Non-viral gene delivery and bone marrow cells in inner ear repair

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BONE MARROW CELLS IN INNER EAR REPAIR**

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

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ABSTRACT

Hearing impairment is the most common sensory deficit in humans and degeneration of auditory hair cells is the major cause of this deficit. Over the past ten years, gene therapy has emerged as a promising approach in the treatment of hearing loss through identification of individual genes and proteins involved in hearing and development of various methods of delivery to the cochlea. Many studies employed the use of viral-mediated gene transfer and delivery through cannulation of the cochlea. Results are encouraging; however the use of viral vectors as gene delivery vehicle (host immunological response) and direct cannulation (surgical trauma) of the cochlea still has potential risks and raises major safety concerns. Hence, the objective of this work is to address these concerns through exploring 1) the use of a non-viral vector, polyethylenimine (PEI) as gene delivery vector and studying 2) the role of the host's intrinsic bone marrow cells' (BMCs') in the restoration process after deafening.

Various delivery methods were compared and assessed to select the optimal method for non-viral vector gene delivery. The rank order of the various delivery routes based on transfection efficacies was - osmotic pump infusion > cochleostomy > round window membrane inoculation > Gelfoam method. Hence PEI-eGFP was introduced with sustained delivery into guinea pig cochleae using an osmotic pump and examined for transgene expression. PEI-mediated gene transfer was demonstrated and the infused cochleae maintained intact cellular and tissue architecture with the absence of inflammation. PEI's relatively lower transfection efficiency limits its potential when compared to viral counterparts; however, we have shown that sustained release of the vector solution improved PEI's transfection efficiency and offers a solution to compensate

for non-viral vectors' relatively low transfection efficiency. These findings indicate that PEI is able to transfect the cochlea *in vivo* and present an alternative for cochlea gene therapy.

To study the role of BMCs in inner ear repair, lethally irradiated mice were transplanted with BMCs from GFP transgenic mice and subjected to acoustic deafening. In a separate experiment, cytokine treatment was administered to test the effect of BMC mobilization on bone marrow-derived cell (BMDC) transdifferentiation. Upon acoustic trauma, robust BMDC migration was observed in the deafened cochlea and was most prominent during the first week after acoustic deafening. These cells accumulated significantly at the spiral ligament, perilymphatic compartment walls and limbus regions. Most BMDCs expressed CD45 and CD68 and were identified as macrophages. Upregulation of SDF-1 was also observed in the spiral ligament during the first week after acoustic deafening. Cytokine treatment resulted in increased BMC mobilization in the systemic circulation. However, the presence of any stem cell progenitors or the differentiation of BMDCs into any cell types expressing cochlea sensory, supporting, fibrocytic or neuronal markers were not detected in the deafened cochlea. Hence, we demonstrated the homing capability of BMDCs to the deafened cochlea and these cells displayed mature hematopoietic properties without spontaneous transdifferentiation to any cochlea cell types after acoustic trauma or bone marrow mobilization.

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LIST OF ABBREVIATIONS

ABR	auditory brainstem response
Ad-eGFP	adenovirus encoding the eGFP gene
APC	allophycocyanin
<i>Atoh1</i>	atonal homolog 1
AVV	adeno-associated virus
Bcl-2	b-cell lymphoma 2
BDNF	brain-derived nerve growth factor
BM	bone marrow
BMC	bone marrow cell
BMDC	bone marrow-derived cell
BMDSC	bone marrow-derived stem cell
BMT	bone marrow transplantation
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CD	cluster of differentiation
CNS	central nervous system
CPE	cytopathic effect
CXCR4	chemokine (C-X-C motif) receptor 4
Cy3	cyanine 3
dB	decibel
DMEM	dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescence protein
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FISH	fluorescent in situ hybridization
G-CSF	granulocyte colony-stimulating factor
GDNF	glial cell line-derived neurotrophic factor
GFP	green fluorescence protein
GFP+	green fluorescence protein positive
HEK 293	human embryonic kidney 293
HepG2	human hepatocellular liver carcinoma cell line
HIV	human immunodeficiency virus
hr	hour
HRP	horse radish peroxidase
HSC	hematopoietic stem cell

HSV-1	herpes simplex virus type 1
IL-6	interleukin-6
LacZ	reporter gene that codes for beta galactosidase
<i>Math1</i>	mammalian atonal homolog 1
MCP	monocyte chemotactic protein
min	minute
MIP-1b	macrophage inflammatory protein
MSC	mesenchymal stem cell
N/P ratio	nitrogen to phosphate ratio
NF200	neurofilament 200
NGF	nerve growth factor
NIH3T3	mouse embryonic fibroblast cell line
NO	nitric oxide
NOS	nitric oxide synthase
NT-3	neurotrophin-3
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethylenimine
PEI-eGFP	dna-vector complex (transfecting unit)
PFU	plaque forming unit
RBC	red blood cell
rtp	room temperature
RWM	round window membrane
SCF	stem cell factor
SD	standard deviation
SDF-1	stromal cell derived factor-1
SDS	sodium dodecyl sulfate
SGN	spiral ganglion neurons
SOD	superoxide dismutase
SPL	sound pressure level
TBS	tris buffered saline
TCID ₅₀	tissue culture infectious dose 50
TGF	transforming growth factor
TRITC	tetramethylrhodamine isothiocyanate
TTS	temporary threshold shift
WBC	white blood cell
wk	week
β-gal	β-galactosidase

CHAPTER 1: INTRODUCTION

1.1 Hearing Problem

Hearing loss/impairment is considered as the most common physical disorders affecting millions worldwide where one out of ten people is hard of hearing. It is estimated that there are 500 million hearing impaired people worldwide, and this number will rise to more than 700 million in 10 years time (figures adapted from <http://www.earinfo.com> and <http://www.hear-it.org>).

Causes of hearing loss include:

- Genetic / heredity (gene defects which interfere with ear's development and function)
- Diseases, injury or infection (such as otitis media and otosclerosis)
- Old age
- Excessive exposure to noise

In developed countries, with lifestyle changes over the years, people suffer from hearing loss at younger ages due to excessive noise exposure. As the proportion of aged increases in many aging societies, incidence of hearing impairment also increases. A survey carried out by the U.S. National Center for Health Statistics identified noise as the main reason for people becoming deaf or hard-of-hearing, followed closely by aging which combines to account for approximately 70% of all hearing impairment cases. (more information can be found at <http://www.nidcd.nih.gov> and <http://www.lhh.org>)

Certain drugs such as aminoglycoside antibiotics (e.g. streptomycin, gentamicin, and kanamycin), anti-cancer drugs (e.g. cisplatin, bleomycin, vincristine) and diuretic

drugs (e.g. acetazolamide, furosemide, ethacrynic acid) may also cause ototoxicity. Hence, the use of such drugs to treat certain ailments or conditions also exposes the patient to ototoxic risks. The extent of ototoxicity varies with the drug, dose, patient's innate conditions and environmental factors. In some cases, there is full recovery after the drug has been discontinued. In other cases, there may be permanent and complete deafness.

There are two types of hearing loss, conductive and sensorineural. When both types exist concurrently, it is termed as mixed hearing loss. In conductive hearing loss, there is impairment in the normal air-conducting mechanism by which sound reaches the inner ear. This impairment can occur in the external auditory canal, tympanic membrane, or middle ear, including the ossicles. In sensorineural hearing loss (the more common type of hearing loss), impairment occurs in the cochlea, cochlear nerve, or brain stem. The most common site of impairment is in the cochlea, where auditory hair cells or spiral ganglion neurons are damaged or lost. Conductive hearing loss can often be corrected surgically, while sensorineural hearing loss requires hearing amplification devices and more complicated measures.

In birds and many cold blooded vertebrates, damage to hair cells induces mature, normally quiescent supporting cells to act as progenitors. They re-enter the cell cycle and divide asymmetrically to give a hair cell and a supporting cell in contrast to the mammalian organ of Corti [1]. It was always thought that mammalian auditory hair cell regeneration was impossible until the early 1990s. It was shown that cells in the utricular sensory epithelia from adult guinea pigs and adult humans proliferate after treatment with ototoxic antibiotics when maintained *in vitro*. The progeny of proliferating cells begin to differentiate as replacement hair cells which suggest that supporting cells of adult

mammalian inner ear retain the capacity for proliferation that can be triggered in response to a loss of sensory hair cells [2]. *In vivo*, ultra-structural features (immature hair bundles in multiple stages of development) characteristic of newly formed hair cells were also observed several weeks after aminoglycoside treatment that cause hair cell loss in the utricle [3]. In the following years, many more research groups involved in regenerative studies also showed promising results in support of the discovery made earlier in the early 1990s. One group showed that existing epithelial supporting cells (Hensen's cells) from embryonic rat cochlear explants can differentiate into outer hair cells or Deiters' cells in cultured explants of the organ of Corti [4]. Gao and his co-workers demonstrated that overexpression of *Math1* gene in postnatal rat cochlear explants induces production of extra hair cells in region of the greater epithelial ridge [5]. Another group proved that early postnatal cochlear tissue contains cells that can divide and generate new sensory hair cells *in vitro* [6].

These results suggest that mammalian sensory hair cell regeneration is no longer a dream but a reality that can be fulfilled in the near future.

1.2 Ear Anatomy

(all pictures in Chapters 1.2 and 1.3 reproduced from: <http://medic.med.uth.tmc.edu>)

The ear consists of the **auditory system**, which is involved in the detection of sound, and the **vestibular system**, which is involved with maintaining equilibrium. The ear is composed of the external ear, the middle ear, and the inner ear (as shown in Figure 1.1). The external and middle ear collect and convey sound to the inner ear where sensory

receptors of the auditory system reside. Sensory receptors for the vestibular system are also in the inner ear and function independently of the external and middle ear.

Figure 1.1: Basic ear anatomy.

1.2.1 External Ear

The external ear, the portion of the ear closest to the external environment, consists of the auricle and external auditory meatus (Figure 1.1 “Outer”)

1.2.1.1 Auricle

The auricle (or pinna) consists of a single irregular plate of elastic cartilage covered by a thin layer of keratinized stratified squamous epithelium containing hairs and sebaceous and sweat glands.

1.2.1.2 External auditory meatus

It extends from the auricle to the tympanic membrane (or the eardrum) of the middle ear. It is lined by stratified squamous epithelium containing hairs, sebaceous glands, and ceruminous glands (modified sweat glands). Secretions by these sebaceous and ceruminous glands combine protect the skin from desiccation and help to keep foreign bodies out of the ear.

1.2.2 Middle Ear

The middle ear acts as the link between the inner ear and the external environment. Its major function is to match the relatively lower impedance airborne sounds to the higher-impedance fluid in the cochlea. Without it, majority of the airborne acoustical energy will be reflected by the higher-impedance fluid within the cochlea. The middle ear houses 3 important ossicles (Figure 1.1 “Middle”, Chapter 1.2.2.2) which transfer sound energy efficiently from the outer ear into the inner ear with minimum energy loss.

1.2.2.1 The Tympanic Cavity

The tympanic cavity is an air-filled space lying within the temporal bone. The lateral wall composed of the tympanic membrane and the medial wall is shared by the inner ear which contains the oval window and the round window.

1.2.2.2 The Ossicles

The ossicles consist of the *malleus* (hammer), the *incus* (anvil), and the *stapes* (stirrup). They form a chain of levers that link the external ear to the inner ear. They composed of compact bone covered by epithelium that is continuous with the epithelium of the tympanic cavity. The malleus is attached to the inner aspect of the tympanic

membrane. The stapes is attached to the oval window of the inner ear by a fibrous ligament. The incus joins the malleus and the stapes. Acoustical energy is efficiently transmitted into the inner ear by this ossicular chain which acts like a lever to mechanically convert eardrum vibrations into amplified pressure waves in the cochlea fluid. The muscles adjoining the ossicles aids in the adjustment of tension in the tympanic membrane and conveying vibrations to the inner ear. It also protects the inner ear in the event of a loud sound by contraction and making the chain of ossicles more rigid, thus allowing less sound to be transmitted.

1.2.2.3 Tympanic Membrane

The tympanic membrane is a thin semitransparent membrane between the external and middle ears. It consists of a central core of connective tissue containing fibrous tissue and elastic fibers. It is covered by epithelium on both sides.

1.2.2.4 Eustachian Tube

The eustachian tube (or the auditory tube) extends from the tympanic cavity to the nasopharynx. The primary function of the auditory tube is to equalize air pressure between the tympanic cavity and the external environment. The walls are normally apposed to one another; however, during swallowing and yawning, the walls separate, allowing air to enter the tympanic cavity.

1.2.3 Inner Ear

The inner ear consists of a series of membranous sacs and ducts (membranous labyrinth) encased within a series of bony cavities and canals (bony labyrinth) (Figure 1.1 “Inner”, 1.2 & 1.3). In it, contains six anatomically separate mechano-sensory epithelia

adapted to interpret different forms of mechanical stimulus, five are part of the vestibular system and one is a part of the auditory system.

1.2.3.1 Bony Labyrinth

Consist of 1) the vestibule (central cavity of the bony labyrinth), which house the utricle and the saccule. 2) The three semicircular canals (superior, lateral, and posterior) oriented at right angles to one another and with a dilatation (ampulla) at the end of each semicircular canal. 3) The cochlea which houses the cochlear (spiral) ganglion and the cochlear nerve. (Figure 1.2)

Figure 1.2: The bony labyrinth

1.2.3.2 Membranous Labyrinth

Consist of the saccule, utricle, semicircular ducts and cochlear duct (scala media) (Figure 1.3). All of these epithelial-lined sacs and ducts communicate with one another, and contain *endolymph*, a fluid similar to intracellular fluids (high K^+ , low Na^+). External to the membranous labyrinth is *perilymph*, a fluid similar to extracellular body fluids (low

K^+ , high Na^+). The mature mammalian inner ear comprises one auditory (organ of Corti in the cochlea duct) and five vestibular organs (three cristae of the semicircular canals and two maculae of the saccule and utricle). The vestibular organs respond to angular and linear acceleration (balance) and the auditory organ respond to sound (hearing).

Figure 1.3: The membranous labyrinth

1.2.3.3 Vestibular (Balance)

The utricle is an irregular, oblong membranous sac located on the medial wall of the vestibule. The saccule is a flattened, irregularly-shaped membranous sac also located in the medial wall of the bony vestibule. Both the utricle and saccule has a macula which is the sensory region. The three semicircular ducts are located within their respective semicircular canals and at the end of each duct is a dilated region called the ampulla which contains the sensory region.

1.2.3.4 Auditory (Hearing)

The cochlea duct lies in the bony cochlear labyrinth between the osseous spiral lamina and the external wall of the bony cochlea (Figure 1.3). It spirals around the modiolus and the basal aspect of duct communicates with the saccule while the apical aspect ends as a blind sac. The scala media is the space within the cochlear duct and is filled with endolymph. The cochlear duct and the osseous spiral lamina divide the cochlea into 2 additional spaces, scala vestibuli and scala tympani (Figure 1.5). These are continuous with one another by means of a tiny opening, called the helicotrema, located at the blind (top) end of the bony cochlea (Figure 1.4). The scala vestibuli and tympani are filled with perilymph.

Referring to Figure 1.5, the cochlea duct has three sides (triangular in shape). One side attaches to the osseous spiral lamina and extends laterally to the bony wall of the cochlea. The basilar membrane forms the floor of the cochlear duct. The side of the basilar membrane facing the scala tympani is lined by epithelial cells. The side facing the scala media is composed of columnar supporting and sensory cells that make up the organ of Corti. The second side of the cochlea duct attach to the external bony wall of the cochlea. This region is called the lateral cochlear wall. It consists of the spiral ligament, stria vascularis and the spiral prominence. The third side of the cochlear duct is *Reissner's membrane*. It extends from the end of the spiral limbus on one side to the spiral ligament above the stria vascularis on the other side.

Figure 1.4: Cross sectioning of the cochlea and zoom-in image of one cochlea turn

1.2.3.5 Organ of Corti

The sensory cells are classified as *inner hair cells* and *outer hair cells*. The inner hair cells lie in a single row along the length of the basilar membrane close to the medial aspect of the duct ("inner"). They are completely surrounded by supporting cells (Figure 1.5). The outer hair cells are cylindrical and lie in 3-5 rows along the basilar membrane. Only their apical and basal surfaces are surrounded by supporting cells; fluid bathes the medial surfaces.

Figure 1.5: Zoom-in image of the cochlea turn

Each hair cell has many stereocilia on its apical surface. At the base of each hair cell are efferent and afferent nerve endings. The hair cells have a highly specialized bundle of modified microvilli on their apical surface, a hair bundle, and this enables them to detect mechanical stimuli and transducer them into electrical signals. There are a bunch of different supporting cells in the organ of Corti (Hensen cells, Deiter's cells, Claudius cells, Boettcher cells, inner phalangeal cells, inner and outer sulcus cells, inner and outer pillar cells). These supporting cells directly or indirectly supports sensory cells by providing the necessary metabolic and electrolytic environmental for sensory cells to transform mechanic energy into bioelectric energy. Recent studies also suggest supporting cells playing an important role in maintaining homeostasis within the organ of Corti and during degenerative and regenerative events of sensory hair cells (see also [7]).

1.2.3.6 Lateral Cochlear Wall

Consist of the spiral ligament, stria vascularis and the spiral prominence. (Figure 1.5) The *spiral ligament* is an organized dense connective tissue made up of a meshwork of fibrils and blood vessels. The *stria vascularis* composed of cells thought to secrete endolymph. The *spiral prominence* is likely to be involved in homeostasis of cochlear fluids.

1.2.3.7 Reissner's Membrane

A delicate structure composed of 2 layers of cells. It separates the perilymphatic space of the scala vestibuli from the endolymphatic space of the scala media.

1.3 Hearing Mechanism

Air-borne vibrations are collected in the pinna and conveyed via the auditory canal to the middle ear. Sound waves reaches the tympanic membrane and ossicles in the middle ear conveys vibrational energy efficiently to the inner ear (refer to Chapter 1.2.2). Vibrational energy transferred from the tympanic membrane, via the ossicles reaches the stapes footplate which rocks in and out against the oval window membrane (entrance for the vibrations). This rocking motion transmits pressure waves through the perilymph of the scala vestibuli to the scala tympani (Figure 1.6, left) with the round window (exit for the vibrations) serving as a pressure release valve which pushes outwards or inwards in the presence or absence of the sound waves. As the waves pass through the perilymphatic compartments, vibrational energy also passes through the roof of the scala media to the endolymph. From there, the waves continue on through the basilar membrane of the scala media to the perilymph of the scala tympani, and are eventually dissipated via the round window (refer to Figure 1.6, right).

Figure 1.6: Pathway taken by sound (vibrational) energy in the cochlea

Each sensory hair cell possesses 100 – 200 cilia which act as mechanosensors for hearing. With each sound cycle, the hair cell cilia deflects and opens gated ion channels allowing charged ions (K^+ and Ca^{+}) to enter the cell. This influx of ions from the endolymph depolarizes the cell and opens voltage gated calcium channels. This results in the entry of calcium ions which trigger the release of neurotransmitters at the basal end of the cell. The neurotransmitters then diffuse across the tight channel between the hair cells and nerve terminals where they bind to receptors and trigger action potentials in the nerve. Hence mechanical sound energy is converted to nerve impulses which travel via the cochlear nerve to the cerebral cortex where the brain interprets them. Repolarization in the hair cell is achieved through the perilymph which possesses a low concentration of positive ions. The electrochemical gradient formed results in the positive ions flowing through the channels into the perilymph.

Different frequencies of sound cause different displacement of the tectorial and basilar membrane. The greater the displacement of the membranes, the more sensory receptors and neurons are stimulated, leading to increased sound intensity. Sound energy of varying frequency transduces different hair cells connected along the length of the basilar membrane in the cochlea, hence allowing the brain to differentiate the pitch of the sound.

CHAPTER 2: LITERATURE REVIEW

2.1 Challenge

More than 80% of hearing loss cases is directly or indirectly related to degeneration and death of sensory hair cells and their associated spiral ganglion neurons (sensorineural hearing loss). Loss of auditory hair cells and hearing loss in mammals has been presumed to be irreversible because the production of these cells ceases before birth [8]. Hence the number of auditory cells (~ 16 000) we are born with is our lifetime supply. This is unlike some lower species such as fishes and amphibians whose inner ear produces sensory hair cells throughout life and any damaged sensory hair cells will be replaced continuously [8-11]. In birds, it has also been found that injured sensory cells are replaced by mitosis of surviving supporting cells [1, 9, 10]. These examples provided some of the most promising insights and increased interest in studying the reparative process with the hope that one day reversible hearing loss in mammals could also be achieved. Currently, clinical approaches are only focused on measures that avoid or alleviate hearing loss.

