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Original Article

DAPT mediates atoh1 expression to induce hair cell-like cells

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Abstract: Hearing loss is currently an incurable degenerative disease characterized by a paucity of hair cells (HCs), which cannot be spontaneously replaced in mammals. Recent technological advancements in gene therapy and local drug delivery have shed new light for hearing loss. Atoh1, also known as Math1, Hath1, and Cath1, is a proneural basic helix-loop-helix (bHLH) transcription factor that is essential for HC differentiation. At various stages in development, Atoh1 activity is sufficient to drive HC differentiation in the cochlea. Thus, Atoh1 related gene therapy is the most promising option for HC induction. DAPT, an inhibitor of Notch signaling, enhances the expression of Atoh1 indirectly, which in turn promotes the induction of a HC fate. Here, we show that DAPT cooperates with Atoh1 to synergistically promote HC fate in ependymal cells in vitro and promote hair cell regeneration in the cultured basilar membrane (BM) which mimics the microenvironment in vivo. Taken together, our findings demonstrated that DAPT is sufficient to induce HC-like cells via enhancing of the expression of Atoh1 to inhibit the progression of HC apoptosis and to induce new HC formation.

Keywords: Hearing loss, DAPT, atoh1, ependymal cells, hair cells

Introduction

The inner ear is a complex and difficult organ to study, and hearing loss is an incurable disease that is not responsive to standard medical and surgical practices [1-3]. A crucial pathological component of hearing loss is the progressive loss of hair cells (HCs), which is followed by the degeneration of spiral ganglion neurons (SGNs). Hearing loss in birds and amphibians can be fully restored because the hair cells can be regenerated [4-6]. However, in mammals, HC loss is irreversible due to the limited mammalian capacity of the cells to regenerate, and the loss of these long-lived cochlear cells leads to permanent hearing impairment [7, 8].

Clinical therapeutics has not proven effective in the treatment of hearing loss because of the complexity and limited understanding of the pathophysiology involved [9, 10]. Gene therapy is emerging as a legitimate and powerful technique to cure some of the most common diseases, such as retinal blindness [11, 12], and Parkinson's disease [13], etc. Progress in the field of gene therapy, including gene vector design, therapeutic gene selection and gene delivery, has renewed in general application and treatment modalities [14].

Atoh1, a mouse homolog of the Drosophila gene atonal, is a proneural basic helix-loop-helix (bHLH) transcription factor essential for inner ear HC differentiation [15]. It has been suggested that the onset of Atoh1 expression correlates with the development of different types of HCs [16]. Therefore, Atoh1 has been used to stimulate HC production and has pro-

vided modest improvements in hearing function [17]. Thus, Atoh1 may be a potential candidate gene to induce HC differentiation and regeneration.

The Notch signaling pathway plays a major role in the distribution of IHCs and outer hair cells (OHCs) within the organ of Corti, these cells are precisely assembled in a mosaic pattern. As we previously described, the Notch signaling pathway is critical for inner ear HC fate during inner ear development [18]. Activation of the Notch signaling pathway leads to the expression of Hes1 and Hes5, which in turn inhibit Atoh1 gene expression [19]. Conversely, as we have described, blockade of the Notch pathway by delivering of an r-secretase inhibitor, such as N-[(3,5-Difluorophen yl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethylester (DAPT) to the organ of Corti results in down regulation of the Hes1 and Hes5 genes. This down regulation releases the Atoh1 promoter and promotes Atoh1 expression, thereby producing supernumerary HCs [20].

Due to the fundamental role of HCs display in hearing function and the irreversibility of their degeneration, various investigations have focused on developing methods to regenerate these non renewable HCs [21]. In previous study, the transcription factor Atoh1 was transfected into various types of stem cells to induce HC-like cells [22]. However, the aforementioned methods showed the limited efficiency. Therefore, in the present study, we delivered Ad-Atoh1-EGFP into ependymal cells [23] and administered DAPT at the same time to induce a hair cell fate. Therefore, we propose that within germinal zone of the adult forebrain, ependymal cells could replace damaged HCs in the auditory system through an epigenetic functional switch. Then, we introduced both DAPT and Ad-Atoh1-EGFP into the cultured basilar membrane. Our findings showed that DAPT not only greatly improved the efficiency of infection but also promote hair cell fate in both the cultured ependymal cells and BM. Taken together, we exploited a promising approach for the future treatment of hearing loss.

Materials and methods

Animals

All investigations were approved by the ethical committee of the Second Xiangya Hospital, Central South University. The ependymal cells and cochlear explants were prepared from C57BL/6J mice neonatal and post-natal day 3-4 (P3-4), respectively. The C57BL/6J mice were housed 2 or 3 per cage, had free access to food and water and were housed under suitable temperature and humidity conditions and a normal 12/12 h light/dark cycle.

