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Viral vector tropism for supporting cells in the developing murine cochlea

Abraham M. Sheffield^a, Samuel P. Gubbels^b, Michael S. Hildebrand^a, Stephen S. Newton^a, John A. Chiorini^c, Giovanni Di Pasquale^c, and Richard J. H. Smith^a

^aDepartment of Otolaryngology, University of Iowa, Iowa City, IA, USA

^bDepartment of Surgery, Division of Otolaryngology, University of Wisconsin, Madison, WI, USA

^cMolecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA

1. INTRODUCTION

Hearing loss (HL) is the most common sensory disorder in developed countries. Bilateral congenital sensorineural HL affects one in every 500 newborns and may lead to significant problems in speech development and educational attainment (Hildebrand et al., 2008; Kochhar et al., 2007). At least half of all congenital HL cases are hereditary. Of these, the majority (~70% of cases) are nonsyndromic hearing loss (NSHL), in which hearing impairment is the sole phenotype, and the remainder (~30% of cases) are associated with syndromes such as Pendred and Usher syndrome (Kochhar et al., 2007). Genetic HL is highly heterogeneous with autosomal recessive inheritance (ARNSHL) in ~80% of cases, autosomal dominant (ADNSHL) inheritance in ~20% of cases, and instances of X-linked (<1%) and mitochondrial (<<1%) inheritance also occurring (Hilgert et al., 2009). To date, 36 ARNSHL genes and 24 ADNSHL genes have been identified. A further 45 loci for recessive and 34 loci for dominant deafness have been mapped (Hereditary Hearing Loss Homepage: <http://hereditaryhearingloss.org>) (Van Camp, 2010).

The inner ear is a complex structure with a large number of unique cell types. Deafness mutations are known to affect a variety of inner ear cells and tissues including: sensory hair cells (e.g. *MYO7A*, *KCNQ4*), non-sensory supporting cells (e.g. *GJB2*, *GJB6*), and the tectorial membrane (e.g. *COL11A2*, *TECTA*) (<http://www.hereditaryhearingloss.org>) (Kochhar et al., 2007; Petit et al., 2001; Van Camp, 2010).

The defect leading to the most common form of ARNSHL at the DFNB1 locus is in the non-sensory supporting cells of the cochlea. The DFNB1 locus contains the deafness genes *GJB2* and *GJB6* that encode connexin 26 (CX26) and connexin 30 (CX30) proteins, respectively (del Castillo et al., 2002; Kelsell et al., 1997). Connexin proteins oligomerize to form gap junction channels between adjacent cells. CX26 and CX30 form gap junction networks between supporting cells in the cochlea that are thought to facilitate transport of ions (e.g. K⁺) and small molecules (e.g. IP₃) vital for maintenance of cochlear homeostasis (Martinez

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Correspondence: Dr R Smith, Department of Internal Medicine, University of Iowa, Iowa City, IA 52242, USA. Tel: +1 319 356 3612; Fax: +1 319 356 4547; richard-smith@uiowa.edu.

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et al., 2009; Nickel et al., 2008). Mutations in *GJB2* account for up to 50% of autosomal recessive forms of isolated deafness in some populations, including an estimated 30–60% in Europe and the United States (Petit et al., 2001). *GJB2* mutations are also associated with ADNSHL at the DFNA3 locus as well as a number of dominant syndromic disorders in which deafness segregates with skin disease such as Keratitis-Ichthyosis-Deafness syndrome (MIM 148210), Vohwinkel syndrome (MIM 124500), and palmoplantar keratoderma with deafness (MIM 148350) (Heathcote et al., 2000; Maestrini et al., 1999; van Steensel et al., 2002).

Current treatment options for sensorineural hearing loss are limited to amplification devices such as hearing aids and cochlear implants. Gene therapy targeting the inner ear to modulate or replace endogenous genes and their products offers the promise of novel treatments for hereditary hearing loss. Given the variety of cell types in the inner ear, viral tropism is an important consideration when selecting a vector for gene transfer (Hildebrand et al., 2008). As some viruses have been shown to have detrimental effects on their targets, the other major consideration for vector selection is toxicity. To date, a variety of viral vectors have been used to target genes in the inner ear, including adenovirus (AV), adeno-associated virus (AAV), herpes simplex virus and lentivirus (Derby et al., 1999; Hildebrand et al., 2008; Izumikawa et al., 2005; Lalwani et al., 2002; Lalwani et al., 1996). A variety of delivery techniques have also been described (Bedrosian et al., 2006; Jero et al., 2001; Lalwani et al., 2002; Praetorius et al., 2002; Yamasoba et al., 1999).

