



This is the author's version of a work that was accepted for publication in the following source:

AK Wise, BO Flynn, PJ Atkinson, JB Fallon, M Nicholson and RT Richardson (2015). Regeneration of cochlear hair cells with Atoh1 gene therapy after noise-induced hearing loss. *Journal of Regenerative Medicine* 4:1

**Notice:** Changes introduced as a result of publishing processes such as copy-editing and formatting may not be reflected in this document. For a definitive version of this work, please refer to the published source:

The final publication is available SciTechnol:

<http://dx.doi.org/10.4172/2325-9620.1000121>

DOI: 10.4172/2325-9620.1000121

Copyright of this article belongs to SciTechnol 2015.

# Regeneration of cochlear hair cells with *Atoh1* gene therapy after noise-induced hearing loss

Andrew K Wise (PhD)

Bionics Institute, Melbourne, Australia

University of Melbourne, Department of Otolaryngology, Melbourne, Australia

University of Melbourne, Department of Medical Bionics, Melbourne, Australia

[awise@bionicsinstitute.org](mailto:awise@bionicsinstitute.org)

Brianna O Flynn (BSc(Hons))

Bionics Institute, Melbourne, Australia

[bflynn@bionicsinstitute.org](mailto:bflynn@bionicsinstitute.org)

Patrick J Atkinson (PhD)

Stanford University, School of Medicine, Department of Otolaryngology, Stanford, CA, USA

[pjatk@stanford.edu](mailto:pjatk@stanford.edu)

James B Fallon (PhD)

Bionics Institute, Melbourne, Australia

University of Melbourne, Department of Otolaryngology, Melbourne, Australia

University of Melbourne, Department of Medical Bionics, Melbourne, Australia

[jfallon@bionicsinstitute.org](mailto:jfallon@bionicsinstitute.org)

Madeline Nicholson (BSc)

Bionics Institute, Melbourne, Australia

[mnichols@bionicsinstitute.org](mailto:mnichols@bionicsinstitute.org)

Rachael Richardson (PhD)

Bionics Institute, Melbourne, Australia

University of Melbourne, Department of Otolaryngology, Melbourne, Australia

University of Melbourne, Department of Medical Bionics, Melbourne, Australia

[rrichardson@bionicsinstitute.org](mailto:rrichardson@bionicsinstitute.org)

## **Corresponding author:**

Dr Rachael Richardson

Bionics Institute

384 Albert Street

East Melbourne

Victoria 3002

Australia

Ph: +613 9667 7594

Fax: +613 9667 7518

E: [rrichardson@bionicsinstitute.org](mailto:rrichardson@bionicsinstitute.org)

**Running head:** Hair cell regeneration in the cochlea

## **Grants/Financial Support:**

Action on Hearing Loss

The Garnett Passe and Rodney Williams Memorial Foundation

National Health and Medical Research Council (GNT1024350)

Victorian Government Operational Infrastructure Support Program

# Regeneration of cochlear hair cells with *Atoh1* gene therapy after noise-induced hearing loss

Andrew K. Wise<sup>1,2,3</sup>, Brianna O. Flynn<sup>1</sup>, Patrick J. Atkinson<sup>4</sup>, James B. Fallon<sup>1,2,3</sup>, Madeline Nicholson<sup>1</sup>, Rachael T. Richardson<sup>1,2,3</sup>

<sup>1</sup>Bionics Institute, Melbourne, Australia

<sup>2</sup>University of Melbourne, Department of Otolaryngology, Melbourne, Australia

<sup>3</sup>University of Melbourne, Department of Medical Bionics, Melbourne, Australia

<sup>4</sup>Stanford University, School of Medicine, Department of Otolaryngology, Stanford, CA, USA

## Abstract

**Background:** Degeneration of hair cells in the mammalian cochlea results in irreversible hearing loss with no current treatment options to regain lost hair cell function. The *Atoh1* gene is necessary for hair cell development and recent research has shown that *Atoh1* gene therapy promotes new hair cell formation and hearing restoration in adult rodent deafness models. **Objective:** The aim of this study was to examine new hair cell formation via *Atoh1* gene therapy in noise-deafened adult guinea pigs. **Methods:** Guinea pigs were deafened by noise exposure (130 dB, 11-13 kHz, 2 hours). After two weeks, the left cochleae were injected with an adenoviral vector containing the *Atoh1* gene. Control animals were injected with a control adenoviral vector. Three weeks after injection cochleae were assessed for hair cell density, maturity and hair cell synaptogenesis with auditory neurons. Hearing thresholds were assessed throughout. **Results:** There were significantly more myosinVIIa-positive hair cells in cochleae that received *Atoh1* gene therapy compared to contralateral cochleae and compared to cochleae that received control gene therapy ( $p < 0.05$  one way ANOVA). However, the number of hair cells in *Atoh1*-treated animals was far below normal. Expression of *Atoh1* had a significant preservation effect on the cytoarchitecture of the sensory epithelium compared to controls ( $p < 0.001$  one way ANOVA). Expression of the synaptic protein CtBP2 was present in some transfected cells from *Atoh1*-injected guinea pigs but at a reduced density compared to normal cochleae. There was evidence of auditory neuron preservation near transfected hair cells in *Atoh1*-injected cochleae ( $p < 0.05$  one way ANOVA), but there were no improvements in hearing thresholds. **Conclusion:** This study supports growing evidence that new hair cell formation is possible in mature cochleae that have been severely damaged, in this case by noise, and demonstrates a protective influence of *Atoh1* gene therapy on the immediate surrounding cellular environment.

**Keywords:** *Atoh1*, cochlea, deafness, gene therapy, hair cells, noise, regeneration, supporting cells

## Introduction

Cochlear hair cells (HCs) convert acoustic sound waves into electrical signals that activate spiral ganglion neurons (SGNs) to convey acoustic information to the auditory brain. Noise, infection or ototoxic drugs can trigger sensorineural hearing loss as a result of degeneration of HCs, supporting cells and SGNs, or the loss of the synapse between the HC and SGN. Therapeutic restoration of hearing after sensorineural hearing loss would therefore require a multi-faceted approach that includes *protection* of residual sensory cells and supporting cells, *regeneration* of lost sensory cells and *reconnection* of denervated HCs with the peripheral fibres of the SGNs.

Inner hair cells (IHCs) and outer hair cells (OHCs) reside in the sensory epithelium of the cochlea

known as the organ of Corti along with supporting cells (border cells, pillar cells, phalangeal cells, Deiters' cells, Hensen's cells and Claudius' cells). IHCs receive innervation from SGNs, with a single IHC being innervated by 20-30 type I afferent SGN fibres. Unmyelinated type II SGNs directly synapse OHCs, with a single afferent nerve fibre innervating many OHCs [1]. IHCs are primarily responsible for the excitation of SGNs whereas OHCs have been implicated in the mechanical amplification of sound, enhancing the responsiveness of the sensory epithelium to selected frequencies and the detection of low intensity sounds [2]. The cochlea is tonotopically organised such that low frequency sounds primarily activate IHCs in the apical turns of the cochlea while high frequency sounds primarily activate IHCs in the basal turns of the cochlea.

Noise induced hearing loss is the most common form of environmental hearing loss. A noise component was associated with 37% of the population with a hearing loss [3]. Acoustic overstimulation causes massive swelling of the IHC nerve terminals 24-48 hours after acoustic overexposure [4, 5] due to glutamate toxicity [6, 7]. The swelling disappears after a few days and thresholds can return to normal [8], a phenomenon known as a temporary threshold shift (TTS). However, even with a TTS, permanent structural changes have occurred to the IHC-SGN synapse [9]. Permanent threshold shift (PTS) results from destruction of cochlear HCs or damage to their stereociliary bundles [10]. OHCs at the base of the cochlea (high frequency) are the most sensitive, but IHCs can also be affected. The HC lesion spreads basally and apically over time stabilising at about two weeks [11-13]. However, neural loss after noise exposure is delayed by months and can continue for years [14, 15].

Viral gene therapy to the cochlea has been widely investigated as a means to introduce genes that protect HCs, SGNs and even trans-differentiate supporting cells into new HCs. The fluid filled scalae of the cochlea provide an ideal environment for localised gene delivery. Transgene delivery to the scala tympani results in gene expression in a broad range of cells (e.g. mesenchymal cells lining the scala tympani, SGNs, supporting cells, HCs) with expression typically observed in all turns of the cochlea [16-21]. Delivery to the scala media, the compartment that houses the sensory epithelium, results in a more localised gene expression pattern (e.g. HCs, supporting cells, interdental cells) that is also more localised to the basal turn of the cochlea [16, 17, 22-25]. But when supporting cells are the target for gene therapy, the progression of deafness-induced degeneration reduces the effectiveness of gene therapy after severe forms of sensorineural hearing loss such as occurs following exposure to ototoxic aminoglycoside drugs [26]. This rapid degeneration of supporting cells becomes particularly problematic for HC regeneration as supporting cells are required for trans-differentiation into new HCs via expression of *Atoh1*, a gene involved in HC development [27, 28].

The role of *Atoh1* in HC development is well documented. Mice that are null for *Atoh1* do not have HCs or supporting cells [29, 30]. Conversely, over-expression of *Atoh1* by gene therapy results in the generation of ectopic HCs that have trans-differentiated from supporting cells [23, 31, 32]. Mouse models with conditional *Atoh1* deletion and conditional periods of *Atoh1* expression elegantly demonstrate that the level and duration of *Atoh1* expression are critical for hair cell viability and differentiation. For example, hair cell death occurs if *Atoh1* expression is prematurely terminated or if *Atoh1* is expressed permanently at inappropriate levels [28, 33-35]. Cells that express *Atoh1* by *in utero* gene transfer in mice appear to attract neuronal processes that trace back to the cochlear nucleus, express synaptic markers and mechanotransduce auditory signals [36] demonstrating that the *Atoh1*-transfected HCs are functional in neonatal animals. However, the efficiency of conversion

of supporting cells to HCs is age-dependent, with Atoh1-mediated conversion of supporting cells to HCs proving more difficult to achieve in adult mice [28]. Furthermore, while the newly generated HCs expressed a multitude of HC markers, they did not express the mature HC markers prestin and oncomodulin [28]. Partial recovery of hearing has been achieved in some studies after acoustic or ototoxic trauma by the forced expression of Atoh1 in supporting cells or by inducing Atoh1 expression through manipulation of the notch signalling pathway [31, 37, 38]. In one study, Atoh1 expression after simulated gun-shot noise exposure was shown to improve hearing thresholds through the repair of stereocilia of residual HCs rather than the regeneration of new HCs, suggesting an additional role of Atoh1 in repair as well as regeneration [39]. After severe ototoxic hearing loss, HCs could not be regenerated if *Atoh1* gene therapy was administered more than 4 days after the initiation of hearing loss in guinea pigs due to loss of supporting cells and a ‘flattening’ of the sensory epithelium [40]. However, the organ of Corti can have extremely variably pathology with regions of surviving supporting cells amid regions of severe damage [41]. Given the importance of residual supporting cells on the success of *Atoh1* gene therapy, models of hearing loss in which supporting cells survive for longer periods warrant further study.