Viral construction

The way of viral construction has been clearly described by our team [24]. In short, replication-deficient recombinant adenoviruses (Ad5) with deleted E1 and E3 regions were used to construct Ad-Atoh1-EGFP using the Adeno-X expression system (K1650-1, Clontech). Atoh1 plasmids was kindly provided by Dr. WeiQiang Gao from Genentech, San Francisco, CA. The sequence and other information about the constructs have been published elsewhere [17, 25]. Undiluted vectors at a concentration of 1×10^{12} total particles purified virus per milliliter were kept at -80°C until thawed for use.

Ependymal cell culture

The isolation and expansion of embryonic ependymal cells from the neonatal day germinal zone followed the protocols described by Grondona [26] and Dong GW [27], respectively. In short, using sterile technique, ependymal cells were dissociated from the ependymal layer of neonatal C57BL/6J mice. Cells were suspended in NEUROBASAL™ (Gibco, New York, NY, US) medium followed by centrifugation. After the cells were separated into a single-cell suspension, primary cultures were plated at a density of 10^5-10^6 cells mL^{-1} in NEUROBASAL™ medium supplemented with basic fibroblast growth factor (bFGF, 20 ng/mL; Gibco), epidermal growth factor (EGF, 20 ng/mL; Sigma) and Insulin growth factor (IGF, 100 ug/mL; Gibco). Two to three days were required for neurosphere formation. The cultures were then passaged every 7-10 days by StemPro® Accutase® Cell Dissociation Reagent (Gibco, 10 μg/mL) digestion.

Scanning electron microscopy (SEM)

To observe the morphology of ependymal cells and compare the recovery of stereocilia, both cultured induction cells and the dissected basilar membrane were prepared for SEM observation. Briefly, the specimens were fixed with

2.5% glutaraldehyde, stored at 4°C for up to 6 hours and then postfixed in 1% osmium tetroxide following decalcification with 10% ethylene diamine tetraacetic acid (EDTA) for 3-5days. After dehydration in a graded ethanol series (50%-70%-90%-100%), the specimens were then critical-point dried by CO_2 using an HCP-2 critical point dryer and mounted on aluminum stubs with silver paint. Gold/palladium was sputter-coated on the specimens using an E-102 ion sputter. Finally, the specimens were observed under a scanning electron microscope (HITACHI S-4800, 15 kV accelerating voltage).

Ependymal cell infection

For gene infection, third-passaged neurospheres were seeded on 24-well plates containing coverslips coated with PL-ornithine (Sigma, 20 μg/mL) and laminin (Gibco, 10 μg/mL). The neurospheres were plated on the coverslips, with 10^5-10^6 cells mL^{-1} in each well. All of the coated cells were divided into three groups as follows: 1) Control group: neither Ad-Atoh1-EGFP nor DAPT was added to the cultured cells. 2) Ad-Atoh1-EGFP infection group: Ad-Atoh1-EGFP (8×10^7) was added to each well. 3) Ad-Atoh1-EGFP and DAPT infection group: Ad-Atoh1-EGFP (8×10^7) and 5 μM DAPT (Sigma, 5 μM) were both added to the medium to infect the cells. The medium of the differentiated cells was replaced with new NEUROBASAL™ medium with 5% fetal bovine serum, bFGF (20 ng/mL), EGF (20 ng/mL) and IGF (100 μg/mL) 24 hours later to miror the survival of the HCs.

Immunofluorescence

Fluorescence immunocytochemistry was carried out to examine the expression of ependymal cell marker (anti-nestin antibody; Sigma, 1:600) and self-proliferation marker (anti-BrdU, Sigma, 1:1,000). The immunocytofluorescence methods that were used to determine the induction results of infection were similar to those reported previously [28-31]. Seven days post-infection, the differentiated cells were fixed with 4% paraformaldehyde and then immersed in 0.3% Triton X-100. The cells were blocked with 10% normal goat serum for 30 minutes at room temperature and then incubated overnight at 4°C in primary antibody: Myosin VIIA (Proteus Bioscience, 1:200), monoclonal anti-β-tubulin III (neuronal) antibody (Sigma, 1:600) and phalloidin (Sigma, 1:50). The specimens were rinsed and then incubated with Alexa Flour 555 and Alexa Flour 647 for 60 minutes at room temperature, except for the philloidin specimens. Fluorescence signals were detected with a laser confocal microscope (LSM780, Carl Zeiss).