However, over the last 15 years, research findings on mammalian sensory hair cell regeneration have been encouraging and optimism has been fuelled by discoveries that hair cells can be replaced in mammalian animals [2, 5, 11-13]. The ability to replace these damaged hair cells holds great promise in fighting deafness and alleviating hearing loss. Finally there are grounds to believe that hearing loss and disorders may one day be reversible.

2.2 Therapeutic approaches for treatment of sensorineural hearing loss

Inner ear disorders have endogenous and exogenous origins. Hearing impairment of endogenous origin is usually of a genetic basis and common examples include age-related and hereditary hearing loss. On the other hand, hearing impairment of exogenous origin is usually caused by noise induced hearing impairment, infectious diseases and ototoxic lesions. In most hearing impaired patients, hearing loss is usually due to the interactions between both exogenous and endogenous factors. For example, some people are more susceptible to hearing loss due to a gene that makes them more vulnerable to environmental factors such as noise, drugs etc. (for complete hereditary hearing loss gene list, please refer to <http://webh01.ua.ac.be/hhh/>). The upcoming sections cover the popular approaches for the alleviation and treatment of sensorineural hearing loss.

2.2.1 Hearing Aids and Cochlear Implants

The main treatments today for sensorineural hearing loss are the use of hearing aids and cochlear implants. These methods do not completely restore our ability to hear but for now are the best options available. For patients with slight to moderate hearing loss, hearing aids allow patients to hear more clearly by picking up, gathering and amplifying sound energy before transmitting to the patient's ear. For patients with severe to profound deafness, cochlear implants are used to electrically stimulate the auditory nerve by bypassing the non-functional component of the auditory system (sensory hair cells) and directly depolarize auditory neurons to effectively restore the sound transduction pathway. These implants consist of a directional microphone which converts sounds into analog electrical signals that are digitized by a miniature speech processor

worn by patient. Signals from the speech processor are transmitted across the skin to a receiving coil and then to a linear multichannel electrode array implanted within the cochlea. The electrodes provide direct electrical stimulation to the afferent neurons and the efficacy of the implant depends largely on the presence of remaining functional afferent neurons. Hence there is a high degree of variation among the individual patient's functional recovery, with some achieving significant improvements, while others experiencing minimal benefits.

2.2.2 Glutamate receptor antagonist, NOS inhibitor, antioxidants

A high concentration of glutamate is released in response to loud noise, and this leads to a flux of large amounts of sodium and potassium ions across the post-synaptic membranes and passive entry of chloride ions into cells. This osmotic imbalance results in entry of fluids into cells which in turn produces swelling and ultimately rupturing of cell membranes and degeneration. Nitric oxide synthase (NOS) production is part of cochlear response to stress where calcium entry activates neuronal NOS in auditory neurons hence driving NO production. This reacts with superoxide to form the highly toxic peroxynitrite radical and ultimately results in neural degeneration. Hence various groups have been assessing the oto-protection potential of several glutamate receptor antagonists [17, 18] and NOS inhibitors [14, 15] and found experimentally that these agents protect the cochlea from various noise trauma and drug-induced (cisplatin and aminoglycoside antibiotics) ototoxicity.

Noise exposure drives mitochondrial activity and free radical production which induces both necrotic and apoptotic cell death in the organ of Corti. Hence pretreatment with agents that increase the amount of endogenous or exogenous antioxidants results in

attenuation of noise induced hearing loss. For example, subjects with reduced glutathione, the primary cellular antioxidant system of the body are more susceptible to hearing loss [16]. And in another case, attenuation of cochlear damage from noise trauma was observed with an iron chelator which acts as a free radical scavenger [17]. Other ways of enhancing antioxidant defense includes the use of other agents such as reversatrol [18], allopurinol [19, 20] and dietary antioxidant nutrients [21-24] which helps to alleviate hearing loss due to noise, drugs and age. A recent study by Yamashita et al. reported the use of a novel neurotrophic agent (T-817MA) which provides protection against oxidative stress-induced neurotoxicity. Acoustic deafened guinea pigs showed improved functional and morphological protection after treatment with this neurotrophic agent [25].

2.2.3 Neurotrophins

Neurotrophic factors are a group of agents vital to neuronal development, including regulation of neuronal survival and affecting nerve fiber elongation and outgrowth. Besides roles in inner ear development and innervation, these neurotrophin also play a role in survival and maintenance of spiral ganglion neurons with their ability to scavenge free radicals, interrupt cell death pathways and modulate calcium homeostasis. Neurotrophins such as glial cell line-derived neurotrophic factor (GDNF), brain-derived nerve growth factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3) have been shown to display certain oto-protective capabilities. GDNF at certain concentrations can effectively protect the inner ear from noise-induced hearing loss and survival of auditory hair cells in GDNF pre-treated ears could be twice as high as compared to control ears in animals that are not pretreated with GDNF [26, 27]. Hakuba et al reported that overexpression of GDNF provides protection against hair cell damage [28]. Other groups

also obtained similar results that support GDNF's oto-therapeutic effect [29, 30]. It has also been demonstrated that BDNF treatment significantly enhanced the survival of spiral ganglion neurons [31]. NGF prevented significant neomycin-induced damage to spiral ganglion neurons and degeneration of the auditory nerve *in vivo* [32]. With combined treatment of NT3 and a N-methyl-D-aspartate antagonist, it is shown that there are beneficial effects in the prevention of hair cell loss and protection against noise-induced excitotoxicity [33]. The use of neurotrophins may prevent or arrest some of the cochlear hair cell death and some of the secondary degeneration of afferent neurons that follows injury to the cochlea. However, hair cells and afferent neurons which have already been damaged in many types of the profound sensorineural hearing loss are usually irreversible and the application of neurotrophins or anti-apoptotic agents does not seem to have the potential to reverse this degeneration.

2.2.4 Gene delivery

Over the last decade, numerous strategies have been developed for gene delivery to the inner ear as a potential clinical treatment. Gene therapy stands out as a new and promising approach in the treatment of hearing disorders, especially with the rapid discovery of genes that regulate the proliferation and differentiation of sensory and supporting cells [34] and the cochlea being an ideal target for gene therapy.

2.2.4.1 The cochlea: An attractive site for gene therapy

The cochlea contains a small localized population of cells (in the organ of Corti) which is responsible for many common hearing disorders; hence, successful treatments targeted to this small cell population are likely to cure many hearing related problems.

Since the cochlea is a confined and isolated space of the inner ear, it limits the spread of gene transfer vectors to surrounding tissue, minimizing unwanted effects following introduction. The limited direct blood supply within the cochlear perilymphatic and endolymphatic spaces also reduces the risk of triggering undesired immune responses. The perilymphatic and endolymphatic fluid spaces allow diffusion and spread of vectors into areas remote to the site of inoculation, but still confined within the cochlea's perimeter. To sum, the cochlea is a low volume fluid-filled space with a relatively small population of specialized cell types that could be potential targets of gene therapy using a small number of vector particles. These advantages combine to make the cochlea an attractive site for gene therapy.

2.2.4.2 Viral vs Non-viral

Gene therapy strategies have been shaped by the choice of agent, vector, and route of delivery. The choice of vectors for gene delivery can be broadly classified into viral and non-viral vectors. In light of some viral vector's immunogenicity and their oncogenic potential, the use of viral vectors to deliver genes without major side effects in the infected host remains a great challenge. The death of an eighteen year old gene therapy patient, Jesse Gelsinger is regarded as the greatest casualty of gene therapy [35]. This tragedy marked the cessation of several viral gene therapy trials. Another major setback in gene therapy research in recent years has been the development of leukemia-like conditions in some patients leading to subsequent death [36]. In inner ear gene therapy, a replication deficient adenovirus even resulted in the lost of outer hair cells and significant reduction in distortion product otoacoustic emissions which monitor the functional status of the outer hair cells [37]. One group working with viral vectors did try to address this

issue through the use of immunosuppressants, however, there were concerns in exposing the body to potentially lethal infections by wild-type viruses [38].

Non-viral gene delivery vectors have emerged as a promising alternative to their viral counterparts. However, in inner ear research, there have been far fewer studies carried out using non-viral vectors [39-42]. The greatest challenge in using non-viral vectors is improving their relatively low transfection efficiency. Currently available non-viral vectors are routinely less efficient than viral vectors. However, non-viral vectors have their own advantages such as the ability to transfer a gene cassette of unlimited size and type, absence of viral component which may evoke a less immunogenic and inflammatory response, inability to replicate or recombine to form infectious agents, inability to integrate into host genome hence minimizing the risk of insertional mutagenesis and lastly, the ease of manufacture. Hence research has been aimed at altering non-viral vector formulations and/or delivery approaches to help compensate for their lower transfection efficiency.

Table 2.1 summarizes the various attempts made by various research groups over the years for inner ear gene therapy.

Table 2.1: Various agents, vectors and route for gene delivery both *in vitro* and *in vivo*.

<i>In vitro</i>	
Agents used	
Atoh1	[5, 43]
Bcl-2	[44]
β -galactosidase (β -gal)/LacZ	[45]
Green fluorescence protein (GFP)	[46-48]
Delivery vector / Technique	
Adeno-associated virus	[47]
Adenovirus	[43, 45, 46]
Baculovirus	[48]
Electroporation	[5]
<i>In vivo</i>	
Agents used	
Antioxidants (human catalase, SOD1, SOD2)	[49]
<i>Atoh1</i>	[12, 13, 50, 51]
Brain-derived neurotrophic factor (BDNF)	[30, 31, 52]
β -galactosidase (β -gal)/LacZ	[37-39, 42, 53-63]
Glial-cell-derived neurotrophic factor (GDNF)	[28-30, 64, 65]
Green fluorescence protein (GFP)	[39-41, 53, 61-63, 66-71]
Transforming growth factor- β 1 (TGF β 1)	[29]
Transforming growth factor- β 2 (TGF β 2)	[30]
X-linked inhibitor of apoptosis protein (XIAP)	[72]
Delivery vector / Technique	
Adeno-associated virus	[53, 61, 62, 65, 67, 68, 71, 72]
Adenovirus	[12, 13, 28-31, 37-39, 41, 49, 50, 54-59, 61-64]
Cationic liposome	[39-42]
Electroporation (<i>in utero</i>)	[51]
Herpes simplex virus type 1 (HSV-1)	[39, 60]
Lentivirus	[66, 67, 70]
Sendai virus	[69]
Vaccinia	[60]

Route of delivery	
Canalostomy	[58]
Cochlear implants with transfected cells	[52]
Cochleostomy	[12, 13, 38, 49, 50, 53, 54, 57, 58, 62-64, 69, 71]
Endolymphatic sac infusion	[56]
Facial canal inoculation	[30]
Gelfoam placement at round window niche	[40, 41]
<i>In utero</i> (Electroporation)	[51]
Osmotic pump	[37, 42, 66]
RWM with phenol damage	[55]
RWM inoculation	[28, 29, 31, 39, 40, 42, 53, 57, 59-61, 65, 68, 70, 72]

2.2.4.2a Adenovirus

Adenoviruses are non-enveloped, double stranded DNA viruses and the most commonly used is based on adenovirus serotype 5 with deletions of early genes E1 and E3. Of the 35kb vector, 30kb can be replaced in current constructs. Adenoviruses can bring about gene transfer in both dividing and non-dividing cell, and most cells are susceptible to infection. The virus enters cells by receptor-mediated endocytosis, and the viral genome enters the host nucleus, where viral reproduction occurs. The nucleic acid is not integrated into the host cell genome, but remains separated and utilize the host machinery to replicate itself. Adenoviral mediated transgene expression is not permanent and can last for up to a few months. Adenovirus can induce immune responses which cause severe inflammation diminishing efficacy and the retention time of DNA in the host is limited. This can be solved by inhibiting the immune response to the vector, either by vector modification or suppression of the recipient's immune response. Advantages of adenoviral vectors include non-disruption of genetic material of host cell and integration

of large foreign genetic sequence (up to 30kb) to the virus vector. Disadvantages include the limited duration of expression and the risk of triggering immune responses with repeated administration. In inner ear gene delivery systems, adenovirus is by far the most commonly studied viral vector [13, 28, 30, 31, 59, 61]. One of the most prominent research groups using adenoviral vectors in inner ear gene delivery studies is a group from the University of Michigan where they delivered the *Math1* gene into damaged cochlea and partially restored hearing [13].

2.2.4.2b Adeno-associated virus

Adeno-associated viruses (AAV) are approximately 4.7kb single stranded DNA parvovirus which replicate in the nucleus in the presence of an adenovirus or a herpes virus. The virus is flanked by terminal repeat sequences needed for the initiation of DNA replication and viral packaging. It is able to integrate in the host chromosome to establish a latent state and in the presence of a helper virus, production of the AAV vector begins. AAV can be used for applications that require long expression periods and 95% of the native genome of AAV can be replaced. However, high titer production is difficult. Duan et al. reported that transgene expression in the spiral ganglion and the stria vascularis in guinea pigs persisted for more than 8 weeks [61]. Safety of AAV mediated gene transfer in the cochlea was assessed by Soochuen and coworkers and they reported that the virus DNA was only detected in the contralateral ear and the cerebellum (not in other organs including the heart, lung, liver, spleen) [53]. Recently, Konishi et al. also reported several AAV serotypes with the ability to transfect sensory cells [62].

2.2.4.2c Lentivirus

Lentivirus belongs to a subclass of retroviruses consisting of non-oncogenic retroviruses that are able to infect both proliferating and non-proliferating cells. Infections by these viral vectors are characterized by long incubation periods and persistent infection. Lentiviruses are unique in that they contain open reading frames between the pol and env genes and in the 3' env region. They are considered more complicated than simple retroviruses, as it contains an additional six proteins, tat, rev, vpr, vpu, nef & vif. The lentiviral vectors used are derived from the human immunodeficiency virus (HIV) and are being evaluated for safety by removing some of the non-essential regulatory genes. The lentiviral vector based on HIV can integrate into chromosomes of both dividing and non-dividing or mitotically quiescent cells leading to a potentially stable, long term expression of a transgene spliced into the viral vector. Researchers at the University of California assessed the efficiency of lentivirus mediated gene transfer in guinea pigs both *in vitro* and *in vivo* [66]. The lentiviral vector did not induce significant inflammation due to limited dissemination of the viral vector (transfection regions *in vivo* were limited to the perilymphatic space). A recent study by Pietola et al. using lentiviral-mediated gene transfer on kanamycin-treated mice also yielded similar results [70].

2.2.4.2d Herpes simplex virus type 1 (HSV-1)

Herpes simplex virus type 1 (HSV-1) is a 152kb double stranded neurotropic DNA virus coated in an envelope consisting of 12 glycoproteins. It is highly infectious and able to infect both dividing and post mitotic cells due to its wide expression pattern of cellular receptors recognized by the virus. It is able to assume a latent state in neuronal cells and exists as an episome in the nucleus of neurons for the duration of the host lifetime.

Advantages of the HSV-1 include prolong transgene expression and being strongly neurotropic. Derby and coworkers reported successful transfection with a HSV-1 vector *in vivo* [60] however, associated immune response may be a concern when using this vector. Staecker et al reported in a study that the HSV-1 was able target neurons most effectively [39] as compared to adenovirus and liposome when assessing these vectors in the mice inner ear.

2.2.4.2e Vaccinia

Vaccinia virus particles are brick-shaped, 300-400 nm in diameter with lipoprotein membranes that surround a complex core structure. Vaccinia was used for vaccination against smallpox and is closely related to cowpox and variola (smallpox) virus. Its exact origin is unknown and it is known for its ability to replicate entirely within the cytoplasm of the infected cell. The only diseases vaccinia causes are complications of vaccination, post vaccinal encephalitis and progressive (spreading) vaccinia. The latter usually occurs in the immunosuppressed or those with multiple skin lesions (severe eczema). Although vaccination with this virus is now recommended only for those who work with it or with smallpox virus, it is also used as a mammalian expression system due to its relative simple methods of construction, ability to infect a wide range of cell types and high expression level not requiring nuclear processing and RNA transport. A vaccinia virus vector, vSC56 was reported to successfully deliver the lacZ transgene to many cells in the cochlea *in vivo* [60].

2.2.4.2f Cationic liposome

Cationic liposomes are vesicles of amphiphilic molecules surrounding an aqueous interior and with an overall positive charge. Due to its opposite surface charge to the DNA (negatively charged), cationic liposomes can form an overall positively charged complex with DNA through ionic interactions. The resulting positively charged biocompatible lipid-DNA complexes (lipoplexes) are able to penetrate the negatively charged biological cell surfaces and are endocytosed by the cell plasma membrane. In addition, cationic liposomes also protect DNA from attack by the deoxyribonucleases. After cell entry, the nucleic acid will migrate to the nucleus and penetrate the nuclear envelope to utilize the cellular transcription/translation machinery for gene expression. The exact mechanism of internalization has not been determined and several possible routes of internalization have been hypothesized, including endocytosis, fusion, and lipid-mediated poration. Endocytosis is generally believed to be the predominant pathway into the cell and further work is underway to define the mechanism for the optimizing of the gene transfer system. Similarly, the entry of DNA plasmid from the cytoplasm into the cell nucleus is poorly understood. Non-viral gene delivery to the inner ear is not as widely studied as compared to its viral counterparts; however, several groups [39, 40, 42] did successfully transfect regions within the cochlea using various cationic liposomal systems. Hence could possibly present be a safer and more clinically viable route for inner ear gene therapy.

2.2.4.2g Electroporation

Electroporation was originally used to transfer DNA to eukaryotic cell and in the electroporative transformation of bacteria cells. To explain it briefly, strong, short pulses of electric currents are applied to punch holes in the cell membrane or alter the structure

of the cell membrane such that molecules that are usually not membrane permeant will be able to enter the cells. Once gaining entry to the cell, DNA will utilize the transcription/translation machinery in the cell and the desired gene will be expressed. This method of gene delivery has been well established and widely used. Towards the late 1990s, this method was then employed *in vivo* to achieve DNA transfer and exogenous gene expression in various cells and tissues *in vivo*. This is a simple method which can transfer almost any DNA construct to animal cells and tissue *in vitro* and *in vivo* and no prior subcloning of the DNA to a vector is required, and is also less likely to invoke adverse immune reactions. In inner ear studies, Gao and coworkers successfully used this method to transfect dissected rat cochlea explants with a pRK5 plasmid (cloned with the gene of interest) [5]. Recently, Gubbels et al. also made use of electroporation (*in utero* gene transfer) to transfect the developing otocyst and demonstrated robust transgene expression within 24 hrs [51].

Table 2.2: Summary of characteristics and properties of various gene delivery vectors / techniques commonly used in inner ear gene therapy studies.