Noise-induced hearing loss presents a less-severe model of deafness with slower degeneration of the sensory epithelium and is more widely clinically relevant compared to ototoxic aminoglycosides used to initiate profound deafness in animal models. The aim of this study was to use *Atoh1* gene therapy to regenerate cochlear HCs from residual supporting cells after noise-induced hearing loss. Guinea pigs were exposed to noise 2 weeks before *Atoh1* gene therapy was introduced via the scala media. Within the noise-induced lesion, HC survival, supporting cell survival and structure, peripheral fibres and synaptic protein expression in HCs were examined.

## Methods

### Animals and experimental timeline

Fourteen young adult pigmented Dunkin–Hartley guinea pigs of either sex were used. All procedures were approved by the Animal Research Ethics Committee of the Royal Victorian Eye and Ear Hospital in accordance with the National Institute of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (NIH publication no. 85-23 Rev. 1985) and the Australian National Health and Medical Research Council guidelines. Guinea pigs were randomly assigned to one of four experimental groups (2 weeks deaf untreated control, 5 weeks deaf untreated control, control gene therapy or *Atoh1* gene therapy). An auditory brainstem response (ABR) was performed 1 week prior to noise exposure (T-1) to ensure normal hearing (see ABR recordings). At time zero (T0), guinea pigs were exposed to 130 dB closed-field noise for 2 hours (see noise deafening). In the untreated groups, guinea pigs were perfused after two weeks (T2) or 5 weeks (T5). In the treated groups, the left cochleae of guinea pigs were inoculated with adenoviral vectors at T2 and the animals were perfused at T5.

### ABR recordings

Click and tone-pip auditory brainstem responses (ABRs) were measured in left and right ears prior to deafening (T-1) under anaesthesia (60 mg/kg ketamine; Parnell Laboratories, Australia; 4 mg/kg xylazine-20; Troy Laboratories, Australia, mixed in a 3:1 ratio) using procedures described previously [42, 43]. Only animals with normal hearing thresholds (click ABR threshold <43 dB peak-equivalent sound pressure level) were used in the study. ABRs were re-measured at T2 (2 weeks post-noise

exposure, just prior to viral injection where applicable), and again at T5 (5 weeks after noise exposure, 3 weeks after viral injection where applicable). Tone-pip ABRs were measured at 1, 2, 8, 16, 24 and 32 kHz.

### Noise deafening

Guinea pigs were placed in a non-traumatic head holder under ketamine/xylazine anaesthesia. The tympanic membrane of each ear was otoscopically examined and determined to be normal for all animals. A speculum was positioned inside the external meatus of each ear and the tympanic membrane was visualised. A calibrated speaker was attached to each speculum to deliver closed-field acoustic stimulation to each ear. Guinea pigs were exposed to 11, 12 and 13 kHz tones at 130 dB for 2 hours.

### Gene therapy

E1/E3/polymerase/terminal protein-deleted adenovirus type 5 vectors were produced and tested as described previously [17, 26]. The vectors contained the gene for green fluorescent protein (GFP) under the control of a cytomegalovirus promoter with or without mouse Atoh1 expressed via an IRES sequence (Ad-GFP or Ad-GFP-Atoh1). The viruses were titered on HT1080 cells by spectrophotometry [ $>10^{11}$  optical particle units (OPU)/ml by  $OD_{260}$ ][44]. Viral stocks were verified by PCR to be free of replication-competent adenovirus. Before the scala media injection, the adenovirus vectors were diluted 1:5 in artificial endolymph (120 mM KCl, 2.5 mM NaCl, 0.5 mM  $MgCl_2$ , 0.28mM  $CaCl_2$ , 7.6 mM  $K_2HPO_4$ , 2.7 mM  $KH_2PO_4$ , pH 7.4) to final concentrations of  $1.1 \times 10^{11}$  OPU/ml (Ad-GFP) and  $2.2 \times 10^{11}$  OPU/ml (Ad-GFP-Atoh1).

Two microliters of Ad-GFP or Ad-GFP-Atoh1 were unilaterally injected into the scala media of the left cochlea of deafened guinea pigs over 5 minutes using aseptic techniques as described previously [17, 26].

### Histology

At the experimental endpoint, guinea pigs were perfused and their cochleae harvested as described previously [26]. The cochleae were decalcified over two weeks in 10% (w/v) ethylenediaminetetraacetic acid (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia) in 0.1M PBS, embedded in optimum cutting compound (OCT; ProSciTech, Kirwan, QLD, Australia) and sectioned on a cryostat. Sections were collected up to and including the mid-modiolar region and half turns were removed from the remaining tissue for surface preparations of the sensory epithelium. Standard immunofluorescent protocols were followed using antibodies to neurofilament heavy chain (NFH; Merck Millipore, Australia) to label SGNs and peripheral fibres, phalloidin (Life Technologies, CA, USA) to stain pillar cells in the sensory epithelium, Ctbp2 (BD BioSciences, New Jersey, USA) for staining the ribbon synapse on HCs (this antibody also stains nuclei), myosinVIIa (Proteus BioScience, CA, USA) for HCs and AlexaFluor secondary antibodies (Life Technologies, CA, USA). Sections were examined on a Zeiss Axioplan II fluorescence microscope (Carl Zeiss, Jena, Germany). Confocal images were taken on a Nikon A1R confocal microscope. Images shown are the maximum projection of a z-series spanning the sensory epithelium. The viewing angle is from the sensory epithelium.

### Data analysis

Hair cells were analysed from the lower basal (LB) and upper basal (UB) turn sensory epithelia using mid-modiolar sections of the cochlea and basal turn surface preparations. Mid-modiolar sections

were taken to enable histological analysis of HCs and supporting cells while surface preparations were used primarily for counting the number of HCs within the noise lesion and examining the neural response to *Atoh1* gene therapy. Having the two modalities of analysis from the same cochlea enabled more information to be extracted from the same animal.

**Mid-modiolar sections:** Sections were collected every 72  $\mu\text{m}$  through the mid-modiolar region and analysed for the presence of HCs via MyosinVIIa. The percentage of sections containing HCs was determined for each group. MyosinVIIa and GFP co-localisation was determined. The noise-induced damage to the sensory epithelium was assessed using a rating scale 0-2: Rating 0 referred to a sensory epithelium with no HCs, supporting cells or tunnel of Corti (flattened epithelium); Rating 1 referred to a sensory epithelium with supporting cells but no tunnel of Corti (HCs present or absent); Rating 2 referred to a sensory epithelium with supporting cells and a tunnel of Corti (HCs present or absent) (See Figure 1). Ratings were averaged for each turn and group and compared using a 2-tailed T-test. Peripheral fibres were analysed in mid-modiolar sections from which it was recorded whether the peripheral fibres extended past the habenula perforata to the anatomical region corresponding to IHC area, the OHC area and whether they synapsed with HCs.

**Surface preparations:** The length of the noise damaged IHC lesion was measured in the image processing program ImageJ (NIH, Bethesda, MD, USA). The number of HCs per mm of the damaged IHC region was counted. MyosinVIIa and GFP co-localisation was determined. Peripheral fibres were analysed from 3 areas of basal turn surface preparations (mid-lesion and the mid-point between this and the two edges of the lesion). Within the selected fields, 8 peripheral fibre length measurements were taken at regular intervals along the sensory epithelium. The lengths of the peripheral fibres were measured from the habenula perforata to the furthest point reached by the peripheral fibre within the field of study, even if the fibre was looping back at that point.

Data is presented as mean  $\pm$  standard error of the mean (SEM).

## Results

Mid-modiolar sections and surface preparations were used to collect data on the number of hair cells, presence of supporting cells, peripheral fibres and the structural integrity of the sensory epithelium. Noise exposure resulted in loss of hair cells in the basal turn of the cochlea. Therefore, the focus of analysis was on the basal turn surface preparations and the lower and upper basal turns from mid-modiolar sections. The schematic in **Figure 1A** shows the LB and UB turns of the cochlea in which the sensory epithelium was analysed.

### Sensory epithelium

First the sensory epithelium was examined for the presence of hair cells, supporting cells and the tunnel of Corti and rated 0, 1 or 2 ('0' being a flattened epithelium with no supporting cells or tunnel of Corti, '1' representing an epithelium with supporting cells but no tunnel of Corti and '2' indicating an epithelium with a tunnel of Corti and supporting cells). Examples of the sensory epithelium in the LB turn are shown in **Figure 1A** with their respective ratings.