Neonatal cochlear explants

The cochlea of P3-4 C57BL/6J mice at were dissected in DMEM/F-12 (Invitrogen). Their crania were divided along the midline and then removed. We viewed the cochlea after the brain tissue was rinsed. To obtain a flat cochlear surface preparation, the modiolus and Reissner's membranes were peeled off. The basilar membrane was plated onto plates containing coverslips coated with PL-ornithine (Sigma, 20 μg/mL) and laminin (Gibco, 10 μg/mL). The cochlear explants were cultured in DMEM/F-12 (Invitrogen) with 5% fetal bovine serum (Gibco). All of the cultures were maintained in a 5% CO_2, 95% O_2 humidified incubator (Thermo Scientific Forma, American).

DAPT and Ad-Atoh1-EGFP infect the basilar membrane explants impaired by gentamicin

To create an HC loss model, 0.25 mM gentamicin was added dropwise to the basilar membrane culture medium for 6 hours; and then, the medium was reconstituted by new DMEM/F-12. Simultaneously, Ad-Atoh1-EGFP (8×10^8) and 5 μM DAPT were added to the medium, and 24 hours later, the medium was changed to DMEM/F-12 with 5% fetal bovine serum, bFGF (Gibco 20 ng/mL), EGF (Gibco 20 ng/mL) and IGF (Gibco 100 ug/mL). The explants were fixed for immunohistofluorescence to determine the role of Atoh1 and/or DAPT on the restoration of lost HCs in vitro.

Immunohistochemistry

The BM both pre-operation and post-operation were fixed with 4% paraformaldehyde and then immersed in 0.3% Triton X-100 and blocked with 10% normal goat serum for 30 minutes at room temperature. Those cells were incubated overnight at 4°C in primary antibody Myosin VIIA (Proteus Bioscience, 1:200). The specimens were rinsed and then incubated with Alexa Flour 555 for 60 minutes at room temperature. All of the cell fluorescence signals

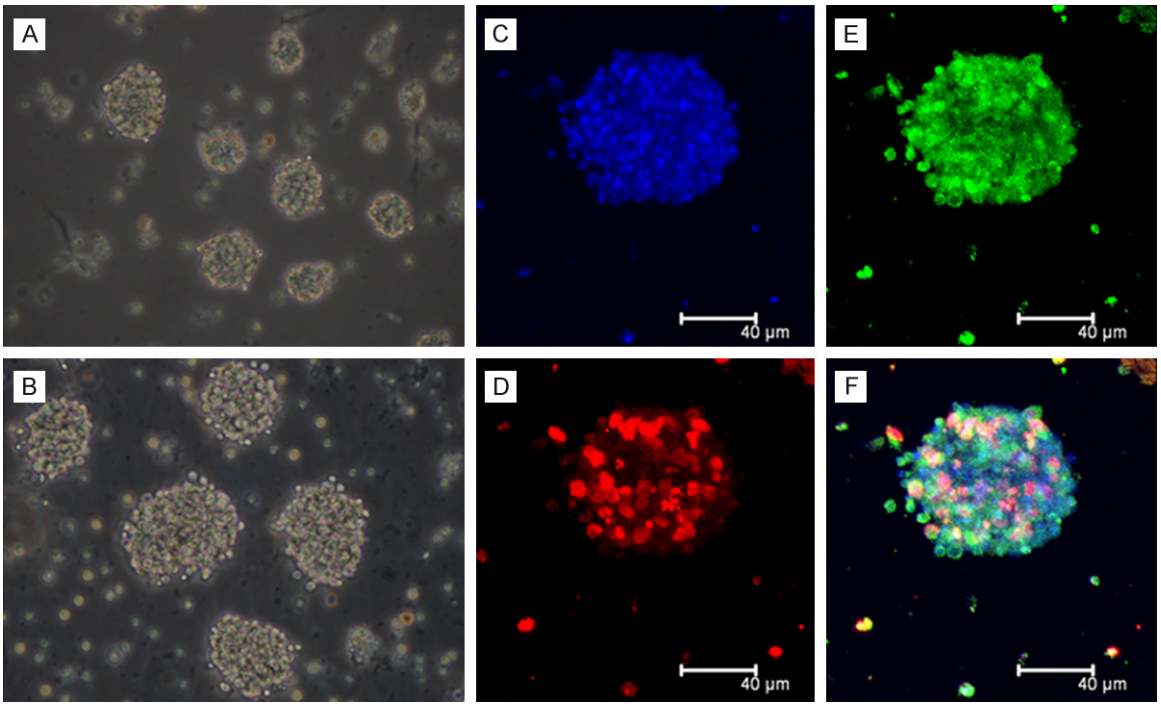

Figure 1. Identification of ependymal cells. (A) Cells were cultured for 3 days. (B) Cells were passaged 3 times. (C) All nuclei were stained with DAPI, blue. (D) Most of cells were Brdu-positive, red. (E) Most of cells were Nestin-positive, green. Scale bars in (A, B), (C-F) and are 200, 40 μm, respectively.

were detected with a laser confocal microscope (LSM780, Carl Zeiss).

