Cellular origin and response of flat epithelium in the vestibular end organs of mice to Atoh1 overexpression

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ABSTRACT

A flat epithelium (FE) may be found in the vestibular end organs of humans and mice with vestibular dysfunction. However, the pathogenesis of FE is unclear and inducing hair cell (HC) regeneration is challenging, as both HCs and supporting cells (SCs) in vestibular FE are damaged. To determine the cellular origin of vestibular FE and examine its response to Atoh1 overexpression, we fate-mapped vestibular epithelial cells in three transgenic mouse lines (vGlut3-iCreERT2:Rosa26tdTomato, GLAST-CreER<sup>T2</sup>:Rosa26tdTomato, and Plp-CreERT2:Rosa26tdTomato) after inducing a lesion by administering a high dose of streptomycin. Atoh1 overexpression in vestibular FE was mediated by an adeno-associated virus serotype 8 (AAV8) vector. Suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor, was administered with AAV8 to enhance Atoh1 overexpression. The transduction efficiency and population of myosin VIIa-positive cells were analyzed. A small number of HCs were present in vestibular FE. FE did not show broad GLAST-Cre or Plp-Cre expression, unlike the original SCs. SAHA dramatically enhanced AAV8-mediated exogenous gene overexpression, and Atoh1 overexpression plus SAHA promoted myosin VIIa expression in FE cells. Our data provide insight into FE formation and will facilitate studies of gene therapy for vestibular FE.

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1. Introduction

Vertigo has a high incidence worldwide and causes varying degrees of disability. In individuals over 60 years of age, the prevalence of vertigo increases with age (Iwasaki and Yamasona, 2015). The presence of vertigo is a strong predictor of falls, the leading cause of accidental death of older adults (Fernández et al., 2015).

Damage to the vestibular sensory epithelium is an important cause of vertigo (Brose et al., 2016; Hizli et al., 2016; McCall et al., 2009). The vestibular sensory epithelium, which comprises hair cells (HCs) and supporting cells (SCs), is sensitive to various insults, including ototoxic drugs, genetic defects, and aging (Fan et al., 2016; Isgrig et al., 2017; Rauch et al., 2001), resulting in vestibular dysfunction. HCs are more vulnerable to insults than SCs. In most cases, insults eliminate HCs but spare SCs (Forge et al., 1993; Golub et al., 2012; Kawamoto et al., 2009; Sayyid et al., 2019; Taylor et al., 2018; Wang et al., 2010). However, a high dose of aminoglycoside damages both HCs and SCs; in that case, the sensory epithelium loses the normal architecture of HCs and SCs, and it is replaced by a flat layer of cells, referred to as flat epithelium (FE) (Kim and Raphael, 2007; Wang et al., 2017). FE has been found in the cochlear and vestibular end organs of patients with severe deafness or intractable Meniere’s disease (McCall et al., 2009; Nadol and Eddington, 2006; Teufert et al., 2006), indicating that FE is an important pathological change in patients with inner-ear diseases. However, the pathogenesis of FE is unclear. Illuminating the mechanism of vestibular FE formation is essential for studies on the prevention and treatment of vestibular dysfunction.

Current therapeutic strategies for vestibular dysfunction are limited. A considerable proportion of patients fail to recover adequate vestibular function following pharmacotherapy or vestibular rehabilitation (Brandt et al., 2010; Gillespie and Minor, 2017).
1999). Functional regeneration of vestibular HCs is considered as a promising approach to restoring vestibular function in individuals with vestibular HC loss due to treatment with cisplatin or amino-glycosides. The vestibular sensory epithelium of mature mammals undergoes limited HC regeneration after damage (Burns and Stone, 2017; Forge et al., 1993; Forge et al., 1998; Golub et al., 2012; Kawamoto et al., 2009; Sayyid et al., 2019; Slowik and Bingham-McDonogh, 2013; Wang et al., 2015). During spontaneous HC regeneration in damaged vestibular organs of adult rodents, atonal homolog 1 (Atoh1), a basic helix-loop-helix transcription factor required for HC development (Birmingham et al., 1999; Fritsch et al., 2005), is upregulated in SCs (Golub et al., 2012; Hicks et al., 2020; Lin et al., 2011; Wang et al., 2010). The regenerated HCs are largely transdifferentiated from surviving SCs (Golub et al., 2012; Kawamoto et al., 2009; Lin et al., 2011; Wang et al., 2015). Because the number of regenerated HCs is small and they lack mature hair bundles (Kawamoto et al., 2009), spontaneous regeneration cannot restore sufficient vestibular function (Sayyid et al., 2019). Deletion of Atoh1 from vestibular SCs prior to damage prevents spontaneous regeneration of HCs (Hicks et al., 2020). By contrast, Atoh1 overexpression in SCs enhances HC regeneration and recovery of the function of the damaged vestibular system (Sayyid et al., 2019; Schlecker et al., 2011; Staecker et al., 2007, 2014). Therefore, Atoh1 is important for the trans-differentiation of SCs to HCs in the damaged vestibular sensory epithelium of mature mice. However, these studies are based on damage that causes loss of vestibular HCs but allows SCs survive. How vestibular FE responds to Atoh1 overexpression is unclear, as differentiated SCs as well as HCs are damaged. In cochlear FE, no sign of HC regeneration is observed following Atoh1 overexpression (Izumikawa et al., 2008), but the outcome may differ in vestibular FE. Those flat cells may be responsive to Atoh1 overexpression and have the potential for induction or conversion into HCs.

