



Research Paper

Cellular origin and response of flat epithelium in the vestibular end organs of mice to *Atoh1* overexpressionLu He, Jing-Ying Guo, Teng-Fei Qu, Wei Wei, Ke Liu, Zhe Peng, Guo-Peng Wang^{**}, Shu-Sheng Gong^{*}

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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-iCreER^{T2}:Rosa26^{tdTomato}*, *GLAST-CreER^{T2}:Rosa26^{tdTomato}*, and *Plp-CreER^{T2}:Rosa26^{tdTomato}*) 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 Yamasoba, 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 (Brosel 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,

Abbreviations: AAV, adeno-associated virus; ANOVA, analysis of variance; *Atoh1*, atonal homolog 1; FE, flat epithelium; GFP, green fluorescent protein; GLAST, glutamate aspartate transporter; HC, hair cell; HDAC, histone deacetylase; PBS, phosphate-buffered saline; Plp, proteolipid protein; SC, supporting cell; SAHA, suberoylanilide hydroxamic acid; TMX, tamoxifen; TE, transitional epithelium; vGlut3, vesicular glutamate transporter 3

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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 aminoglycosides. 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 Bermingham-McDonogh, 2013; Wang et al., 2015). During spontaneous HC regeneration in damaged vestibular organs of adult rodents, *atoh1* homolog 1 (*Atoh1*), a basic helix-loop-helix transcription factor required for HC development (Bermingham 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 transdifferentiation 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^{T2}:Rosa26^{tdTomato}* mice (Li et al., 2018), *GLAST-CreER^{T2}:Rosa26^{tdTomato}* mice (Stone et al., 2018) and *Plp-CreER^{T2}:Rosa26^{tdTomato}* 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^{T2}* mice were provided by Dr. Zhiyong Liu (Chinese Academy of Sciences, Shanghai, China) (Li et al., 2018). *GLAST-CreER^{T2}* mice were purchased from the Jackson Laboratory (Bar Harbor, ME, US; stock number, 012586) (Stone et al., 2018). *Plp-CreER^{T2}* mice were provided by Dr. Guoqiang Wan (Model Animal Research Center of Nanjing University, Nanjing, China) (Wan and Corfas, 2017).

Rosa26^{tdTomato} 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^{T2}:Rosa26^{tdTomato}* mice, male *vGlut3-iCreER^{T2}* mice were crossed with female *Rosa26^{tdTomato}* 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^{T2}:Rosa26^{tdTomato}* and *Plp-CreER^{T2}:Rosa26^{tdTomato}* mice, male *GLAST-CreER^{T2}* and *Plp-CreER^{T2}* mice, respectively, were crossed with female *Rosa26^{tdTomato}* 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^{12} vg/mL (AAV8-*Atoh1*-GFP) and 2×10^{12} 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 (S9137, 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 + *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 (ZSGB-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 VIIa 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 647-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-iCreER^{T2}; Rosa26^{tdTomato}* mice, *GLAST-CreER^{T2}; Rosa26^{tdTomato}* mice, and *Plp-CreER^{T2}; Rosa26^{tdTomato}* mice), we captured images using a 63 × objective lens with a 2 × 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 × 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 all 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-CreER^{T2}; Rosa26^{tdTomato}* mice and *Plp-CreER^{T2}; Rosa26^{tdTomato}* mice), three views (90 μm × 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 VIIa-positive cells, GFP-positive cells, and FE cells in the utricular FE, we captured images of the whole utricle using a 20 × objective lens with a 0.9 × 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 $2^{-\Delta\Delta CT}$ 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 VIIa-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-iCreER^{T2}; Rosa26^{tdTomato}* mice were used for lineage tracing of vestibular HCs following lesion generation. In normal *vGlut3-iCreER^{T2}; Rosa26^{tdTomato}* mice (Fig. 1A–C and Table 1), TMX administration induced tdTomato expression in approximately half of vestibular HCs ($53.06 \pm 4.12\%$). 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-iCreER^{T2}; Rosa26^{tdTomato}* mice (Fig. 1D–F and Table 2), a few HCs ($5.42 \pm 2.92\%$) 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-CreER^{T2}; Rosa26^{tdTomato}* mice (Fig. 2A–C and Table 1), TMX induced tdTomato expression in most SCs ($73.15 \pm 2.49\%$), a few HCs ($0.32 \pm 0.16\%$), and cells in the TE area ($1.83 \pm 0.51\%$) of the vestibular sensory epithelium. After lesion generation (Fig. 2D–F and Table 2), some HCs ($33.33 \pm 20.41\%$) and a small proportion of flat cells ($1.46 \pm 0.88\%$) expressed tdTomato.

In normal *Plp-CreER^{T2}; Rosa26^{tdTomato}* mice (Fig. 3A–C and Table 1), tdTomato labeled a majority of SCs ($58.42 \pm 4.62\%$) and a few HCs ($0.28 \pm 0.2\%$), comparable to in *GLAST-CreER^{T2}; Rosa26^{tdTomato}* mice (Fig. 2A–C and Table 1). However, a higher proportion of tdTomato-positive TE cells was detected in *Plp-CreER^{T2}; Rosa26^{tdTomato}* mice than *GLAST-CreER^{T2}; Rosa26^{tdTomato}* mice (Table 1; $29.68 \pm 5.7\%$ vs. $1.83 \pm 0.51\%$, $P < 0.01$ by *t*-test). In FE (Fig. 3D–F and Table 2), few HCs were tdTomato-positive ($1.37 \pm 0.94\%$). The proportion of tdTomato-positive flat cells was higher in the FE of *Plp-CreER^{T2}; Rosa26^{tdTomato}* mice than in that of *GLAST-CreER^{T2}; Rosa26^{tdTomato}* mice (Table 2; $17.33 \pm 3.71\%$ vs. $1.46 \pm 0.88\%$, $P < 0.01$ 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 intraperitoneally administered for 8 consecutive days, starting 12 days after

