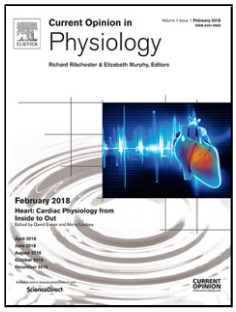

Restoring the balance: regeneration of hair cells in the vestibular system of the inner ear

Andrew Forge, Daniel Jagger, Jonathan Gale

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Restoring the balance: regeneration of hair cells in the vestibular system of the inner ear.

Andrew Forge, Daniel Jagger and Jonathan Gale

UCL Ear Institute, 332, Gray's Inn Road, London WC1X 8EE, UK

Corresponding author:
Prof Andrew Forge
e-mail:a.forge@ucl.ac.uk
Phone (+44) 207 679 8983

Abstract
Loss of the sensory "hair cells" (HCs) from the vestibular (balance) system of the inner ear results in dizziness and balance dysfunction contributing to falls. In the inner ears of non-mammalian vertebrates, there is spontaneous and complete replacement of lost HCs. The regenerates derive from the non-sensory supporting cells (SCs) that surround each HC either from the daughter cells following SC division or by direct, non-mitotic conversion of SCs. In mammals, there is a very limited capacity to regenerate vestibular HCs but only a small percentage is replaced. They arise exclusively by SC conversion. Recent work in mice, and some in humans, has shown possibilities for inducing SCs to convert to cells expressing some HC characteristics, but that differentiation to fully functional HCs is incomplete. Identification of necessary transcription factors, and/or epigenetic modifiers as well as targets to promote SC proliferation is ongoing.

Keywords: Supporting cells, phenotypic conversion, Atoh1, Notch, Lgr5

Introduction

Hair cells (HCs) are the mechanotransducing sensory cells of the hearing and balance (vestibular) systems in the inner ears of vertebrates, and in the neuromasts of the lateral lines in fish and amphibians. They derive their name from the organised bundle of elongated, modified microvilli known as stereocilia that project from their apical surfaces (Fig.1B,C). Deflection of this "hair" bundle gates mechanosensitive ion channels to modulate ionic current flow through the HC to initiate physiological responses (reviewed in [1]). Each HC is surrounded by and separated from its neighbours by intervening non-sensory, glia-like supporting cells (SCs) (Fig 1C,D) that maintain the physiological environment necessary for HCs to function [2]. HCs and SCs derive during development from a common precursor following terminal mitotic events (reviewed in [3]). Expression of the transcription factor Atoh1 directs precursors towards the HC fate. Those cells that first begin to do so exert lateral inhibition on their contacting neighbours by activation of the Notch-Delta signalling system [4,5], which blocks expression of *Atoh1*. The inhibited cells differentiate as SCs.

HC loss is the major cause of vestibular dysfunction, the consequences of which are dizziness, vertigo and balance disequilibrium contributing to postural instability and falling. These are serious, underappreciated disabilities with significant health care implications [6]. Vestibular HCs are lethally damaged by certain “ototoxic” chemicals including aminoglycoside antibiotics, still a mainstay of treatment for tuberculosis, and are lost progressively with age. Age-related vestibular dysfunction is a major underlying factor in falls in the elderly. While a very limited capacity for spontaneous HC regeneration *in vivo* has been demonstrated in the vestibular organs of adult mammals [7-11], possibly including humans [12] fewer than 20% of lost HCs are replaced over a period of several weeks [9,13*] so there is no significant functional recovery after HC losses. Regenerating vestibular HCs might offer the only therapeutic intervention to ameliorate the disabling conditions, and investigation of HC regeneration in the balance organs may also offer insights into the prospects and challenges for regenerative therapies for acquired hearing loss.