The goals of the present study were to determine: 1) the cellular origin of vestibular FE, and 2) the response of Atoh1 overexpression in vestibular FE. Three transgenic mouse lines were used for fate-mapping of vestibular epithelial cells (vGlut3-iCreER<sup>T2</sup>; Rosa26<sup>tdTomato</sup> mice (Li et al., 2018), GLAST-CreER<sup>T2</sup>; Rosa26<sup>tdTomato</sup> mice (Stone et al., 2018) and Plp-CreER<sup>T2</sup>; Rosa26<sup>tdTomato</sup> mice (Bucks et al., 2017) after induction of a severe lesion with a high dose of streptomycin. Atoh1 overexpression was mediated by the adeno-associated virus serotype 8 (AAV8) vector, which shows strong tropism for the vestibular sensory epithelium (Isgrig et al., 2017; Wang et al., 2014). Moreover, we used suberoylanilide hydroxamic acid (SAHA), a histone deacetylase (HDAC) inhibitor, together with AAV8, to enhance the level of Atoh1 overexpression, as SAHA promotes gene transfer by viral vectors in tumor cells (Kia et al., 2013; Okada et al., 2006) and in cultured inner-ear tissue (Taura et al., 2010). The transduction efficiency and the population of myosin VIIa-positive cells were evaluated to examine the response to Atoh1 overexpression in vestibular FE.

2. Materials and methods

2.1. Animals

FVB/N mice at 4–5 weeks of age were purchased from SPF Biotechnology Co., Ltd. (Beijing, China). The vGlut3-iCreER<sup>T2</sup> mice were provided by Dr. Zhiyong Liu (Chinese Academy of Sciences, Shanghai, China) (Li et al., 2018). GLAST-CreER<sup>T2</sup> mice were purchased from the Jackson Laboratory (Bar Harbor, ME, US; stock number, 012586) (Stone et al., 2018), Plp-CreER<sup>T2</sup> mice were provided by Dr. Guoqiang Wan (Model Animal Research Center of Nanjing University, Nanjing, China) (Wan and Corfas, 2017). Rosa26<sup>G2A</sup> reporter mice were obtained from Vital River Laboratory Animal Technology (Beijing, China). All experiments involving animals were approved by the Animal Care and Use Committee of Capital Medical University of China and the mice were housed in the Laboratory Animal Department of the university.

2.2. Lineage tracing

To generate vGlut3-iCreER<sup>T2</sup>; Rosa26<sup>tdTomato</sup> mice, male vGlut3-iCreER<sup>T2</sup> mice were crossed with female Rosa26<sup>G2A</sup> mice. Tamoxifen (TMX; T5648, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in corn oil (Solarbio, Beijing, China) and administered intraperitoneally (3 mg/40 g body weight) once daily at P10 and P11 (Li et al., 2018). To assess tdTomato expression in the normal utricle, mice were euthanized at 5–6 weeks of age. To trace tdTomato expression in FE, streptomycin solution was inoculated into the inner ear at the age of 5–6 weeks and utricles were harvested 1 month after surgery.

To generate GLAST-CreER<sup>T2</sup>; Rosa26<sup>tdTomato</sup> and Plp-CreER<sup>T2</sup>; Rosa26<sup>tdTomato</sup> mice, male GLAST-CreER<sup>T2</sup> and Plp-CreER<sup>T2</sup> mice, respectively, were crossed with female Rosa26<sup>tdTomato</sup> mice. TMX (9 mg/40 g body weight) was administered twice at 6 weeks of age at an interval of 24 h (Bucks et al., 2017; Stone et al., 2018). To assess tdTomato expression in the normal utricle, mice were euthanized 1 week after TMX treatment. To trace tdTomato expression in the FE, streptomycin solution was injected into the ear 1 week after TMX administration, and utricles were analyzed 1 month after surgery.

