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Research Report

Reprogramming with *Atoh1*, *Gfi1*, and *Pou4f3* promotes hair cell regeneration in the adult organ of Corti

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Abstract

Cochlear hair cells can be killed by loud noises, ototoxic drugs, and natural aging. Once lost, mammalian hair cells do not naturally regenerate, leading to permanent hearing loss. Since the mammalian cochlea lacks any intrinsic ability to regenerate, genetic reprogramming of cochlear supporting cells that lie adjacent to hair cells is a potential option for hearing restoration therapies. We targeted cochlear supporting cells with three hair cell transcription factors: *Atoh1*, or *Atoh1* + *Gfi1*, or *Atoh1* + *Gfi1* + *Pou4f3* and found that 1- and 2-factor reprogramming is not sufficient to reprogram adult supporting cells into hair cells. However, activation of all three hair cell transcription factors reprogrammed some adult supporting cells into hair cell-like cells. We found that killing endogenous hair cells significantly improved the ability of supporting cells to be reprogrammed and regenerated numerous hair cell-like cells throughout the length of the cochlea. These regenerated hair cell-like cells expressed myosin VIIa and parvalbumin, as well as the mature outer hair cell protein prestin, were innervated, expressed proteins associated with ribbon synapses, and formed rudimentary stereociliary bundles. Finally, we demonstrate that supporting cells remained responsive to transcription factor reprogramming for at least 6 weeks after hair cell damage, suggesting that hair cell reprogramming may be effective in the chronically deafened cochlea.

Keywords: inner ear, reprogramming, hair cells

Significance Statement

Hearing loss results from the loss of sensory hair cells due to aging, noise or ototoxic insults. Mammals are unable to naturally regenerate lost hair cells and there are currently no regenerative therapies for hearing loss. Here we show that cochlear supporting cells can be reprogrammed into hair cell-like cells after death of endogenous hair cells by ectopic expression of *Atoh1*, *Gfi1*, and *Pou4f3*. We suggest that supporting cells of the organ of Corti are a potential target for reprogramming as a means of hearing restoration.

Introduction

Hair cells in the mammalian organ of Corti detect and transduce auditory stimuli into electrochemical signals that are interpreted by the brain as sound. There are two types of cochlear hair cells that each has a unique function: inner hair cells detect sound and convey this information to the central nervous system through their afferent innervation, while outer hair cells contribute to amplification of auditory input and tuning of the basilar membrane on which the organ of Corti sits. Loss of either inner hair cells or outer hair cells results in hearing deficits, with substantial depletion leading to a complete loss of function. Environmental insults, such as loud noises (1, 2), ototoxic drugs (3–6), and aging (7) can result in the loss of hair cells, with outer hair cells being more susceptible than inner hair cells. Unlike nonmammalian vertebrates, mammals cannot naturally replace lost hair cells by cellular regeneration (8–10) and the only current treatments for hair cell loss are hearing aids or cochlear implants.

Nonmammalian vertebrates such as birds and fish can regenerate lost hair cells by mobilizing supporting cells that lie next to hair cells. After hair cells die, nonmammalian supporting cells rapidly divide, differentiate into hair cells, and restore hearing or balance organs of the ear to a functional state (8). Mammalian cochlear supporting cells retain a very limited capacity for hair cell regeneration in young animals, but this regenerative ability is rapidly lost before the onset of hearing (11, 12). Therefore, strategies to genetically reprogram supporting cells into hair cells in mature mammals that have lost the capacity to regenerate may provide a therapeutic alternative for hearing restoration.

Atoh1 is a transcription factor that is both necessary and sufficient for the differentiation of developing hair cells (13).

Competing Interest: The authors declare no competing interests.
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Overexpression of *Atoh1* can reprogram supporting cells and nonsensory cells of the cochlea into hair cells, however reprogramming of supporting cells with *Atoh1* is extremely inefficient at all ages (14–23), and the efficiency of *Atoh1*-mediated reprogramming of nonsensory cells on either side of the organ of Corti drops rapidly after birth (16, 24). Co-expression of *Atoh1* with a second critical hair cell transcription factor, *Pou4f3*, was reported to enhance the reprogramming of supporting cells into hair cells (15); however, these reprogrammed hair cells were morphologically abnormal and lacked organized stereocilia. In addition to *Atoh1* and *Pou4f3*, *Gfi1* has been identified as a critical transcription factor in the development of all cochlear hair cells (25, 26). It acts in conjunction with *Atoh1* to promote the expression of hair cell genes, and represses neuronal and other fates, thereby promoting hair cell identity (25). Adenoviral co-expression of *Atoh1* and *Gfi1* in deafened adult mice increased the production of regenerated hair cells compared with *Atoh1* alone (19). Additionally, the combined expression of *Atoh1*, *Gfi1*, and *Pou4f3* reprograms ES cells (27), chick epithelial cells (27), fibroblasts (28), and immature cochlear cells into hair cell-like cells (16, 29). We also recently showed that the expression of *Atoh1*, *Gfi1*, and *Pou4f3* in nonsensory cells of the neonatal and mature cochlea reprograms these cells into hair cell-like cells by activating hair cell networks in nonhair cells (16, 24).

To investigate the feasibility of transcription factor reprogramming in promoting hair cell regeneration from adult cochlear supporting cells, we used transgenic mice to overexpress (i) *Atoh1*, (ii) *Atoh1* + *Gfi1*, or (iii) *Atoh1* + *Gfi1* + *Pou4f3* in supporting cells of the intact cochlea or in supporting cells from cochleas in which all hair cells were killed. We found that *Atoh1* alone or in combination with *Gfi1* was unable to reprogram supporting cells in the intact cochlea, but combined expression of *Atoh1*, *Gfi1*, and *Pou4f3* reprogrammed a small number of supporting cells into hair cells. However, when endogenous hair cells were killed, the co-expression of *Atoh1*, *Gfi1*, and *Pou4f3* reprogrammed significantly more supporting cells into hair cell-like cells. The regenerated hair cells were located throughout the length of the cochlea and displayed stereocilia bundles, ribbon synapses, and were innervated by afferent inputs.

Since mature mammals cannot replace cochlear hair cells, the organ of Corti responds to hair cell loss by the surrounding supporting cells clearing hair cell debris and remodeling their apical surfaces to reestablish the integrity of the surface of the organ of Corti (30). Very little is known about the short- and long-term genetic and epigenetic changes that occur in mammalian cochlear supporting cells after hair cell loss. A recent study showed that hair cell loci may become somewhat more accessible in cochlear supporting cells weeks after hair cell loss (31), suggesting that supporting cells might be amenable to reprogramming weeks after hair cell loss. In agreement with this, we found that supporting cells are also responsive to reprogramming for at least 6 weeks after hair cells were killed. Our results show that new hair cells can be produced in the acutely and chronically deafened organ of Corti by TF reprogramming of supporting cells.

