Published in final edited form as:

Neuroscience. 2010 April 14; 166(4): 1185–1193. doi:10.1016/j.neuroscience.2009.12.064.

Histone Deacetylase Inhibition Enhances Adenoviral Vector Transduction in Inner Ear Tissue

Akiko Taura¹, Kojiro Taura³, Yun-Hoon Choung¹, Masatsugu Masuda¹, Kwang Pak¹, Eduardo Chavez¹, and Allen F. Ryan^{1,2}

¹Department of Surgery, Division of Otolaryngology, UCSD School of Medicine and VA Medical Center, La Jolla, CA 92093

²Department of Neurosciences, UCSD, La Jolla, CA 92093

³Department of Medicine, UCSD, La Jolla, CA 92093

Abstract

Adenovirus vectors (AdVs) are efficient tools for gene therapy in many tissues. Several studies have demonstrated successful transgene transduction with AdVs in the inner ear of rodents (Kawamoto et al., 2003). However, toxicity of AdVs (Morral et al., 2002) or lack of tropism to important cell types such as hair cells (Shou et al., 2003) appears to limit their experimental and potential clinical utility. Histone deacetylase inhibitors (HDIs) are known to enhance AdV-mediated transgene expression in various organs (Dion et al., 1997), but their effects in the inner ear have not been documented. We investigated the ability of one HDI, trichostatin A (TSA), to enhance AdV-mediated transgene expression in inner ear tissue. We cultured neonatal rat macular and cochlear explants, and transduced them with an AdV encoding green fluorescent protein (Ad-GFP) under the control of a constitutive promoter for 24hrs. In the absence of TSA, GFP expression was limited, and very few hair cells were transduced. TSA did not enhance transduction when applied at the onset of Ad-GFP transduction. However, administration of TSA during or just after Ad-GFP application increased GFP expression in supporting cells approximately 4-fold. Moreover, vestibular hair cell transduction was enhanced approximately 6-fold, and that of inner hair cells by more than 17-fold. These results suggest that TSA increases AdV-mediated transgene expression in the inner ear, including the successful transduction of hair cells. HDIs, some of which are currently under clinical trials (Bakke et al., 2002), could be useful tools in overcoming current limitations of gene therapy in the inner ear using Ad-GFP.

Keywords

histone acetylation; HDI; TSA; adenovirus vector; inner ear; gene therapy; hair cell

Introduction

The major cause of sensorineural hearing loss and vestibular disorders is the loss of hair cells (HCs) in the inner ear (Nadol, 1993). This can occur for many reasons, including aging,

Address correspondence and reprint requests to: Allen F. Ryan, Ph.D. ENT, 0666 UCSD School of Medicine 9500 Gilman Drive La Jolla, CA 92093 Tel.: (858) 534-4594 Fax: (858) 534-5319 afryan@ucsd.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

genetic disorders and exposure to noise or toxins. Moreover, although HC regeneration occurs in birds and fish, the loss of HCs in mammals is currently irreversible. It is thus important to develop therapies that can prevent HC loss or induce HC regeneration in patients.

Potential treatments for inner ear disorders include gene therapy. It has been demonstrated experimentally that gene transfer into the inner ear can induce the expression of protective substances (Kawamoto et al., 2004), correct genetic disorders (Fukushima et al., 2007), and induce HC regeneration (Izumikawa et al., 2005). Gene transfer into the inner ear also has the advantage that this site is relatively isolated from other tissue and the spread of gene transfer vectors to other organs is likely to be limited.

Adenovirus vectors (AdVs) have been widely explored for gene therapy because of their ability to mediate transgene expression in many cell types. However, adenoviral toxicity restricts their use for clinical medicine, and they do not efficiently transduce all types of cells. This is particularly true of HCs, which are one of the most important targets for gene therapy of the inner ear. Augmentation of gene expression efficacy from AdVs may be helpful for inner ear gene therapy because it would potentially decrease the quantity of vector required for therapy and thus decrease the potential for toxicity. It may also increase the numbers and types of inner ear cells that can effectively be transduced.