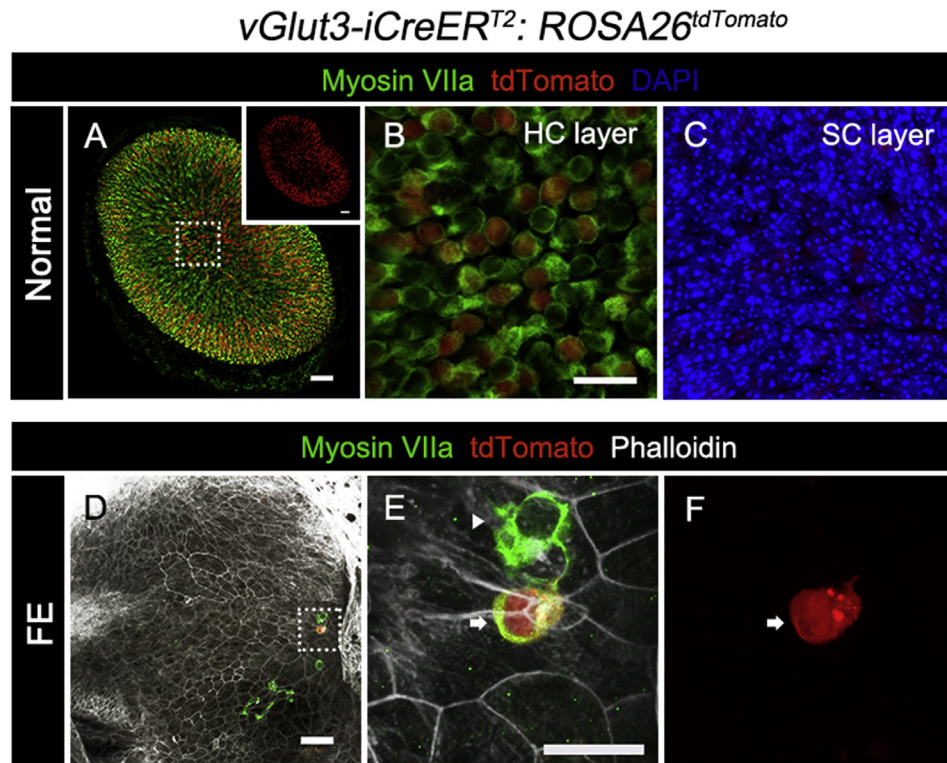


Fig. 1. Cre activity and lineage tracing of hair cells (HCs) in *vGlut3-iCreER^{T2}:Rosa26^{tdTomato}* mice. (A–C) Confocal images of normal utricles showing the distribution of tdTomato in the utricular sensory epithelium of mice treated with tamoxifen. Inset: the tdTomato channel of the image in (A). (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 μ m (A, inset to A, and D) and 20 μ m (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.

Normal		<i>vGlut3-iCreER^{T2}: Rosa26^{tdTomato}</i> (n = 3) ^a	<i>GLAST-CreER^{T2}:Rosa26^{tdTomato}</i> (n = 3) ^a	<i>Plp-CreER^{T2}:Rosa26^{tdTomato}</i> (n = 3) ^a
Hair cell (HC) layer	Supporting cell (SC)			
	HC number ^b	445 $\pm$ 42.34	515 $\pm$ 26.21	568.33 $\pm$ 34.67
	Labeled HC number ^b	239.33 $\pm$ 41.38	1.67 $\pm$ 0.88	1.67 $\pm$ 1.2
	% of HCs labeled	53.06 $\pm$ 4.12%	0.32 $\pm$ 0.16%	0.28 $\pm$ 0.2%
	SC number ^b	—	1086.67 $\pm$ 58.17	1048.67 $\pm$ 81.14
Transitional epithelium (TE)	Labeled SC number ^b	0	797.67 $\pm$ 70.07	609.33 $\pm$ 48.79
	% of SCs labeled	0	73.15 $\pm$ 2.49%	58.42 $\pm$ 4.62%
	TE cell number ^c	—	337 $\pm$ 29.02	360.33 $\pm$ 17.85
	Labeled TE cell number ^c	0	6.33 $\pm$ 2.03	107 $\pm$ 22.23
	% of TE cells labeled	0	1.83 $\pm$ 0.51%	29.68 $\pm$ 5.7%

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

^b Cell counts in six randomly selected views (90 μ m $\times$ 90 μ m per view) in the sensory-epithelium area were summed for each utricle.

^c Cell counts in three randomly selected views (90 μ m $\times$ 90 μ m per view) in the TE area were summed for each utricle.

Table 2
Quantification of tdTomato-labeled cells in the utricular flat epithelium of transgenic mice.

Flat epithelium	<i>vGlut3-iCreER^{T2}: Rosa26^{tdTomato}</i> (n = 3) ^a	<i>GLAST-CreER^{T2}:Rosa26^{tdTomato}</i> (n = 4) ^a	<i>Plp-CreER^{T2}:Rosa26^{tdTomato}</i> (n = 6) ^a
HC number	10 $\pm$ 3.46	2 $\pm$ 1.41	9.33 $\pm$ 6.44
Labeled HC number	0.67 $\pm$ 0.33	1.5 $\pm$ 1.19	0.33 $\pm$ 0.21
% of HCs labeled	5.42 $\pm$ 2.92%	33.33 $\pm$ 20.41%	1.37 $\pm$ 0.94%
Flat cell number	—	564 $\pm$ 37.79	637 $\pm$ 49
Labeled flat cell number	0	8.25 $\pm$ 5.11	110.33 $\pm$ 26
% of flat cells labeled	0	1.46 $\pm$ 0.88%	17.33 $\pm$ 3.71%