Many forms of genetic deafness, including those caused by *GJB2* or *GJB6* mutations, have pre-lingual onset. In these cases, early intervention is required to achieve effective gene correction or replacement. Mouse models of connexin-related deafness display significant hearing loss at the time of cochlear maturation, requiring that therapeutic strategies be targeted at the developing cochlea (Ahmad et al., 2007; Cohen-Salmon et al., 2002; Hildebrand et al., 2008; Kudo et al., 2003; Teubner et al., 2003). Recently, Bedrosian and colleagues developed and optimized a method for *in utero* gene transfer, whereby an adeno-associated virus pseudotype (AAV2/1) vector was shown to safely and efficiently transduce sensory hair cell progenitors in the murine otocyst (Bedrosian et al., 2006).

While *in utero* targeting of sensory hair cells with AAV2/1 provides therapeutic potential for many types of genetic hearing loss, there are important reasons to identify a virus with tropism for supporting cells in the developing cochlea. Aside from their role in the most common form of ARNSHL, cochlear supporting cells serve as the primary targets of intervention to induce hair cell regeneration (Izumikawa et al., 2005). One significant advance in the application of gene therapy to restore auditory function was the discovery that adenoviral delivery of *Atoh1* (*Math1*) to supporting cells in the guinea pig cochlea resulted in the formation of “hair cell-like” cells (Izumikawa et al., 2005). The promise of hair cell regeneration and the potential treatment of common connexin-related deafness disorders are compelling reasons to identify vectors with tropism for inner ear supporting cells (Ballana et al., 2008; Brigande et al., 2009).

AV and bovine adeno-associated virus (BAAV) vectors have previously been used for successful transduction of cochlear supporting cells in adult rodents, but have never been delivered to the developing cochlea *in vivo* (Dazert et al., 2001; Di Pasquale et al., 2005; Ishimoto et al., 2002; Izumikawa et al., 2005; Ortolano et al., 2008; Shibata et al., 2009). In this study we used the transuterine microinjection approach to examine the safety and inner ear tropism of three previously untested vectors delivered to the developing murine cochlea: Ad5.CMV.GFP (early-generation adenovirus), Adf.11D (late-generation adenovirus) and BAAV.CMV.GFP (bovine adeno-associated virus).

2. MATERIALS AND METHODS

2.1. Virus Production

Early-generation replication-deficient adenovectors (deleted of E1A and a partial deletion of E1B and E3) were constructed in the Gene Transfer Vector Core Laboratory at the University of Iowa as described previously (Vasquez et al., 1998). The green fluorescent protein (GFP) reporter gene expression was driven by the human cytomegalovirus (CMV) promoter (Ad5.CMV.GFP). Recombinant Ad5.CMV.GFP viruses were grown in human embryonic kidney (HEK) 293 cells that complement the E1 early viral promoters. Virus titers of 1.0×10^{11} plaque-forming units per milliliter (pfu/ml) were suspended in phosphate-buffered saline (PBS) with 3% sucrose and stored at -80°C until use.

An advanced-generation adenovirus vector, Adf.11D, was provided by Dr. Douglas Brough at GenVec (Gaithersburg, MD, USA). The E1/E3/E4-deleted adenovector contains an expression cassette of GFP driven by a human CMV promoter. Construction and production of Adf.11D has been reported previously (Staecker et al., 2007). Total particles (pu) are analyzed by a spectrophotometric assay that has been standardized to reliably quantify the total particles within a given lot of adenovector. The lot of Adf.11D used for these experiments measured 9.6×10^{11} total particles per milliliter (pu/ml), with 10.7×10^{10} focal forming units per ml (ffu/ml), a measure of adenovector activity. Adf.11D was purified and aliquots stored at -80°C until use.

Recombinant BAAV expressing GFP driven by a human CMV promoter was provided by Drs. John A. Chiorini and Giovanni Di Pasquale at the National Institutes of Health (Bethesda, MD, USA). The vector was produced as previously described, purified using CsCl gradients and particle titers were determined by qPCR (Di Pasquale et al., 2005; Kaludov et al., 2002). The recombinant BAAV particle titers were 5.0×10^{11} DNase-resistant particles per milliliter (DRP/ml). Purified BAAV.CMV.GFP was dialyzed in PBS and stored at -80°C until use.

2.2. Animals and Virus Administration

All procedures were performed in adherence with institutional and national guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Iowa. Six-week to three-month old BALB/C mice maintained at the University of Iowa animal care facility underwent timed breeding to generate the fetuses for transuterine microinjection. For aging of the embryos, noon of the cervical plug date was considered to be day E0.5.