Results

The morphology of the ependymal cells

An inverted microscope and scanning electron microscope were used to determine the status of the cultured ependymal cells. The cultured ependymal cells developed and aggregated as neurospheres, which began as cell clusters derived from single cells (**Figure 1A**) and then development in the following days (**Figure 1B**). In addition, the ependymal cells were phase-bright, highly cellular, round and positively stained by anti-BrdU antibody (Sigma, US, 1:1000) (**Figure 1D**) and anti-Nestin antibody (Sigma, US, 1:600) (**Figure 1E**), indicating the self-proliferation potential of the ependymal cells.

DAPT promotes the transdifferentiation of ependymal cells into hair -cell-like cells with stereocilia bundles by improving atoh1 infection efficiency

Ependymal cells were seeded on coverslips just before infection. Cell processes extended around the neurospheres one day after induction. The newly differentiated cell bodies in the control group radiated around the neurospheres and appeared to be filamentous (**Figure 2A**), whereas the induced cells became cylinder-shaped and were well-stacked, especially three days after induction, suggesting a similarity between the induced cells and HCs (**Figure 2B**, **2C**). In the control group, a few cells were positive for EGFP (**Figure 2F**) and Myosin VIIA (**Figure 2G**), but most of the differentiated cells were stained by β-tubulin III (**Figure 2E**), indicating that there were no HC cells. Nevertheless, there were far more induced cells expressing EGFP in the Atoh1+DAPT group (**Figure 2P**) than in the Atoh1 group (**Figure 2K**), suggesting that DAPT greatly improved the efficiency of Atoh1 infection. In addition, we noted that the induced cells also infected by Ad-Atoh1-EGFP also expressed Myosin VIIA (**Figure 2I**, **2Q**). However, there were far more induced cells that expressed both EGFP and Myosin VIIA but were negative for β-tubulin III in the Atoh1+DAPT group (**Figure 2R**) than in the Atoh1 group (**Figure 2M**). That is, under the influence of DAPT/Atoh1, ependymal cells were differentiated/induced into neurons that only expressed β-tubulin III and HC like cells that