Vectors / Techniques	Characteristics	Advantages	Disadvantages
Adenovirus	<ul style="list-style-type: none"> - Double stranded, non-enveloped DNA virus - Commonly based on serotype 5 - Most widely used vector in inner ear gene therapy studies 	<ul style="list-style-type: none"> - Do not integrate to host cell genome - Infects both dividing and non dividing cells - Ease of production - High transfection efficiency - Availability of high titers 	<ul style="list-style-type: none"> - Host immunological response - Cytotoxicity - Transient expression
Adeno-associated virus (AAV)	<ul style="list-style-type: none"> - A parovirus - Can exist in a lytic life cycle or integrate into host's genome as a provirus - Helper virus needed to complete lytic cycle or for production of AAV vector 	<ul style="list-style-type: none"> - Not known with any known human disease - Yield long term stable expression of transferred gene - Infects both dividing and non dividing cells 	<ul style="list-style-type: none"> - Host immunological response (less compared to adenovirus) - Integrates to host genome - Packaging limit of DNA - High tier production is difficult
Lentivirus	<ul style="list-style-type: none"> - Subclass of retrovirus - RNA virus - First virus used for gene therapy 	<ul style="list-style-type: none"> - Yield long term stable expression of transferred gene - Can infect both dividing and post-mitotic cells 	<ul style="list-style-type: none"> - Host immunological response (less compared to adenovirus) - Integrates to host genome - Possible insertional mutagenesis
Herpes simplex virus (HSV)	<ul style="list-style-type: none"> - Most common is HSV type 1 - Neurotropic DNA virus - Transfect primarily the spiral ganglion neurons 	<ul style="list-style-type: none"> - Broad tissue tropism - Can transfect dividing and post mitotic cells - Yield long term stable expression of transferred gene - Suitable for targeting neurons - Large gene payload 	<ul style="list-style-type: none"> - Host immunological response - Difficulty of production - Pathogenic virus
Cationic Liposome	<ul style="list-style-type: none"> - Charged lipids or polymer that condense DNA to be transferred 	<ul style="list-style-type: none"> - No DNA limit - Relatively easy to produce - Low host immunological response 	<ul style="list-style-type: none"> - Relatively low efficiency - Transient expression
Electroporation	<ul style="list-style-type: none"> - Creating transient pores in cell membrane facilitating DNA entry into cells 	<ul style="list-style-type: none"> - Simple method - No DNA limit 	<ul style="list-style-type: none"> - Low efficiency - Significant results mainly <i>in vitro</i> - Optimization required for each cell type

2.2.4.3 Critical genes in inner ear gene therapy

Perhaps the most successful studies in inner ear gene therapy research involved the use of the mouse atonal homologue (*Math1*) gene, a basic helix-loop-helix transcription factor, homolog of the *Drosophila* atonal gene. In 1999, Bermingham and coworkers established the importance of the *Math1* gene in genesis of sensory hair cells in the mammalian inner ear where complete hair cell loss was observed in *Math1*-null mice [11]. During development, the *Math1* gene is also necessary for the differentiation of sensory hair cells and is vital in the overall formation of the sensory epithelia [73, 74]. Experimentally, both *in vitro* and *in vivo* overexpression of the *Math1* gene resulted in the formation of hair cells triggered by the transdifferentiation of postmitotic, postnatal supporting cells in the cochlea [5, 12, 13, 74]. In addition, these newly formed hair cells are capable of mechanoelectrical transduction [51] and aided functional recovery in deafened guinea pigs [13]. Hence, the *Math1* gene will be an important gene in future studies involving mammalian sensory hair cell regeneration and brings much hope in achieving reversible hearing loss in human beings.

A group of genes encoding for a set of neurotrophic factors including NT-3, BDNF and GDNF are also important and are widely studied in inner ear gene therapy research. In the developing inner ear, NT-3 and BDNF are essential for both vestibular and spiral ganglion neuron development and mutants in these two genes resulted in significant impairment in maintaining afferent and efferent innervations and loss of neurons [75]. Direct inoculation of these factors have shown to exhibit short term sensory hair cell protection and spiral ganglion neuron survival in various ototoxic or acoustic deafened models [26, 27, 76]. Overexpression of these factors has demonstrated

long term neuronal survival, hair cell protection and functional preservation in several deafened animal models [28-31, 64, 65].

Several other candidate genes not as widely employed / studied in inner ear gene therapy research but play important roles in regulating hair cell generation and protection has been identified (see also [34]). In addition, genetic hearing loss models allowed the characterization of various genetic mutations, better appreciation of the normal cochlea physiology and identification of various deafness genes (refer also to <http://webhost.us.ac.be/hhh/>). The list of potential targets for gene therapy is large and success in inner ear gene therapy lies upon the identification of key regulators in sensory hair cell determination and generation. In future studies, further discovery and manipulation of these candidate genes that control and regulate hair cell fate would give us a better understanding in achieving mammalian hair cell restoration via gene therapy.

2.2.5 Cell therapy

Cell therapy includes the use of cell, stem cell or gene modified stem cell implants. Research strategies are currently directed towards these cell transplants to restore or replace the degenerating auditory neurons following hearing loss. In recent years, techniques for cultivating and transplanting stem cells have improved dramatically which spurred the use of stem cells for various exciting applications, including osteogenesis treatment, differentiation to a variety of blood cell types, astrocytes, neurons and many others [77, 78]. The main stem cell types investigated in inner ear studies include the bone marrow stem cells, neural stem cells and embryonic stem cells. In addition, transplantation methodologies and sites among various research group varies (refer to Table 2.2 for summary). Most reported the survival of exogenous stem cells in the inner

ear within a month after transplantation with a few recording survival for up to 9 weeks after transplantation [79, 80]. Migration of transplanted cells was also observed in the cochlea, and regions near the auditory nerve and brain stem, including detection within the Rosenthal's canal and scala media, close to the sensory organ [80-85]. Several studies noted a more extensive migration of transplanted cells following inner ear damage [81, 85] and differentiation of stem cells to cochlear cell types was also observed [81, 82, 85-90]. These reports lay ground for future application to treat hearing disorders and suggest that there is great potential in using cell therapy for the treatment of sensorineural hearing loss.

Table 2.3: Various inner ear cell transplantation studies

Cell type transplanted	
Dorsal root ganglion cells	[79, 84, 91-94]
Embryonic stem cells	[79-81, 84, 89, 90, 92, 93, 95-98]
Hematopoietic stem cells	[99]
Mesenchymal stem cells	[88, 100-103]
Neural stem cells	[82, 83, 85-87, 92, 93, 104]
NIH3T3 cells transfected with BDNF	[105]
Transplantation site	
Auditory nerve	[79, 81, 90, 93]
Modiolus	[88, 89, 97, 100, 103, 104]
Rosenthal's Canal	[96]
Scala media	[80, 82]
Scala tympani	[83-86, 91, 92, 94, 95, 97, 99-101, 103]
Semicircular canals	[87, 98, 102, 105]
Recipient Species	
Chinchilla	[88]
Gerbil	[83, 90, 96, 99, 100, 103]
Guinea pig	[80, 84-86, 89, 91-93, 95, 97]
Mouse	[82, 87, 98, 101, 104, 105]
Rat	[79, 81, 92-94, 102]
Mode of Injury	
Acoustic Deafening	[85]
Cochlear ischemia	[83, 99]
Drug Induced Ototoxicity	[80, 82, 84, 86, 88-92, 94-97, 102-104]
No additional insult	[79, 87, 98, 100, 101, 105]
Surgical induced trauma of auditory nerve	[81, 93]

2.2.5.1 Concerns

Extended survival of transplanted cells and extensive migration throughout the inner ear system illustrates the promise of this therapy, however, many questions remain unanswered as current experimental methodologies are highly empirical and there are various concerns regarding the use this therapy. It is imperative that these concerns are

resolved before cell transplantation therapies will be clinically feasible and applicable for humans in the future.

1) Stage of differentiation of transplanted stem cells

Undifferentiated stem cells possess similar properties to tumors and when transplanted into animals, have the ability to form teratocarcinomas *in vivo*. High density of differentiating stem cells could have released signals to each other that resulted in uncontrolled proliferation and tumor formation. Several works suggest that in order for successful replacement/regenerative therapy to occur, cells at a specific stage of differentiation is required. While less differentiated cells are preferable for avoiding immunorejection, problems arising due to uncontrolled proliferation must be avoided at all cost.

2) Surgical Manipulation

The primary goal in developing surgical procedures for cell transplantation therapy is to achieve even distribution and survival of transplanted cells while minimizing damage to the delicate cochlear architecture during surgery. Hence the selection of the site of transplantation and development of safe surgical techniques that can be applied clinically is the key in balancing between mechanical damage sustained during transplantation and the subsequent integration of the transplanted cells in the inner ear system.

3) Microenvironment and environmental cues

The correct microenvironment of transplanted cells is vital for the survival and transdifferentiation of the transplanted cells. This is especially challenging in the inner ear system as the perilymph within the fluid-filled space of the cochlea lacks

nutrition and factors required by these cells. In addition, the fluid environment lacks a structural matrix for cell growth. Hence the identification of proper intrinsic and extrinsic factors capable of sustaining cell survival and induction cell differentiation will determine the successful integration of transplanted cells.

4) Immunological response and side effects

Successful incorporation of transplanted cells without immunological rejections or undesirable side effects is vital in determining the clinical feasibility of any treatment. In order to reduce the susceptibility for immunorejection, the use of immunosuppressants have been employed, however, though these immunosuppressive drugs improve the immuno-compatibility of transplanted stem cells, they also result in complications including impaired wound healing, infections and drug toxicities. Another approach to tackle this problem includes the *in vitro* engineering of cell membrane proteins of implanted cells to eliminate recognition by the host's immune system thus avoiding immunological rejections or inflammation.

5) Synaptogenesis and cochleotopic connectivity of transplanted cells

Upon successful integration and transdifferentiation of transplanted cells, the functional connectivity of newly formed cells with the host auditory system to replace the function of degenerated or absent afferent neurons is the key in determining function recovery. An improved understanding of factors or stimulus involved in the auditory system development in terms of how synapses form at the connection will provide essential information on functional recovery and aid in

determining the ability of these cells to form new synapses with existing neurons in an appropriate cochleotopic manner.

2.3 Polyethylenimine in gene transfer studies

Polyethylenimine (PEI) is well known as a superior non-viral vector for gene transfection both *in vitro* and *in vivo*. Transfection results using PEI were impressive from the beginning compared to many existing non-viral vectors then [106]. It is often regarded as the gold standard in non-viral gene delivery due to its strong DNA compaction capacity and high endosomolytic competence resulting from the high density of protonable amino nitrogen atoms. The high cationic charge density of PEI results in an improved membrane destabilizing potential and DNA condensing capacity which protects the DNA from degradation hence increasing the probability of DNA reaching the nucleus [107]. While PEI intrinsically possesses these essential features necessary for efficient transfection, additional modifications can also be introduced to add specificity [108, 109] and improve biocompatibility [110, 111] for *in vivo* applications. There is a wide variety of PEI with various architectures (linear, branched, dendrimeric etc.) each bearing different advantages and disadvantages, hence currently there is no general favorite and the discovery of various PEI-based vectors still relies heavily on empirical approaches [112].

2.3.1 Mechanism of PEI-mediated gene transfer

The anionic DNA and the cationic PEI spontaneously collapse into a DNA-vector complex with a net cationic charge, which is the transfecting unit. The complex interacts non-specifically and binds to the polyanionic glycosaminoglycans of the cell membrane

leading to endocytosis. Every third atom of PEI is a protonable amino nitrogen atom, which makes the polymeric network an effective "proton sponge" at virtually any pH [113]. Accumulation of protons is coupled to an influx of Cl^- anions within the endosome. These results in large increase in ionic concentration and substantial water entry, leading to rapid osmotic swelling and membrane rupture facilitating endosomal escape. Therefore it protects the DNA against nucleases during trafficking and aids translocation to the nucleus. Finally the complex migrates through the nuclear membrane and disassembles where transcriptional apparatus in the nucleus works on the DNA for gene expression [106, 112, 114] (see also Figure. 2.1 for mechanism).

Figure 2.1: Schematic of the proton sponge hypothesis postulating the mechanism of PEI-mediated gene transfer.

2.4 Bone marrow cell transplantation in gene transfer and transdifferentiation studies

The bone marrow contains two types of stem cells, the hematopoietic (HSCs) and mesenchymal stem cells (MSCs). Traditionally, HSCs give rise to blood cells and MSCs give rise to stromal cells, which include the muscle, epithelial, neural cells etc. When an injury is inflicted in the body, distant stem cells migrate towards the injury site, undergo stem cell differentiation and promote structural and functional repair [115]. Stem cells' ability to divide (self renewal) and differentiate (plasticity) allows it to act as a repair system for the body, replenishing other cells as long as the host is alive.

2.4.1 Transdifferentiation studies

Although controversial [116, 117], bone marrow derived stem cells' high degree of plasticity [118] has created a great deal of attention among various research groups. The ability of bone marrow stem or progenitor cells to home areas of injury and transdifferentiate to organ specific cells has been displayed successfully in the heart [119], liver [120, 121], muscle [122, 123], brain [124, 125], kidney [126] and pancreas [127]. These studies provide increasing evidence that the bone marrow may serve as a reservoir for a renewable source of cells that contributes to organ repair after cellular injury. At the same time, this highlights the possibility of harnessing the regenerative ability of bone marrow derived cells (BMDCs) for treatment of various degenerative diseases. The ability of bone marrow stem or progenitor cells to home areas of injury and transdifferentiate to organ specific cells has been displayed successfully in many studies, however, failure to

reproduce BM contribution to many tissue types has raised a lot of doubts about the entire concept of adult stem cell plasticity. In addition, a major controversy has emerged claiming that the plasticity / transdifferentiation observed were due to cell-cell fusion instead of BM stem cell plasticity [128, 129]. Hence, the use of bone marrow cells as reparative cells has elicited a huge interest coupled with major skepticism which raised several important questions with regards to adult bone marrow cells' role in tissue repair and regeneration.

Table 2.3 is a non-exhaustive list which summarizes the studies made in the various *in vivo* systems.

Table 2.4: Summary of transdifferentiation studies with bone marrow transplantation (hematopoietic reconstitution of irradiated or immuno-deficient recipients) in various in vivo systems (non-exhaustive).

Organ/s assessed	Prior Insult (excluding irradiation before BMT)	Transdifferentiation / Recovery	Author, Year
Heart	Hypoxia / Aortic constriction	Transdifferentiation to cardiomyocytes	Endo et al., 2007 [130]
Colon	Dextran sulfate sodium induced colitis	Transdifferentiation to colonic interstitial cells	Hayashi et al., 2007 [131]
Pancreas	Streptozotocin-induced diabetes	β cell regeneration coupled with improved hyperglycemia	Hasegawa et al., 2007 [127]
Retina	Laser-induced Bruch's membrane rupture	Transdifferentiate to astrocytes, microglia, mural and retinal pigment epithelia cells	Chan-ling et al., 2006 [132]
Retina	Mechanical rupture of Bruch's membrane	Transdifferentiate to retinal pigment epithelia cells	Harris et al., 2006 [133]
Kidney	Ischemic renal injury	Transdifferentiate to renal tubular epithelial cells	Kale et al., 2003 [126]

Retina	Laser-induced Bruch's membrane rupture	Choroidal neovascularization observed	Sengupta et al., 2003 [134]
Lung, stomach, esophagus, bowel, colon, intestine, liver, kidney, and skin	No	Transdifferentiate to liver, lung, gastrointestinal tract and skin-related cell	Krause et al., 2001 [118]
Heart	Myocardial infarction by coronary artery ligation	Neovascularization and cardiac function improvement	Kocher et al., 2001 [135]
Heart	Ischemic by coronary artery occlusion	Transdifferentiation to cardiomyocytes and endothelial cells	Jackson et al., 2001 [136]
Brain	No	Transdifferentiation to neuronal cells	Brazelton et al., 2000 [124]
Brain	No	Transdifferentiation to neuronal cells	Mezey et al., 2000 [125]
Liver	No	Transdifferentiation to hepatocytes	Theise et al., 2000 [137]
Liver	No	Transdifferentiation to hepatocytes	Lagasse et al., 2000 [138]
Pancreas	Pancreatic lesion and streptozotocin-induced diabetes	No transdifferentiation to β cells but hyperglycemia improvement observed	Lavazais et al., 2007 [139]
Liver	α -naphthylisothiocyanate / carbon tetrachloride induced liver damage	No transdifferentiation to hepatic cells	Rountree et al., 2007 [140]
Brain	Neurotoxin induced brain injury	No transdifferentiation to neuronal cells	Keshet et al., 2007 [141]
Adipose tissue	Surgical induced wound	No transdifferentiation to adipocytes	Koh et al., 2007 [142]
Heart	Cardiac infarction	No transdifferentiation to cardiomyocytes, only scar tissue neoangiogenesis	Möhlmann et al., 2006 [143]
Kidney	Folic-acid induced renal injury	No transdifferentiation to renal tubule cells	Szczypka et al., 2006 [144]
Lung	Naphthalene induced airway epithelial injury	No transdifferentiation to airway epithelial cells	Loi et al., 2006 [145]

Cornea	No	No transdifferentiation to corneal or neuronal cells	Nakamura et al., 2005 [146]
Heart	Puncturing of cardiac apex	No transdifferentiation to cardiomyocytes, only fusion	Ishikawa et al., 2005 [147]
Heart	Myocardial infarction	No transdifferentiation to cardiomyocytes, only fusion	Nygren et al., 2004 [148]
Pancreas	Streptozotocin-induced diabetes	No transdifferentiation to insulin-expressing cells	Mathews et al., 2004 [149]
Pancreas	Streptozotocin-induced diabetes	No transdifferentiation to β cells	Lechner et al., 2004 [150]
Brain and liver	Genetic induced leukodystrophy	No transdifferentiation to neuronal, astrocytic or hepatocytic cells	Yagi et al., 2004 [151]
Liver	Carbon tetrachloride induced liver damage	No transdifferentiation to hepatocytes	Cantz et al., 2004 [152]
Pancreas	Streptozotocin-induced diabetes	No transdifferentiation to β cells	Choi et al., 2003 [153]
Brain	Cerebral cortex lesion	No transdifferentiation to neuronal, microglial or endothelial cells	Valli�res et al., 2003 [154]
Liver, brain and heart	No	No transdifferentiation, only fusion	Alvarez-Dolado et al., 2003 [128]
Brain	No	No transdifferentiation to neural cells	Ono et al., 2002 [155]
Liver	No	No transdifferentiation to hepatocytes	Wang et al., 2002 [156]
Blood, brain, kidney, intestine, lung, liver and muscle	No	No transdifferentiation observed in the brain, kidney, intestine, lung, liver and muscle	Wagers et al., 2002 [116]

It is of utmost importance to understand the signals that enable the transdifferentiation of bone marrow cells and more efforts are still required to improve our understanding of the mechanisms involved in regeneration before bone marrow cells can be employed as a stable and standard therapy for tissue repair. Questions that must be

addressed include the genetic and cellular regulatory mechanisms involved and the physiological role of transdifferentiation and cell fusion during tissue repair

2.4.2 Gene transfer studies

The ideal gene transfer vector for gene therapy is one which is accessible, readily available, able to be manipulated *in vitro*, and most importantly, capable of efficiently delivering a gene to its target without inducing an immune response from the recipient. Recently, with advances in the ability to isolate, genetically manipulate and transfer BM cells into recipient host, BMSC has become a vector to deliver therapeutic genes to non-hematopoietic tissues. Palma et al. demonstrated the use of genetically modified HSC to target a certain suicide gene to tumors using BMT which resulted in inhibition of angiogenesis and slower tumor growth [157]. Chan et al. also displayed the protective effects of transplanting genetically modified bone marrow cells which express proinsulin II that protects mice against insulinitis [158]. Grove et al. made use of retrovirally transduced BMSC encoding a reporter gene and demonstrated these BMSC's ability to differentiate into lung epithelium cells [159]. In the central nervous system, Priller et al. displayed the presence of genetically modified hematopoietic cells which differentiated to microglia after BMT [160] and Makar et al reported significant increase in BDNF expression in the brain after transplanting BDNF-transduced bone marrow cells into irradiated mice [161]. Similar therapeutic effects were also demonstrated by Park et al. using GDNF engineered bone marrow cells [162].

Therefore, with the integration of BMSC's plasticity and advances in genetic modifications, this provide a novel non-invasive approach to target potential therapeutic factors which will help restore injured tissues and cells through the expression of

therapeutic proteins and transdifferentiate of stem cells. The challenge in this therapy is to determine if the transgene expression achieved by this method is adequate for protection and select a suitable vector which will allow the efficient transduction of HSC and stable gene expression at therapeutic levels that will persist throughout the duration of the patient's disease.

CHAPTER 3: RESEARCH OBJECTIVES

Although in recent years, encouraging results from various research groups has sparked hope that mammalian inner ear repair could be a possible accomplishment in the near future, current methodologies are still plagued with skepticism and are still a long way before clinical implementation. The success of mammalian inner ear repair will depend on our understanding and development of novel therapeutics for the inner ear that are of clinical relevance (safe, effective and direct). Hence with the extensive basic research in the area of sensorineural hearing loss for the past 10 years, recent efforts are focused on its translation to clinical applications.

In view of the limitations of current methods (the use of viral gene delivery vectors and invasiveness of administration) in inner ear therapeutic studies, the objective of this research is to explore the use of PEI to achieve polycationic mediated cochlea gene transfer and to make use of the host's intrinsic capability and BMDSCs' high degree of plasticity to investigate the role of an individual's own bone marrow cells (BMCs) in the restoration processes after deafening.