In the 2 week group the LB sensory epithelium contained supporting cells in all cases and a tunnel of Corti was present in 53% of sections. The average rating of the sensory epithelium was  $1.29 \pm 0.13$  (**Figure 1B**). By 5 weeks post-noise exposure (in the absence of control or *Atoh1* gene therapy) the sensory epithelium in the LB turn was significantly more degenerated compared to the 2 week group

( $p < 0.001$ ; one way ANOVA on Ranks; **Figure 1B**). In the 5 week untreated group, there was collapse of the tunnel of Corti in all mid-modiolar sections examined in the LB turn (average rating  $0.3 \pm 0.08$ ). In the control gene therapy group the epithelium was flattened (rating 0) in 45% of mid-modiolar sections and the tunnel of Corti was absent (rating 0 or 1) in 97% of sections. The average rating for the control gene therapy group was  $0.59 \pm 0.074$ . In the *Atoh1* gene therapy group, the average sensory epithelium rating was  $1.17 \pm 0.085$ : significantly greater than the 5 week and control gene therapy groups and not significantly different to the 2 week group ( $p < 0.001$ ; one way ANOVA) (**Figure 1B**). The sensory epithelium rating on the injected side of *Atoh1* gene therapy group animals was also significantly greater than the contralateral (untreated) side ( $1.12 \pm 0.098$  vs  $0.74 \pm 0.066$ ;  $p < 0.005$  one-way ANOVA). Transgene expression was not uniformly distributed within the lesion with 'patches' of gene expression present. The rating of the sensory epithelium was significantly greater in regions of transgene expression compared to regions without transgene expression ( $1.52 \pm 0.18$  vs  $0.88 \pm 0.19$ ;  $p < 0.05$ ; 2-tailed T-test). Areas of transgene expression in the sensory epithelium of the control gene therapy group did not significantly change the sensory epithelium rating compared to areas where there was no transgene expression (2-tailed T-test). In the UB turn, all animals had average epithelium ratings close to 3 (all supporting cells and tunnel of Corti were present) indicating that the noise-induced lesion was restricted to the LB region in all animals.

### Transgene expression after gene therapy

In the control gene therapy and *Atoh1* groups, transgene expression was observed in the sensory epithelium (IHCs, OHCs, inner and outer pillar cells, Hensen's cells, Deiters' cells and inner phalangeal cells), greater epithelial cells, interdental cells of the spiral limbus, spiral ligament and, less commonly, stria vascularis and cells lining the scala tympani or scala vestibuli (**Figure 2**). Expression was predominantly in the LB turn with minor expression in the UB turn.

### Hair cell survival

Hair cell survival was quantified from basal turn surface preparations in which myosin VIIa-positive cells were counted within the noise-damaged region. Hair cells were also assessed in mid-modiolar sections. Examples from each group are shown in **Figure 3**.

The apical end of the basal turn surface preparations had intact IHCs in all but one case showing that the IHC noise lesion was restricted to the LB turn in most cases. The IHC lesion began at varying distances from the apical end and extended beyond the basal end of the surface preparation (except the 2 week group). Nine out of 14 surface preparations were missing all OHCs demonstrating that the OHC lesion was larger than the IHC lesion and extended to the UB turn in some cases. HCs were counted within the IHC lesion that was present on the surface preparation (expressed as number of HCs per mm of IHC lesion).

The two week untreated group was analysed to show the extent of cell damage or loss at the time of gene therapy delivery in the treated groups. Two weeks after noise exposure the region of IHC loss was  $1.9 \pm 0.4$  mm. Within the notch of IHC loss, there were  $1.2 \pm 0.61$  IHCs/mm and no OHCs (**Figure 3B and Figure 4**). In mid-modiolar sections, IHCs were present in  $61.7 \pm 11.4\%$  of sections in the LB turn (**Figure 3B**) and no OHCs were present. The OHC lesion spanned the LB and UB turns of the cochlea.

Five weeks after noise exposure analysis of the surface preparations revealed that the length of the

IHC lesion had expanded (but could not be measured as it extended beyond the edge of the surface preparation). Analysis of the HCs within the IHC lesion showed that on average the IHC count was reduced to  $0.1 \pm 0.1$  IHCs/mm (**Figure 3C and Figure 4**). Mid-modiolar sections showed that the IHC lesion was predominantly in the LB turn (with IHCs present in only  $35.3 \pm 22.5\%$  LB mid-modiolar sections (t-test:  $p=0.06$  compared to the 2 week group) with additional sporadic losses of IHCs in the UB turn. The OHC lesion spanned both the LB and UB turns, with complete OHC loss in the LB turn in all animals and some OHC loss in the UB turn. The contralateral side was not significantly different.

In surface preparations from the control gene therapy group, there were  $1.6 \pm 0.76$  HCs/mm within the region of damage, none of which were positive for GFP (**Figure 4**). On the contralateral side there were  $2.2 \pm 1.0$  HCs/mm in the lesion (**Figure 4**). In mid-modiolar sections,  $48.9 \pm 18.5\%$  of the sections from the injected side contained IHCs, of which  $4.2\%$  co-localised with GFP expression (only one residual HC), indicating only minimal transfection of residual HCs was observed (**Figure 3D**). OHCs were absent in all cases. On the contralateral side,  $30.0 \pm 20.1\%$  of sections contained IHCs (all negative for GFP).

In the *Atoh1* group all animals had GFP-positive (GFP<sup>+</sup>) IHCs. In the regions of IHC damage, surface preparations revealed there were on average  $14.1 \pm 5.4$  HCs/mm in the *Atoh1* group (average  $19 \pm 4.5$  HCs per animal), which was significantly more than all other groups ( $p<0.05$ , one Way ANOVA, Holm-Sidak) (**Figure 3E and Figure 4**). Importantly,  $53.4\% \pm 11.6\%$  of these HCs were GFP<sup>+</sup> indicating that there was a much greater expression of the *Atoh1* transgene in HCs compared to the HC expression of the control vector in the control gene therapy group (present in only one HC). On the contralateral side there was only  $1.0 \pm 0.44$  HC/mm in the damaged region. This was not significantly different to the contralateral sides of any other group ( $p=0.128$ , one-way ANOVA). On average,  $61.2 \pm 5.9\%$  of mid-modiolar sections from the injected cochleae of the *Atoh1* group contained 1 or more IHCs of which  $51.7 \pm 14.5\%$  co-localised with GFP expression.

In the *Atoh1* group, 1 out of 4 injected cochleae had OHCs (present in  $35.3\%$  of mid-modiolar sections in the LB turn) all of which co-localised with GFP expression. No other group had OHCs in the LB turn of mid-modiolar sections except the 2 week group.

HCs that expressed GFP also expressed *Ctbp2* in  $37.5\%$  cases analysed ( $n=16$  cells) (**Figure 5**). Interdental cells in the spiral limbus that expressed GFP were also observed to express myosinVIIa in  $71\%$  of cases (from sections) (**See Figure 2C**). Cells in any other region of the cochlea that expressed GFP (e.g. cells lining the scala vestibuli or the spiral ligament) were never observed to express myosinVIIa.

### Peripheral fibres

The length of peripheral fibres was measured as a metric of degeneration and resprouting. In the 2 week group, peripheral fibres extended past the habenula perforata in all cases ( $n=28$  mid-modiolar cross sections), and in  $25\%$  of analysed sections the fibres crossed the tunnel of Corti towards the OHC region (**Figure 6A**). The average length of peripheral fibres from the habenula perforata to the furthest point of the fibre in basal turn surface preparations was  $59.6 \mu\text{m} \pm 10.1 \mu\text{m}$ . In the 5 week group, peripheral fibres extended past the habenula perforata in only  $18.75\%$  of mid-modiolar sections analysed ( $n=16$ ) and were never observed to cross towards the OHC region, indicative of the retraction of the type I and type II fibres typically observed following damage to the organ of Corti. Fibres often appeared to loop back at the point of the habenula perforata (**Figure 6B**). The

average fibre length in basal turn surface preparations in the 5 week group was  $17.1 \pm 5.7 \mu\text{m}$ . In the control gene therapy group, peripheral fibres extended past the habenula perforata in 71.8% of sections analysed (n=39). The average length of peripheral fibres measured in surface preparations of the sensory epithelium was  $41.9 \pm 3.0 \mu\text{m}$ . In the *Atoh1* group, peripheral fibres extended past the habenula perforata in 91.1% of sections analysed (n=56), and were observed to extend towards the OHC region when HCs were present in this region. In cases when there were ectopic HCs within the IHC region, fibres were observed to extend towards these HCs (**Figure 6C**). The average length of peripheral fibres measured in surface preparations of the sensory epithelium from the *Atoh1* group was significantly greater than the 5 week group at  $71.5 \pm 1.9 \mu\text{m}$  ( $p < 0.05$  one-way ANOVA, Holm-Sidak comparisons), while there was no significant difference between the control gene therapy group and the 5 week untreated group ( $p = 0.58$  one-way ANOVA, Holm-Sidak comparisons). In regions that were more distal to GFP expression in the *Atoh1* group, the length of peripheral fibres was  $34.9 \pm 4.6 \mu\text{m}$  and was not significantly different to any other group.

### Hearing thresholds

Prior to noise exposure (T-1), all GPs were normal hearing with both ears displaying similar ABR thresholds for click and tone-pip stimuli (1 and 32 kHz). Two weeks following noise exposure (T2) there was a significant elevation in click thresholds ( $p < 0.001$ , 2-tailed T-test) and a significant increase in tone-pip thresholds for 8 kHz and above ( $p < 0.05$  one-way ANOVA). The effects of noise exposure were consistent across the experimental cohorts (T-1 and T2 in **Figure 7**).

To assess functional changes in hearing during and following gene therapy, ABR thresholds were remeasured at 4 weeks (T4) and 5 weeks (T5) after noise exposure (equating to 2 and 3 weeks of gene therapy). As expected there was no change in the 5 week deaf control (untreated) group indicating a permanent elevation of hearing thresholds following noise exposure. Similarly, there was no change in hearing thresholds during and following delivery of the control vector. Importantly, there was no change in ABR thresholds during, or at the completion, of the *Atoh1* gene therapy indicating that there was no restoration of hearing sensitivity.

### Intra-scala fibrous tissue

In the control gene therapy group, 75% of animals had fibrotic tissue in the LB turn, occupying 50-70% of the scala tympani. Fifty percent of animals in the *Atoh1* group had fibrotic tissue in the scala tympani. One case exhibited damage to the basilar membrane and fibrous tissue growth in the scala tympani but still had GFP<sup>+</sup> HCs and surrounding supporting cells. All fibrous tissue growth was restricted to the LB turn.