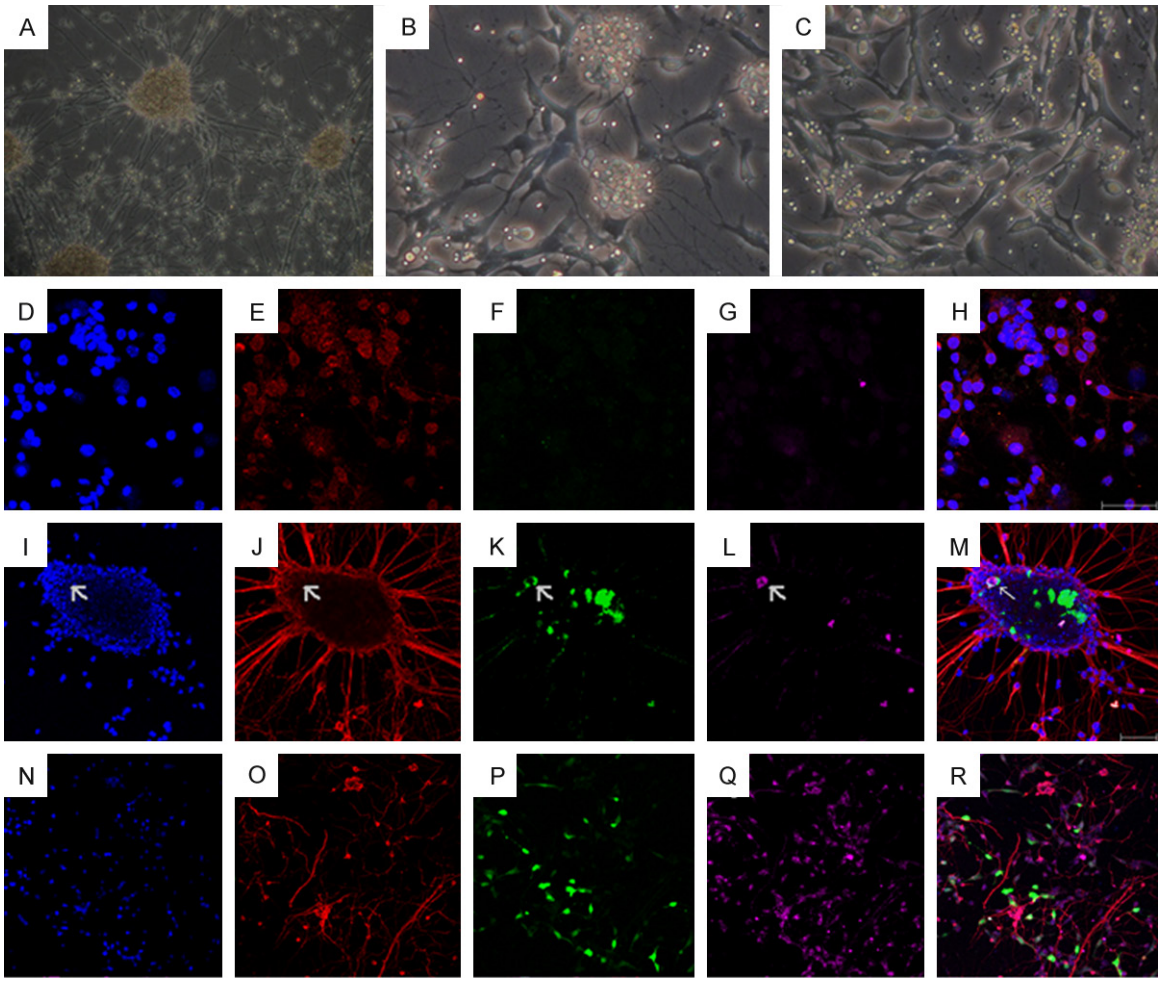

Figure 2. Induction of hair cell-like cells in vitro. (A, D-H) Control group: ependymal cells without Atoh1 and DAPT. (B, I-M) Atoh1 group: ependymal cells induced by Atoh1. (C, N-R) Atoh1+DAPT group: ependymal cells induced by Atoh1 and DAPT. (D, I, N) All nuclei were stained with DAPI, blue. (E, J, O) Some of the induced cells was immune-positive for β-tubulin III, red. (F, K, P) Cells were EGFP positive. (G, L, R) Myosin VIIA positive cells. Scale bars in (A-C) and (D-R) are 200, 100 μm.

expressed both EGFP and Myosin VIIA but were negative for β-tubulin III. Together, the results demonstrated that DAPT promots hair cell induction by improving the efficiency of Ad-Atoh1-EGFP infection, which in turn leads to the upregulation of Atoh1.

Withdrawal of growth factors, such as bFGF and IGF, is an effective way to initiate the induction of HC-like cells. The FGF signaling pathway and IGF signaling pathway were activated after the addition of bFGF and IGF, respectively. Thus, the two signaling pathway not only acted to upregulate HC markers but to also adopt morphology that is typical of HCs. (1) Expression of Myosin VIIA and phalloidin were evaluated after 7 days of cultivation. A subpopulation of EGFP-positive induced cells was immuno-positive for the HC marker Myosin VIIA (**Figure 3A-E**). Simultaneously, we also found that nearly all of the EGFP-positive cells expressed hair bundle markers, such as phalloidin (**Figure 3F-I**). (2) The occurrence of asymmetrically distributed phalloidin immunoreactivity raised a question regarding whether there were stereocilia bundles appeared in the induced cells. Stereocilia-like structures were examined by using scanning electron microscope. Few differentiated neurospheres adopted stereocilia-like structures (**Figure 3J**). However, a number of EGFP-positive differentiated cells possessed stereocilia bundles protruding from the surface of the clusters (**Figure 3K**).

