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Richardson RT, Atkinson PJ. Atoh1 gene therapy in the cochlea for hair cell regeneration. *Expert opinion on biological therapy*. 2015 Mar 4;15(3):417-30.

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The final publication is available at Expert Opinions on Biological Therapy:

<http://informahealthcare.com/doi/abs/10.1517/14712598.2015.1009889>

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Atoh1 gene therapy in the cochlea for hair cell regeneration

1. Abstract

Introduction: The sensory epithelium of the cochlea is a complex structure containing hair cells, supporting cells and auditory nerve endings, all of which degenerate after hearing loss in mammals. Biological approaches are being considered to preserve and restore the sensory epithelium after hearing loss. Of particular note is the ectopic expression of the Atoh1 gene which has been shown to convert residual supporting cells into hair cells with restoration of function in some cases. *Areas covered:* In this review, hair cell development, spontaneous regeneration and hair cell regeneration mediated by Atoh1 gene therapy in the cochlea will be discussed. *Expert opinion:* Gene therapy can be safely delivered locally to the inner ear and can be targeted to the sensory epithelium of the cochlea. Expression of the Atoh1 gene in supporting cells results in their transformation into cells with the appearance and function of immature hair cells, but with the resulting loss the original supporting cell. While the feasibility of Atoh1 gene therapy in the cochlea is largely dependent on the severity of the hearing loss, hearing restoration can be achieved in some situations. With further advances in Atoh1 gene therapy, hearing loss may not be as permanent as once thought.

Key words

Adenovirus, Atoh1, Cochlea, Gene therapy, Hair cell, Hearing loss, Regeneration, Supporting cell.

Article Highlights

- The Atoh1 gene directs hair cell and supporting cell development in the cochlea
- Multiple developmental roles of the Atoh1 gene have been uncovered, including hair cell differentiation, hair cell maturation and stereocilia formation
- There are critical stages during developing when Atoh1 expression is required for normal hair cell development
- In neonatal animals there is spontaneous regeneration of hair cells after damage to the sensory epithelium, but this becomes very limited as the cochlea matures
- Acquired sensorineural hearing loss results in permanent loss of hair cells and supporting cells and degeneration of spiral ganglion neurons
- Viral-based gene therapy in the cochlea, particularly via the scala media or the endolymphatic sac, enables efficient transduction of residual supporting cells of the sensory epithelium of the cochlea, but must be performed before the supporting cells have fully degenerated after hearing loss
- Atoh1 gene therapy in the mature cochlea forces supporting cells to trans-differentiate into immature hair cells that express multiple hair cell markers with functional mechanotransducer channels
- Mixed reports on hearing recovery after Atoh1 gene therapy indicate a complex set of conditions are required for functional connections to be re-established

2. Hearing loss

Hearing impairment represents a major public health problem worldwide with an estimated 360 million people suffering from this sensory disorder (World Health Organisation, 2013).

Furthermore, as the population ages, the prevalence of hearing impairment is expected to rise.

Hearing loss can arise from a number of causes: normal ageing, noise trauma, disease, genetic disorders, as well as a subset of antibiotics (ototoxic drugs) and chemotherapy drugs. The

impact of hearing impairment on society is large but no more so than for children who often

experience delays in the development of speech, cognitive and social skills¹. At present there is

no cure for sensorineural hearing loss. The current clinical treatments—hearing aids and cochlear implants—do provide benefit; however, patient outcomes are varied^{2,3} and these devices fail to address the underlying pathology, the loss of sensory hair cells and the degeneration of auditory neurons. The regeneration of these cellular populations is therefore thought to be the panacea for hearing restoration.

3. The sensory epithelium of the cochlea

The sensory epithelium of the cochlea, or the organ of Corti, is a mosaic of mechanosensory hair cells (inner and outer hair cells) and non-sensory supporting cells (border cells, pillar cells, phalangeal cells, Deiters' cells, Hensen's cells and Claudius' cells). Each hair cell is separated from the next hair cell by a supporting cell. Inner hair cells are responsible for the excitation of spiral ganglion neurons in response to sound. They receive innervation from type I spiral ganglion neurons and a single inner hair cell is innervated by numerous type I afferent nerve fibers. Outer hair cells are responsible for the mechanical amplification of sound, enhancing the responsiveness of the sensory epithelium to selected frequencies and the detection of low intensity sound⁴. They are innervated by unmyelinated type II spiral ganglion neuron fibers and a single efferent nerve fiber innervates many outer hair cells⁵ (Figure 1A&B).

Degeneration of hair cells, supporting cells and spiral ganglion neurons occurs after a trauma to the cochlea, for example exposure to noise, ototoxicity, ageing, cochlear implantation or a hereditary pathology. Loss of hair cells occurs first, following which there is extensive tissue reorganization to close the lesion. Non-specialised cells migrate from lateral regions of the sensory epithelium and cover the Deiters' cells which eventually degenerate as well. The pillar cells collapse with loss of the distinctive tunnel of Corti. They are also covered by expanding inner phalangeal cells and inner border cells. The sensory epithelium eventually flattens and is

replaced by a simple cuboidal epithelium^{6,7} (Figure 1C). In adult mammals, spontaneous regeneration of hair cells is extremely limited and hearing loss is permanent.

4. Hair cell development

Cochlear hair cells and supporting cells are derived from a common progenitor⁸. The mammalian homolog of the *Drosophila melanogaster* gene *atonal homolog 1* (*Atoh1*) is a proneural basic helix-loop-helix transcription factor and is the earliest known gene expressed in differentiating hair cells^{9,10}. *Atoh1* is also referred to as *Math1* (mouse *atonal* homolog) and *Hath1* (human *atonal* homolog). *Atoh1* is first detected in the base of the cochlea at E12.5. Cellular commitment and differentiation progresses in a wave from the base to the apex of the cochlea from about day E13.5 in mice and occurs over a period of several days¹⁰.

The expression of *Atoh1*, and subsequent hair cell specification in progenitor cells during development, is mediated by canonical Wnt/beta-catenin signalling. Wnt/beta-catenin signalling pathways are involved in controlling proliferation, specification and differentiation in development via frizzled receptors. *Atoh1* was found to be a downstream target of Wnt/beta-catenin signalling^{11,12}. There were fewer hair cells after beta-catenin disruption and supernumerary hair cells after beta-catenin over expression in mice. Without beta-catenin, the gap between inner and outer hair cells did not form implicating its role in cell patterning in the developing sensory epithelium, particularly the pillar cells. The lack of pillar cells in beta-catenin knockout mice may have been due to a deficit in FGF8 that is normally released by inner hair cells¹³ or a direct effect on pillar cells which express leucine-rich-repeat-containing, G-protein-coupled receptor 5 (*Lgr5*), a Wnt target gene and potentiator of Wnt signalling. Once hair cell fate was established, beta-catenin was not required to maintain *Atoh1* expression¹¹.