2.3. Transuterine Otocyst Microinjection

Injection of vectors into the left otocyst of E12.5 mouse embryos was performed via transuterine microinjection as described previously (Bedrosian et al., 2006; Brigande et al., 2009). Briefly, E12.5 dams were anesthetized with 9 mg/ml Nembutal at a dose of $7 \mu\text{l/g}$ body weight. Using a Leica M220 F12 surgical microscope (Leica Microsystems Inc., Bannockburn, IL, USA), a 1.5-cm midline laparotomy incision was made and the uterine horns were exposed. Embryos were transilluminated to identify anatomical landmarks of the otocyst. Prior to delivery, $8 \mu\text{l}$ of vector was mixed with $4 \mu\text{l}$ of 2.5% fast green dye in PBS and loaded into a specially pulled glass micropipette (Brigande et al., 2009). Approximately 50 nl of this vector preparation (Ad5.CMV.GFP, Adf.11D, or BAAV.CMV.GFP) was delivered to the left otocyst of accessible embryos (average of 3 to 5 embryos per dam) using a glass micropipette, M33 roller bearing micromanipulator (Stoelting Co., Wood Dale, IL, USA) and PLI-100 pico-injector pump system (Harvard Apparatus Inc., Holliston, MA, USA). Contralateral (right) otocysts were not injected and served as non-injected controls.

Uterine horns were replaced inside the peritoneal cavity, incisions sutured, and dams allowed to give birth vaginally and to nurse their young to maturity.

2.4. Auditory Brainstem Response Testing

At five weeks of age, mice were anesthetized by intraperitoneal injection of (10 μ l/g body weight) [10 mg/ml] ketamine/xylazine and placed in an acoustic test chamber (Acoustic Systems, Glendale Heights, IL, USA) for testing of auditory-evoked brainstem response (ABR) thresholds. ABR testing was carried out as previously described (Zheng et al., 1999). Briefly, after anesthetic had taken effect, active, reference and ground electrode needles were inserted subcutaneously and the SmartEP software system (Intelligent Hearing System Inc., Miami, FL, USA) was used for the stimulus presentation, ABR acquisition, and data management. The first stimulus was presented at an intensity of 70 decibels sound pressure level (dB SPL) and, depending upon the response, was followed by increasing or decreasing the decibel level, initially in 10 dB- and subsequently in 5 dB-increments, to determine the auditory threshold (the lowest sound level at which an ABR pattern is recognizable). Click-stimulus ABR testing presents clicks that cover a range of frequencies (2–8 kHz) and is used for general screening of auditory function. Pure-tone ABR testing presents a series of tones to test ability to hear at specific frequencies: 4, 8, 16, and 32 kHz for these experiments.

2.5. Sample Preparation and Immunofluorescent Imaging

At P35 anesthetized mice were sacrificed and the inner ears were isolated, immediately fixed for two hours in 4% paraformaldehyde (PFA), rinsed in PBS, decalcified in EDTA and prepared for cryosectioning as previously described (Whitlon et al., 2001). 10 μ m mid-modiolar cryosections were prepared for immunohistochemistry. For surface preparations of the sensory epithelium, P5–P7 mouse pups were decapitated, the cochleae dissected, and the sensory epithelium isolated and placed on a glass slide as previously described (Parker et al., 2010). The sensory epithelium was immediately fixed in 4% PFA for 20 minutes, rinsed in PBS, and stored at 4°C in preparation for immunohistochemistry. Immunohistochemistry was performed according to a previously established methodology (Whitlon et al., 2001). For anti-GFP immunostaining, sections from each of the left cochleae and several of the right cochleae were incubated with a 1:500 dilution of rabbit polyclonal anti-GFP primary antibody (#AB3080 Lot LV1407277; Chemicon, Temecula, CA) followed by incubation with a 1:1000 dilution of a secondary Alexa Fluor 568-conjugated goat anti-rabbit antibody (#A11036, Invitrogen, Carlsbad, CA, USA). Immunofluorescence imaging using a Leica DM IRE2 fluorescent microscope (Leica Microsystems Inc., Bannockburn, IL, USA) was performed to verify GFP expression. Sections from confirmed GFP-positive cochleae were incubated with a 1:150 dilution of rabbit polyclonal anti-myosin VIIA primary antibody (#25–6790, Proteus Biosciences Inc., Ramona, CA, USA) which is specific for inner and outer hair cells. This was followed by incubation with Alexa Fluor 568 antibody as described above. All slides were treated with ProLong Gold antifade reagent with DAPI (#P36931; Invitrogen, Carlsbad, CA, USA), followed by placement of a coverslip and allowed to set for 24 hours prior to imaging. Immunofluorescence imaging using the Leica DM IRE2 fluorescent microscope or the Leica TCS SP5 confocal microscope (Leica Microsystems Inc., Bannockburn, IL, USA) was used to identify transduced cell types.

3. RESULTS

3.1. *In utero* delivery of adenovirus to the developing murine inner ear

We performed transuterine microinjection of the murine otocyst with early-generation (E1/E3-deleted) adenoviral (AV5) vector expressing GFP under the control of a human CMV promoter (Ad5.CMV.GFP). Otocyst injections were performed on E11.5–12.5 mouse embryos as described by Bedrosian et al. (Bedrosian et al., 2006; Brigande et al., 2009).