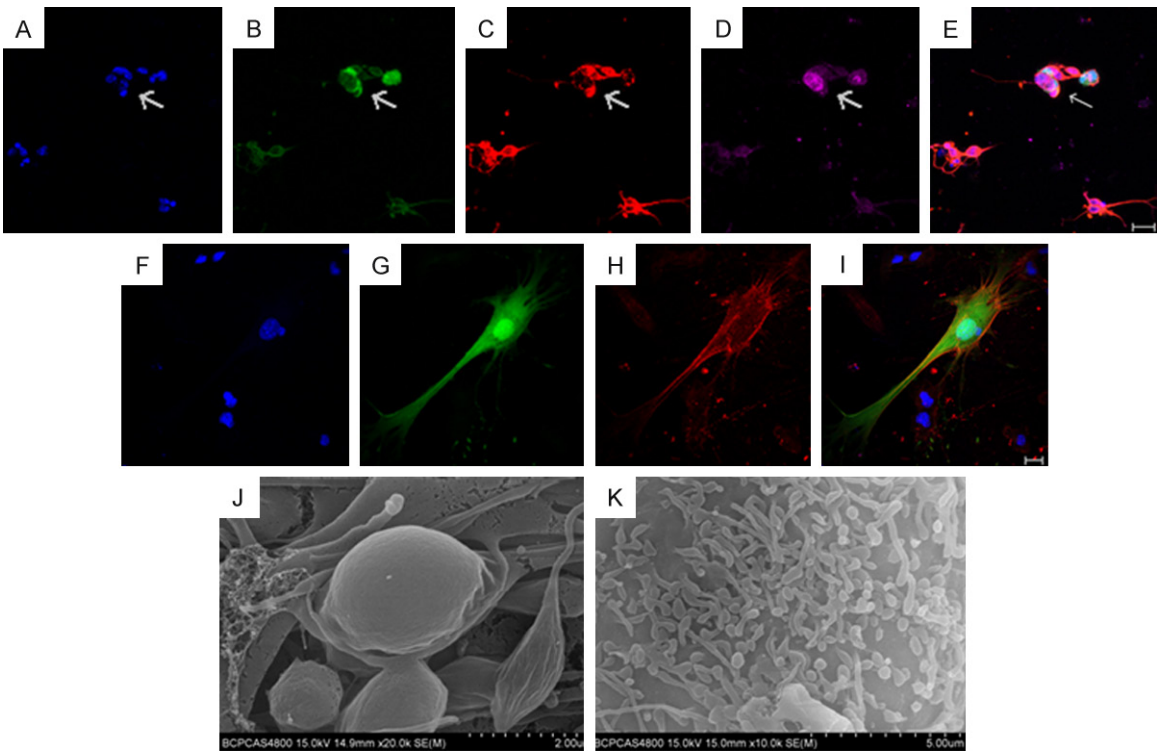

Figure 3. Identification of hair cell-like cells. (A-E) A subpopulation of EGFP-positive induced cells was immunopositive for HC marker Myosin VIIA. (G, H) Nearly all of the EGFP-positive cells express hair bundle markers such as phalloidine. Scanning electron microscope results: (J) Few undifferentiated neurospheres adopt stereocilia-like structures; (K) A number of EGFP-positive differentiated cells possessed stereocilia bundles which protruding from the surface of the clusters. Scale bars in (A-I) are 30 μm.

Delivery of Ad-Atoh1-EGFP and/or DAPT into basilar membrane explants impaired by gentamicin promotes the replacement of HCs or reverses the progression of HC loss

Ad-Atoh1-EGFP and/or DAPT were added dropwise into the cultured organs of Corti isolated from the P3-4 C57BL/6J mice, whose HCs had been partially lost after the gentamicin treatment. The explants were cultured for 10 days and were collected and fixed for immunohistofluorescence to observe the infection efficiency of Ad-Atoh1-EGFP and to confirm the effects of Ad-Atoh1-EGFP and the r-secretase inhibitor, DAPT, on the recovery of HCs. HC growth was detected using a laser confocal microscope. In the control group, there were no extra rows of HCs in the cultured basilar membranes (**Figure 4a-d**). In the Atoh1 group, there were EGFP-positive cells that expressed Atoh1 but were immuno-negative for Myosin VIIA in the basilar membrane (**Figure 4f-i**), some EGFP-positive cells that expressed Atoh1 were also immuno-positive for Myosin VIIA in the apical turn of the cochlea after the addition of DAPT (**Figure 4k-n**). However, the results for the middle and basal turn were similar to those in the Atoh1 group.

Systemic Ad-Atoh1-EGFP and/or DAPT administration are much more potent in repairing the stereocilia bundles of the sensory epithelia

To further investigate the ultra-structural changes in the stereocilia bundles, we prepared SEM specimens dissected from C57BL/6J mice, including preoperative and BMs 7 days post-operation. In the control group, there were 4 regular rows of HCs including 1 row of IHCs and 3 rows of OHCs (**Figure 4e**). In the Atoh1 group, an increase in Myosin VIIA-positive OHCs only rarely increased from 3 rows to 4 rows, but inner ear rows were not yet detected (**Figure 4j**). In the DAPT+Atoh1 group, not only were there many more Myosin VIIA-positive OHCs from 3 rows to 4 rows, but ectopic IHCs also appeared (**Figure 4o**). The results mimicked the morphology findings from the laser confocal microscopy.