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

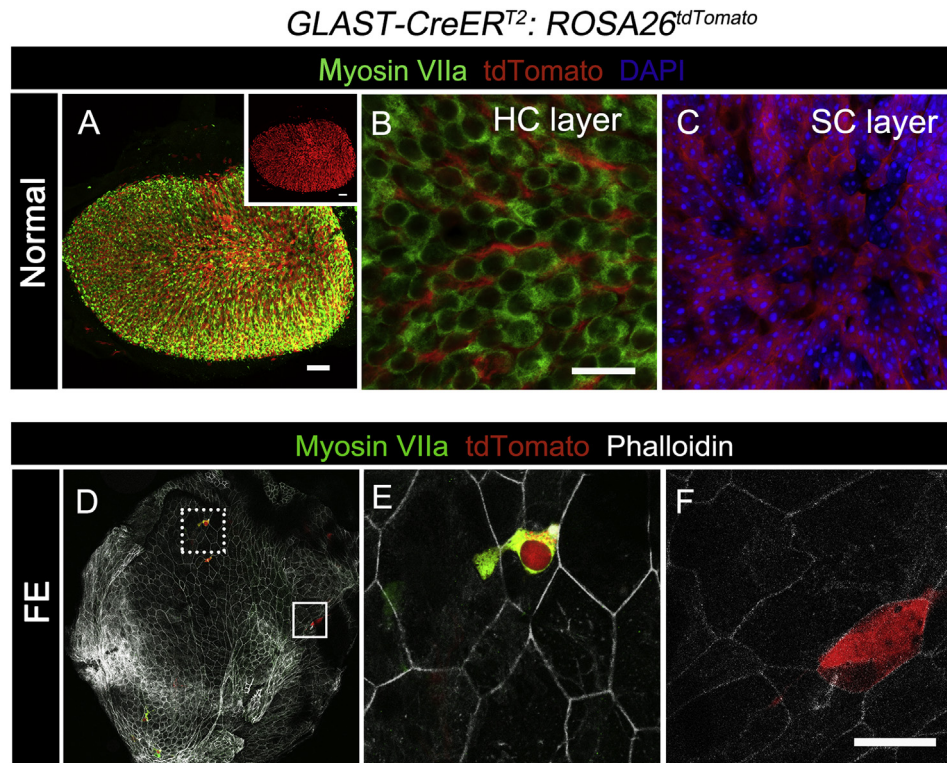


Fig. 2. Cre activity and lineage tracing in *GLAST-CreER^{T2};Rosa26^{tdTomato}* mice. (A–C) Confocal images of normal utricles showing the distribution of tdTomato in the utricular sensory epithelium of mice treated with tamoxifen. (A) Low-magnification image showing a large portion of cells labeled with tdTomato. Inset: the tdTomato channel of the image in (A). (B–C) High-magnification images of the hair cell (HC) layer and supporting cell (SC) layer showing SCs, but not HCs, labeled with tdTomato. (D–F) Lineage tracing of SCs in flat epithelium (FE). (D) A few tdTomato-positive cells are present. (E) High-magnification image of the dotted square in (D) shows a tdTomato-positive HC. (F) High-magnification image of the solid square in (D) shows a tdTomato-positive flat cell. Scale bars, 50 μ m (A and inset to A), 20 μ m (B and F). Scale bar in (A) applies to (A) and (D). Scale bar in (B) applies to (B), (C) and (E).

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; $46.53 \pm 4.54\%$ vs. $68.66 \pm 3.51\%$, $P < 0.01$ 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–A'), 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–B' 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 ,

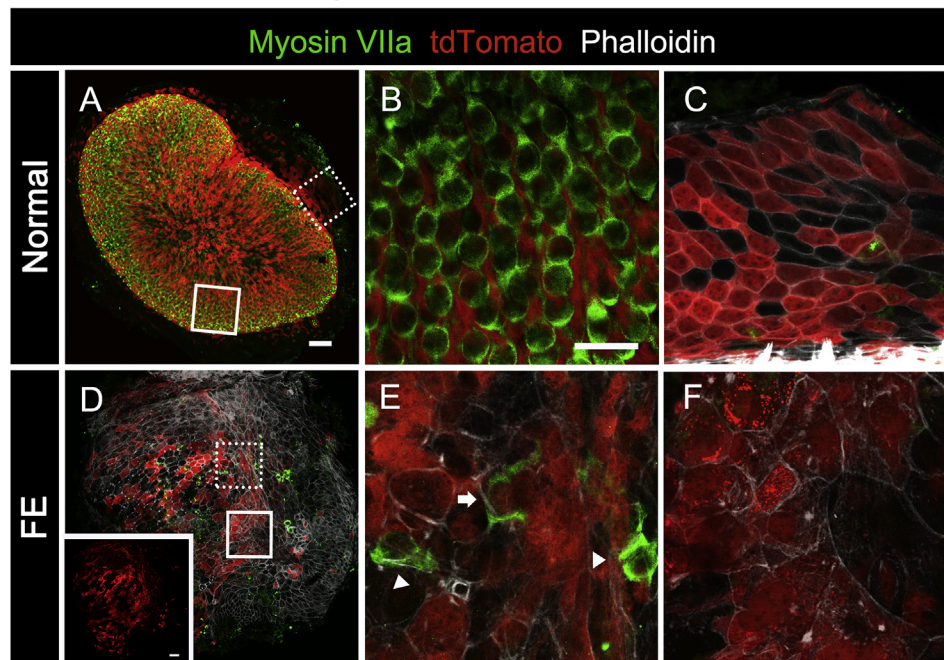
Plp-CreER^{T2}: Rosa26^{tdTomato}

Fig. 3. Cre activity and lineage tracing in *Plp-CreER^{T2}:Rosa26^{tdTomato}* mice. (A–C) Confocal images showing the distribution of tdTomato in the normal utricles of mice treated with tamoxifen. (B) High-magnification image of the solid 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 bars, 50 μ m (A and inset to D) and 20 μ m (B). Scale bar in (A) applies to (A) and (D). Scale bar in (B) applies to (B), (C), (E) and (F).