The outline of the methodologies is as follows:

Non-Viral Gene Delivery Studies

- To overcome non-viral vectors' inherent low transfection efficiencies, we compare and assess efficiencies of various delivery routes with adenovirus-mediated gene transfer in the guinea pig.
- Select the optimal method of delivery for non-viral gene delivery vectors.
- Achieve PEI-mediated cochlea gene delivery with the appropriate delivery method.

Bone Marrow Cell Studies

- Transplant whole BMC from GFP transgenic mice into irradiated recipient mice to track and investigate the homing capacity of BMC or BMDC to the cochlea.
- Investigate BMC homing response to deafening and characterize infiltrated BMDCs in the cochlea.
- Investigate transdifferentiation potential of BMC in a deafened mice model.
- Investigate the effect of cytokine mobilization on the transdifferentiation potential of BMDCs into cochlear cell types in a deafened mice model.

CHAPTER 4: MATERIALS AND METHODS

4.1 Animals

Male Albino Hartley guinea pigs (250 – 350 g) were used in all gene delivery surgeries. The guinea pig model was used because of its relatively large cochlea size as compared to those of mice and rats hence allowing greater ease for surgical manipulation without causing significant injury. (n = 5 for each experimental group + 1 sham operated guinea pig for each experimental group)

Male C57BL/6J male recipient mice (7 weeks) and female C57BL/6J transgenic donor mice (16 weeks) homozygous for the eGFP were used for bone marrow transplantation. The mice were obtained from a breeding colony established in the institutional animal care facilities. The eGFP transgenic mice ubiquitously express enhanced GFP under the control of a chicken beta-actin promoter and cytomegalovirus enhancer. 1 donor mouse was required to supply bone marrow cells for 10 – 12 recipient mice. (n = 4 for each experimental timepoint + 1 control mouse for each experimental timepoint)

The animals had free access to water and a regular diet. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Biological Resource Centre – Biopolis (Singapore). (IACUC#: 050006, 060182)

Table 4.1: Summary of animals used for each experiment.

	No. of animals used
Chapter 5 (Adenoviral-mediated gene transfer)	
Gelfoam method	6 guinea pigs (5 experimental + 1 sham operated)
RWM inoculation	6 guinea pigs (5 experimental + 1 sham operated)
Cochleostomy	6 guinea pigs (5 experimental + 1 sham operated)
Chapter 6 (PEI-mediated gene transfer)	
Cochleostomy	6 guinea pigs (5 experimental + 1 sham operated)
Osmotic pump method	6 guinea pigs (5 experimental + 1 sham operated)
Chapter 7 (BMDC migration in deafened model)	
Hematopoietic reconstitution	4 donor mice
Deafened animal	35 mice (4 experimental + 1 control per timepoint)
Chapter 8 (BMDC migration in cytokine treated deafened model)	
Hematopoietic reconstitution	4 donor mice
Deafened animal	40 mice (4 experimental + 1 control per timepoint)

4.2 Vectors and Plasmid DNA

4.2.1 Adenovirus construct and generation of active virus

Figure 4.1: Schematic construct of the virus construct (Ad-eGFP)

A replication deficient adenoviral vector (Ad-eGFP) (derived from wild type Ad5 genome) containing the eGFP gene driven by a cytomegalovirus promoter was provided by Dr Weiqiang Gao (Genentech, San Francisco, California). Viral suspensions (0.8×10^7 pfu/ml) were kept at -80°C until thawed for use.

The adenoviral vector was constructed using the Adeno-X expression system (K1650-1, Clontech). Briefly, the eGFP coding region was isolated by PCR amplification and inserted into the pShuttle vector. The expression cassette was then cut from the pShuttle vector and subsequently ligated into pAdeno-X vector, derived from the wild-type Ad5 genome with deletions in the E1 and E3 regions, to generate the replication incompetent recombinant adeno-eGFP construct. The viral DNA was linearized with *Pac* I digestion and transfected into early-passage human embryonic kidney 293 (HEK 293)

cells. Adenovirus were propagated in E1A, E1B-trans-complementing HEK-293 cells and purified from sonicated cell pellets by double cesium chloride density gradients.

4.2.2 Polyethylenimine

The non-viral vector used (polycationic polymer, ExGen500, #R0521, Fermentas, Burlington, Ontario) was a 22 kDa linear polyethylenimine (PEI) with nitrogen concentration of 100 mM in every 1 μ l of ExGen500. Before use, DNA (plasmid DNA encoding the eGFP reporter gene) and PEI were first diluted in sterile 5% (w/v) glucose solution to obtain the appropriate dilutions.

4.2.3 Plasmid construct

Figure 4.2: Schematic construct of the plasmid (pRK5-eGFP)

The plasmid (pRK5-eGFP) was also provided by our collaborator Dr Weiqiang Gao (Genentech, San Francisco, California). Upon receipt, the plasmid was amplified in DH5 α strain of *E. coli*, and purified using the NucleoBond PC500 EF (Endotoxin-free plasmid purification kit) according to manufacturer's instructions. DNA stock was kept at -20°C until thawed for use.

4.3 Surgical Procedures

Before surgery, all guinea pigs were checked to have normal Preyer's reflex and no abnormalities in physical appearance. Guinea pigs were anesthetized with ketamine: xylazine mixture (40 mg/kg: 3-5 mg/kg, Intramuscular) and temgesic was injected (0.05 mg/kg, Intramuscular) as analgesic. Four delivery methods (Gelfoam, RWM inoculation, Cochleostomy, Osmotic Pump) were assessed (n = 5 each) using two surgical approaches, namely the postauricular and ventral approach.

Figure 4.3: (Left) A guinea pig cochlea and location of its round window membrane.

(Right) A chinchilla cochlea with bony shell been removed to reveal the fluid filled spiral chambers and various methods of delivery A) Gelfoam method, B) RWM Inoculation, C) Cochleostomy. (chinchilla cochlea photograph reproduced from www.sickkids.on.ca)

4.3.1 Gelfoam

Route of surgery followed the postauricular approach where the guinea pig was placed in the side-lying position and an incision was made behind the ear. The connective tissues and veins were retracted until the tympanic bulla was exposed. The bulla was opened with forceps to expose the cochlea laterally at the basal turn with the round window membrane (RWM). A small piece of Gelfoam big enough to cover the RWM is placed at the bony groove of the round window niche in contact with the RWM. 10 μ l of the virus vector solution is injected and absorbed on the Gelfoam (refer to Figure 4.3A, right). The bony defect in the bulla was then sealed with dental cement (Fuji I glass ionomer luting cement, Alsip, IL) and the wound was sutured closed.

(The Gelfoam is a sterile, pliable, surgical sponge prepared from specially treated purified gelatin solution and capable of absorbing and holding within its meshes many times its weight. In clinical applications, it has been used as a hemostatic to arrest bleeding or hemorrhage.)

4.3.2 Round Window Membrane Inoculation

Route of surgery also followed the postauricular approach as described in Chapter 4.3.1 to expose the guinea pig's RWM. Using a microsyringe (Hamilton, Reno, Nevada) connected with a 25G needle, 10 μ l of the virus vector solution was injected across the RWM manually from a microsyringe mounted on a free-standing clamp over a period of 10 min (refer to Figure 4.3B, right). To prevent leakage of inner fluid and vector solution, a small piece of thinned fascia was placed on the bony niche to seal the opening on the RWM after needle retraction. The cavity in the bulla was then sealed with dental cement (Fuji I glass ionomer luting cement, Alsip, IL) and the wound was sutured closed.

4.3.3 Cochleostomy

Route of surgery followed the ventral approach where the guinea pig was placed in the supine position and ventral paramedian skin incisions were made extending from the jawbone to collar bone. The jugular vein, submandibular gland and connective tissue were retracted medially. The tympanic bulla was exposed and opened with forceps, revealing the cochlea laterally. A small orifice was made in the cochlea bony shell (middle turn), penetrating the scala tympani. 10 μ l of the vector (viral vector or DNA complex) solution was injected through the orifice over 10 min into the scala tympani (refer to Figure 4.3C, right). The needle was then carefully removed and the orifice was sealed with dental cement to prevent backflow of the vector solution. Upon completion, the cavity in the bulla was closed with dental cement and the wound was sutured closed.

4.3.4 Osmotic Pump

Route of surgery also followed the ventral approach as described in Chapter 4.3.3 where the guinea pig's cochlea was revealed laterally and a small orifice was made in the cochlea bony shell (middle turn), penetrating the scala tympani (refer to Figure 4.3C, right). A catheter (Instech Solomon, PUFC-C30-10, Plymouth Meeting, PA) connected to an osmotic pump (Alzet, Cupertino, CA, 1007D, Flow rate: 0.5 μ l/hr, Reservoir volume: 90 μ l containing 10 μ g of DNA complexed with PEI at N/P ratio = 6) pre-activated by overnight incubation at 37°C was inserted into the orifice made in the cochlea bony shell and secured by means of dental cement and a topical tissue adhesive (NEXABAND, Abbott, Chicago, IL). The osmotic pump reservoir was then inserted subcutaneously under the neck region of the guinea pig. Upon completion, the cavity in the bulla was closed with dental cement and the wound was sutured closed.

4.4 Primary Cell culture

The cochleae of the guinea pig (300 – 350 g) were harvested and dissected carefully in cold extra-cellular solution (142 NaCl, 5.37 KCl, 1.47 MgCl₂, 2 CaCl₂, 10 HEPES in mM, 300 mOsm, pH 7.2). After the spiral ligament, stria vascularis tissues and tectorial membrane were removed, the sensory epithelia was carefully dissected and collected for enzymatic disaggregation in a mixture of 0.125% trypsin and 0.125% collagenase for 5 – 10 min at 37°C with shaking. The enzyme was inactivated with equal volume of culture medium containing 20% fetal bovine serum (FBS). The dissociated cells were added to a modified Keratinocyte medium (containing basic Keratinocyte medium (Gibco, Grand Island, NY), L-glutamine, human recombinant epidermal growth factor, bovine pituitary extract and 10% FBS), triturated and seeded with fresh modified Keratinocyte medium in 48-well plate (approximately 10 wells for every two cochleae). Cells were cultured at 37°C in a 5% CO₂ incubator and the media were changed every 3 – 4 days. The cells were subsequently passaged using trypsin-EDTA.

4.5 *In vitro* transfection with polyethylenimine

DNA and PEI were diluted in serum free media with gentle vortexing and incubated at rtp for 10 min before combination. Diluted PEI was added to the DNA solution, vortexed vigorously and incubated at rtp for at least 10 min before use. Primary/HepG2 cells were plated in 24 well plates the previous day to obtain 70% confluency. On the day of transfection, existing medium was removed and cells were rinsed with serum free media. The complex solution containing DNA and PEI were added

dropwise into each well (1 µg of DNA per well complexed at various N/P ratios). DNA-PEI complex at various N/P ratios are prepared by mixing various amount of DNA and PEI together based on manufacturer's instructions (using sterile solution of 5% glucose as diluent). The cells were then incubated at 37°C in CO₂ incubator for 6 hrs. After incubation, the cells were replaced with fresh complete medium and incubated for 3 days before proceeding for flow cytometry analysis.

4.6 Bone Marrow Transplantation

BMCs were aseptically flushed in bulk with RPMI 1640 (containing 10% fetal bovine serum) from the medullary cavities of femurs and tibias of donor GFP transgenic mice (16 weeks old). The marrow cells was mechanically dissociated and filtered through a 40µm nylon mesh to obtain a single cell suspension. Erythrocytes were lysed and the recovered cells were counted ($\sim 3 \times 10^7$ cells per donor mice). On the same day, recipient mice (7 weeks old) were lethally irradiated (900 cGy) with a gamma emitting source and reconstituted with a single intravenous infusion of 2×10^6 BMCs from the donor mice. In addition, 2 mice were sham transplanted with whole BMCs from a wild type mouse. The percentage of GFP-positive (GFP⁺) cells among recipient BMCs was determined by fluorescence-activated cell sorting (FACS), using a FACS Caliber with CellQuest software (BD PharMingen, San Diego, CA). Engraftment efficiency in recipients was determined in the peripheral blood and bone marrow by donor chimerism (%) = (% GFP⁺ cells of BMT recipient / % GFP⁺ cells of normal GFP-transgenic mice) x 100%

4.7 Acoustic Deafening

Acoustic deafening was conducted 3 months after BMT. The mice, unanaesthetized and unrestrained within cages in a reverberant exposure chamber, were exposed continuously to 120 dB SPL white noise for 2 h. The sound stimulus was produced by a RP2 enhanced real-time processor (Tucker-Davis Technologies, Gainesville, FL), filtered through a PA5 programmable attenuator (Tucker-Davis Technologies) and delivered via a free-field monitor speaker (Yamaha Corporation, Hamamatsu Shizuoka, Japan). The sound pressure level was measured through a 1/2-inch prepolarised free-field microphone (Type 4189, Bruel and Kjaer, Naerum, Denmark) connected to an integrating sound level meter (Type 2239A, Bruel and Kjaer) and the sound pressure level was monitored at 120 ± 2 dB SPL throughout the entire noise exposure period. Prior to the experimental deafening procedure, the exposure levels measured at four positions within each cage varied by < 0.5 dB.

4.8 BMC Mobilization by Cytokine Treatment

Bone marrow cells were mobilized from the bone marrow via cytokine treatment (SCF and G-CSF). To prevent sequestration of the circulating BMDCs, the spleen was removed in all animals. Under general anesthetic, a small lateral incision was made in the inner body wall. The spleen was exteriorized and excised after ligation of the blood vessels. The wound was closed with stitches and the mice were left at least 2 weeks before any subsequent procedures. After recovery, BMCs were mobilized by daily subcutaneous injections of recombinant rat stem cell factor (SCF) at $200 \mu\text{g/kg/day}$, and recombinant

human granulocyte colony-stimulating factor (G-CSF) at 50µg/kg/day (Amgen Biologicals, Thousand Oaks, CA) for a total of ten days. Control mice were treated with PBS (pH 7.3) containing 0.1% bovine calf serum. Deafening was scheduled at day 6 of the cytokine injection period.

4.9 Bromodeoxyuridine (BrdU) administration

BrdU was dissolved in saline at a concentration of 10mg/ml and filtered at 0.2µm before use. To identify the presence of any proliferating cells in the cochlea, mice in both the control and experimental groups were injected intraperitoneally with 3 doses of BrdU (100mg/kg) at 2hr intervals the day before sacrifice.

4.10 Cochlea Harvesting and Processing

Under general anesthetic (ketamine/xylazine mix), the subject was decapitated and the skull plate was opened using a bone breaking forceps to expose the brain. Upon removing the brain tissue from the skull, the temporal bones containing the cochleae were exposed at the lateral sides of the skull. Using a pair of surgical tongs, the temporal bones are carefully twisted out from the skull and broken to retrieve the cochleae. The RWM of the cochlea was perforated and the cochlea was perfused thoroughly with ice cold 4% paraformaldehyde in PBS. The harvested cochleae were then kept in the same fixative overnight at 4°C before subsequent procedures.

4.10.1 Surface Mount Preparation

After fixation, the cochleae were removed from the fixative and rinsed with PBS. The cochlear bony shell was carefully broken and chipped off piece by piece to expose the cochlea modiolus. Using a fine forceps, the tectorial membrane was removed and the basilar membrane was carefully dissected from the cochlear modiolus. The basilar membranes were collected, mounted on glass slides and prepared for fluorescence microscopy.

4.10.2 Cryosectioning

After fixation, the cochleae were removed from the fixative, rinsed and stored in 10% EDTA at rtp (in the dark). Guinea pig cochleae are stored for 7 days and mice cochleae are stored for 3 days with daily solution changes. After decalcification, the cochleae were transferred to 20% sucrose solution at 4°C and stored overnight for cryoprotection. The cochleae were then infiltrated by a gelatin embedding technique as described by Hurley and coworkers [163] for better histological processing to achieve both anatomical resolution and antigen preservation.. Briefly, the cochleae were pre-warmed in PBS at 37°C, and immersed in 15% gelatin solution for at least 2 hrs at 37°C to fill up the internal compartments of the cochleae. The gelatin was then cooled down and solidified to form gelatin blocks which are trimmed and frozen at -20°C. Cross sections of the cochleae were obtained using the cryostat (CM 3050S, Leica, Bensheim, Germany) at 10µm per section and mounted on poly-l-lysine coated slides. Slides were blow dried at room temperature and kept in the dark at -20°C for long term storage before subsequent immunostaining or microscopy procedures.

4.11 Western Blot (plasmid/adenovirus analysis of transgene expression)

The plasmid, complexed with PEI (22kDa linear polyethylenimine, ExGen500, #R0521) (N/P ratio = 6) and adenovirus (Ad-eGFP) were transfected on HepG2 cells *in vitro* for a total of 3 days. The cells were lysed with a protein lysis buffer, resuspended in Laemmli loading buffer and denatured by boiling for 5 min. The lysate was then loaded onto 10% SDS-polyacrylamide separating gel. After electrophoresis, the proteins were transferred to nitrocellulose membrane (BioRAD, Richmond, CA). Blots were then blocked in 5% non-fat milk in TBS/0.1% Tween 20. Primary antibody used is rabbit anti-eGFP (8367, BD Bioscience, San Jose, CA). After incubation with primary and HRP-conjugated secondary antibody (6440-05, Southern Biotech, Birmingham, AL) and extensive rinses, the membrane was dipped in ECL chemi-luminescence substrate and visualize in the ChemiGeniusQ system (Syngene, Cambridge, UK). Control protein used is the EGFP protein (BD Bioscience, San Jose, CA)

4.12 Virus titering (Tissue Culture Infectious Dose 50 Method)

The TCID₅₀ is a method that has been used to determine the titer of many different types of viruses including the adenovirus where dilutions of the virus are incubated with cells in 96-well plates and the presence or absence of cytopathic effect (CPE) in each well is determined. The TCID₅₀ method offers several advantages over the PFU assay such as being twice as fast to perform and providing results that are easier to interpret and more consistent between individuals. Cells were collected from a flask of fresh HEK293 cells, counted on a hemocytometer and seeded 10^4 per well in two 96-well flat bottom plates.

The cells were cultured overnight to allow adhesion. The following day, serial dilutions of the virus from 10^{-1} to 10^{-10} dilution was prepared in duplicates. For each row of the 96-well plate, 0.1 ml of the diluted virus was added into each well #1 to #10 (10 wells per dilution) for the eight highest dilutions. Columns #11 and 12 were used for the negative controls where DMEM was added to each well to test for cell viability. The cells were then incubated at 37°C in a CO_2 incubator for 10 days. After 10 days, the plates were read using an inverted microscope where the number of cells with observable CPE was counted per row by comparison with the negative control wells. A well was counted as positive even if only a small spot or a few cells show CPE. The test is only valid if the negative controls do not show any CPE or cell growth problems and the lowest dilution shows 100% infection while the highest dilution shows 0% infection.

Figure 4.4: Schematic of the 96-well plate set-up.

4.13 Sensory hair cell labeling

After surface mount preparation of basilar membranes, the tissues were then washed with PBS and stained with 1.25µg/ml Phalloidin-TRITC in 0.2% Triton X-100 and 1% BSA in PBS for half an hour at rtp. Upon completion, the tissues were rinsed in PBS at least 3 times and mounted onto slides for viewing under fluorescence microscopy.

4.14 Immunochemistry

4.14.1 Immunohistochemistry

Table 4.2: List of antibodies (Immunohistochemistry)

Antibody	Source	Host	Dilution
T lymphocyte	Serotec, Oxford, UK	Mouse	1:10
CD45	Serotec, Oxford, UK	Rat	1:500
CD68	Serotec, Oxford, UK	Rat	1:150
BrdU	Santa Cruz Biotechnology, Santa Cruz, CA	Rat	1:50
SDF-1	eBioscience, San Diego, UK	Rabbit	1:200
CD117	Chemicon, Temecula, CA	Rat	1:50
CD34	Abcam, Cambridge, UK	Rat	1:50
Myosin VIIa	Novus Biologicals, Littleton, CO	Rabbit	1:200
Calretinin	Chemicon, Temecula, CA	Goat	1:1000
Jagged1	Santa Cruz Biotechnology, Santa Cruz, CA	Goat	1:50
S100A1	Labvision, Fremont, CA	Rabbit	1:75

NF200	Sigma, St. Louis, MO	Mouse	1:400
Na, K-ATPase	Development Studies Hybridoma Bank, Iowa, IA	Mouse	1:800

Slides were removed from storage at -20°C, air-dried and rinsed in 37°C PBS to remove residual gelatin and tissue mounting medium. To block unspecific binding, the sections were incubated in blocking solution (10% serum in PBS) and then incubated with a primary antibody diluted in PBS at rtp for 2 hr. The primary antibodies used in this study are listed in Table 3.1. After at least three wash cycles, the sections were incubated at rtp for 1 hr with the appropriate AlexaFluor594 secondary antibody (Molecular Probes, Eugene, OR, 1:400). After secondary antibody incubation, the slides were washed again for at least three wash cycles and visualized by fluorescence microscopy. Negative control was performed by using cochlea sections with no primary antibody and another with no secondary antibody.