Some background: hair cell regeneration in non-mammalian vertebrates

In all vertebrates, when HCs die, the SCs that surround each one expand to close the lesion to maintain epithelial integrity [12,14-17]. The SCs also act as phagocytes to remove the bodies of dying hair cells [16-18*]. In birds and all other non-mammalian vertebrates the supporting cells then spontaneously give rise to new replacement hair cells, which, even after extensive losses, results in almost complete recovery of HC number and function in a few days (reviewed in [19]). Some regenerated HCs arise from the daughter cells following division of SCs stimulated to re-enter the cell cycle by HC loss; others are generated by direct phenotypic conversion (non-mitotic transdifferentiation) of SCs into HCs [20-22] (Fig 1E). In the avian basilar papilla (auditory epithelium) the two mechanisms may operate in spatially separated regions of the epithelium, and phenotypic conversion may precede initiation of division [20]. Asymmetric divisions of SCs produce HCs and replacement SCs, whereas symmetric divisions generate only SC pairs presumably to maintain SC numbers as some convert to HCs [23]. Transcriptomic analyses of the progression of HC loss-regeneration in birds [24,25], complemented by functional studies, have shown inactivation of the Notch signalling system underlies spontaneous SC conversion. This supports earlier work [4] showing pharmacological blocking of the Notch signalling pathway with gamma-secretase inhibitors (GSIs) such as DAPT, stimulates SC conversion to HCs after damage. Transcriptomics has also revealed signalling pathways, known to be active during development, that modulate proliferative responses during regeneration in the avian inner ear and zebrafish neuromasts [4,24-26]: activation of the Wnt signalling pathway promotes proliferation; FGF and BMP signalling may antagonise cell division. It remains to be resolved for the avian inner ear whether there are subsets of SCs - i) with the progenitor-like capacity to convert directly to HCs; (ii) with the stem cell-like characteristic of asymmetric division; or (iii) which divide symmetrically – *or* if there is a uniform population of SCs with the capacity to activate any one of those programs depending on the local environment or signalling. However, single-cell RNA sequencing applied to regenerating zebrafish neuromasts, identified 5 different supporting cell types including quiescent and activated stem cells [26].

The mammalian vestibular sensory epithelia.

There are two HC types in mammalian vestibular organs, Type I and Type II, (Fig. 1C) distinguished by morphology, innervation patterns, ion channel composition, and distinctive molecular markers [27-30**]. They differ in the speed of their physiological responses [31]. In utricular maculae (utricles), the most favoured of the 5 vestibular sensory patches for studies, HCs are distributed across an epithelial sheet (macula) in which there is a differentiated band in the middle, the striola, which occupies ca.10-20% of the macula area and is shaped to match the outline of the macula (Fig 1A). It is distinguished by various molecular makers as well as by the physiological properties of HCs [30,31]. Transgenic mice expressing a fluorescent protein reporter driven by the promoter for proteolipid protein1 (PLP1; which is expressed by myelin-producing cells in the nervous system) show that PLP1 is expressed by SCs in the extrastriolar regions, but not across the striola [18*,30**,32,33]. Conversely, SCs in reporter mice for Lgr5, a target for Wnt signalling that promotes cellular proliferation and is highly expressed in stem cells, are located exclusively across the striolar region during development [30**,34,35]. Lgr5 is then down-regulated and is absent from the mature vestibular sensory epithelia of adults. Explants of the mature utricular macula from a several species [36,37], can be maintained *ex corporeally* for up to 4 weeks providing the only opportunity for experimental studies of inner ear tissues from humans [12,38*].

Regeneration of vestibular hair cells by phenotypic conversion.