At first, Atoh1 is expressed in the cytoplasm and the nucleus of hair cells. As the hair cells mature and begin to express other hair cells markers such as MyosinVI, Atoh1 expression translocates to the nucleus¹⁴. Atoh1 expression is sustained until after birth suggesting a role in maturation and function of the hair cells, in addition to differentiation¹⁴. In this elegant study, a conditional knockout system was developed in which Atoh1 could be deleted from hair cells at different stages of development. There was a critical time period during which Atoh1 was required for hair cell survival, about 2 days after initiation of Atoh1 expression (E15.5 to E17.5). Loss of supporting cells mirrored the loss of hair cells in the absence of Atoh1 within this time frame. The pattern of Atoh1 dependence followed a basal to apical gradient, whereby hair cells in the basal regions of the cochlea are no longer dependent on Atoh1 by E16.5, while apical hair cells continue to require Atoh1 expression up to at least E17.5. Deletion of Atoh1 at later time points did not affect hair cell number but resulted in defective and disorganised hair cell stereocilia formation, although mechanotransducer channels were still present and functional¹⁴. The hair cells are presumed to express additional survival factors by this stage. Possible identities of these factors are Pou4f3 and Gfi1 as mice that are null for these genes exhibit hair cell loss later in development^{15, 16}.

5. Supporting cell development

Specification of supporting cells is secondary to hair cell differentiation and occurs by lateral inhibition via Notch signalling¹⁷. Lateral inhibition ensures that the correct number and patterning of hair cells and supporting cells are formed during development. Notch signalling blocks differentiation of hair cells¹⁷⁻¹⁹, while inhibition of Notch signalling generates supernumerary hair cells in embryonic and neonatal mammals^{18, 20-22}.

Notch1 is expressed throughout the developing prosensory epithelium between E12 and E14.5. Expression of the Notch ligands, JAG2 and DLL1, appear in a subset of these cells^{17, 21}. When JAG2 and DLL1 bind to the Notch receptor of a neighbouring cell, gamma secretase-mediated cleavage of the intracellular domain of Notch occurs, which then translocates to the nucleus to activate the expression of Hes1 and Hes5^{17, 23}. Hes1 and Hes5 are transcription factors that down-regulate the expression of Atoh1 and commit cells to differentiate into supporting cells²⁴. By E18, Notch1 is down-regulated in cells that will become inner and outer hair cells and maintained in cells that will become supporting cells. The Notch-mediated lateral inhibition results in the mosaic patterning of the sensory epithelium in which hair cells do not contact other hair cells. It has been demonstrated that hair cell death is accompanied by loss of supporting cells suggesting a level of reciprocal signalling between the hair cell and the surrounding supporting cells. Further to this, it has been shown that supporting cells up-regulate Atoh1 expression after hair cell loss through a loss of lateral inhibition. This Atoh1 up-regulation is theorized to play a role in the subsequent death of supporting cells through an ill-fated attempt to differentiate into hair cells¹⁴.

6. Atoh1 knockout studies

Deletion of Atoh1 has been shown to result in a complete loss of cochlear and vestibular hair cells and supporting cells^{9, 10, 25}. Although supporting cells are absent in Atoh1 knockout mice, Atoh1 was shown to not be directly required for supporting cell formation, instead supporting cell development was induced by the hair cells¹⁰. In support of this, ectopic expression of Atoh1 resulted in ectopic hair cell formation with clusters of surrounding supporting cells in the greater epithelial ridge or Kolliker's organ in neonatal rat or mouse cochlear cultures^{10, 26}. Transient expression of Atoh1 during development was not sufficient to prevent most hair cell death and

could not support the proper function of remaining hair cells²⁷. A conditional knockout study to generate viable post-natal mice showed that hair cell precursors undergo apoptosis in the absence of Atoh1 and, as a result, the organ of Corti is transformed into a flattened epithelium in postnatal animals²⁸. Interestingly, a recent study has shown that up to 30% of cells in the organ of Corti that are initially Atoh1-positive, become supporting cells. Atoh1 expression, therefore does not irreversibly commit prosensory cells to a hair cell fate, but rather it represents a dynamic interaction with other signalling pathways. In these alternative pathways, levels of Atoh1 are initially low and either gradually increase or are inhibited, determining whether a hair cell will form²⁹. An example of the interaction with other signalling pathways is the antagonistic relationship of Atoh1 and SRY (sex determining region Y)-box 2 (Sox2). Following a period of co-expression, Sox2 becomes down-regulated in cells that will become hair cells. The down-regulation of Sox2 correlates, temporally, with an increase in Atoh1 expression in the same cells³⁰. Further supporting this antagonistic relationship is the observation that when Atoh1 is transfected into the cochlea, ectopic expression of the hair cell markers, myosinVI and myosinVIIa, was observed in approximately 90% of transduced cells. Conversely, when Sox2 and Atoh1 were co-transfected, the number of transduced cells that expressed myosinVI was reduced to just over 50%, showing that Sox2 acts to inhibit the ability of Atoh1 to induce hair cell formation³⁰.

7. Spontaneous hair cell regeneration

In non-mammalian vertebrates such as birds and zebrafish, auditory and vestibular hair cells are known to regenerate after insult. The regeneration of hair cells observed in non-mammalian vertebrates after hair cell loss has been shown to be through two distinct mechanisms; direct trans-differentiation of supporting cells into new hair cells and mitotic regeneration. Direct

trans-differentiation is when supporting cells directly convert to hair cells without entering the cell cycle. In mitotic regeneration, supporting cells re-enter the cell cycle and then one or both of the daughter cells become hair cells^{31, 32}.

Mitotic regeneration has also been demonstrated in the mammalian vestibular epithelium, with significant hair cell regeneration shown after gentamicin-induced damage³³. In contrast, the hair cells of the mammalian cochlea have very limited regenerative capacity after insult. The mechanism behind this difference in regenerative capacity between the mammalian cochlea and vestibular system is not clear; whether mammalian cochlear supporting cells are intrinsically unable to divide and differentiate or whether this is due to an absence or the inhibition of regenerative signals³⁴. A seminal study exploring these factors has shown that mammalian supporting cells are indeed capable of dividing and differentiating into hair cells when isolated and placed in culture, suggesting that the lack of regeneration observed after trauma is due to the inhibition of regenerative signalling *in vivo*³⁴. A recent study by Wang and colleagues examining the signalling which may underlay the regeneration observed in the utricle has shown that after damage there is an both an up-regulation of Atoh1 combined with a down-regulation in the Notch target gene, Hes5³⁵, suggesting that a similar interplay between pro hair cell factors and inhibitory factors is necessary for spontaneous hair cell regeneration in the vestibular system.