Initial screening of injected embryos demonstrated robust transduction throughout the sensory epithelium on postnatal day 7 (P7) (Figure 1a). In addition to supporting cells, outer hair cells were also transduced (Figure 1b). Next, we assessed expression in injected mice (n=4) at 5 weeks of age (approximately P35). We observed robust transduction throughout the organ of Corti with strong tropism for supporting cells (Figure 2a–f). Transduced cells were also observed in the stria vascularis, the supralimbal region of the spiral limbus, Reissner's membrane, and spiral ganglion neurons (SGNs) (Figure 2a–c,g). We observed no evidence of immune cell infiltrates or hair cell damage in histologic cross-sections. None of the cochleae showed significant or consistent vestibular expression of GFP, nor did any of the mice display circling or head-tilting behavior which would indicate vestibular dysfunction.

3.2. Effect of *in utero* adenoviral transduction on hearing

We assessed the hearing of injected mice at 5 weeks of age using click-stimulus ABR testing to determine if transduction of cochlear cells with AV was associated with ototoxicity. Using the two-sample t-test for equal variances we determined that hearing thresholds of the ears injected with Ad5.CMV.GFP were significantly elevated ($97.5 \text{ dB} \pm 5$, n=4) when compared with contralateral non-injected ears ($45.0 \text{ dB} \pm 0$, n=4; $t(6)=21.0$, $p=7.6 \times 10^{-7}$) and with non-injected littermates ($45.8 \text{ dB} \pm 7.4$, n=6; $t(8)=12.2$, $p=1.9 \times 10^{-6}$). These results suggest that Ad5.CMV.GFP transduction is strongly ototoxic to cells of the developing cochlea (Figure 3a).

Because early-generation AVs have been reported to be ototoxic to hair cells *in vivo* as assessed by distortion-product otoacoustic emission (DPOAE) testing (Luebke et al., 2001a), we hypothesized that the use of advanced-generation (E1/E3/E4-deleted) AV would result in diminished ototoxicity (Praetorius et al., 2003). Following the same protocol as with Ad5.CMV.GFP, we injected 9 embryos with Adf.11D, an E1/E3/E4-deleted adenovector expressing GFP from a CMV promoter. GFP expression at 5 weeks of age was localized to the organ of Corti region. However, expression was less intense and more variable than with Ad5.CMV.GFP, making it difficult to definitively identify uniformly transduced cell types (data not shown). There was no morphologic evidence of hair cell damage. We again performed ABR testing at 5 weeks of age and, as with early-generation AV, the two-sample t-test for unequal variances showed that hearing thresholds of the ears injected with Adf.11D were significantly elevated ($99.4 \text{ dB} \pm 19.8$, n=9) when compared with contralateral non-injected ears ($56.1 \text{ dB} \pm 15.4$, n=9; $t(15)=5.19$, $p=1.0 \times 10^{-4}$) and with non-injected littermates ($51.3 \text{ dB} \pm 4.3$, n=20; $t(8)=7.24$, $p=8.9 \times 10^{-5}$) (Figure 3a). These results indicate that both early- and advanced-generation AV vectors induce ototoxicity when delivered to cells of the developing murine cochlea.

3.3. *In utero* delivery of bovine adeno-associated virus to the developing murine inner ear

We next tested bovine adeno-associated virus (BAAV) vector expressing GFP under the control of a CMV promoter (BAAV.CMV.GFP), initially injecting the left ear in two embryos and assessing cochlear expression at 5 weeks of age. We observed strong transduction in the organ of Corti with tropism for supporting cells (Figure 4a–f). Although transduction occurred in all turns of the cochlea, we noted preferential transduction toward the apex.

To confirm these findings we injected six additional embryos with BAAV.CMV.GFP. Two of the injected mice were sacrificed at P5 and a whole mount preparation of the sensory epithelium was visualized to determine transduction efficiency along the length of the cochlea (Figure 5a,b). GFP-positive cells were counted along the entire length of each cochlea. The mean transduction efficiency was 29.4 ± 16.6 (ranging from 3 to 56)

transduced cells/500 μm . To assess whether a statistically significant difference in transduction occurred along the length of the cochlea we measured the average number of transduced cells in 1 mm segments from the most apical, middle and most basal regions of the cochleae. The average transduction efficiency for each of these segments was 39.8 ± 5.3 , 29.5 ± 13.1 , and 8.3 ± 5.6 transduced cells/500 μm in the apical, middle, and basal cochlear turns, respectively (Figure 5d). The mean transduction efficiencies at the apex and at the middle of the cochlea are each significantly higher than the efficiency at the base according to the two-sample t-test for equal variances (Apex: $t(6) = 8.24$, $p = 1.7 \times 10^{-4}$; Middle: $t(6) = 2.99$, $p = 0.02$). The transduction efficiency between the apex and the middle were not significantly different ($t(6) = 1.45$, $p > 0.05$). The trend of preferential transduction toward the apex can be appreciated by graphs showing the number of transduced cells along the linear distance of individual cochlear preparations (Figure 5c).