### Discussion

This study has demonstrated successful transgene expression in a damaged sensory epithelium two weeks after exposure to noise (130 dB for 2 hours). Within the noise-induced lesion of the organ of Corti there was extensive loss of inner and outer hair cells but importantly supporting cells, vital for hair cell trans-differentiation, were still present 2 weeks after noise exposure. Forced expression of the *Atoh1* gene by adenoviral gene therapy in supporting cells such as inner pillar cells or Deiters' cells resulted in the expression of HC markers such as myosin VIIa. There were significantly more myosin VIIa<sup>+</sup> HCs within the noise lesion of guinea pigs inoculated with the *Atoh1* gene compared to controls, but hearing thresholds were not improved in this group at 3 weeks post gene therapy. There was significant preservation of both the spiral ganglion neuron peripheral fibres and the

cytoarchitecture of the sensory epithelium (supporting cell survival and a preserved tunnel of Corti) in the *Atoh1* group, suggesting a protective role for *Atoh1* gene therapy against the progressive degeneration of the organ of Corti.

### Noise exposure

A two hour exposure to 130 dB noise at frequencies between 11 and 13 kHz produced stable and permanent threshold shifts between 8 and 32 kHz, corresponding to the LB and UB turns of the cochlea. In the basal turn at 2 weeks post noise-exposure there was loss of OHCs and IHCs and regions in which the tunnel of Corti had collapsed, but importantly for *Atoh1* gene therapy, the supporting cells (pillar cells, Deiters' cells and Hensen's cells) were still present. By 5 weeks, thresholds were 100 dB SPL or higher (100 dB was the stimulus maximum intensity used). Histologically, the area of damage to the organ of Corti had spread and the extent of damage had worsened compared to the 2 week noise-exposed group. There was greater OHC loss (extending apically to the UB turn), greater IHC loss in the LB turn and significantly greater loss of supporting cells in the noise damaged region. Other studies are consistent with these findings with threshold shifts reported to stabilise at about day 10 post noise-exposure but with the area of damage and the severity of damage continuing to progress and spread to adjacent regions of the sensory epithelium over a period of weeks to months in guinea pigs and chinchillas [13, 41, 45]. As HCs and supporting cells die, they are replaced by non-specialised squamous cells that cover the basilar membrane between 30-100 days post noise-exposure [11, 41]. There are often areas of surviving supporting cells among the flattened squamous epithelium and as long as these are present there exists the potential to generate new HCs [41].

### Transgene expression

The LB (high frequency) region of the cochlea is the area of the cochlea that is most affected by noise exposure in humans. Gene therapy via the scala media resulted in gene expression that was largely localised to the sensory epithelium in the LB turn of the cochlea, with only minor gene expression in the UB turn. This expression pattern is consistent with other studies in which gene therapy is given via the scala media of the basal turn [16, 17, 46]. Gene therapy to the scala media enhances the targeting of the transgene to supporting cells of the sensory epithelium [17]. However, gene therapy in the scala media could disrupt the maintenance of ionic imbalance between the endolymph and perilymph that is essential for mechanotransduction and result in hearing loss. There was hearing loss associated with gene therapy to the scala media in this and other studies [16, 17, 25] as well as histological evidence of surgery-induced trauma (fibrous tissue formation or damage to the basilar membrane) in the LB turn in some injected animals (3 from control gene therapy group and 2 from *Atoh1* group). From a translational point of view, it is possible to observe the basilar membrane during surgical exposure for cochlear implantation if performing a cochleostomy, however, it may be less damaging to use an intra-labyrinthine approach which has shown to result in gene expression within supporting cells of the cochlea with minimal damage to hearing [47]. Alternatively, some types of viral vectors can cross the round window membrane and transfect the sensory epithelium [48], avoiding the breaching of the cochlea and the associated hearing loss.

### *Atoh1* gene therapy after noise exposure

*Atoh1* gene therapy after noise exposure has been examined prior to this study, but with several important differences in the level of noise exposure and the time between exposure and treatment, both of which can impact on the presence of residual HCs and supporting cells at the time of gene therapy and the expected level of HC and hearing recovery. Expression of *Atoh1* in the sensory epithelium via adenoviral gene therapy 7 days after noise exposure in guinea pigs (via injection

through the round window membrane) resulted in some hearing recovery [39]. The noise exposure was severe enough to damage the stereocilia on the HCs, but not to result in loss of HCs at the time of *Atoh1* gene therapy. Interestingly, *Atoh1* transgene expression was adjacent to HCs rather than in the damaged HCs themselves and appeared to result in the repair of the stereocilia on the damaged HCs suggesting an additional role of *Atoh1* beyond trans-differentiation of supporting cells to HCs [39]. In support of this, conditional deletion of *Atoh1* at different development periods in mice demonstrated roles of *Atoh1* in HC bundle development, HC survival and indirectly, supporting cell survival [49]. In another study, a  $\gamma$ -secretase inhibitor was locally administered to noise damaged 4-week old mouse cochleae 1 day after noise exposure (116 dB SPL, 8-16 kHz) to inhibit Notch signalling and thereby promote *Atoh1* gene expression [37]. Improved numbers of HCs and moderate hearing improvements in the treated group were observed [37].

Other studies that have shown hearing recovery after *Atoh1* gene therapy had much longer treatment times compared to the 3 weeks used in this study. In Izumikawa 2005, guinea pigs were deafened by ototoxic drugs and were treated with *Atoh1* gene therapy (via the middle cochlear turn) 4 days after ototoxic exposure. Four weeks post-treatment, the ABR thresholds were high or unmeasurable, but at 8 week post-treatment, the ABR thresholds in the *Atoh1* treated ears were lower than the contralateral ears at all frequencies indicating functional improvement [31]. This demonstrated that considerable time post-inoculation is required before improvements to hearing are measurable. A different study tested an adenovirus serotype 28 viral vector containing the *Atoh1* gene for HC regeneration after profound hearing loss in mice. The viral vector was injected into the posterior canal of mature mice 10 days after administering kanamycin and furosemide to profoundly deafen the mice. A separate group of 10 day-deafened mice demonstrated complete loss of HCs and collapse of the tunnel of Corti at the time of inoculation. Regeneration of HCs was reported and a moderate hearing recovery in the more apical regions of the cochlea was found when assessed at 2 months post intervention, but not at earlier time points [38].

### Hair cell trans-differentiation

In this study it is difficult to differentiate between HC trans-differentiation due to *Atoh1* expression in supporting cells and expression of the transgene in surviving IHCs. In surface preparations HCs were counted strictly within the IHC lesion where residual HC survival was minimal but not completely absent at 2 weeks post-noise exposure. Not every HC within the noise damaged region was GFP<sup>+</sup> in the *Atoh1* group. It is possible that GFP expression was low or undetectable in some cells but it is also possible that there were some surviving IHCs at the time of gene therapy and at the end of the treatment period, as found in the control gene therapy and 5 week groups. Nevertheless, the overall higher numbers of HCs in the *Atoh1* group, in particular the number of GFP<sup>+</sup> HCs, strongly indicate that *Atoh1* was contributing to HC trans-differentiation and the possibility of additional HC protection or repair of residual HCs by *Atoh1* transgene expression cannot be ruled out. There is strong evidence for the conversion of supporting cells to HCs in many studies [28, 50, 51]. In our study, evidence for conversion was especially prominent in one animal in the *Atoh1* group in which GFP<sup>+</sup> HCs were detected in the OHC location within the noise lesion. OHCs are particularly susceptible to noise damage and no OHCs were detected in any other group, not even at 2 weeks post-noise exposure. In the control gene therapy group, GFP expression in the sensory epithelium was higher in regions where residual IHCs were present, but was rarely seen in IHCs themselves.

MyosinVIIa expression was observed in cells which were positive for GFP in sensory epithelium of the *Atoh1* group. This occurred in only 1 cell in the control gene therapy group which was likely to be a residual hair cell. MyosinVIIa expression was also observed in GFP<sup>+</sup> cells in the interdental cells of the spiral limbus in the *Atoh1* group. We have previously found that interdental cells expressing the *Atoh1* transgene express myosinVIIa, but rarely express any other HC markers indicating incomplete or aborted trans-differentiation of cells in non-sensory areas compared to cells within the sensory epithelium [46]. This suggests either an environmental influence of the sensory epithelium on the differentiation of the cell or an inherent inability of cells outside of the sensory epithelium to fully trans-differentiate into HCs. Even within the sensory epithelium, the location of the *Atoh1* transgene expression has a strong influence on the conversion towards a new HC. For example, cells in the IHC region expressed more HC markers than cells in the OHC region [46].

Many studies on *Atoh1*-mediated hair cell regeneration have been performed in neonatal or immature animals, with supporting cells observed to trans-differentiate into hair cells with functional characteristics and functional hearing improvements [36, 52, 53]. The effectiveness of *Atoh1*-mediated HC regeneration decreases with age as the molecular environment of the immature cochlea is more conducive to hair cell regeneration than the mature sensory epithelium [50]. Nevertheless, a handful of studies have reported HC regeneration and/or hearing improvements in mature cochleae after damage [28, 31, 38, 46].

### Sensory epithelium

The epithelium dedifferentiates into a simple cuboidal epithelium after hearing loss [54, 55]. If *Atoh1* gene therapy was given at this stage of degeneration (for example 7 days after unilateral deafening by neomycin in guinea pigs), no new HCs were formed when observed at 2 months or 10 weeks following *Atoh1* injection (via the second turn scala media) [40]. In the model of hearing loss used in this study, supporting cells were still present 2 weeks after noise exposure, but the epithelium had degenerated significantly by 5 weeks after noise exposure. Significant preservation of the sensory epithelium in the *Atoh1* group at 5 weeks post noise exposure was observed. HCs indirectly regulate the survival of supporting cells [49] such that supporting cells die when their neighbouring hair cells die. Therefore, it is not surprising that *Atoh1* expression (and subsequent HC formation or preservation) led to preservation of supporting cells, especially in a noise damage model of hearing loss when there was significant survival of supporting cells at the time of gene therapy. There are many signalling pathways activated by *Atoh1* expression that could enable supporting cells survival. For example, pillar cells which form the boundary of the tunnel of Corti, are disrupted if the gene for either the FGF8 signalling molecule or its receptor FGFR3 is deleted [56, 57]. During development, FGF8 is expressed in hair cells while FGFR3 is expressed by the pillar cells and this expression pattern is maintained in mature mammals [56, 58-60] suggesting that ongoing FGFR3 activation may be required to maintain pillar cells in their differentiated state. *Atoh1* gene therapy may enable the survival of pillar cells and therefore the preservation of the tunnel of Corti by reintroducing FGF8 expression via newly regenerated HCs. Regenerated HCs have been shown to express FGF8 [53], but the longevity of expression is unknown and the effect on residual pillar cells is yet to be tested.

