BDNF gene therapy induces auditory nerve survival and fiber sprouting in deaf Pou4f3 mutant mice

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Current therapy for patients with hereditary absence of cochlear hair cells, who have severe or profound deafness, is restricted to cochlear implantation, a procedure that requires survival of the auditory nerve. Mouse mutations that serve as models for genetic deafness can be utilized for developing and enhancing therapies for hereditary deafness. A mouse with Pou4f3 loss of function has no hair cells and a subsequent, progressive degeneration of auditory neurons. Here we tested the influence of neurotrophin gene therapy on auditory nerve survival and peripheral sprouting in Pou4f3 mouse ears. BDNF gene transfer enhanced preservation of auditory neurons compared to control ears, in which nearly all neurons degenerated. Surviving neurons in treated ears exhibited pronounced sprouting of nerve fibers into the auditory epithelium, despite the absence of hair cells. This enhanced nerve survival and regenerative sprouting may improve the outcome of cochlear implant therapy in patients with hereditary deafness.

Hearing loss can result from hereditary and/or environmental causes. The latter may involve overstimulation (acoustic trauma), ototoxic drugs, infections, autoimmune disease or aging. Genetic predisposition appears to influence the severity of most if not all environmental causes of hearing loss. The two most common cochlear tissues involved in hearing loss are the sensory epithelium and the auditory nerve. Since the mammalian auditory epithelium is unable to replace lost sensory cells, and neuronal loss also is permanent, sensorineural (inner ear) deafness due to loss of these cells is irreversible1–4. The loss of sensory cells (hair cells) often leads to secondary degeneration of nerve fibers from the sensory epithelium, and eventually to the degeneration of spiral ganglion neurons (SGNs)5–8. However, primary neuronal damage may also occur in the cochlea, in the absence of hair cell loss9,10.

The organ of Corti, the sensory portion of the auditory epithelium, contains two types of sensory cells: inner and outer hair cells. The auditory neurons are bipolar with one ending in the organ of Corti and the other in the cochlear nucleus. Cell bodies of these bipolar neurons reside in Rosenthal’s canal in the cochlea. The inner hair cells receive 90–95% of all afferent SGN fibers11. Neurotrophins, specifically brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), are required for the development and maintenance of normal innervation of hair cells. These neurotrophins are expressed both in hair cells and supporting cells, and their relative levels of expression in each cell type vary during development and in the mature tissue12–19. Therefore, loss of hair cells or supporting cells in the auditory epithelium results in reduced levels of BDNF and NT-3 expression, causing degenerative changes in the peripheral fibers and somata of SGNs. The role of supporting cells in maintaining auditory nerve fibers and somata has been demonstrated by blocking the ErbB receptor in these cells20,21. In some cases, SGNs can survive for months or years after inner hair cell loss, indicating that other cells, including supporting cells or central auditory neurons also could be sources of SGN survival factors22,23.

The only therapy currently available for hearing loss secondary to a severe or complete loss of hair cells is the cochlear implant auditory prosthesis. In the absence of hair cells, cochlear implant electrodes can directly stimulate SGN soma and possibly their central axons, providing partial hearing restoration to patients with severe or profound hearing loss24. In such cases, it is essential to maximally preserve the population of SGNs, both qualitatively and quantitatively. Following a severe ototoxic lesion that results in the loss of hair cells and supporting cells in guinea pigs, neurotrophin gene transfer has been shown to induce regrowth of auditory nerve...
fibers into the auditory epithelium, as well as enhance preservation of SGNs22,26. Because many of the patients aided by cochlear implant prostheses have hereditary hearing loss, it is important to determine the efficacy of neurotrophin therapy in genetic deafness conditions as well. Genetic mouse models of human inherited inner ear disease serve as excellent research systems to test the influence of neurotrophin gene therapy on auditory nerve regeneration. In this study, we characterized the outcome of neurotrophin gene therapy on the cochleae of Pou4f3 mutant mice.

The transcription factor Pou4f3 is necessary for the maturation and survival of hair cells in the inner ear. Mutations in Pou4f3, the human ortholog of Pou4f3, are responsible for DFNA15, an autosomal dominant disorder characterized by progressive, non-syn-dromic, sensorineural hearing loss. DFNA15 patients demonstrate a high degree of clinical variability in age of onset and degree of progression27–29. In contrast, the Pou4f3 mutation used in the present study is recessive, requiring homozygosity to exhibit a phenotype. Affected mice are profoundly deaf from birth, and exhibit poor balance, circling behavior, low weight and decreased fertility30. In addition to the loss of hair cells and other abnormalities of the auditory epithelium, most spiral ganglion cells in these mice degenerate between 2 and 6 weeks of age31–32. Despite the differences between human DFNA15 and mouse Pou4f3 mutants in the genetics and phenotypic manifestation of the disease, these mice serve as a valuable model for several deafness mutations where hair cells and hearing are absent and the auditory nerve degenerates over time.