Next we analyzed mid-modiolar cross-sections of the injected cochleae in order to evaluate transduced cell types. GFP expression was predominantly localized to the organ of Corti region with tropism for supporting cells (pillar and Deiters cells, inner and outer sulcus cells) (Figure 4c,f). A small number of inner and outer hair cells were also transduced in each cochlea (Figure 4c). Sporadic transduction was seen in cells of the spiral prominence, Reissner's Membrane and stria vascularis. SGNs were transduced with high efficiency ($51.1\% \pm 8.0\%$) (Figure 4g).

Although we observed no apparent structural abnormalities or evidence of immune cell infiltrates in the transduced cochleae, we screened the hearing of injected mice at 5 weeks of age. Hearing thresholds determined by click-stimulus ABR showed no significant difference ($p > 0.05$) between injected ears ($43.5 \text{ dB} \pm 7.5$, $n=4$), contralateral non-injected ears ($46.3 \text{ dB} \pm 4.8$, $n=4$) and non-injected littermate ears ($44.0 \text{ dB} \pm 5.7$, $n=12$) (Figure 3a). Click-stimulus ABR assesses hearing across a range of frequencies. Hair cells in the cochlea are tuned to respond maximally to different sound frequencies depending on their position along the length of the cochlea. The apex of the cochlea is attuned to respond to low frequency stimulation and the base to high frequency stimulation. Because of the linear transduction gradient observed with BAAV we tested pure-tone ABRs at 4, 8, 16, and 32 kHz (Figure 3b). There was no significant difference ($p > 0.05$) in hearing threshold at any of the tested frequencies between injected ears (4 kHz = $88.3 \text{ dB} \pm 2.6$, 8 kHz = 54.2 ± 2.0 , 16 kHz = 35.8 ± 2.0 , 32 kHz = 34.2 ± 5.8 ; $n=6$), contralateral non-injected ears (4 kHz = $88.3 \text{ dB} \pm 2.6$, 8 kHz = 55.0 ± 3.2 , 16 kHz = 35.8 ± 2.0 , 32 kHz = 37.5 ± 5.2 ; $n=6$), and non-injected littermate ears (4 kHz = $89.5 \text{ dB} \pm 3.2$, 8 kHz = 54.8 ± 3.4 , 16 kHz = 36.3 ± 3.9 , 32 kHz = 35.8 ± 6.3 ; $n=20$). These results indicate that BAAV transduction of cells of the developing cochlea has no detrimental effects on hearing function to at least 5 weeks of age.

4. DISCUSSION

Gene therapy offers a promising means for modulating gene expression in the inner ear with the ultimate goal of altering the deafness phenotype. Viral tropism and the potential for ototoxic effects are important considerations when selecting a vector for gene transfer. Bedrosian and colleagues have identified AAV2/1 as a safe vector with tropism for sensory hair cells of the organ of Corti when delivered to the embryonic otocyst (Bedrosian et al., 2006). Here we report cellular tropism and potential ototoxicity profiles of early- and advanced-generation adenoviral vectors as well as bovine adeno-associated virus.

Our data for AV are consistent with previous *in vitro* and *in vivo* studies that show tropism for supporting cells (Husseman et al., 2009; Ishimoto et al., 2002; Izumikawa et al., 2005; Shou et al., 2003; Yamasoba et al., 1999). Although both sensory hair cells and supporting cells of the sensory epithelium were transduced at P7, mid-modiolar sections at P35 showed

transduction favoring supporting cells. AV does not integrate into the host genome and is only capable of achieving transient transgene expression. The inconsistent transduction profiles we observed in the mid-modiolar sections of AV-injected cochleae and weak transduction observed with Adf.11D suggest that the five-week time point may have surpassed peak transduction time for adenovectors. The five-week time point was selected because *in vitro* transduction of the cochlea by AV reaches its peak at approximately P35 (Holt et al., 1999). Izumikawa and colleagues reported persistent *in vivo* GFP expression in the guinea pig cochlea two months after inoculation with AV (Izumikawa et al., 2005). However, it appears likely that peak expression with AV delivered to the embryonic otocyst occurred earlier than P35. In the future, therefore, earlier analysis of AV cochlear tropism would be beneficial.

