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Auditory hair cell replacement and hearing improvement by *Atoh1* gene therapy in deaf mammals

Masahiko Izumikawa[1,2], Ryosei Minoda[1,3], Kohei Kawamoto[2], Karen A Abrashkin[1], Donald L Swiderski[1], David F Dolan[1], Douglas E Brough[4] & Yehoash Raphael[1]

In the mammalian auditory system, sensory cell loss resulting from aging, ototoxic drugs, infections, overstimulation and other causes is irreversible and leads to permanent sensorineural hearing loss. To restore hearing, it is necessary to generate new functional hair cells. One potential way to regenerate hair cells is to induce a phenotypic transdifferentiation of nonsensory cells that remain in the deaf cochlea. Here we report that *Atoh1*, a gene also known as *Math1* encoding a basic helix-loop-helix transcription factor and key regulator of hair cell development, induces regeneration of hair cells and substantially improves hearing thresholds in the mature deaf inner ear after delivery to nonsensory cells through adenovectors. This is the first demonstration of cellular and functional repair in the organ of Corti of a mature deaf mammal. The data suggest a new therapeutic approach based on expressing crucial developmental genes for cellular and functional restoration in the damaged auditory epithelium and other sensory systems.

The most common reason for sensorineural hearing loss is degeneration of cochlear sensory (hair) cells, resulting from overstimulation, ototoxic drugs, infections, autoimmune disease or aging. Sensorineural hearing loss affects millions of people worldwide. This impairment is irreversible because lost auditory hair cells cannot be spontaneously replaced. Treatment for replacing lost hair cells is currently unavailable.

During mammalian embryogenesis, cochlear hair cells and supporting cells have common cellular precursors[1–3]. Precursor cell differentiation is regulated by several genes; key among them is the gene *Atoh1* (also known as *Math1*), a mouse homolog of the *Drosophila* gene *atonal*, that encodes a basic helix-loop-helix transcription factor. *Atoh1* has been shown to act as a 'pro-hair cell gene' and is required for the differentiation of hair cells from multipotent progenitors[4,5]. Experimental overexpression of *Atoh1* or *ATOH1* (the human *atonal* gene) in nonsensory cells of the normal cochlea generates new hair cells, both *in vitro*[6] and *in vivo*[7]. Here we test the influence of *Atoh1* overexpression on hair cell regeneration and hearing restoration in the mature deaf cochlea.

Young adult guinea pigs with normal hearing were deafened by systemic administration of ototoxic drugs, resulting in complete bilateral hair cell loss in the high- and mid-frequency regions of the cochlea. In animals killed 3 d after the deafening procedure, scanning electron microscopy (SEM) analysis of the organ of Corti surface showed complete absence of hair cells (**Fig. 1a**). At this time point, whole mounts of the organ of Corti stained with phalloidin showed a complete lack of hair cells at the upper (luminal) surface of the epithelium (**Fig. 1b**). Hair cell features such as stereocilia and cuticular plate were absent, whereas pillar cells remained in the tissue (**Fig. 1a**,**b**). In normal cochleae stained with bisbenzimide (Hoechst) and analyzed with fluorescence microscopy, outer hair cell (OHC) nuclei were arranged in three distinct rows (**Fig. 1c**) with supporting cell nuclei similarly arranged, at a lower focal plane (data not shown). OHC nuclei were absent 3 d after deafening (**Fig. 1d**); however, supporting cell nuclei were present at the lower level (data not shown), showing that the original OHCs were eliminated by this deafening protocol. Cochlear whole mounts stained 3 d after the ototoxic insult with antibodies to myosin VIIa, a hair cell–specific marker[8], showed a complete lack of myosin VIIa (data not shown). Auditory brain-stem response (ABR) thresholds in all deafened animals were either unmeasurable or extremely high (>95 dB), indicative of profound deafness. The battery of post-deafening analyses we have used showed absence of stereocilia bundles and apical surfaces of hair cells with SEM, absence of actin-rich hair cell features with phalloidin staining, lack of myosin VIIa staining with immunocytochemistry and lack of nuclei at the OHC plane. These analyses were performed in cochlear turns one through three, and showed that these three turns were consistently devoid of inner hair cells (IHCs) and OHCs by day 3 after the ototoxic insult.