In mice, maturation of the vestibular sensory epithelia occurs postnatally, with division of progenitor cells continuing into the first postnatal week and the majority of precursors differentiating into HCs and SCs until about postnatal day (P)10 [30**,39]. When HCs are ablated in the early postnatal utricle, post-mitotic SCs retain an ability to re-enter the cell cycle, but this capacity is lost by ca. P5 [32]. The loss of this capacity coincides with the development of thick actin bands at the level of the adherens junctions around the necks of SCs (Fig 1B). These bands are thought to rigidify and stabilise the mature sensory epithelia, and the unique thickness of these bands in mammals in comparison with their counterparts in non-mammals, has been suggested to be one factor suppressing regenerative responses in the mature mammalian utricle [32]. There is no SC division following HC loss in the mature tissue. The regenerated HCs in vestibular sensory epithelia of adult mammals derive by direct phenotypic conversion of SCs, demonstrated most convincingly by Golub et al. [13*]. In a transgenic mouse in which tamoxifen induces expression of diphtheria toxin receptor only in hair cells (DTR mice), following injection of diphtheria toxin almost all HCs were ablated. The new HCs, which were generated over a period of several weeks, i) did not incorporate markers of entry into the S-phase of the cell cycle; ii) up-regulated Atoh1 (which is expressed in differentiating HCs but is down regulated when they mature); and iii) increased in number in parallel with decreases in SC number. Induction of SC conversion is, thus, one strategy investigated for regenerating vestibular HCs and two approaches have been examined: viral-mediated transduction with the gene encoding Atoh1; and pharmacological inhibition of the Notch pathway. Studies of adenoviral vector delivery of *Atoh1* to utricle explants from mature mice [40**] and from humans [38*] after ablation of hair cells with

aminoglycoside, both showed generation of cells expressing the HC marker myosin (myo)7a. Transcriptomic analyses in both cases revealed up-regulation of many HC-related genes, and several genes characteristic of SCs were down-regulated. However, many HC genes were not expressed and morphological analyses indicated that organised hair bundles were not generated. Inhibition of Notch signalling in explants of adult mouse utricles has also been reported to induce supporting cell conversion. Using a GFP reporter for Atoh1 expression, Lin et al. [41] found that after extensive aminoglycoside-induced hair cell death, disruption of the Notch pathway with GSIs led to generation of cells expressing the Atoh1-reporter and myo7a in the absence of any SC division. However, these cells did not produce mature stereociliary bundles over the period examined. In contrast, in the human vestibular tissue, inhibition of Notch activation did not lead to generation of cells expressing myo7a [38*]. Whether the apparent difference in responses between mouse and human are species inherent or a result of the experimental conditions used remains to be tested.

Regeneration of hair cell types

After ablating hair cells in DTR mice, cell fate mapping both in early post-natal [30] and mature (P60) [18*] animals has shown that PLP1-expressing SCs give rise by conversion *in vivo* exclusively to Type II HCs, identified not only by morphology and molecular markers but also by detailed electrophysiological assessment [30**]. This revealed that baso-lateral ion channel composition and synaptic specialisation of regenerated HCs appeared mature-like, but stereociliary bundles were immature or absent and mechanotransduction was defective. Type I HCs did not arise from Type II HCs [18*]. They may arise from the subset of SCs across the striola that express Lgr5. In mice, Lgr5 is normally down-regulated by P1 but after ablation of HCs in DTR/Lgr5 reporter mice at P3, SCs expressing GFP driven by the Lgr5 promoter re-appeared predominantly across the striola. These SCs divided - as shown by incorporation of mitotic markers - and both Type I and Type II HCs expressing GFP differentiated [34]. Detailed analyses at a mature stage (P30) showed they had the electrophysiological characteristics of Type I HCs, but again stereociliary bundles were not fully differentiated [30**].

Stimulating proliferation of Lgr5+SCs by activating Wnt signalling has been proposed as a potential basis for HC regeneration [6,30**,34]. A cocktail of factors that appear to stimulate proliferation of Lgr5+ cells isolated from inner ear tissues, including those of primates and humans, and which promotes differentiation of daughters into HCs has been reported [42]. However, Wnt activation in explants of mature utricles after HC loss failed to induce SC proliferation [43]. A very small number of SCs incorporating mitotic markers were, however, observed when Wnt activation and inhibition of the Notch pathway were applied together. This combination appears to promote significant SC proliferation and regeneration of Type I and Type II HCs in the immature utricle [35] and its therapeutic potential has been discussed [6,44].