Acetylation of core histones is an important regulator of gene expression in eukaryotic cells, since it reduces the density of nucleosomes and enhances exposure of DNA to the transcriptional machinery of the cell. Histones may be involved in efficient transduction with vector DNA, as well. Viruses produce histone-like proteins and recruit them to their DNA, which can increase transcriptional access (Grove and Saavedra, 2002). A histone deacetylase inhibitor (HDI): trichostatin A (TSA) has been shown to enhance AdV-mediated transgene expression in a number of cell types (Dion et al., 1997), and this property may be helpful in overcoming some limitations of gene therapy using AdVs. However HDIs have not been evaluated for their potential to enhance AdV-mediated gene expression in the inner ear. The present study was designed to assess the effects of TSA on transgene expression mediated by AdVs in inner ear cells, and to determine whether TSA altered core histone acetylation.

Experimental Procedures

Adenoviral vectors (AdVs)

Replication-incompetent AdVs (E1/E3 deleted, CMV promoter, serotype 5 expressing green fluorescent protein (Ad-GFP) or β -galactosidase (Ad-LacZ) were used.

Cell culture and treatment

As a positive control for the effects of TSA, the Rat-1 fibroblast cell line was infected with Ad-GFP at 30 PFU/cell. After infection, the cells were cultured in the presence or absence of 500 nM TSA for 48 hours. The effect of TSA on GFP expression was evaluated by fluorescence microscopy.

Tissue culture of vestibular maculae and organ of corti

Utricular and saccular maculae and organs of Corti were dissected from Wistar rat pups at postnatal days 3-5 (P3-5). The vestibular explants were then transferred into a 24-well plate and cultured with the HC layer uppermost. The explants were maintained in culture medium composed of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum

(FBS) and 30 U/ml penicillin, to which HEPES buffer had been added to a concentration of 25 mM.

Culture wells contained 500 μ l of medium and were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The experimental protocol adopted for this study was approved by the Animal Subjects Committee of the San Diego VA Medical Center.

Administration of TSA and Ad-GFP or Ad-LacZ

The explants were incubated in the serum-free medium containing 1×10^7 PFU/ml of Ad-GFP or Ad-LacZ for 24 hrs. The medium was then replaced with fresh DMEM containing 10% FBS, and TSA (500 nM final concentration) was added to the medium at designated times (Figure 1).

Measurement of transduction efficiency

Forty-eight hours after transduction with Ad-GFP, the explants were fixed with 4% paraformaldehyde (PFA) for 30 min. Explants were first evaluated as whole-mounts under fluorescence and confocal microscopy. They were then embedded in OCT compound and sectioned at 10 μ m on a cryostat. The sections were nuclear-stained with DAPI and immunostained for myosin 7A to distinguish HCs from supporting cells. The percentages of GFP-positive cells were normalized to the number of DAPI-positive cell nuclei. Expression of Ad-LacZ was examined by whole mount X-gal staining followed by sectioning. Explants were fixed with 4% PFA and immersed in X-gal solution. They were then embedded in OCT and sectioned at 10 μ m.

Western blotting for detection of histone acetylation

TSA-treated and normal control vestibular maculae were evaluated for acetylation of histones by Western blotting. Twenty vestibular organs from P4 rats were lysed in 400 μ l T-PER (Tissue Protein Extraction Reagent). The samples were then spun down at 1000 rpm for 2 min and the supernatant discarded. The tissue was then homogenized. NuPAGE LSD sample buffer and NuPAGE Reducing Agent (Invitrogen, Carlsbad, CA USA) were mixed with the samples and they were heated to 70°C for 3 min. The samples were then kept on ice for a few minutes. Thirty μ l of tissue lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% gels and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat dried milk in TBS-Tween [50 mM Tris-HCL (pH 7.4), 150 mM NaCl, 0.1% Tween 20] for 60 min at room temperature. The blots were incubated with a primary antibody (mouse monoclonal anti-acetyl-lysine antibody AKL5C1, (sc-32268; Santa Cruz Biotechnology, Santa Cruz, CA USA) that detects N-epsilon-acetylated lysine residues) in blocking buffer overnight at 4°C and then incubated with horseradish peroxidase-labeled anti-mouse IgG antibody (sc-2005; Santa Cruz) as a secondary antibody, followed by chemiluminescent detection of labeling at the molecular weights corresponding to core histones H3 and H4. To verify protein loading, the PVDF membranes were immediately stripped by placing the membrane in stripping buffer (0.5 M NaCl and 0.5 M acetic acid) for 30 min at room temperature. The membrane was washed once for 10 min in TBS-Tween, reblocked, and blotted with an antibody to β -actin as an internal control for protein quantity.