To deliver the *Atoh1* transgene into nonsensory cells in the deafened auditory epithelium, an adenoviral vector was infused into the left cochlea 4 d after the ototoxic lesion. Animals received adenovirus alone (Ad.empty), with a *GFP* cassette (Ad.*GFP*), with *Atoh1* (Ad.*Atoh1*) or with a dual cassette (Ad.*Atoh1-GFP*). To test the pattern of *Atoh1* expression, animals were killed 4 d after Ad.*Atoh1* inoculation and processed for Atoh1 immunocytochemistry. We found numerous Atoh1-positive cells among the nonsensory cells that

[1]Kresge Hearing Research Institute, Department of Otolaryngology, University of Michigan, MSRB 3, Room 9303, 1150 W. Medical Center Drive, Ann Arbor, Michigan 48109-0648, USA. [2]Department of Otolaryngology, Kansai Medical University, 10-15 Fumizono-Cho, Moriguchi, Osaka 570–8506, Japan. [3]Department of Otolaryngology–Head and Neck Surgery, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860–8556, Japan. [4]Department of Vector Sciences, GenVec Inc., 65 W. Watkins Mill Road, Gaithersburg, Maryland 20879-4021, USA. Correspondence should be addressed to Y.R. (yoash@umich.edu).

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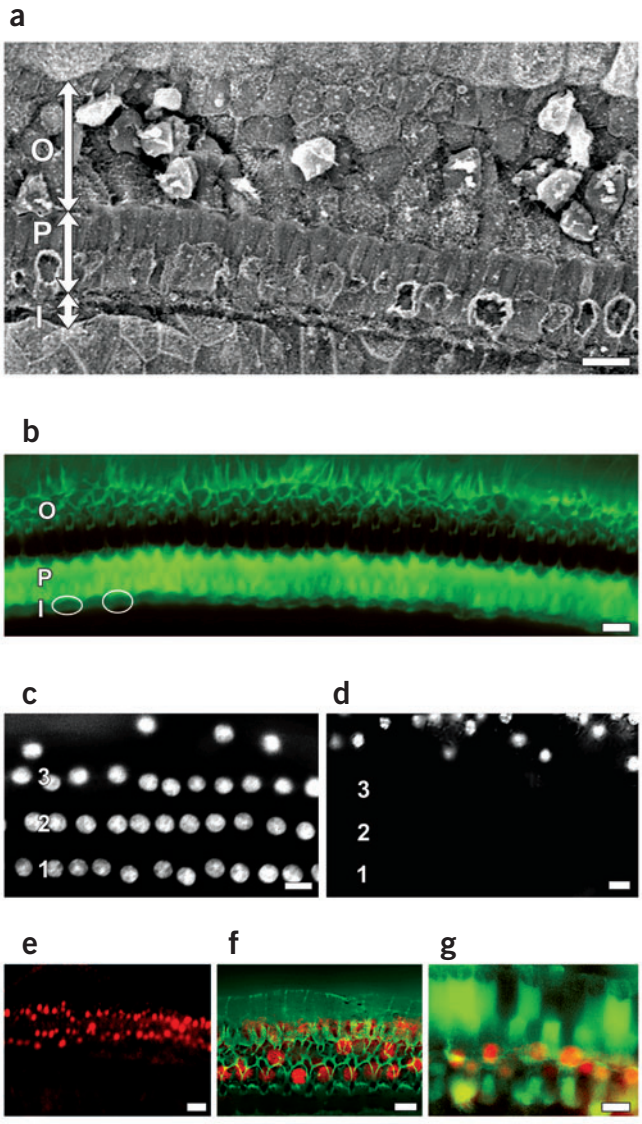

Figure 1 Hair cell elimination and *Atoh1* expression in deaf cochleae. SEM (**a**) or epifluorescence (**b**–**g**) showing ototoxic lesions with complete hair cell loss (**a**,**b** and **d**), nuclei in the organ of Corti (**c**,**d**) and *Atoh1* expression after Ad.*Atoh1* inoculation (**e**–**g**) in the second cochlear turn. (**a**) Complete hair-cell loss was observed 3 d after systemic (bilateral) deafening, with nonsensory cells replacing degenerated hair cells in the IHC (I) and OHC (O) region. Pillar cells (P) survive the insult. (**b**) Distribution of F-actin shows lack of hair cells 3 d after deafening. Ovals encircle representative sites of missing IHCs and O points to cells missing in OHC area. Pillar cells (P) survive the insult. (**c**) In the normal organ of Corti, Hoechst stain depicts nuclei of the three rows of OHCs. (**d**) All OHC nuclei are missing 3 d after an ototoxic insult. (**e**) Antibody to Atoh1 is seen in nuclei of nonsensory cells that replaced hair cells (first turn) 4 d after Ad.*Atoh1* inoculation. (**f**) Atoh1 (red) in nonsensory cells in second cochlear turn, with phalloidin (green) showing actin in cell-cell junctions. (**g**) Atoh1 (red, nuclear stain) and GFP (green, cytoplasmic stain) are coexpressed in nonsensory cells (Deiters and pillar cells) 4 d after Ad.*Atoh1-GFP* inoculation. Additional positive nuclei are present out of the focal plane shown in **e**–**g**. Scale bars, 25 μm in **e** and 10 μm in all other micrographs.

remained in the organ of Corti (**Fig. 1e**,**f**). We observed additional Atoh1-positive nuclei in other focal planes (data not shown). We observed the highest efficiency of *Atoh1* transduction in the first and second cochlear turns, within the normal boundaries of the organ of Corti. We determined that in 2 mm flanking the inoculation site, an average of 865 Atoh1-positive cells were found in the organ of Corti 4 d after Ad.*Atoh1* inoculation (n = 10 ears, s.d. = 196.6). Right (contralateral) ears and left ears inoculated with Ad.empty or Ad.*GFP* were Atoh1-negative (data not shown). Deafened animals killed 4 d after inoculation with Ad.*Atoh1-GFP* showed widespread expression of both genes (**Fig. 1g**). These data show efficient transduction and *Atoh1* transgene expression in nonsensory cells of the deaf cochlea and absence of *Atoh1* expression in control ears.