2.3. AAV vector

The purified AAV8 vector carrying Atoh1 and the gene for green fluorescent protein (GFP) (AAV8-Atoh1-GFP) and the AAV8-GFP vector were produced by BioMiao Biological Technology Co. Ltd. (Beijing, China). The expression of the genes carried was driven by the cytomegalovirus promoter. The vector was generated using a tripartite plasmid transfection system with a pSNAV-Atoh1-EGFP plasmid, an Ad helper plasmid, and an AAV Rep2/Cap8 plasmid. The viral particles were purified using ion-exchange column chromatography; the physical titers were 4 × 10<sup>12</sup> vg/mL (AAV8-Atoh1-GFP) and 2 × 10<sup>12</sup> vg/mL (AAV8-GFP). The vector was stored in phosphate-buffered saline (PBS) at ~80 °C.

2.4. Surgery and SAHA administration

The canalostomy procedure has been described in detail previously (Guo et al., 2018). The surgery was performed only on the left ear of mice. To induce a severe lesion in the utricle, streptomycin powder (91317, Sigma-Aldrich) was dissolved in normal saline at 400 g/L and 1 µL streptomycin solution was injected into the inner ear through the lateral semicircular canal of the left ear. At 2 weeks following lesion induction, the viral vector was inoculated into the ear through the left posterior semicircular canal.

SAHA (Selleck, Shanghai, China) was dissolved in dimethyl sulfoxide (Sigma-Aldrich) at 50 mg/mL. Mice in the SAHA and Atoh1 and SAHA groups were administered SAHA solution (0.1 mg/g body weight) intraperitoneally once daily for 8 consecutive days.

2.5. Swim test

A swim test was performed to evaluate the vestibular function of mice as described previously (Hardisty-Hughes et al., 2010). Swimming was scored as follows: 0 = normal swimming; 1 = irregular swimming; 2 = immobile floating; and 3 = underwater tumbling. After removal from the water, the mice
were warmed on a heat mat.

2.6. Immunofluorescence staining

Mice were decapitated under deep anesthesia, and the temporal bones were removed and fixed in 4% paraformaldehyde in PBS for 2 h. After rinsing three times with PBS, the utricles were carefully dissected out and treated with 0.3% Triton X-100 (Sigma-Aldrich) and 5% normal goat serum (ZSG-BIO, Beijing, China) in PBS for 2 h at room temperature. Next, the samples were incubated overnight at 4 °C with the following primary antibodies: rabbit anti-myosin Vllα antibody (diluted 1:300, Proteus Biosciences Inc., Ramona, CA, USA) and mouse anti-GFP antibody (diluted 1:100, Santa Cruz Biotechnology Inc., Dallas, TX, USA). After rinsing in PBS, samples were incubated with fluorescence-labeled secondary antibodies tagged with Alexa Fluor 488 or 568 (diluted 1:300; Invitrogen, Carlsbad, CA, USA) for 2 h at room temperature. Alexa Fluor 487-conjugated phalloidin (diluted 1:300; Invitrogen) was used for F-actin labeling. The DNA-binding fluorescent stain 4′,6-diamidino-2-phenylindole (diluted 1:1000; AppliChem, Darmstadt, Germany) was added for 5 min when nuclear visualization was required. After rinsing with PBS, samples were mounted on glass slides with Fluoromount-G (Southern Biotech, Birmingham, AL, USA) and examined using a Leica scanning confocal microscope (Leica Camera AG, Solms, Hessen, Germany). Images were labeled and normalized using Photoshop software (Adobe Systems Inc., San Jose, CA, USA).

2.7. Cell counts

To enumerate tdTomato-positive HCs and SCs in normal transgenic mice (vGlut3-iCreERT2; Rosa26tdTomato mice, GLAST-CreERT2; Rosa26tdTomato mice, and Plp-CreERT2; Rosa26tdTomato mice), we captured images using a 63 x objective lens with a 2 x digital zoom. The numbers of tdTomato-positive HCs or SCs and all HCs or SCs in each utricle were counted in six randomly selected views (90 μm x 90 μm per view): three in the extrastriolar area and three in the striolar area. Subsequently, the number of cells in each of the six views was summed for each utricle. The proportion of tdTomato-labeled HCs or SCs among all HCs or SCs was calculated to yield the tdTomato-labeled HC or SC rate of each utricle.

When calculating the number of tdTomato-labeled cells in the area of transitional epithelium (TE) of normal transgenic mice (GLAST-CreERT2; Rosa26tdTomato mice and Plp-CreERT2; Rosa26tdTomato mice), three views (90 μm x 90 μm per view) were randomly selected in the TE of each utricle. The numbers of tdTomato-labeled TE cells and all TE cells in each view were counted and summed for each utricle. The tdTomato-labeled TE cell rate was calculated as the proportion of tdTomato-labeled TE cells among all TE cells.

To count tdTomato-labeled cells, myosin Vllα-positive cells, GFP-positive cells, and FE cells in the utricular FE, we captured images of the whole utricle using a 20 x objective lens with a 0.9 x digital zoom. Cells were counted throughout the field of view for each FE sample. The GFP transduction efficiency was determined as the proportion of GFP-positive cells among all FE cells.