Additional factors and emerging protocols

A common finding from these studies of induced HC regeneration is the failure to generate mature, functional stereociliary bundles. This suggests that additional factors are required to obtain fully functional hair cells. Atoh1 transduction of human tissue led to changes in the expression of a number of genes involved in epigenetic modulation [38*], emphasising that such modifications are also likely to be critical in the regulation of "HC genes" in SCs. A comprehensive analysis of chromatin accessibility in the mature mouse utricle [39**] identified several HC genes that remained inaccessible after *Atoh1* transduction. Although this might indicate the potential additional factors necessary for inducing generation of fully differentiated HCs, the chromatin accessibility of many HC genes that were expressed after *Atoh1* transduction was no different from those that were not, This demonstrates that further work is required to identify those essential transcription factors and/or chromatin modifiers necessary to generate differentiated HCs from mature SCs.

An *in vitro* investigation of hair cell production from embryonic stem cells identified transcription factors, Gfi1 and Pou4f3, in addition to Atoh1 as being necessary to induce cells with an HC-like transcriptome and generation of an organised hair-bundle-like structure [45]. Targets for epigenetic modifications is also an area of increasing interest for inducing hair cell regeneration in the mature inner ear [44,46,47]. The application of single-cell RNA sequencing to the development of vestibular tissues [48] should also enable determination of the changes in gene expression patterns as immature post-mitotic SCs that retain phenotypic plasticity and proliferative potential progress to maturity where these capacities are lost. This could identify those transcription factors and epigenetic modifiers that could enhance HC regeneration in the mature vestibular sensory epithelia. In addition, organoid systems derived from human pluripotent stem cells [49*] are likely to provide platforms for future investigations into HC regeneration. To date the HCs generated through such organoids show characteristics, both anatomical and physiological, exclusively of Type II HCs, similar to HCs regenerated *in vivo* [18]. Understanding the mechanisms regulating the generation of sub-populations of SCs (e.g. PLP1+ and LGR5+) could be a necessary step in determining how to regenerate Type I HCs. HCs with Type I chararacteristics are present only in terrestrial vertebrates; comparisons of the developmental generation of SCs in fish or amphibia with that in mammals using contemporary single-cell molecular profiling will provide important insight.

Declarations of interest: None

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References and recommended reading

References

1. Fettiplace R: **Hair Cell Transduction, Tuning, and Synaptic Transmission in the Mammalian Cochlea**. *Compr Physiol* 2017, **7**:1197-1227.
2. Gale JE, Jagger DJ: **Cochlear supporting cells**. In *Oxford Handbook of Auditory Science -The Ear* Edited by Fuchs P: Oxford University Press; 2010:307-327.
3. Basch ML, Brown RM, 2nd, Jen HI, Groves AK: **Where hearing starts: the development of the mammalian cochlea**. *J Anat* 2016, **228**:233-254.
4. Daudet N, Gibson R, Shang J, Bernard A, Lewis J, Stone J: **Notch regulation of progenitor cell behavior in quiescent and regenerating auditory epithelium of mature birds**. *Dev Biol* 2009, **326**:86-100.
5. Kiernan AE: **Notch signaling during cell fate determination in the inner ear**. *Semin Cell Dev Biol* 2013, **24**:470-479.
6. Sayyid ZN, Kim GS, Cheng AG: **Molecular therapy for genetic and degenerative vestibular disorders**. *Curr Opin Otolaryngol Head Neck Surg* 2018, **26**:307-311.
7. Burns JC, Stone JS: **Development and regeneration of vestibular hair cells in mammals**. *Semin Cell Dev Biol* 2017, **65**:96-105.
8. Forge A, Li L, Corwin JT, Nevill G: **Ultrastructural evidence for hair cell regeneration in the mammalian inner ear**. *Science* 1993, **259**:1616-1619.
9. Forge A, Li L, Nevill G: **Hair cell recovery in the vestibular sensory epithelia of mature guinea pigs**. *J Comp Neurol* 1998, **397**:69-88.
10. Kawamoto K, Izumikawa M, Beyer LA, Atkin GM, Raphael Y: **Spontaneous hair cell regeneration in the mouse utricle following gentamicin ototoxicity**. *Hear Res* 2009, **247**:17-26.
11. Lopez I, Honrubia V, Lee SC, Schoeman G, Beykirch K: **Quantification of the process of hair cell loss and recovery in the chinchilla crista ampullaris after gentamicin treatment**. *Int J Dev Neurosci* 1997, **15**:447-461.
12. Taylor RR, Jagger DJ, Saeed SR, Axon P, Donnelly N, Tysome J, Moffatt D, Irving R, Monksfield P, Coulson C, et al.: **Characterizing human vestibular sensory epithelia for experimental studies: new hair bundles on old tissue and implications for therapeutic interventions in ageing**. *Neurobiol Aging* 2015, **36**:2068-2084.
13. Golub JS, Tong L, Ngyuen TB, Hume CR, Palmiter RD, Rubel EW, Stone JS: **Hair cell replacement in adult mouse utricles after targeted ablation of hair cells with diphtheria toxin**. *J Neurosci* 2012, **32**:15093-15105.