Early-generation (E1/E3-deleted) adenovectors have been shown to induce hair cell toxicity *in vitro* and *in vivo*. Early-generation AV transduction of neonatal mouse cochlear cultures resulted in loss of hair cell bundles after 36 hours (Holt et al., 1999). When delivered *in vivo* to the guinea pig cochlea, this same early generation AV resulted in diminished DPOAE magnitudes and eventual outer hair cell death (Luebke et al., 2001a). As AV had not previously been administered *in vivo* to the embryonic cochlea and an adverse immune response was not expected at this early stage of development, we delivered Ad5.CMV.GFP to the embryonic otocyst and found that it was ototoxic. This ototoxicity was consistent with other reports of ototoxicity associated with delivery of early-generation AV (Luebke et al., 2009).

Late-generation adenovectors have been shown to transduce the cochlea without ototoxic effects (Luebke et al., 2009). For example, E1/E3/E2b-deleted AV preserved hair cell bundles and mechanotransduction in murine cochlear cultures and transduced adult guinea pig hair cells without diminishing cochlear function (Holt, 2002; Luebke et al., 2001b). Similarly, E1/E3/E4-deleted AV injected into the round window of adult mice did not diminish hearing function, as measured by ABR (Praetorius et al., 2003). Based on these previous reports, we delivered an E1/E3/E4-deleted late-generation AV (Adf.11D) to the murine embryonic otocyst. However, in contrast with the reports in which E1/E3/E4-deleted adenovectors were delivered to the inner ear of adult mice without toxicity, delivery of Adf.11D to the E12.5 otocyst resulted in significant ototoxicity. Despite the significantly elevated hearing thresholds in AV-injected cochleae, we did not observe morphologic damage to the organ of Corti or loss of hair cells in mid-modiolar sections of the cochleae. The significant ototoxicity associated with advanced-generation adenovirus was unexpected. Potential reasons for the ototoxicity include: pressure from the injection, GFP expression, triggering of immune responses, high titers of AV delivered, and increased sensitivity of the rapidly developing otocyst cells to adenovirus.

Pressure toxicity, or toxicity from the otocyst injection technique, is not likely because each of the injections for AV as well as BAAV was performed by the same person following an identical protocol that allows for safe delivery to the otocyst (Brigande et al., 2009). In the report by Bedrosian and colleagues lentivirus had ototoxic effects while none of the pseudotyped AAVs were associated with toxicity (Bedrosian et al., 2006). There are reports that GFP can be toxic to living cells (Liu et al., 1999), but there are currently no reports of GFP-induced ototoxicity. Viruses expressing GFP from a CMV promoter have been safely delivered to the cochlea of adult rodents (Praetorius et al., 2003) and Atoh1-GFP plasmid constructs have been injected and electroporated into the developing murine otocyst without detriment to hearing (Gubbels et al., 2008).

AV is known to induce a potent immune response that may produce ototoxicity (Hildebrand et al., 2008; Ishimoto et al., 2003; Luebke et al., 2001a). Early-generation adenovectors

deleted of the E1/E3 regions have been shown to be toxic to cochlear hair cells *in vivo*, likely related to immune responses (Luebke et al., 2009). To overcome this toxicity, later generation adenovectors have been generated that have reduced immunostimulatory effects (Luebke et al., 2009; Staecker et al., 2004). For example, advanced-generation adenovectors deleted of the E1/E3/E2b regions have been reported to be less immunogenic than earlier generations, as have E1/E3/E4-deleted adenovectors (Holt, 2002; Izumikawa et al., 2005; Luebke et al., 2001b; Praetorius et al., 2003). The E4 region of AV encodes proteins that modulate function of the host cells and removal of this region leads to decreased cytotoxicity and immunostimulation (Luebke et al., 2009; Praetorius et al., 2003; Staecker et al., 2004). In histological cross-sections of the cochlea we observed no evidence of immune cell infiltrates. Additionally, the blood-labyrinth barrier maintains the inner ear as a relatively immune-protected region, minimizing concern for immune-mediated complications in the intact cochlea (Juhn et al., 1981). Ototoxicity may also depend on the titer of virus delivered, with lower titers being safer but achieving less robust transduction (Luebke et al., 2001a; Luebke et al., 2001b). Because of the limited volume of the otocyst, we delivered high titer AV (7.1×10^{10} ffu/ml). It may be possible to achieve sufficient transduction of the organ of Corti and diminish ototoxic effects by using lower titers of virus. However, the dynamic range for *in vivo* AV transduction in the cochlea is reportedly narrow and significantly reducing the titer may result in negligible transduction (Luebke et al., 2009). In addition to delivering lower titers of AV to the embryonic otocyst, future efforts may include delivery of other advanced-generation adenovectors, such as E1/E3/E2b-deleted AV or a “gutted” AV that contains only essential packaging sequences and has no immunostimulatory effects (Luebke et al., 2009).