2.8. Quantitative real-time PCR

Three to four independent RNA pools were prepared for each group. For each pool, 2–3 utricles were dissected out in RNAlater (Qiagen, Germany). Total RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and cDNA was synthesized by FastQuant RT Super Mix reverse transcription (Tiangen Biotech Co., Ltd.). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a real-time PCR system (ABI 7900HT). Applied Biosystems, Foster City, CA, USA) with SYBR Green (Tiangen Biotech Co., Ltd, Beijing, China). The Atoh1 transcript level was examined. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. The 2^−ΔΔCT method was used to evaluate changes in mRNA levels.

2.9. Statistical analyses

Data are expressed as means ± standard errors. Statistical tests were performed using Graphpad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA). The unpaired Student’s t-test was used to evaluate differences in the proportion of tdTomato-positive TE cells and the GFP transduction efficiency. Statistical differences in the numbers of myosin Vllα-positive cells in FE, swim test scores, and Atoh1 mRNA levels were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparisons test. Differences were considered statistically significant at P < 0.05.

3. Results

3.1. Few HCs survive in the vestibular FE

The vGlut3-iCreERT2; Rosa26tdTomato mice were used for lineage tracing of vestibular HCs following lesion generation. In normal vGlut3-iCreERT2; Rosa26tdTomato mice (Fig. 1A–C and Table 1), TMX administration induced tdTomato expression in approximately half of vestibular HCs (53.06 ± 4.12%). No tdTomato expression was detected in vestibular SCs or TE cells (Fig. 1A–C), suggesting that tdTomato was exclusively expressed in vestibular HCs in this mouse strain. After application of a high dose of streptomycin, the normal cytoarchitecture of the vestibular sensory epithelium was replaced by FE, as reported previously (Wang et al., 2017). In the FE of vGlut3-iCreERT2; Rosa26tdTomato mice (Fig. 1D–F and Table 2), a few HCs (5.42 ± 2.92%) expressed tdTomato, suggesting that they survived in the vestibular FE.

3.2. FE cells do not exhibit broad GLAST-Cre or Plp-Cre expression

In normal GLAST-CreERT2; Rosa26tdTomato mice (Fig. 2A–C and Table 1), TMX induced tdTomato expression in most SCs (73.15 ± 2.49%), a few HCs (0.32 ± 0.16%), and cells in the TE area (1.83 ± 0.51%) of the vestibular sensory epithelium. After lesion generation (Fig. 2D–F and Table 2), some HCs (33.33 ± 20.41%) and a small proportion of flat cells (1.46 ± 0.88%) expressed tdTomato. In normal Plp-CreERT2; Rosa26tdTomato mice (Fig. 3A–C and Table 1), tdTomato labeled a majority of SCs (58.42 ± 4.62%) and a few HCs (0.28 ± 0.2%), comparable to in GLAST-CreERT2; Rosa26tdTomato mice (Fig. 2A–C and Table 1). However, a higher proportion of tdTomato-positive TE cells was detected in Plp-CreERT2; Rosa26tdTomato mice than GLAST-CreERT2; Rosa26tdTomato mice (Table 1: 29.68 ± 5.7% vs. 18.3 ± 0.51%, P < 0.01 by t-test). In FE (Fig. 3D–F and Table 2), few HCs were tdTomato-positive (1.37 ± 0.94%). The proportion of tdTomato-positive flat cells was higher in the FE of Plp-CreERT2; Rosa26tdTomato mice than in that of GLAST-CreERT2; Rosa26tdTomato mice (Table 2: 17.33 ± 3.71% vs. 14.6 ± 0.88%, P < 0.01 by t-test), suggesting TE cells to be the source of flat cells.

3.3. SAHA enhances the AAV8 transduction efficiency in FE

Following lesion generation, the mice were divided into the following four groups: AAV8-Atoh1-GFP (Atoh1 group, n = 11), SAHA + AAV8-Atoh1-GFP (SAHA + Atoh1 group, n = 13), SAHA group (n = 8), and control group (no AAV8-Atoh1-GFP or SAHA; n = 5) (Fig. 4A). In the SAHA + Atoh1 group, SAHA was intraependymally administered for 8 consecutive days, starting 12 days after
Fig. 1. Cre activity and lineage tracing of hair cells (HCs) in vGlut3-iCreERT2:Rosa26tdTomato mice. (A-C) Confocal images of normal utricles showing the distribution of tdTomato in the utricular sensory epithelium of mice treated with tamoxifen. Inset: the tdTomato channel of the image in (A). (B-C) High-magnification images of the dotted square in (A) showing HCs (myosin VIIa-positive cells), but not supporting cells (SCs), labeled with tdTomato. (D-F) Lineage tracing of HCs in flat epithelium (FE) at 1 month after streptomycin injection. (D) A few tdTomato-positive cells are scattered in the utricular FE. (E-F) High-magnification images of the dotted square in (D) showing a tdTomato-positive HC (arrow) and a tdTomato-negative HC (arrowhead). (F) tdTomato channel of the image in (E). Scale bars, 50 μm (A, inset to A, and D) and 20 μm (B and E). Scale bar in (B) applies to (B) and (C). Scale bar in (E) applies to (E) and (F).

