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AAV mediated gene delivery to the inner ear an in vitro study

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AAV Mediated Gene Delivery to the Inner Ear, an In Vitro Study

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Adeno-associated virus (AAV) is generally considered to be an attractive vector for gene delivery. Previous attempts to transduce auditory hair cells and support cells with AAV have not been successful. New AAV serotypes are now available that have not yet been tested in the auditory system. These vectors are able to target a wide range of cells, and in some cases, may use different cell surface receptors and co-receptors for viral entry into cells. For example, AAV2 has been shown to bind heparan sulfate proteoglycans with alpha2beta1 integrin and fibroblast growth factor receptor 1 as a co-receptor. AAV5 binds sialic acid and uses platelet derived growth factor as a co-receptor.

To assess the transduction efficiency of AAV in specific cell types within the cochlea, primary cochlear explants from CD1 mice age P0-1 were transduced with AAV serotypes 1, 2 or 5 carrying the GFP gene expression cassette. GFP gene expression was detected in hair cells and support cells following transduction with AAV1 and AAV2 when the ubiquitous CAG promoter. AAV5 transduced fibroblasts, neurons, and few support cells within the cochlear explants but not sensory epithelial cells. To determine whether support cell specific expression would occur, primary cochlear explants were treated with AAV1, 2, or 5 carrying the GFP gene driven by the astrocyte-specific GFAP promoter. Under these conditions, GFP expression was observed specifically in support cells but not hair cells following transduction with AAV1 and AAV2. No GLAST positive cells showed GFP expression following transduction with AAV1 or AAV5. However, GFP expression was observed within cell bodies of GLAST positive cells following transduction with AAV2. A gradient of GFP expression was observed in a basal to apical preference for each serotype with both promoters. When the GFP marker gene was driven by the CAG promoter, robust expression in hair cells was observed following transduction of primary mouse cochlear explants with AAV1 and AAV2. However, AAV5 transduced hair cells and support cells inefficiently. Using AAV1 and AAV2, we further demonstrated that GFP gene expression could be directed specifically to support cells using the GFAP promoter.
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Introduction

Auditory System

In mammals, the cochlea is the sensory end organ of the auditory system. The cochlea is encased in a bony structure called the otic capsule (Figure 1). It is located in the inner ear and forms a coiled cavity of two to four turns in most species. The basal end transduces high frequency sounds while the apex transduces low frequencies. The organ of Corti is the sensory organ of the cochlea. It sits on the basilar membrane and is covered by the Vestibular (or Reissner’s) membrane. This forms the scala media, a fluid-filled compartment containing endolymph. Endolymph is similar to intracellular fluids containing a high concentration of potassium (Raphael 2003). The two adjacent compartments are the scala vestibuli above and the scala tympani below, both of which contain perilymph. Perilymph is similar to extracellular fluid, high in sodium, and is continuous with cerebral spinal fluid (CSF) via the cochlear aqueduct (Raphael 2003).

Hair cells and support cells are the predominant cell types within the organ of Corti and exhibit a distinct architectural organization (see Figure 1). Hair cells, named for the bundles of stereocillia at their apical ends, are divided into two distinct populations based on their location within the organ of Corti. The inner hair cells form a single row and are the primary sensory cells of the cochlea. The three rows of outer hair cells amplify the mechanical activity of the basilar membrane and increase sensitivity to sound. Inner hair cells are innervated by a single type I neuron, while the outer hair cells are innervated by 5-100 type II neurons (Raphael 2003; Squire LR 2003). The inner hair cells are considered to transduce the initial, rapid response to sound and outer hair cells magnify and fine-tune the signal.
Support cells are a population of highly differentiated epithelial cells located immediately beneath the hair cells and rest on the basilar membrane (Ashmore 2000; Rubel 2002; Raphael 2003). There are several types of support cells based on morphology and location within the organ of Corti including; Interdental, Pillar, Dieters’, and Hensen cells. The function of mammalian support cells is unclear but they may be involved in the homeostasis of the ionic environment and stiffness of the cochlear partition (Raphael 2003).

In avian systems, a population of support cells can differentiate into hair cells after noise or aminoglycoside antibiotic damage (Rubel 1991; Cotanche 1997; Ishimoto 2002). After damage to the sensory epithelia, specific support cells that are in direct contact to the damaged hair cells are induced to return to the cell cycle and divide (Cotanche 1997). Cotanche (1997) demonstrated that bromodeoxyuridine (BrdU) labeled support cells could give rise to numerous daughter cells that could become either hair cells or support cells (Cotanche 1997). These new hair cells were found to develop stereocilia and form functional neuronal connections. However, hair cells have not been observed to spontaneously regenerate in mammals.
Figure 1. Cochlea.
Figure 1. Cochlea.

A schematic illustration of the mammalian inner ear. The different regions and cell types are labeled. (a) Cochlea and vestibular region of inner ear. (b) Cross section of the cochlea. (c) Scala media. (d) Single inner hair cell. These images have been reproduced from *The Essentials of Human Anatomy and Physiology 3rd Ed.* McGraw Hill 2000.
Hearing loss

More than 20 million Americans suffer from some type of hearing loss (Kho 2000; Luebke 2001). There are five major causes of hearing loss (Kotecha 1994; Wersall 1995; Hudspeth 2000). First, infections such as meningitis and rubella, have been shown to cause severe hearing loss by and can cause inflammation leading to damage of the sensory epithelia (Lai 2002). Second, acute acoustic trauma which can occurs after exposure to loud sound. The mechanism for this type of damage is mediated in part by glutamate excitotoxicity. Third, gradual deterioration from chronic exposure to a loud sound or the breakdown of small blood vessels due to atherogenesis (Hudspeth 2000). The fourth major cause of hearing loss is due to drug-related ototoxicity.

Aminoglycoside antibiotics are charged, poly-cationic molecules which include gentamicin and streptomycin (Kotecha 1994). These antibiotics are known to be both ototoxic and nephrotoxic. Aminoglycoside antibiotics cause hair cell death in the cochlea and vestibular regions with the most basal regions affected first (Wersall 1995). Gentamicin is widely used to treat Gram-negative bacterial infections. The damage induced by gentamicin toxicity results in lesions of the outer hair cells leading to the ultimate destruction of those cells (Kotecha 1994). Another class of drugs with ototoxic side effects is platinum-based chemotherapeutics. These drugs are used to treat solid and disseminated forms of cancer (McFadden 2003). An example of this class of drugs is Cisplatin, which is known to be ototoxic, neurotoxic and nephrotoxic. The side effects of cisplatin include irreversible bi-lateral sensorineural hearing loss and tinnitus (ringing in the ear) (Sergi 2003). Cisplatin-induced otoxicity is observed at the high frequency regions of the cochlea and is dose-dependent. Damage involves destruction of outer hair
cells and sporadic loss of inner hair cells. Degeneration of the stria vascularis and a significant decrease in spiral ganglion cells has also been reported (Sergi 2003).

The last major cause of hearing loss is due to genetic disease. There are more than 100 genetically-based hearing disorders. Two of the most common are Usher’s syndrome and mutations in the connexin 26 gene. Usher’s syndrome, an example of syndromic hearing loss, features deafness and vestibular dysfunction along with blindness (Steel 1997; Hone 2003). This syndrome affects 4.4 per 100,000 people in the US and accounts for 2-4% of profound deafness and 50% of the deaf-blind population (Nance 2003). Non-syndromic hearing loss makes up 70-80% of genetic deafness (Nance 2003). Mutations in the connexin 26 gene lead to the most prevalent form of non-syndromic hearing loss (Avraham 2003). This is responsible for up to half of autosomal-recessive non-syndromic hearing loss and a significant amount of sporadic hearing loss with mutations identified in over 60 different loci (Hone 2003).