The goal of this study was to determine whether BDNF supplementation in the cochlea of Pou4f3 mutants promotes auditory nerve fiber growth and SGN survival. To locally elevate BDNF levels, we delivered the BDNF gene to the auditory sensory epithelium using an adenovirus vector with the BDNF gene insert (Ad.BDNF). Pou4f3 homozygous mutant mice had no hair cells and few peripheral nerve fibers at 4 weeks of age. Animals that received Ad.BDNF exhibited regeneration of peripheral nerve fibers and enhanced survival of SGNs compared to untreated ears 2 weeks following inoculation. These data demonstrate for the first time the ability to manipulate an ear with genetic deafness in a manner that would potentially improve its hearing performance with cochlear implant stimulation.

**Results**

The cochlear phenotype of Pou4f3 mutant mice. The Pou4f3 mouse exhibits abnormal cochlear epithelium and innervation (Figure 1). In the wild-type organ of Corti whole-mount analysis of the auditory epithelium demonstrated intact sensory hair cells and non-sensory cells (supporting cells) (Figure 1a). In contrast, the auditory epithelium of mutant ears contained no hair cells, based on the lack of stereocilia, cuticular plate or the typical hair cell contour (Figure 1b), as well as the absence of myosin VIIa staining (not shown). In addition to the complete lack of hair cells, the organized pattern of the supporting cells was also absent (Figure 1b). In the area of the auditory epithelium where pillar cells and Deiters cells usually reside, the phalloidin-stained whole-mounts also showed clusters of actin-rich cells (AR cells) that were approximately evenly spaced along the cochlear duct (Figure 1b). In cross-sections, these cells appeared to have densely stained cytoplasm (probably due to abundant cytoskeletal proteins) and often resembled the morphology of pillar cells (Figures 1c and d).

Analysis of neural components demonstrated that in wild-type cochlea, many nerve fibers extended from the habenula perforata to the inner and outer hair cells (Figure 1a). In contrast, considerable elimination of nerve fibers was found in homozygous Pou4f3 mutant ears (Figure 1b). The few fibers present in the tissue were extremely thin and tended to terminate in the vicinity of the AR cells. No nerve fibers were present in the auditory epithelium beyond the AR cells (Figure 1b).

Mid-modiolar cross-sections of the wild-type organ of Corti revealed the presence of a highly organized sensory epithelium (Figure 1c), with the typical array of inner and outer hair cells and non-sensory cells23. In contrast, sections from Pou4f3 homozygous mutant mice at 6 weeks of age revealed an epithelium in which no hair cells could be recognized (Figure 1d). In addition, cells with characteristic features (cell shape, cytoskeletal protein aggregations, etc.) of Deiters cells, pillar cells and other supporting cells were
also not detected. Rather, the cross-sectional view resembled a flat epithelium, composed of a single layer of cuboidal epithelial cells. No morphological aberrations were found in the overall architecture of Reissner’s membrane or the stria vascularis (not shown), suggesting that the pathology associated with the Pou4f3 mutation phenotype is restricted to the area of the auditory epithelium.

Cross-sections of Rosenthal’s canal in wild-type ears at 6 weeks of age showed densely packed SGNs (Figure 1e) that occupied the entire cross-sectional area of the canal. Schwann cells adhered to the neurons and myelin was observed in areas where fibers were present. In contrast, the number of SGNs was greatly reduced in Rosenthal’s canal of mutant mice (Figure 1f, basal cochlear turn). Similar losses of neurons and nerve fibers were also detected in the apical cochlear turn (not shown). The SGNs that remained appeared smaller than normal, and their outer perimeters were irregular. The area of Rosenthal’s canal that leads to the osseous spiral lamina (and the habenula perforata) contained a dense array of myelinated fibers in wild-type ears (Figure 1e), whereas the mutant ears had very few fibers in this area (Figure 1d).