### Neural response to *Atoh1* gene expression

Nerve fibre degeneration begins approximately 1-2 days after noise exposure [11]. Recruitment of peripheral fibres towards HCs formed via *Atoh1* expression has been demonstrated [23, 30, 36, 52]. In our study, peripheral fibres were present near GFP<sup>+</sup> HCs in the *Atoh1* group but not in the control

gene therapy group while peripheral fibres were significantly degenerated in the 5 week deaf control group. Collectively these data indicate that the *Atoh1*-induced HCs have a recruitment or protective effect on peripheral fibres. However, the reduced expression of *Ctbp2* protein in new HCs suggests that the fibres may not be synapsing with the HCs. A detailed study on HC differentiation found that newly generated HCs by *Atoh1* expression are not fully differentiated even 4 months after they are formed [53]. *Ctbp2* puncta has been reported to be lower in new HCs compared to endogenous HCs [46, 53] and despite the presence of peripheral fibres near the new HCs, the post-synaptic protein GluR2 was absent surrounding the *Ctbp2* puncta suggesting that no new synaptic connections were made between new HCs and the peripheral fibres [53].

## Conclusions

*Atoh1* gene therapy two weeks after exposure to noise resulted in greater HC numbers within the noise lesion compared to control groups. Survival of supporting cells at the time of gene therapy via a noise model of hearing loss enabled successful HC trans-differentiation to occur after a longer period of deafness than in profound ototoxic models of hearing loss. *Atoh1* transgene expression had a positive impact on the survival of HCs and supporting cells as well as the survival of peripheral fibres within the sensory epithelium. At 3 weeks post-treatment, the hair cells did not have a mature HC morphology or have normal expression of the ribbon synapse protein *Ctbp2*, and hearing thresholds were not improved. However, with longer treatment times, *Atoh1* gene therapy has the potential to reverse hearing loss through HC repair or trans-differentiation from supporting cells. Identifying the conditions in which efficient HC conversion takes place, such as after a noise induced hearing loss with supporting cell survival, will help enable the development of clinical therapies for *Atoh1* gene therapy.

## Acknowledgements

This study was generously funded by Action on Hearing Loss, the Garnett Passe and Rodney Williams Memorial Foundation and the National Health and Medical Research Council (GNT1024350). The Bionics Institute acknowledges the support it receives from the Victorian Government through its Operational Infrastructure Support Program. We are grateful to Prof. Cliff Hume of the University of Washington for providing the viral vectors used in this paper and to Rodney Millard for technical assistance with the noise deafening hardware and software.

## References

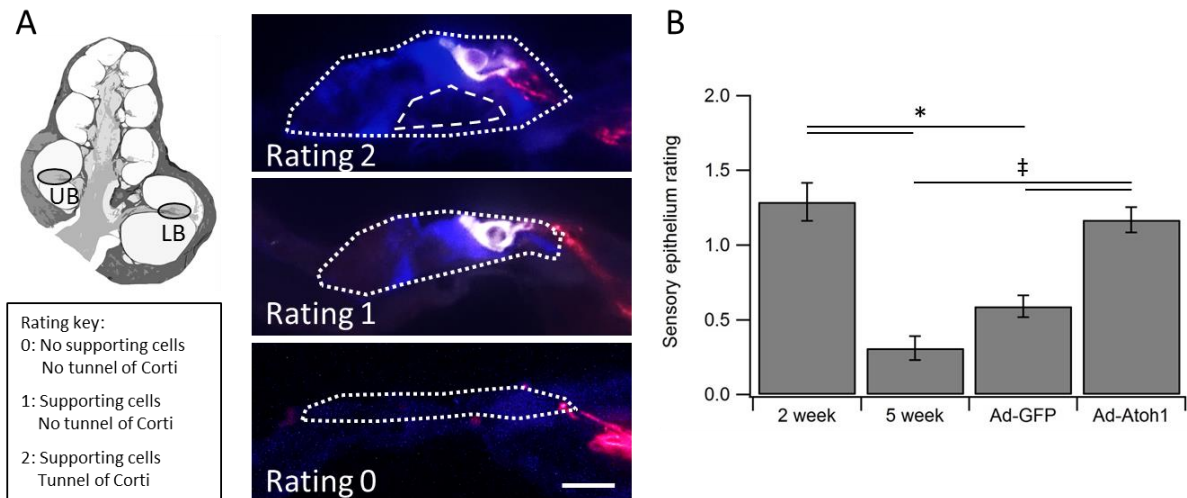
1. Spoendlin, H (1973) The innervation of the cochlea receptor. Basic Mechanisms in Hearing. Academic Press, New York.
2. Pickles, JO (1988) An introduction to the physiology of hearing. Academic press, London
3. Wilson, DH, Walsh, PG, Sanchez, L and Reed, P (1998) Hearing impairment in an Australian population. Disability Services: 14.
4. Robertson, D (1983) Functional significance of dendritic swelling after loud sounds in the guinea pig cochlea. Hear Res 9: 263-278.
5. Wang, Y, Hirose, K and Liberman, MC (2002) Dynamics of noise-induced cellular injury and repair in the mouse cochlea. J Assoc Res Otolaryngol 3: 248-268.
6. Puel, JL, Pujol, R, Tribillac, F, Ladrech, S and Eybalin, M (1994) Excitatory amino acid antagonists protect cochlear auditory neurons from excitotoxicity. J Comp Neurol 341: 241-256.
7. Pujol, R and Puel, JL (1999) Excitotoxicity, synaptic repair, and functional recovery in the mammalian cochlea: a review of recent findings. Annals of the New York Academy of Sciences 884: 249-254.

8. Puel, JL, Ruel, J, Gervais d'Aldin, C and Pujol, R (1998) Excitotoxicity and repair of cochlear synapses after noise-trauma induced hearing loss. *Neuroreport* 9: 2109-2114.
9. Kujawa, SG and Liberman, MC (2009) Adding insult to injury: cochlear nerve degeneration after "temporary" noise-induced hearing loss. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29: 14077-14085.
10. Liberman, MC and Dodds, LW (1984) Single-neuron labeling and chronic cochlear pathology. III. Stereocilia damage and alterations of threshold tuning curves. *Hear Res* 16: 55-74.
11. Lawner, BE, Harding, GW and Bohne, BA (1997) Time course of nerve-fiber regeneration in the noise-damaged mammalian cochlea. *International Journal of Developmental Neuroscience* 15: 601-617.
12. Bohne, BA, Harding, GW, Nordmann, AS, Tseng, CJ, Liang, GE, et al. (1999) Survival-fixation of the cochlea: a technique for following time- dependent degeneration and repair in noise-exposed chinchillas. *Hear Res* 134: 163-178.
13. Yamashita, D, Jiang, HY, Schacht, J and Miller, JM (2004) Delayed production of free radicals following noise exposure. *Brain Res* 1019: 201-209.
14. Kujawa, SG and Liberman, MC (2006) Acceleration of age-related hearing loss by early noise exposure: evidence of a misspent youth. *J Neurosci* 26: 2115-2123.
15. Lin, HW, Furman, AC, Kujawa, SG and Liberman, MC (2011) Primary neural degeneration in the Guinea pig cochlea after reversible noise-induced threshold shift. *J Assoc Res Otolaryngol* 12: 605-616.
16. Shibata, SB, Di Pasquale, G, Cortez, SR, Chiorini, JA and Raphael, Y (2009) Gene transfer using bovine adeno-associated virus in the guinea pig cochlea. *Gene Ther*:
17. Wise, AK, Hume, CR, Flynn, BO, Jeelall, YS, Suhr, CL, et al. (2010) Effects of localized neurotrophin gene expression on spiral ganglion neuron resprouting in the deafened cochlea. *Mol Ther* 18: 1111-1122.
18. Li Duan, M, Bordet, T, Mezzina, M, Kahn, A and Ulfendahl, M (2002) Adenoviral and adeno-associated viral vector mediated gene transfer in the guinea pig cochlea. *Neuroreport* 13: 1295-1299.
19. Raphael, Y, Frisancho, JC and Roessler, BJ (1996) Adenoviral-mediated gene transfer into guinea pig cochlear cells in vivo. *Neurosci Lett* 207: 137-141.
20. Lalwani, A, Walsh, B, Reilly, P, Carvalho, G, Zolotukhin, S, et al. (1998) Long-term in vivo cochlear transgene expression mediated by recombinant adeno-associated virus. *Gene Ther* 5: 277-281.
21. Liu, Y, Okada, T, Sheykholslami, K, Shimazaki, K, Nomoto, T, et al. (2005) Specific and efficient transduction of Cochlear inner hair cells with recombinant adeno-associated virus type 3 vector. *Mol Ther* 12: 725-733.
22. Ishimoto, S, Kawamoto, K, Kanzaki, S and Raphael, Y (2002) Gene transfer into supporting cells of the organ of Corti. *Hear Res* 173: 187-197.
23. Kawamoto, K, Ishimoto, S, Minoda, R, Brough, DE and Raphael, Y (2003) Math1 gene transfer generates new cochlear hair cells in mature guinea pigs in vivo. *J Neurosci* 23: 4395-4400.
24. Wenzel, GI, Xia, A, Funk, E, Evans, MB, Palmer, DJ, et al. (2007) Helper-dependent adenovirus-mediated gene transfer into the adult mouse cochlea. *Otol Neurotol* 28: 1100-1108.
25. Atkinson, PJ, Wise, AK, Flynn, BO, Nayagam, BA, Hume, CR, et al. (2012) Neurotrophin gene therapy for sustained neural preservation after deafness. *PLoS One* 7: e52338.
26. Wise, AK, Tu, T, Atkinson, PJ, Flynn, BO, Sgro, BE, et al. (2011) The effect of deafness duration on neurotrophin gene therapy for spiral ganglion neuron protection. *Hear Res* 278: 69-76.
27. Fekete, DM, Muthukumar, S and Karagogeos, D (1998) Hair cells and supporting cells share a common progenitor in the avian inner ear. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 18: 7811-7821.