Results

Co-expression of *Atoh1*, *Gfi1*, and *Pou4f3*, reprograms mature cochlear supporting cells into hair cells

We previously showed that nonsensory cells on either side of the mature organ of Corti (the inner and outer sulci and spiral limbus) can be reprogrammed to hair cells by co-expression of *Atoh1*, *Gfi1*, and *Pou4f3* (24). However, this study did not specifically target supporting cells. To understand the ability of supporting cells to activate hair cell gene networks, we targeted them with reprogramming factors using the *Lfng-Cre*^{ERT2} BAC transgenic mouse line (*Lfng-Cre*^{ERT2}; (32)), which is expressed specifically in organ of Corti supporting cells in the first postnatal week (33). To confirm this transgene is still active in adult supporting cells, we bred *Lfng-Cre*^{ERT2} mice with *Rosa26*$^{loxP\text{-}stop\_loxP\_tdTomato}$ mice (hereafter referred to as *Rosa26*^{tdTomato}; also called *Ai9* (34)); and induced recombination at 3 weeks of age by administration of tamoxifen (TM). We collected the cochlea at 5 weeks of age and quantified the number of tdTOMATO/SOX2-double positive cells in the organ of Corti. In control cochleas that did not receive TM, almost no supporting cells were labeled with tdTOMATO (3.4 ± 2.3% of inner phalangeal and border cells, 0.0 ± 0.0% of inner pillar cells, 0.6 ± 1.1% of outer pillar cells, 0.5 ± 0.3% of Deiters' cells, and 0.0 ± 0.0% of Hensen's cells; Fig. S1). Following TM-induced recombination, we found a significant increase in tdTOMATO-positive supporting cells, where 41.0 ± 14.1% of inner phalangeal and border cells ($P = 0.0013$), 29.7 ± 22.9% inner pillar cells ($P = 0.0090$), 34.1 ± 21.7% outer pillar cells ($P = 0.0037$), and 47.9 ± 19.7% Deiters' cells ($P = 0.0001$) were labeled with tdTOMATO following recombination. As expected from previous reports of Lfng expression the cochlea, Hensen's cells remained unlabeled (0.0 ± 0.0%, $P \geq 0.9999$; Figs. 1A and S1, $N = 3$ control and 3 TM induced; $P < 0.0001$ as determined by a two-way ANOVA with a Tukey's multiple comparisons test). No tdTomato-positive hair cells were observed in any mice.

We observed a pattern of inheritance suggesting that the *Lfng-Cre*^{ERT2} allele was X-linked. When the *Lfng-Cre*^{ERT2}-positive breeding animal was male, all female progeny were positive for *Lfng-Cre*^{ERT2} and all male progeny were negative. However, when the *Lfng-Cre*^{ERT2}-positive breeding animal was female, ~50% of both male and female progeny were *Lfng-Cre*^{ERT2}-positive. This could lead to increased variability in CreER activity where in female animals there is a 50% chance that any cell will silence the *Lfng-Cre*^{ERT2}-positive allele by X inactivation. We consistently observed variability of tdTOMATO expression when activated by *Lfng-Cre*^{ERT2}. Based on quantification of these samples, we observed a range of expression where some samples had low expression (less than ~25% of supporting cells labeled), average expression (between ~25 and 50% of supporting cells labeled), and high expression (more than 50% of supporting cells labeled; Fig. S1). We elected to exclude samples that had low expression (~25% or fewer labeled supporting cells) of tdTOMATO (Fig. S1) in all subsequent analyses.

Previously, *Atoh1* was shown to reprogram supporting cells into hair cells in the immature cochlea as well as other nonsensory cells in the mature ear at low efficiency (22, 35). Therefore, we first asked whether expression of *Atoh1* alone could reprogram supporting cells in the mature organ of Corti into hair cell-like cells. We bred *Lfng-Cre*^{ERT2} mice to Rosa26$^{loxp\text{-}stop\text{-}loxp\text{-}Atoh1}$ (Rosa-A (16)); mice and gave TM to activate *Atoh1* in Lfng-positive supporting cells on 2 consecutive days at 3 weeks of age. Cochleas were collected and analyzed at 4, 5, or 6 weeks of age (Fig. 1A). The tissue was stained with anti-MYO7A and anti-SOX2 antibodies to detect hair cells and supporting cells respectively. Because hair cells of the mature cochlea occur in well-defined rows (1 row of inner hair cells and 3 rows of outer hair cells), we were able to quantify the number of ectopic hair cells. We found no ectopic inner or outer hair cells following *Atoh1* expression among supporting cells (0.0 ± 0.0, $N = 5$ at 4 weeks; 0.4 ± 0.9, $N = 5$ at 5 weeks; 0.0 ± 0.0 $N = 3$