**Gene Therapy**

Current treatments such as hearing aids and cochlear implants are not always available or useful for people with inherited or acquired hearing disorders. These treatments use the remaining functioning part of the auditory system to correct or enhance hearing for patients that have previously had normal or slightly less than normal hearing. The lack of effective treatment has prompted interest in the potential application of newly emerging gene transfer methods that may provide an additional option for the treatment of hearing loss. The compartmentalization of the inner ear is ideal for localized gene therapy treatments. The fluid-filled compartments allow secreted gene products to
diffuse throughout the cochlea making them available to all cell types. Most mammals have a cochlear aqueduct that connects directly to the cerebral spinal fluid. However, most humans don’t have a cochlear aqueduct, making the inner ear a closed system and thus limiting dissemination of introduced therapeutic genes to unwanted tissues outside of the cochlea itself (Van de Water 1999).

Gene transfer, or gene therapy, can be defined as the transfer of nucleic acids for the treatment or prevention of disease. The objective of gene transfer is to replace a defective gene with a correct, exogenous copy or to deliver a therapeutic gene. The ideal vehicle for gene therapy would: 1) be easily produced and purified, 2) be non-immunogenic and non-oncogenic, 3) allow unlimited gene packaging capacity, 4) be tissue or cell type-specific, 5) have prolonged, controllable expression, and 6) the ability to transfect both dividing and non-dividing cells (Staecker 2001; Grimm 2002; Lu 2004).

Methods of gene transfer include non-viral vectors and a variety of viral vectors (Robbins 1998). So far, none of these methods meets the criteria as the “ideal” gene transfer vehicle. However, each has their share of advantages and disadvantages.

Non-viral Vectors

Naked nucleic acids (DNA) can be directly delivered to cells in several ways. Electroporation uses an electric current to force DNA into cells. This method can damage or kill large numbers of cells and may not be practical for in vivo use. DNA can also be delivered to cells using a “gene gun”. This method uses a helium driven gun to “shoot” gold-plated beads covered with DNA into cells (Robbins 1998). However, using these methods, the efficiency and duration of expression is highly variable. Finally, the
direct addition of “naked” nucleic acids is limited by permissive cell types, such as muscle, and has a short duration of expression (Robbins 1998; Poulsen 2002).

Liposomes are positively charged molecules that can be treated to form pseudo-membranes. The advantages of liposomes are that they are non-infectious, easy to make, and have a virtually unlimited packaging capacity. However, they have been shown to be relatively inefficient in gene transfer and associated with a short duration of transgene expression (approximately 1 week) (Van de Water 1999; Poulsen 2002). Another major disadvantage is there is almost no tissue-targeting capability. Jero et al. (2001) used gelfoam soaked with a liposome-GFP plasmid complex to deliver GFP to the mouse cochlea via round window application. Increased GFP expression was observed in the ipsilateral cochlea specifically within the spiral limbus, spiral ganglion cells and Reissner’s membrane (Jero 2001).

**Viral Vectors for Gene Therapy**

Viral mediated gene delivery, or transduction, is a highly efficient means of gene transfer. However, there are several factors that affect gene expression including gene size and maintenance, immune response, and promoter selection. Gene size is an important consideration and the packaging capacities of viral vectors differ greatly. Therapeutic genes for hearing loss, or any disease, are highly variable in size, therefore the gene therapy vector needs to be flexible in its packaging capacity. For example, adeno-associated virus (AAV) has a relatively small packaging size (4.5kb) compared to herpes simplex virus (HSV) with a relatively large capacity (50kb).
How the transgenes are maintained has a large impact on the duration of gene expression. This depends in part on the viral vector used. Genes can integrate randomly, in a site-specific manner into the host cell chromosome, or can be maintained episomally within the nucleus. Random integration into the host chromosome can cause gene disruption and/or oncogenic transformation of the transduced cell. Both of which are undesirable and could cause the ultimate destruction of the transduced cell or uncontrolled cell proliferation. However, chromosomal integration can lead to permanent transduction of a cell and subsequent maintenance after cell division.

The ability of the virus to elicit an immune response also determines the duration of gene expression. The viral proteins and/or the antigenicity of the transgene itself can play a role in the potential immune response. This could be considered a beneficial effect in the case of gene transfer for cancer treatment. If the vector is targeted to cancer cells, the immune system may then recognize the cancerous cells as foreign thus mounting an immune response against the tumor itself. However, if long-term expression is the goal, an immune response could decrease the duration of expression by the ultimate elimination of the transduced cells. An immune response could also inhibit the ability to re-administer the vector if multiple doses are required.

The promoter selected can determine which cell type(s) express the transduced gene and the level of expression. Traditionally, the human cytomegalovirus immediate early promoter (CMV) has been the promoter of choice for most gene transfer studies. It is a ubiquitous, constitutive promoter that has been shown to drive very high levels of gene expression. The CAG promoter is a hybrid promoter of the chicken beta-actin (CBA) and CMV promoters (Xu 2001; Klein 2002). This is a ubiquitous promoter and
has been shown to drive robust expression in liver and brain (Xu 2001; Klein 2002). The glial fibrillary acidic protein (GFAP) promoter drives expression in a more selective subset of glial cells with an astrocytic phenotype (Rio 2002). Thus, promoter selection is one way of targeting tissue or cells of interest and minimizing possible complications of nonspecific transduction.

Regulatory elements are sequences within the DNA that control transcription, either negatively or positively, by binding to a particular site(s) on the DNA. An example of an enhancer regulatory element is the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). This gene has evolved to stimulate the expression of intronless viral messages by aiding in transport out of the nucleus (Loeb 1999). WPRE has been shown to increase the stability and level of gene expression, both in vitro and in vivo (Loeb 1999; Klein 2002).

There are many viral vectors available for use in gene transfer. These include retrovirus, HSV, adenovirus and AAV. As with non-viral vectors these too have both pros and cons for use as gene therapy vectors.

Retrovirus

Retroviruses, including lentivirus murine leukemia virus, contain a single-stranded RNA genome (Berns 1991; Robbins 1998). These viral vectors have a packaging capacity of 6-8kb (Poulsen 2002). Unlike most retroviruses, lentivirus can transduce both mitotic and post-mitotic cells. This class of vectors is relatively easy to produce in moderate titers (Robbins 1998). Both the advantages and disadvantages of retroviral vectors stem from their ability to integrate into the host chromosome. The
advantages of integration include expression of the transgene for the life of the cell and maintenance of that gene after mitotic divisions (Robbins 1998). The major drawback of this is the possibility of disrupting a functional gene or oncogenic transformation of the transduced cell after random integration (Berns 1991; Van de Water 1999; Poulsen 2002). Lentivirus has been shown to transduce cells within the inner ear. However, transduction was restricted to the perilymphatic space (Han 1999).