To determine the effects of *Atoh1* overexpression in deafened ears, ABR thresholds were recorded and animals killed 4, 5 or 8 weeks after inoculation. We prepared cochleae for SEM or whole-mount fluorescence microscopy. Ad.*Atoh1*-treated cochleae showed large numbers of IHCs and OHCs 8 weeks after inoculation (**Fig. 2a**–**c**). All right ears (**Fig. 2d**) and all left ears treated with Ad.empty (**Fig. 2e**) or Ad.*GFP* (not shown) were devoid of hair cells. Higher-magnification SEM analysis showed that the surface morphology of the hair cells in *Atoh1*-treated ears was relatively normal (**Fig. 2f**,**g**) but the supporting cells that separated one hair cell from its neighbor were narrow and not well defined.

Hair cells detected in Ad.*Atoh1*-treated deafened cochleae showed normal surface morphology and orientation in the organ of Corti (**Fig. 2a**,**f**,**g**), suggesting that the positional cues for cellular organization remain in the mature traumatized tissue. Organization of third-row OHCs was poorer than other rows, suggesting that cues for organizing the tissue are closer to the first row of OHCs. Ectopic hair cells found outside the organ of Corti (**Fig. 2a**) were neither well differentiated nor correctly oriented. Based on their location in the organ of Corti and their highly polarized morphology, we speculate that pillar cells provide cues for reorganizing the auditory epithelium.

Hair cells in *Atoh1*-treated ears expressed myosin VIIa, providing further evidence for hair-cell phenotype (**Fig. 3a**). Ad.empty-inoculated ears (data not shown) and contralateral ears (**Fig. 3b**) were negative for myosin VIIa, confirming that all original hair cells have been eliminated. In the normal auditory epithelium there is a 1:1 ratio of OHCs to Deiters cells (supporting cells), with OHC nuclei positioned above the supporting-cell nuclei. In deafened ears, OHC nuclei were absent (**Fig. 1d**) and the total number of nuclei in this area was half that of normal ears. Two months after Ad.*Atoh1* inoculation, nuclei reappeared at a plane above the supporting cells (**Fig. 3c**). An average number of 1,548 nuclei was observed in the same area of *Atoh1*-treated ears, compared to an average of 830 nuclei in contralateral ears (n = 5 each, s.d. = 209 and 51, respectively), a statistically significant increase (P = 0.0006). The increase in nuclei number probably is not a direct result of *Atoh1* expression, because *Atoh1* acts as a differentiation factor. Therefore, we hypothesize that new hair cells recruit nonsensory cells from outside the organ of Corti to migrate and become supporting cells, as described *in vitro*[9]. Supporting cells may also induce proliferation of other nonsensory cells as part of a self-organizational capacity of the regenerating cochlea[9]. The *Atoh1*-induced transdifferentiation from a nonsensory to sensory phenotype can be either direct, with no mitosis, or indirect, with mitosis preceding the appearance of the hair cell features.

The numbers and appearance of hair cells were similar in Ad.*Atoh1* and Ad.*Atoh1-GFP* inoculated ears. Some Ad.*Atoh1-GFP*–inoculated left ears contained hair cells positive for GFP, establishing a direct relationship between transgene expression in surviving nonsensory cells and transdifferentiation into hair cells (**Fig. 3d**). Although adenoviral-mediated gene expression is not expected to last for 2 months, the GFP gene product probably persisted in transduced cells because of slow degradation.