Acetylation of histones in tissue

We also evaluated histone acetylation in TSA-treated explants by immunohistochemistry using an antibody against acetylated histone. Vestibular macula explants were embedded in OCT compound and sectioned at 10 μ m on a cryostat. The sections were then reacted with

rabbit monoclonal anti-acetylated histone H3 (Lys23) antibody (#9674; Cell Signaling Technology, Beverly, MA USA) and FITC-labeled anti-rabbit IgG (#0936; DAKO, Glostrup, Denmark) as a secondary antibody, followed by fluorescence microscopy.

Effects of TSA on the expression of an endogenous gene using a transgenic reporter mouse

In order to examine the effects of acetylation on an endogenous gene encoding the same protein encoded by Ad-GFP, we used transgenic mice in which GFP is expressed in HCs under the control of a *pou4f3* promoter construct (Erkman et al., 1996). Vestibular maculae and organ of Corti from P3-P5 mice were cultured with or without TSA for 2 days. The intensity of HC GFP fluorescence was then evaluated by image analysis.

PI evaluation of cell viability

In order to assess the toxicity of Ad-GFP itself and of Ad-GFP plus TSA, we evaluated the viability of cells using propidium iodide (PI) exclusion. Explants were treated with Ad-GFP, with or without TSA as above. They were then incubated with PI (P-3566, 1:2000; Molecular Probes, Invitrogen) in PBS solution for 15 min at room temperature. The explants were washed with HBSS 3 times and fixed with 4% PFA for 30 min, followed by evaluation under light and fluorescence microscopy. Cells exhibiting nuclear staining with PI were scored as nonviable.

Statistical analysis

Quantitative differences were evaluated using Student's t test. Significance was evaluated at a level of $P < 0.05$. Data are presented as means and standard errors, along with the number of explants used in each condition.

Results

Effect of TSA in cell lines

Rat1-fibroblasts, which were obtained from American Type Culture Collection, were infected with Ad-GFP and cultured in the presence or absence of TSA. As shown in Figures 2A and 2B, TSA significantly augmented expression of GFP, even though there was no significant difference in the density of the cultured cells themselves.

Effect of TSA in inner ear tissues

The effect of TSA on Ad-GFP expression in inner ear tissues is illustrated in Figures 3 to 8. In the absence of TSA, GFP-positive cells were primarily localized in peripheral regions of vestibular maculae (Figure 3A). However, in explants treated simultaneously with TSA, the number of GFP positive cells was substantially increased and GFP positive cells were observed not only in the periphery but also in the central region of the macula (Figure 3B). Sections revealed that both supporting cells and HCs were transduced with Ad-GFP in the absence of TSA (Figure 3A'), although HCs were much less commonly transduced in agreement with previous studies (Dazert et al., 2001; Jero et al., 2001; Yagi et al., 1999). TSA significantly increased Ad-GFP expression in both supporting cells and HCs (Figure 4B'), with the result that substantial numbers of HCs were transduced. In the HC layer, transduction efficiency rate increased from 3.09 ± 0.84 to 17.6 ± 1.35 percent (mean $\pm$ SE); that is, by 6 fold ($P < 0.05$, $n=7$) (Figure 3C). In the supporting cell layer, it increased from 7.18 ± 1.35 to 33.18 ± 1.66 percent (mean $\pm$ SE); that is by 4.2 fold ($P < 0.05$, $n=7$) (Figure 3C). Thus the enhancement in HC transduction was somewhat greater than that seen in supporting cells. TSA also enhanced Ad-LacZ transduction (Figure 4).

In organ of Corti explants, we used the same treatment paradigm as in the vestibular epithelium. TSA was added to the media at the same time as Ad-GFP. TSA also enhanced Ad-GFP transduction in both supporting cells and HCs (Figures 5). However, inner HCs were consistently transduced at higher levels than were outer HCs with TSA (Figures 5B' and B''). In inner HCs, TSA enhanced transduction efficiency from 3.33 ± 1.86 to 58.33 ± 2.94 percent (mean $\pm$ SE); that is, by more than 17 fold ($P < 0.001$, $n = 6$). For outer HCs, it increased transduction from 3.33 ± 0.97 to 5.83 ± 0.64 ; that is by less than 2 fold ($P = 0.2$, $n = 6$).