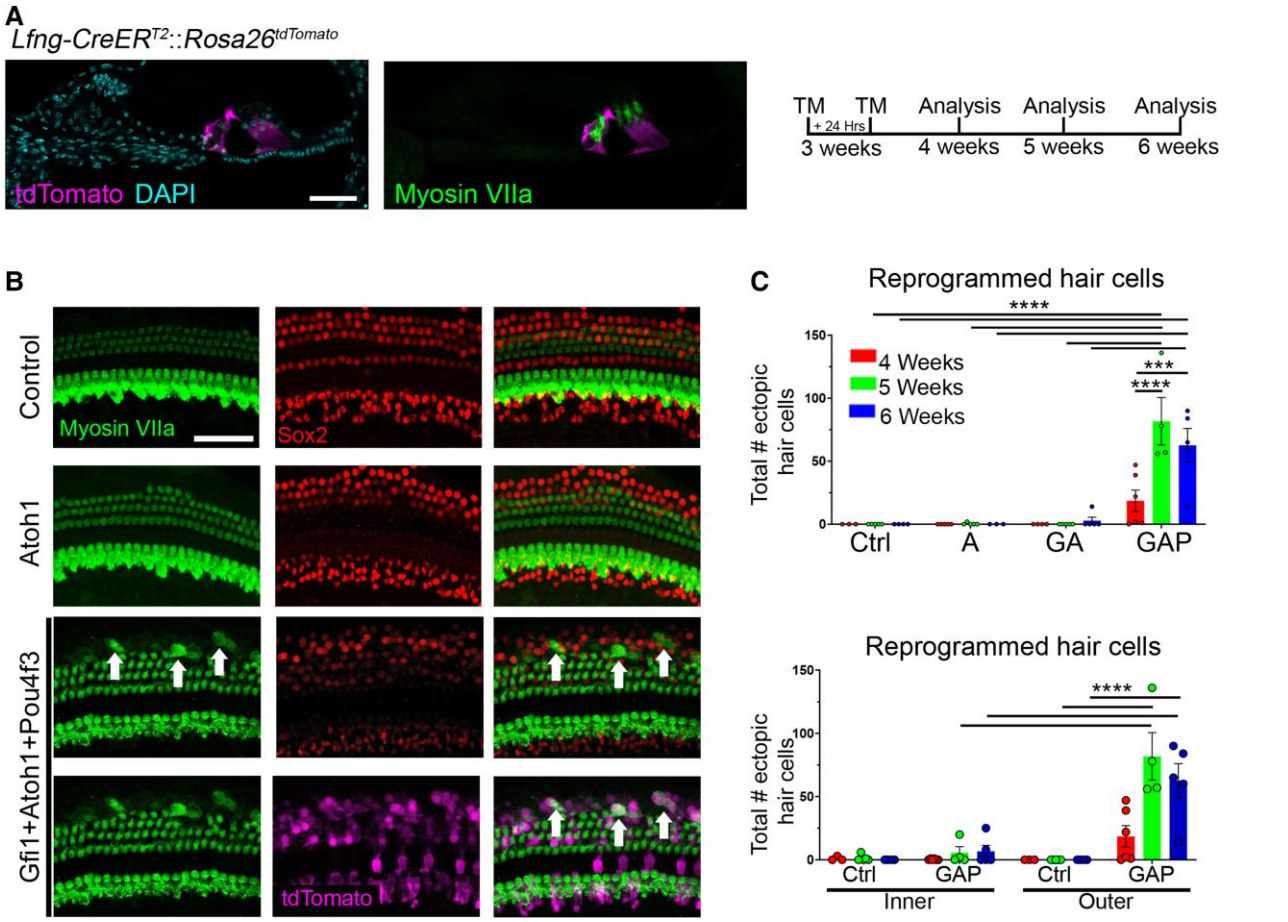

Fig. 1. Combined *Atoh1*, *Gfi1*, and *Pou4f3* reprogram supporting cells into hair cell-like cells in the mature cochlea. A) Recombination in *Lfng-Cre*^{ERT2}::*Rosa26*^{tdTomato} in the mature organ of Corti following TM induction. tdTOMATO expression is observed in supporting cells adjacent to hair cells. CreER-mediated recombination was activated at 3 weeks of age with 2 TM injections (9 mg/40 g) 24 h apart. Samples were collected at 4, 5, or 6 weeks of age; the image is from a sample at 5 weeks of age. TM B) Myosin VIIA positive hair cells and SOX2-positive supporting cells are observed in the cochlear epithelium. Reprogrammed hair cells were observed in the cochlea after *Atoh1*, *Gfi1*, and *Pou4f3* misexpression (arrows) but not in cochleas after *Atoh1* misexpression alone. Arrows indicate reprogrammed cells, scale bar = 50 µm. C) Quantification of ectopic hair cells at 4, 5, or 6 weeks of age in control cochlea as well as reprogramming with either *Atoh1*, *Gfi1* + *Atoh1*, or *Gfi1* + *Atoh1* + *Pou4f3*. Significantly more reprogrammed hair cells are observed with *Gfi1* + *Atoh1* + *Pou4f3* reprogramming compared with *Atoh1* or *Gfi1* + *Atoh1*. In addition, significantly more reprogrammed cells are observed in the outer hair cell region compared with the inner hair cell region and more regenerated hair cells were observed at 5 and 6 weeks of age compared with 4 weeks. Ctrl = control, A = *Atoh1*, GA = *Gfi1* + *Atoh1*, GAP = *Gfi1* + *Atoh1* + *Pou4f3*. Significance indicated by asterisks ****$P < 0.0001$.

at 6 weeks) compared with controls (1.0 ± 1.7, $N = 3$ at 4 weeks; 1.4 ± 2.6, $N = 5$ at 5 weeks; 0.0 ± 0.0 $N = 3$ at 6 weeks $P > 0.9999$ as determined by a 2-way ANOVA with a Tukey's multiple comparison's test), indicating that Atoh1 alone is insufficient to reprogram cochlear supporting cells in 3-week-old mice (Fig. 1B and C).

We next asked whether the co-activation of *Atoh1* and *Gfi1* was sufficient to reprogram supporting cells into hair cells in the mature mouse cochlea. We bred *Lfng-Cre*^{ERT2} mice to *Rosa26*$^{loxp\text{-}stop\text{-}loxp\text{-}Gfi1\text{-}Atoh1}$ (Rosa-GA (16)); mice and injected TM (9 mg/40 g) on 2 consecutive days at 3 weeks of age. We analyzed the tissue at 4, 5, or 6 weeks of age and again found no evidence for ectopic hair cells in the mature inner ear (1.0 ± 1.7 ectopic hair cells at 4 weeks $N = 3$, 1.4 ± 2.6 at 5 weeks $N = 5$, 0.0 ± 0.0 at 6 weeks $N = 5$, as determined by a 2-way ANOVA followed by a Tukey's multiple comparisons test) indicating that *Atoh1* + *Gfi1* combined are insufficient to reprogram cochlear supporting cells into hair cells (Fig. 1C).

We previously showed that the expression of *Atoh1*, *Gfi1*, and *Pou4f3* reprograms significantly more mature nonsensory cells into hair cell-like cells than *Atoh1* alone (24). Therefore, we next asked whether mature supporting cells are responsive to reprograming at 3 weeks of age with combined *Atoh1*, *Gfi1*, and *Pou4f3* expression. We observed ectopic myosin VIIa positive cells along the lateral edge of the organ of Corti at 5 and 6 weeks of age, but not at 4 weeks of age (18.8 ± 20.7, $N = 6$, P = n.s. vs. control at 4 weeks; 87.3 ± 39.1, $N = 4$, $P < 0.0001$ vs. control at 5 weeks; 69.2 ± 37.0, $N = 5$, $P \leq 0.0001$ vs. control at 6 weeks; as determined by a 2-way ANOVA with a Tukey's multiple comparison test). To confirm that these cells were in fact reprogrammed cells, we included the Rosa26^{tdTomato} reporter in our breeding scheme to lineage trace ectopic cells. Ectopic myosin VIIa-positive cells expressed both SOX2 as well as tdTOMATO indicating that these cells originated as supporting cells and were reprogrammed into immature hair cell-like cells following the expression of *Atoh1*, *Gfi1*, and *Pou4f3* (Fig. 1B and C).

