration in the mammalian inner ear. *Science* **259**, 1616–1619.

Freshney, R. I. 1994 *Culture of animal cells. A manual of basic technique*. New York: Wiley–Liss Inc.

Gibson, F., Walsh, J., Mburu, P., Varela, A., Brown, K. A., Antonio, M., Beisel, K. W., Steel, K. P. & Brown, S. D. M. 1995 A type VII myosin encoded by the mouse deafness gene *shaker-1*. *Nature* **374**, 62–64.

Glowatski, E., Wild, K., Brandle, U., Fakler, G., Fakler, B., Zenner, H.-P. & Ruppersberg, J. P. 1995 Cell-specific expression of the α9 n-ACh receptor subunit in auditory hair cells revealed by single-cell RT-PCR. *Proc. R. Soc. Lond.* B **262**, 141–147.

Gonos, E. S. (and 10 others) 1996 Rat embryo fibroblasts immortalized with simian virus 40 large T antigen undergo senescence upon its inactivation. *Molec. Cell Biol.* **16**, 5127–5138.

Hasson, T., Gillespie, P. G., Garcia, J. A., MacDonald, R. B., Zhao, Y., Yee, A. G., Mooseker, M. S. & Corey, D. P. 1997 Unconventional myosins in inner-ear sensory epithelia. *J. Cell Biol.* **137**, 1287–1307.

Holley, M. C. & Lawlor, P. W. 1997 Production of conditionally immortalised cell lines from a transgenic mouse. *Audiol. Neuro-Otol.* **2**, 25–35.

Holley, M. C., Nishida, Y. & Grix, N. 1997 Conditional immortalisation of hair cells from the inner ear. *Int. J. Dev. Neurosci.* **15**, 541–552.

Hopfer, U., Jacobberger, J. W., Gruenert, D. C., Eckert, R. L., Jat, P. S. & Whisett, J. A. 1996 Immortalisation of epithelial cells. *Am. J. Physiol.* **270**, C1–C11.

Housley, G. D. & Ashmore, J. F. 1991 Direct measurement of the action of acetylcholine on isolated outer hair cells of the guinea pig cochlea. *Proc. R. Soc. Lond.* B **244**, 161–167.

Housley, G. & Ryan, A. F. 1997 Cholinergic and purinergic neurohumoral signalling in the inner ear: a molecular physiological analysis. *Audiol. Neuro-Otol.* **2**, 92–110.

Ikram, Z., Norton, T. & Jat, P. S. 1994 The biological clock that measures the mitotic life-span of mouse embryo fibroblasts continues to function in the presence of simian virus 40 large tumor antigen. *Proc. Natn. Acad. Sci. USA* **91**, 6448–6452.

Jagger, D. J., Rivolta, M. N., Holley, M. C. & Ashmore, J. F. 1998 Cholinergic and voltage-activated currents expressed in a cochlear cell line derived from the Immortomouse *J. Physiol.* 509.P, 189–190P.

Jat, P. S., Noble, M. D., Ataliotis, P., Tanaka, Y., Yannoutsos, N., Larsen, L. & Kioussis, D. 1991 Direct derivation of conditionally immortal cell lines from an H-2Kb-tsA58 transgenic mouse. *Proc. Natn. Acad. Sci. USA* **88**, 5096–5100.

Jones, J. E. & Corwin, J. T. 1996 Regeneration of sensory cells after laser ablation in the lateral line system: hair cell lineage and macrophage behaviour revealed by time-lapse video microscopy. *J. Neurosci.* **16**, 649–662.

Kelley, M. W., Xu, X.-M., Wagner, M. A., Warchol, M. E. & Corwin, J. T. 1993 The developing organ of Corti contains retinoic acid and forms supernumerary hair cells in response to exogenous retinoic acid in culture. *Development* **119**, 1041–1053.

Kelley, M. W., Talreja, D. R. & Corwin, J. T. 1995 Replacement of hair cells after laser microbeam irradiation in cultured organs of Corti from embryonic and neonatal mice. *J. Neurosci.* **15**, 3013–3026.

Kuijpers, W., Tonnaer, E. L. G. M., Peters, T. A. & Ramaekers, F. C. S. 1992 Developmentally-regulated coexpression of vimentin and cytokeratins in the rat inner ear. *Hearing Res.* **62**, 1–10.

Lewis, T. M., Harkness, P. C., Sivilotti, L. G., Colquhoun, D. & Millar, N. S. 1997 The ion channel properties of a rat recombinant neuronal nicotinic receptor are dependent on the host cell type. *J. Physiol.* **505**, 299–306.

Liu, X.-Z, Walsh, J., Mburu, P., Kendrick-Jones, J., Cope, M. J. T. V., Steel, K. P. & Brown, S. D. M. 1997 Mutations in the myosin VIIA gene cause non-syndromic recessive deafness. *Nat. Genet.* **16**, 188–190.