Cross-sections of the auditory epithelium from Pou4f3 mice obtained at 6 weeks of age were used to investigate the auditory epithelium at higher magnification, within AR clusters and between them (Figure 2). In sections cut through an AR cell cluster, the AR cells often consisted of one or two cells (Figure 2a), and in other clusters several dark cells were observed (Figure 2b). The tunnel of Corti was present in some cases (Figure 2a) and absent in others (Figure 2b). In many cases, the nuclei of the AR cells were not perfectly round (Figure 2), which differs from normal pillar and Deiters cells (Figure 1c). Sections through areas between clusters of AR cells (devoid of AR cells) exhibited a nearly flat epithelium without any sign of differentiated supporting cells (Figure 2c). To determine if AR cells survive in the auditory epithelium of Pou4f3 mutant mice over time, we assessed whole-mounts of homozygous mutant ears at ages up to 6 months, and determined that these cells did survive and appeared similar to those seen in ears of younger animals (not shown).

**Nerve fiber growth.** To investigate the spatial and temporal pattern of adenovirus-mediated transgene expression, we inoculated an adenoviral vector containing eGFP (Ad.eGFP) into the scala media of mature wild-type and Pou4f3 mutant mice. One week after the surgery, mice were sacrificed and the whole-mounts were stained with phalloidin (red) and GFP-specific antibody (green). In wild-type mouse ears, the mesothelial cells expressed GFP throughout the apical turn (Figure 3a). In the basal turn mesothelial cells were also GFP-positive, along with some epithelial cells such as pillar cells and Deiters cells (Figure 3b). The pattern of GFP distribution was similar in mutant mice despite the different cochlear morphology, with robust expression in mesothelial cells throughout the cochlear duct (Figures 3c–d). However, fewer epithelial cells were transduced in the mutant, and the AR cells appeared negative.

Pou4f3 mutant mice inoculated with the Ad.empty or Ad.BDNF vectors at 4 weeks of age were allowed to survive for 2 weeks. Cochleae were then harvested, stained for neurofilament and actin, and prepared as whole-mounts. Low magnification epi-fluorescence analysis demonstrated thin peripheral nerve fibers after Ad.empty inoculation (Figures 4a and b). Ad.empty (control)-treated ears appeared similar to untreated ears (Supplementary Figure S1). In contrast, we observed robust nerve fiber generation at both apex and base of cochlea that were Ad.BDNF-inoculated (Figures 4c and d), with more prominent fiber growth in the apex. The shape, size and distribution of AR cells are similar between Ad.empty and Ad.BDNF inoculation ears, suggesting that BDNF does not directly influence the morphology of these cells.

We analyzed Ad.BDNF-treated ears as well as the contralateral (control, non-inoculated) ears at higher magnification (Figure 5).
Neurofilament staining revealed numerous nerve fibers extending from the habenula perforata in a radial fashion, with fibers extending both laterally (toward the auditory epithelium) and medially (towards the inner sulcus) under higher magnification. The diameter and number of nerve fibers were dramatically increased compared to those in control ears (Figures 5a–d). The regenerated nerve fibers appeared to grow preferentially toward the AR cells. Some fibers terminated in the vicinity of the AR cells while others extended beyond these cells and continued on a lateral trajectory (Figure 5b). Fibers that extended from the habenula perforata into the auditory epithelium in Ad.BDNF-treated ears appeared to travel between the epithelial cells. Near the medial aspect of the AR cells, several fibers exhibited enlarged endings resembling growth cones or axon terminals (Figures 5b and d). Combined with data shown in Figure 4, the comparison between ears treated with Ad.BDNF and control ears (contralateral untreated, or Ad.empty-treated) is striking, demonstrating a robust effect of transgenic BDNF on sprouting of nerve fibers into the epithelium of these mutant deaf ears.

Neurofilament positive fibers extending beyond the AR cells were counted in the entire cochlear duct, from the apical turn to the hook, using whole-mounts of the auditory epithelium under the epi-fluorescence microscope, and the distance each fiber extended into the auditory epithelium was noted. We defined three regions in the auditory epithelium (Levels, Figure 6a), bounded by the medial and lateral margins of the AR cells. We counted all fibers in Level 1, including those that extended beyond the medial margins of the AR cells (open arrow heads), but not in AR cells (filled arrow heads). Scale bar indicates 100 μm.

![Figure 3](image-url) Low magnification epi-fluorescence images showing the distribution of GFP in wild-type (a, b) or Pou4f3 homozygotes (Pou4f3−/−) cochleae (c-d) following inoculation of Ad.eGFP. Whole-mounts are double-stained with phalloidin (red) and GFP (green) and show cochlear apex (a and c) and base (b and d). (a) GFP can be detected in numerous mesothelial cells (elongated or spindle shaped cells) throughout the entire cochlear apex. (b) Expression of GFP is seen in several types of cells including supporting cells and inner hair cells, as well as inner mesothelial cells. (c, d) In Pou4f3 mutants, GFP expression is detected in a large number of mesothelial cells (open arrow heads), but not in AR cells (filled arrow heads). Scale bar indicates 100 μm.