4.14.2 Immunocytochemistry

Table 4.3: List of antibodies (Immunocytochemistry)

Antibody	Source	Host	Dilution
Jagged1	Santa Cruz Biotechnology, Santa Cruz, CA	Goat	1:50
S100A1	Labvision, Fremont, CA	Rabbit	1:50
Pan-Cytokeratin	Sigma, St. Louis, MO	Mouse	1:100

Anti-Jagged1[164], anti-S100A1[165] and anti-pan cytokeratin[166] primary antibodies were used as cochlear supporting cell markers. Cells were rinsed and fixed with 2% paraformaldehyde in PBS (pH 7.4) for 30 min at 4°C. After washing, the cells

were blocked with blocking solution for 30 min at rtp and incubated with primary antibodies diluted in PBS with 0.1% Triton X-100 overnight at 4°C. After three washing cycles, the cells were incubated with appropriate diluted secondary antibodies (Jackson ImmunoResearch, WestGrove, PA) conjugated to Cyanine 3 (Cy3) for 1 hr at rtp. The cells were then washed for three washing cycles and imaged by fluorescence microscopy.

4.15 Fluorescence Microscopy

The surface mount preparations, cryosections and cells were viewed and photographed using the Olympus Fluoview Confocal Laser Scanning Microscope (Olympus, Tokyo, Japan), Leica TCS-SP2 confocal laser scanning microscope (Leica, Bensheim, Germany) or Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan). The images were stored and processed with Adobe Photoshop 7.0 (Adobe, San Jose, CA). Supplementary magenta-green fluorescence figures were created by duplicating the red channel images into the blue channel.

4.15.1 Gene transfection studies

Surface mount preparations and cryosections were assessed based on the gene marker product of Ad-eGFP and PEI-eGFP observed. Cryosections were assessed by a semi-quantitative score system that evaluated eGFP transfection distribution across the cochlear turns and relative intensity of transgene expression in each turn (see Table 5.1). Sections were scored twice by different observers blinded to the group identities.

4.15.2 Colocalization studies

Validation of colocalization was achieved by first examining the sections using standard epifluorescence microscopy. Sections with suspected colocalization were then marked and visualized using confocal microscopy where z-stacks of 1µm optical sections were obtained digitally throughout the entire depth of the sample for confirmation of any suspected colocalization.

4.15.3 GFP⁺ cell migration quantification

Transverse cryosections of the cochlea were obtained (10µm thickness) in the mid-modiolar plane. The four most mid-modiolar sections (each separated by 100µm, defining a reference space of 350µm) were selected from each cochlea and subjected to quantitative analysis of the GFP⁺ cells. Each section was divided into 9 anatomic subdivisions, namely the spiral ligament, limbus, spiral ganglion, modiolus, perilymphatic walls, stria vascularis, Reissner's membrane, basilar membrane, organ of Corti. For the spiral ligament, limbus, spiral ganglion and modiolus, extent of GFP⁺ cell infiltration was determined by measuring the total GFP relative fluorescence density (GFP fluorescence area / Total area of interest) of each of the anatomic subdivision of the four selected cochlea sections using Image Pro Plus software (Media Cybernetics, Silver Spring, MD). For the perilymphatic compartment walls, stria vascularis, Reissner's membrane, basilar membrane, and organ of Corti, GFP⁺ cell quantification was carried out by manual cell counting where the total number of GFP⁺ cells was determined in each of the anatomic subdivision of the four selected cochlea sections.

4.16 Auditory Brainstem Response

Recording of ABR was performed at 4, 8, 12, 16, 20 and 24 kHz. Animals were anesthetized and needle electrodes were placed subcutaneously (Active: On vertex, Reference: Beneath pinna of measured ear, Ground: Beneath contralateral ear) in a sound proof chamber. The sound stimulus consisted of 15 ms tone bursts, with a rise-fall time of 1 ms and sound intensity was varied in 5 dB intervals. Threshold was defined as the lowest intensity level at which a clear waveform was detected. Stimulus presentation and response recording were done using the TDT system hardware and SigGen/Biosig software (Tucker-Davis Technologies, Gainesville, FL). ABR of test subjects were measured at the various experimental timepoints (Day 0, 1, 3, Week 1, 2, 4 and 8) of the experiment. Day 0 measurements were recorded ~10 hrs after acoustic deafening procedure; hence no TTS component was recorded.

4.17 White blood cell counting and Geimsa staining

Under general anesthesia, blood was collected into heparinized tubes. For leukocyte counting, red blood cells (RBC) were lysed in RBC lysis buffer (160 mM NH_4Cl , 10 mM KHCO_3 , and 0.1 mM EDTA, pH 7.4) and the remaining white blood cells were counted on a standard hemocytometer. For Giemsa staining, thin blood smears were obtained on glass slides, air dried, fixed with methanol for 3 – 5 min, stained with Giemsa's solution (Merck, Darmstadt, Germany) for 10 – 15 min and visualized via standard microscopy.

4.18 Flow cytometry analysis

4.18.1 Gene transfection studies

On the day of harvesting, cells were trypsinized, collected and fixed in ice cold 2% paraformaldehyde in PBS. Transfected cells were observed using fluorescence microscopy. Transfection efficiency was assessed based on the percentage of cells expressing the marker gene product of PEI-eGFP using flow cytometry analysis with a minimum of 10,000 cells interrogated in each sample. Data was derived from experiments repeated on three separate occasions with each data point conducted in triplicates. Quantitative data was compared using the Student's t-test.

4.18.2 Cytokine mobilization studies

Blood and marrow cells were collected and incubated with allophycocyanin (APC)-conjugated anti-mouse CD117 and APC-Cy7 conjugated anti-mouse Gr-1 (BD Pharmingen) for 30 min at rtp. After RBC lysis with PharmLyse (BD Pharmingen), the cells were washed in 2% FBS in PBS and fixed in 2% paraformaldehyde. Analyses were performed on a FACSCalibur (Becton Dickinson) and a minimum of 10,000 cells were interrogated in each sample. Flow cytometry data were analyzed using CellQuest software (Becton Dickinson).

4.19 Statistical analysis

Data were reported as mean $\pm$ SD. Statistical analyses were performed with the Student's t test for unpaired samples. Differences were considered statistically significant when $p < 0.05$.

CHAPTER 5: EVALUATION AND COMPARISON OF VARIOUS METHODS OF DELIVERY (ADENOVIRAL- MEDIATED)

Since inner ear gene delivery was reported in 1996 [59], much work and great progress have been achieved in the field of inner ear gene therapy over the past 10 years. Numerous studies have been published investigating the use of various vectors, genes and routes of delivery for the treatment of hearing related disorders or the protection of hearing function. Much progress has been reported in terms of sensory hair cell protection [28, 29, 31, 49, 167], regeneration [12, 13, 50], and functional restoration [13, 50] with the use of viral vectors in various gene therapy protocols. The most commonly used viral vectors are the adenovirus [13, 31, 49] and adeno-associated virus [47, 67, 167]. Compared to viral vectors, there have been far fewer reports on the use of non-viral vectors in inner ear gene delivery studies. These studies were carried out mainly by Lalwani and co-workers [40-42] in the late 1990s and early 2000s using cationic liposomes as delivery vector. Since then, limited work involving the use of non-viral vectors in inner ear gene delivery studies has been reported. However with safety issues being the top priority in clinical trials, and with rising concerns using viral vectors since reported deaths in viral gene therapy trials, non-viral vectors remain a relevant approach to be further explored for inner ear gene therapy.

Due to non-viral vectors' relatively low transfection efficiency compared to their viral counterparts, the choice of delivery route into the cochlea is particularly important. With the right choice of delivery method, it would compensate non-viral vectors' relative

lower transfection efficiency and maximize non-viral vectors' transfection capacity to meet or come close to that of viral vectors.

Therefore, exploiting the adenovirus's high transfection efficiency, an adenoviral vector (Ad-eGFP) was used to compare the relative transfection efficiencies of the various methods of delivery (Gelfoam, RWM inoculation and Cochleostomy), in order to select the most efficient method of delivery for non-viral vectors.

5.1 Adenovirus (Ad-eGFP)

5.1.1 Virus transgene expression

Upon receipt of the adenoviral vector (Ad-eGFP) from Genentech Inc., transfection was carried out on HepG2 cells. Subsequently, the faithfulness of the vector was assessed by Western Blot analysis of the cell lysate from the infected HepG2 cells, using a polyclonal antibody raised against eGFP.

Figure 5.1: Western blot for eGFP expression in HepG2 cells 3 days after transfection with Ad-Hath1-eGFP (three biological replicates labeled Ad-eGFP 1, 2 & 3).

From Figure 5.1, a band at ~27kDa was observed in all lanes representing the eGFP protein, with verification against the control eGFP protein obtained commercially.

Hence the infected cells expressed the eGFP protein of the correct size and the viral vector transgene expression was verified.

5.1.2 Virus titer value

The TCID₅₀ method was used to determine the titer value of the adenovirus. This method was used as it is fast to perform, provide results that are easy to interpret and consistent between tests. The method is based on the development of observable cytopathic effects in HEK293 cells using end-point dilutions to derive the titer value (refer to Chapter 4.12 for details).

Figure 5.2: Schematic of the 96-well plate displaying the results of the TCID₅₀ test. Wells with observable cytopathic effects were indicated with “CPE”.

Using KÄRBER statistical method:

For 100µl of dilution, the titer is $T = 10^{1+d(S-0.5)}$

d = Log 10 of the dilution (= 1 for a ten-fold dilution)

S = the sum of ratios (always starting from the first 10⁻¹ dilution)

$$= 1+1+1+1+1+1+0.1+0+0+0 = 6.1$$

Titer: $T = 10^{1 + 1(6.1 - 0.5)} = 10^{6.6}$ (for 100 μ L aliquot of virus)

$$T = 10^{7.6} \text{ TCID}_{50} / \text{mL}$$

To transform $\text{TCID}_{50} / \text{mL}$ in PFU/mL:

(Based on verification by the manufacturer that the titer as measured by TCID_{50} is 0.7 log higher than the titer by standard plaque assay)

$$T = 1 \times 10^{7.6} \text{ TCID}_{50} / \text{mL}$$

$$= 1 \times 10^{7.6 - 0.7} \text{ PFU} / \text{mL}$$

$$= 1 \times 10^{6.9} \text{ PFU} / \text{mL}$$

or $= 0.8 \times 10^7 \text{ PFU/mL}$

Table 5.1: TCID_{50} test carried out in triplicate.

Test	S	T / TCID_{50} per mL	T / $\times 10^7$ PFU per mL
1	6.1	$10^{7.6}$	0.8
2	6.2	$10^{7.7}$	1.0
3	6.1	$10^{7.6}$	0.8
Average Titer Value			$0.87 \times 10^7 \text{ PFU/mL}$

Hence the average titer value of the virus used in subsequent *in vivo* experiments (Chapter 5.2 – 5.4) is $0.87 \times 10^7 \text{ PFU/mL}$.

5.2 Gelfoam

Successful delivery using the Gelfoam method involves diffusion of the vector transgene complex across an intact round window membrane. This is carried out by mere surgical placement of a vector solution-soaked biodegradable gelatin sponge on the round window membrane. Compared to many other implantation and infusion methods, the Gelfoam method is considered non-invasive with minimal surgical manipulation and results in minimal inner ear damage. If gene transfer is achieved successfully, this method presents a more clinically viable route for cochlear gene delivery compared to any other existing delivery methods.

The Gelfoam method was first employed (refer to Chapter 4.3.1 for details) to verify the RWM's permeability to the vector solution. No signs of transfection were observed within the various regions in the cochleae including the organ of Corti, stria vascularis and spiral ganglion neurons (Figure 5.3A). Closer observations on the RWM also displayed no signs of transfection (Figure 5.3B). Based on current observations, the vector solution was unable to diffuse across the RWM to transfect the cells within the cochlea.

Figure 5.3: Confocal images showing the (A) OOC and the (B) round window membrane with no signs of transfection observed. Scale bar in B = 100µm.

The inability of the vector solution to penetrate the RWM for successful transfection of cells within the cochleae was in line with results reported by Suzuki and coworkers where transfection across the RWM in the guinea pig was only possible when the RWM was permeabilized by a local anesthetic solution containing phenol [55]. Difficulty in penetrating the RWM could be the resultant effect of (1) the defensive structure of the RWM where the outer epithelium consist of interdigitating cell boundary and tight junctional complex [168]; (2) the size of the vector particles and (3) the lack of intimate contact between the Gelfoam and the RWM. On the contrary, in another study by Lalwani and co-workers, successful gene transfer was achieved across an intact RWM in a mouse [41]. This suggest that factors such as the animal model, surgical procedures, and type of vectors used also play an important role in determining the RWM's permeability to the applied vector solution. If transfection via an intact RWM using a non-viral vector could be achieved, this technique would definitely prove to be a safer and clinically viable

route of delivery in humans as compared to current invasive delivery methods used in inner ear gene delivery studies.

5.3 RWM inoculation

In order to determine if the RWM was the barrier to successful transfection within the cochlea *in vivo*, the virus vector solution was injected directly across the RWM (refer to Chapter 4.3.2 for details). One out of five injected cochleae showed transfection in every turn (Figure 5.4A), while the rest showed transfection in the lower turns and weaker transfection in the upper turns (Figure 5.4B). Among the successfully transfected cochleae, GFP fluorescence was restricted to the perilymphatic fluid space and occasionally to the Reissner's membrane. Cells that were preferentially transfected include the fibrocytes lining the scala vestibuli and scala tympani, mesenchymal and epithelial cells of the Reissner membrane, fibrocytes in the supratricle zone of the spiral ligament, mesothelial cells of the basilar membrane, Hensen's cells, Deiters' cells, inner and outer hair cells (Figure 5.4A & B). No GFP fluorescence was observed in the spiral ganglion neurons and stria vascularis.

Figure 5.4: Fluorescence and corresponding overlay images of cryosections of transfected cochleae 3 days after RWM inoculation of Ad-eGFP. (A) Transfection observed in every turn (all the way to the apical turn), (B) Transfection observed in the basal and middle turn. SV: Scala Vestibuli, SM: Scala Media, ST: Scala Tympani.

RWM inoculation resulted in a gradient of transfection along the scala tympani after inoculation. This is likely the result of vector solution backflow from the damaged RWM and the longitudinal concentration gradient of the vector solution along the scala

tympani after inoculation [169, 170]. The failure to retain the injected viral vector solution resulted in insufficient number of the viral particles reaching the apical turns, leading to transfection only at the lower turns. Another possible reason for the varied transfection results obtained could be the effect of the time lapse and force used during the injection process. This could be improved by using an injector system to bring about a lower force and longer time of inoculation. However, more importantly, the varied transfection results confirmed the longitudinal concentration gradient of the vector particles along the scala tympani after inoculation. Besides the method of delivery, particle diffusion rate, particle clearance rate from the perilymph and perilymph flow rate could also contribute to different rates of transfection along the cochlear turns. The guinea pig's RWM is approximately 1 – 1.5 mm in diameter [171], hence RWM inoculation should be carried out at dimensions preferably 0.2 mm diameter and below. Ideally, the aperture created should be minimal in order to retain the vector solution by surface tension but large enough for vector particle passage without causing significant damage to the particles. Other measures include the use of a micromanipulator and injector system which allows greater precision in achieving a low inoculation rate ($< 0.5 \mu\text{l/min}$) at a consistent inoculation force.

5.4 Cochleostomy

Since it is difficult to seal the aperture made in the RWM (to prevent backflow of vector solution) without risking further damage to the delicate membrane, cochleostomy was investigated (refer to Chapter 4.3.3 for details). Delivery via cochleostomy allows sealing of the orifice created on the cochlea bony shell after inoculation and minimized excessive backflow upon needle retraction.

Successful and consistent transfection was obtained in all cochlear turns of all subjects in the experimental batch. On inspection of the surface mount preparations and cryosections, transfection was restricted to the lining of the perilymphatic fluid space. Cells that were preferentially transfected include the fibrocytes lining the scala vestibuli and scala tympani, mesenchymal and epithelial cells of the Reissner membrane, fibrocytes in the suprachiasmatic zone of the spiral ligament (Figure 5.5 & 5.6). The endolymphatic fluid space, including the organ of Corti and stria vascularis were not transfected. However, transfection efficiency is the highest compared to the previous two methods.

Figure 5.5: Fluorescence and corresponding overlay images of surface mounts (plan view of basilar membrane) of cochleae 3 days after administration of Ad-eGFP by

cochleostomy. GFP fluorescence observed concentrated near the spiral lamina regions.

BsM: Basilar Membrane, SLa: Spiral Lamina.

Figure 5.6: Fluorescence and corresponding overlay images of cryosections of cochleae 3 days after administration of Ad-eGFP by cochleostomy. Widespread transfection restricted within the walls of the perilymphatic fluid spaces. (SV: Scala Vestibuli, SM: Scala Media, ST: Scala Tympani)

With the same amount of vector solution used, transfection efficiencies differ significantly according to the method of delivery employed. Based on results from all cochleae that were successfully transfected, no qualitative differences in the cell types being transfected were observed. Majority of the transfected cells were fibrocytes lining the perilymphatic compartments, spiral ligament and epithelial cells on the Reissner membrane. The rank order of the various methods of delivery based on ease of transfection is as follows, Cochleostomy > RWM inoculation > Gelfoam method. Therefore, in order to maximize non-viral vectors' transfection capacity and compensate for its inherent low transfection efficiency, the choice of delivery method used is important.

CHAPTER 6: POLYETHYLENIMINE DELIVERY

Polyethylenimine (PEI), a cationic polymer well known as a superior non-viral vector for gene transfection is often regarded as the gold standard in non-viral gene delivery. It possesses both strong DNA compaction capacity and high endosomolytic competence which aids in endosomal escape and translocation of DNA to the nucleus. Owing to its superior transfection properties as a non-viral vector, PEI was selected as the non-viral vector for our cochlea gene delivery studies.

6.1 *In vitro*

6.1.1 DNA transgene expression

After receiving the plasmid (pRK5-eGFP) from Genentech Inc., transfection was carried out on HepG2 cells with PEI complexation. Subsequently, the faithfulness of the plasmid was assessed by Western Blot analysis of the cell lysate from PEI transfected HepG2 cells, using a polyclonal antibody raised against eGFP.

Figure 6.1: Western blot for eGFP expression in HepG2 cells 3 days after transfection with pRK5-eGFP (complexed with PEI) (two biological replicates labeled PEI-eGFP 1 & 2).

From Figure 6.1, a band at ~27kDa was observed in all lanes representing the eGFP protein, with verification against the control eGFP protein obtained commercially. Hence the transfected cells expressed the eGFP protein of the correct size and the plasmid transgene expression was verified.

6.1.2 Transfection with PEI (HepG2 cells)

HepG2 (human liver carcinoma cell line) cells were initially used to test the transfection capability of PEI. N/P ratio represents the ratio of PEI nitrogen to DNA phosphate within the PEI-DNA complex transfecting unit. It determines the transfection activity of the vector complex and has a direct impact on the transfection efficiencies. Excessively high N/P ratio is associated with cytotoxicity and excessively low N/P ratio results in incomplete compaction and formation of the complex unit. Hence, the transfection efficiency of PEI at various N/P ratios was assessed to determine the appropriate N/P ratio to be used in subsequent experiments.

GFP⁺ cells were observed (Figure 6.2A) in all experimental wells with PEI-DNA complex added irregardless of N/P ratios used. Percentage of cells transfected varied with N/P ratio and results were quantified by flow cytometry analysis (Figure 6.2B). From N/P ratio 2 to 6, the percentage of GFP transfected cells increased gradually and significantly. At an N/P ratio of 8, the percentage of cells transfected appeared to decrease slightly though the drop was not of statistical significance.