Supporting cells can be more efficiently reprogrammed after hair cells are killed

Although we were able to generate hair cells using combined expression of *Atoh1*, *Gfi1*, and *Pou4f3*, we only observed

reprogrammed hair cell-like cells on the lateral aspect of the organ of Corti (Fig. 1B). This suggested that only some of the supporting cells could respond to reprogramming while the majority of supporting cells were still refractory to the reprograming factors. Cell fate in the developing organ of Corti is regulated in part by cell–cell signaling from hair cells preventing the activation of hair cell gene networks in neighboring supporting cells (36). Therefore, it is possible that hair cells still exert control over supporting cell identity in the mature cochlea and may prevent activation of hair cell networks following *Atoh1*, *Gfi1*, and *Pou4f3* misexpression in supporting cells. We next asked whether the presence of the original hair cells was preventing supporting cells from being reprogrammed efficiently. We killed hair cells in the mature cochlea using the *Pou4f3*^{DTR} mouse line (37), which has been reported to kill hair cells with the degree of death varying with the background strain of the mouse (38). We also observed variability in the elimination of cochlear hair cells following the injection of diphtheria toxin (DT), however we excluded animals with less than ∼90% hair cell loss, determined by visual inspection. We first asked whether the activation of *Atoh1* alone in supporting cells following hair cell loss was sufficient to reprogram them into hair cell-like cells. We injected *Lfng-Cre*^{ERT2}::*Rosa26*^{tdTomato}::*Pou4f3*^{DTR} control mice and *Lfng-Cre*^{ERT2}::*Rosa26*^{tdTomato}::*Rosa26-A*::*Pou4f3*^{DTR} mice with TM (9 mg/40 g, IP) on 2 consecutive days at 3 weeks of age to activate *Atoh1* and *tdTomato* expression in supporting cells. We then injected DT (DT; 25 ng/g, IP) ∼6 h after the second TM injection to kill the endogenous hair cells. Cochleas were collected at 5 weeks of age and labeled with antibodies detecting MYO7A and SOX2, and we quantified the number of tdTOMATO/MYO7A-double positive cells (Fig. 2A). We found only 2.8 ± 6.2 tdTOMATO/MYO7A-double positive cells in control cochleas, and we found no increase in the number of regenerated hair cells when we activated expression of *Atoh1* alone (46.0 ± 17.1, $P = 0.9250$; Fig. 2A and B). We next asked whether the combined activation of *Atoh1*, *Gfi1*, and *Pou4f3* in supporting cells following damage would lead to the regeneration of hair cells. Activation of *Atoh1*, *Gfi1*, and *Pou4f3* produced significantly more tdTOMATO/MYO7A-double positive cells (479.9 ± 213.4, $P = 0.0004$ compared with control and $P = 0.0034$ compared with *Atoh1* alone; Fig. 2A and B). We observed a clear distinction between regenerated hair cells in both the medial region (175.5 ± 17.1) as well as the lateral region (304.4 ± 205.5) compared with both control cochleas (0.0 ± 0.0 medial and 2.8 ± 6.3 lateral) and *Atoh1* over expression alone (46.0 ± 17.1 medial and 0.0 ± 0.0 lateral; Fig. 2A and B). The new tdTOMATO+ hair cells were observed throughout the entire length of the organ of Corti (Figs. 2C and S2).

Previously, we found that the number of reprogrammed hair cells in nonsensory regions of the cochlea on either side of the organ of Corti increased with additional programming time (24). Therefore, we next asked whether additional regenerated hair cell-like cells would be added with additional time after supporting cell reprogramming. We again killed hair cells at 3 weeks of age and analyzed cochleas at 8 weeks of age, allowing 5 weeks of reprogramming time and quantified the number of regenerated hair cells throughout the length of the cochlea. Unfortunately, we observed a significant death of animals due to seizures between 5 and 8 weeks of age, likely due to expression of CreER in CNS astrocytes and stem cells. We were only able to collect two animals for these experiments. We found no difference between the number of regenerated cells at 5 and 8 weeks of age (175.5 ± 50.5 medial and 304.4 lateral regenerated hair cells at 5 weeks, $N = 8$, vs. 240.5 ± 58.6 medial and 361.5 ± 150.6 lateral regenerated hair cells at 8 weeks, $N = 2$, statistical analysis not performed due to insufficient numbers). This suggests that there is no increase in the number of regenerated hair cells from cochlear supporting cells after 2 weeks of reprogramming time.

Regenerated hair cells develop features of endogenous cochlear hair cells

Inner hair cells normally receive afferent innervation from spiral ganglion neurons, so we next investigated whether regenerated hair cells were innervated. We stained *Lfng-Cre*^{ER}::*Rosa26*^{tdTomato}::*Rosa26-GAP*::*Pou4f3*^{DTR} animals, as well as damaged and undamaged controls lacking *Rosa26-GAP* or both *Rosa26-GAP* and *Pou4f3*^{DTR} with TUBB3 and MYO7A antibodies. In undamaged control cochleas, TUBB3 labeling of spiral ganglion neurons and afferent projections was observed in both inner and outer hair cell compartments in the organ of Corti (Fig. 3A). Following hair cell killing, MYO7A and TUBB3 labeling was absent from the organ of Corti (Fig. 3A); this retraction of afferent processes is typically seen after hair cell loss (39). Following genetic reprogramming, TUBB3-positive spiral ganglion neurons were observed contacting MYO7A/tdTomato double-positive regenerated hair cells (Fig. 3A), suggesting that the new hair cells can recruit the processes of afferent neurons.

Hair cells communicate with spiral ganglion neurons with specialized synaptic features called ribbon synapses (40). We stained *Lfng-Cre*^{ER}::*Rosa26*^{tdTomato}::*Rosa26-GAP*::*Pou4f3*^{DTR} animals, as well as *Lfng-Cre*^{ER}::*Rosa26*^{tdTomato} (undamaged) and *Lfng-Cre*^{ER}::*Rosa26*^{tdTomato}::*Pou4f3*^{DTR} (damaged) controls with antibodies to recognize the ribbon synapse protein ribeye (anti-CTBP2) and MYO7A. In undamaged control cochlea, anti-CTBP2 labeling was observed as puncta in both inner and outer hair cells (Fig. 3B). Following hair cell killing, CTBP2-positive puncta were largely absent from the organ of Corti, but were observed in surviving hair cells (Fig. 3B). However, following the expression of *Atoh1*, *Gfi1*, and *Pou4f3* in cochlear supporting cells, CTBP2 puncta were observed in regenerated hair cells (Fig. 3B), suggesting that regenerated hair cells can reconstitute parts of the synaptic machinery by which they communicate with spiral ganglion neurons.

Hair cells convert sound waves into electrochemical signals by the mechanical perturbation of elongated microvilli on their apical surface called stereocilia (41). We asked whether regenerated hair cells developed stereocilia using scanning electron microscopy (SEM). We fixed cochleas from *Lfng-Cre*^{ER}::*Rosa26*^{tdTomato}::*Rosa26-GAP*::*Pou4f3*^{DTR} animals and damaged controls lacking *Rosa26-GAP*, and analyzed them by SEM. We found that following hair cell killing, stereocilia, and other microvilli were largely absent from the apical surface of the organ of Corti (Fig. 3C). However, following the misexpression of *Atoh1*, *Gfi1*, and *Pou4f3* in cochlear supporting cells, the apical surface of the organ of Corti was covered in microvilli (Fig. 3C). In addition, there were distinct groupings of elongated microvilli resembling immature cochlear hair cell stereocilia in both the inner and outer hair cell regions, although very few cells possessed the characteristic staircase-like arrangement of stereocilia found in mature hair cells (Fig. 3C). Some of these structures also contained a single elongated projection resembling the kinocilium that is a transient feature found in immature cochlear hair cells (Fig. 3C).