* A definitive demonstration that HC regeneration in mammalian vestibular sensory epithelia occurs by direct non-mitotic phenotypic conversion of supporting cells. Provides the first description of the

development and use of transgenic mice expressing diphtheria toxin receptor in hair cells as a model with which to induce vestibular hair cell loss.

14. Jagger DJ, Nickel R, Forge A: **Gap junctional coupling is essential for epithelial repair in the avian cochlea**. *J Neurosci* 2014, **34**:15851-15860.
15. Li L, Nevill G, Forge A: **Two modes of hair cell loss from the vestibular sensory epithelia of the guinea pig inner ear**. *J Comp Neurol* 1995, **355**:405-417.
16. Monzack EL, May LA, Roy S, Gale JE, Cunningham LL: **Live imaging the phagocytic activity of inner ear supporting cells in response to hair cell death.** *Cell Death Differ* 2015 **22**: 1995-2005.
17. Bird JE, Daudet N, Warchol ME, Gale JE: **Supporting cells eliminate dying sensory hair cells to maintain epithelial integrity in the avian inner ear**. *J Neurosci* 2010, **30**:12545-12556.
18. Bucks SA, Cox BC, Vlosich BA, Manning JP, Nguyen TB, Stone JS: **Supporting cells remove and replace sensory receptor hair cells in a balance organ of adult mice**. *Elife* 2017, **6**.

* Identifies the hair cell type regenerated in the mature utricular macula and the sub-population of supporting cells that give rise to them.

19. Rubel EW, Furrer SA, Stone JS: **A brief history of hair cell regeneration research and speculations on the future**. *Hear Res* 2013, **297**:42-51.
20. Cafaro J, Lee GS, Stone JS: **Atoh1 expression defines activated progenitors and differentiating hair cells during avian hair cell regeneration**. *Dev Dyn* 2007, **236**:156-170.
21. Roberson DW, Alosi JA, Cotanche DA: **Direct transdifferentiation gives rise to the earliest new hair cells in regenerating avian auditory epithelium**. *J Neurosci Res* 2004, **78**:461-471.
22. Taylor RR, Forge A: **Hair cell regeneration in sensory epithelia from the inner ear of a urodele amphibian**. *J Comp Neurol* 2005, **484**:105-120.
23. Scheibinger M, Ellwanger DC, Corrales CE, Stone JS, Heller S: **Aminoglycoside Damage and Hair Cell Regeneration in the Chicken Utricle**. *J Assoc Res Otolaryngol* 2018, **19**:17-29.
24. Jiang L, Xu J, Jin R, Bai H, Zhang M, Yang S, Zhang X, Zhang X, Han Z, Zeng S: **Transcriptomic analysis of chicken cochleae after gentamicin damage and the involvement of four signaling pathways (Notch, FGF, Wnt and BMP) in hair cell regeneration**. *Hear Res* 2018, **361**:66-79.
25. Ku YC, Renaud NA, Veile RA, Helms C, Voelker CC, Warchol ME, Lovett M: **The transcriptome of utricle hair cell regeneration in the avian inner ear**. *J Neurosci* 2014, **34**:3523-3535.
26. Lush ME, Diaz DC, Koenecke N, Baek S, Boldt H, St Peter MK, Gaitan-Escudero T, Romero-Carvajal A, Busch-Nentwich EM, Perera AG, et al.: **scRNA-Seq reveals distinct stem cell populations that drive hair cell regeneration after loss of Fgf and Notch signaling**. *Elife* 2019, **8**.
27. Desai SS, Zeh C, Lysakowski A: **Comparative morphology of rodent vestibular periphery. I. Saccular and utricular maculae**. *J Neurophysiol* 2005, **93**:251-266.
28. Oesterle EC, Campbell S, Taylor RR, Forge A, Hume CR: **Sox2 and JAGGED1 expression in normal and drug-damaged adult mouse inner ear**. *J Assoc Res Otolaryngol* 2008, **9**:65-89.
29. Pujol R, Pickett SB, Nguyen TB, Stone JS: **Large basolateral processes on type II hair cells are novel processing units in mammalian vestibular organs**. *J Comp Neurol* 2014, **522**:3141-3159.