**Herpes simplex virus**

Herpes simplex virus type I (HSV) is a large double-stranded DNA virus with a genome of approximately 150kb. As a viral vector, HSV has a packaging capacity of approximately 50kb and preferentially targets neuronal derived tissue (Van de Water 1999). The main disadvantage of HSV as a vector is the possibility of the virus entering into a lytic versus a latent life cycle. In the lytic cycle, large quantities of the virus are produced and the transduced cell is ultimately destroyed by the host immune system (Van de Water 1999). However, modifications have been made rendering HSV vectors replication deficient. Thus, recombinant HSV vectors enter only the latent stage and evade the host immune system (Robbins 1998; Van de Water 1999). A major disadvantage of HSV is the associated short duration of transgene expression, often less than two months. Another disadvantage is the relative difficulty in production of high titers thus a larger volume of virus must be used compared to other viral vectors (Staecker 2001). This virus has been used extensively in gene transfer studies in the mammalian nervous system and auditory system (Derby 1999). When HSV was injected into the round window of the guinea pig, cells in the spiral ligament, Reissner's
membrane, scala vestibuli, and some support cells within the organ of Corti were transduced (Kawamoto 2001). However, expression decreased to undetectable levels in only 6 days (Derby 1999).

**Adenovirus**

Adenovirus is a double-stranded DNA virus with a genome of 36 kb in length (Lai 2002). Adenoviral vectors accommodate relatively large inserts (8-10kb) and mediate high levels of transient protein expression (Van de Water 1999; Lai 2002; Li Duan 2002). These vectors have been shown to transduce a broad range of both mitotic and post-mitotic host cells. Transduced genes are maintained episomally (Lai 2002; Li Duan 2002). A major drawback in using adenoviral vectors is the fact that they introduce viral genes into the host cell that can stimulate an immune response. This limits the duration of gene expression due to the ultimate destruction of the transduced cells as well as prohibiting re-administration of the viral vector (Robbins 1998; Van de Water 1999; Lai 2002).

Adenovirus has been used extensively for gene transfer studies in many systems, including the auditory system, and has been shown to transduce cells within the cochlea of mouse and guinea pig (Stover 2000; Kawamoto 2001; Ishimoto 2002; Kanzaki 2002; Kawamoto 2003). Adenovirus has been shown to transduce the scala tympani of the basal and second turn, over 90% of inner hair cells, and more than 50% of outer hair cells and some supporting cells of the guinea pig *in vivo*. (Stover 2000; Luebke 2001; Luebke 2001). Adenovirus containing glial-derived neurotrophic factor (GDNF) has been shown to protect hair cells against sound damage (Kawamoto 2001). Ishimoto et al. (2002)
showed gene transfer into supporting cells of guinea pig using adenovirus. The results revealed damage to the hair cells when adenovirus was directly injected into the scala media. However, using immunostaining, it was shown that all supporting cells within the organ of Corti and cells in the inner and outer sulcus were positive for the transgene (Ishimoto 2002).

Adeno-associated Virus

Adeno-associated virus (AAV) is a single-stranded DNA virus from the family Parvoviridae and genus dependovirus (Tal 2000; Smith-Arica 2001; Rabinowitz 2002; Xie 2002). The genome of AAV is approximately 4700 nucleotides in length (Tal 2000; Sanlioglu 2001; Lai 2002; Monahan 2002). AAV has many properties that make it an attractive viral vector for gene transfer. Although approximately 85% of humans are seropositive, the virus has not been associated with any pathology or disease (Lalwani 1998; Carter 2000; Stover 2000; Lai 2002; Xie 2002; Tenenbaum 2003). One of the most appealing qualities is its ability to transduce a wide range of both mitotic and post-mitotic cells (Lalwani 1998; Tal 2000; Sanlioglu 2001; Smith-Arica 2001; Lai 2002). The majority of the AAV viral genome (96%) has been removed from the current recombinant AAV vectors (rAAV). Only the 145bp inverted terminal repeats (ITR) are retained and are the minimal sequence required for packaging (Carter 2000; Monahan 2002). Because of this fact, transduced cells do not produce AAV antigens (Smith-Arica 2001; Monahan 2002). This limits the immune response to transduced cells and allows transgene expression to persist for up to at least 5 years (Stover 2000; Lai 2002; Monahan 2002). However, neutralizing antibodies have been generated against the capsid protein
of AAV serotype 2 and immune response to specific gene products has been observed (Monahan 2002; Tenenbaum 2003). Clinical trials using AAV are currently underway for treatment of cystic fibrosis, hemophilia B, and limb girdle muscular dystrophy (Monahan 2002; Xie 2002; Tenenbaum 2003). These clinical trials have found no apparent pathological effects on cell growth and differentiation.

Currently, there are eight recognized serotypes of AAV based on their respective capsid proteins (Smith-Arica 2001; Gao 2002; Lai 2002; Tenenbaum 2003). AAV 1, 2, and 3 have approximately 85% amino acid sequence homology to each other, while AAV 4 and 5 have only 55% homology to AAV2 (Xie 2002). AAV1 and 6 are 99% homologous and are therefore not considered to be functionally distinct. AAV7 and 8 have recently been isolated from Rhesus monkeys showing 63-85% amino acid sequence identity with the other AAV serotypes (Gao 2002). AAV serotype 2 was the first to be isolated and has been the most extensively studied. The recent isolation of other serotypes has expanded the potential utility of this virus as a gene delivery system. AAV has been used as a gene transfer vector in many different cell types, including muscle, lung, liver and neurons (Sanlioglu 2001; Lai 2002). Alternative serotypes appear to be selective for different cell surface markers thus changing the respective transduction efficiency between cell types. For example, AAV serotype 2 has been shown to bind heparan sulfate proteoglycans (HSPG) using alpha2beta1 integrin and fibroblast growth factor receptor 1 (FGFR1) as co-receptors (Sanlioglu 2001; Xu 2001; Lai 2002; Pasquale 2003; Smith 2004). While AAV serotype 4 and 5 bind sialic acid with AAV5 using platelet-derived growth factor receptor (PDGFR-alpha polypeptide) as a co-receptor (Stover 2000; Walters 2001; Monahan 2002; Xie 2002; Pasquale 2003; Tenenbaum
2003). Cell specificity between the different serotypes has been well documented. For example, AAV1 transduces skeletal muscle and retina with high efficiency, while AAV5 is highly efficient at transducing cells in lung and central nervous tissue (Gao 2002).

Multiple studies have been done in guinea pig cochlea in vivo using low titer AAV2 (10^6 infectious particles) carrying β-galactosidase or GFP reporter genes (Lalwani 1996; Lalwani 1997; Lalwani 1998; Kho 2000). The reporter gene product was observed in the organ of Corti, spiral ligament, spiral limbus, and spiral ganglion cells. Previous studies have suggested that AAV2 is unable to transduce hair cells or support cells in vivo and in vitro (Kho 2000; Jero 2001; Luebke 2001). Several possible reasons for this are the low titers used in the studies (10^5 or 10^8 infectious particles) and the use of alternate promoters.

**Specific Aims**

The overall goal of this project was to explore the utility of AAV for gene transfer in the murine auditory system. Primary cochlear explant cultures represent an attractive in vitro model system because the explants maintain intact physiological morphology. Much of the cochlear research currently being done is in guinea pig and chicken models. However, we chose to use a murine model to take advantage of the many transgenic mutants available that model hearing loss.