![Figure 4](image-url) Whole-mounts of Pou4f3 mutant cochleae treated with Ad.empty (a, b) or Ad.BDNF (c, d), stained for actin (green) and neurofilament (red) and imaged with epi-fluorescence (Figures a and c for apex, b and d for base). (a, b) After Ad.empty inoculation, there is no visible effect on AR cells or on nerve fiber distribution throughout the cochlear duct (arrow heads). (c, d) Following Ad.BDNF inoculation, many nerve fibers are observed around AR cells (open arrows) throughout the cochlear duct, as seen in both the apex (c) and the base (d). The shapes of AR cells in Ad.empty cochleae are similar to that seen in Ad.BDNF-treated ears (arrow heads). Scale bar indicates 100 μm.

![Figure 5](image-url) Whole-mounts of Pou4f3 mutant contralateral ears (untreated with the neurotrophin vector, a, c) or ipsilateral ears treated with Ad.BDNF (b, d), stained for neurofilament (red) and actin (green). (a) In the cochlear apex, a few nerve fibers are seen in the auditory epithelium and appear to preferentially grow toward the AR cells, but not beyond them. (b) In the Ad.BDNF-treated ear apex, many nerve fibers grow to the AR cells and some grow beyond them. Some of the fibers loop (arrow) and some exhibit bulging areas (open arrowheads) (also see (d)). (c) In the cochlear base the distribution of neurofilament positive fibers is similar to the apex (a) but the density appears lower. There are no fibers extending beyond the AR cells. (d) In the basal cochlea of a treated ear, there is a large number of fibers many of which display bulging areas (arrow head), and some of them traverse beyond the AR cells (arrow). Scale bar indicates 20 μm.
the basal turn. In contralateral ears, we observed a similar number of fibers in apex and base, and only slightly less in the hook. We also counted the number of nerve fibers in Level 1 after Ad.empty inoculation and determined that Ad.empty treatment was not different from contralateral (no treatment) ears (data not shown). We also determined the proportion of total fibers (Level 1) that extended to Level 2 (Figure 6c), or Level 3 (Figure 6d). The Level 2 nerve fiber count suggested that more than 60% reached at least as far as the AR cells, in contrast to less than 30% in contralateral ears (Figure 6c). The number of nerve fibers extending beyond the AR cells to Level 3 was 2 or 3 times greater than in controls (Figure 6d). For both Level 2 and Level 3, the increased number of fibers in BDNF-treated ears was statistically significant.

**SGN survival.** The density and qualitative morphology of SGNs were examined in mid-modiolar sections, comparing the contralateral control ears to ears treated with Ad.BDNF, in order to determine the influence of BDNF on SGN survival. The population of SGNs in both apical and basal cochlear turns of contralateral (no treatment) ears (data not shown). We also determined the proportion of total fibers (Level 1) that extended to Level 2 (Figure 6c), or Level 3 (Figure 6d). The Level 2 nerve fiber count suggested that more than 60% reached at least as far as the AR cells, in contrast to less than 30% in contralateral ears (Figure 6c). The number of nerve fibers extending beyond the AR cells to Level 3 was 2 or 3 times greater than in controls (Figure 6d). For both Level 2 and Level 3, the increased number of fibers in BDNF-treated ears was statistically significant.

**SGN survival.** The density and qualitative morphology of SGNs were examined in mid-modiolar sections, comparing the contralateral control ears to ears treated with Ad.BDNF, in order to determine the influence of BDNF on SGN survival. The population of SGNs in both apical and basal cochlear turns of contralateral (no treatment) ears was similar to contralateral ears (not shown). In contrast, two weeks after Ad.BDNF inoculation, treated ears showed a remarkable preservation of SGNs (Figures 7a and c). The population of SGNs in the apex appeared not only denser than in contralateral ears, but also healthier, with nearly round nuclei and a smooth plasma membrane without ruffles (Figure 7b). In the Ad. BDNF-inoculated ears, SGNs in the base appeared morphologically healthy with normal appearing nuclei and cell membrane, similar to that seen in the treated apex (Figure 7d). However, Ad. BDNF-induced preservation of SGNs in the base was less complete than in the apex and Rosenthal’s canal was not filled with SGNs (Figure 7d). The density of SGNs was low in the hook, base and apex region of cochleae of mutant ears that were not treated with BDNF (Figure 8a). The density in the base was lowest. The average of SGN density in Ad.BDNF-treated ears was significantly higher than in untreated ears. The most complete preservation of SGNs was noted in the apex. In addition, BDNF treatment significantly increased average size of SGN somata in the apex as compared to the contralateral ears (Figure 8b).