Prior treatment with TSA has no effect on Ad-mediated transgene expression

When TSA was administered 24 hours after Ad-GFP application, HDAC inhibition enhanced Ad-GFP expression to a degree comparable to that seen with simultaneous treatment when compared to Ad-GFP alone (Figure 6B). Thus in vestibular sensory epithelium, transduction efficiency in the HC layer increased from 3.92 ± 0.05 to 19.47 ± 5.98 percent (mean $\pm$ SE); that is, 5 fold ($P < 0.05$, $n = 11$). In the supporting cell layer efficiency increased from 12.4 ± 0.48 to 32.75 ± 6.33 (mean $\pm$ SE); that is, 2.5 fold ($P < 0.05$, $n = 11$). However, when TSA was added to the media 24 hours prior to Ad-GFP but then discontinued upon Ad-GFP treatment, there was no effect of TSA on transduction efficiency (Figure 6A). These results suggest that the effect of TSA on adenoviral transgene expression is not mediated by an increase in uptake of Ad-GFP, but rather is mediated by the augmentation of transgene expression.

Association of core histone hyperacetylation with the enhancement of Ad gene expression

Histones are acetylated at their lysine residues. Their acetylation was detected by Western blotting of macular explants using an anti-acetyl-lysine antibody. TSA induced substantially more acetylation of histones H3 and H4 in inner ear cells (Figure 7). The amounts of β actin protein observed in the blots were similar. We also evaluated histone acetylation by immunohistochemistry, using an anti-acetylhistone antibody (Figure 8). In explants without TSA, few cells displayed visible immunolabeling (Figure 8A). However, in explants exposed to TSA, the nuclei of almost all cells were labeled (Figures 8B), indicating extensive hyperacetylation.

Influence of TSA on expression of an endogenous gene

In order to assess whether TSA might influence the expression of an endogenous gene, we used explants from transgenic mice in which an integrated gene construct directs the expression of GFP to HCs. No significant differences in HC GFP expression were observed with TSA (Figures 9A and 9B), suggesting that TSA does not influence the regulatory DNA of the integrated transgene, nor does it influence the fluorescence of GFP directly.

The effect of TSA on adenoviral toxicity

We determined the viability of sensory epithelial cells using a PI exclusion assay. Exposure to AdV increased PI staining in explants, suggesting toxicity to inner ear cells (Figure 10A) although it was not severe with the amount of Ad-GFP used for the present study. The addition of TSA had no significant effect on PI labeling of explants (Figure 10B) ($P = 0.3$, $n = 7$), indicating that decreased toxicity is not responsible for the observed effects of TSA on transduction.

Discussion

AdVs are powerful tools for gene therapy. Several studies have demonstrated successful gene transduction of cells in the inner ear using AdVs, indicating their potential utility at this site. Luebke et al. (2001) reported effective transduction and no apparent ototoxicity using

AdVs. Staecker et al. (2007) observed functional HC regeneration following Math1 gene transfer using AdVs. However inconsistent results have been reported, especially in terms of the tropism of AdVs for HCs; for example, Holt et al. (1999) reported successful Ad-GFP transduction in HCs but in contrast, Shou et al. (2003) found that Ad-GFP preferentially transduced supporting cells, but rarely if ever HCs. Our present study is consistent with the latter finding. Very few HCs were transduced in the absence of TSA, while moderate numbers of supporting cells showed reporter gene expression. AdV transduction efficiency can also be dependent upon age (Umegaki et al., 2003), and it is possible that the effects of TSA might also change with the developmental stage. In preliminary experiments for this study, we found that in vitro Ad-GFP transduction of inner ear sensory epithelia was more efficient at P1 than at P3-P5 (data not shown). It will be of interest to evaluate the effects of TSA on AdV transduction at both earlier and later stages of development than used for the present study.

Treatment with TSA significantly enhanced Ad-GFP gene expression in supporting cells. In addition, to our surprise, TSA induced transgene expression in many HCs as well. Also, in the cochlea, TSA enhanced gene expression to a much greater extent in inner HCs than in outer HCs. Little is known about differences in the TSA sensitivity of cells, but it appears that HCs are much more sensitive to TSA than supporting cells and that inner HCs are more sensitive than outer HCs. The differential sensitivity does not appear to be related to differences in histone acetylation between the various cell types, since we observed similar enhancement of histone acetylation in supporting cells and HCs (Figure 8), and since Chen et al. (2009) recently observed that treatment of neonatal mouse organ of Corti with TSA induced robust acetylation of histones in outer HCs as well as inner HCs.