30. Wang T, Niwa M, Sayyid ZN, Hosseini DK, Pham N, Jones SM, Ricci AJ, Cheng AG: **Uncoordinated maturation of developing and regenerating postnatal mammalian vestibular hair cells**. *PLoS Biol* 2019, **17**:e3000326.

** A detailed analysis of the origins of the different hair cell types from different sub-populations of supporting cells in the mouse utricular macula, both during initial development and when regenerated. Demonstrates the use of the most comprehensive and contemporary range of techniques, including electrophysiology, necessary to provide the most convincing identification of hair cell types and functionality.

31. Eatock RA, Songer JE: **Vestibular hair cells and afferents: two channels for head motion signals**. *Annu Rev Neurosci* 2011, **34**:501-534.

32. Burns JC, Cox BC, Thiede BR, Zuo J, Corwin JT: **In vivo proliferative regeneration of balance hair cells in newborn mice**. *J Neurosci* 2012, **32**:6570-6577.

33. Gomez-Casati ME, Murtie J, Taylor B, Corfas G: **Cell-specific inducible gene recombination in postnatal inner ear supporting cells and glia**. *J Assoc Res Otolaryngol* 2010, **11**:19-26.

34. Wang T, Chai R, Kim GS, Pham N, Jansson L, Nguyen DH, Kuo B, May LA, Zuo J, Cunningham LL, et al.: **Lgr5+ cells regenerate hair cells via proliferation and direct transdifferentiation in damaged neonatal mouse utricle**. *Nat Commun* 2015, **6**:6613.

35. You D, Guo L, Li W, Sun S, Chen Y, Chai R, Li H: **Characterization of Wnt and Notch-Responsive Lgr5+ Hair Cell Progenitors in the Striolar Region of the Neonatal Mouse Utricle**. *Front Mol Neurosci* 2018, **11**:137.

36. Cunningham LL: **The adult mouse utricle as an in vitro preparation for studies of ototoxic-drug-induced sensory hair cell death**. *Brain Res* 2006, **1091**:277-281.

37. Forge A, Li L: **Apoptotic death of hair cells in mammalian vestibular sensory epithelia**. *Hear Res* 2000, **139**:97-115.

38. Taylor RR, Filia A, Paredes U, Asai Y, Holt JR, Lovett M, Forge A: **Regenerating hair cells in vestibular sensory epithelia from humans**. *Elife* 2018, **7**

*****. First experimental study of vestibular epithelia of humans. Examines means to induce SC conversion. Transcriptome analysis provides data on changes in gene expression following hair cell loss and subsequent transduction with ATOH1.

39. Burns JC, On D, Baker W, Collado MS, Corwin JT: **Over half the hair cells in the mouse utricle first appear after birth, with significant numbers originating from early postnatal mitotic production in peripheral and striolar growth zones**. *J Assoc Res Otolaryngol* 2012, **13**:609-627.