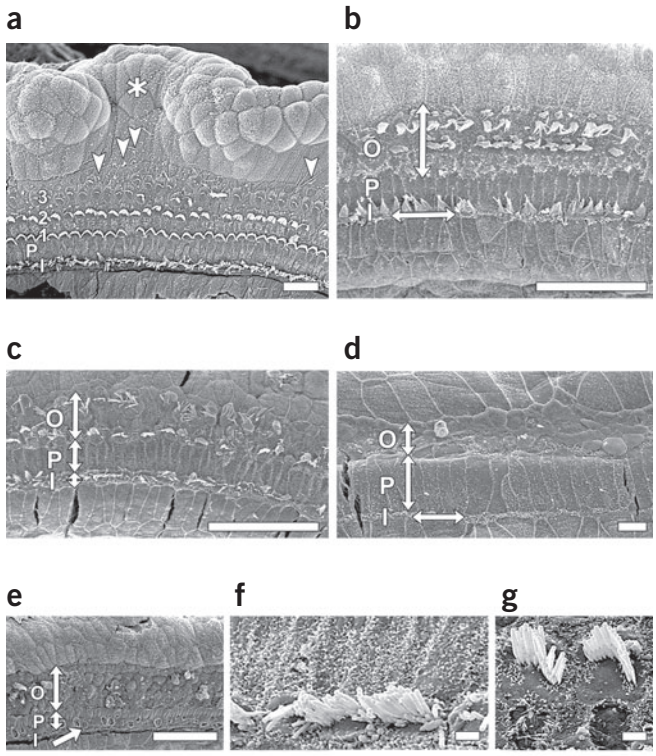

Figure 2 Hair cells reappear in deaf ears treated with Ad.*Atoh1*. SEM view of deafened cochleae 2 months after *Atoh1* inoculation (**a**–**c**), contralateral cochlea (**d**), Ad.empty-inoculated ear (**e**), and higher magnification of IHC (**f**) and OHC (**g**) 2 months after *Atoh1* inoculation. (**a**) The site of inoculation in the second cochlear turn (asterisk) is shown along with numerous stereocilia bundles at the normal sites of IHCs (I) and OHCs (rows 1–3). Pillar cells (P) are present between IHCs and OHCs. Ectopic bundles (arrowheads) are seen lateral to the third row of OHCs. (**b**) In some *Atoh1*-treated ears the morphology of IHC (I) and OHCs (O) is less well differentiated. (**c**) In other *Atoh1*-treated ears, hair cell reappearance is incomplete and third row OHCs are missing. (**d**,**e**) second cochlear turns of right (**d**, contralateral to **a**) or Ad.empty-inoculated left cochlea (**e**), showing complete absence of hair cells. (**f**,**g**) Stereocilia bundle organization is relatively normal in IHCs (**f**) and OHCs (**g**) but supporting cells between neighboring hair cells are narrow and not well defined. Scale bars, 25 μm in **a**; 50 μm in **b**, **c** and **e**; 10 μm in **d** and 5 μm in **f** and **g**.

Phalloidin epifluorescence facilitated identification of hair cells based on the presence of stereocilia (data not shown) and the distribution of actin in the cell border areas and the cuticular plate, and the absence of actin in the centrosomal region (**Fig. 3d**). Quantitative analysis of these phalloidin-stained cochleae 2 months after *Atoh1* treatment showed the number of new hair cells in the 2 mm of the organ of Corti flanking the inoculation site to be 256 IHCs and 691 OHCs ($n = 5$ animals, s.d. = 35 and 159 for IHCs and OHCs, respectively). The number of hair cells in the *Atoh1*-treated ears was significantly greater than contralateral ears, which were devoid of hair cells ($P < 0.0006$). The restoration of hair cells in *Atoh1*-treated ears was best near the site of inoculation (**Figs. 2a** and **3a**,**d**). These data show that many new hair cells appear in the 8 weeks after *Atoh1* expression in deafened mature cochleae.

Cross-sections of *Atoh1*-treated cochleae showed that some cells in the OHC area displayed a mixed phenotype with features of both OHC and supporting cell. These mixed-phenotype cells showed a luminal projection similar to stereocilia on their apical surface and a prominent cuticular plate, yet spanned the distance from the luminal surface to the basement membrane, suggesting that they also retained several supporting-cell features (**Fig. 3e**). In some sections, two layers of nuclei were found above the basement membrane (**Fig. 3f**), in agreement with data obtained with Hoechst staining in whole mounts (**Fig. 3c**). IHC morphology appeared relatively normal (**Fig. 3g**). The presence of cells with a mixed phenotype suggests that, at least in some cases, the process of transdifferentiation can occur directly, without a preceding step of de-differentiation or mitosis. The increase in the number of nuclei compared to the contralateral ears may be a result of mitosis or of migration from adjacent regions in the epithelium.

We analyzed Ad.*Atoh1*-treated cochleae at intermediate time points between deafening and 2 months. Cochleae analyzed with SEM 4 weeks after Ad.*Atoh1* inoculation showed numerous bundles of very tall stereocilia that resembled the apical surfaces of immature hair cells (**Fig. 4a**). The high density of immature bundles observed at 4 weeks corroborates the high efficiency of *Atoh1* transduction shown at day 4. In the contralateral cochleae there were no surviving hair cells and no immature bundles, and the area where OHCs used to reside often appeared narrow and greatly diminished (**Fig. 4b**). The pattern of myosin VIIa distribution in *Atoh1*-treated cochlea analyzed 5 weeks after the inoculation of Ad.*Atoh1* delineated both IHCs and OHCs (**Fig. 4c**). The myosin VIIa–positive cells in the OHC area were rather disorganized, in agreement with the SEM image obtained 1 week earlier (**Fig. 4a**). Contralateral ears were negative for myosin VIIa (**Fig. 4d**).