Recent studies have shown spontaneous regeneration in the neonatal mouse cochlea after damage or hair cell loss^{36, 37}. In the Bramhall study, supporting cells spontaneously differentiated into new hair cells (predominantly outer hair cells) in the neonatal mouse after ototoxic deafening by aminoglycosides, mostly by trans-differentiation with a limited percentage arising from mitotic regeneration. Inhibition of Notch signalling increased the level

of hair cell regeneration³⁶. Spontaneous hair cell regeneration was also reported in the neonatal mouse with an inducible hair cell ablation model. The level of spontaneous regeneration followed a basal to apical gradient, with a greater number of regenerated hair cells observed in the more apical regions of the cochlea. This gradient of spontaneous regeneration follows the gradient of hair cell maturation in the cochlea, with basal regions maturing before the apical regions. Thus the apical regions are more permissive to hair cell regeneration in their less mature state. Supporting cells that neighbored the ablated hair cells spontaneously acquired a hair cell fate through direct trans-differentiation and, to a lesser extent, mitotic differentiation, but the newly regenerated hair cells were short lived³⁷. Postnatal hair cells and supporting cells are normally mitotically quiescent³⁸, but after hair cell loss mitotic cells were detected between P2 and P5, some of which acquired a hair cell fate³⁷. However, spontaneous hair cell regeneration was not observed after P7. Thus, spontaneous hair cell differentiation occurs in neonatal mammalian cochleae by direct trans-differentiation and mitotic regeneration, with the capacity to differentiate being very limited by adulthood when the cochlea has matured.

8. Which cells become new hair cells?

Supporting cells express markers similar to neural stem cells and neonatal supporting cells divide and differentiate into hair cells when placed in culture³⁴. It was noticed that specific subsets of supporting cell populations generated the new hair cells after damage to the sensory epithelium. When *Lgr5*, a Wnt responsive gene, was used to isolate cells from the sensory epithelium, it was found that these *Lgr5* positive cells had a 10-fold higher rate of spontaneous conversion to hair cells after damage and an enhanced capacity for self-renewal *in vitro*³⁹.

Lineage tracing of *Lgr5* and *Sox2* positive cells within the sensory epithelium was performed using *Lgr5* and *Sox2-Cre* mouse lines. Lineage models showed that the new hair cells arose from

Lgr5-expressing inner pillar and the third row Deiters' cells^{36,39}. Lgr5 expressing subsets of supporting cells were therefore determined to be progenitors for hair cells after damage^{36,39}. Some new hair cells in the inner pillar cell and outer hair cell regions stained for prestin (a mature outer hair cell protein), while others co-expressed the supporting cell marker Sox2 with immature hair cell markers and immature stereociliary bundles. Inhibition of notch signalling with a gamma secretase inhibitor increased the production of new hair cells in the damaged cochlea in newborn mice. Lgr5 is part of the Wnt signalling positive feedback loop. Beta-catenin and Wnt signalling were necessary for the trans-differentiation of the supporting cells into new hair cells following damage and notch inhibition. While the majority of the regenerated hair cells arose from direct trans-differentiation, BrdU staining indicated that mitotic regeneration of hair cells also occurred to a limited extent³⁶.

9. Characterisation of Atoh1-induced hair cells

In early studies, Atoh1-transduced cells were initially characterised by the expression of the structural hair cell marker myosinVIIa, however, with the advent of more sophisticated labeling techniques and a greater understanding of the molecular makeup of hair cells, regenerated hair cells have been characterised using a much broader cohort of markers.

The maturity of regenerated hair cells can be assessed by markers, with early markers of hair cells being myosinVI, myosinVIIa and calbindin^{40,41}, followed by parvalbumin⁴² and oncomodulin or prestin being used to label late and terminally differentiated outer hair cells⁴³⁻⁴⁶. The stereocilia can also be used to assess the maturity of the hair cell, whereby immature stereocilia are shorter and more disorganised than mature stereocilia. Stereocilia can further be used to differentiate inner and outer hair cells in that inner hair cells have a straight row of stereocilia while outer hair cells have a classical "V" shaped pattern of stereocilia. Functionality

of hair cells is assessed in a number of ways, including the presence of mechanotransduction channels visualised through the uptake of styryl dyes, age appropriate mechanotransduction currents and the presence of ribbon synapses.

To date, regenerated hair cells have been identified predominantly as outer hair cells by their pattern of stereocilia and expression of prestin³⁶. However, using a slightly different approach, hair cell regeneration was targeted to the inner hair cell region of the sensory epithelium, in particular the inner border cells and inner phalangeal cells, using Cre-mouse transgenic lines⁴⁷. When *Atoh1* expression was introduced into inner border and inner phalangeal cells at birth, hair cells were formed with an immature inner hair cell phenotype, as determined by the shape of the stereociliary bundle, *FGF8* expression, calretinin expression (normally stronger in inner hair cells compared to outer hair cells) and lack of outer hair cell-specific prestin expression. The hair cells had functional mechanotransduction channels but their outward current amplitudes remained immature. Furthermore, they did not express the terminal differentiation marker *vGlut3*⁴⁷. The new hair cells did express the synaptic vesicle protein *Otoferlin*, but had lower expression of the pre-synaptic ribbon protein *Ctbp2*. Neuronal fibres were also observed near the new inner hair cells, however, synapses did not form as there was an absence of the post synaptic marker glutamate receptor type 2 (*GluR2*) surrounding the *Ctbp2* puncta of the new inner hair cells⁴⁷. Interestingly, the trans-differentiation process was faster in the inner border and inner phalangeal cells (as little as 6 days) compared to pillar cells and Deiters' cells (about 3 weeks)^{47,48}.