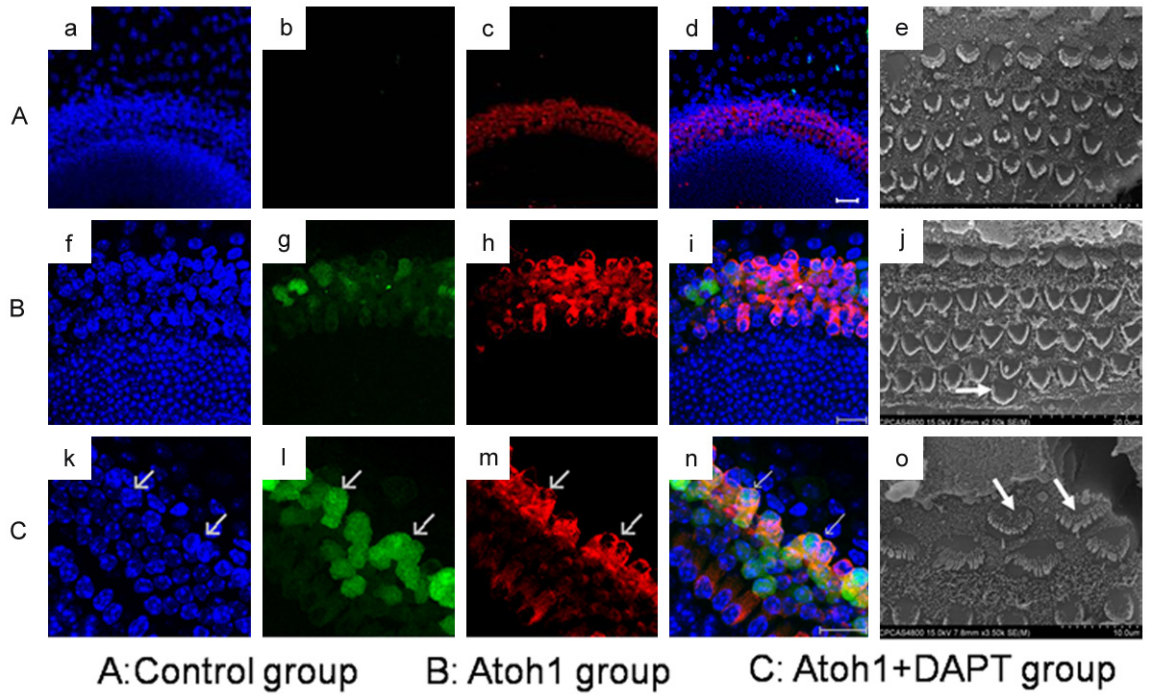

Figure 4. Ad-Atoh1-EGFP or/and DAPT administration in the recovery of cultured organs (a-d). In the control group, there are no extra rows of HC in the cultured basilar membrane and the hair cell was negative for EGFP. (f-i) In the Atoh1 group, there are EGFP-positive cells expressing Atoh1 but immunn-negative for Myosin VIIA in every turns of the basilar membrane; (k-n) In the DAPT+Atoh1 group, some EGFP-positive cells expressing Atoh1 were also detected immunpositive for Myosin VIIA in the apical turn after the droplet of DAPT. SEM results: (e) In the control group, there are 4 regular rows of HC including 1 role of IHCs and 3 role of OHCs; (j) In the Atoh1 group, only rare Myosin VIIA positive OHCs increased from 3 rows to 4 rows (arrowheads), but inner ear rows is not yet; (o) In the DAPT+Atoh1 group, not only much more Myosin VIIA positive OHCs increased from 3 rows to 4 rows, but also the ectopic IHCs appeared (arrowheads); Scale bars in (a-d, f-i, k-n) are 30 μm.

Discussion

Our results showed that AAVs for gene transfer, conjunction with DAPT droplets, can be delivered safely and effectively with no evidence of substantial toxic effects or adverse events. Moreover, all of our findings demonstrated that DAPT is sufficient to induce HC-like cells by promotion of Atoh1 expression.

Although Atoh1 gene therapy, as a novel therapeutic strategy, has incurred some unforeseen setbacks [32-35]. We enhanced the Atoh1 expression level in HCs by introducing exogenous Ad-Atoh1-EGFP and/or DAPT into the cultured ependymal cells and BMs via viral infection. In addition, DAPT administration may inhibit the endogenous Notch signaling pathway to indirectly promote expression of both endogenous and exogenous Atoh1 expression. In brief, we aimed to assess the safety, tolerability, and efficacy of the delivery of Ad-Atoh1-EGFP and/or DAPT in BM to pave the way for hearing loss related gene therapy.

The Notch signaling pathway is capable of specifying sensory domains within the otic placode and later the differentiation of the hair cells and supporting cells of the sensory epithelium. γ-secretase is involved in the activation of Notch signaling and initiates the expression of downstream genes such as Hesl and Hes5. However, Hesl and Hes5 play an inhibitory role in the expression of Atoh1, which plays a key role in the differentiation of hair cells. Subsequently, Notch signaling can be inhibited by a γ-secretase inhibitor. Thus, downregulation of Hes1 and Hes5 can remove the suppression of Atoh1 expression. As a result, Atoh1 can promote the differentiation of hair cells. Several studies have shown that inner ear stem cells treated with a γ-secretase inhibitor can be induced to differentiate into hair cells by blockade of the Notch signaling pathway and upregu-

lated expression of Atoh1 [36]. Moreover, another study demonstrated that the inhibition of Notch after noise damage leads to the transdifferentiation of supporting cells into hair cells, which was dependent on the expression of Atoh1 [37]. Thereby, our results showed that DAPT, as a γ-secretase inhibitor, inhibited Notch signaling and increased the differentiation of hair cells by controlling the expression of native Atoh1.