$P < 0.05$; 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-iCreER^{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}* mice 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 than in *GLAST-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: Sox10^{fl/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 mice. 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; Staecker et al., 2007,

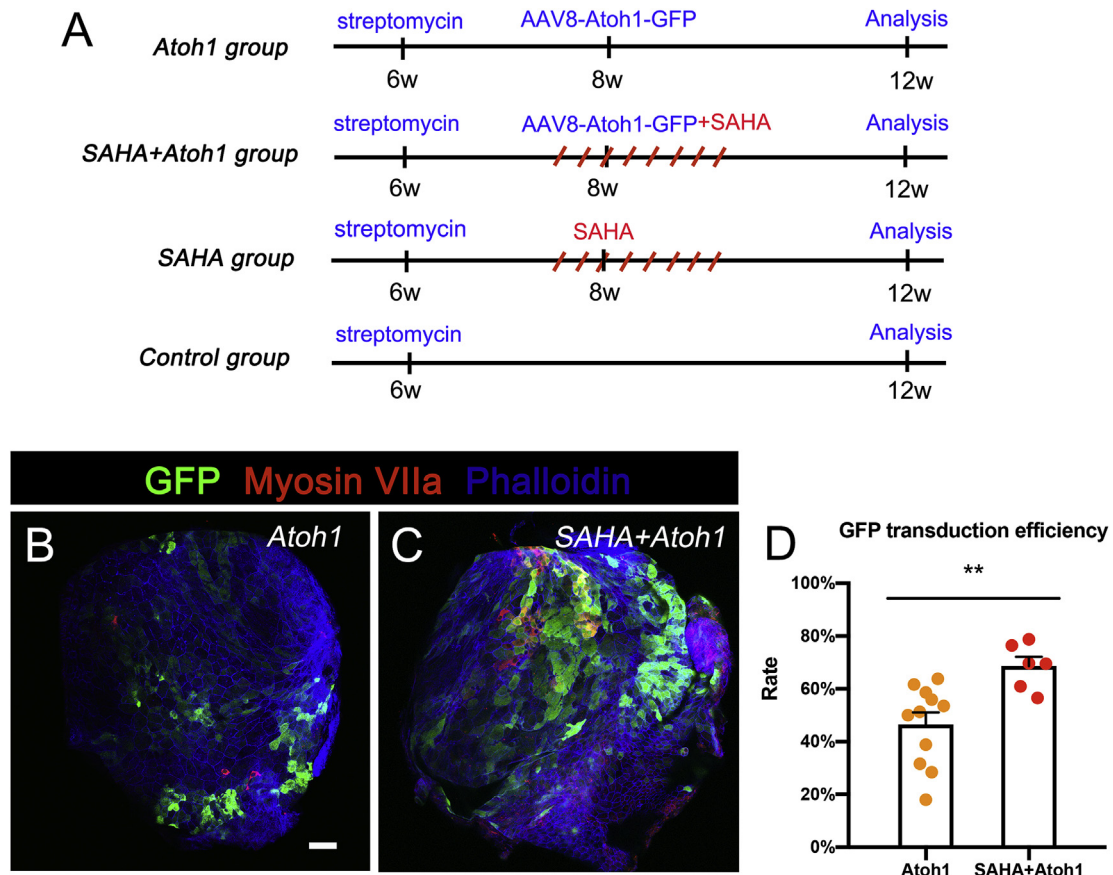


Fig. 4. SAHA enhances the efficiency of AAV8 transduction into the utricular flat epithelium (FE). (A) Treatment flowchart: AAV8-Atoh1-GFP (Atoh1), SAHA + AAV8-Atoh1-GFP (SAHA + Atoh1), SAHA, and control groups. (B) Representative whole mount of FE of the Atoh1 group showing scattered GFP expression. (C) Representative whole mount of FE of the SAHA + Atoh1 group showing widespread GFP expression. (D) The GFP transduction efficiency of the SAHA + Atoh1 group is significantly higher than that of the Atoh1 group. ** $P < 0.01$ by t -test. Scale bar, for B-C, represents 50 μm.

2014). 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. Overexpression 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 VII-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* HCs is unclear. Previous work has showed 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. 4–7 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