*Figure 6.2: In vitro PEI transfection (A) Transfection of HepG2 cells with PEI-eGFP at N/P ratio = 6 after 3 days (Original optical magnification x 200) (A') Vector transfected control cells under the same exposure demonstrating minima background signal. (B) Quantification of percentage of cells transfected at various N/P ratios using flow cytometry. (** $P < 0.005$, * $P < 0.05$)*

Hence PEI transfected the HepG2 cells efficiently and acts as an effective non-viral gene delivery vector in this cell line.

6.1.3 Primary cell culture of sensory epithelia

Currently, there is yet a representative *in vitro* model or cell line available for cochlea gene transfection studies. Several groups reported the isolation and culture of several primary inner ear cell types such as Marginal and Claudius cells [172], stria vascularis [173] and sensory epithelia [166] from mature animals. Depending on the target of gene delivery, these primary cultures isolated from the cochlea could be useful as an *in vitro* model to assess various vectors' transfection efficiency prior to *in vivo* tests. This produces better correlated *in vitro* and *in vivo* results and also reduces the number of animal subjects required for tests. For our purpose, a dissociated cochlear cell culture was derived from the guinea pig sensory epithelia (Figure 5.3) to assess PEI's transfection efficiency *in vitro* (refer to Chapter 4.4 for details). Figure 6.3A shows the cochlea cells derived from the dissected sensory epithelia one day after seeding of the disaggregated epithelia. Epithelial-like cells could be seen growing and proliferating from the disaggregated epithelia. Figure 6.3B shows the cells at 3rd passage approximately two weeks after initial seeding.

Figure 6.3: Cochlea cells in dissociated culture (A) the next day after seeding and (B) at 3rd passage. Cells display epithelial-like morphology (Original optical magnification $\times$ 200).

6.1.4 Immunostaining for cochlea markers

The cells that were derived from the primary culture were immunostained with antibodies specific to cochlea supporting cells markers and shown to express three supporting cell markers namely Jagged1, Cytokeratin and S100A1 (Figure 6.4A, B, C).

Figure 6.4: Primary cells from dissociated cochlea cell culture were immunolabeled with cochlea supporting cell markers, namely (A) Cytokeratin, (B) Jagged1 and (C) S100A1. Scale bar = 50 μ m

6.1.5 Transfection with PEI (primary cells)

The cells isolated were then used to assess the transfection efficiencies at various N/P ratios to select the optimum N/P ratio for subsequent *in vivo* experiments. The transfection efficiency trend with varying N/P ratios was similar to that when HepG2 cells

were used as test subjects. However, this time, the absolute number of cells transfected was much lower in the range of less than 10% of the total cells available. Similar results were also obtained previously by Ito and coworkers where transfection results of various tumor and normal cells were investigated [174].

A steady increase in cells transfected was also observed from N/P ratio 2 to 6 as in previous transfection experiments using the HepG2 cell line. There was no significant increase in transfection efficiency observed between N/P ratio 6 and 8 (Figure 6.5B). Hence in view of the possible cytotoxic effects *in vivo* with increasing amount of PEI used, an N/P ratio of 6 was employed in subsequent *in vivo* experiments.

Figure 6.5: In vitro PEI transfection (A) Transfection of primary cochlea cells with PEI-eGFP at N/P ratio = 6 after 3 days (Original optical magnification x 200). (B) Quantification of percentage of cells transfected at various N/P ratios using flow cytometry. ($P < 0.001$)(The primary cochlea cells used were isolated and cultured from the primary culture of dissociated cochlea sensory epithelia obtained previously in Chapter 6.1.3)*

6.2 *In vivo*

6.2.1 Cochleostomy

Among the three methods of delivery (Gelfoam, RWM inoculation and cochleostomy) assessed previously with the viral vector, cochleostomy was the most effective based on the transfection efficiency observed. Hence to assess the transfection efficiency of PEI, cochleostomy was used as the initial method of delivery.

Only three out of 5 injected cochleae showed signs of transfection and lower transfection efficiencies were observed compared to the previous viral transfection as expected. On inspection of surface mount preparations and cryosections, few transfected cells were observed and transfected cells were mostly mesenchymal cells lining the perilymphatic fluid spaces (Figure 6.6 & 6.7).

Figure 6.6: Surface mounts (plan view of basilar membrane) of cochleae 3 days after administration of PEI-eGFP by cochleostomy. Minimal transfection observed on the lateral side of basilar membrane. On inspection of this image with corresponding cryosections images in Figure 6.7, the transfected cells shown in Figure 6.6 are identified as mesenchymal cells lining the perilymphatic spaces.

Figure 6.7: PEI-eGFP transfected cochlea 3 days after administration of complex solution by cochleostomy. Representative fluorescence confocal images of cryosections (A) and plan view of surface mounts of basilar membrane (B). (A, B) Sparse and weak transfection observed in the various cochlear turns and transfection restricted to the perilymphatic fluid spaces. (Arrowhead: Transfected cells on the lining of perilymphatic fluid spaces) (darker regions of the surface mounts depict the spiral lamina regions, lighter region with membranous-like morphology depict the basilar membrane) SV: Scala Vestibuli, SM: Scala Media, ST: Scala Tympani.

6.2.2 Osmotic Pump

In order to compensate for PEI's lower transfection efficiency compared to viral vectors, sustained delivery using an osmotic pump via cochleostomy was employed. The osmotic pump delivered the vector solution at 0.5 $\mu\text{l/hr}$ over a period of one week, providing a constant supply of vector particles into the cochlear fluid spaces.

At the end of one week, all 5 guinea pigs showed improved transfection efficiency compared to the single inoculation of PEI-eGFP via cochleostomy (Figure 6.8 & 6.9). Cells which are preferentially targeted include the fibrocytes lining the scala vestibuli and scala tympani, mesenchymal and epithelial cells of Reissner's membrane and fibrocytes in the supracribral zone of the spiral ligament (Figure 6.9, 6.10). No transfection was observed in the organ of Corti and stria vascularis. In addition, absence of T lymphocyte labeling at various regions within the infused cochleae, including the spiral ganglion cells and the organ of Corti indicates minimal inflammatory response.

Figure 6.8(A – C): Surface mounts (plan view of basilar membrane) of cochleae after 1 week sustained release of PEI-eGFP by osmotic pump. Improvement in transfection efficiency compared to a single inoculation of PEI-eGFP vector solution with transfection observed near the limbus and spiral lamina regions (darker regions of the surface mounts depict the spiral lamina regions, lighter region with membranous-like morphology depict the basilar membrane) On inspection of this image with corresponding cryosections images in Figure 6.9-10, the transfected cells shown in Figure 6.8 are identified as mesenchymal cells lining the perilymphatic spaces.

Figure 6.9: Cryosections of cochleae after 1 week sustained release of PEI-eGFP by osmotic pump. Improvement in transfection efficiency with transfection observed on the lining of the perilymphatic fluid spaces. (SV: Scala Vestibuli, SM: Scala Media, ST: Scala Tympani)

Figure 6.10: Cryosections of cochleae after 1 week sustained release of PEI-eGFP by osmotic pump. Regions of transfection restricted to regions outside the scala media. (Double arrow: Suprastrial zone of the spiral ligament, Arrowhead: Scala Vestibuli lining, Arrow: Reissner's Membrane, SM: Scala Media, SV: Scala Vestibuli) Scale bar = 100 μ m

Our results displayed GFP expression restricted to the perilymphatic fluid spaces in both sets of experiments where Ad-eGFP (Figure 5.6) and PEI-eGFP (Figure 6.9, 6.10) were evaluated and is consistent with previous gene delivery models reported [57, 64, 66]. This is likely due to vector inoculation via the scala tympani which only exposes the walls of the perilymphatic fluid spaces and the basolateral domain of the organ of Corti to the vector particles. This excluded the spiral ligament and the apical surface of the organ of Corti cells. Therefore, it appears that contact between the vector particles inoculated and regions of transfection are particularly important in obtaining successful transfection within the scala media as there is little or no dissemination of vector particles from the perilymphatic into the endolymphatic fluid space after inoculation. This is especially true

for non-viral PEI or cationic liposomes - DNA complexes (~ 300 – 600 nm) which tend to be larger than viral vectors particles such as adenovirus and adeno-associated virus (< 100 nm). In order to increase the accessibility of vector particles to the organ of Corti and spiral ligament within the scala media, inoculation of the vector solution into the scala media leading to the endolymphatic fluid space is critical. However, locating the scala media from the exterior of the cochlea bony shell or the RWM is challenging due to the relatively small size of the scala media even in the guinea pig model. Two possible techniques reported include vector inoculation into the endolymphatic sac [56] or cochleostomy through the fenestra via a microcannula reaching the scala media [54]. Targeting the scala media for vector inoculation would aid the transfection of cells in the organ of Corti and also reduces the amount of vector solution required, thus improving efficiency and reducing possible cytotoxicity. However, limited transfection in the cochlea apical turns and hair cell loss associated with scala media inoculation are two major drawbacks which may limit its future clinical application.

The difference in transfection efficiencies among the various delivery methods and vectors were apparent. Hence, to estimate the effectiveness of transgene expression among various experimental groups, we employed a semi-quantitative score system to compare the distribution as well as the relative intensity of transfection in each animal. Table 5.1 summarizes the relative transfection efficiencies of the various experimental groups.

Table 6.1: Transgene expression scores for each animal

	Subject	Transfected Turn		
	Number	(Transfection scores on a scale of 3)		
		Apex	Middle	Basal
Adenovirus				
Gelfoam	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
	5	-	-	-
RWM inoculation	1	###	###	###
	2	-	#	##
	3	-	##	##
	4	-	##	###
	5	-	#	###
Cochleostomy	1	###	##	##
	2	##	##	###
	3	###	###	###
	4	###	##	###
	5	##	###	##
PEI				
Cochleostomy	1	-	#	#
	2	-	#	-
	3	-	-	-
	4	#	-	#
	5	-	-	-
Osmotic pump infusion	1	##	##	##
	2	#	##	#
	3	#	##	##
	4	##	##	#
	5	##	##	#

- No transfected cells observed

Few transfected cells observed

Number of transfected cells between # and

Numerous transfected cells

Adenoviral-mediated gene transfer has been extensively studied by various research groups over the years [54, 59, 61, 175]. The sole purpose of including the adenoviral vector in our study was only to exploit its high transfection efficiency to discern the relative transfection efficiencies of the various methods of delivery. Hence, allowing us to select the most efficient route of delivery for PEI. In addition, it was apparent that sustained release of the adenovirus over a week will yield higher transfection efficiency than the single inoculation of the virus vector. Hence with the high transfection efficiency of the adenovirus and toxicity associated with a large dose of adenovirus using the osmotic pump infusion method, the method was not assessed with the adenovirus. In our pilot study, PEI-mediated gene transfer was already evaluated via the Gelfoam and RWM inoculation method. However, due to the innate lower transfection efficiency of PEI compared to the adenovirus and limitations of the two delivery methods as discussed earlier, expectedly negative results was obtained (results not included in Table 5.1).

Clearly, non-viral vectors' lower transfection efficiency limits its potential in competing with its viral counterparts. Besides the selection of vector used for inner ear gene delivery, the vectors' route of delivery into the cochlea also plays a role in determining gene transfer efficiency. Based on our series of experiments involving the various routes of delivery and results reported by others [57, 176], the delivery methods assessed could be ranked according to transfection efficiency, with the osmotic pump method being the top of the list, followed by cochleostomy, RWM inoculation and lastly, the Gelfoam method (delivery through intact RWM), being the least invasive and the least effective [see also 57]. In non-viral vector gene delivery studies, the route of delivery is

especially important due to its generally lower transfection efficiency, and selection of an ideal delivery method could compensate for such a disadvantage. In our experiments, prolonged and sustained delivery using an osmotic pump improved the transfection efficiency of PEI and presents a possible way to augment the transfection efficiency of non-viral vectors gene transfer in the cochlea. In addition, the osmotic pump method enables the testing of candidate gene delivery vectors (especially non-viral vectors) that require relatively larger volumes and cannot be assessed by other delivery methods. This provides more opportunities in the screening of various candidate vectors and giving us a better idea of the potency of these vectors in terms of transfection efficiency and cytotoxicity.

The high density of positive charges in PEI enables the tight compaction of DNA into DNA-PEI complex particles of nanometer range, which aids in endocytosis. However, high PEI nitrogen to DNA phosphate (N/P) ratio also results in cellular toxicity, possibly due to high levels of free PEI present in the system [177]. Hence, due to PEI's potential cytotoxicity [178, 179], particular attention was made in observing the cellular structure of the inoculated cochlea to check for any signs of extensive cell deaths and other cellular abnormalities within the cochlea. During surgical implantation of the catheter, an orifice was made on the cochlea bony shell. Regions in the vicinity of the orifice (inoculation site) underwent mechanical trauma, and cytostructural damage was observed near the site of catheter implantation and cochleostomy. As distance increase from the inoculation site towards the extreme apical and basal turns, the cochlea displayed intact cellular and tissue cytoarchitecture with no pathological changes. Transfection results also revealed that GFP transfected cells were observed in the various turns of the cochlea and not only restricted

to regions near the catheter implantation site. This demonstrates that the vector particles were well-distributed within the cochlea across the various turns and not restricted only to the vicinity of the implantation site. Hence, the cytostructural damage observed near the implantation site is likely due to mechanical trauma during catheter implantation and surgical manipulations, rather than the toxicity of the vector particles itself. In addition, previous *in vivo* studies conducted by several groups involving viral gene delivery vectors, revealed apparent lymphocytic infiltration within the cochlea after vector administration [59, 60]. In these studies, the influx of lymphocytic cells within the infused cochlea reveals an apparent inflammatory response elicited by these viral vectors. In our experimental setup, no cellular infiltration was observed in the infused cochlea and there was absence of positive T lymphocytes staining at various regions across all turns, including the organ of Corti and spiral ganglion cells. Hence, with the current experimental dosage administered, there was minimal cytotoxic effect and inflammatory response within the infused cochlea. Also, the dosage administered is well-tolerated in the infused cochlea with absence of detrimental effects observed in the cochlea during analysis.

In this study, the effects of PEI-mediated gene transfer on ABR response was not evaluated; however, the effects of sustained delivery using an osmotic pump on hearing have been investigated extensively by several groups. Carvalho and coworkers conducted ABR studies on the osmotic pump infusion method and demonstrated ABR threshold preservation at low frequencies. Although elevated ABR thresholds in the mid and high frequency due to surgical trauma was observed, one long term animal did show significant recovery of thresholds after a month [180]. Luebke and coworkers monitored the

distortion product otoacoustic emissions measurements throughout the infusion period of an adenoviral vector and found no loss in cochlear function [37, 181]. Hence based on these previous findings, combining with the minimal cytotoxicity and inflammatory response found in our study, we believe the infusion period of the PEI vector solution via the osmotic pump will be well tolerated with minimal functional compromise on hearing.

In our experiments, it was also noted that proximity to the site of inoculation does not necessarily lead to greater amount of transfection as transfected regions was observed across the various turns in the cochlea. No significant difference in the number of transfected sites was observed among the various turns in the cochlea. This is likely due to (1) the position of the catheter implantation site (cochlea middle turn), (2) long and steady period of time for vector inoculation by the osmotic pump and (3) relatively efficient circulation of the perilymphatic fluids in the cochlea which resulted in efficient diffusion and propagation of inoculated vector particles throughout the cochlea turns.

Results obtained by previous studies using adenoviral vectors indicated greater susceptibility of transfection in a deafened cochlea [57, 182]. The reason and mechanism for such a trend is unclear. However one possible explanation could be an increase in tissue permeability due to kanamycin (administered for deafening) - induced trauma that led to a higher DNA intake. Alternatively, in non-viral gene delivery, kanamycin, a natural polyamine known to bind with nucleic acid could have interacted with “stray” DNA released in the system. Such interaction resulted in greater DNA uptake due to increased transport and nuclease activity protection, hence, aided the overall transfection efficiency [183, 184]. Subsequent tests could be carried out to investigate if this trend is vector or trauma dependent.

PEI is less efficient in delivering DNA and initiating gene expression when compared to viral vectors, particularly in cochlea gene delivery studies. However, strategies to improve transgene expression levels that match, if not, come close to levels observed in viral vectors could be employed. One such strategy as shown in this study is the choice of delivery method. Since PEIs are polymers, they provide great flexibility and ease in altering their structure to improve transfection efficiency. Some strategies previously reported include conjugating groups to improve stability [185, 186] or incorporating receptor specific ligands to target receptors on the cell surface [187-189]. In addition, PEI can be produced at almost any quantity in large scale polymerization reactors, and purification is easy and inexpensive compared to that of viruses. This is important since large quantities, high reproducibility and acceptable cost are required for direct clinical applications. PEI and DNA may form complexes which include more than one plasmid or DNA of virtually any size; however, this is limited by aggregation as concentration increases. Non-viral gene delivery systems at therapeutic doses require high concentrations of polymer-DNA complexes; hence, preparation protocol and conditions such as pH, ionic strength and temperature should be empirically controlled and stabilization measures implemented for extended circulation times [190, 191]. Due to compact complex formation, PEI works well for cell uptake and nuclease protection; however, dissociation is difficult and poses difficulties in releasing the gene material within the cytosol. These two opposing phenomena could be resolved with the introduction of pH or temperature sensitive groups onto polymer chains to produce environmental sensitive polymers capable of targeted delivery and intracellular trafficking of DNA at the releasing stage [192, 193].

In summary, we demonstrated successful PEI-mediated gene transfer in the cochlea and displayed improved transfection efficiency with sustained release of the vector solution. This was achieved in the absence of inflammation and maintenance of intact cellular architecture within the infused cochlea. Currently, no ideal vector exists in cochlea gene therapy and these findings present a novel alternative for non-viral gene delivery vector in cochlea gene transfer.

CHAPTER 7: BONE MARROW-DERIVED CELL MIGRATION IN DEAFENED ANIMAL MODEL

Sensory hair cell loss is generally considered irreversible in the mammalian inner ear [194]. Unlike in birds and amphibians [195, 196], supporting cells (i.e. Deiters' cells) [197] in mammals are unable to reconstitute the degenerated tissues hence resulting in permanent functional deterioration. The high degree of bone marrow stem cell plasticity [118] and BMDC transdifferentiation have been reported in several injured organ models, including the heart, brain, muscle and liver [119-127]. In inner ear studies, Lang and coworkers provided the first evidence for the origin of inner ear cells from the bone marrow, specifically from HSCs and suggested that mesenchymal cells including fibrocytes in the adult inner ear may be derived continuously from HSCs [198]. Yoshida and coworkers made use of HSCs to prevent hair cell death and to ameliorate hearing impairment after transient cochlea ischemia [99]. Iwai and coworkers employed bone marrow transplantation (BMT) to replace abnormal HSCs with normal HSCs to treat presbycusis [199]. MSCs from the bone marrow has been shown to differentiate into inner ear hair cells *in vitro* [200] and transplantation aided cochlea fibrocyte recovery in a deafened rat model [102]. Taken together, these studies highlight the potential for bone marrow derived stem cells (BMDSCs) in the treatment of hearing loss. Several inner ear cell therapy studies involve the direct injection and transplantation of embryonic [81, 95], neural [85, 86] or mesenchymal [101] stem cells into the cochlea compartments. Results obtained from these studies have so far been encouraging. However, the methods of administration are invasive and may limit future clinical applications.

Hence the motivation of our study was to make use of the host's intrinsic capability and BMDSCs' plasticity to investigate the role of an individual's BMC in the restoration process after deafening. In this study, we investigated the characteristics and distribution of BMDCs in the deafened mouse model, followed by its transdifferentiation potential after acoustic damage.

Figure 7.1 presents the schematic depicting the experiment protocol for this study.

Figure 7.1: Schematic representation of experimental setup (timeline not drawn to scale).

7.1 Hematopoietic reconstitution

To investigate the transdifferentiation potential of BMC in a deafened animal model, we engrafted whole BMC from the green fluorescent protein (GFP) transgenic donor mice into irradiated recipient mice in order not to exclude any kind of stem cells (refer to Chapter 4.6 for details). Transplanting BMCs from GFP transgenic mice allowed us to track and distinguish cells of bone marrow origin from those resident in the recipient mice tissue. We confirmed the hematopoietic reconstitution of transplanted recipient mice using fluorescence microscopy and flow cytometry.