Bovine adeno-associated virus (BAAV) has recently been used *in vivo* to transduce cells in the adult guinea pig cochlea (Shibata et al., 2009). We were able to recapitulate these results when BAAV was delivered to the murine otocyst. BAAV expression after embryonic delivery showed consistent tropism for the organ of Corti and for SGNs. In the organ of Corti, supporting cells were preferentially transduced with occasional transduction of inner and outer hair cells. Other cochlear cell types were sporadically transduced. While transduction occurred in each turn of the cochlea, we observed a preference for transduction toward the apex. Both click-stimulus and pure-tone ABR tests indicated functionally normal hearing in the BAAV-treated ears at 5 weeks of age. Additionally, none of the injected mice displayed phenotypes indicative of vestibular dysfunction prior to sacrifice.

The apical transduction preference is unique to the transuterine delivery of BAAV to the otocyst. No apical preference has been previously observed with BAAV delivery *in vivo* or *in vitro*. Neither has an apical preference been reported for any other vector delivered via transuterine injection to the otocyst, including AAV2/1, lentivirus, and AV. This finding suggests that the observed apical preference is a consequence of properties unique to BAAV transduction of the developing cochlea.

Cell surface receptors are an important determinant of virus entry, infection, and tropism (Di Pasquale et al., 2010). AAVs are known to utilize a variety of cell surface carbohydrates as attachment receptors. For example, AAV2 binds heparan sulfate proteoglycans, while AAV4 and AAV5 each recognize different forms of sialic acid for cell attachment and transduction (Kaludov et al., 2001; Summerford et al., 1998). The platelet-derived growth factor receptor, an integral membrane glycoprotein with a terminal 2–3-linked sialic acid, is a co-receptor for AAV5 transduction (Di Pasquale et al., 2003). BAAV has unique tropism compared with other AAVs and is able to transduce cells of the inner ear with high efficiency (Di Pasquale et al., 2005; Ortolano et al., 2008; Shibata et al., 2009). BAAV transduction appears to occur in a two-step process: cell-binding followed by cell entry. Non-terminal sialic acid groups mediate cell binding and terminal sialic acid groups are

essential for cell entry and transduction (Schmidt et al., 2006). Unlike AAV5, which uses a glycoprotein as its receptor, BAAV requires glycosphingolipids for transduction. Gangliosides, glycosphingolipids containing sialic acid, are essential for BAAV entry into cells (Schmidt et al., 2006). Multiple gangliosides are expressed in the mammalian cochlea (Maguchi et al., 1991; Santi et al., 1994; Yoshikawa et al., 2009). GM3, the most abundant ganglioside in the inner ear, is the first product in the biosynthetic pathway of the ganglio-series gangliosides and serves as a common precursor to many other gangliosides. GM3 and GT1b, another ganglioside expressed in the inner ear, are found throughout the cochlea at P3 but after P14 localize to specific regions in the inner ear including the organ of Corti (Yoshikawa et al., 2009).

The most likely cause of the apical transduction preference observed with BAAV is differential expression of cellular BAAV receptors during cochlear development. There is ample evidence for differential expression gradients along the length of the cochlea during development. Terminal mitosis of cells in the organ of Corti begins apically and proceeds towards the base between E12.5 and E18.5 (Ruben et al., 1967). Cytological differentiation of the organ of Corti occurs in the opposite direction, beginning in the base of the cochlea and proceeding apically (Sher, 1971). A cDNA microarray analysis identified 141 genes that were differentially expressed between the apex and the base of the mouse cochlea (Sato et al., 2009). Individual gangliosides have unique spatiotemporal distribution within the cochlea and their localization is known to dramatically change during early postnatal development (Yoshikawa et al., 2009). The transduction gradient observed with BAAV is likely a result of the spatiotemporal distribution pattern of cellular attachment and internalization receptors during cochlear development. In order to better understand BAAV's apical transduction preference in the developing cochlea it will be important to identify specific cellular receptors for BAAV transduction and determine the expression patterns of these receptors during development.

In summary, BAAV has minimal impact on inner ear function compared to AV. It displays strong tropism for inner ear sensory epithelium and has the ability to penetrate barrier epithelial cells via transcytosis (Ortolano et al., 2008). These properties, together with BAAV's efficient and non-toxic transduction of organ of Corti cells via embryonic otocyst injection, make BAAV an attractive vector for delivery of long-term gene therapy to the inner ear to treat connexin-related (e.g. DFNB1) and other forms of deafness associated with pathology of cochlear supporting cells.

ABBREVIATIONS

AAV	adeno-associated virus
ABR	auditory brainstem response
ADNSHL	autosomal dominant nonsyndromic hearing loss
ARNSHL	autosomal recessive nonsyndromic hearing loss
AV	adenovirus
BAAV	bovine adeno-associated virus
CMV	cytomegalovirus
CX26	connexin 26
CX30	connexin 30
dB	decibel

GFP	green fluorescent protein
NSHL	nonsyndromic hearing loss
SGN	spiral ganglion neuron

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Figure 1. Adenovector transduction of inner ear

Transduction of the mouse sensory epithelium at age P7 following *in utero* microinjection of Ad5.CMV.GFP at E12.5. Cells throughout the sensory epithelium (marked by bracket) were robustly transduced (a). Adjusting the focal plane to the hair cell region clearly shows transduction of outer hair cells by Ad5.CMV.GFP (b).