The aim of this project was to identify which AAV serotype (1, 2, or 5) would most efficiently transduce hair cells and support cells in vitro. Our primary objectives were to: 1) produce high titers of rAAV virus serotypes 1, 2, and 5; 2) establish postnatal cochlear explants; 3) transduce explants with AAV-CAG-GFP serotypes 1, 2 or 5 to
examine overall gene expression; and 4) transduce explants with AAV-GFAP-GFP serotypes 1, 2, and 5 to examine support cell specific gene expression.
Materials and Methods

Virus production

Adeno-associated virus (AAV) serotypes 1, 2, and 5 were packaged in human embryonic kidney 293T cells (ATCC, CRL-11268). Cells were maintained in growth medium consisting of Dulbecco’s modification of Eagle’s medium (DMEM, Cellgro) supplemented with 10% fetal bovine serum (FBS, Hyclone), 0.05% penicillin/streptomycin (5000U/mL, Cellgro), 0.1 mM MEM nonessential amino acids (Cellgro), 1 mM MEM sodium pyruvate (Cellgro), and gentamicin (25 mg/mL, Cellgro). The day before transfection, approximately 1.5x10^7 cells were plated on 150mm dishes containing growth medium. Twenty-four hours later, medium was changed to DMEM containing 5% FBS and antibiotics and cells were transfected using Polyfect transfection reagent (Qiagen). All plasmids were purified using the Endo-Free Mega kits (Qiagen) or High Purity Plasmid Maxiprep kits (Marligen Biosciences). A triple transfection method was used with a plasmid ratio of 2:1:1 (Lai 2002). Plasmids used for transfection were: 1) pFA6 (containing the required adenoviral helper gene); 2) pRVI (capsid and rep genes for serotype 2), pH21 (capsid gene for serotype 1 and rep gene for serotype 2), or pH25a (capsid gene for serotype 5 and rep gene for serotype 2); and 3) trans-gene, GFP cassette flanked by the AAV ITR’s (Figure 2). These plasmids were obtained from the laboratory of Dr. Matthew During Jr. from the University of Auckland, New Zealand.
Figure 2. GFP Cassette.
Figure 2. GFP Cassette.

A schematic representation of the GFP cassettes packaged into the AAV virus. ITR = inverted terminal repeats, CAG = chicken beta actin/ Cytomegalovirus hybrid promoter, GFAP = glial fibrillary acidic protein promoter, hrGFP = humanized renilla green fluorescent protein, WPRE = woodchuck hepatitis post-transcriptional regulatory element, and BGH-PA = bovine growth hormone poly-adenylation signal.
Cells were harvested 48-72 hours later using freeing solution (700mM NaCl, 
13mM KCl, 102mM Na₂PO₄, 3mM Na₂EDTA). Cells were pelleted by centrifugation at 
6,000XG for 15 minutes and re-suspended in 150mM NaCl plus 20mM Tris pH 8.0 
(50mL for every 10 X 150mm dishes). Cells were lysed by three freeze-thaw cycles in a 
dry ice-ethanol bath followed by treatment with 50U benzonase (Novagen) and 0.5% 
sodium deoxycholate. The cell debris was pelleted at 6,000XG for 15 minutes at 4°C. 
The supernatant was then incubated at 56°C for 15 minutes to heat inactivate the 
benzonase. The cell lysate was buffer exchanged with 150mM NaCl plus 20mM Tris pH 
8.0 to remove the deoxycholate and was then condensed to approximately 7.5mL using 
JumboSep centrifugation devices (Pall). The virus was then purified as previously 
described by Zolotukhin et al. (2002). Briefly, discontinuous iodixanol (OptiPrep, 
Accurate Chemical) step gradients were used to displace the less dense cell lysate. Each 
gradient consisted of 5mL of 60%, 5mL of 40%, 6mL of 25%, and 8mL of 15% 
iodixanol. Iodixanol was diluted with PBS containing 1mM MgCl₂ and 2.5mM KCl. 
The 15% solution also contained 1M NaCl to prevent aggregation of viral particles. 
Sealed gradient tubes (25x89 mm, Beckman) were centrifuged at 65,000XG in a Ti-70 
rotor for 2.5 hour at 18°C. Using an 18-gauge needle, the virus was collected by 
removing approximately 5-7mL just below the 60-40% interface. The purified virus was 
buffer exchanged (3X) with PBS to remove the iodixanol and condensed in JumboSep 
centrifugation device followed by Biomax-100 concentrators (Millipore) to a final 
volume of approximately 100-300µL. 

Purified virus was titered using quantitative PCR. Briefly, 25µL of purified virus 
was treated with 50U of benzonase (Novagen) at 37°C for 30 min followed by heat
inactivation at 95°C for 30 min. Proteinase K (10μg, Promega) was added and the mixture incubated at 50°C for 1 hour and heat inactivated at 95°C for 20 min to expose packaged single-stranded DNA. Forward primer (TTC CGG GAC TTT CGC TTT C), reverse primer (AAG GCA GGC GGC GAT AG) and probe (/5TET/CCT CCC TAT TGC CAC GGC GGA/36-TAMRAph/) were designed from the WPRE sequence, an element contained in the GFP plasmid. A standard of the pAM-CAG-hrGFP-WPRE-BGH polyA plasmid was diluted to known concentrations. Virus titers (genomic particles (gp) per ml) were determined by RT-PCR on an ABI 7700 (Applied Biosystems).

**Cochlear cultures**

Primary cochlear explants were prepared from P0-P1 CD1 mice (Charles River). The day of birth was designated post-natal day 0 (P0). All animal procedures were performed in strict accordance with NIH Guide for Care and Use of Laboratory Animals and were approved by the University of Montana Institutional Animal Care and Use Committee. Cochleas were dissected as described previously (Raz 1999; Mueller 2002). Briefly, the cochlea and vestibular region were aseptically dissected away from the skull in cold dissection medium composed of 1X HBSS containing 5mM HEPES and 0.6% glucose. The vestibular region was pinned down and the bony outer capsule was carefully dissected away from the rest of the cochlea. Since the cochlea exceeds more than one turn at P0, the cochlea was cut into 3 pieces and carefully transferred to Mat-tek dishes coated with 0.05mg/mL poly-D-Lysine (BD) followed by 3.75% matrigel (BD). Culture medium was DMEM supplemented with 10% FBS, N2 (1:100, Invitrogen),
Penicillin G (1500U/mL, CalBiochem) plus Fungizone (9μg/mL, Calbiochem) or Ciprofloxacin (10μ/mL, Cellgro). AAV1-CAG-hrGFP, AAV2-CAG-hrGFP, AAV5-CAG-hrGFP, AAV1-GFAP-hrGFP, AAV2-GFAP-hrGFP or AAV5-GFAP-hrGFP was added to the media for a final concentration of 10^10 gp per dish at the time of plating. Cultures were maintained at 37°C, 5% CO₂ for 5 days in a humidified incubator.

**Immunocytochemistry**

**Hair cells and Support cells**

After 5 days, the medium was carefully aspirated and cultures were fixed in cold 4% paraformaldehyde (PFA) at room temperature for 30 minutes followed by a post-fix in ice-cold methanol for 2 minutes at -20°C. To remove residual PFA and methanol, cultures were rinsed with PBS (3 X 15 minutes). Tissue was permeabilized with PBS plus 0.5% Triton X (PBS-Tx) for 1 hour at room temperature. Tissue was then blocked with 5% normal goat serum (NGS) for 1 hour at room temperature. All immunocytochemical reagents were diluted in PBS-Tx unless otherwise noted. Antibodies used, their specificity, and dilutions are outlined in [table 1](#).

All primary antibodies were diluted in PBS-Tx containing 1% NGS and were incubated overnight at 4°C. Unbound primary antibodies were removed by washing tissue with PBS-Tx (3 X 20 minutes). Primary antibodies were detected by incubation with secondary Alexa-Fluor 546 (1:2000, Molecular Probes) for 1 hour at room temperature. To remove any unbound secondary antibody and to remove residual salts, tissues were then washed with PBS-Tx (3 X 10 minutes), PBS (2 X 5 minutes), sterile water, and then mounted using Fluorsave (Calbiochem). Cover slips were sealed with
clear fingernail polish to prevent dessication. Stained cultures were stored at 4°C in the
dark.