The stage of maturity reached by regenerated hair cells has been variable depending on the age of the animal at the time of induction of *Atoh1* expression and the method by which *Atoh1* is induced or expressed. In general, newly formed outer hair cells in mature animals did not

express terminal markers of differentiation^{48,49}. A recent study examining the ability to convert supporting cells into hair cells using an inducible Atoh1 model demonstrated that in the neonatal and juvenile mouse approximately 10% of pillar and Deiters' cells underwent trans-differentiation⁴⁸. The trans-differentiated cells expressed 11 hair cell and synaptic proteins, a process that took around 3 weeks. New hair cells, which were found within the outer hair cell region, also formed stereocilia, contained mechano-electrical transduction channels and survived for more than 2 months *in vivo*. The transduced cells, however, lacked expression of mature outer hair cell proteins, prestin and oncomodulin, as well as mature hair cell morphology such as the cylindrical cell body shape and the characteristic "V" shaped stereocilia⁴⁸.

10. Gene Therapy

Gene therapy has the unique potential to regenerate the sensory components of the cochlea after hearing loss. This is achieved through the targeted introduction and regulated expression of a transgene in a desired cell or tissue to produce a biological or therapeutic effect. The use of gene therapy has generated a large amount of interest in the last two decades, with over 1800 clinical trials completed, currently underway or approved worldwide at the end of 2012⁵⁰. Gene therapy has been utilised in numerous conditions ranging from cancer to cardiovascular disease. Neurological diseases have also been targeted with 36 registered phase I, I/II and II trials aimed to treat a variety of diseases including multiple sclerosis, Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis⁵⁰. Many of the clinical and preclinical trials have shown potential. A noteworthy example is in the treatment of haemophilia B, whereby four of the six patients that underwent gene therapy were able to discontinue their prophylactic treatment⁵¹. Unfortunately, this effect was shown to be transient due to an immune response, indicating the

necessity for preclinical testing to ensure efficacy and safety within the context of the targeted condition and tissue.

Gene therapy has a number of benefits over other possible therapies; it allows for localised introduction of transgene(s) to specific cells (via cell-specific promoters), the desired protein is produced for longer periods than can be safely provided by most long-term release devices and, in the case of the Atoh1 gene, gene therapy can be used to replace cells that were thought to be permanently lost.

To achieve the most desirable outcome when utilising gene therapy a number of factors have to be considered. Initially, the intended biological effect needs to be ascertained; whether that be the replacement of a missing gene, the production of a therapeutic protein or the expression of an inhibitory gene. Another important determinate is the tissue of interest, its accessibility and its ability to be transduced. The gene must also be targeted to the desired cell population. This can be crudely achieved by injecting into the specific cochlear compartment in which the cells reside or more specifically via molecular targeting using cell specific promoters or viral vectors.

11. Gene Therapy in the Cochlea

The cochlea is ideal for localized gene therapy. The scala tympani is easily accessible and is the site of cochlear implantation. It can be directly accessed via the round window membrane or via a cochleostomy. After injection into the scala tympani, reporter genes are expressed in cells lining the perilymphatic space, spiral ganglion neurons and within cells of the sensory epithelium^{52,53} (Figure 2A,B), with the distribution being dependent on the viral vector type and surgical approach. There is some hearing loss associated with this approach, but not significantly more than occurs after cochlear implantation⁵⁴. The scala tympani can also be indirectly accessed via the vestibular system. After injection of adenovirus into the posterior semi-circular canal,

reporter gene expression is detected within the perilymphatic space of the cochlea with no associated hearing loss⁵⁵.

The bony wall of the cochlea need not necessarily be compromised to express genes within cochlear cells. Gene therapy vectors such as adenovirus can cross the round window membrane, but with reduced efficiency compared to direct injection into cochlear fluids^{56,57}. Enhanced transgene expression was observed when hyaluronic acid was added to the round window membrane prior to delivering the adenoviral vector⁵⁸.

Accessing the scala media of the cochlea containing the endolymphatic fluid is surgically more challenging. It is a smaller compartment with tight junctions surrounding the cells.

Experimentally it can be accessed through the basilar membrane or through the lateral wall of the cochlea^{52,59,60}, but not without risks of damaging the delicate sensory cells near the injection site and the cells of the stria vascularis, which are involved in the homeostatic regulation of potassium in the cochlea. Some hair cell loss, and in turn, hearing loss occurs using these approaches^{52,61}. After injection into the scala media, there is localized reporter gene expression in the sensory epithelium, including hair cells, supporting cells, as well as interdental cells of the cochlea⁵² (Figure 2C,D). Gene therapy with the glial fibrillary acidic protein reporter gene has been used to target supporting cells of the sensory epithelium⁶². To avoid hearing loss during gene therapy, the cochlear endolymph can be accessed via the endolymphatic sac of the vestibular system with resulting gene expression in the supporting cells of the sensory epithelium⁶³.

After a trauma to the cochlea, the sensory hair cells and supporting cells die and the epithelium rapidly degenerates until it becomes a flattened epithelium. There is a limited time frame during which the Atoh1 transgene can be introduced into supporting cells of the sensory epithelium

before the supporting cells degenerate. The efficacy of gene therapy to the sensory epithelium was reduced 1 week after severe ototoxic injury in guinea pigs compared to normal hearing guinea pigs. Reporter gene expression within the sensory epithelium was even more compromised when gene therapy was given at 8 weeks after injury⁶⁴ (Figure 3). The period of the window of opportunity for gene therapy to the sensory epithelium would vary greatly between different pathologies of hearing loss and between species. The degeneration of the sensory epithelium may be slower in humans, however, it eventually becomes a flattened epithelium as observed in animal studies^{65, 66}.

12. Hair Cell Regeneration using Gene Therapy

Due to its role in hair cell differentiation during development, Atoh1 has been a prime candidate for regenerating hair cells after damage to the sensory epithelium. Atoh1 has been delivered to the cochlea of embryonic animals^{10, 67}, neonatal animals^{26, 68, 69} and mature animals^{49, 61, 70, 71} with hair cell regeneration being achieved to differing degrees of success. Ectopic expression of Atoh1 in embryonic and neonatal animals resulted in greater trans-differentiation of supporting cells to hair cells and greater recovery of hearing after hearing loss compared to mature animals.

Embryonic expression of the Atoh1 gene induces functional ectopic hair cell formation^{10, 67}.