To test the influence of *Atoh1* treatment on hearing, we measured ABR thresholds at 4, 8 and 10 weeks after Ad.*Atoh1* inoculation. Measurements at 4 weeks indicated profound deafness with thresholds high or unmeasurable, similar to post-deafening thresholds (not shown). These data are consistent with the absence of mature hair cells at 4 weeks after *Atoh1* treatment (**Fig. 4a**). At 8 weeks, the average ABR thresholds in a group of five Ad.*Atoh1*-treated ears was lower (better) than contralateral ears at all frequencies, and ABR waveforms contained the normal four to five vertex-positive peaks (**Fig. 5**). The considerable improvement in thresholds (in some cases to near baseline) at the high frequency region of the guinea pig cochlea (4–24 kHz) is in agreement with the restoration of IHC morphology in this area[10]. Nevertheless, in the absence of a normal OHC population, frequency selectivity is unlikely to be normal[11]. When present, the thresholds recorded in the contralateral (right) ears probably represent hearing from *Atoh1*-treated left ears and reflect interaural crossover[12]. ABR thresholds measured in four animals at 10 weeks after Ad.*Atoh1* inoculation appeared similar to their 8-week thresholds (data not shown) suggesting that functional recovery is stable, at least up to 10 weeks. In Ad.empty-treated ears, no thresholds could be recorded (data not shown). These data provide the first demonstration of a therapeutic approach leading to substantial recovery of hearing in deaf mammalian ears.

Our results show generation of new hair cells and improvement of hearing in deaf animals treated with *Atoh1*. New OHCs are incompletely differentiated and unlikely to provide the functions of the active cochlear amplifier. In contrast, regenerated IHCs seem normal and the restoration of thresholds attests to their functionality. We show that differentiated cells (nonsensory cells of the auditory epithelium) can be induced to alter their phenotype by expression of the developmental gene, *Atoh1*. Together with loss-of-function and *in vitro* experiments[4,5,9,13], our data suggest that *Atoh1* is a master regulatory gene that is both necessary and sufficient for producing hair cells in the mammalian cochlea. The competence of mature and differentiated nonsensory cells to respond to *Atoh1* is notable because the structural and functional changes that accompany differentiation of mammalian cells are usually irreversible. Our data, therefore, sug-