Finally, we investigated the expression of two additional hair cell proteins critical for hair cell function: parvalbumin (PVALB) and prestin. PVALB is a calcium buffer typically expressed in both immature and mature hair cells in the cochlea while prestin is expressed specifically in mature outer hair cells and is

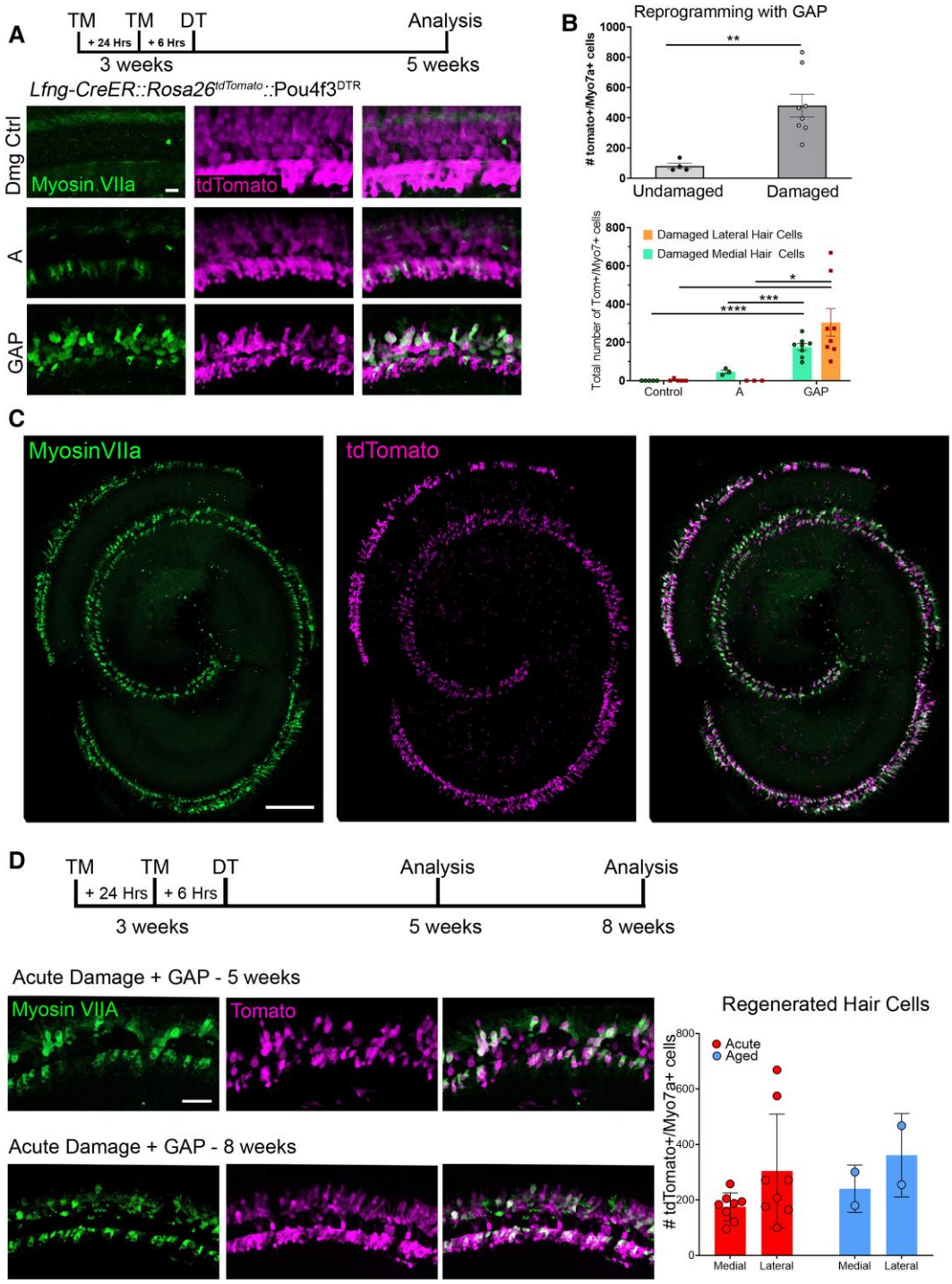

Fig. 2. Hair cell death increases the number of reprogrammed hair cell-like cells. A) Reprogramming and fate mapping of cochlear supporting cells was activated at 3 weeks of age with 2 TM injections (TM, 9 mg/40 g) ~24 h apart. Endogenous hair cells were killed at 3 weeks of age by injecting DT into Pou4f3^{DTR} -positive animals ~6 h after the second TM injection. Supporting cell fate was traced with tdTOMATO. Near complete loss of endogenous hair cells was observed following damage. Expression of *Atoh1* (A) alone did not reprogram supporting cells into hair cell-like cells while *Gfi1* + *Atoh1* + *Pou4f3* (GAP) reprogrammed significantly more supporting cells into hair cells thus regenerating them. Regenerated hair cells were observed both medial and lateral to the pillar cells. TM = tamoxifen, DT = diphtheria toxin, hrs = hours. A = *Atoh1*, GAP = *Gfi1* + *Atoh1* + *Pou4f3*. Scale bar = 20 µm. B) Significantly more hair cells were reprogrammed following GAP expression when hair cells are killed compared with when the original hair cells are present. *Atoh1* expression alone was not sufficient to regenerate hair cells, however, GAP combined expression reprogrammed hair cells both medial and lateral to the pillar cells. C) Regenerated hair cells were observed throughout the entire length of the cochlea. D) Regenerated hair cells were observed in similar numbers following 2 weeks of reprogramming and 5 weeks of reprogramming. Scale bar = 200 µm. Significance indicated by asterisks $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

necessary for their electromotility and cochlear amplification (42, 43). We stained *Lfng-Cre*^{ER}::*Rosa26*^{tdTomato}::*Rosa26-GAP*::*Pou4f3*^{DTR} animals, as well as undamaged controls lacking both *Rosa26-GAP* and *Pou4f3*^{DTR} with MYO7A and PVALB or prestin antibodies at 5–6 weeks of age. tdTomato was detected via its endogenous fluorescence. PVALB and MYO7A-double positive hair