Cochlear explant cultures were established from E13.5-14.5 embryos and individual cells within the developing sensory epithelium (Kölliker's organ) were transfected with Atoh1 by electroporation. New hair cells were generated with clusters of supporting cells surrounding the hair cells, reminiscent of the sensory mosaic structure of the normal mature sensory epithelium. The supporting cells surrounding the ectopic hair cells were not positive for Atoh1 suggesting that they were induced by neighbouring Atoh1-positive cells. The induction of supporting cells

was inhibited with a gamma-secretase inhibitor indicating the role of Notch signalling in supporting cell formation¹⁰. The level of induction of supporting cells around Atoh1-positive transfected cells decreased with the age of the embryo. It was also shown that only transient ectopic expression of Atoh1 is required for hair cell formation, as a positive feedback loop is initiated in the Atoh1-positive cells¹⁰. In another study, embryonic (E11.5) mice that were microinjected with an expression plasmid containing the Atoh1 gene also developed hair cells⁶⁷. Detailed examination of the ectopic hair cells revealed that they had phalloidin-positive stereociliary bundles with an immature phenotype. The ectopic hair cells attracted neuronal fibres that terminated at the bases of the hair cells. Approximately half of the new hair cells expressed correctly localized ribbon synapse proteins (Ctbp2) suggesting that the nerve fibres could be synapsing with the new hair cells. The new hair cells were also found to have mechanotransduction properties that were similar to normal developing hair cells⁶⁷.

The sensory epithelium of neonatal animals appears to be just as permissive to Atoh1-induced hair cell formation as embryonic tissue²⁶. Cochlear explants were prepared from P0-P3 rats and the Atoh1 gene was transfected by electroporation or introduced with an adenoviral vector. Ectopic hair cells were observed in the greater and lesser epithelial ridges that normally develop into inner sulcus cells and Hensen's and Claudius' cells, respectively. The new hair cells were positive for the hair cell marker myosinVIIa, had stereociliary bundles, were derived from direct trans-differentiation^{26, 72} and attracted neurite outgrowths despite the abnormal location of the new hair cells⁷³.

Viral gene delivery methods have been used to ectopically express the Atoh1 gene in the cochlea of mature animals^{49, 61, 68, 70, 71, 74}. Initially, young adult guinea pigs with normal hearing were injected with the Atoh1 adenoviral vector via the scala media. Immature hair cells were

observed in the sensory epithelium in ectopic locations, some with recruited neuronal fibres⁶¹. In a subsequent study, guinea pigs were deafened by ototoxic drugs and the cochleae were injected with adenoviral vectors containing the Atoh1 gene 4 days later. Hair cells exhibited stereociliary bundles and stable, albeit partial, hearing recovery was seen⁷⁰. However, it could not be determined if the new hair cells were regenerated hair cells or whether they were hair cells that recovered or were repaired by the Atoh1 gene. A study in which Atoh1 was expressed in the adult mammalian cochlea of noise-exposed guinea pigs revealed an additional role for Atoh1 in hair cell repair⁷⁴. In this study, adenoviral delivery of the Atoh1 gene via the round window membrane into the perilymphatic fluid resulted in expression in supporting cells only (not in hair cells). Hair cells were still present at the time of gene therapy (7 days after noise exposure) and hearing recovery was reported as a result of repair to the damaged stereocilia. This study highlights the importance of distinguishing between the role of Atoh1 in hair cell repair and hair cell regeneration with regard to hearing recovery. It is necessary to ascertain the level of hair cell degeneration at the time of gene therapy. In Atkinson et al., 2014, guinea pigs were deafened by kanamycin for 4 days. A 4-day deaf control group demonstrated that most hair cells were absent at the time of gene therapy, but there were about 10 residual hair cells per millimeter of the sensory epithelium, just under 10% of the number of hair cells seen in normal hearing guinea pigs. After Atoh1 gene therapy, the number of hair cells had increased significantly indicating trans-differentiation of supporting cells (Figure 4), but it is also possible that some repair to existing hair cells could also have taken place. No hearing recovery was observed in this study, but the study did not extend beyond 3 weeks after Atoh1 inoculation⁴⁹, and other studies indicate that this is the minimum time is needed for supporting cells to convert to hair cells and hearing recovery was observed to take place between 1 and 2 months post-transfection^{48, 71}.

The induction of hair cell differentiation after ototoxic deafness in mature guinea pigs, however, has only been possible when Atoh1 was introduced within 4-days post ototoxic deafening. Supporting cells are known to be lost with increasing periods of deafness⁶⁴ suggesting that the presence of differentiated supporting cells are needed for trans-differentiation to occur⁷⁵. The need for differentiated supporting cells may limit the clinical viability of Atoh1 gene therapy for hair cell regeneration.

Direct trans-differentiation of supporting cells into hair cells depletes the supporting cell population and could compromise the stability and function of the sensory epithelium. Expanding the supporting cell population prior to hair cell regeneration could therefore be required to maintain a stable sensory epithelium that contains appropriately patterned supporting cells and hair cells. The Wnt-responsive Lgr5-expressing supporting cells have the capacity for proliferation and differentiation into hair cells, but this does not occur after hair cell loss in mammals^{39,76}. Other approaches to encourage cellular proliferation might be needed. A study conducted by Chen and colleagues, for example, using cultured sensory epithelia from neonatal mice demonstrated that expression of Pax2 (implicated in proliferation of progenitor cells in the cochlea) and Atoh1 by adenoviral gene delivery increased supporting cell proliferation in both damaged and non-damaged cochleae and these cells differentiated into hair cells with functional mechanotransduction channels⁶⁹.

Assessing the most appropriate vector for gene delivery is also a critical step in evaluating the viability of gene therapy in the cochlea. It is known, for example, that 65-75% of the population have neutralizing antibodies to Adenovirus type 5^{77,78}, which may lower the safety and efficacy profiles of this vector. In a recent study, Adenovirus type 28 has been shown to have superior transfection kinetics to Adenovirus type 5 and have preferential uptake into supporting cells⁷⁹.

Kraft and colleagues trialed the use of Adenovirus serotype 28 rather than the more commonly used Adenovirus type 5 to transfect cells with Atoh1. Injection of Atoh1 into the perilymph via the posterior canal of profoundly deafened mature mice was shown to result in the regeneration of hair cells and a moderate hearing recovery in the more apical regions of the cochlea when assessed at 2 months post intervention ⁷¹.

13. Clinical translation of Atoh1 gene therapy

Scientists are now embarking on a clinical trial for Atoh1 gene therapy at the University of Kansas Medical School, led by Hinrich Staecker. About 45 volunteers with severe hearing loss will have the Atoh1 gene injected into their cochlea. The trial will involve the use of adenovirus type 5 with the Atoh1 gene expressed from the glial fibrillary acidic protein promoter which has been shown to restrict gene expression to supporting cells in the cochlea ⁶². The trial will give us important safety information about gene therapy in the cochlea and the efficacy of the Atoh1 gene in restoring hearing in people with severe hearing loss. An intra-labyrinthine approach is proposed which has been shown to result in gene expression within supporting cells of the cochlea with minimal damage ⁶³.