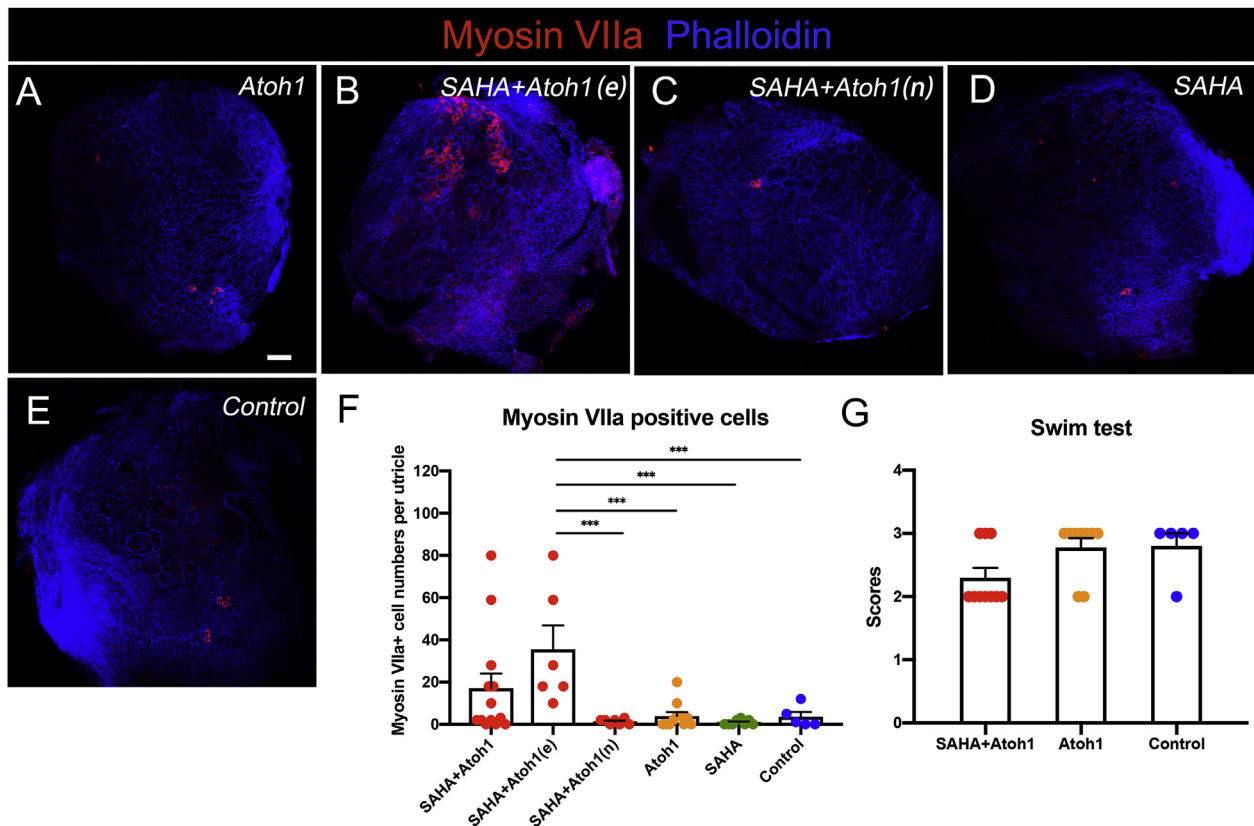


Fig. 5. *Atoh1* overexpression plus SAHA promotes myosin VIIa expression in the utricular flat epithelium (FE) of some samples but does not induce functional recovery. (A-E) Representative whole mounts of FE showing myosin VIIa-positive cells in the Atoh1 (A), SAHA + Atoh1 (B-C), SAHA (D), and control (E) groups. In the SAHA + Atoh1 group (B-C), numerous myosin VIIa-positive cells are present in 6 of 13 samples in which SAHA appears effective (SAHA + Atoh1[e]) (B); however, few myosin VIIa-positive cells are present in the other 7 samples in which SAHA shows noneffective (SAHA + Atoh1[n]) (C). (F) Enumeration of myosin VIIa-positive cells. The number of myosin VIIa-positive cells in the SAHA + Atoh1(e) group is significantly greater than that in the SAHA + Atoh1(n), Atoh1, SAHA, and control groups. *** $P < 0.001$ by one-way ANOVA followed by Bonferroni's multiple comparisons test. (G) Swim test scores do not differ significantly among the groups by one-way ANOVA. Scale bar, for A-E, represents 50 μ m.

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

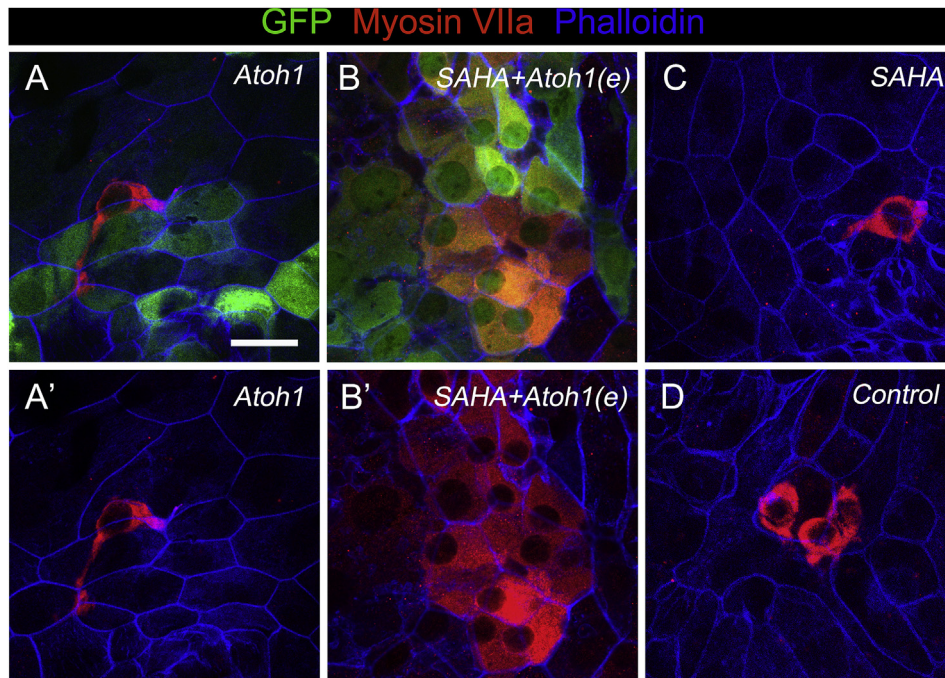


Fig. 6. High-magnification images of the Atoh1, SAHA + Atoh1(e), SAHA, and control groups. (A-A') In flat epithelium (FE) of the Atoh1 group, a myosin VIIa-positive cell has two cytoplasmic processes, and it does not resemble FE cells morphologically. (B-B') Myosin VIIa-positive cells in the SAHA + Atoh1(e) group are co-labeled with GFP and had a cuboidal or trapezoidal shape similar to that of FE cells. (C-D) Myosin VIIa-positive cells in the SAHA (C) and control (D) groups show similar features to those in the Atoh1 group (A-A') but lack GFP expression. Scale bar, for all images, represents 20 μ m.