The mechanism by which HDAC inhibitors influence Ad-GFP transduction is not clear. Some authors suggest that transduction efficacy is increased via the up-regulation of the coxsackie and adenovirus receptor (CAR) on target cells (Kitazono et al., 2001). However in our experiment, treatment with TSA prior to Ad-GFP transduction had no effect on AdV-mediated gene expression, suggesting that the augmentative effect of TSA is not likely to involve an increase in Ad-GFP uptake, which could be mediated by CAR.

Alternatively, as noted above, acetylation of histones is known to play an important role in the regulation of gene transcription in eukaryotic cells. Acetylation and deacetylation are catalyzed by specific enzyme families, histone acetyltransferases (HATs) and deacetylases (HDACs) and hyperacetylation of core histones in the nucleus is associated with enhanced gene expression. Much less is known about the role of histones in Ad-GFP gene transcription. The present study demonstrated that TSA caused both hyperacetylation of core histones and enhanced AdV-mediated transgene expression in inner ear cells. However, the adenoviral genome is not integrated into the host genome and may not use histones of host origin, although adenovirus has its own histone-like proteins (Lischwe and Sung, 1977; Sung et al., 1977), which are known to be acetylated (Fedor and Daniell, 1980). While a direct causal relationship between enhanced gene expression and hyperacetylation of Ad-GFP histone-like proteins was not demonstrated, our study suggests that acetylation may be important for the regulation of Ad-GFP insert transcription. This could occur in a manner analogous to the effects of core histone acetylation on host cell gene expression. However, as noted above cells with similar levels of histone acetylation showed different levels of transduction enhancement. Moreover, it should be noted that there are many other cellular and nuclear proteins whose acetylation can be enhanced by TSA, including a number whose acetylation state influences gene transcription. This includes both specific and general transcription factors (Das and Kundu, 2005).

It is also not clear why HCs should be more sensitive to TSA than other inner ear cells. However, Gaetano et al. (2000) reported that TSA enhancement of AdV-mediated gene expression is particularly strong in cells bearing retinoic acid receptor (RAR) and retinoid X receptor (RXR) complexes. The CMV promoter used in Ad-GFPs contains repeated RAR elements, and these authors concluded that RA may co-operate with TSA on the CMV promoter, with TSA enhancing access to RAR elements by RA. RARs and RXRs are known to be present in the developing sensory epithelia of the inner ear (Romand et al., 1998). Moreover, HCs have been found to be particularly sensitive to the effects of RA (Kelley et al., 1993; Raz and Kelley, 1999).

While AdV-transduced expression of GFP, as well as of LacZ, was amplified by TSA treatment, endogenous GFP expression in the HCs of a transgenic mouse was not affected. This is perhaps not surprising, since GFP is expressed at high levels in the HCs of these mice, and thus the regulatory and transcriptional DNA of active genes is presumably highly acetylated (Verdone et al., 2006; Heintzman et al., 2007). While TSA has been found to regulate some endogenous genes (Dombrowsky and Uhlig, 2007), the effects of HDAC inhibition vary depending upon the gene. In fact, Van Lint et al. (1996) reported that the expression of only approximately 2% of cellular genes is affected by histone hyperacetylation due to HDIs. This may be because epigenetic downregulation of eukaryotic genes is more likely to be caused by histone or DNA methylation than by hypoacetylation (Zhang and Reinberg, 2001).

Whatever the mechanism by which TSA enhances Ad-GFP transduction, this effect is not restricted to Ad-GFPs. Similar effects of TSA have been reported in other viral vectors and even nonviral transfection methods such as lipofectamine [our data not shown; Tobias et al., 2000; Kitazono et al., 2001; Yamano et al., 2000].

In conclusion, the present study demonstrates the potential utility of HDAC inhibitors for overcoming limitations of AdV-mediated gene therapy targeting HCs of the inner ear.

Acknowledgments

Supported by grants from the Research Service of the US Veterans Administration, the NIH/NIDCD (DC000139) and the National Organization for Hearing Research.