One hurdle to clinical application of gene therapy technologies is the inability to control and modulate the level of gene expression within cells. This could be a critical factor in the regeneration of mature hair cells after damage, as it is known during development and into early postnatal stages in the mouse cochlea that Atoh1 expression is dynamic. Atoh1 is up-regulated as early in development as E12.5 followed by a subsequent down-regulation between E17 and P5 as hair cells mature and become fully integrated and functional ²⁹. In multiple mouse transgenic studies, the duration and level of Atoh1 expression have been found to be important for the outcomes of hair cell differentiation, the resulting type of hair cell (type I or type II),

function and long-term viability^{27, 48, 80}. In a transgenic model in which Atoh1 expression was down-regulated earlier than normal, non-functional hair cells were formed which degenerated over time, with inner and outer hair cells affected differently²⁷. In another study, brief ectopic expression of Atoh1 in neonatal transgenic mice led to ectopic hair cell induction in the supporting cells of the sensory epithelium, but extended Atoh1 expression was necessary to force more mature supporting cells to trans-differentiate into new hair cells⁸⁰. Also using transgenic mouse models, another study demonstrated that permanent Atoh1 expression in mature hair cells resulted in their death, while permanent expression of Atoh1 in immature hair cells did not⁴⁸. Hence, if gene therapy is to one day generate mature hair cells, a mechanism to switch off Atoh1 gene expression may be required. Parker et al have developed a system in which the Atoh1 gene is regulated via a helper gene. Cells express an inactive form of the Atoh1 gene, but when tamoxifen is added, Atoh1 can be activated and deactivated in a regulated manner. Atoh1 need only be expressed to transform supporting cells into hair cells, and once done, the gene can be turned off⁸¹.

The use of Atoh1 gene therapy in the cochlea may be applied across a larger range of conditions and not just specifically for the regeneration of hair cells. Indeed, Atoh1 gene therapy enabled hearing recovery in guinea pigs deafened with simulated gun-shot exposure. In this case Atoh1 was shown to repair damaged hair cell stereocilia, demonstrating a more diverse function of the Atoh1 gene in hair cells than purely driving hair cell specification⁷⁴.

14. Conclusion

Hearing loss is the most common sensory deficit and arises from the loss of sensory hair cells of the cochlea. The loss of hair cells is followed by the degeneration of supporting cells and peripheral fibres of the auditory neurons. The regeneration of hair cells is therefore critical for

restoring hearing function. To achieve this outcome studies have examined the factors necessary for hair cell formation during development. Atoh1, a transcription factor, is necessary for hair cell specification. In concert with hair cell development surrounding cells become supporting cells through Notch-mediated lateral inhibition and a down-regulation of Atoh1. It has been demonstrated that if Atoh1 gene therapy is provided to supporting cells before they degenerate after hearing loss, they can be forced to trans-differentiate into new hair cells, but at the expense of the supporting cell. Atoh1 gene therapy in very young animals results in a high rate of conversion of supporting cells to hair cells. The new hair cells have many of the markers of mature hair cells and are functional. Atoh1 gene therapy in mature mammals has not been as successful to date, with the conversion rate of supporting cells to hair cells being lower and with fewer mature hair cell markers. It is possible that hearing recovery observed in some of the studies to date is mediated through repair of existing hair cells rather than acquired function in the newly converted hair cells.

15. Expert Opinion

The sensory epithelium of the cochlea is a complex structure containing a mosaic of sensory hair cells and supporting cells with afferent and efferent innervation. Acquired or hereditary deafness results in permanent loss of hair cells, supporting cells and innervation. The cochlear implant does a remarkable job of restoring hearing to people with severe to profound deafness. The cochlear implant does have some limitations in certain situations, such as hearing with background noise and conveying tonal languages and the complex characteristics of music. Reversing hearing loss through the regeneration of sensory cells in the cochlea would be a remarkable feat that could benefit millions of people. However, there are many obstacles to overcome to achieve this goal.

The human cochlea is accessible to gene therapy. The best route of application of Atoh1 gene therapy to the cochlea will be one that takes into account the need for the vector to reach the supporting cells of the sensory epithelium and the specificity of the resulting gene expression pattern while minimising side effects from the surgical approach such as dizziness or loss of residual hearing. The fluids of the endolymphatic sac are continuous with the cochlear endolymphatic space (the scala media) , allowing access to the supporting cells of the cochlea. Gene therapy to the endolymphatic sac in animals results in gene expression in supporting cells in the cochlea with little to no hearing loss or dizziness. Other routes of administration to the cochlea include gene delivery to the scala tympani. This option is especially attractive if surgery to access the scala tympani is already being considered (e.g. for cochlear implantation). Gene expression has been reported in supporting cells of the sensory epithelium after injection into the scala tympani, but with lower efficiency than direct administration to the endolymphatic space. Direct injection into the scala media of the cochlea, although performed experimentally, would not be ideal due to the loss of hair cells in sensory epithelium near the injection site, the temporary loss of the endocochlear potential and the ionic balance of the cochlear fluids and the potential to damage residual hearing.

Viral gene therapy is finding more and more acceptance as clinical trials continue to demonstrate its safety and efficacy. New generation viral vectors are less immunogenic than their earlier counterparts with very little viral genetic material remaining in the vectors. There are many different serotypes of viral vectors, each with different cell type specificities and this can be used to some advantage when targeting cells such as supporting cells in the cochlea. Gene expression from some viral vectors has been reported to last for many years⁸²⁻⁸⁴ which is of advantage for some types of gene therapy applications, but not necessarily for Atoh1-induced

hair cell regeneration. If we are to follow the developmental pattern of Atoh1 expression, Atoh1 should be expressed for a short period before being down-regulated in hair cells.

There are three main approaches to transform existing supporting cells into new hair cells after hearing loss: expression of the Atoh1 gene by viral gene therapy; delivering an inhibitor of Notch signalling to the cochlea or, simultaneously stimulating the proliferation of supporting cells to boost the population of supporting cells in the cochlea and inducing hair cell differentiation with the Atoh1 gene. In examining the outcomes of studies performed in adult mammalian cochleae, both Atoh1 gene therapy and the use of Notch inhibitors after damage to the cochlea have merit. There is no doubt that new hair cells are formed from residual supporting cells and that the new hair cells have many of the hallmark features and functions of hair cells. However, the rate of Atoh1-mediated trans-differentiation is low in mature mammals and evidence suggests that new hair cells do not become mature hair cells after Atoh1-induced trans-differentiation in mature mammals. Most reports suggest the new hair cells do attract or recruit spiral ganglion neurons peripheral fibres and synaptic connections appear to be made in some cases. It is difficult to assess whether the new hair cells are functional in mature animals as many studies are hampered by the fact that residual hair cells may have been present at the time of gene therapy giving opportunity for hair cell repair or recovery. Nevertheless, significant hearing improvements have been reported and even a small enhancement in hearing (e.g 20 dB) would make an enormous difference to cochlear implant users or people with moderate to severe hearing loss.