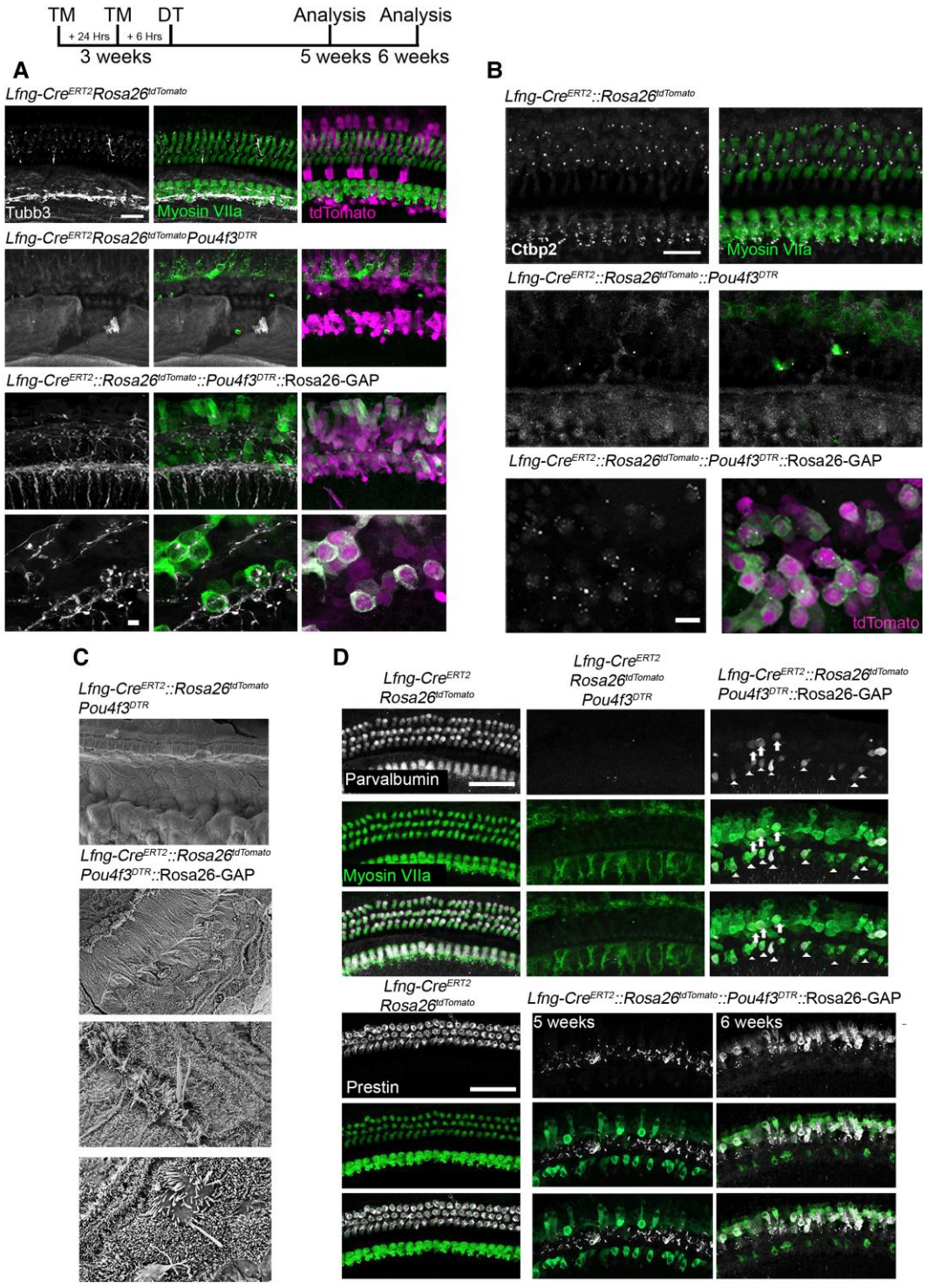

Fig. 3. Regenerated hair cells develop structural components of endogenous hair cells. A) Endogenous hair cells (Myosin VIIa) of the intact cochlea are innervated by spiral ganglion neurons (TUBB3). Following hair cell killing, hair cells and neurons are absent from the organ of Corti. Neurites reinnervate the organ of Corti, making contact with regenerated hair cells. B) Inner and outer hair cells in the intact organ of Corti express the ribbon synapse protein ribeye (anti-CTBP2). Hair cell killing results in the loss of both hair cells and CTBP2, while regenerated hair cells exhibit CTBP2-positive puncta. C) SEM image showing that hair cell killing results in the loss of stereocilia and minimal presence of microvilli on SC apical surfaces. Following induced *Atoh1*, *Gfi1*, and *Pou4f3* expression in the SCs, disorganized and immature stereocilia are present. D) More regenerated hair cells in the medial compartment express parvalbumin at 5–6 weeks of age. Regenerated hair cells turn on Prestin by 6 weeks of age, but not 5 weeks of age. Scale bars in A, B, and D = 20μm.

cells were observed as expected in the undamaged cochlear epithelium (Fig. 3D). Following regeneration, we quantified the number of regenerated hair cells expressing PVALB in both the inner and outer compartments. We found that 63.8% ± 3.7% ($N = 2$) of regenerated hair cells in the inner hair cell compartment expressed PVALB while significantly fewer regenerated hair cells express PVALB in the outer hair cell compartment (8.8% ± 0.1%, $N = 2$; Fig. 3D). PRESTIN was detected in outer hair cells in undamaged

control cochlea at both 5 and 6 weeks of age, however PRESTIN expression was only detected in regenerated hair cells at 6 weeks of age, with 45.6 ± 5.5% of regenerated hair cells co-labeled for MYO7A+ PRESTIN+ tdTomato at 6 weeks of age ($N = 2$; Fig. 3D).

Supporting cells can still respond to reprogramming several weeks after damage

In order for supporting cells to be a potential target for therapeutic hair cell reprogramming, they need to remain responsive to reprogramming factors well after hair cell death has occurred. Therefore, we next asked whether supporting cells would still be responsive to genetic reprogramming several weeks after hair cell killing. We injected *Lfng-Cre^{ER}:: Rosa26^{tdTomato}::Rosa26-GAP:: Pou4f3^{DTR}* animals with DT (25 ng/g, IP) to kill hair cells at 6 weeks of age and TM (9 mg/40 g, IP) to induce reprogramming 6 weeks later, at 12 weeks of age. We collected the cochleas at 16 weeks of age and stained them with antibodies against MYO7A (Fig. 4A). We quantified the number of MYO7A/tdTOMATO-double positive cells and found 141.3 ± 57.5 regenerated hair cells in the medial compartment ($N = 4$, $P = 0.0006$ vs. control) and 130.5 ± 55.2 regenerated cells in the lateral compartment ($N = 4$, $P = 0.0012$ vs. control; as determined by a two-way ANOVA with a Sidak's multiple comparison test; Fig. 4B and C). This indicates that supporting cells in the mature cochlea are still responsive to reprogramming when initiated 6 weeks after hair cell loss.