**Stereocillia**

After 5 days, cultures were fixed in cold 4% PFA at room temperature for 30
minutes. To remove any residual PFA, tissue was rinsed with PBS-0.1% Tween 20 (2
X10 minutes). Phalloidin was diluted in PBS-0.1% Tween 20 and was incubated with the
tissue for 40 minutes at room temperature. The tissue was then washed with PBS (3 X10
minutes), once with sterile water, and then mounted using Fluorsave. Cover slips were
sealed with clear fingernail polish to prevent dessication. Stained cultures were stored at
4°C in the dark.

**Immunocytochemical analysis**

Images were taken on Bio-Rad Radiance 2000 MP laser scanning confocal or
Olympus fluorescence microscopes (model MT-2), magnification ranged from 20X to
100X. Z stack images 1.5-2 microns thick were taken on the confocal microscope.
Images were merged and analyzed using MetaMorph software (Universal Imaging).
Positive identification of transduced cells was determined by green GFP fluorescence at
488nm. The specific cell types were identified by red immunofluorescence at 543nm.
Cell counts were expressed as a ratio of red immunolabel and green GFP expression over
the cell specific red-immunolabel. A dual label was defined as a positive red
immunolabel and GFP positive cell. Three to four fields, with a total length of
approximately 250 microns of apex or base, were counted at 40X magnification.
Preliminary experiments revealed a difference in transduction between inner and outer hair cells, so separate counts were obtained for both. Counts were also obtained for apical and basal portions of the cochlea due to the fact that transduction appears to follow a basal to apical preference (personal communication with Dr. Matthew Kelley). Data were analyzed using ANOVA using Graphpad Instat (Version 3.0a for Macintosh 2001). A p value of less than 0.05 was considered statistically significant.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cell type</th>
<th>Recognizes</th>
<th>Stock Concentration</th>
<th>Working Dilution</th>
<th>Suppliers</th>
</tr>
</thead>
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<td>1:1500</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Myosin VI</td>
<td>Hair cells</td>
<td>Myosin VI</td>
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<td>1:1000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Connexin 26</td>
<td>Support cells</td>
<td>Gap junctions</td>
<td>1 μg/μL</td>
<td>1:500</td>
<td>Alpha Diagnostics</td>
</tr>
<tr>
<td>GFAP</td>
<td>Support cells</td>
<td>Glial fibillary acidic protein</td>
<td>4.1 g/L</td>
<td>1:500</td>
<td>Chemicon</td>
</tr>
<tr>
<td>GLAST</td>
<td>Support cells</td>
<td>Glutamate/Aspartate transporter</td>
<td>0.5 μg/μL</td>
<td>1:100</td>
<td>Alpha Diagnostics</td>
</tr>
<tr>
<td>Jagged 1</td>
<td>Support cells</td>
<td>Jagged (Notch ligand)</td>
<td>2 μg/mL</td>
<td>1:200</td>
<td>Santa Cruz Biotech</td>
</tr>
<tr>
<td>p75</td>
<td>Support cells</td>
<td>Nerve growth factor p75</td>
<td>N/A</td>
<td>1:1000</td>
<td>Chemicon</td>
</tr>
<tr>
<td>S100a</td>
<td>Support cells</td>
<td>S100 (Ca+ binding protein)</td>
<td>45.6 mg/mL</td>
<td>1:200</td>
<td>DAKO</td>
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</table>
Table 1. **Antibody Information.**

Antibodies used in this study. Phalloidin is a directly conjugated phallotoxin that binds directly to the F-actin of the stereocilia. All primary antibodies are rabbit polyclonal.
Results

Immunocytochemical analysis

Phalloidin, a phallotoxin, binds to the F-actin of stereocillia bundles on the tips of hair cells (Mueller 2002). Phalloidin was used to assess the overall health of the explants and to orient cultures when stained for support cells (Figure 3A). The inner rows of hair cells have “V” shaped bundles. The outer rows, 3 top rows, have more of a “U” shape.

There are four known unconventional myosins (1β, V, VI, and VIIa) found in the sensory epithelia of the inner ear (Hasson 1997). Myosin 1β, VI, and VIIa are located in the hair cells while myosin V is located in the afferent nerve cells that innervate the hair cells. Myosin VI was used to identify hair cells (Figure 3B). Inner hair cells can be seen as the bottom, single row of columnar shaped cells. The outer hair cells form 3-4 rows adjacent to the inner hair cells and are more spiracle shaped. The round unstained portion on the hair cell is the cells’ nuclei.

There are many different support cell antibodies including: Connexin 26, Glial fibrillary acidic protein (GFAP), Glutamate/ Aspartate transporter (GLAST), Jagged1, p75, or S100a (Table 1). However, most of these are not very selective in the type of support cells they recognize and have been shown to give irregular results. In our culture system, the anti-GLAST antibody was the only antibody that gave consistent results. In addition, GLAST is only expressed in border and inner phalangeal cells allowing us to specifically identify these support cells. GLAST positive cells were found to ring the inner hair cells and in a subset of unidentified round peripheral cells (Figure 3C). The long thread-like staining are processes that are projected from the support cell bodies. The “holes” are where the inner hair cells rest.
Figure 3. Confocal Images of Cell Specific Immunostaining

A

B

C
Figure 3. Confocal Images of Cell Specific Immunostaining.

Representative confocal images of P0-1 explant cultures immunostained using Phalloidin-546, anti-Myosin VI or anti-GLAST antibodies and Alexa-Fluor-546 secondary. A, Phalloidin-546 stained stereocilia. The three rows of outer hair cells are at the top of the image and the single row of inner hair cells is immediately below. B, Myosin VI stained hair cells. The bottommost row shows the inner hair cells with 2-3 rows of outer hair cells immediately above. C, GLAST stained support cells. GLAST is specifically expressed only in border and inner phalangeal cells. The “holes” are where the inner hair cells are located. Magnification 40X. Scale bar = 10μm.
AAV transduction of murine hair cells in cochlear explants

Previous studies have concluded that AAV2 does not transduce cochlear hair cells because of the lack of heparin sulfate on the cell surface (Luebke 2001). AAV5 does not require heparin sulfate for cell surface attachment. Therefore, we hypothesized that AAV5 would transduce hair cells most efficiently. However in our hands, we observed that AAV2 and AAV1 could efficiently transduce both inner and outer hair cells in postnatal cultures, while AAV5 does not.

AAV2 transduction leads to robust GFP reporter gene expression in both the inner and outer hair cells. Figure 4 shows a representative image of an AAV2-CAG-GFP transduced basilar region cochlear explant. Inner hair cells are along the top of the culture and the three rows of outer are immediately below. Yellow cells, or co-localized cells, expressed both the GFP gene and are myosin VI positive hair cells. Approximately 61% of outer hair cells and 37% of inner hair cells expressed GFP (Table 2). GFP positive cells were found in both the base and apex with a gradient of expression observed from the base to the apex with more GFP positive cells observed in the base (Figure 5). In the apex, 43% of outer and 15% of inner hair cells expressed GFP (Table 2). GFP expression was also observed in several populations of support cells, possibly interdental, Hensen, and Dieters’ cells in both the base and apex. This expression of GFP in supporting cells also followed the basal to apical gradient.