Three months after whole BMT, the cochlea, spleen, peripheral blood and bone marrow were harvested to assess the hematopoietic reconstitution in the recipient mice. Using fluorescence microscopy, BMDC (GFP⁺ cells) were observed in the cochlea confirming the homing ability of transplanted BMCs to migrate and incorporate into the recipient cochlea. Areas of population included the stria vascularis, perilymphatic compartment walls, spiral ligament, limbus, spiral ganglion neurons and modiolus (Figure 7.2A). In addition, a high prevalence of GFP⁺ cells in both the bone marrow regions of the otic capsule (Figure 7.2B) and spleen (Figure 7.2C) were observed. Sham-transplanted mice displayed an absence of GFP⁺ cells in all regions mentioned above.

Figure 7.2: Cryosections of (A) Cochlea (B) Bone Marrow Region in the otic capsule (C) Spleen, from recipient mice 3 months after whole BMT. Resident population of GFP⁺ cell marked by arrowheads in (A). StV: stria vascularis, ST: scala tympani, SL: spiral ligament, L: limbus, SG: spiral ganglion neurons, M: modiolus. Scale bar: 100µm.

Nucleated cells in the peripheral blood and bone marrow were assessed with flow cytometry. In transplanted recipients, an average of 70.5% and 41.0% of nucleated cells in the peripheral blood and bone marrow were GFP⁺. In transgenic donors, an average of 77.5% and 43.2% of nucleated cells in the peripheral blood and bone marrow were GFP⁺ (Figure 7.3). 91.0% and 94.9% chimerism were achieved in the peripheral blood and bone marrow.

Figure 7.3: %GFP⁺ nucleated cells in the blood and bone marrow obtained by FACS analysis. Values in mean $\pm$ SD. WT: Wild Type (20 weeks old), GFP+: GFP transgenic donor (20 weeks old), BMT: Bone Marrow Transplantation recipient (3 months after transplantation).

Hence hematopoietic reconstitution (> 90% chimerism) was achieved and with the replacement of recipient bone marrow with that of donor BMCs, it allowed the identification and tracking of transplanted donor cells in the recipient mice.

7.2 Deafened animal model

A deafened animal model is commonly produced by infusing an aminoglycoside antibiotic (kanamycin, neomycin or other ototoxic drugs) into the cochlea through surgery or subjecting the subjects to prolonged high noise levels. Both methods were carried out and compared to select the method appropriate for our study (please refer to Appendix for procedures and results). Surgical administration of drugs to induce oto-toxicity resulted in a larger response variation in the sample population due to difficulty in ensuring consistency of each deafening surgery. Such consistency is furthered challenged by the varied wound healing response and the possible bacterial infection (additional inflammatory response) arising from surgical manipulations. Acoustic deafening procedures are more easily carried out, less time-consuming, less room for errors and resulted in a more consistent deafening effect among the test subjects within a sample population.

Hence, to study the effects of BMDCs in a deafened animal model, recipient mice were subjected to acoustic deafening (120 dB SPL white noise, 2hrs continuous exposure) three months after BMT (refer to Chapter 4.7 for details). A noise induced hearing loss model was produced in the current experiment where significant elevated ABR thresholds were recorded in the deafened mice compared to control (non-deafened) mice at all defined frequencies ($p < 0.01$) (Figure 7.4).

Figure 7.4: Effect of acoustic deafening on transplanted recipient mice. ABR thresholds of deafened mice at various frequencies up to 8 weeks. Non-deafened mice (open circle). Values in mean $\pm$ SD.

Acoustic deafening occurred with an average threshold shift ranged between 25.5 – 56.0dB at all timepoints across all frequencies (Table 6.1).

Table 7.1: Average ABR threshold shifts at various frequencies and timepoints after acoustic deafening

	Frequency					
Timepoint	4	8	12	16	20	24
Day 0	53.5	46.5	49.5	30.5	37.5	34.0
Day 1	52.7	40.7	45.3	34.7	36.7	30.7
Day 3	56.0	34.0	35.3	28.0	36.7	34.0
Wk 1	52.7	37.3	38.7	34.7	40.0	44.0
Wk 2	56.0	39.0	42.0	30.5	42.5	41.5
Wk 4	56.0	39.0	39.5	25.5	35.0	31.5
Wk 8	56.0	44.0	48.7	28.0	36.7	34.0

Average ABR threshold shifts are calculated by subtracting average ABR thresholds of control subjects from average ABR threshold of all deafened subjects within an experimental group.

No sign of ABR threshold recovery was observed after deafening at all timepoints up to 8 weeks suggesting the deafening conditions were intense enough to produce a permanent threshold shift in the deafened mice.

Phalloidin labeling was also carried out to label the hair cells on the sensory epithelia in both the control and deafened cochlea. In the control cochlea, both the inner and outer hair cells were intact and an orderly outline of three rows of outer hair cells and one row of inner hair cells was observed. The structural arrangements of both the outer and inner hair cells were well-preserved. Whereas, in the deafened cochlea, obvious

destruction of the outer and inner hair cells and lesions in the nearby regions were observed. The orderly structural arrangement of the sensory hair cells was also badly disrupted compared to that in the control cochlea (Figure 7.5). The presence of more subtle type of damages on the hair cells was not possible to evaluate by the microscopic assessment.

Figure 7.5: Representative surface mount preparations of basilar membranes stained with Phalloidin-TRITC. (A) Control cochlea (B) Deafened cochlea 3 days after deafening. Scale bar: 50µm

7.3 GFP⁺ cell infiltration

A bone marrow-derived population of cells (GFP⁺ cells) resides in the inner ear at normal/steady state (i.e. in the non deafened control cochlea) (Figure 7.6C, D). These cells were present in the spiral ligament, perilymphatic compartment walls, basilar membrane, Reissner's membrane, limbus, spiral ganglion neurons and modiolus regions (Figure 7.6D). No GFP⁺ cells were found in the sensory epithelial i.e. organ of Corti. Regions of population observed were similar to those obtained in previous models [198, 201-203].

In the deafened cochleae, the number of GFP⁺ cells observed increased significantly (Figure 7.6A, B) as compared to the control (non-deafened) cochleae (Figure 7.6C, D). Acoustic deafening resulted in significant infiltration and accumulation of GFP⁺ cells in most regions within the deafened cochlea (Figure 7.7). Areas with marked increase of GFP⁺ cells included the spiral ligament, perilymphatic compartment walls, limbus, spiral ganglion and modiolus. Negative staining obtained from BrdU immunohistochemistry (results not shown) indicated that the increase in BMDCs was a result of BMDC migration rather than proliferation. This is further supported by previous report by Okano and coworkers where Ki67 immunohistochemistry revealed no significant statistical difference between deafened and non-deafened groups [202].

Figure 7.6: Cryosections of (A, B) deafened cochlea and (C, D) control (non-deafened), 3 days after acoustic deafening. Significant migration of GFP⁺ cells in deafened cochlea compared to control cochlea. SL: Spiral ligament, L: Limbus, SG: Spiral Ganglion, M: Modiolus, SV: Scala Vestibuli, SM: Scala Media, ST: Scala Tympani. Scale bar: 200 μ m

Figure 7.7: Zoom-in images of various regions within (A) control and (B) deafened cochleae, 3 days after acoustic deafening. Significant migration of GFP⁺ cells in various regions within the deafened cochlea compared to the control. (1) Spiral ligament, (2) Limbus, (3) Spiral ganglion neuron region, (4) Modiolus. Scale bar: 100μm

Quantitative assessment of GFP⁺ cell infiltration was carried out by GFP⁺ cell counting and GFP relative fluorescence density calculation (Figure 7.8) (refer to Chapter 4.15.3 for details).

Generally, GFP⁺ cell infiltration was most prominent during the first week after deafening, increasing from Day 0 (day of deafening) to Day 3, followed by a decrease at Week 1. Similar trends were observed in most regions in the cochlea except the stria vascularis and organ of Corti where GFP⁺ cell infiltration is minimal. GFP⁺ cell migration is most prominent in the spiral ligament, limbus and perilymphatic compartment walls with more than five fold increase compared to the control group whereas the spiral ganglion, modiolus, Reissner's membrane and basilar membrane showed a 2 to 5 fold increase compared to the control group.

Figure 7.8: Quantitative data of GFP⁺ cell infiltration over time at various regions in the cochlea. SL: Spiral ligament, L: Limbus, SG: Spiral Ganglion, M: Modiolus, Pwall: Perilymphatic compartment walls, StV: Stria Vascularis, RM: Reissner's membrane, BsM: Basilar Membrane, OOC: Organ of Corti. *p < 0.01, **p < 0.001 compared with control.

During the initial post deafening period (Day 0 and 1), GFP⁺ cells in the spiral ligament were observed mainly in the Type IV fibrocytes and from Day 3 onwards, they were found throughout the whole spiral ligament (Figure 7.9). Loss of fibrocytic cells at the spiral ligament and limbus were also observed in the deafened cochleae.

Figure 7.9(A – H): GFP⁺ cell infiltration in the spiral ligament from day 0 to week 8 after deafening. Scale bar: 100 μ m.

In our study, significant donor derived leukocyte infiltration was observed after acoustic trauma during the early post-deafening period at Day 0 and 1 (Figure 7.8). Previous reports also demonstrated the early nature (2h post-exposure) of chemokine expression (MCP-1, MCP-5, MIP-1b) suggesting that the resident cochlea cells rather than the non resident inflammatory cells are responsible for the upregulation [204]. Hence inflammatory response in the deafened cochlea triggered the secretion of a number of factors that act on the bone marrow, causing the release of large number of BMDCs into

the circulation that migrate to the site of injury [204-206]. Previous reports highlighted the lateral wall, specifically the spiral ligament fibrocytes involvement in producing chemoattractants for recruiting inflammatory cells [206, 207]. In Fujioka and coworkers' deafened cochlea model, IL6 immunoreactivity was observed initially in and around the Type IV fibrocytes and then expanded diffusely and broadly towards the stria cells [205]. In our model, we observed intense GFP⁺ cell infiltration at the spiral ligament with initial accumulation at the lower regions of the spiral ligament, followed by migration to the whole spiral ligament over time (Figure 7.9). Taken together, the spiral ligament, specifically the Type IV fibrocytes are likely to be the initiator of the local inflammatory response to acoustic trauma in the cochlea. Previously, the main entry route for cells participating in inner ear inflammatory process was thought to be the spiral modiolar vein [208]. However, with the greater extent of leukocyte infiltration at the spiral ligament compared to the modiolus regions (Figure 7.8), the highly vascularised nature of the lateral wall and cochlea lateral wall fibrocytes being the initiator of local inflammatory response [206, 207], it further supports the notion that the main route of entry of cells participating in inner ear inflammatory process is through the vasculature at the lateral wall comprising of the stria vascularis and spiral ligament [201].

7.4 Characterization of BMDC

To elucidate the nature of the infiltrated BMDCs, immunohistochemistry was carried out to identify the GFP⁺ cell lineage. Most of the GFP⁺ cells were identified as leukocytes by positive labeling with the pan-leukocyte marker CD45 and approximately 95% of the GFP⁺ cells were positively labeled. Figure 7.10 – 7.12 shows the colocalization of CD45 with the GFP⁺ cells. (Figure 7.10 – spiral ligament, Figure 7.11 – limbus and spiral ganglion region, Figure 7.12 – perilymphatic compartment wall).

7.4.1 CD45

In the deafened cochlea, the spiral ligament (Figure 7.10) and perilymphatic compartment walls (Figure 7.12) contained the most CD45 labeling, coinciding with the significant accumulation of GFP⁺ cells. GFP⁺/CD45⁺ cells were observed up to 8 weeks after deafening. Although most BMDCs in the deafened cochleae are GFP⁺/CD45⁺, there was a subpopulation of GFP⁺/CD45⁻ cells in both the deafened and undisturbed cochleae which indicate a resident population of non-leukocyte BMDCs. Abundant CD45⁺ labeling was also observed in the bone marrow regions in the otic capsule (Figure 7.13).

Figure 7.10: CD45 immunohistochemistry in spiral ligament 3 days after deafening. Majority of GFP⁺ cells colocalized with CD45 labeling. SV: Scala Vestibuli, SM: Scala Media, ST: Scala Tympani, SL: Spiral Ligament, Scale bar: 100 μ m. (supplementary magenta-green image available in Appendix II, Figure IIIC)

Figure 7.11: CD45 immunohistochemistry in limbus and spiral ganglion neuron regions 3 days after deafening. Majority of GFP⁺ cells colocalized with CD45 labeling. SV: Scala Vestibuli, SM: Scala Media, L: Limbus, ST: Scala Tympani, SG: Spiral Ganglion, Scale bar: 100µm. (supplementary magenta-green image available in Appendix II, Figure IIIA)

Figure 7.12: CD45 immunohistochemistry in perilymphatic compartment wall 3 days after deafening. Majority of GFP⁺ cells colocalized with CD45 labeling. SV: Scala Vestibuli, Scale bar: 100 μ m. (supplementary magenta-green image available in Appendix II, Figure IIIB)

Figure 7.13: CD45 immunohistochemistry in bone marrow region in the otic capsule. Majority of GFP⁺ cells colocalized with CD45 labeling. Scale bar: 100μm.

7.4.2 CD68

To further characterize the bone marrow derived leukocytes, we carried out CD68 immunohistochemistry. Labeling with CD68 yielded results similar to those observed with CD45 labeling where most GFP⁺ cells were CD68⁺ (Figure 7.14) hence identifying the BMDCs as macrophages. The bone marrow regions in the otic capsule also contain abundant GFP⁺/CD68⁺ cells (Figure 7.15).

Figure 7.14: CD68 immunohistochemistry in the cochlea 1 day after deafening. Majority of the GFP^{+} cells colocalized with CD68 labeling. (A – C) Low magnification view of CD68 labeling at the lateral wall region, (B') High magnification view of the suprastrial zone of the spiral ligament and the perilymphatic compartment wall, (C') High

magnification view of Type IV fibrocytes in the spiral ligament. SV: Scala Vestibuli, SM: Scala Media, SL: Scala Ligament, ST: Scala Tympani, Scale bar: 100µm. (supplementary magenta-green image available in Appendix II, Figure IV)

Figure 7.15: CD68 immunohistochemistry in bone marrow region in the otic capsule. Majority of GFP⁺ cells colocalized with CD68 labeling. Scale bar: 100µm.

Immunohistochemical analysis identified the majority of BMDCs as leukocytes by their positive staining with the pan-leukocyte marker, CD45. Further characterization identified these cells primarily as macrophages. Our results are in agreement to those reported previously where a significant migration of inflammatory cells was observed especially at the spiral ligament and the fluid-filled spaces of the scala tympani and scala vestibuli [201, 202, 204, 209]. Beside the deafened cochlea, the homing and accumulation of BMDCs has also been displayed in injured organs such as the retinal [133], brain [154], kidney [144], pancreas [149] and heart [143]. In some cases, contribution to the healing process was observed [126, 127, 132, 133] where BMDCs promoted regeneration and contributed to repair at the injury site, whereas in other cases, no healing was observed [143-145, 149, 154]. GFP⁺ cells that were not stained positively with CD45 or CD68 comprised of approximately less than 5% of the total GFP⁺ cell population observed. These cells are rare and seem to appear randomly among the GFP⁺ cells. They do not occur specifically in a particular location or in any particular fashion. A portion of these cells could have arisen from less than perfect staining procedures or image capturing methodology. The remaining of these non-hematopoietic cells, if they exist, is likely to be very rare. At the moment, though we are unable to account for the exact identity of this rare population of cells, we do know that these cells are of donor origin since they are GFP⁺. They are not of cochlea origin since they do not stain positively for any of the cochlea sensory, supporting, fibrocytic and neuronal markers assessed. Most importantly, they do not result in observable morphological or functional improvement in the deafened cochlea.

By observing the kinetics of the GFP⁺ cell population, the onset of inflammatory response was observed on the day of acoustic deafening, peaking about 3 days after, and gradually decreasing with time until Week 8 where a slight increase in GFP⁺ cells was still observed compared to the control cochleae. The majority of these cells were CD45⁺/CD68⁺ and the post deafening inflammatory healing response period was similar to previous acoustic deafened models [201, 204]. The decrease in GFP⁺ cell population with time is most likely the result of the phagocytosis of these inflammatory cells that underwent cell death or the exit of these cells back into circulation. Similar inflammatory response was also demonstrated in the infarcted heart model where the accumulation of inflammatory cells was observed during the first 7 days after infarction followed by a decrease, which eventually ended at day 21 [143]. The time course of GFP⁺ cell infiltration was very similar to that seen in traumatized organs [210, 211]. Hence this represents a typical time course of a post-injury inflammatory healing response and BMT did not interfere with the basic inflammatory response.

7.5 SDF-1 upregulation

Following acoustic trauma, positive staining of SDF-1 was observed in the Type II fibrocyte region within the spiral ligament and was excluded from cells that express GFP. SDF-1 expression was absent in all regions within the undisturbed cochlea hence, SDF-1 is upregulated only in the deafened subject (Figure 7.16). SDF-1 up-regulation was most prominent at Day 3, declining at Week 1 and was undetected in all samples from Week 2 onwards. This trend during the first week was similar to that of the GFP⁺ cell infiltration

trend, where the extent of SDF-1 upregulation corresponded with that of GFP⁺ cells infiltration. Similar positive labeling and up-regulation trend was also observed in deafened wild type mice (without BMT).

Figure 7.16: SDF-1 immunohistochemistry in cochlea 3 days after deafening. SDF-1 labeling observed at the spiral ligament. SM: Scala Media, SL: Spiral Ligament. Scale bar: 100 μ m. (supplementary magenta-green image available in Appendix II, Figure V)

SDF-1 and its receptor CXCR4 are emerging as a common axis for directing the migration of BMSCs associated with injury repair in many systems. Given the apparent function of SDF-1 to serve as a chemoattractant for BMDCs, including progenitor and stem cells associated with repair [212-214], SDF-1 expression is increased to act as a signaling mediator to guide BMDCs to the injury site [215-217]. Consistent with that, our studies confirmed the up-regulation of SDF-1 at the Type II fibrocytic regions of the spiral ligament in the deafened cochlea during the first week after deafening, peaking at approximately Day 3. Similar results observed in wild type mice without BMT showed that this up-regulation is a common response to acoustic trauma and not the BMT procedure. Several systems have also shown similar effects specifically in the heart, eye and brain where the temporary up-regulation of SDF-1 was observed at the site of injury [218-220]. The post injury SDF-1 expression suggests that SDF-1 mediated cell homing may be the most effective during the first week after injury and may provide a portal of access for circulating reparative cells to migrate to the areas of injury [221, 222]. In the present study, together with this up-regulation of SDF-1 expression, we observed robust BMDC infiltration. Hence, this brief time span of SDF-1 expression may be critical for the acquisition of reparative cells and provide a defined time frame in which therapeutically transplanted cells (engineered cells that express specific factors) or autologous stem cells are mobilized from the bone marrow to home, engraft and differentiate into needed cell types.

7.6 Check for transdifferentiation to cochlear cell types

To investigate whether BMDCs transdifferentiated to any cochlear cell types, we carried out immunolabeling with several markers, followed by an extensive search for colocalization. None of the GFP⁺ cells colocalized with various cochlea sensory (MyosinVIIa, Calretinin), supporting (Jagged1, S100A1), fibrocytic (Na, K-ATPase) and neuronal markers (NF200) at all timepoints up to 8 weeks (results not shown). Hence abundant BMDCs were detected in the deafened cochlea after acoustic trauma but none displayed cochlea sensory, supporting, neuronal, fibrocytic characteristics.

We conclude that direct differentiation of BMDCs is negligible or at most a rare occurrence in the acoustic deafened cochlea, which is in line with the general lack of regenerative potential in the mammalian cochlea. Taken together, under steady state, the deafened mammalian cochlea does not provide the specific microenvironment where regenerative signals are present or activated to initiate both migration and differentiation of BMDCs.

CHAPTER 8: BONE MARROW CELL MOBILIZATION BY CYTOKINE TREATMENT

Since BMDSCs are naturally rare in peripheral circulation, the amount of medullar progenitors in the peripheral blood could be increased with mobilization from the bone marrow using cytokine treatment [223].

Figure 8.1 presents the schematic depicting the experiment protocol for this study.

Figure 8.1: Schematic representation of experimental setup (timeline not drawn to scale).