Sections of ears that received Ad.BDNF displayed connective tissue in scala tympani. The tissue appeared to contain fibroblasts and extracellular material in a loose organization. The connective tissue was limited to the area adjacent to the modiolus and did not obliterate the entire fluid space of Rosenthal’s canal (Figures 7b and d). The fluid spaces (endolymph and perilymph) appeared clear and intact in untreated ears (Figures 7a and c).

**Discussion**
We used Pou4f3 mutant mice as a model for testing whether neurotrophin treatment can induce nerve fiber regeneration and SGN preservation in ears with hereditary deafness. In untreated Pou4f3 homozygotes at 6 weeks of age, the number of peripheral nerve fibers and the density of SGNs were much lower than in normal mice. Ad.BDNF inoculations into the cochlear fluids of these mutant mice lead to additional growth of nerve fibers into the auditory epithelium and preservation of SGNs in Rosenthal’s canal.

The Pou4f3 mutant mouse serves as a model of hereditary hearing loss. Pou4f3 homozygotes are completely deaf because cochlear hair
supporting cell survival and neuronal survival and postulated that SCIENTIFIC REPORTS

Figure 7 | Light microscope micrographs of cross-sections through Rosenthal’s canal in the apex (a-b) and base (c-d) of contralateral ears (a and c), and 2 weeks after inoculation of Ad.BDNF (b and d). (a) SGNs are much less dense than normal in the apical cochlear turns of untreated ears. (b) Rosenthal’s canal of the treated ear appears densely packed with SGNs and morphologically healthy, with normal appearing nuclei and cytoplasm. (c) SGNs in the base appear similar to those seen in the apex in the absence of BDNF treatment (a). (d) BDNF-induced preservation of SGNs in the base is less complete than in the apex (compare to (b)). In some of the Ad.BDNF-inoculated ears, connective tissue could be observed in the scala tympani (open arrow-heads in b and d). Scale bar indicates 20 µm.

cells fail to develop31,34,35. The normal organization of supporting cells is also absent. Instead, the auditory epithelium appears nearly flat, with the exception of one type of cell, an AR cell based on its appearance in phalloidin-stained whole-mounts. We demonstrate that clusters of AR cells are scattered periodically along the cochlear duct. The identity of the AR cells is difficult to determine. Given the lack of signals from adjacent hair cells, it is possible that the AR cells do not express pillar cell or Deiters cell markers, or might even mis-express them. We could not detect other marker proteins, such as p75 in Pou4f3-null ears (data not shown). Based on their location, AR cells most likely represent the same cells previously referred to as “pillars”36. Although AR cells are not a typical or general finding in ears with early hair cell loss37, similar clustering of pillar cells has been observed in other deaf ears that had an Atoh1 conditional deletion38. While the AR cells (regardless of their identity, pillar and/or Deiters) appear to play a role in attracting a few fibers in mutant ears without neurotrophin treatment, the Ad.BDNF treatment appears to dramatically increase both the number of nerve fibers in the auditory epithelium and the survival of the auditory neurons in Rosenthal’s canal.

Sugawara et al.10 previously described the relationship between supporting cell survival and neuronal survival and postulated that pillar cells may be at least as important as hair cells for the purpose of maintenance of nerve survival10,20. Our data generated using a mouse model of a mutation that affects hair cell differentiation and survival support the findings of Sugawara et al.10. The mechanism by which supporting cells maintain nerve fibers is not completely clear, but a likely explanation involves an elevated level of neurotrophins expressed by these cells13,19. The ability of nerve fibers to sprout in response to neurotrophins has been shown in deaf ears of guinea pigs23,41,42, and now we demonstrate similar findings in the mouse. While the AR cells (regardless of their identity, pillar and/or Deiters) appear to play a role in attracting a few fibers in mutant ears without neurotrophin treatment, the Ad.BDNF treatment appears to dramatically increase both the number of nerve fibers in the auditory epithelium and the survival of the auditory neurons in Rosenthal’s canal.

Our results showed that in mutant cochleae treated with Ad.BDNF, the sprouting of nerve fibers was significantly greater in the apical turn than in the basal turn. This was surprising because the inoculation of the viral vector was into the basal turn, likely yielding a higher concentration of BDNF in the base. There are several possible explanations for this unexpected finding. First, SGN survival in the apical cochlea was better than in the base in both control and treated ears, providing more neurons for sprouting. In addition, it is possible that apical neurons are more responsive to BDNF than basal neurons. This would be in agreement with the developmental role of BDNF in determining the innervation pattern of the cochlea, where BDNF plays a greater role in the apical turn than in the base43. Finally, it is possible that the concentration of BDNF in the apex was lower, but closer to normal physiological levels than in the base, and that excess neurotrophin is not necessarily better for nerve fiber...
sprouting. Because the rate of viral vector-mediated transgene expression is unregulated, it is possible that the concentration of neurotrophins attained in the cochlea is higher than normal.