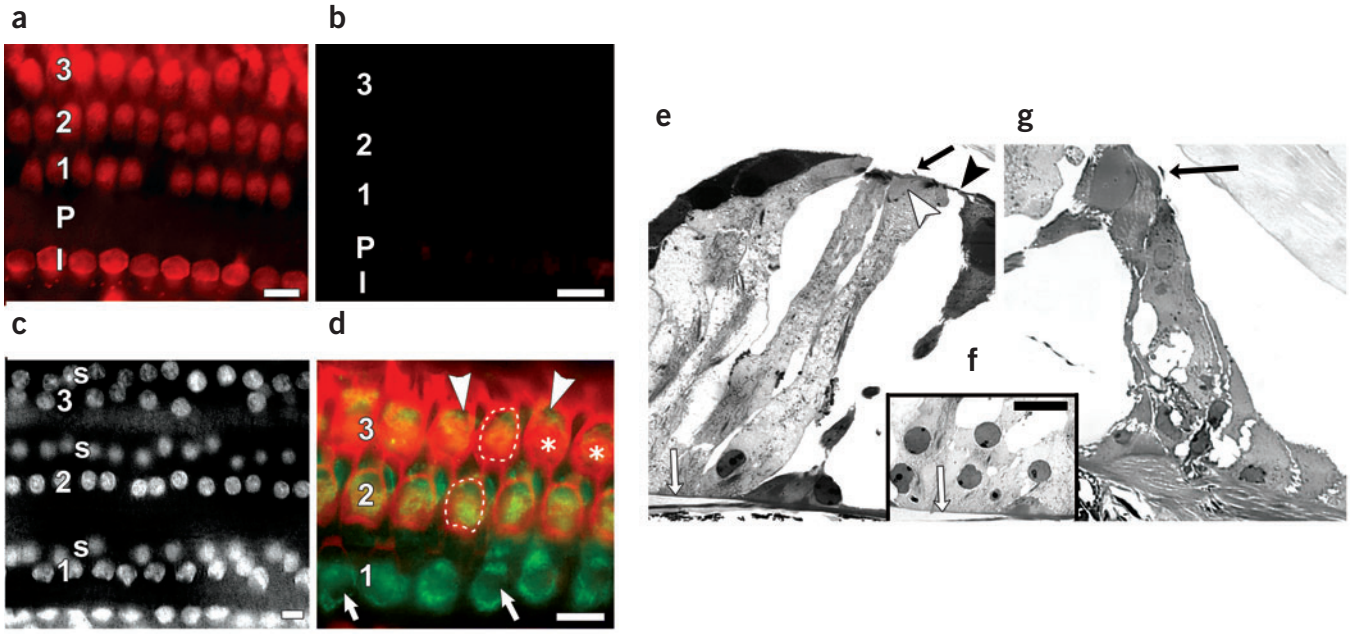

Figure 3 New hair cells and nuclei in the deafened cochleae inoculated with Ad.*Atoh1*. *Atoh1*-treated ears (**a** and **c**–**g**) and contralateral cochlea (**b**) processed 2 months later for myosin VIIa immunocytochemistry (**a**,**b**), Hoechst stain (**c**), epifluorescence for actin and GFP (**d**) or plastic sections (**e**–**g**). (**a**) Pillar cells (P) are flanked by IHCs (I) and OHCs (rows 1–3) positive for myosin VIIa in the second cochlear turn. (**b**) Lack of myosin VIIa–positive hair cells in the contralateral (right) ear. (**c**) Nuclei are present in rows at the supporting cell focal plane (S), and above, closer to the OHC focal plane (rows 1–3). (**d**) Two months after inoculation with Ad.*Atoh1-GFP*, actin (red), is seen in junctional complexes delineating the contour of OHCs (dotted circles) and in cuticular plates (asterisks), but not in the area of the centrosomes which is actin-free (arrowheads). GFP (green, cytoplasmic) is seen in all three rows of OHCs (rows 1–3). First row OHCs are shown in focal plane beneath apical junctions. Nuclei visible in this focal plane are unstained for GFP (arrows). (**e**) A cell in the OHC area of the second turn has a cuticular plate (white arrowhead) and a lumenal projection (black arrow) protruding from the cuticular plate. The cell body extends from the lumenal surface (black arrowhead) to the basement membrane (white arrow), and the nucleus is basal. (**f**) In some regions of OHC area a dual layer of nuclei can be seen above the basement membrane (arrow). (**g**) An IHC featuring a luminal projection (arrow) and normal morphology. Scale bars, 10 μm in **a**, **b**, and **d**–**g**, and 5 μm in **c**.

gest that re-expression of developmental regulatory genes in mature tissues is a potential strategy for cell replacement therapy in the cochlea and elsewhere.

The increase in number of nuclei in the sensory epithelium after *Atoh1* treatment can be explained either by cell migration from areas flanking the organ of Corti, as seen in birds[14], or by a proliferative response of nonsensory cells that occurs secondary to the generation of new hair cells. *Atoh1* by itself is unlikely to induce mitosis[13]. The cochlea may also have a population of unidentified stem cells that divide after deafening, a possibility suggested by recent findings in the utricle[15]. It is unknown whether specific subpopulations of supporting cells are capable of (i) generating new hair cells following *Atoh1* expression (ii) migrating into the organ of Corti and/or (iii) dividing and repopulating the epithelium.

The *Atoh1*-induced restoration of the luminal surface of the auditory epithelium generates both hair cell and supporting cell-like areas which, in some cases, resemble the normal cellular mosaic in the organ of Corti. We speculate that dual-phenotype cells contribute in part to the generation of areas with surface features of supporting cells. Newly added cells that derive from proliferation or migration probably also contribute to the restoration of the surface morphology.