8.1 Successful BMC mobilization

In a separate experiment, to explore the possibility of transdifferentiation of BMDCs after BMC mobilization, cytokine treatment was administered over a period of ten days with acoustic deafening carried out on the sixth day (refer to Chapter 4.8 for details). Geimsa-wright staining of blood smears from cytokine treated and non-cytokine treated mice was carried out (Figure 8.2). Staining of white blood cells in the blood smears demonstrated the significant increase in white blood cells within the peripheral circulation of the cytokine treated mice.

Figure 8.2: Geimsa-wright staining of blood smears of (A) non-cytokine and (B) cytokine treated mice. Scale bar: 100 μ m

White blood cell count was also carried out in the mice at all experimental timepoints (Figure 8.3). A significant increase in white blood cell count was recorded in the cytokine treated mice. This increase was most evident during the injection period and

by the third recorded timepoint (i.e. 1 week), white blood cell count was comparable to that in the control mice.

Figure 8.3: Effect of cytokine injections on transplanted mice with a daily dose of SCF (200µg/kg/day) & G-CSF (50µg/kg/day) over 10 days. White blood cell count in the peripheral blood of cytokine and non-cytokine treated mice at various timepoints. Timepoints, Day 1 and 3 lies within the 10 day injection period.

Flow cytometry analysis was also carried out to measure the percentage of GFP⁺, CD117⁺ (hematopoietic progenitor marker) and Gr1⁺ (granulocyte marker) cell count in the peripheral blood (Figure 8.4). Flow cytometry measurements for all three markers showed a similar spike in percentage positive cells upon initial doses of cytokine injection and subsequent normalization of readings as in the white blood cell count results (Figure 8.3).

Figure 8.4: %GFP⁺, CD117⁺, Gr1⁺nucleated cells in the blood obtained by FACS analysis. Comparison of non-cytokine treated and cytokine treated mice over time. Timepoints, Day 1 and 3 lies within the 10 day injection period. Values in mean $\pm$ SD. *p < 0.05 compared with control.

Taken together, there was successful mobilization of BMDCs from the bone marrow into the peripheral blood and effects of cytokine treatment were transient as readings started to revert to that of control animals with the cessation of injections.

8.2 GFP⁺ cell infiltration and characterization

The mice were subjected to the same acoustic deafening conditions to produce the deafened mice model. Upon acoustic deafening, robust GFP⁺ cell migration was also observed. Although the number of mobilized BMDCs increased in circulation after cytokine treatment, there was no subsequent increase in cell infiltration within the deafened cochlea. BMDC infiltration and characterization results were comparable to those obtained previously in Chapter 7.3 and 7.4. Hence no further quantification and characterization results of these GFP⁺ cells are presented here. The reasons for a lack of subsequent increase in BMDC cell infiltration after cytokine treatment is unclear at this point. Possible reasons include the innate limited vascularisation within the cochlea that impeded the surge in BMDCs from circulation to the deafened cochlea or the lack of specific cues generated in our acoustic deafened model to direct more BMDCs to the already ‘saturated’ deafened cochlea.

8.3 Check for transdifferentiation to cochlear cell types

Similarly, to investigate whether BMDCs transdifferentiated to any cochlear cell types, we carried out immunohistochemical analysis and an extensive search for colocalization. However none of the GFP⁺ cells colocalized with the various cochlea sensory (MyosinVIIa, Calretinin), supporting (Jagged1, S100A1), fibrocytic (Na, K-ATPase) and neuronal markers (NF200) at all timepoints up to 16 weeks (Figure 8.5, 8.6).

Figure 8.5: Immunohistochemical labeling of various cochlea sensory, supporting, fibrocytic and neuronal markers in cochlea 3 days after deafening. Absence of colocalization with all GFP⁺ cells. (A): Calretinin, (B): MyosinVIIa, (C): Jagged1, (D): S100A1, (E): Na, K-ATPase, (F): NF200, SV: Scala Vestibuli, SM: Scala Media, ST: Scala Tympani, Scale bar: 100μm

Figure 8.6: Immunohistochemical labeling of various cochlea sensory, supporting, fibrocytic and neuronal cell markers in the cochlea 3 days after deafening. Absence of colocalization with all GFP⁺ cells. High magnification view of the (A) lateral wall and (B) organ of Corti, limbus and spiral ganglion neuron region (1): Calretinin, (2): MyosinVIIa,

(3): NF200, (4): S100A1, (5): Na, K-ATPase. Scale bar: 100 μ m. (supplementary magenta-green image available in Appendix II, Figure VI)

Cytokine treatment expectedly increased the number of circulating white blood cells, including CD117⁺ hematopoietic progenitor cells. However, after rounds of immunohistochemical staining and extensive search for colocalization, colocalization of the GFP⁺ cells with assessed markers was absent via immunohistochemical labeling nor was there any compelling evidence to show that any of these BMDCs had undergone differentiation. In addition, the high hematopoietic donor chimerism is indicative of the successful replacement of recipient bone marrow with the transplanted donor BMCs and the lack of donor derived cochlea cell types was not caused by incomplete chimerism. At this point, we are unable to exclude the possibility of GFP transgene silencing resulting in the failure to detect BMDC transdifferentiation [224, 225] as there is no completely reliable method to detect female derived cells in male tissue sections. However, with the abundance of GFP⁺ leukocytes and macrophages observed within the deafened cochlea and the absence of ectopic expression of specialized cochlea cell markers assessed, we believe that down-regulation of GFP expression is unlikely to explain the absence of BMDC transdifferentiation here. Hence, after mobilization of BMC by SCF and G-CSF injection, transdifferentiation was not observed even when deafening was induced at the time of BMC mobilization. BMC mobilization has minimal or no effect on cochlea repair. Although BMDC may be able to contribute to cardiac [218, 226] or neural [227, 228] cell regeneration after bone marrow mobilization, it seems unlikely to be the case here and transdifferentiation was not observed even when deafening was induced at the time of

BMC mobilization. Possible reasons include the innate limited vascularisation in the cochlea and the absence of specific cues in the injured cochlea to direct pluripotent cells from circulation to the site of injury.

In our model, regardless of cytokine administration, no colocalization of Na, K-ATPase and GFP⁺ cells was observed in the cochlea including the spiral ligament under confocal microscopy. The Type II, and IV fibrocytes of the spiral ligament labeled with the antibody $\alpha 6F$ (anti-Na, K-ATPase) in red clearly represents a cell population that is separate from the GFP⁺ BMDCs infiltrating the spiral ligament. This result is similar to that obtained by Hirose and coworkers where none of the inflammatory cells in the acoustic deafened cochlea exhibited any trace of colocalization with Na, K-ATPase [201, 209]. On the contrary, in Lang and coworkers' model, some colocalization of GFP⁺ cells with Na, K-ATPase was observed in the spiral ligament [198]. This discrepancy is unclear at this point and it could be due to a difference in interpretation of results or a difference in experiment protocols. Firstly, non hematopoietic cells could be incorrectly identified as donor derived in histological sections as marrow derived hematopoietic cells overlay the cell of interest. Furthermore, in their whole BMT model, only one mouse was assessed for Na, K-ATPase colocalization without quantitative data to account for the number of such cells and the GFP-expressing cells were presented at a magnification level which does not enable identification of possible colocalization. Secondly, there is a high possibility of partial nuclei sampling in their fluorescence in situ hybridization (FISH) analysis due to insufficient sections assessed. Hence even if GFP⁺/Na, K-ATPase⁺ cells were present, it does not completely exclude the possibility of cell fusion between BMDCs with the fibrocytic cells [128, 147]. Alternatively, the difference in results obtained could be due to

subtle differences in experimental design. Firstly, the age of the donor and recipient mice used for BMT were different in both studies. Although age did not affect the overall engraftment potential during BMT, the difference in phenotype of the HSC transplanted due to age [229] may have an effect on the plasticity potential of the BMDCs. Secondly, the time taken post-BMT for transdifferentiation to occur is unclear at this point and the detection of BMC transdifferentiation in Lang's model could be due to a longer survival period (up to twenty months post-BMT) before sacrifice compared to our model where all mice assessed were harvested within a much shorter period (seven months post-BMT). Hence, we would like to emphasize that under the experimental conditions described here; we did not detect BMDC transdifferentiation into any cochlea cell types. Also, the induction of specific cues required for transdifferentiation of BMCs remains to be determined.

Injury is identified as the single most important factor for transdifferentiation of adult bone marrow cells to non-hematopoietic cells of multiple tissues [230, 231]. Total body irradiation of test subjects during BMT can enhance the effect of injury either as a second injury to aid in such a conversion or eliminate environmental cues for transdifferentiation and inhibit the ability of injured tissues to regenerate. Various studies have been carried out to study the overall effects of irradiation on bone marrow stem cell plasticity after BMT [137, 232-234]. Results obtained collectively suggest a significant role of irradiation on the homing and transdifferentiation of these stem cells in the injured tissue primarily by promoting chimerism [235]. Hence, it is unlikely that the absence of bone marrow transdifferentiation in our study is due to the effects of irradiation on the recipient mice. More studies could be carried out to evaluate the critical aspects of

irradiation on bone marrow cell transdifferentiation in the cochlea including the irradiation method (systemic or local), dosage and timing.

Immunolabeling with CD34 and CD117 were also carried out and there were absence of hematopoietic progenitors or stem cells detected in the deafened cochlea (results not shown). Hence further supporting the notion that BMDCs may incorporate to the deafened cochlea upon BMC mobilization but they rarely contribute to repair and transdifferentiation of BMDCs is a negligible event in the normal repair process after deafening and bone marrow mobilization.

Although we did not observe the transdifferentiation potential of the BMDCs, we demonstrated the migration ability of these BMDCs to the cochlea under normal steady conditions and in the presence of acoustic trauma. Such findings will aid in the design of appropriate gene delivery systems using these BMDCs or endogenous / exogenous macrophages for cochlea gene delivery [236-238]. In addition, this natural ability of BMDCs to infiltrate the cochlea could be exploited and allow the use of these BMDCs as vehicles to introduce therapeutic genes into the cochlea [239, 240]. By retroviral transfection, donor bone marrow cells could be transfected with cochlea therapeutic genes prior to transplantation into irradiated recipients. Cues generated in the deafened cochlea after acoustic damage will direct BMDCs encoding these therapeutic genes of interest into the cochlea and the effect of these genes of interest could be assessed in terms of functional restoration and protection. Examples of these therapeutic genes include the various neurotrophic factors such as BDNF, GDNF and TGF- β 1. Several studies have reported that over expression of these neurotrophic factors such as the BDNF [30, 31], GDNF [28-30, 64] and TGF- β 1 [29, 30] in the cochlea have resulted in the protection of

sensory hair cells and spiral ganglion neuron against various cochlea damages (ischemia, aminoglycoside ototoxicity, nerve avulsion). Overexpression of various antioxidants in the cochlea has also been reported to reduce inner ear oxidative stress and protect sensory hair cells against ototoxicity [49]. In other organ systems, such as the central nervous system [161, 241], retinal [242] and pulmonary airway etc. [158, 159], BMDCs have also been used as gene delivery vectors via *ex-vivo* gene manipulation and results have been promising.

Mechanisms underlying tissue repair by BMCs are not fully understood, and the question remains if these BMDCs have tissue repair capacity. They are able to mobilize, migrate to the site of injury, and possibly differentiate, but the concept of transdifferentiation or plasticity of these cells remains controversial. In this study, we demonstrated BMDCs readily home the cochlea in response to acoustic deafening, coupled with the upregulation of SDF-1 expression at the spiral ligament however the infiltrated BMDCs rarely contribute to regeneration of any cochlear cell types under steady conditions, after tissue injury nor after BMC mobilization. We attributed the BMDC migration to extensive donor derived leukocyte infiltration after deafening, consistent with the known mechanism of progression and recovery of cochlea injury. The experimental model used is appropriate in assessing the role of the host's bone marrow cells in the restoration process after acoustic trauma and results are in agreement with the findings of several authors. However, the transdifferentiation potential of BMCs in other species and the induction of specific cues or experimental settings required for successful regeneration need to be determined and would prompt further investigation to give us a better understanding of BMDC's role in the hearing recovery process. A possible future

application of this model is to harness the robust migration ability of BMDCs to be used as a vehicle for gene delivery in the deafened cochlea.

CHAPTER 9: CONCLUSIONS AND FUTURE PERSPECTIVES

Considering the extent of sensorineural hearing loss affecting people worldwide, the extent of research being conducted is fairly modest. However, with the recent advancements in techniques within the various fields (such as stem cell technology, genetic manipulation, gene therapy etc.) and exciting results involving mammalian hair cell development and repair, the restoration of sensorineural hearing loss in humans could be an obtainable dream in the near future. Currently, mammalian inner ear repair still has to overcome several obstacles before fulfillment despite recent advancements achieved by several research groups. These advancements in gene therapy and cell therapy has raised hopes for recovery of mammalian hearing loss but results obtained so far are still at the basic science level and with safety being the top priority in all clinical trials; much work is still needed before clinical implementations. Current gene therapy techniques involve the use of viral vectors for gene delivery which may invoke host immunological response and current cell therapy techniques involve invasive direct transplantation which results in mechanical trauma and damage to the cochlea. Hence in view of these shortcomings, we approach the issue of hearing loss restoration through the use of a non-viral vector (a safer alternative instead of the commonly used viral vector) to achieve transgene expression in the mammalian cochlea and to investigate the body's intrinsic capacity for restoration using bone marrow cells.

In the area of non-viral gene delivery, we assessed and compared the various methods of delivery in cochlea gene delivery studies and identified the osmotic pump infusion method as a way of augmenting non-viral vector transfection efficiency. We also

carried out transfection studies using PEI and displayed improved transfection efficiency with sustained release of the vector solution over a week. Currently, no ideal vector exists in cochlea gene therapy and different systems need to be integrated and optimized to overcome many obstacles limiting the success of non-viral cochlea gene delivery. Hence this study presents another novel and safe approach for non-viral gene transfer in the cochlea using linear PEI as an alternative to viral vectors. In future studies, further modifications can be carried out on such a versatile non-viral gene vector to improve its overall transfection performance in areas such as cellular uptake, nuclear transport and cytotoxicity. Conjugation of side-groups or ligands on the polymer backbone to enhance specificity and targeting to specific cochlea cells of interest will also improve its overall efficiency and relevance to cochlea gene delivery studies. Delivery of important genes such as the *Math1* gene (transcription factor which has gained a tremendous amount of attention in inner ear studies) using PEI and comparing its transfection effects to that achieved by the Michigan group (Raphael Yehosh's lab) using the adenoviral vector [13] would also be an important approach for future studies.

In the area of bone marrow cell's role in inner ear repair, we displayed the homing capability of BMDCs to the deafened cochlea and these cells displayed mature hematopoietic properties without spontaneous transdifferentiation into cochlear cell types after acoustic trauma and bone marrow mobilization induced by cytokines. In this study, robust migration of GFP⁺ BMDCs to the cochlea in response to acoustic deafening, coupled with the upregulation of SDF-1 expression at the spiral ligament was observed. However, the infiltrated BMDCs rarely contribute to regeneration of any cochlear cell types under steady conditions, after tissue injury nor after BMC mobilization. We

characterized the GFP⁺ BMDCs and attributed the BMDC migration to extensive donor derived leukocyte infiltration after deafening, consistent with the known mechanism of progression and recovery of cochlea injury. Molecular players involved in homing, growth and differentiation of stem cells are likely to differ depending on the tissue and degree of injury. In future studies, more work could be done to improve our understanding on the mechanism and factors required for migration and transdifferentiation of BMDSC in the inner ear. With current controversies in the regenerative potential of BMDSC/BMDC, this improved understanding will definitely aid in deriving more stringent experimental conditions and criteria to prove or reject plasticity *in vivo*. In addition, exploitation of BMDC migration for gene delivery purposes by *ex-vivo* manipulation could also be an important approach for future studies where therapeutic potential of genes of interest delivered via this method could be evaluated.

With continued advancements in the area of genetic manipulation, gene therapy and cell transplantation, hopefully, the mammalian cochlea capacity for functional restoration and structural regeneration can be unlocked. Clearly, at the current stage, no single method is truly effective and the future direction would be to employ combination therapy involving both gene and cell therapy, such as harnessing BMDCs' migration ability and exploit these cells as a vector for gene delivery with *ex-vivo* gene manipulation. In addition, gene and cell therapy could also be employed with current drug treatments and cochlear electrode implantation to devise therapy customized for individual patients to aid functional recovery. Hence the success of mammalian hearing restoration in the near future will depend heavily on 1) the increase understanding of the molecular mechanism of development, pathology, repair and regeneration in the cochlear tissue, and

2) translation and combination of basic science research to clinical applications by improving the safety and effectiveness of delivery vehicles, transgene expression and transplantation.

Summary of achievements in this study:

Non-Viral Gene Delivery Studies

- 1) Assessed and compared the various methods of delivery in cochlea gene delivery studies.
- 2) Identified the osmotic pump method as a way of augmenting non-viral vectors' inherent low transfection efficiency
- 3) Carried out transfection using a non-viral vector, PEI and displayed improved transfection efficiency with sustained release of the vector solution (along with intact cellular architecture and absence of inflammation in the infused cochleae).

Bone Marrow Cell Studies

- 4) Displayed the homing capability of BMDC to the cochlea under normal / steady state.
- 5) Demonstrated the robust migration of GFP⁺ BMDC in a deafened mouse model.
- 6) Characterized the GFP⁺ BMDC in the deafened mouse model.
- 7) Demonstrated the up-regulation of SDF-1 in the cochleae post-deafening.
- 8) Identified the possibility of harnessing migration ability of BMDC to carry gene of interest to the deafened cochlea.
- 9) Studied the transdifferentiation possibility of these BMDC cells in the deafened mouse and BMC mobilized model.

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APPENDICES

Appendix 1 (Drug induced deafening versus noise induced deafening)

Subjects

C57BL/6J mice, 3 months after hematopoietic reconstitution (n = 8 for each group).

Mice were sacrificed for assessment at 1 week after deafening procedure.

Procedures

1) Drug induced deafening

Route of surgery followed the postauricular approach where the mouse was placed in the side-lying position and an incision was made behind the ear. The connective tissues and veins were retracted until the semi-circular canal was exposed. An orifice was drilled into the posterior and lateral semi-circular canals and 1 μ of kanamycin (100 mg/mL) was injected into the posterior semicircular canal slowly via the orifice. After injecting the drug, the holes in the semicircular canals were plugged with connective tissue and were then covered with adhesive agent. Surgical procedures were completed by suturing of the skin incision.

2) Noise induced deafening

Deafening Conditions: 120 dB SPL white noise, 2 hours

(For more details, please refer to Materials and Methods, Acoustic Deafening)

Results

ABR

Deafened cochleae showed a range of 0 – 50 dB SPL threshold shifts 1 week after kanamycin inoculation surgery across the three assessed frequencies (Figure I). Some

mice experienced a greater extent of deafening compared to others. In addition, within the same mouse, the extent of deafening across the frequencies was different. This is a result of varied deafening effect from the deafening surgeries conducted on the mice.

Figure I: ABR threshold shifts resulted from drug-induced (kanamycin inoculation) deafening, $n = 8$

GFP⁺ cell quantification

From the error bar comparisons between the kanamycin and acoustic deafened group, there was greater response variation for kanamycin deafened mice compared to acoustic deafened mice (Figure II). This is especially significant in regions where GFP⁺ cell infiltration is intense such as the spiral ligament, modiolus and the perilymphatic compartment walls.

Figure II: Quantitative data of GFP⁺ cell infiltration at 1 week after deafening of the various experimental groups

Conclusion

Hence with the difficulty in ensuring reasonable consistency in the surgical procedures, acoustic deafening will be the preferred deafening choice. Acoustic deafening would result in greater consistency and elimination of inflammatory response due to any surgical procedures.

Appendix 2 (Magenta-green images of selected red-green fluorescence images)

Figure III: CD45 immunohistochemistry. (A): Limbus, (B): Perilymphatic compartment wall, (C): Spiral ligament, (D) Spiral ganglion neurons, (E) Bone marrow region in the otic capsule. Scale bar: 100µm.

Figure IV: CD68 immunohistochemistry.

Figure V: SDF-1 immunohistochemistry.

Figure VI: Immunohistochemical labeling of various cochlea sensory, supporting, fibrocytic and neuronal cell markers.