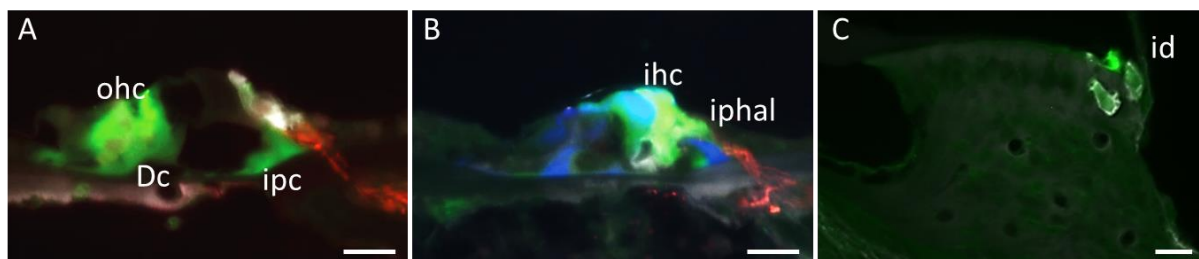
28. Liu, ZY, Dearman, JA, Cox, BC, Walters, BJ, Zhang, LL, et al. (2012) Age-Dependent In Vivo Conversion of Mouse Cochlear Pillar and Deiters' Cells to Immature Hair Cells by Atoh1 Ectopic Expression. *Journal of Neuroscience* 32: 6600-6610.
29. Bermingham, N, Hassan, B, Price, S, Vollrath, M, Ben-Arie, N, et al. (1999) Math 1: An essential gene for the generation of inner ear hair cells. *Science* 284: 1837-1841.
30. Woods, C, Montcouquiol, M and Kelley, MW (2004) Math1 regulates development of the sensory epithelium in the mammalian cochlea. *Nature Neuroscience* 7: 1310-1318.
31. Izumikawa, M, Minoda, R, Kawamoto, K, Abrashkin, KA, Swiderski, DL, et al. (2005) Auditory hair cell replacement and hearing improvement by Atoh1 gene therapy in deaf mammals. *Nature Medicine* 11: 271-276.
32. Zheng, JL and Gao, WQ (2000) Overexpression of math1 induces robust production of extra hair cells in postnatal rat inner ears. *Nat Neurosci* 3: 580-586.
33. Pan, N, Jahan, I, Kersigo, J, Duncan, JS, Kopecky, B, et al. (2012) A novel Atoh1 "self-terminating" mouse model reveals the necessity of proper Atoh1 level and duration for hair cell differentiation and viability. *PLoS One* 7: e30358.
34. Pan, N, Jahan, I, Kersigo, J, Kopecky, B, Santi, P, et al. (2011) Conditional deletion of Atoh1 using Pax2-Cre results in viable mice without differentiated cochlear hair cells that have lost most of the organ of Corti. *Hearing Research* 275: 66-80.
35. Jahan, I, Pan, N, Kersigo, J and Fritzsche, B (2013) Beyond generalized hair cells: molecular cues for hair cell types. *Hear Res* 297: 30-41.
36. Gubbels, SP, Woessner, DW, Mitchell, JC, Ricci, AJ and Brigande, JV (2008) Functional auditory hair cells produced in the mammalian cochlea by in utero gene transfer. *Nature* 455: 537-541.
37. Mizutari, K, Fujioka, M, Hosoya, M, Bramhall, N, Okano, HJ, et al. (2013) Notch inhibition induces cochlear hair cell regeneration and recovery of hearing after acoustic trauma. *Neuron* 77: 58-69.
38. Kraft, S, Hsu, C, Brough, DE and Staecker, H (2013) Atoh1 induces auditory hair cell recovery in mice after ototoxic injury. *The Laryngoscope* 123: 992-999.
39. Yang, SM, Chen, W, Guo, WW, Jia, S, Sun, JH, et al. (2012) Regeneration of stereocilia of hair cells by forced Atoh1 expression in the adult mammalian cochlea. *PLoS One* 7: e46355.
40. Izumikawa, M, Batts, SA, Miyazawa, T, Swiderski, DL and Raphael, Y (2008) Response of the flat cochlear epithelium to forced expression of Atoh1. *Hear Res* 240: 52-56.
41. Taylor, RR, Jagger, DJ and Forge, A (2012) Defining the cellular environment in the organ of Corti following extensive hair cell loss: a basis for future sensory cell replacement in the Cochlea. *PLoS One* 7: e30577.
42. James, DP, Eastwood, H, Richardson, RT and O'Leary, SJ (2008) Effects of round window dexamethasone on residual hearing in a Guinea pig model of cochlear implantation. *Audiol Neurootol* 13: 86-96.
43. Richardson, RT, Wise, AK, Thompson, BC, Flynn, BO, Atkinson, PJ, et al. (2009) Polypyrrole-coated electrodes for the delivery of charge and neurotrophins to cochlear neurons. *Biomaterials* 30: 2614-2624.
44. Mittereder, N, March, KL and Trapnell, BC (1996) Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. *J Virol* 70: 7498-7509.
45. Bohne, BA and Harding, GW (2000) Degeneration in the cochlea after noise damage: primary versus secondary events. *Am J Otol* 21: 505-509.
46. Atkinson, PJ, Wise, AK, Flynn, BO, Nayagam, BA and Richardson, RT (2014) Hair cell regeneration after ATOH1 gene therapy in the cochlea of profoundly deaf adult guinea pigs. *PLoS One* accepted:
47. Yamasoba, T, Yagi, M, Roessler, BJ, Miller, JM and Raphael, Y (1999) Inner ear transgene expression after adenoviral vector inoculation in the endolymphatic sac. *Hum Gene Ther* 10: 769-774.

48. Shibata, SB, Cortez, SR, Wiler, JA, Swiderski, DL and Raphael, Y (2012) Hyaluronic acid enhances gene delivery into the cochlea. *Hum Gene Ther* 23: 302-310.
49. Cai, T, Seymour, ML, Zhang, H, Pereira, FA and Groves, AK (2013) Conditional deletion of Atoh1 reveals distinct critical periods for survival and function of hair cells in the organ of Corti. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 33: 10110-10122.
50. Kelly, MC, Chang, Q, Pan, A, Lin, X and Chen, P (2012) Atoh1 directs the formation of sensory mosaics and induces cell proliferation in the postnatal mammalian cochlea in vivo. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 32: 6699-6710.
51. Chen, Y, Yu, H, Zhang, Y, Li, W, Lu, N, et al. (2013) Cotransfection of Pax2 and Math1 promote in situ cochlear hair cell regeneration after neomycin insult. *Scientific reports* 3: 2996.
52. Yang, J, Bouvron, S, Lv, P, Chi, F and Yamoah, EN (2012) Functional features of trans-differentiated hair cells mediated by Atoh1 reveals a primordial mechanism. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 32: 3712-3725.
53. Liu, Z, Fang, J, Dearman, J, Zhang, L and Zuo, J (2014) In vivo generation of immature inner hair cells in neonatal mouse cochleae by ectopic Atoh1 expression. *PLoS One* 9: e89377.
54. Forge, A, Li, L and Nevill, G (1998) Hair cell recovery in the vestibular sensory epithelia of mature guinea pigs. *J Comp Neurol* 397: 69-88.
55. Kim, YH and Raphael, Y (2007) Cell division and maintenance of epithelial integrity in the deafened auditory epithelium. *Cell Cycle* 6: 612-619.
56. Jacques, BE, Montcouquiol, ME, Layman, EM, Lewandoski, M and Kelley, MW (2007) Fgf8 induces pillar cell fate and regulates cellular patterning in the mammalian cochlea. *Development* 134: 3021-3029.
57. Puligilla, C, Feng, F, Ishikawa, K, Bertuzzi, S, Dabdoub, A, et al. (2007) Disruption of fibroblast growth factor receptor 3 signaling results in defects in cellular differentiation, neuronal patterning, and hearing impairment. *Dev Dyn* 236: 1905-1917.
58. Pirvola, U, Cao, Y, Oellig, C, Suoqiang, Z, Pettersson, RF, et al. (1995) The site of action of neuronal acidic fibroblast growth factor is the organ of Corti of the rat cochlea. *Proc Natl Acad Sci U S A* 92: 9269-9273.
59. Pirvola, U, Ylikoski, J, Trokovic, R, Hebert, JM, McConnell, SK, et al. (2002) FGFR1 is required for the development of the auditory sensory epithelium. *Neuron* 35: 671-680.
60. Bermingham-McDonogh, O, Stone, JS, Reh, TA and Rubel, EW (1998) FGFR3 expression in the developing and mature chick cochlea. *Assn Res Otolaryngol Abstr* 21: 167.