Milton, N. G. N., Bessis, A., Changeux, J.-P. & Latchman, D. S. 1996 Differential regulation of neuronal nicotinic acetylcholine receptor subunit gene promoters by Brn-3 POU family transcription factors. *Biochem. J.* **317**, 419–423.

Morris, P. J., Lakin, N. D., Dawson, S. J., Ryabinin, A. E., Kilimann, M. W., Wilson, M. C. & Latchman, D. S. 1996 Differential regulation of genes encoding synaptic proteins by members of the Brn-3 subfamily of POU transcription factors. *Molec. Brain Res.* **43**, 279–285.

Nicolson, T., Rusch, A., Friedrich, R. W., Granato, M., Ruppersberg, J. P. & Nusslein-Volhard, C. 1998 Genetic analysis of vertebrate sensory hair cell mechanosensation: the zebrafish circler mutants. *Neuron* **20**, 271–283.

Ninkina, N. N., Stevens, G. E. M., Wood, J. N. & Richardson, W. D. 1993 A novel Brn-3 like POU transcription factor expressed in subsets of rat sensory and spinal cord neurones. *Nucl. Acids Res.* **21**, 3175–3182.

Nishida, Y., Rivolta, M. N. & Holley, M. C. 1998 Timed markers for the differentiation of the cuticular plate and stereocilia in hair cells from the mouse inner ear. *J. Comp. Neurol.* **395**, 18–28.

Pack, A. K. & Slepecky, N. B. 1995 Cytoskeletal and calcium-binding proteins in the mammalian organ of Corti: cell-type specific proteins displaying longitudinal and radial gradients. *Hearing Res.* **91**, 119–135.

Petit, C. 1996 Genes responsible for human hereditary deafness: symphony of a thousand. *Nat. Genet.* **14**, 385–391.

Puchacz, E., Buisson, B., Bertrand, D. & Lukas, R. J. 1994 Functional expression of nicotinic acetylcholine receptors containing rat α7 subunits in human SH-SY5Y neuroblastoma cells. *FEBS Lett.* **354**, 155–159.

Ruben, R. J. 1967 Development of the inner ear of the mouse: a radioautographic study of terminal mitoses. *Acta Otolaryngol. Suppl.* **220**, 1–44.

Ryan, A. F. 1997 Transcription factors and the control of inner ear development. *Semin. Cell Dev. Biol.* **8**, 249–256.

Seguela, P., Wadiche, J., Dineley-Miller, K., Dani, J. A. & Patrick, J. W. 1993 Molecular cloning, functional properties, and distribution of rat brain α7: a nicotinic cation channel highly permeable to calcium. *J. Neurosci.* **13**, 596–604.

Steinberg, M. L. & Defendi, V. 1985 Altered pattern of keratin synthesis in human epidermal keratinocytes transformed by SV40. *J. Cell Physiol.* **123**, 117–125.

Suh, E. & Traber, P. G. 1996 An intestine-specific homeobox gene regulates proliferation and differentiation. *Molec. Cell Biol.* **16**, 619–625.

Vahava, O. (and 14 others) 1998 Mutation in transcription factor POU4F3 associated with inherited progressive hearing loss in humans. *Science* **279**, 1950–1954.

Warchol, M. E., Lambert, P. R., Goldstein, B. J., Forge, A. & Corwin, J. T. 1993 Regenerative proliferation in inner ear sensory epithelia from adult guinea pigs and humans. *Science* **259**, 1619–1622.

Weil, D. (and 18 others) 1995 Defective myosin VIIA gene responsible for Usher syndrome type 1B. *Nature* **374**, 60–61.

Weintraub, H., Tapscott, S. J., Davis, R. L., Thayer, M. J., Adam, M. A., Lassar, A. B. & Miller, A. D. 1989 Activation of muscle-specific genes in pigment, nerve, fat, liver and fibroblast cell lines by forced expression of MyoD. *Proc. Natn. Acad. Sci. USA* **86**, 5434–5438.

Xiang, M., Zhou, L., Macke, J. P., Yoshioka, T., Hendry, S. H. C., Eddy, R. L., Shows, T. B. & Nathans, J. 1995 The Brn-3 family of POU-domain factors: primary structure, binding specificity, and expression in subsets of retinal ganglion cells and somatosensory neurons. *J. Neurosci.* **15**, 4762–4785.

Xiang, M., Gan, L., Daqing, L., Chen, Z.-Y., Zhou, L., O'Malley, B. W. Jr, Klein, W. & Nathans, J. 1997 Essential role of POU-Domain factors Brn3c in auditory and vestibular cell development. *Proc. Natn. Acad. Sci. USA* **94**, 9445–9450.

Zheng, J. L., Lewis, A. K. & Gao, W.-Q. 1998 Establishment of conditionally immortalised rat utricular epithelial cell lines using a retrovirus-mediated gene transfer technique. *Hearing Res.* **117**, 13–23.

Zine, A. & de Ribaupierre, F. 1998 Replacement of mammalian auditory hair cells. *NeuroReport* **9**, 263–268.

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