Table 1
Quantiﬁcation of tdTomato-labeled cells in the normal utricle of transgenic mice.

<table>
<thead>
<tr>
<th></th>
<th>vGlut3-iCreERT2: Rosa26tdTomato (n = 3)</th>
<th>GLAST-CreERT2: Rosa26tdTomato (n = 3)</th>
<th>Plp-CreERT2: Rosa26tdTomato (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hair cell (HC)</td>
<td>445 ± 42.34</td>
<td>515 ± 26.21</td>
<td>568.33 ± 34.67</td>
</tr>
<tr>
<td>Supporting cell (SC)</td>
<td>239.33 ± 41.38</td>
<td>1.67 ± 0.88</td>
<td>1.67 ± 1.2</td>
</tr>
<tr>
<td>% of HCs labeled</td>
<td>53.06 ± 4.12</td>
<td>0.32 ± 0.16</td>
<td>0.28 ± 0.2%</td>
</tr>
<tr>
<td>SC number</td>
<td>1086.67 ± 58.17</td>
<td>1048.67 ± 81.14</td>
<td>1.67 ± 1.2</td>
</tr>
<tr>
<td>% of SCs labeled</td>
<td>0</td>
<td>79.72 ± 70.07</td>
<td>65.53 ± 46.79</td>
</tr>
<tr>
<td>Transitional epithelium (TE)</td>
<td>73.15 ± 2.49</td>
<td>58.42 ± 4.62</td>
<td>17.33 ± 3.71</td>
</tr>
<tr>
<td>TE cell number</td>
<td>337 ± 29.02</td>
<td>360.33 ± 17.85</td>
<td>29.68 ± 5.7%</td>
</tr>
<tr>
<td>Labeled TE cell</td>
<td>0</td>
<td>6.33 ± 2.03</td>
<td>107 ± 22.23</td>
</tr>
<tr>
<td>% of TE cells labeled</td>
<td>1.83 ± 0.51%</td>
<td>1.46 ± 0.88%</td>
<td>29.68 ± 5.7%</td>
</tr>
</tbody>
</table>

* n, Number of mice analyzed. One utricle per animal was assessed.

<table>
<thead>
<tr>
<th></th>
<th>GLAST-CreERT2: Rosa26tdTomato (n = 4)</th>
<th>Plp-CreERT2: Rosa26tdTomato (n = 6)</th>
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<tr>
<td>Flat epithelium</td>
<td>2 ± 1.41</td>
<td>9.33 ± 6.44</td>
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<tr>
<td>Labeled HC number</td>
<td>0.67 ± 0.33</td>
<td>0.33 ± 0.21</td>
</tr>
<tr>
<td>% of HCs labeled</td>
<td>5.42 ± 2.92%</td>
<td>3.33 ± 20.41%</td>
</tr>
<tr>
<td>Flat cell number</td>
<td>564 ± 37.79</td>
<td>637 ± 49</td>
</tr>
<tr>
<td>% of flat cells labeled</td>
<td>0.82 ± 5.11%</td>
<td>110.33 ± 26</td>
</tr>
</tbody>
</table>

* n, Number of mice analyzed. One utricle per animal was assessed.
streptomycin treatment. An AAV8-Atoh1-GFP virus suspension was inoculated into the inner ear 2 weeks after streptomycin treatment. Mice in the Atoh1 and SAHA groups were administered only the virus and only SAHA, respectively. Mice in the control group were not treated with SAHA or AAV8-Atoh1-GFP following streptomycin-mediated lesion generation.

In the Atoh1 group, scattered GFP expression was observed throughout the FE after viral injection (Fig. 4B and Supplementary Fig. 1). By contrast, robust and widespread GFP expression was detected in the SAHA+ Atoh1 group (Fig. 4C). The GFP transduction efficiency differed significantly between those two groups (Fig. 4D and Supplementary Table 1; 46.53 ± 4.54% vs. 68.66 ± 3.51%, P < 0.01 by t-test), suggesting that SAHA administration enhances the AAV8-mediated overexpression of exogenous genes in FE.