40. Jen HI, Hill MC, Tao L, Sheng K, Cao W, Zhang H, Yu HV, Llamas J, Zong C, Martin JF, et al.: **Transcriptomic and epigenetic regulation of hair cell regeneration in the mouse utricle and its potentiation by Atoh1**. *Elife* 2019, **8**.

** Comprehensive analysis of patterns of gene expression and of transcriptional accessibility in mouse utricular macula after loss of hair cells and following transduction with *Atoh1*.

41. Lin V, Golub JS, Nguyen TB, Hume CR, Oesterle EC, Stone JS: **Inhibition of Notch activity promotes nonmitotic regeneration of hair cells in the adult mouse utricles**. *J Neurosci* 2011, **31**:15329-15339.
42. McLean WJ, Yin X, Lu L, Lenz DR, McLean D, Langer R, Karp JM, Edge AS: **Clonal Expansion of Lgr5-Positive Cells from Mammalian Cochlea and High-Purity Generation of Sensory Hair Cells**. *Cell Rep* 2017, **18**:1917-1929.
43. Wu J, Li W, Lin C, Chen Y, Cheng C, Sun S, Tang M, Chai R, Li H: **Co-regulation of the Notch and Wnt signaling pathways promotes supporting cell proliferation and hair cell regeneration in mouse utricles**. *Sci Rep* 2016, **6**:29418.
44. Samarajeewa A, Lenz DR, Xie L, Chiang H, Kirchner R, Mulvaney JF, Edge ASB, Dabdoub A: **Transcriptional response to Wnt activation regulates the regenerative capacity of the mammalian cochlea**. *Development* 2018, **145**.
45. Costa A, Sanchez-Guardado L, Juniat S, Gale JE, Daudet N, Henrique D: **Generation of sensory hair cells by genetic programming with a combination of transcription factors**. *Development* 2015, **142**:1948-1959.
46. Layman WS, Zuo J: **Epigenetic regulation in the inner ear and its potential roles in development, protection, and regeneration**. *Front Cell Neurosci* 2014, **8**:446.
47. Stojanova ZP, Kwan T, Segil N: **Epigenetic regulation of Atoh1 guides hair cell development in the mammalian cochlea**. *Development* 2015, **142**:3529-3536.
48. McInturff S, Burns JC, Kelley MW: **Characterization of spatial and temporal development of Type I and Type II hair cells in the mouse utricle using new cell-type-specific markers**. *Biol Open* 2018, **7**.
49. Koehler KR, Nie J, Longworth-Mills E, Liu XP, Lee J, Holt JR, Hashino E: **Generation of inner ear organoids containing functional hair cells from human pluripotent stem cells**. *Nat Biotechnol* 2017.

*Building on the previous work using mouse ESCs this paper describes the use of 3D approaches to generation of human inner ear organoids in vitro, providing a potential platform for investigation of inner ear development and hair cell regeneration.