Trans-differentiation of supporting cells into new hair cells necessitates the presence of residual supporting cells after the onset hearing loss. The pathologies of hearing loss vary remarkably between individuals. In severe cases of hearing loss, the sensory epithelium is flattened and

contains only simple cuboidal cells that do not respond to forced expression of the Atoh1 gene. However, even after a severe insult to the cochlea, there is a window of opportunity for gene therapy in the cochlea where the supporting cells are still present. People with hearing loss would need to be screened for the likelihood of having residual supporting cells as well as the aetiology of hearing loss. People with a genetic defect in a hair cell gene could not undergo Atoh1 gene therapy alone as the resulting hair cells would be equally defective for that gene. An additional therapy would be required to first repair the genetic defect.

Direct trans-differentiation of supporting cells into new hair cells depletes the supporting cell population in the cochlea which may become problematic for the long-term stability of the sensory epithelium. In order to regenerate the sensory mosaic pattern of the cochlea, supporting cells would need to be expanded prior to introducing the Atoh1 gene. This has been achieved in animals, but induction of mitosis in humans is risky and would need further investigation.

So much is now known about hair cell formation during development and after various manipulations of the Atoh1 or Notch signalling pathways. Atoh1 gene therapy studies have demonstrated that loss of hair cells and hearing loss in mammals are not as irreversible as once thought. New hair cells can be produced by directly trans-differentiating supporting cells but with a corresponding loss of the complex structure of the sensory epithelium and it is unknown at this stage whether the incredibly intricate structure of the cochlea can be completely restored after hearing loss with gene therapy.

16. Acknowledgements

The authors of this manuscript would like to acknowledge Action on Hearing Loss (G39), the Garnett Passe and Rodney Williams Memorial Foundation, the National Health and

Medical Research Council (GNT1024350) and the U.S. Department of Health and Human Services-National Institutes of Health for research funding. The Bionics Institute acknowledges the support it receives from the Victorian Government through its Operational Infrastructure Support Program.

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Figures

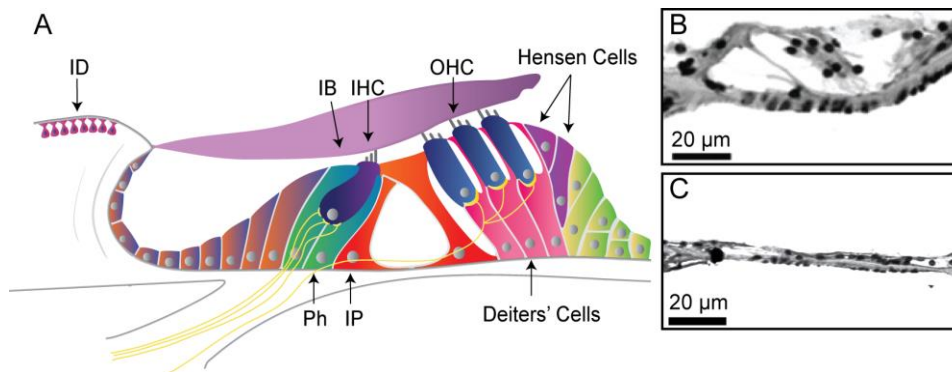


Figure 1. The sensory epithelium of the cochlea. **(A)** Schematic of the organ of Corti and surrounding structures showing inner hair cells (IHC), outer hair cells (OHC), supporting cells such as phalangeal cells (Ph), inner pillar cells (IP), inner border cells (IB), Deiters' cells and Hensen's cells. Interdental cells (ID) of the spiral limbus are also shown. Type I and type II afferent nerve fibres (yellow) can be seen synapsing with inner and outer hair cells, respectively. **(B)** Histological image of the sensory epithelium in a normal hearing guinea pig with intact inner and outer hair cells and supporting cells. **(C)** Histological image of the sensory epithelium 8 weeks after exposure to ototoxic drugs. The inner and outer hair cells and supporting cells have degenerated, leaving a 'flattened' epithelium.