3.4. Atoh1 overexpression plus SAHA promotes myosin VIIa expression in FE but does not induce functional recovery

Myosin VIIa-positive cells were enumerated in FE (Fig. 5A–F and Supplementary Table 2). There were no significant differences in the number of myosin VIIa-positive cells among the Atoh1 (3.91 ± 1.85), SAHA + Atoh1 (17.08 ± 7), SAHA (0.88 ± 0.44), and control (3.60 ± 2.29) groups by one-way ANOVA. However, variation was found among samples from the SAHA + Atoh1 group (Fig. 5B, C, F, and Supplementary Table 2): 6 of the 13 samples showed a larger number of myosin VIIa-positive cells (35.5 ± 11.33) than the other 7 samples (1.29 ± 0.47). This suggested that SAHA was effective for those six samples, which were defined as the SAHA + Atoh1(e) group (Fig. 5B), but noneffective in the other seven samples (SAHA + Atoh1[n] group, Fig. 5C). The number of myosin VIIa-positive cells in the SAHA + Atoh1(e) group was significantly greater than that in the Atoh1, SAHA, and control groups (Fig. 5F). No significant difference in the number of myosin VIIa-positive cells was found between the SAHA and control groups (Fig. 5F). Nevertheless, no significant difference in swim test scores was detected among the SAHA + Atoh1 (2.3 ± 0.15), Atoh1 (2.78 ± 0.15) and control (2.8 ± 0.2) groups by one-way ANOVA (Fig. 5G), suggesting that either Atoh1 overexpression or Atoh1 overexpression plus SAHA does not restore the swimming ability of the mice.

High-magnification images indicated differences among the groups in the features of myosin VIIa-positive cells (Fig. 6). In the Atoh1 group (Fig. 6A–A0), myosin VIIa-positive cells had one or several cytoplasmic processes. They were GFP-negative, and their morphology was distinct from that of FE cells. By contrast, myosin VIIa-positive cells in the SAHA + Atoh1(e) group (Fig. 6B–B0 and Supplementary Fig. 2) were co-labeled with GFP and had a cuboidal or trapezoidal shape, which resembled that of FE cells. Those myosin VIIa-positive cells had no hair bundles. Myosin VIIa-positive cells in the SAHA (Fig. 6C) and control (Fig. 6D) groups exhibited similar features to those of the Atoh1 group, but no GFP expression was observed.

3.5. SAHA increases the Atoh1 mRNA level after AAV8 injection

The Atoh1 mRNA level was examined by qRT-PCR and compared among the Atoh1, SAHA + Atoh1, and control groups at 4 weeks after Atoh1 injection. As shown in Fig. 7, the Atoh1 mRNA level was significantly elevated in the SAHA + Atoh1 group compared to the Atoh1 and control groups (150.05 ± 32.68 vs. 52.81 ± 11.87,
We detected a small number of tdTomato-positive HCs in FE of Glut3-CreER T2:Rosa26 tdTomato mice in which tdTomato was exclusively expressed in vestibular HCs (Fig. 1, Tables 1 and 2), suggesting that a few HCs survive the lesion induced by a high dose of streptomycin. In FE of GLAST-CreER T2:Rosa26 tdTomato and Plp-CreER T2:Rosa26 tdTomato mice, a few HCs were labeled by tdTomato (Figs. 2 and 3 and Table 2). Whether those tdTomato-positive HCs survived or regenerated is unclear, because both GLAST-CreER T2:Rosa26 tdTomato and Plp-CreER T2:Rosa26 tdTomato mice had a small number of tdTomato-positive HCs prior to damage (Table 1) (Bucks et al., 2017; Stone et al., 2018). Alternatively, it is possible that the small number of HCs in these two lines could have formed from tdTomato-positive SCs, as part of ongoing HC turnover, but not regeneration, as in the normal utricle (Bucks et al., 2017). Therefore, whether spontaneous HC regeneration occurs in the vestibular FE requires further investigation.

The cellular origin of FE cells is heterogeneous. SCs are a source of FE cells in vestibular sensory organs, because some flat cells are myosin VIIa-negative/Sox2-positive (Wang et al., 2017). Our data showed that only a small portion of cells expressed tdTomato in the FE of GLAST-CreER T2:Rosa26 tdTomato and Plp-CreER T2:Rosa26 tdTomato mice (Figs. 2 and 3 and Table 2), suggesting that FE does not share important properties of SCs (broad GLAST-Cre or Plp-Cre expression). We observed more tdTomato-labeled cells in the FE of Plp-CreER T2:Rosa26 tdTomato mice than that of GLAST-CreER T2:Rosa26 tdTomato mice (Figs. 2 and 3 and Table 2). Because more TE cells were labeled in Plp-CreER T2:Rosa26 tdTomato mice prior to damage (Figs. 2 and 3 and Table 1), we speculate that TE cells migrate into the adjacent sensory epithelium region and become flat cells after lesion induction, similar to the FE of chicken (Cotanche et al., 1995). In addition, as Plp is also expressed in Schwann cells of the inner ear (Bucks et al., 2017; Morris et al., 2006), Schwann cells may be another source of flat cells. Therefore, specific CreER lines are needed for fate-mapping of TE or Schwann cells and to determine whether they are the origin of flat cells. Conditional deletion of the floxed Sox10 gene with Wnt1-cre results in loss of Schwann cells of the inner ear in Wnt1-Cre: Sox10fl/fl mice (Mao et al., 2014), which may serve as a useful mouse model for investigating the role of Schwann cell in FE formation.