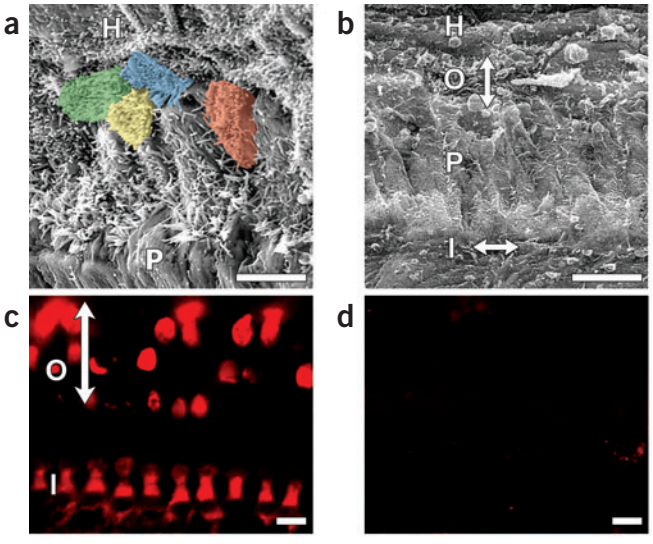

Figure 4 Cochleae analyzed with SEM at 4 weeks after *Atoh1* treatment (**a**) or myosin VIIa immunocytochemistry at 5 weeks after treatment (**c**) and the respective contralateral ears (**b** and **d**). (**a**) Several immature stereocilia bundles (some colored to highlight the morphology) are observed lateral to the pillar cells (P). The OHC region is wide and the tall surface projections cover much of the surface. (**b**) In the contralateral cochlea the distance between pillar cells (P) and Hensen cells (H) where OHCs had resided before deafening (O, vertical double-arrow) is greatly diminished. Neither OHCs nor IHCs (I and horizontal double-arrow) are present. (**c**) In a *Atoh1*-treated ear, a well-organized single row of IHCs and relatively disorganized rows of OHCs are seen with cytoplasmic staining of myosin VIIa in the second turn. (**d**) Myosin VIIa–positive cells are absent in contralateral ear. Scale bars, 5 μm in **a** and **b** and 10 μm in **c** and **d**.

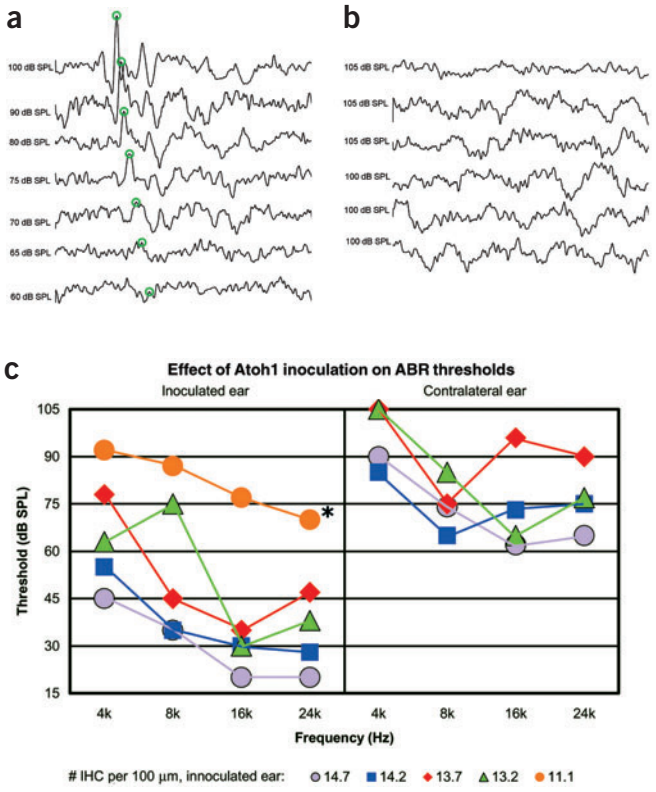

Figure 5 *Atoh1* treatment improves ABR thresholds. (**a**) ABR waves for Ad.*Atoh1*-treated ear represented in **c** by orange circle with asterisk. Waves show marked peaks in response to input as low as 65 dB sound pressure level (SPL). The waveform shows that the latency of the marked peak increases with decreasing level of the stimulus, as expected in normal ABR response[20]. (**b**) In right (contralateral) ear no consistent peaks are seen. (**c**) Data points for thresholds at each frequency for the left (*Atoh1*-inoculated) and right (contralateral) ears of five animals. ABRs could not be obtained from the right ear of the orange animal at the limits of the sound delivery system (105 dB SPL). Average dB SPL thresholds in treated ears are significantly lower (better) than in contralateral ears at each frequency ($P < 0.004$). The number of IHCs per 100 µm is given for each animal. Thresholds are significantly correlated with the number of IHCs in *Atoh1*-treated ears. The weakest correlation was at 4 kHz ($r^2 = 0.78$, $P < 0.05$).

The organization of the tissue is likely to depend on the ability of hair cells to dictate the phenotype of their neighbors and to attract supporting cells, as shown *in vitro*[9]. The ability of the tissue to respond to *Atoh1* overexpression and to reorganize the new hair cells generated by *Atoh1* may depend on the mechanism of deafening, the timing of treatment after deafening and the degree of differentiation of the surviving nonsensory cells. Better understanding of the response of supporting cells to *Atoh1* expression will also shed light on the process of post-trauma repair of the mosaic-like cytoarchitecture at the luminal surface of the auditory epithelium.

A clinical restoration of threshold sensitivity similar to that shown here in guinea pigs would be an extremely attractive therapeutic outcome for patients with profound deafness. Addition of new hair cells may also enhance the outcome of cochlear implantation, because presence of some hair cells improves implant function[16]. Our findings support the feasibility of genetic manipulation for cell replacement therapies based on inducing transdifferentiation of endogenous cells in the inner ear and in other systems.