Like AAV2, AAV1 was also able to efficiently transduce both inner and outer hair cells (Figure 6). The same basal to apical expression gradient was also observed with AAV1 (Figure 7). Outer hair cell transduction was observed at approximately 67% and 66% in basal and apical regions, respectively. While inner hair cells showed a
significant decrease in GFP expression from 52% in the base to 17% in the apex (Table 2). AAV1 transduction also resulted in robust GFP expression in several populations of support cells in the base and apex, possibly interdental, Hensen, and a few Dieters' cells. These support cells appear to be more peripheral to the hair cells than what is seen following AAV2 transduction.

While AAV5 can transduce murine cochlear hair cells, it is a rare and inefficient event (Figure 8). Hair cell transduction efficiency was less than 1% in either base or apex (Table 2). When GFP expression was observed in hair cells it was not to the magnitude of either of the other two serotypes examined in this study. AAV5 also transduced some support cells and neurons within the periphery of the cultures. GFP expression was only observed at very low levels in the apex (Figure 9).

AAV1 and AAV2 demonstrated similar levels of transduction in the basal and apical regions (Table 2). Approximately 45% of inner and 64% outer hair cells were transduced in the basilar region following transduction with AAV1 or AAV2. Robust GFP expression was observed in outer hair cell and to a lesser extent in inner hair cells. Both AAV1 and AAV2 showed significantly more transduction of IHC at the base than in the apex (p<0.001 and p<0.05, respectively). In addition, both AAV1 and AAV2 showed significantly more transduction (p<0.001) in the outer hair cells than the inner hair cells in the apex. The only major statistically significant difference between AAV1 and AAV2 was the percentage of GFP positive OHC transduced in the apex. AAV1 showed significantly more transduction (p<0.05) of OHC in the apex when compared to AAV2. These results clearly show that AAV1 and AAV2 can transduce murine cochlear hair cells and that the CAG promoter is active within these cells.
Figure 4. CAG Promoter Drives Gene Expression in AAV2 Transduces Basilar Cochlear Hair Cells
Figure 4. CAG Promoter Drives Gene Expression in AAV2 Transduced Basilar Cochlear Hair Cells.

Representative confocal images of AAV2-CAG-GFP transduced P0 cochlear explants from the basilar region. AAV2-CAG treated cultures show robust GFP expression in inner and outer hair cells. GFP expression is also observed in some supporting cells. Top three panels show 40X magnification of fluorescent images; GFP (A), merge (B) and Myosin VI respectively (C). Lower three panels show 100X magnification of same images of GFP (D), merge (E) and Myosin VI (F). Scale bars = 25μm for A, B, and C; 5μm for D, E, and F.
Figure 5. CAG Promoter Drives Gene Expression in AAV2 Transduced Apical Cochlear Hair Cells
Figure 5. **CAG Promoter Drives Gene Expression in AAV2 Transduced Apical Cochlear Hair Cells.**

Representative confocal images of AAV2-CAG-GFP transduced P0 cochlear explants from the apical region. AAV2-CAG treated cultures also show GFP expression in inner and outer hair cells and some support cells. Top three panels show 40X magnification of fluorescent images; GFP (A), merge (B) and Myosin VI respectively (C). Lower three panels show 100X magnification of same images of GFP (D), merge (E) and Myosin VI (F). Scale bars = 25μm for A, B, and C; 5μm for D, E, and F.
Figure 6. CAG Promoter Drives Gene Expression in AAV1 Transduced Basilar Cochlear Hair Cells
Figure 6. **CAG Promoter Drives Gene Expression in AAV1 Transduced Basilar Cochlear Hair Cells.**

Representative confocal images of AAV1-CAG-GFP transduced P0 cochlear explants from the basilar region. AAV1-CAG treated cultures show robust GFP expression in inner and outer hair cells and subpopulations of support cells. Top three panels show 40X magnification of fluorescent images; GFP (A), merge (B) and Myosin VI respectively (C). Lower three panels show 100X magnification of same images of GFP (D), merge (E) and Myosin VI (F). Scale bars = 25μm for A, B, and C; 5μm for D, E, and F.
Figure 7. CAG Promoter Drives Gene Expression in AAV1 Transduced Apical Cochlear Hair Cells
Figure 7. **CAG Promoter Drives Gene Expression in AAV1 Transduced Apical Cochlear Hair Cells.**

Representative confocal images of AAV1-CAG-GFP transduced P0 cochlear explants from the apical region. AAV1-CAG treated cultures show GFP expression in inner and outer hair cells and subpopulations of support cells. Top three panels show 40X magnification of fluorescent images; GFP (A), merge (B) and Myosin VI respectively (C). Lower three panels show 100X magnification of same images of GFP (D), merge (E) and Myosin VI (F). Scale bars = 25μm for A, B, and C; 5μm for D, E, and F.
Figure 8. AAV5-CAG Transduction in Basilar Cochlear Explants
Figure 8. **AAV5-CAG Transduction in Basilar Cochlear Explants.**

Representative confocal images of AAV5-CAG-GFP transduced P0 cochlear explants from the basilar region. AAV5-CAG treated cultures show sparse GFP expression in only a few support cells. Top three panels show 40X magnification of fluorescent images; GFP (A), merge (B) and Myosin VI respectively (C). Lower three panels show 100X magnification of same images of GFP (D), merge (E) and Myosin VI (F). Scale bars = 25μm for A, B, and C; 5μm for D, E, and F.
Figure 9. AAV5-CAG Transduction in Apical Cochlear Explants
Figure 9. AAV5-CAG Transduction in Apical Cochlear Explants.

Representative confocal images of AAV5-CAG-GFP transduced P0 cochlear explants from the apical region. AAV5-CAG treated cultures shows little to no GFP expression in hair cells and support cells. Top three panels show 40X magnification of fluorescent images; GFP (A), merge (B) and Myosin VI respectively (C). Lower three panels show 100X magnification of same images of GFP (D), merge (E) and Myosin VI (F). Scale bars = 25μm for A, B, and C; 5μm for D, E, and F.
Table 2. Percentage of Transduced Hair Cells

<table>
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<tr>
<th></th>
<th>Transduced IHC Base</th>
<th>Transduced OHC Base</th>
<th>Transduced IHC Apex</th>
<th>Transduced OHC Apex</th>
</tr>
</thead>
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<tr>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>CAG</td>
<td>52.14 ± 7.5 (7)</td>
<td>67.57 ± 6.17(7)</td>
<td>17.50 ± 7.14 (4)</td>
<td>66.75 ± 3.71(4)</td>
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<td>GFAP</td>
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<td>0 ± 09(4)</td>
<td>0 ± 0 (3)</td>
<td>0 ± 0(3)</td>
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<tr>
<td>CAG</td>
<td>37.40 ± 4.65 (5)</td>
<td>61.40 ± 5.54(5)</td>
<td>15.50 ± 5.23 (6)</td>
<td>43.83 ± 8.48(6)</td>
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<tr>
<td>GFAP</td>
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<tr>
<td>CAG</td>
<td>0 ± 0 (5)</td>
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<td>0 ± 0 (2)</td>
<td>0 ± 0 (4)</td>
<td>0 ± 0(4)</td>
</tr>
</tbody>
</table>
Table 2. **Percentage of Transduced Hair Cells.**

The percentage of transduced hair cells ± standard error mean (SEM) is shown. Each AAV serotype and promoter is listed in the leftmost column. N values, in parentheses, are given for each treatment group for both basal and apical regions. Averages were determined as a ratio of Myosin VI red hair cells and GFP positive labeled cells over single Myosin VI labeled hair cells. N = number of images analyzed, IHC = inner hair cells, OHC = outer hair cells, CAG = chicken beta actin/ cytomegalovirus promoter, GFAP = glial fibrillary acidic protein promoter.
AAV transduction of murine support cells in cochlear explants

To assess the transduction of support cells within murine cochlear explants, we created another GFP construct in which the CAG promoter was replaced with the GFAP promoter. We chose this promoter based on a report by Rio et al. (2002) which indicated that GFAP was expressed in all support cell populations immediately surrounding hair cells in mice. Robust transduction of support cells were observed with both AAV1 and AAV2, but not with AAV5.