Discussion

In this study, we show that 3 hair-cell-inducing transcription factors, *Atoh1*, *Gfi1*, and *Pou4f3*, can act together to convert mature mammalian supporting cells into hair cell-like cells. In normal animals where the original hair cells are intact, *Atoh1*, *Gfi1*, and *Pou4f3* convert a subset of supporting cells on the lateral edge of the organ of Corti into hair cell-like cells (Fig. 1). However, we show that loss of endogenous cochlear hair cells significantly enhances the ability of supporting cells to respond to reprogramming (Fig. 2). When the 3 transcription factors were expressed in mice where endogenous hair cells were killed, we observed regenerated hair cells derived from supporting cells throughout the length of the cochlear epithelium. These hair cell-like cells, developed stereocilia bundles and ribbon synapses, attracted innervation, and possessed the mature outer hair cell protein prestin (Fig. 3). In addition, these cells survived for at least 5 weeks of age. We also show that cochlear supporting cells remain responsive to transcription factor reprogramming at least 6 weeks following hair cell killing (Fig. 4). Previous work on reprogramming of the mouse cochlea showed that reprogramming of a small number of cochlear supporting cells could be achieved by activation of *Atoh1* and *Pou4f3* in intact cochleas (15). In addition, we previously showed that activation of *Atoh1*, *Gfi1*, and *Pou4f3* in cells of the inner sulcus of undamaged P1 or P8 cochleas reprograms inner phalangeal and border cells into hair cell-like cells (16). However, here we show that when we activate reprogramming at 3 weeks of age, inner phalangeal and border cells are no longer responsive to *Atoh1*, *Gfi1*, and *Pou4f3* reprogramming when the original hair cells are still intact. This indicates that inner phalangeal and border cells lose plasticity between 1 and 3 weeks of age, suggesting that they are still undergoing fate consolidation in this period. Taken together, these data suggest that inner phalangeal and border cells mature between birth and 3 weeks of age making them less responsive to genetic reprogramming. Further work will be required to better understand the mechanisms of the cell fate restrictions that are placed on these cells as they mature.

We found that the loss of endogenous hair cells enhances the ability of *Atoh1*, *Gfi1*, and *Pou4f3* to reprogram 3-week-old cochlear supporting cells into hair cell-like cells. While a small number of reprogrammed hair cells were found along the lateral edge of the organ of Corti in the apical turn of undamaged cochleas at 5 weeks of age, following hair cell killing and reprogramming of supporting cells, significantly more supporting cells were reprogrammed into hair cells. These regenerated hair cells were found across the radial axis of the organ of Corti, deriving from inner phalangeal and border cells, pillar cells, and Deiters' cells and were found throughout the length of the cochlea in the apical, middle and basal turns. This indicates that the presence of endogenous hair cells restricts the ability of supporting cells to respond to transcription factor reprogramming. We also found that these supporting cells remain competent for regeneration weeks after the hair cells were killed. This effect was not seen in previous reprogramming models lacking Gfi1 and/or Pou4f3 (15, 35), suggesting that the combination of reprogramming factors will be critical to the regeneration achieved through genetic reprogramming.

In the undamaged cochlea, only cells on the lateral aspect of the organ of Corti could respond to reprogramming factors. This is similar to previous work in which regeneration was attempted by expressing *Atoh1* and *Pou4f3* (15). The cells along this lateral aspect, the third row of Dieters' cells, are adjacent to the outer most row of outer hair cells. Because hair cell damage increases responsiveness of supporting cells, it is possible that hair cells are signaling to their neighbors restricting their hair cell fate. If this is the case, then the third row of Dieters' cells may be more plastic because they receive signaling from only one row of outer hair cells. This contrasts somewhat with cells adjacent to inner hair cells, inner phalangeal, and border cells. These cells did not respond to reprogramming when the original hair cells were intact. Because these cells are adjacent only to inner hair cells, they also would receive input from only one row of hair cells. It is possible then that inner hair cells have an increased ability to restrict the fate of their neighboring cells.

There is currently little known about cell–cell signaling between hair cells and supporting cells in the mature cochlea. However, one very well characterized signaling pathway in the developing cochlea, the Notch signaling pathway, signals from hair cells to supporting cells to control cell fate decisions. Specifically, this pathway makes use of Notch ligands (DLL1 and JAG2) that signal from hair cells to supporting cells and restrict their ability to become hair cells through lateral inhibition (36). This interaction activates the expression of Notch downstream effectors in progenitor and supporting cells, including the downstream effector *Hes5*, which directly inhibits hair cell identity (44). Inhibition of Notch signaling in the neonatal cochlea converts supporting cells into hair cells in an age dependent manner (45, 46), whereas by 1 week of age, supporting cells can no longer convert into hair cells following Notch inhibition. In addition, down-regulation of Notch signaling is necessary for the natural regeneration observed in the neonatal mammalian cochlea at birth (47), as well as in birds and fish. In the mammalian cochlea, members of the Notch signaling pathway are largely down-regulated by the end of the first postnatal week correlating with the loss of regenerative potential (45). However, one study found that inhibition of Notch signaling following hair cell damage in mature mouse cochleas increased the regeneration of hair cells (48), suggesting that cell-signaling might be involved in the loss of supporting cell plasticity in the