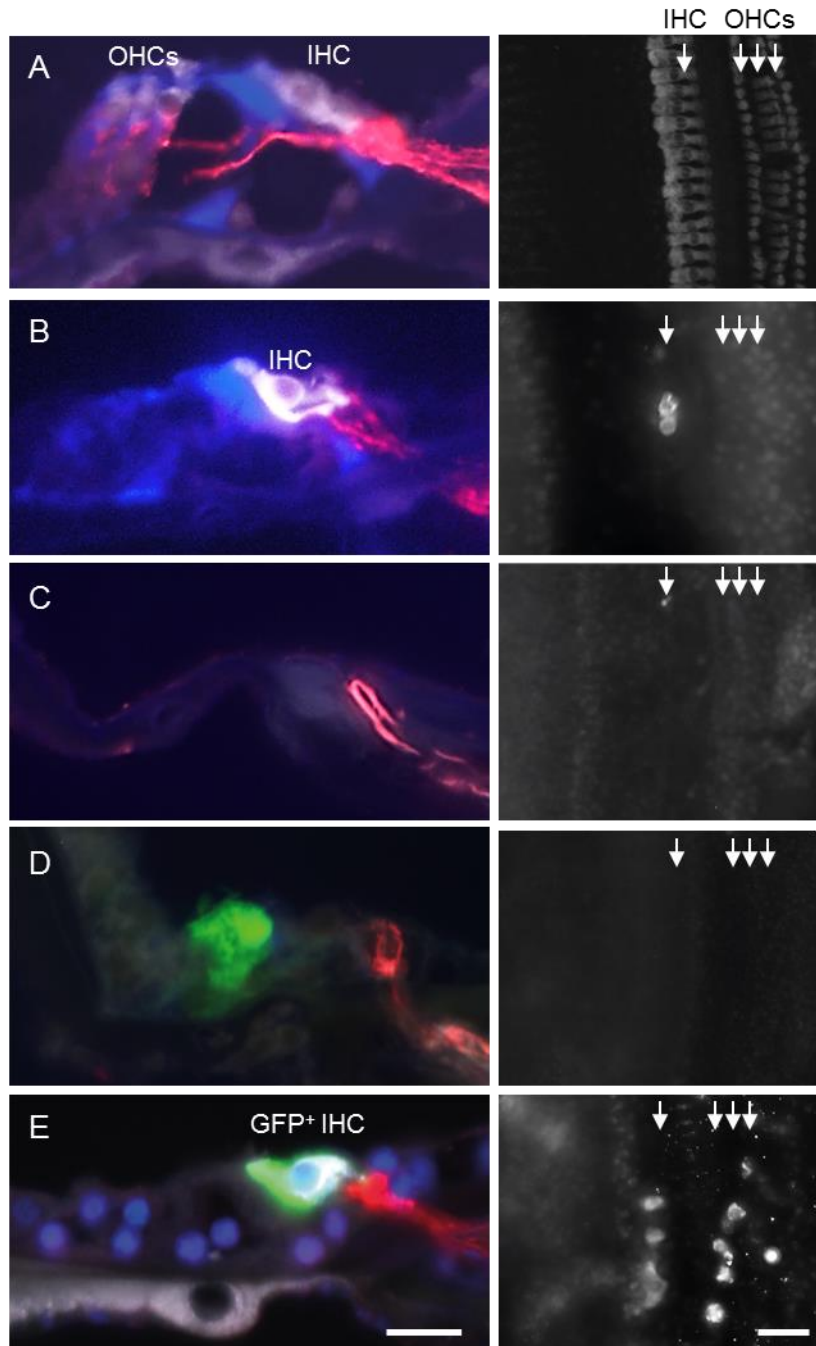
## Figure Legends



**Figure 1.** The sensory epithelium in the LB turn (from mid-modiolar sections). **(A)** Schematic of a mid-modiolar section of the cochlea showing the location of the sensory epithelium in the lower basal turn (LB) and upper basal turn (UB). The rating key is shown with photomicrographs depicting examples of each rating (phalloidin staining of supporting cells is shown in blue, peripheral fibres in red, hair cells in white; sensory epithelium is outlined by short dashes; tunnel of Corti is outlined by long dashes; scale bar is 20  $\mu$ m) **(B)** The epithelium was significantly more degenerated in the 5 week deaf and control gene therapy group compared to the 2 week deaf group ( $p < 0.001$  one way ANOVA on Ranks). The sensory epithelium was more preserved in the Atoh1 group compared to the 5 week and control gene therapy groups ( $\ddagger p < 0.001$  one way ANOVA on Ranks).

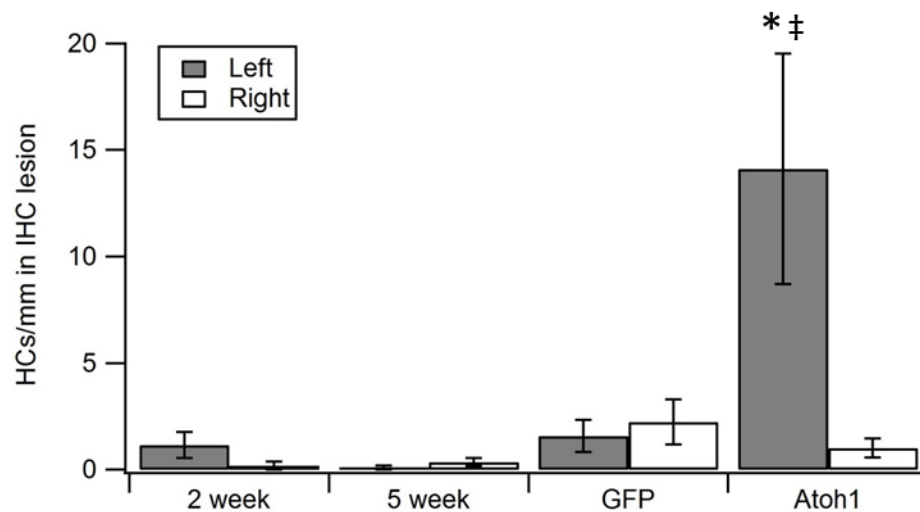


**Figure 2:** Transgene expression in the cochlea (Atoh1 group). **(A)** Examples of Atoh1 transduction (green) in outer hair cells (ohc), Deiters' cells (Dc) and inner pillar cell (ipc). **(B)** Example of Atoh1 transduction (green) in an inner hair cell (ihc) and inner phalangeal cell (iphal) **(C)** Example of Atoh1 transduction (green) in interdental cells of the spiral limbus. Green = GFP reporter gene from Atoh1 gene therapy, white = myosinVIIa, red = NFH in A and B, blue = phalloidin in B; Scale bars = 20  $\mu$ m.

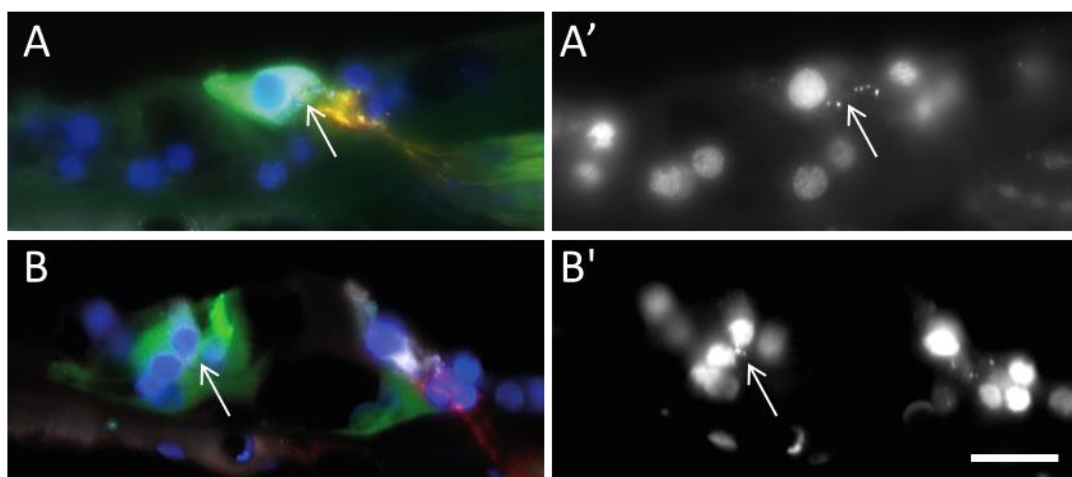


**Figure 3:** HCs in LB turn mid-modiolar sections (left column) and basal turn surface preparations (right column). **(A)** Inner and outer HCs adjacent to the non-noise-affected region of the cochlea showing supporting cells (blue) and neural innervation (red). In this region of the surface preparation, some OHCs were affected but the majority of inner and outer HCs remained **(B)** Two weeks after noise exposure, there was loss of OHCs in the LB turn. IHCs (arrow) were present in 61.7% mid-modiolar sections (left panel). A notch of IHC loss was observed in the basal turn surface preparation (right panel). Some residual IHCs were present in this example. **(C)** Five weeks after noise exposure, only 35.3% mid-modiolar sections displayed IHCs (absent in this image). Supporting cells had degenerated as indicated by an absence of phalloidin staining and the tunnel of Corti had collapsed. In surface preparations, the noise affected lesion was devoid of inner and outer HCs. **(D)** When control gene therapy was introduced two weeks after noise exposure, GFP expression (green)

was observed in the sensory epithelium, but there was no difference in HC numbers compared to the 5-week noise-only control group. No HCs were present in the mid-modiolar section or surface preparation shown here. **(E)** When Atoh1 gene therapy was introduced two weeks after noise exposure, GFP<sup>+</sup> HCs (white in left panel, magenta in right panel for clarity) were observed within the noise-induced IHC lesion. **Left panels:** Green = GFP from control gene therapy (D) or Atoh1 gene therapy (E), red = NFH, blue = phalloidin (A-D) or DAPI (E), white = myosinVIIa; scale bar is 20  $\mu$ m. **Right panels:** Hair cells stained with myosinVIIa; scale bar is 50  $\mu$ m