Surprisingly, we noted that most of the hair cells, especially the OHCs, were EGFP positive. Why was the infection efficiency so high? We surmised that the inhibition of Notch signaling by DAPT may increase the expression of Atoh1-EGFP following viral infection. It is difficult to explain the infection efficiency of hair cells in our investigation if we only based on the explanation on the reasons. Nevertheless, this hypothesis needs further preclinical studies.

Previous studies have demonstrated that Atoh1 plays a central role in HC formation, but the factors that regulate its expression have yet to be identified [38]. Nevertheless, the FGF, IGF, Shh (sonic hedgehog), BMP, and Wnt signaling pathways are all necessary and sufficient for the induction of HCs [39, 40]. Reflecting the important role of the FGF and IGF signaling pathways, we achieved in vitro HC induction by exposing ependymal cells to bFGF and IGF. Meanwhile, we delivered Ad-Atoh1-EGFP and/or DAPT into the cultured ependymal cells. The results showed that ectopic Atoh1 expression could efficiently induce the ependymal cells into a HC fate, possibly through the FGF and IGF signaling pathways. The infection efficacy was greatly improved following the addition of DAPT, which leads to the inhibition of Notch signaling. These findings proved that manipulation of FGF and IGF signaling pathway could alter the number of HCs, either by direct regulation of Atoh1 transcription or by indirect activation of other factors that could themselves regulate Atoh1 [38].

HC recovery was assessed in the cultured basilar membranes that were impaired by gentamicin. We found that in the apical turn in Atoh1+DAPT group, some of the EGFP-positive cells expressing Atoh1 were also immuno-positive for Myosin VIIA. However, there were no extra rows of HCs or EGFP-positive cells that expressing Myosin VIIA in the cultured organs of Corti in the Atoh1 group and control groups. There were EGFP-positive cells that expressed Atoh1 but were not immuno-positive for Myosin VIIA in every turn of the basilar membrane in the Atoh1 group. It is worthy for us to explore the origin of those EGFP-positive cells that expressed both Atoh1 and the typical HC marker Myosin VIIA. Two possible pathways should be taken into account as follows: first, supporting cells approximately within to the lost HC area were infected with Atoh1; then, the administration of DAPT activated Atoh1 transcription to specify a HC fate and replace the lost HCs. However, the solitary role of Atoh1 may not be enough to induce both Atoh1- and Myosin VIIA-positive cells in such a short time in the Atoh1 group, not to mention in the control group. Second, the HCs that were undergoing the process of apoptosis after gentamicin were EGFP-positive and expressed Atoh1. The delivery of DAPT promoted Atoh1 transcription which in turn reversed the progression of HC apoptosis in the neonatal organ of Corti.

Although improvements in hair cell regeneration provide a useful early marker of successful gene transfer, it worth noting the following problems: 1) Although pharmacological (e.g., DAPT) inhibition of Notch signaling can rapidly upregulate Atoh1 expression and subsequently promote HC induction, the duration is limited. 2) Once a sufficient number of HCs are triggered, we did not clearly observe specific HC subtypes such as auditory or vestibular HCs, IHCs or OHCs, or even type I or type II HCs or identify the subsequent signaling pathway that were activated or suppressed by the procedure. 3) It is essential to design an approach that will yield supernumerary HCs that can survive, connect with local SGNs and function.

Ultimately, our aim was to determine whether Atoh1 with or without DAPT could promote a HC fate in ependymal cells in vitro, resulting in hair cell regeneration in the cultured membrane which mimics the microenvironment in vivo. The exact morphological differences between the Atoh1 group and the Atoh1+DAPT group were not detected in our study (data not shown), except for the rare ectopic HC. However, we delivered Ad-Atoh1-EGFP and/or DAPT in to the BM and demonstrated that DAPT is sufficient to induce HC-like cells by enhancing the expression of Atoh1 in non-functioning HCs and supporting cells to inhibit the progression of HC apoptosis and to induce new HC formation,

respectively. Although the application of gene therapy to the most difficult-to-treat disorders is rapidly maturing, the application of this technology to hearing loss in vivo will require breakthroughs in research and continued commitment from researchers in the field. Clinical studies with these therapies will not be possible until additional reliable preclinical experiments have been conducted.

Disclosure of conflict of interest

There are no competing financial interests during the process of writing and publication. All authors have contributed to, read and approved the final manuscript for submission. The experiment was approved by the Second Xiangya Hospital Central South University.

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