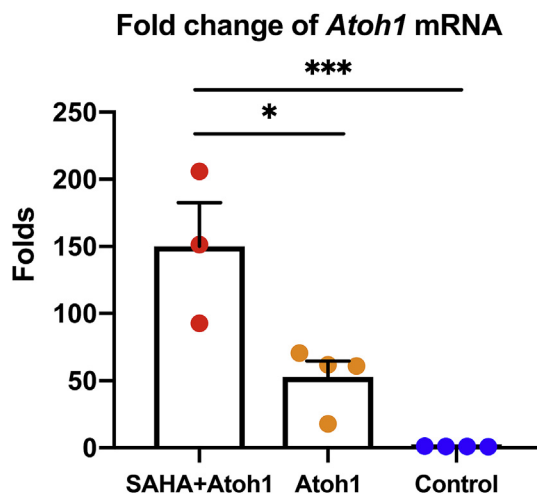


Fig. 7. *Atoh1* mRNA levels in the SAHA + Atoh1, Atoh1, and control groups. * $P < 0.05$ and *** $P < 0.001$ by one-way ANOVA followed by Bonferroni's multiple comparisons test.

transdifferentiation into myosin VIIa-positive cells by overexpression 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

Lu He: Conceptualization, Methodology, Writing - original draft, Software. **Jing-Ying Guo:** Formal analysis, Data curation. **Teng-Fei Qu:** Investigation, Formal analysis. **Wei Wei:** Investigation. **Ke Liu:** Software, Validation. **Zhe Peng:** Data curation, Validation. **Guo-Peng Wang:** Conceptualization, Visualization, Writing - review & editing. **Shu-Sheng Gong:** Conceptualization, Writing - review & editing, Supervision.

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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

- Bermingham, N., Hassan, B., Price, S., Vollrath, M., Ben-Arie, N., Eatock, R., Bellen, H., Lysakowski, A., Zoghbi, H., 1999. Math1: an essential gene for the generation of inner ear hair cells. *Science* 284, 1837–1841.
- Brandt, T., Huppert, T., Hufner, K., Zingler, V.C., Dieterich, M., Strupp, M., 2010. Long-term course and relapses of vestibular and balance disorders. *Restor. Neurol. Neurosci.* 28, 69–82.