Unfortunately, the lack of a good support cell marker prevented us from identifying and quantifying the specific support cell populations transduced. We therefore used the myosin VI staining to orient the cultures as well as examine GFP expression in hair cells with this promoter. No hair cell expression was observed with any serotype using the GFAP promoter. However, a distinctly different distribution of GFP expression within the support cells was observed depending on the serotype used.

Based on location relative to hair cells, robust GFP expression is seen in what appeared to be Dieters’, interdental and pillar cells following transduction with AAV2 (Figure 10). High magnification of AAV2–GFAP transduced cochleas, show GFP expression is not in hair cells obvious by the void in the shape of the hair cells (Figure 10D and E). In contrast, a very robust GFP expression was only observed in what appeared to be Hensen, interdental cells and a few Dieters’ cells following transduction with AAV1 (Figure 11). Transduction with AAV5 showed GFP expression in peripheral support cells, possibly Hensen and a few interdental cells (Figure 12). A similar basal to apical gradient that was observed in the GFP expression in hair cells was also seen in the GFP expression within support cells following transduction with all serotypes (data not
shown).
Figure 10. GFAP Promoter Drives Gene Expression in AAV2 Transduced Basilar Cochlear Support Cells
Figure 10. **GFAP Promoter Drives Gene Expression in AAV2 Transduced Basilar Cochlear Support Cells.**

Representative confocal images of AAV2-GFAP-GFP transduced P0 cochlear explants from the basilar region. AAV2-GFAP treated cochleas show GFP expression in almost all types of supporting cells surrounding the hair cells including interdental (I), Dieters’ (D), and pillar or inner phalangeal (*) cells. Top three panels show 40X magnification of fluorescent images; GFP (A), merge (B) and Myosin VI respectively (C). Lower three panels show 100X magnification of same images of GFP (D), merge (E) and Myosin VI (F). Scale bars = 25μm for A, B, and C; 5μm for D, E, and F.
Figure 11. GFAP Promoter Drives Gene Expression in AAV1 Transduced Basilar Cochlear Support Cells
Figure 11. **GFAP Promoter Drives Gene Expression in AAV1 Transduced Basilar Cochlear Support Cells.**

Representative confocal images of AAV1-GFAP-GFP transduced P0 cochlear explants from the basilar region. AAV1-GFAP treated cochleas show robust GFP expression in interdental (I), Hensen (H) and few Dieters’ cells. Top three panels show 40X magnification of fluorescent images; GFP (A), merge (B) and Myosin VI respectively (C). Lower three panels show 100X magnification of same images of GFP (D), merge (E) and Myosin VI (F). Scale bars = 25µm for A, B, and C; 5µm for D, E, and F.
Figure 12. GFAP Promoter Drives Gene Expression in AAV5 Transduced Basilar Cochlear Support Cells
Figure 12. GFAP Promoter Drives Gene Expression in AAV5 Transduced Basilar Cochlear Support Cells.

Representative confocal images of AAV5-GFAP-GFP transduced P0 cochlear explants from the basilar region. AAV5-GFAP treated cochleas show expression in a limited sub-population of peripheral support cells, possibly Hensen (H) and interdental (I) cells. Top three panels show 40X magnification of fluorescent images; GFP (A), merge (B) and Myosin VI respectively (C). Lower three panels show 100X magnification of same images of GFP (D), merge (E) and Myosin VI (F). Scale bars = 25μm for A, B, and C; 5μm for D, E, and F.
**GFP expression within GLAST positive cells**

As previously stated, we were unable to specifically identify which support cells were transduced by AAV 1, 2, and 5 using a cell specific stain. All conclusions of types of supporting cells are based on proximity to hair cells and basic morphology. Anti-GLAST antibody specifically labels only inner phalangeal and border cells that immediately surround the inner hair cells (Furness 2003; Jin 2003; Rebillard 2003). This was the only support cell antibody that gave us consistent and reliable results.

Using GLAST immunostaining, we observed no co-localization of GLAST positive cells and GFP expression with neither AAV1 nor AAV5 using the CAG or GFAP promoter (data not shown). This suggests that AAV1 and AAV5 do not transduce inner phalangeal cells and border cells. However, AAV2 appears to be able to transduce these cell types. **Figure 13** shows a representative image of the basilar region of a cochlear explant transduced with AAV2-GFAP-GFP. The GFP expression appears to be localized in the cell bodies but not within the extended processes. Robust GFP expression was also observed in what appears to be the interdental cells flanking the inner hair cell region. The apical region shows decreased transduction with little to no dual labeling (Data not shown). Other populations of transduced support cells are also visible.
Figure 13. Anti-GLAST Staining of AAV2-GFAP-GFP Transduced Basilar Cochlear Explants
Figure 13. **Anti-GLAST Staining of AAV2-GFAP Transduced Basilar Cochlear Explants.**

Representative confocal images of AAV2-GFAP-GFP transduced P0 cochlear explants stained with anti-GLAST antibody. Anti-GLAST stains only border and inner phalangeal cells. The "holes" between the GLAST positive cells are hair cells. Dual positive anti-GLAST staining and GFP expression is observed within the cell bodies but not the extended processes. Top three panels show 40X magnification of fluorescent images; GFP (A), merge (B) and GLAST respectively (C). Lower three panels show 100X magnification of same images of GFP (D), merge (E) and GLAST (F). Scale bars = 25µm for A, B, and C; 5µm for D, E, and F.
Discussion/Conclusions

In previous studies, AAV was shown not to transduce hair cell and support cells within the cochlea (Lalwani 1996; Lalwani 1997; Kho 2000; Luebke 2001; Li Duan 2002). AAV2 was the only serotype used in these studies. The overall conclusion was that the cell surface receptor, HSPG, was not present, therefore no transduction could occur (Luebke 2001). However, in our study we have shown that AAV2 and AAV1 carrying the CAG-GFP expression cassette can efficiently transduce murine hair cells in vitro. AAV1 appears to be the most efficient at transducing hair cells in the apex. The primary cell surface receptor for AAV1 has not yet been identified. Surprisingly, AAV5 which binds sialic acid, does not transduce hair cells even though sialic acid is thought to be present on hair cells (Tachibana 1990). Further studies are needed to determine the transduction efficiency of the remaining AAV serotypes (3, 4, 7, and 8) in the cochlea. These serotypes may, or may not, show a more selective tropism based on their cell surface receptors. HSPG and sialic acid may only serve to facilitate the initial contact between virus and cell, thus enhancing the efficiency of transduction. This may be overcome with the use of higher titers.

In addition, the difference in the ability of AAV to transduce these cells and for cells to express the transgene could be due to the different titers used in earlier experiments. Most of the previous studies used low titer (10^5 or 10^8 infectious particles) virus, whereas in this study we used a relatively higher titer (10^10 gp). Our study clearly demonstrates that AAV can transduce cells within the cochlea at this titer. Notably, we did observe a decreased and inconsistent transduction of hair cells with AAV1-CAG when lower titers (10^8 gp) were used. Interestingly, a very high titer (10^{13} gp) did not
change the transduction efficiency of AAV5-CAG (data not shown). This suggests that titer was not the only factor for efficiency of AAV5 within the cochlea. Therefore, titer can affect the efficiency of AAV transduction but there appears to be a threshold as well as other determining factors.