HCR = hair cell region; OHC = outer hair cell

Figure 2. Adenoviral transduction of cochlear cells in the adult mouse inner ear

Microinjection of Ad5.CMV.GFP into the mouse otocyst was performed at E12.5 and injected mice were sacrificed at 5 weeks. Transduction of cochlear cells is still evident six weeks after injection of Ad5.CMV.GFP as shown by GFP fluorescence (a) and immunofluorescence with an anti-GFP antibody (b) in mid-modiolar cross section of the cochlea. A merged image is shown in (c). Cells lining the scala media cavity (c) were predominantly transduced by the AV with sparse transduction of spiral ganglion neurons (data not shown). Targeted transduction of supporting cells in the organ of Corti is shown by GFP fluorescence (d). Immunofluorescence with an anti-myosin VIIA antibody was conducted to specifically mark the inner and outer hair cells (e). A merged image is shown in (f). Deiters cells that support the outer hair cells appear to be readily transduced. Adf.11D expression was also localized to the organ of Corti, but was less intense than Ad5.CMV.GFP expression (data not shown).

IHC = inner hair cell; OHC = outer hair cell; DC = Deiters (supporting) cells; SV = stria vascularis; SL = spiral limbus; RM = Reissner's membrane; IS = inner sulcus

Figure 3. Auditory assessment of mice injected with viral vectors

Five-week old mice were anesthetized and placed in an acoustic test chamber for measurement of ABR thresholds. (a) Average click-stimulus hearing thresholds of mice injected with virus compared with non-injected contralateral ears and non-injected littermates, respectively: Ad5.CMV.GFP (97.5 dB ± 5, n=4; 45 dB ± 0, n=4; 45.8 dB ± 7.4, n=6), Adf.11D (99.4 dB ± 19.8, n=9; 56.1 dB ± 15.4, n=9; 51.3 dB ± 4.3, n=20), BAAV.CMV.GFP (43.5 dB ± 7.5, n=4; 46.3 dB ± 4.8, n=4; 44 dB ± 5.7, n=12). Using the two-sample t-test, both early- and advanced-generation AV injected ears had significant ototoxicity (indicated by asterisks) compared with contralateral non-injected ears (Ad5.CMV.GFP: $t(6)=21.0$, $p=7.6 \times 10^{-7}$; Adf.11D: $t(15)=5.19$, $p=1.0 \times 10^{-4}$), while

BAAV injections were non-ototoxic ($p>0.05$). (b) Pure-tone frequency (4, 8, 16, and 32 kHz) audiograms of mice injected with BAAV-CMV-GFP ($n=6$) compared with contralateral ears ($n=6$) and non-injected littermates ($n=20$) showed no significant threshold differences at any of the tested frequencies by single factor ANOVA calculations ($p>0.05$).

Figure 4. BAAV transduction of cochlear cells in the adult mouse inner ear

BAAV.CMV.GFP transduction of supporting cells in the organ of Corti (a–f) and spiral ganglion neurons (g) is shown by GFP fluorescence in 5-week old mice following otocyst injection at E12.5. Occasional inner (c) and outer hair cell transduction occurs. Immunostaining with anti-myosin VIIA antibody marks the inner and outer hair cells (b, e). Merged images are shown in (c, f). Nuclei stained with DAPI.

IHC = inner hair cell; OHC = outer hair cells; SGN = Spiral ganglion neurons

Figure 5. Gradient of BAAV transduction from cochlear apex to base

(a,b) BAAV.CMV.GFP transduction of supporting cells is shown by GFP fluorescence in P5 explants of cochlear sensory epithelium. Immunostaining with anti-myosin VIIA antibody marks the inner and outer hair cells. (c) Graphical representation of the number of transduced cells in 500 μm segments extending from the apex to the base of BAAV11-2, a representative explant from a BAAV-injected cochlea. (d) Mean transduction efficiencies from the most apical, middle, and most basal 1mm segments of cochleae were 39.8 ± 5.3 , 29.5 ± 13.1 , and 8.3 ± 5.6 transduced cells/500 μm, respectively. Transduction efficiencies at the apex and at the middle of the cochlea were each significantly higher than the efficiency at the base according to the two-sample t-test for equal variances (Apex:

$t(6)=8.24$, $p=1.7\times 10^{-4}$ (**); Middle: $t(6)=2.99$, $p=0.02$ (*). The transduction efficiencies between the apex and the middle did not significantly differ ($t(6) = 1.45$, $p > 0.05$).