- Brosel, S., Laub, C., Averdam, A., Bender, A., Elstner, M., 2016. Molecular aging of the mammalian vestibular system. *Ageing Res. Rev.* 26, 72–80.
- Bucks, S.A., Cox, B.C., Vlosich, B.A., Manning, J.P., Nguyen, T.B., Stone, J.S., 2017. Supporting cells remove and replace sensory receptor hair cells in a balance organ of adult mice. *Elife* 6, e18128.
- Burns, J.C., Stone, J.S., 2017. Development and regeneration of vestibular hair cells in mammals. *Semin. Cell Dev. Biol.* 65, 96–105.
- Chen, J., Hill, K., Sha, S.H., 2016. Inhibitors of histone deacetylases attenuate noise-induced hearing loss. *J. Assoc. Res. Otolaryngol.* 17, 289–302.
- Cotanche, D., Messina, E., Ofstie, M., 1995. Migration of hyaline cells into the chick basilar papilla during severe noise damage. *Hear. Res.* 91, 148–159.
- Cox, B.C., Chai, R., Lenoir, A., Liu, Z., Zhang, L., Nguyen, D.H., Chalasani, K., Steigelman, K.A., Fang, J., Rubel, E.W., Cheng, A.G., Zuo, J., 2014. Spontaneous hair cell regeneration in the neonatal mouse cochlea in vivo. *Development* 141, 816–829.
- Danielsson, A., Dzojic, H., Rashkova, V., Cheng, W.S., Essand, M., 2011. The HDAC inhibitor FK228 enhances adenoviral transgene expression by a transduction-independent mechanism but does not increase adenovirus replication. *PLoS One* 6, e14700.
- Fan, C., Zou, S., Wang, J., Zhang, B., Song, J., 2016. Neomycin damage and regeneration of hair cells in both mechanoreceptor and electroreceptor lateral line organs of the larval Siberian sturgeon (*Acipenser baeri*). *J. Comp. Neurol.* 524, 1443–1456.
- Fernández, L., Breinbauer, H., Delano, P., 2015. Vertigo and dizziness in the elderly. *Front. Neurol.* 26, 144.
- Forge, A., Li, L., Nevill, G., 1998. Hair cell recovery in the vestibular sensory epithelia of mature Guinea pigs. *J. Comp. Neurol.* 397, 69–88.
- Forge, A., Li, L., Corwin, J., Nevill, G., 1993. Ultrastructural evidence for hair cell regeneration in the mammalian inner ear. *Science* 259, 1616–1619.
- Fritzsch, B., Matei, V., Nichols, D., Birmingham, N., Jones, K., Beisel, K., Wang, V., 2005. Atoh1 null mice show directed afferent fiber growth to undifferentiated ear sensory epithelia followed by incomplete fiber retention. *Dev. Dynam.* 233, 570–583.
- Gillespie, M., Minor, L., 1999. Prognosis in bilateral vestibular hypofunction. *Laryngoscope* 109, 35–41.
- Golub, J.S., Tong, L., Nguyen, T.B., Hume, C.R., Palmiter, R.D., Rubel, E.W., Stone, J.S., 2012. Hair cell replacement in adult mouse utricles after targeted ablation of hair cells with diphtheria toxin. *J. Neurosci.* 32, 15093–15105.
- Guo, J.Y., Liu, Y.Y., Qu, T.F., Peng, Z., Xie, J., Wang, G.P., Gong, S.S., 2017. Cochleovestibular gene transfer in neonatal mice by canalostomy. *Neuroreport* 28, 682–688.
- Guo, J.Y., He, L., Qu, T.F., Liu, Y.Y., Liu, K., Wang, G.P., Gong, S.S., 2018. Canalostomy as a surgical approach to local drug delivery into the inner ears of adult and neonatal mice. *JoVE* 135, e57351.
- Hardisty-Hughes, R.E., Parker, A., Brown, S.D., 2010. A hearing and vestibular phenotyping pipeline to identify mouse mutants with hearing impairment. *Nat. Protoc.* 5, 177–190.
- He, Y., Cai, C., Tang, D., Sun, S., Li, H., 2014. Effect of histone deacetylase inhibitors trichostatin A and valproic acid on hair cell regeneration in zebrafish lateral line neuromasts. *Front. Cell. Neurosci.* 8, 382.
- Hicks, K.L., Wisner, S.R., Cox, B.C., Stone, J.S., 2020. Atoh1 is required in supporting cells for regeneration of vestibular hair cells in adult mice. *Hear. Res.* 385, 107838.
- Hizli, O., Kaya, S., Schachern, P.A., Kwon, G., Paparella, M.M., Cureoglu, S., 2016. Quantitative assessment of vestibular otopathology in otosclerosis: a temporal bone study. *Laryngoscope* 126, E118–E122.
- Isgrig, K., Shteamer, J.W., Belyantseva, I.A., Drummond, M.C., Fitzgerald, T.S., Vijayakumar, S., Jones, S.M., Griffith, A.J., Friedman, T.B., Cunningham, L.L., Chien, W.W., 2017. Gene therapy restores balance and auditory functions in a mouse model of usher syndrome. *Mol. Ther.* 25, 780–791.
- Iwasaki, S., Yamasoba, T., 2015. Dizziness and imbalance in the elderly: age-related decline in the vestibular system. *Ageing Dis.* 6, 38–47.
- Izumikawa, M., Batts, S., Miyazawa, T., Swiderski, D., Raphael, Y., 2008. Response of the flat cochlear epithelium to forced expression of Atoh1. *Hear. Res.* 240, 52–56.
- Jahan, I., Pan, N., Fritzsch, B., 2015. Opportunities and limits of the one gene approach: the ability of Atoh1 to differentiate and maintain hair cells depends on the molecular context. *Front. Cell. Neurosci.* 9, 26.
- Jen, H.L., Hill, M.C., Tao, L., Sheng, K., Cao, W., Zhang, H., Yu, H.V., Llamas, J., Zong, C., Martin, J.F., Segil, N., Groves, A.K., 2019. Transcriptomic and epigenetic regulation of hair cell regeneration in the mouse utricle and its potentiation by Atoh1. *Elife* 8, e44328.
- Kawamoto, K., Izumikawa, M., Beyer, L.A., Atkin, G.M., Raphael, Y., 2009. Spontaneous hair cell regeneration in the mouse utricle following gentamicin ototoxicity. *Hear. Res.* 247, 17–26.
- Kia, A., Yata, T., Hajji, N., Hajitou, A., 2013. Inhibition of histone deacetylation and DNA methylation improves gene expression mediated by the adeno-associated virus/phage in cancer cells. *Viruses* 5, 2561–2572.
- Kim, Y.H., Raphael, Y., 2007. Cell division and maintenance of epithelial integrity in the deafened auditory epithelium. *Cell Cycle* 6, 612–619.
- Kitazono, M., Goldsmith, M.E., Aikou, T., Bates, S., Fojo, T., 2001. Enhanced adenovirus transgene expression in malignant cells treated with the histone deacetylase inhibitor FR901228. *Cancer Res.* 61, 6328–6330.
- Layman, W.S., Williams, D.M., Dearman, J.A., Saucedo, M.A., Zuo, J., 2015. Histone deacetylase inhibition protects hearing against acute ototoxicity by activating the NF-kappaB pathway. *Cell Death Dis.* 1, 15012.
- Li, C., Shu, Y., Wang, G., Zhang, H., Lu, Y., Li, X., Li, G., Song, L., Liu, Z., 2018. Characterizing a novel vGlut3-P2A-iCreER knockin mouse strain in cochlea. *Hear. Res.* 364, 12–24.
- Lin, V., Golub, J.S., Nguyen, T.B., Hume, C.R., Oesterle, E.C., Stone, J.S., 2011. Inhibition of notch activity promotes nonmitotic regeneration of hair cells in the adult mouse utricles. *J. Neurosci.* 31, 15329–15339.