The promoters and enhancer sequences used in the current study are another major difference that needs to be considered when comparing our results to previous studies. Xu et al. (2001) compared AAV mediated expression of coagulation protein human factor X driven by the CAG, CMV, or elongation factor 1 alpha (EF1α) promoters. That study concluded that the CAG promoter resulted in 9.5-fold higher expression than CMV promoter alone and 137-fold higher than EF-1α promoter alone (Xu 2001). The majority of previous cochlear AAV studies relied upon the CMV promoter (Lalwani 1997; Lalwani 1998; Luebke 2001; Li Duan 2002). These experiments resulted in expression in the spiral ligament, spiral ganglion, spiral limbus, and within the organ of Corti. However, the cell types within the organ of Corti were not specified. Poulsen et al. (2002) compared the transduction of AAV2 and AAV5 in the spinal cord using the neuron specific enolase (NSE) and GFAP promoters. This study found that high titers of AAV2 and AAV5 transduced similar levels of neurons when the NSE promoter was used to drive GFP expression. However, using the GFAP promoter to drive GFP expression, AAV5 showed more transduction of astrocytes compared to AAV2. Luebke et al. (2001) studied AAV transduction using the CBA, CMV, NSE, platelet derived growth factor (PDGF), and EF-1α promoters to drive expression in the guinea pig cochlea. That study showed that AAV did not transduce hair cells, but it did transduce support cells (Luebke 2001). Furthermore, the support cell expression was
promoter dependent, thus supporting observations in the current study. The NSE promoter drove expression in nerve fibers while EF1α drove expression in the non-interdental cells of the spiral limbus. Interestingly, the CBA and CMV promoters did not drive expression in any cochlear tissue except cochlear blood vessels. This is very interesting considering the CAG promoter used in our study is a hybrid of the CBA and CMV promoters. Using the CAG promoter and WPRE element, we observed GFP expression in several different types of cells within the cochlea following AAV transduction.

Recently, Rio et al. (2002) examined GFAP expression in cochlear cells. Using anti-GFAP immunostaining and transgenic mice expressing a reporter gene under control of the GFAP promoter, the authors demonstrated a distinct localization of GFAP in the support cells directly associated with the hair cells but not within the hair cells themselves. Due to the type of image analysis and lack of a good support cell specific antibody, it was not clear exactly which support cell types express GFAP. However, the results state that all supporting cells surrounding hair cells express GFAP even though the magnification used made it difficult to distinguish between the inner phalangeal and inner pillar cells. Figure 14 shows a transmission electron micrograph image of an inner hair cell (Furness 2003). This previous study used immunogold staining along with TEM microscopy to identify GLAST expression in the cochlea. In this figure, the boundaries of the border cell, inner phalangeal cell, and the inner pillar cell surrounding the inner hair cell are clearly visible. Thus, it seems reasonable that using the GLAST promoter AAV2 transduction could lead to transgene expression in only the border and inner phalangeal cells.
Figure 14. Transmission Electron Micrographs of Immunogold Labeling for GLAST Around Inner Hair Cells of Guinea Pig Cochlea.
Figure 14. Transmission Electron Micrographs of Immunogold Labeling for GLAST Around Inner Hair Cells of Guinea Pig Cochlea.

A, Inner hair cell (IHC) flanked on both sides by support cells; inner phalangeal cells (IPhC), Pillar cell (PC), border cell (BC). (B and C), are higher power images of outlined boxes in A. B, Labeling of GLAST (arrow heads) is primarily confined to cell membrane. C, GLAST is confined to the membrane of inner phalangeal cells as well (arrow heads). A greater number of particles can be seen in the inner phalangeal cell. Copyright 2003 by Society for Neuroscience.
Cell specific expression will be important for future gene delivery studies and clinical trials. Targeting only the cells or tissue of interest will allow for gene expression only where it is needed. As shown with the GFAP promoter, specific cells can be targeted using AAV. While AAV1-GFAP and AAV2-GFAP have both been shown to transduced support cells, AAV1 shows a more limited cellular tropism. This data along with the previous studies suggests that AAV can be targeted to drive expression in specific cell types based on the promoter selected. This is important when only certain populations of cells are of interest. The Myo7A promoter has recently been cloned and could be used for gene expression specifically within hair cells (Boeda 2001). The introduction of otoprotective genes directly to the hair cells could increase survival after ototoxic or sound damage.

Following transduction with AAV, a pattern of decreasing GFP expression was observed in a basal to apical gradient. This gradient is not only seen in the frequency of sound transduced by the cochlea but with endogenous gene expression as well (Rio 2002). Sensitivity to damage (ototoxic or noise related) also follows the same gradient. It has been speculated in several in vivo gene transfer studies that the region for high transduction in the base versus the apex was due to mode of delivery and dissemination of virus (Lalwani 1997; Stover 2000; Li Duan 2002). In vivo studies rely on direct injection of the virus to the cochlea via two main routes. The site of inoculation can either be by direct injection into the round window or pumped into the basal portion of the scala tympani via cochleostomy (Lalwani 1997; Stover 2000; Li Duan 2002). Thus, the conclusion made for the in vivo studies may be valid but the basal to apical gradient should also be considered. In this study, both regions were treated with the same titer and
duration of virus and the gradient was still observed. In all viral treatment groups, less GFP was expressed in the apex versus the base.

Future Studies

This in vitro project is a first step in using AAV as a gene delivery vector in the murine auditory system. The next logical step will be to use the AAV serotype(s) found to transduce the explants in an in vivo model. Once an efficient mode of delivery to the scala media is established for the mouse, AAV can be used to introduce functional genes into the cochlea. These could include developmental genes such as the Math-1. Math-1 is a developmental transcription factor that is required for hair cell development (Zuo 2002; Kawamoto 2003). Using adenoviral vectors, Kawamoto et al. (2003) successfully introduced the functional Math1 gene into guinea pig cochlea. Math-1 positive cells were found in non-sensory epithelia within the organ of Corti and within Hensen cells, inner sulcus, and interdental cells. Furthermore, over-expression of the Math-1 gene lead to immature hair cells in the organ of Corti and non-sensory epithelia (Kawamoto 2003). Other neuroprotective genes could also be introduced including glial derived neurotrophic factor (GDNF) or neurotrophic factor 3 (NT3) (Li Duan 2002). These genes have been shown to increase survival of neurons after hair cell damage due to ototoxic drugs or noise (Kawamoto 2001; Kanzaki 2002). This type of gene transfer in combination with cochlear implants could help preserve the neurons that would ultimately deteriorate after hair cell loss and thus increase the chance of restoring hearing.

Adeno-associated viral vectors appear to be promising viral vectors for gene therapy in the inner ear. It is important that the remaining AAV serotypes be tested in the
inner ear to evaluate their transduction ability. Using AAV1 and AAV2, the CAG promoter was shown to drive expression in many cell types while the GFAP promoter was able to drive expression in only supporting cells. Our study has therefore shown that AAV can be used to efficiently transduce cells within the murine cochlea in vitro.
Literature Cited


Xu, L., T. Daly, et al. (2001). "CMV-beta-actin promoter directs higher expression from an adeno-associated viral vector in the liver than the cytomegalovirus or elongation factor 1 alpha promoter and results in therapeutic levels of human factor X in mice." Hum Gene Ther 12(5): 563-73.