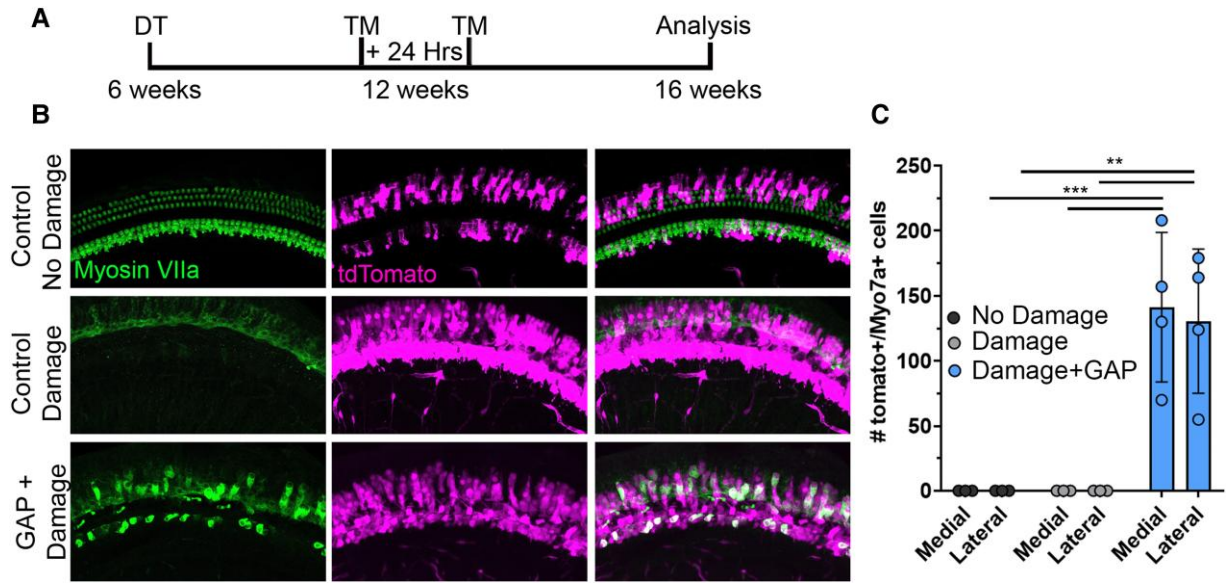

Fig. 4. Mature cochlear supporting cells are reprogrammed into hair cell-like cells 6 weeks after hair cell loss. A) Timeline for hair cell killing and reprogramming. Hair cells were killed at 6 weeks of age. Reprogramming and tdTomato were activated in supporting cells via TM injections at 12 weeks of age followed by analysis at 16 weeks of age. B) In control cochlea with no damage, hair cells are observed in the expected three rows of outer hair cells and one row of inner hair cells with adjacent tdTOMATO-positive supporting cells. Following DT mediated damage, myosin VIIa + cells are absent however supporting cells remain. Following combined hair cell damage + GAP reprogramming, regenerated hair cells were observed in both the medial and lateral compartment of the organ of Corti. Scale bar = 50 µm. C) Quantification of the total number of MYO7A/tdTOMATO-double positive cells in undamaged and damaged samples without reprogramming as well as samples with both damage and reprogramming. Significance is indicated by asterisks $^{**}P < 0.01$, $^{***}P < 0.001$.

mature cochlea. It is still unclear how Notch might be affecting hair cell regeneration in the adult cochlea, as the only known Notch ligand still expressed in the mature cochlea is Jag1 and is not normally associated with lateral inhibition (49). Here, we see that the killing of endogenous hair cells leads to an increased response of supporting cells to genetic reprogramming suggesting that some form of signaling—whether through the Notch pathway or other mechanisms—is still occurring between hair cells and supporting cells in the mature cochlea. Further work is required to better understand how hair cells control the identity of supporting cells, how supporting cells respond to hair cell loss, and how expression of *Atoh1*, *Gfi1*, and *Pou4f3* affect hair cell fate in supporting cells.

Currently, the transcriptional and epigenetic changes in supporting cells following hair cell death and whether supporting cells continue to change weeks or months after damage is not well understood. We recently collaborated on a study that used ATAC-seq to monitor changes in chromatin accessibility in supporting cells following hair cell death. We found that animals that were deafened at 3 weeks of age showed an increase in the accessibility of several hair cell loci 7 weeks later compared with undamaged supporting cells (31). Unfortunately, the technical difficulties in isolating sufficient supporting cells militated against a comprehensive survey of chromatin accessibility. Nevertheless, while several studies have characterized the morphological and cellular changes that occur in mammalian supporting cells after hair cell killing (30, 50), this was the first study to suggest that changes in chromatin accessibility can occur after hair cell damage.

While the specific mechanism underlying the increase in supporting cells responsiveness to reprogramming following hair cell loss is yet unknown, our work shows that genetic reprogramming of mature cochlear cells with *Atoh1*, *Gfi1*, and *Pou4f3* can overcome genetic barriers to hair cell generation and produce numerous hair cell-like cells throughout the length of the adult organ of Corti. These hair cells express some mature hair cell markers, can attract innervation, and have rudimentary stereocilia bundles. We also show that these cells remain responsive to reprogramming for several weeks after hair cell loss, meaning that the therapeutic potential of genetic reprogramming is not limited to the time of insult. Although additional work is necessary to improve the maturation and function of hair cells generated by reprogramming, our data represent a step forward toward hearing restoration in the mature cochlea.

Methods

Full methods can be found in the supporting documentation. The following mouse lines were used for this study: *Lfng-Cre*^{ERT2} (Jax strain # 035554); *Rosa2* $^{loxp\text{-}stop\text{-}loxp\text{-}tdTomato}$ (Ai9; Jax strain # 007909), (51); *Rosa26*$^{loxp\text{-}stop\text{-}loxp\text{-}Atoh1}$ (Jax strain # 038583) (16); Rosa26 $^{loxp\text{-}stop\text{-}loxp\text{-}Gfi1\text{-}Atoh1}$ (Jax strain # 038584) (16) Rosa26 $^{loxp\text{-}stop\text{-}loxp\text{-}Gfi1\text{-}Atoh1\text{-}Pou4f3}$ (Jax strain # 038585) (16); *Pou4f3*^{DTR} (Jax strain # 028673) (Golub et al. (37)). Animals were injected with TM (9 mg/40 g) at 3, 6, or 12 weeks of age and analyzed at 4, 5, 6, 8, or 16 weeks of age. All animal work was conducted in accordance with an animal protocol approved by the Baylor College of Medicine IACUC. Fluorescence histology was performed as previously described (52, 53) and samples were imaged using Zeiss 780, 880, 900, or 980 confocal microscopy systems. SEM samples were prepared as previously described (54, 55) and imaged on a ZEISS Supra 40 SEM.

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Supplementary Material

Supplementary material is available at *PNAS Nexus* online.

Author Contributions

M.M.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, validation, visualization, writing—original draft. S.G.: data curation, formal analysis, investigation, methodology, validation, visualization, writing—revised drafts. C.D.: investigation, methodology, validation. B.W.: data curation, formal analysis, investigation, methodology, validation, visualization, writing—revised drafts, funding acquisition. A.G.: conceptualization, funding acquisition, project administration, writing—original draft.

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Data Availability

Four lines of mice used in this paper were generated by the Groves lab and our collaborators and have been deposited with the Jackson Laboratory. These are: *Lfng-Cre^{ERT2}* mice (B6;FVB-Tg(Lfng-cre/ERT2)1Mmsa/J), strain #035554; *Rosa26$^{loxp\text{-}stop\text{-}loxp\text{-}Atoh1}$ (Gt(ROSA)26Sor$^{em1(CAG\text{-}Atoh1/EGFP)Akg}$/J)* strain # 038583 ; Rosa26$^{loxp\text{-}stop\text{-}loxp\text{-}Gfi1\text{-}Atoh1}$ (*Gt(ROSA)26Sor$^{em2(CAG\text{-}Gf1i,\text{-}Atoh1/EGFP)Akg}$/J*), strain #038584; Rosa26 $^{loxp\text{-}stop\text{-}loxp\text{-}Gfi1\text{-}Atoh1\text{-}Pou4f3}$ (*Gt(ROSA)26Sor$^{em3(CAG\text{-}Gf1i,\text{-}Atoh1/EGFP,\text{-}Pou4f3)Akg}$/J*), strain # 038585.

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