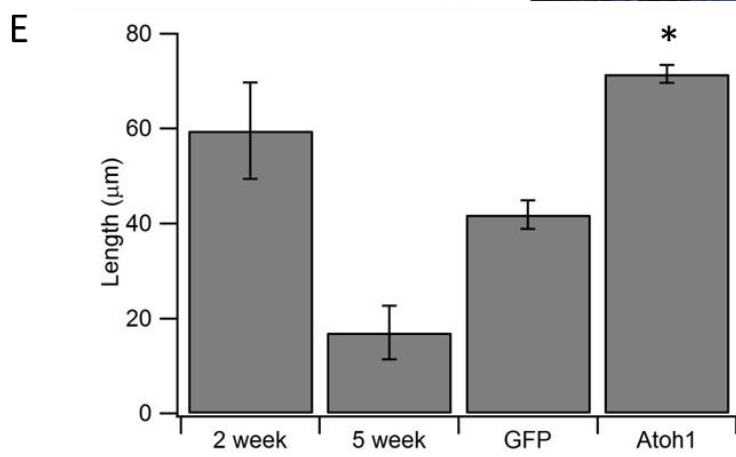
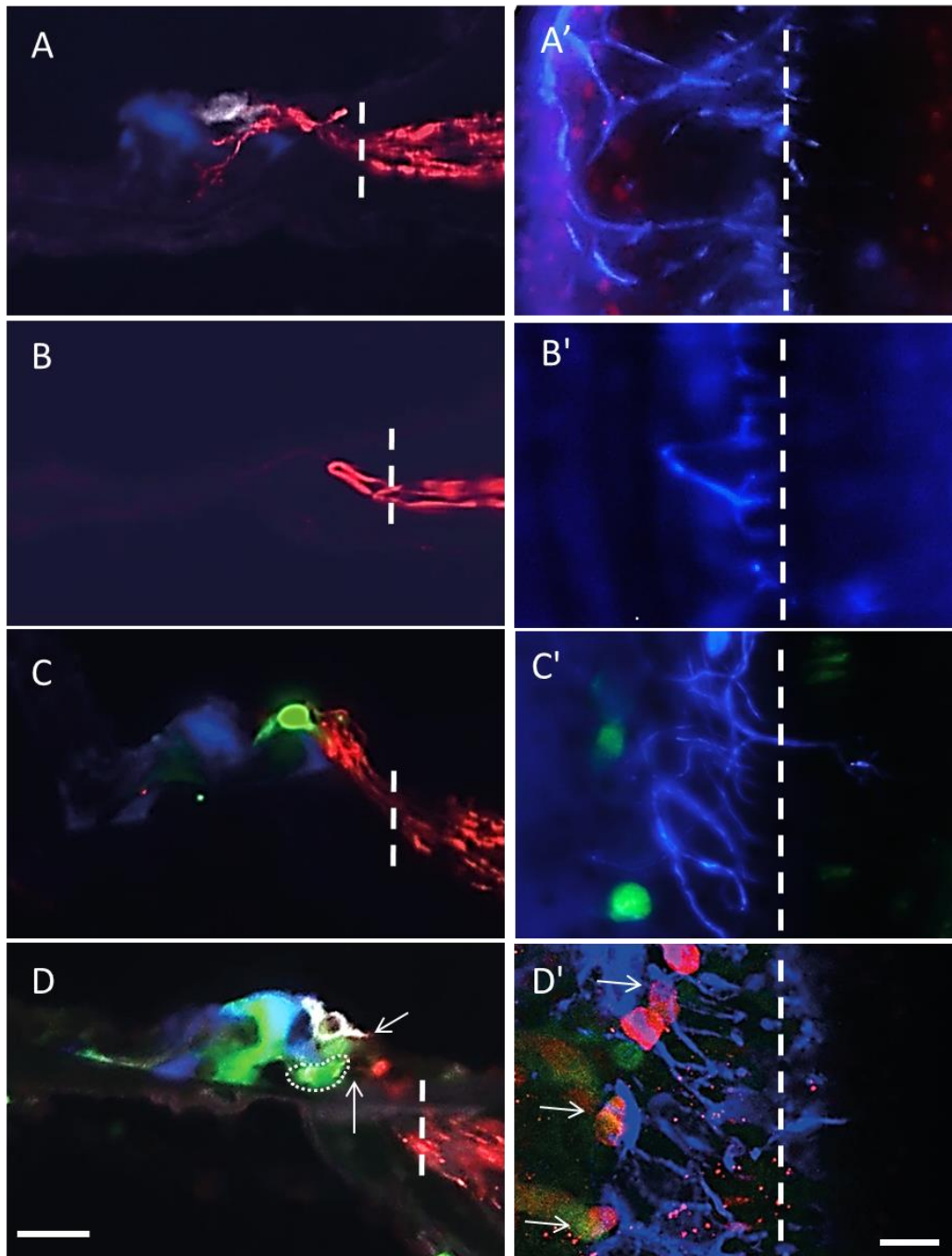


**Figure 4.** Average number of HCs per mm of the IHC lesion in basal turn surface preparations. There were significantly more HCs in the injected (left) cochleae of the Atoh1 group compared to the left/injected sides of all other groups (\*  $p < 0.05$ , one way ANOVA, Holm-Sidak comparisons) and compared to the contralateral (right) cochleae of the Atoh1 group (‡  $p < 0.05$  2-tailed t-test). Error bars show the standard error of the mean.

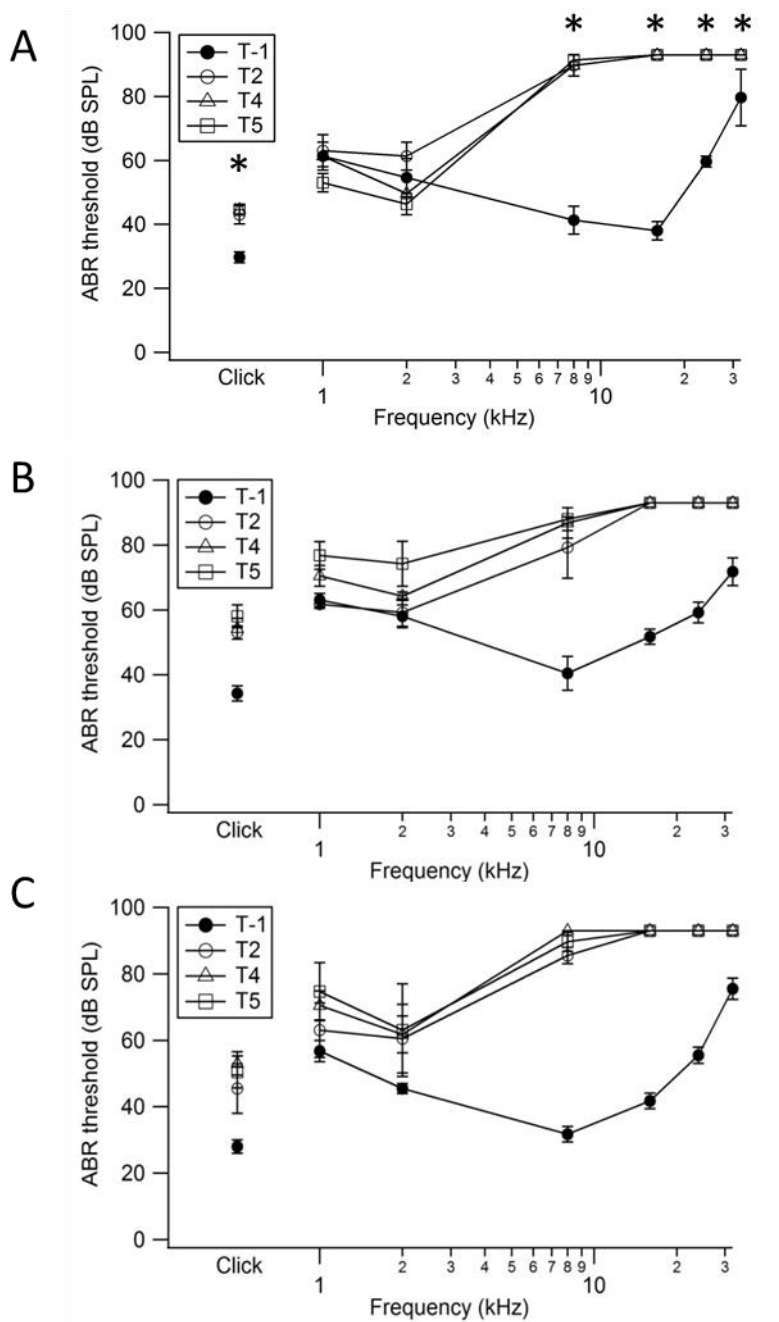


**Figure 5.** Ctbp2 expression in GFP<sup>+</sup> cells from the Atoh1 group. **(A)** Merged image showing GFP expression (green) in an IHC (myosinVIIa; white), peripheral fibres (NFH; red) and Ctbp2 expression (blue). The Ctbp2 channel is shown separately in **A'** (shown in greyscale for clarity), with punctate Ctbp2 expression (arrow) within a GFP<sup>+</sup>/myosinVIIa<sup>+</sup> cell. **(B)** GFP<sup>+</sup>/myosinVIIa<sup>+</sup> HC in the outer

sensory region of the organ of Corti. The Ctbp2 channel is shown separately in **B'**. Ctbp2 expression is present in both the GFP<sup>+</sup>/myosinVIIa<sup>+</sup> cell (arrow) and the GFP<sup>-</sup>/myosinVIIa<sup>+</sup> cell. Scale bar is 20 μm and applies to all images.



**Figure 6.** Peripheral fibres in the sensory epithelium. **(A)** 2 week deaf group **(B)** 5 week deaf group **(C)** control gene therapy group **(D)** Atoh1 group. Dotted lines indicate the habenula perforata with the sensory region to the left of the dotted line. In **A-D** it can be seen that peripheral fibres (red) have retracted in the 5 week group compared to the 2 week group, but in the Atoh1 group peripheral fibres (arrows) are seen near the two hair cells (white), one of which is GFP<sup>+</sup> (green). Blue = phalloidin. Similarly, in **A'-D'**, peripheral fibres (blue) within the noise lesion are also retracted in the 5 week group, but are seen among hair cells (red) in the Atoh1 group. Arrows indicate myosinVIIa<sup>+</sup> HCs (red) that are also positive for GFP (green). Scale bars are 20  $\mu$ m and apply to all like images. **(E)** The length of peripheral fibres from the habenula perforata to the furthest position was significantly longer in the Atoh1 group compared to the 5 week group (\* p<0.05 one way ANOVA).



**Figure 7:** Average click and tone-pip ABR thresholds at T-1, T2, T4 and T5. **(A)** 5 week group (left side)

**(B)** Control gene therapy group (injected side) **(C)** Atoh1 group (injected side). All T4 and T5 thresholds were significantly greater than the T-1 threshold ( $p < 0.05$  RM ANOVA). All groups had a significant increase in tone-pip ABR threshold between 8 and 32 kHz at T2 and later compared to T-1. Error bars represent standard error of the mean. (\* $p < 0.05$ ; paired t-test for click ABR and two-way RM ANOVA for tone-pip frequencies)