List of Abbreviations

AdV	Adenovirus vector
Ad-GFP	adenovirus vector encoding green fluorescent protein
Ad-LacZ	adenovirus vector encoding β -galactosidase
CAR	coxsackie and adenovirus receptor
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
GFP	green fluorescent protein
HATs	histone acetyltransferases
HCs	hair cells
HDACs	histone deacetylases
HDI	histone deacetylase inhibitors

PAGE	polyacrylamide gel electrophoresis
PFA	paraformaldehyde
PI	propidium iodide
PVDF	polyvinylidene difluoride
RAR	retinoic acid receptor
RXR	retinoid $\times$ receptor
T-PER	tissue protein extraction reagent
TSA	trichostatin A

References

- Chen FQ, Schacht J, Sha SH. Aminoglycoside-induced histone deacetylation and hair cell death in the mouse cochlea. *J Neurochem* 2009;108:1226–1236. [PubMed: 19141081]
- Das C, Kundu TK. Transcriptional regulation by the acetylation of nonhistone proteins in humans -- a new target for therapeutics. *IUBMB Life* 2005;57:137–149. [PubMed: 16036576]
- Dazert S, Aletsee C, Brors D, Gravel C, Sendtner M, Ryan A. In vivo adenoviral transduction of the neonatal rat cochlea and middle ear. *Hear Res* 2001;151:30–40. [PubMed: 11124449]
- Dion LD, Goldsmith KT, Tang DC, Engler JA, Yoshida M, Garver RI Jr. Amplification of recombinant adenoviral transgene products occurs by inhibition of histone deacetylase. *Virology* 1997;231:201–209. [PubMed: 9168882]
- Dombrowsky H, Uhlig S. Steroids and histone deacetylase in ventilation-induced gene transcription. *Eur Respir J* 2007;30:865–877. [PubMed: 17626110]
- Fedor MJ, Daniell E. Acetylation of histone-like proteins of adenovirus type 5. *J Virol* 1980;35:637–643. [PubMed: 7420537]
- Gaetano C, Catalano A, Palumbo R, Illi B, Orlando G, Ventoruzzo G, Serino F, Capogrossi MC. Transcriptionally active drugs improve adenovirus vector performance in vitro and in vivo. *Gene Ther* 2000;7:1624–1630. [PubMed: 11083470]
- Grove A, Saavedra TC. The role of surface-exposed lysines in wrapping DNA about the bacterial histone-like protein HU. *Biochemistry* 2002;41:7597–7603. [PubMed: 12056890]
- Heintzman ND, Stuart RK, Hon G, Fu Y, Ching CW, Hawkins RD, Barrera LO, Van Calcar S, Qu C, Ching KA, Wang W, Weng Z, Green RD, Crawford GE, Ren B. Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. *Nat Genet* 2007;39:311–318. [PubMed: 17277777]
- Holt JR, Johns DC, Wang S, Chen ZY, Dunn RJ, Marban E, Corey DP. Functional expression of exogenous proteins in mammalian sensory hair cells infected with adenoviral vectors. *J Neurophysiol* 1999;81:1881–1888. [PubMed: 10200223]
- Izumikawa M, Minoda R, Kawamoto K, Abrashkin KA, Swiderski DL, Dolan DF, Brough DE, Raphael Y. Auditory hair cell replacement and hearing improvement by Atoh1 gene therapy in deaf mammals. *Nat Med* 2005;11:271–276. [PubMed: 15711559]
- Jero J, Mhatre A, Tseng C, Stern R, Coling D, Goldstein J, Hong K, Zheng W, Hoque A, Lalwani A. Cochlear gene delivery through an intact round window membrane in mouse. *Hum. Gene Ther* 2002;12:539–548. [PubMed: 11268286]
- Kawamoto K, Ishimoto SI, Minoda R, Brough DE, Raphael Y. Math1 gene transfer generates new cochlear hair cells in mature guinea pigs in vivo. *J Neurosci* 2003;23:4395–4400. [PubMed: 12805278]
- Kelley MW, Xu XM, Wagner MA, Warchol ME, Corwin JT. The developing organ of Corti contains retinoic acid and forms supernumerary hair cells in response to exogenous retinoic acid in culture. *Development* 1993;119:1041–1053. [PubMed: 8306874]