Because the type I afferents are the relevant population of neurons for receiving cochlear implant stimulation and transmitting electrically evoked signals to the brain stem, it is important to identify the fibers that grow into the auditory epithelium following neurotrophin transgene expression. Previous studies examined resprouting nerve fibers in ears treated with BDNF and acidic FGF and identified them as afferent nerve fibers\(^{47,48}\). In addition, experiments involving transgenic mice expressing *BDNF* have indicated that the neurotrophin is the most important molecule for inner ear afferent fiber guidance\(^{49-56}\). In the current study, both narrow and thick nerve fibers were observed growing into the auditory epithelium after Ad.*BDNF* inoculation. This result suggests that transgenic *BDNF* expression in the area of the auditory epithelium may induce growth of more than one population of neurons. Possibilities include both Type I and Type II afferent dendrites as well as olivocochlear efferent nerve fibers. Developing Type II afferent fibers have recently been shown to respond preferentially to BDNF in vitro\(^{57}\). Another explanation for the presence of thick and thin fibers is that some afferent fibers may respond to the BDNF treatment by changing their diameter. Better characterization of the type of fibers that sprout into the deaf ear after BDNF treatment will need to be performed in future studies.

We observed several fibers that exhibited localized swelling, most commonly near the medial aspect of the AR cells. These swellings sometimes appear as an enlarged nerve ending, and other times as an enlarged region away from the ending. Previous reports have described similar varicosities in guinea pig SGN fibers treated with neurotrophins\(^{58,59}\). There are other examples of bulging areas in neurons\(^{60-62}\) and their nature is unclear. One possible explanation provided by Lee et al.\(^{63}\) is that enlarged regions serve to orient neurons in the tissue, but it is also possible that these enlarged endings are associated with stress or a constant search for a target for synaptogenesis. Alternatively it has been proposed that such varicosities form whenever anterograde transport of organelles and other growth cone substrates temporarily exceed the rate of growth cone extension\(^{64}\).

The degeneration of SGNs in *Pou4f3* mutant mice has been described\(^{65}\). Here we show that inoculation with Ad.*BDNF* resulted in the maintenance of a significantly higher density of SGNs in *Pou4f3* mutant mouse ears. To our knowledge this is the first demonstration of the ability to prevent a loss of inner ear neurons due to hereditary disease. The induced neurotrophin expression most likely had several parallel effects, inducing nerve fiber extension, preventing degeneration of the SGN somata and increasing cell size. BDNF-induced survival of the SGNs was more effective in the apical turn than in the base, although the density of SGNs in Rosenthal’s canal in the hook was higher than in the base. BDNF also increased SGN somata size only in the apex. We can only speculate on the reason for this surprising pattern. One possibility is that the inoculation caused mechanical trauma in the base. Another possibility is that the concentration of BDNF in the base, close to the inoculation site, was excessive, whereas the hook and the apex, flanking the base on each side, received a concentration of the neurotrophin that is closer to optimal. The fact that the nerve fiber degeneration was quite different between the base and the hook was also understood. The fact that the nerve fiber degeneration was relatively less pronounced in the base (compared with the apex) may also be related to the finding that the density of SGNs in the base was the lowest in the cochlea (lower than the hook and much lower than the apex).

Potential negative side effects must be considered in the development of any clinical applications of our work. We noted that two weeks after Ad.*BDNF* inoculation, connective tissue could be observed in the scala tympani. Prior studies have shown similar findings following viral vector inoculation\(^{66}\) or exogenous BDNF inoculation and electrode stimulation\(^{67}\). Growth of fibrous tissue in the cochlear fluid spaces is an undesirable side effect that may lead to several negative outcomes, including an increase in electrode impedance\(^{68}\) and difficulty in re-insertion of an electrode if revision surgery becomes necessary. It is unclear how elevated levels of BDNF in endolymph (following inoculation into the scala media) causes changes in cells lining the scala tympani. However, BDNF is a pleiotropic factor that can originate from more than one source and influence diverse cell types. It is expressed by inflammatory cells including macrophages and T cells\(^{69-71}\), and in the lung it can be associated with airway obstruction\(^{72}\). Once the dynamics of neurotrophin diffusion in the cochlear fluids are better understood, it will also be possible to design a strategy to prevent or limit this side effect. A possible solution is to lower the viral titer to reduce neurotrophin levels, assuming a lower concentration of neurotrophins decreases connective tissue growth. Alternatively, a specific reagent that can antagonize connective tissue growth could be added at the time of the neurotrophin therapy. Other methods for gene delivery could also be considered, as previously shown with electrode coatings with allogeneic fibroblasts\(^{73}\) or using alginate capsules\(^{74}\). Due to the minute size of the mouse ear and correspondingly small volume of its cochlear fluids, accurate measurements of concentrations of neurotrophins in the ear cannot be accomplished but work on larger animals may help correlate gene delivery methods with resulting neurotrophin concentrations, to advance these methods towards clinical applications.