There are other possible reasons for the lower expression of tdTomato in the FE of GLAST-CreER T2:Rosa26 tdTomato mice than Plp-CreER T2:Rosa26 tdTomato mice. SCs may survive damage, but the damage may induce downregulation of GLAST-Cre expression and loss of tdTomato protein. By contrast, Plp-Cre expression may be maintained, so new tdTomato protein is made in SCs in this mouse line. In addition, GLAST-Cre-expressing SCs may die after streptomycin treatment but Plp-Cre-expressing SCs may survive, resulting in loss of tdTomato in only GLAST-Cre mouse. Further work is required to clarify the underlying mechanism.

Atoh1, previously known as Math1, is essential for the generation of differentiated HCs (Bermingham et al., 1999; Fritzsch et al., 2005). In the mature vestibular sensory epithelium, Atoh1 expression is upregulated in SCs during spontaneous HC regeneration after damage (Golub et al., 2012; Hicks et al., 2020; Lin et al., 2011; Wang et al., 2010). Forced expression of Atoh1 in the SCs of damaged vestibular sensory epithelium induces their transdifferentiation into HCs and improves the balance function (Sayyid et al., 2019; Schlecker et al., 2011; Staeker et al., 2007).

P < 0.05; 150.05 ± 32.68 vs. 1.0 ± 0.06, P < 0.001 by one-way ANOVA followed by Bonferroni’s multiple comparisons test).

4. Discussion

We detected a small number of tdTomato-positive HCs in FE of vGlut3-CreER T2:Rosa26 tdTomato mice, which was treated with tamoxifen. (B) High-magnification image of the dotted square in (A) shows tdTomato labeling of SCs but not HCs. (C) High-magnification image of the dotted square in (A) shows transitional epithelial cells expressing tdTomato. (D-F) Lineage tracing of tdTomato-positive cells in flat epithelium (FE), (D) A number of tdTomato-positive flat cells are present; Inset: the tdTomato channel of the image. (E) High-magnification image of the dotted square in (D) showing a tdTomato-positive HC (arrow) and tdTomato-negative HCs (arrowhead). (F) High-magnification image of the solid square in (D) showing tdTomato-positive flat cells. Scale bar, 50 μm (A and inset to D) and 20 μm (B). Scale bar in (A) applies to (A) and (D). Scale bar in (B) applies to (B), (C), (E) and (F).
The FE differs from the damaged state described above, as SCs, in addition to HCs, are damaged. Our data showed that Atoh1 overexpression did not impact the number of myosin-VIIa positive cells in vestibular FE (Fig. 5 and Supplementary Table 2). As the myosin VIIa-positive cells in the Atoh1 group were GFP negative, and they morphologically resembled HCs in FE of the control group (Fig. 6D) and transgenic mice (Figs. 1E, 2E and 3E), they were likely surviving rather than regenerated HCs. Over-expression of Atoh1 plus SAHA induced some flat cells to become myosin VIIa-positive cells, indicating a transition towards HCs. Those cells had a similar morphology to the original flat cells, with irregular large cell bodies and no cytoplasmic processes (Fig. 6 and Supplementary Fig. 2). Because those myosin VIIa-positive cells have different features than spontaneously regenerated vestibular HCs, which exhibit multipolar cell bodies and short bundles (Golub et al., 2012), whether they can transition into bona fide HC remains uncertain. Previous work has shown that Atoh1 deletion ablates almost all HCs in Atoh1 null mice, except that some undifferentiated precursors remain and express Atoh1 and BDNF, a marker for HCs in embryos (Bermingham et al., 1999; Fritzsch et al., 2005). In Atoh1 conditional knockout mice, some remaining cells express myosin VIIa and attract nerve fibers, but do not differentiate normal stereocilia (Pan et al., 2011, 2012). Pou4f3 (formerly, Brn3c) null mutants show a few immature HC, expressing myosin VIIa, and long term retention of afferents in the inner ear (Xiang et al., 2003). Therefore, HC differentiation requires an essential set of genes, including Atoh1, Pou4f3, Gfi1, and miRNA-183 (Jahan et al., 2015; Pauley et al., 2008). Future work on manipulating multiple genes or signaling pathways could further help to generate differentiated HCs in vestibular FE (Burns and Stone, 2017; Jahan et al., 2015; Pauley et al., 2008; Wu et al., 2016).