- Kitazono M, Chuman Y, Aikou T, Fojo T. Construction of gene therapy vectors targeting thyroid cells: enhancement of activity and specificity with histone deacetylase inhibitors and agents modulating the cyclic adenosine 3,5 -monophosphate pathway and demonstration of activity in follicular and anaplastic thyroid carcinoma cells. *J Clin Endocrinol Metab* 2001;86:834–840. [PubMed: 11158054]
- Kitazono M, Goldsmith ME, Aikou T, Bates S, Fojo T. Enhanced adenovirus transgene expression in malignant cells treated with the histone deacetylase inhibitor FR901228. *Cancer Res* 2001;61:6328–6330. [PubMed: 11522619]
- Lischwe MA, Sung MT. A histone-like protein from adenovirus chromatin. *Nature* 1977;267:552–554. [PubMed: 876376]
- Luebke AE, Steiger JD, Hodges BL, Amalfitano A. A modified adenovirus can transfect cochlear hair cells in vivo without compromising cochlear function. *Gene Ther* 2001;10:789–794. [PubMed: 11420643]
- Maeda Y, Fukushima K, Kawasaki A, Nishizaki K, Smith RJ. Cochlear expression of a dominant-negative GJB2R75W construct delivered through the round window membrane in mice. *Neurosci Res* 2007;58:250–254. [PubMed: 17462767]
- Erkman L, McEvilly RJ, Luo L, Ryan AE, Hoosmand F, O'Connell SM, Keithley EM, Rappaport DH, Ryan AF, Rosenfeld MG. Role of transcription factors Brn-3.1 and Brn-3.2 in auditory and visual system development. *Nature* 1996;381:603–606. [PubMed: 8637595]
- Morral N, O'Neal WK, Rice K, Leland MM, Piedra PA, Aguilar-Cordova E, Carey KD, Beaudet AL, Langston C. Lethal toxicity, severe endothelial injury, and a threshold effect with high doses of an adenoviral vector in baboons. *Human Gene Ther* 2002;13:143–154. [PubMed: 11779418]
- Nadol JB Jr. Hearing loss. *N Engl J Med* 1993;329:1092–102. [PubMed: 8371732]
- Nakaizumi T, Kawamoto K, Minoda R, Raphael Y. Adenovirus-mediated expression of brain-derived neurotrophic factor protects spiral ganglion neurons from ototoxic damage. *Audiol Neurotol* 2004;9:135–143. [PubMed: 15084818]
- Raz Y, Kelley MW. Retinoic acid signaling is necessary for the development of the organ of Corti. *Dev Biol* 1999;213:180–193. [PubMed: 10452855]
- Romand R, Sapin V, Dolle P. Spatial distributions of retinoic acid receptor gene transcripts in the prenatal mouse inner ear. *J Comp Neurol* 1998;393:298–308. [PubMed: 9548551]
- Sandor V, Bakke S, Robey RW, Kang MH, Blagosklonny MV, Bender J, Brooks R, Piekarz RL, Tucker E, Figg WD, Chan KK, Goldspiel B, Fojo AT, Balcerzak SP, Bates SE. Phase I trial of the histone deacetylase inhibitor, depsipeptide (FR901228, NSC 630176) in patients with refractory neoplasms. *Clin Cancer Res* 2002;8:718–728. [PubMed: 11895901]
- Shou J, Zheng JL, Gao WQ. Robust generation of new hair cells in the mature mammalian inner ear by adenoviral expression of *Hath1*. *Mol Cell Neurosci* 2003;23:169–179. [PubMed: 12812751]
- Staecker H, Praetorius M, Baker K, Brough DE. Vestibular hair cell regeneration and restoration of balance function induced by *math1* gene transfer. *Otology Neurotol* 2007;10:223–231.
- Sung MT, Lischwe MA, Richards JC, Hosokawa K. Adenovirus chromatin I. Isolation and characterization of the major core protein VII and precursor Pro-VII. *J Biol Chem* 1977;252:4981–4987. [PubMed: 873925]
- Tobias CA, Kim D, Fischer I. Improved recombinant retroviral titers utilizing trichostatin A. *Biotechniques* 2000;29:884–890.
- Umegaki H, Ishiwata K, Ogawa O, Ingram DK, Roth GS, Oda K, Kurotani S, Kawamura K, Wang WF, Ikari H, Senda M, Iguchi A. Longitudinal follow-up study of adenoviral vector-mediated gene transfer of dopamine D2 receptors in the striatum in young, middle-aged, and aged rats: a positron emission tomography study. *Neurosci* 2003;121:479–486.
- Van Lint C, Emiliani S, Verdin E. The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. *Gene Expr* 1996;5:245–253. [PubMed: 8723390]
- Verdone L, Agricola E, Caserta M, Di Mauro E. Histone acetylation in gene regulation. *Brief Funct Genomic Proteomic* 2006;5:209–21. [PubMed: 16877467]
- Yagi M, Magal E, Sheng Z, Ang K, Raphael Y. Hair cell protection from aminoglycoside ototoxicity by adenovirus-mediated overexpression of glial cell line-derived neurotrophic fac. *Hum. Gene Ther* 1999;10:813–823. [PubMed: 10210148]