- Liu, Z., Dearman, J.A., Cox, B.C., Walters, B.J., Zhang, L., Ayrault, O., Zindy, F., Gan, L., Roussel, M.F., Zuo, J., 2012. Age-dependent in vivo conversion of mouse cochlear pillar and deiters' cells to immature hair cells by Atoh1 ectopic expression. *J. Neurosci.* 32, 6600–6610.
- Mao, Y., Reiprich, S., Wegner, M., Fritzsch, B., 2014. Targeted deletion of Sox10 by Wnt1-cre defects neuronal migration and projection in the mouse inner ear. *PLoS One* 9, e94580.
- McCall, A.A., Ishiyama, G.P., Lopez, I.A., Bhuta, S., Vetter, S., Ishiyama, A., 2009. Histopathological and ultrastructural analysis of vestibular endorgans in Meniere's disease reveals basement membrane pathology. *BMC Ear Nose Throat Disord.* 9.
- Morris, J.K., Maklad, A., Hansen, L.A., Feng, F., Sorensen, C., Lee, K.-F., Macklin, W.B., Fritzsch, B., 2006. A disorganized innervation of the inner ear persists in the absence of ErbB2. *Brain Res.* 1091, 186–199.
- Nadol, J., Eddington, D., 2006. Histopathology of the inner ear relevant to cochlear implantation. *Adv. Oto-Rhino-Laryngol.* 64, 31–49.
- Oesterle, E.C., 2013. Changes in the adult vertebrate auditory sensory epithelium after trauma. *Hear. Res.* 297, 91–98.
- Okada, T., Uchibori, R., Iwata-Okada, M., Takahashi, M., Nomoto, T., Nonaka-Sarukawa, M., Ito, T., Liu, Y., Mizukami, H., Kume, A., Kobayashi, E., Ozawa, K., 2006. A histone deacetylase inhibitor enhances recombinant adeno-associated virus-mediated gene expression in tumor cells. *Mol. Ther.* 13, 738–746.
- Pan, N., Jahan, I., Kersigo, J., Duncan, J.S., Kopecky, B., Fritzsch, B., 2012. A novel Atoh1 "self-terminating" mouse model reveals the necessity of proper Atoh1 level and duration for hair cell differentiation and viability. *PLoS One* 7, e30358.
- Pan, N., Jahan, I., Kersigo, J., Kopecky, B., Santi, P., Johnson, S., Schmitz, H., Fritzsch, B., 2011. Conditional deletion of Atoh1 using Pax2-Cre results in viable mice without differentiated cochlear hair cells that have lost most of the organ of Corti. *Hear. Res.* 275, 66–80.
- Pauley, S., Kopecky, B., Beisel, K., Soukup, G., Fritzsch, B., 2008. Stem cells and molecular strategies to restore hearing. *Panminerva Med.* 50, 41–53.
- Rauch, S.D., Velazquez-Villasenor, L., Dimitri, P.S., Merchant, S.N., 2001. Decreasing hair cell counts in aging humans. *Ann. N. Y. Acad. Sci.* 942, 220–227.
- Sayyid, Z.N., Wang, T., Chen, L., Jones, S.M., Cheng, A.G., 2019. Atoh1 directs regeneration and functional recovery of the mature mouse vestibular system. *Cell Rep.* 28, 312–324.
- Schlecker, C., Praetorius, M., Brough, D.E., Presler Jr., R.G., Hsu, C., Plinkert, P.K., Staeker, H., 2011. Selective atonal gene delivery improves balance function in a mouse model of vestibular disease. *Gene Ther.* 18, 884–890.
- Slattery, E.L., Speck, J.D., Warchol, M.E., 2009. Epigenetic influences on sensory regeneration: histone deacetylases regulate supporting cell proliferation in the avian utricle. *J. Assoc. Res. Otolaryngol.* 10, 341–353.
- Slowik, A., Birmingham-McDonogh, O., 2013. Hair cell generation by notch inhibition in the adult mammalian cristae. *J. Assoc. Res. Otolaryngol.* 14, 813–828.
- Staeker, H., Praetorius, M., Baker, K., Brough, D., 2007. Vestibular hair cell regeneration and restoration of balance function induced by Math1 gene transfer. *Otol. Neurotol.* 28, 223–231.
- Staeker, H., Schlecker, C., Kraft, S., Praetorius, M., Hsu, C., Brough, D.E., 2014. Optimizing atoh1-induced vestibular hair cell regeneration. *Laryngoscope* 124 (Suppl. 5), S1–S12.
- Stone, J.S., Wisner, S.R., Bucks, S.A., Mellado Lagarde, M.M., Cox, B.C., 2018. Characterization of adult vestibular organs in 11 CreER mouse lines. *J. Assoc. Res. Otolaryngol.* 19, 381–399.
- Taura, A., Taura, K., Choung, Y.H., Masuda, M., Pak, K., Chavez, E., Ryan, A.F., 2010. Histone deacetylase inhibition enhances adenoviral vector transduction in inner ear tissue. *Neuroscience* 166, 1185–1193.
- Taura, K., Yamamoto, Y., Nakajima, A., Hata, K., Uchinami, H., Yonezawa, K., Hatano, N., Nishino, N., Yamaoka, Y., 2004. Impact of novel histone deacetylase inhibitors, CHAP31 and FR901228(FK228), on adenovirus-mediated transgene expression. *J. Gene Med.* 6, 526–536.
- Taylor, R.R., Filia, A., Paredes, U., Asai, Y., Holt, J.R., Lovett, M., Forge, A., 2018. Regenerating hair cells in vestibular sensory epithelia from humans. *Elife* 7, e34817.
- Teufert, K., Linthicum, F.J., Connell, S., 2006. The effect of organ of corti loss on ganglion cell survival in humans. *Otol. Neurotol.* 27, 1146–1151.
- Wan, G., Corfas, G., 2017. Transient auditory nerve demyelination as a new mechanism for hidden hearing loss. *Nat. Commun.* 8, 14487.
- Wang, G.P., Guo, J.Y., Peng, Z., Liu, Y.Y., Xie, J., Gong, S.S., 2014. Adeno-associated virus-mediated gene transfer targeting normal and traumatized mouse utricle. *Gene Ther.* 21, 958–966.
- Wang, G.P., Chatterjee, I., Batts, S.A., Wong, H.T., Gong, T.W., Gong, S.S., Raphael, Y., 2010. Notch signaling and Atoh1 expression during hair cell regeneration in the mouse utricle. *Hear. Res.* 267, 61–70.
- Wang, G.P., Basu, I., Beyer, L.A., Wong, H.T., Swiderski, D.L., Gong, S.S., Raphael, Y., 2017. Severe streptomycin ototoxicity in the mouse utricle leads to a flat epithelium but the peripheral neural degeneration is delayed. *Hear. Res.* 355, 33–41.
- Wang, T., Chai, R., Kim, G.S., Pham, N., Jansson, L., Nguyen, D.H., Kuo, B., May, L.A.,

- Zuo, J., Cunningham, L.L., Cheng, A.G., 2015. Lgr5+ cells regenerate hair cells via proliferation and direct transdifferentiation in damaged neonatal mouse utricle. *Nat. Commun.* 6, 6613.
- Wu, J., Li, W., Lin, C., Chen, Y., Cheng, C., Sun, S., Tang, M., Chai, R., Li, H., 2016. Coregulation of the Notch and Wnt signaling pathways promotes supporting cell proliferation and hair cell regeneration in mouse utricles. *Sci. Rep.* 6.
- Xiang, M., Maklad, A., Pirvola, U., Fritzsche, B., 2003. Brn3c null mutant mice show long-term, incomplete retention of some afferent inner ear innervation. *BMC Neurosci.* 4, 2.
- Xu, W.S., Parmigiani, R.B., Marks, P.A., 2007. Histone deacetylase inhibitors: molecular mechanisms of action. *Oncogene* 26, 5541–5552.