Among the advantages of adenoviral vectors are their ability to infect a broad range of cell types with high efficiency, and the rapid onset of gene expression following infection. However, adenovirus gene expression is transient, and therefore elevated levels of transgenes cannot be sustained over several weeks. Therefore, the data we present can serve as a proof for the principle that nerve fibers can be regenerated in the mutant ear, but for future clinical application, it would be better to use a long-term expressing vector such as adeno-associated virus which can sustain gene expression for a long time\(^{75}\).

In many human deafness cases, SGNs survive for many years, despite the loss of most hair cells and the degeneration of peripheral nerve fibers from the auditory epithelium\(^7\). This finding is not limited to environmental hair cell loss, but also holds for many of the mutation-caused hereditary deafness cases. However, there are mutations in which spiral ganglion neurons do not survive, and these patients could benefit from a treatment for enhancing neuronal survival. One important example is neurofibromatosis 2 (NF2) which involves severe degeneration of SGNs. In many of the NF2 patients SGN degeneration makes the cochlear implant impractical leaving a brainstem implant as the only therapeutic option. When a small number of SGNs survive, a traditional cochlear implant is of some benefit\(^{77}\), suggesting that any therapy to enhance preservation of the neurons would further improve the cochlear implant therapy outcome. There are other mutations with variable outcome of cochlear implant therapy, such as DFN3, where the variable outcome is not yet understood\(^{78}\). It is possible that enhancing preservation of SGNs would increase the success of the cochlear implant procedure, especially in patients who otherwise do poorly with their implant. Even in cases where SGNs survive in human cochlea for a long time, cochlear implant patients may sometimes do very poorly with their prosthesis\(^{79}\). It is possible that inducing auditory nerve fiber regeneration in such ears could improve the outcome of the cochlear implant therapy. The concept relating the cochlear substrate with cochlear implant outcome has been demonstrated\(^{80-82}\). In addition, transgenic *BDNF* over-expression with concurrent electrical stimulation has been shown to improve cochlear implant thresholds and survival of auditory neurons\(^{83}\).

The data we report, showing substantial neural preservation and regeneration in a mouse model for human hereditary deafness, join other recent advances in restoring structure and function of ears with hereditary disease\(^{84,85}\). These advances provide milestones in
the progress towards treating humans who suffer from severe hereditary inner ear disease. We provide a proof of principle that ears affected by genetic diseases that do not target neurons directly, are amenable to treatment aimed at enhancing nerve survival, thereby providing an avenue for enhancing cochlear implant outcomes in patients with hereditary hearing loss. In addition, improving nerve survival and fiber regeneration will also augment the outcome of future hair cell replacement therapies based on transdifferentiation or stem cell transplantation.

Methods

Animals and groups. All animal care and handling were approved by the University of Michigan Institutional Committee on the Use of Care of Animals and performed using standard protocols. We used Adenovirus mutant mice, which have been described previously.17. Pou3f1 homozygous mutant mice of either gender were 4 weeks old at the onset of the experiments. Because there are differences in the severity of nerve degeneration between mice, but SGN survival rate is consistent between the two cochleae of the same animal, contralateral ears were used as internal controls. We examined whole-mounts of Ad.BDNF-inoculated cochleae, untreated contralateral cochleae and Ad.empty inoculated cochleae of 10 animals, and cross-sections of Ad.BDNF or Ad.empty-inoculated cochleae and untreated contralateral cochleae of 10 animals. Four wild-type 4-weeks-old mice were inoculated with Ad.eGFP into the scala media of the left cochleae and processed for transgene expression 4 days later.