- Yamano T, Ura K, Morishita R, Nakajima H, Monden M, Kaneda Y. Amplification of transgene expression in vitro and in vivo using a novel inhibitor of histone deacetylase. *Mol Ther* 2000;1:574–580. [PubMed: 10933982]
- Zhang Y, Reinberg D. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. *Genes Dev* 2001;15:2343–2360. [PubMed: 11562345]

1) Co-incident TSA Administration

2) Pre- and Post-Infection TSA Administration

a) Before Ad-GFP

b) After Ad-GFP

Fig. 1.

Experimental protocols used in the present study. TSA was applied during, before or after exposure of the tissue to Ad-GFP.

Fig. 2. Effect of TSA on Ad-GFP transduction of rat fibroblasts. A) Ad-GFP alone: Only a few GFP-positive cells are observed. B) Ad-GFP + TSA: Many more GFP-positive cells are present and transduction levels are increased, as has been reported previously [4].

Fig. 3. Effect of simultaneous TSA administration on Ad-GFP transduction of neonatal vestibular maculae, surface preparations (A, B), sections (A', B') and cell counts (C). **Ad-GFP alone:** Cells are transduced primarily in the peripheral region (A). Sections reveal that only two HCs and one supporting cell are transduced (A'). **Ad-GFP with TSA:** GFP-positive cells are present throughout the epithelium (B). Sections reveal that there are many GFP-positive HCs and supporting cells (B'). (Green:GFP; Red:Myosin 7A; Blue:DAPI.) C) Graph illustrating Ad-GFP transduction efficiency by cell type in vestibular maculae: TSA enhanced GFP expression in both the hair cell (HC) layer and the supporting cell (SC) layers. However, the enhancement of HC transduction was somewhat greater.

Fig. 4. Typical TSA effect on transduction with Ad-LacZ in vestibular maculae. A) Ad-LacZ alone: Only stromal cells are strongly transduced. B) Ad-LacZ plus TSA: Both HCs and supporting cells (SC) are strongly transduced. Because the number of samples used was small (n=3), quantification was not performed.

Fig. 5. Effect of TSA on AdV transduction of cochlear explants, surface preparations (A-A', B-B') and sections (A'', B'') and cell counts (C). Ad-GFP alone: Cochlear cells are transduced, but expression is weak and few hair cells are transduced (A, A', A''). Ad-GFP plus TSA: Many more cells are transduced, and expression levels are higher. Inner HCs are preferentially transduced (B, B', B''). (Green: Ad-GFP; Red: Myosin 7A; Blue: DAPI) C) Graph illustrating Ad-GFP transduction efficiency by cell type in the organ of Corti: TSA enhanced GFP expression in HCs and supporting cells. However, the enhancement of inner HC transduction was much greater.

Fig. 6. Effect of time of TSA administration on AdV transduction in vestibular maculae. A) TSA administration before Ad-GFP: Only a few cells are transduced in the epithelial region. B) TSA administration after Ad-GFP: Many more GFP-positive cells are present. (Green:Ad-GFP; Red:Myosin 7A;Blue:DAPI)

Fig. 7.

Acetylation of histones in vestibular sensory epithelia was analyzed by Western blotting using anti-acetyllysine antibody. H3 and H4 indicate the position of histone H3 and H4, respectively. These data suggest that, in the tissue treated with TSA, histones are highly acetylated.

Fig. 8.
The acetylation of histones in tissues was analyzed by staining with anti-acetylated Histone 3. A) an untreated explant: There are a few acetylated nuclei in the epithelial layer. B) TSA-treated explant: almost all cells show acetylated histones in their nuclei.

Fig. 9. Lack of effect of TSA on endogenous gene expression in the vestibular maculae of transgenic mice expressing GFP in HCs. A) Untreated macula. B) TSA-treated macula. There is no difference in gene expression between the two conditions.

Fig. 10.

Effects of TSA on cell viability in the vestibular macula. A) Ad-GFP alone: While some cells are labeled with PI, the number is small. B) Ad-GFP plus TSA-treated explant: the number of PI-positive cells was not significantly different from ($P = 0.3$, $n=7$).