METHODS

Animals. All animal experiments were approved by the University of Michigan Institutional Committee on the Use and Care of Animals and performed using accepted veterinary standards. We used young adult guinea pigs (Elm Hill Breeding Laboratory) weighing 250–400 g with normal ABR baseline thresholds of 15–50 dB sound pressure level (SPL). Animal groups were Ad.*Atoh1* ($n = 30$), Ad.empty ($n = 12$), Ad.*Atoh1-GFP* ($n = 5$), Ad.*GFP* ($n = 5$) and deafening alone ($n = 4$). Inner ears were analyzed using epifluorescence, SEM or plastic cross-sections of the organ of Corti. Animals that did not have a complete loss of hair cells in the three lower cochlear turns, as determined by observing the right (contralateral) ear, were excluded from the study.

Deafening surgery and viral inoculation. Guinea pigs were deafened bilaterally with a single systemic dose of kanamycin (500 mg/kg, subcutaneous) followed 2 h later by ethacrynic acid (50 mg/kg, intravenous). We verified deafening by measuring ABR thresholds. This ototoxic drug regimen is designed to consistently eliminate hair cells but spare the pillar cells. Hair-cell elimination is complete in the first three cochlear turns, although a few hair cells may survive in the apical turn. We excluded animals that did not show a threshold greater than 95 dB SPL (numbers of animals given above do not include excluded animals). Viral vectors were inoculated into the left cochlea 4 d after the deafening surgery, using a procedure previously described[17], except that the inoculation was into the second cochlear turn.

Adenovirus vector. Replication-deficient recombinant adenoviruses with deleted E1, E3, and E4 regions[18] were Ad.*Atoh1*, Ad.*Atoh1-GFP*, Ad.empty and Ad.*GFP*. The *Atoh1* cDNA was obtained from H. Zoghbi (Baylor College of Medicine). The insert in Ad.*Atoh1* was driven by the human cytomegalovirus promoter and the GFP by the chicken beta-actin promoter. We used undiluted vectors at a concentration of 1×10^{12} total particles purified virus per milliliter. Viral suspensions were kept at –80 °C until thawed for use.

ABR measurement. To assess auditory thresholds, we recorded ABRs at 4, 8, 16, and 24 kHz (tone bursts, 15 ms duration, 1 ms $\cos^2$-shaped rise-fall times) as previously described[19]. Measured frequencies correspond to the basal and lower second turn of the guinea pig cochlea, close to the site of inoculation. When present, the thresholds recorded in the contralateral (right) ears probably represent hearing from *Atoh1*-treated left ears. For this reason, an animal with lesser threshold recovery in the left ear was selected for showing ABR waveforms in the contralateral ear in **Fig. 5**. Lower frequency thresholds are not reliably measurable with ABR audiometry[20]. The researcher who measured ABRs was blinded to the identity of the animal.

Immunocytochemistry and SEM. We stained whole mounts of the auditory epithelium with antibodies to myosin VIIa or Atoh1 as described[7], except that tissues were double stained with phalloidin (Molecular Probes) to stain actin[21]. To augment GFP fluorescence, we stained tissues with a GFP-specific rabbit antibody (Chemicon, diluted 1:500). Antibody to myosin VIIa was purchased from the University of California San Diego. Antibody to Atoh1 was purchased from the Tissue Culture Hybridoma Core at the University of Iowa. To stain nuclei, we used the DNA-specific label Hoechst as previously described[22]. The specimens were examined and photographed using a Leica DMRB epifluorescence microscope (Leica) with a CCD Cooled SPOT-RT digital camera (Diagnostic Instruments). For SEM, samples were prepared as described[23]. The samples were mounted on stubs using silver paste and photographed digitally using a Philips XL30 Field Emission Gun SEM (FEI).

Plastic sections. We decalcified cochleae, embedded them in Epon as described[24] and sectioned (1 µm) them with a glass knife. Sections were photographed using a Leica DMRB epifluorescence microscope and a 100× objective lens.

Data analysis. We performed the statistical analysis of the ABR data using Excel. Conventional t-tests were performed to determine the significance of the difference between treated and nontreated ears at each tested frequency. Because we performed multiple tests, we applied sequential Bonferroni adjustment

to keep the table-wide error rate below 0.05. ABR thresholds for each animal were regressed on the number of IHC to obtain the correlation for each tested frequency. A *t*-test was performed on the 4 kHz data to determine whether the correlation was significant (slope > 0).

Evaluation of the morphology of *Atoh1*-inoculated ears 8 weeks after the inoculation showed three animals that were completely devoid of hair cells. These animals were assumed to have had failed inoculation surgeries and were excluded from these statistical analyses.

We performed quantitative analysis of whole mounts of the organ of Corti to determine the number of hair cells (phalloidin-stained cochleae) or positively stained nuclei (Atoh1 or Hoechst stains). Nuclei counts in Hoechst-stained whole mounts were performed in the OHC area, where nuclei of hair and supporting cells can be distinguished from each other based on their organization in distinct focal planes. We counted a segment of 1 mm on each side of the inoculation site for a total of 2 mm. Conventional *t*-tests were performed to determine the significance of differences between treated and nontreated cochleae.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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