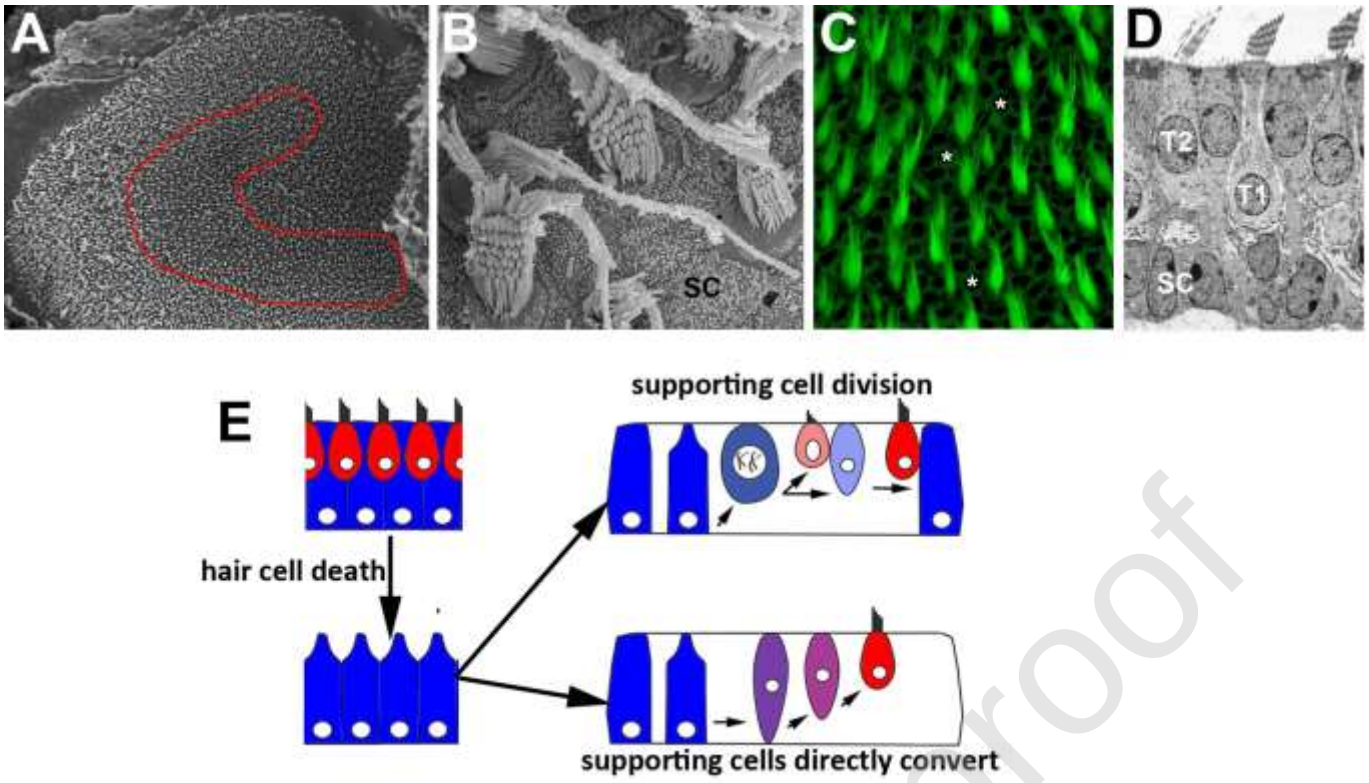

Figure Legend.

A. Utricular macula of guinea pig vestibular system. Hair cells, the hair (stereociliary) bundles of which are the white puncta, are distributed across the epithelial sheet. The approximate limit of the striola is depicted by the red line.

B. Hair bundles in utricular macula of a ferret. A hair bundle is formed of rows of stereocilia that increase in height in one direction across the apical surface of the HC defining a morphological polarity. Supporting cells (SC) between each hair cell bear short microvilli at their apical surface

C. Fluorescently-tagged phalloidin labelling of f-actin in mouse utricular macula. Stereocilia are composed of f-actin, and labelling of f-actin at the adherens junctions around the necks of all cells delineates the apical surfaces of the SCs (asterisks) revealing that SCs separate neighbouring HCs from each other. The actin bands associated with adherens junctions in SCs are unusually thick.

D. Section through utricular macula of a guinea pig. Two types of HC can be distinguished by morphology and innervation patterns. Type I (T1) HCs are flask shaped with entire baso-lateral surface enclosed with a calyx nerve ending of a single afferent nerve fibre. Type II (T2) are approximately cylindrical with several bouton nerve- endings synapsing at the base. (Type II HCs may have numerous basally projecting elongations [28]). The supporting cells (SC) sit on the basement membrane and send projections between HCs to the luminal surface. The bodies of the SCs intervene between the base of each HC and the basement membrane; HCs do not contact the underlying basement membrane.

E. Diagrammatic representation of the two modes of hair cell regeneration observed in non-mammalian vertebrates: SC division and differentiation of HCs from one of the daughter cells; and direct, non-mitotic phenotypic conversion of SCs.