Surgery. Pou3f1 mutant mice were inoculated with 1 ml of viral vector solution into the scala media endolymph via a cochleostomy. Before opening the cochleostomy, the following devices were prepared. A polyethylene tube was connected to a fine polyimide tube and the end of the polyethylene tube was connected by a 30G needle to a 5 μl Hamilton syringe filled with sterile water. The mice were anesthetized with ketamine (120mg/kg, i.p.) and xylazine (7mg/kg, i.p.). The left postauricular region was shaved and an incision was made behind the left pinna. The muscles overlying the bulla were divided, and the tympanic bulla was exposed without damaging the facial nerve. The bulla was perforated with a fine gauge needle (26G) and the hole was expanded with forceps. After the stapedial artery was identified as it coursed over the basal cochlear turn, the cochlear lateral wall was thinned just beneath the stapedial artery with a surgical drill. The tip of the polyimide tube was inserted into the cochlea and advanced toward the scala media. To identify the angle and depth of insertion for scala media inoculation, we first performed exploratory surgeries in which the tube was cemented to the bone and the ears later opened to determine the location of the tip. The infusion speed was controlled by a syringe pump. After the inoculation was complete, the tube was removed and the hole in the cochlea was sealed immediately with muscle tissue and fat. The skin was closed with 5-0 Ethilon suture. It took approximately 30 minutes to complete this procedure. The mice were allowed to awaken from anesthesia, and their pain was controlled with ketoprofen (5mg/kg, s.c.).

Adenoviral vector. Animals in the experimental group received an adenoassociated vector with a mouse BDNF gene insert, driven by the cytomegalovirus (CMV) promoter.66. The effectiveness of the vector and its specific BDNF activity were previously established.8. We inoculated 1μl of Ad.BDNF at a titer of 4 × 10^7 adenoviral particles per ml into each cochlea. The inoculated Ad.empty-emulated Ad.BDNF vector with the CMV promoter. The viral suspension was preserved at −80 °C and thawed on ice before use.

Immunohistochemistry. Two weeks after surgery, animals were euthanized, temporal bones removed, placed in fixative solution (4% paraformaldehyde in PBS, at 4°C) and advanced toward the scala media. To identify the angle and depth of insertion for scala media inoculation, we first performed exploratory surgeries in which the tube was cemented to the bone and the ears later opened to determine the location of the tip. The infusion speed was controlled by a syringe pump. After the inoculation was complete, the tube was removed and the hole in the cochlea was sealed immediately with muscle tissue and fat. The skin was closed with 5-0 Ethilon suture. It took approximately 30 minutes to complete this procedure. The mice were allowed to awaken from anesthesia, and their pain was controlled with ketoprofen (5mg/kg, s.c.).

Quantitative analyses of neural preservation. Peripheral fiber outgrowth was evaluated by counting fibers in the auditory epithelium while examining the tissue under the microscope. Direct examination was preferred over imaging because it permitted tracking fibers to their ends as they meandered in 3 dimensions. Fibers were counted separately for each region of the cochlear duct (apex, base and hook). Fibers were scored based on the distance they extended from the habenula perforata and divided into three groups: (1) inner hair cells (AR cells at the AP level and beyond); (2) outer hair cells (AR cells at marker cell levels of extension were recognized; Figure 6.a). Level 1 included all fibers extending beyond the habenula perforata. Level 2 were those that reached the AR cells but not beyond. Level 3 are those that did reach beyond the AR cells. To compare total numbers of fibers across specimens and regions, the length of the dissected segment from that region was measured using tpsDig2 software (F. James Rohlf, Ecology & Evolution, SUNY at Stony Brook) and the number of cells per mm was computed. For Levels 2 and 3, the proportions of cells continuing to each level was computed by dividing the number reaching that level by the total (Level 1). For each level, differences between treated and contralateral controls for each region were tested using Student’s t-test for paired samples. Bonferroni adjustment for multiple comparisons was used to ensure table-wide α < 0.05.

To evaluate SGN survival, images of 5 cross-sections were randomly selected out of the total of ~44 sections obtained for each cochlea. SGNs that exhibited a clear nucleus and cytoplasm in Rosenthal’s canal of the apex, base and hook were counted. The area of Rosenthal’s canal was measured using tpsDig2. The number of SGNs in each turn was calculated per 10,000 μm². Differences between treated ears and contralateral controls were tested using Student’s t-test for paired samples, with Bonferroni adjustment for multiple comparisons. Somata areas were measured in the same sections used for cell counting. Areas were measured by tpsDig2 and determined by taking the square root. In sections with numerous cells (usually treated ears), a grid was randomly selected to place 20 cells for measuring; in sections with less than 20 cells, all cells were included. Average cell size for all cells in all sections was determined for each side of each individual. Because some individuals had no cells in the measured sections, comparison between treated and untreated ears was performed using a t-test for unpaired samples, assuming unequal variances.

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