The AAV8 vector exhibits strong tropism for the normal or moderately damaged vestibular sensory epithelium (Guo et al., 2017; Isgrig et al., 2017; Wang et al., 2014); however, in the FE, the transduction efficiency of AAV8 was not satisfactory (Fig. 4 and Supplementary Fig. 1) and the number of myosin VIIa-positive cells did not increase in the absence of SAHA (Fig. 5 and Supplementary Table 2). When SAHA was administered with AAV8-Atoh1-GFP, the GFP transduction efficiency and Atoh1 mRNA level were markedly increased, and the number of myosin VIIa-positive cells increased in 6 of 13 samples (Figs. 4e–7 and Supplementary Tables 1–2), suggesting that cell transdifferentiation due to Atoh1 overexpression depends on the magnitude of the expression. By contrast, the other seven samples showed no increase in the number of myosin VIIa-positive cells (Fig. 5 and Supplementary Table 2). We speculated that the level of overexpression of Atoh1 in those samples was insufficient to trigger transdifferentiation. The variation may be due to differences among animals in the transduction efficiency of AAV8 or absorption of SAHA. Cochlear FE reportedly does not respond to Atoh1 overexpression (Izumikawa et al., 2008). There are several possible reasons for the discrepancy with our finding. First, vestibular sensory epithelium maintains a limited capacity for spontaneous HC
regeneration after damage, whereas the mammalian cochlea fails to regenerate new HCs after the onset of hearing (Cox et al., 2014; Liu et al., 2012; Oesterle, 2013). These findings suggest that the properties of vestibular FE may differ from those of cochlear FE. Second, the regenerative response of the utricle is associated with a more accessible chromatin structure in vestibular SCs than in cochlear SCs (Jen et al., 2019). Third, different viral vectors (AAV vs. adenovirus), injection approaches (canalostomy vs. scala media) and SAHA treatment (with SAHA vs. without SAHA) were used in the two studies, which may have resulted in different expression levels of the exogenous gene. Determining how the cochlear FE responds to Atoh1 overexpression plus SAHA treatment is an interesting subject for future research.

HDAC inhibitors can improve the transduction efficiency of viral vectors in various cell types (Danielsson et al., 2011; Kitazono et al., 2001; Taura et al., 2004). HDAC inhibitors enhance the levels of viral receptors on the cell surface (Okada et al., 2006) and boost gene expression in cancer cells in a manner dependent on the promoter (Kia et al., 2013), indicating that such compounds enhance exogenous gene expression by multiple mechanisms. In cultured inner-ear tissue, an HDAC inhibitor improves adenoviral vector transduction (Taura et al., 2010). We showed here for the first time that an HDAC inhibitor enhanced AAV-mediated gene transfer in the inner ear in vivo. We speculate that SAHA augments viral transduction, enhances transcription of the Atoh1 transgene, increases Atoh1 transcript and protein stability, and/or curtails death of transduced FE cells. The underlying mechanism needs to be investigated. Furthermore, SAHA is the first HDAC inhibitor to be approved by the United States Food and Drug Administration for clinical use in cancer treatment (Xu et al., 2007). Following systemic administration, SAHA crosses the blood-labyrinth barrier and protects against the HC loss and hearing impairment caused by ototoxic drugs or noise (Chen et al., 2016; Layman et al., 2015). Therefore, SAHA will facilitate inner-ear gene therapy and otoprotection studies.

HDAC inhibitors are negative regulators of HC regeneration in the zebrafish lateral line and chick utricle (He et al., 2014; Slattery et al., 2009). In this study, there was no significant difference in the number of myosin VIIa-positive cells between the SAHA and the control groups (Fig. 5 and Supplementary Table 2). This suggests that SAHA alone likely has no effect on myosin VIIa expression in the mouse FE.

The major limitation of this study is that some but not all samples in the SAHA + Atoh1 group showed an increased number of myosin VIIa-positive cells, but no behavioral improvement (Fig. 5G). Those myosin VIIa-positive flat cells had no hair bundles and were morphologically different from mature HCs, which may explain the lack of restoration of vestibular function. Further studies should focus on generating functional HCs in FE. Our data reveal that vestibular FE cells can be induced to undergo
transdifferentiation into myosin VIIa-positive cells by over-expression of Atoh1 and SAHA treatment. SAHA markedly increases the expression levels of exogenous genes in vestibular FE, which will facilitate studies of gene therapy for the inner ear.

5. Conclusions

Our findings demonstrate that a few HCs survive in vestibular FE induced by a high dose of streptomycin. FE cells do not show broad GLAST-Cre or Plp-Cre expression, unlike the original SCs. SCs and TE cells are potential sources of vestibular FE. SAHA markedly increases AAV8-mediated overexpression of exogenous genes in vestibular FE. Atoh1 overexpression plus SAHA administration induces vestibular flat cells to become myosin VIIa-positive cells, which will facilitate further studies of gene therapy in vestibular FE.

CRediT authorship contribution statement


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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heares.2020.107953.

References


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