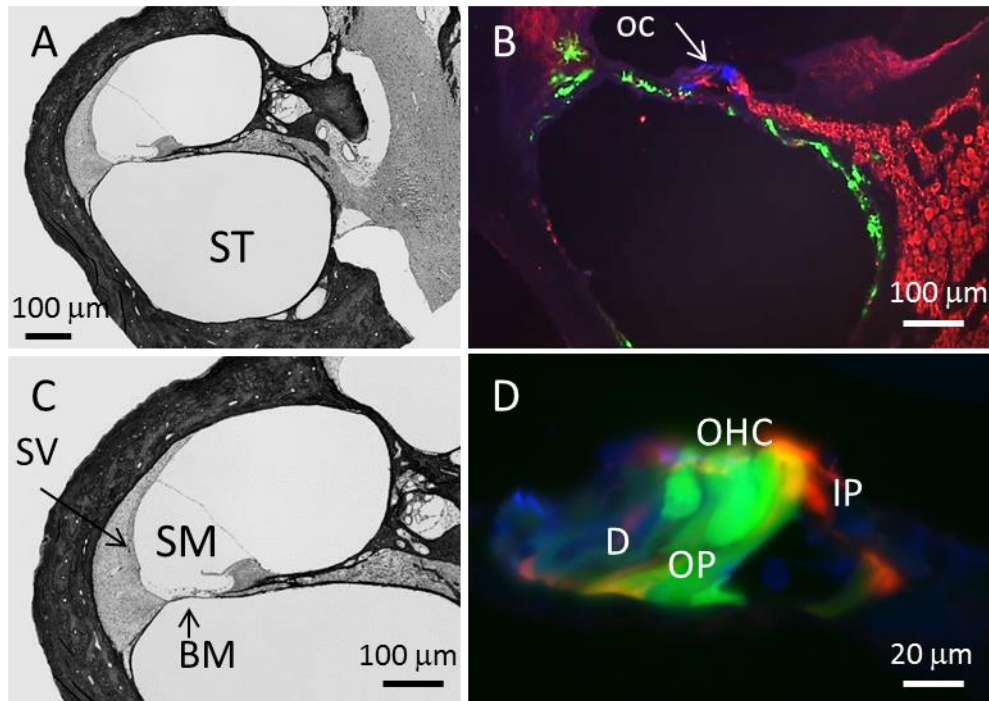


Figure 2. Gene therapy in the cochlea. **(A)** Histological image of the cochlea in cross section showing the scala tympani (ST), a readily accessible part of the cochlea containing perilymphatic fluid. Viral vectors can be introduced into the scala tympani via a cochleostomy, through the round window membrane or via the posterior semi-circular canal. **(B)** Following delivery of an adenoviral vector into to the scala tympani, transgene expression (green) is often observed in the cells surrounding the perilymphatic space, but also the sensory epithelium (organ of Corti; OC) in some studies (red=neurofilament heavy chain (spiral ganglion neurons), blue=phalloidin (inner and outer pillar cells)). **(C)** Image of the cochlea depicting the scala media (SM), a smaller compartment of the cochlea containing endolymphatic fluid. The scala media is accessible via the lateral wall through the stria vascularis (SV), through the basilar membrane (BM) or via the endolymphatic sac (not shown). **(D)** Following delivery of an adenoviral vector to the scala media, transgene expression (green) is observed within the sensory epithelium, including the

hair cells and supporting cells (D=Deiters' cells, OHC=outer hair cells, OP=outer pillar cells, IP=inner pillar cell (red=phalloidin (pillar cells), blue=calretinin (Deiters' cells))).

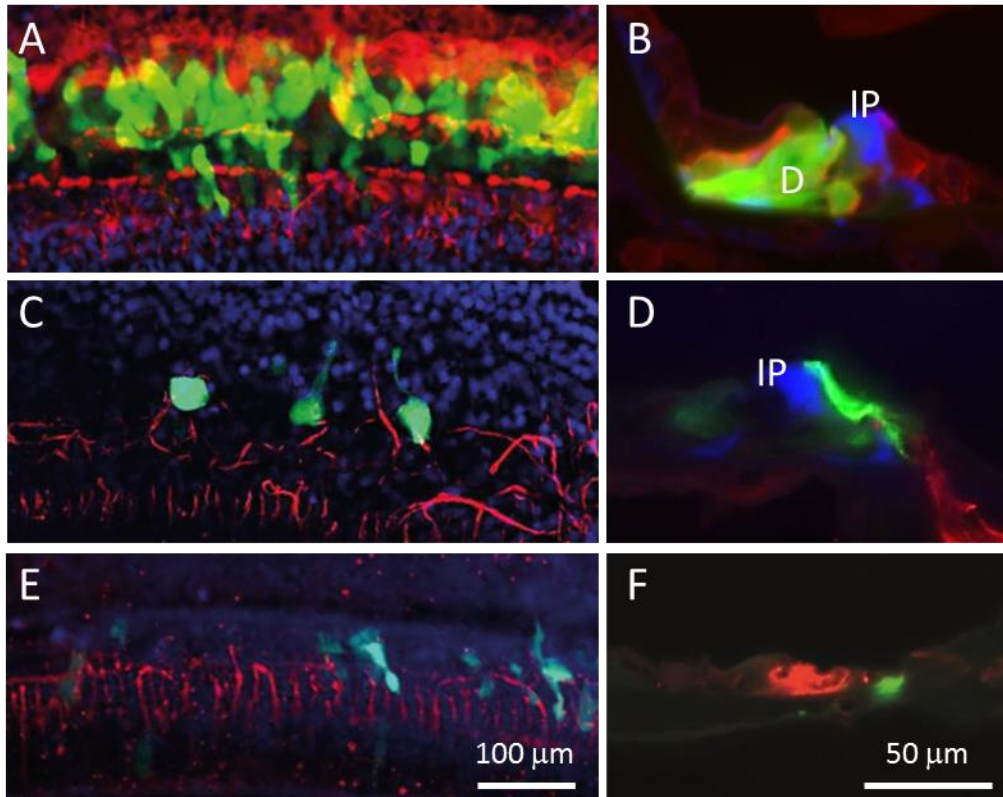


Figure 3. Effect of deafness on transgene expression in the sensory epithelium. **(A, B)** In a normal hearing guinea pig, the green fluorescent protein reporter gene from an adenoviral vector is expressed in numerous cells in the sensory epithelium (green) **(A=**top down view, **B=**cross section; IP=inner pillar cell (blue), D=Deiters' cells). **(C, D)** When gene therapy is given 1 week after ototoxic hearing loss in guinea pigs, the level of transgene expression in supporting cells is significantly reduced due to deafness-induced degeneration of cells in the sensory epithelium (IP=inner pillar cell (blue)). **(E, F)** When gene therapy is delayed by 8 weeks after hearing loss, the number of residual cell types in the sensory epithelium is severely limited and transgene expression is similarly rare. A, C, E: Red=peripheral fibres, blue=nuclei. B, F: Red=calretinin, blue=pillar cells. D: Red=peripheral fibres, blue=pillar cells.

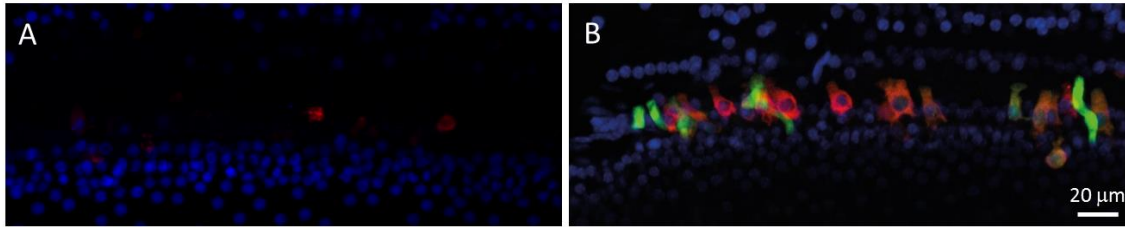


Figure 4. Atoh1 gene therapy after ototoxicity by aminoglycosides in the guinea pig **(A)** Four days after ototoxicity there are very few residual hair cells (red) in the sensory epithelium of the cochlea. **(B)** When Atoh1 gene therapy is given 4 days after administration of aminoglycosides, numerous hair cells are apparent within the sensory epithelium after 3 weeks, most of which co-localise with the GFP reporter gene from the adenoviral vector. Red=myosinVIIa, blue=nuclei, green=green fluorescent protein